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PRODUCTION OF TRANSGENIC CHANNEL CATFISH

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Forestry, Wildlife, and Fisheries

by
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ABSTRACT

The channel catfish industry has experienced tremendous growth over the last several years. This growth has triggered more intensive culture practices resulting in disease outbreaks and devastating mortalities. Medicated feeds and vaccination have been of limited use. This research presents studies on techniques to produce disease resistant channel catfish by gene transfer. The gene used was the cecropin B gene from the giant silkworm moth Hyalophora cecropia, controlled by an acute phase response (APR) promoter also from H. cecropia.

The objectives of this study were to: (1) determine if an early maturing population of catfish from Lake Maurepas, Louisiana, could be used as a model fish for genetic research; (2) develop techniques for the collection of unfertilized catfish eggs; (3) determine the effect of electroporation of eggs on the resulting embryos, and (4) develop screening methods of embryos to determine the percentage transgenic fish.

The early maturing channel catfish population from Lake Maurepas, Louisiana was determined to be a normal population of channel catfish in all respects other than maturing at an early age and small size, and spawning later in the year when compared to other populations of channel catfish in southern Louisiana.

An alternative channel catfish spawning method in which females are grouped rather than paired with males is described. The proportion of successful spawns for paired females (41%) was not significantly different ($P = 0.64$) from that of the grouped females (58%). Percent fertilization was significantly different ($P = 0.02$) for eggs stripped from paired females ($43 \pm 37\%$) and grouped females ($16 \pm 20\%$). The grouped method has promise if timing for collection of high quality eggs can be determined.

The effect of electroporation of unfertilized eggs on fertilization and hatching rate was significant ($P \leq 0.0001$). However, there was no effect ($P = 0.32$) on percentage of fry surviving to 2 weeks after fertilization.

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Methods for the isolation of potentially transgenic treatment groups prior to screening were developed. The polymerase chain reaction was used to screen embryos for the presence of the cecropin gene which was evident in 47% of the embryos tested.

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CHAPTER 1
Introduction

The pond spawning method for production of channel catfish Ictalurus punctatus dates back to the early 1920's (Clapp 1929), when broodfish were found to enter and spawn in cans that were placed in ponds. Today, seven decades later, the channel catfish industry uses this same basic method to produce seed stock (Avault 1996). The beef, dairy, pork, and poultry industries have realized enormous gains in productivity due to genetic improvement (Hafez 1993). Genetic improvement will require changes in spawning methods. The need for improved production methods for channel catfish has been recognized for many years (United States Department of the Interior 1970, Smitherman et al. 1978).

Commercial culture of channel catfish has increased dramatically over the last several years. In 1979, a total of ~18 million kg of live catfish were processed in the United States. Processing of live catfish in 1995 was over 240 million kg (Jensen 1995). Producers have adopted more intensive culture practices to meet the demand for channel catfish and to remain competitive. Stressors present in the pond, combined with extremes in temperature and oxygen concentration, play an important role in causing disease in fish.

Pathogenic organisms are always present in pond or tank water and are able to infect fish weakened by stress. Among the many organisms pathogenic to channel catfish, Edwardsiella ictaluri (Hawke 1979) has been found to cause commercially devastating mortalities (Huner and Dupree 1984). Peak incidence of the disease caused by E. ictaluri, enteric septicemia of catfish (ESC), occurs in June and September in the southern United States. During this time, water temperatures in ponds range between 22 and 28 C, the optima for growth of E. ictaluri (Avault 1996). Disease outbreaks are further encouraged by intensive management practices, requiring an added expense in medication of fish. Only two antibiotics are registered by the food and drug administration (FDA) for

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treatment of channel catfish raised for food. The registered drugs are ormetroprim-sulfadimethoxine (Romet®-30) and oxytetracycline (Terramycin®). Resistant strains to these drugs have been isolated (McPhearson et al. 1991). Recent research has focused on genetic improvement of disease resistance in channel catfish (Dunham and Smitherman 1985; Wolters and Johnson 1995). This research, however, is hampered by the long generation time of channel catfish.

The 3-to-4 year generation time of commercial strains of channel catfish is an impediment to genetic research. Fish with shorter generation times have been used as models for research. Some examples are the zebrafish Brachydanio rerio (Buono and Linser 1992) and the medaka Oryzias latipes (Inoue et al. 1992) which spawn at a small size, a few months of age and year-round. The reproductive characteristics of these fish make them attractive experimental animals for genetic research. However, these fish have limited commercial value. A compromise between using a strictly experimental model species and a commercially valuable species would be an early reproducing strain of the commercial species. Populations of catfish found in Lake Maurepas and Lac Des Allemands in southeast Louisiana show promise for use as models in genetic research. Half of the catfish in these populations reach sexual maturity at less than 2 years of age and as small as 170 mm total length (TL) (McElroy et al. 1990) rather than the normal age of 3 to 4 years and TL of $\geq$ 500 mm for channel catfish.

In addition to the long generation time of channel catfish, the commercial catfish industry has not developed methods necessary for stock improvement. Such methods will be required to produce more productive strains of fish. Artificial spawning, the collection of eggs from a female followed by fertilization with sperm from one or more males, is a powerful method for genetic improvement, hybridization, and the production of transgenic fish. However, the currently used method requires continuous monitoring of broodfish paired in aquaria, and stripping of eggs from hormonally treated females actively involved in spawning. Artificial spawning of other cultured species of fish

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typically involves egg collection from large groups of hormonally treated females at a specified time after hormone injection. This method has been used only rarely for channel catfish, and therefore requires development before it can be useful at the commercial level or in the production of large numbers of transgenic fish.

Methods to produce transgenic organisms have been developed for animals (Burns et al. 1993) and plants (Tarczynski et al. 1993). Microinjection, the injection of purified DNA into a cell nucleus or cytoplasm, was the original technique used to produced transgenic fish. Early transgenic research utilized microinjection to introduce reporter genes into fish embryos or eggs to study gene expression (Inoue 1992; McEvoy et al. 1988). The reporter genes offered the advantage of giving the researcher an easily detectable signal if the gene was being expressed. This allowed determination of promoter function and control. Current transgenic efforts are applying what was learned in the early work and are directed toward improvement of culture traits such as growth rate (Dunham et al. 1987) or cold-tolerance (Shears et al. 1991) (Table 1-1). Disease resistance has been suggested as a possible trait for improvement by transgenic methods (Abel et al. 1986; Cooper 1993). Disease resistance can be improved in fish by selective breeding techniques (Wolters and Johnson 1995), however, this can require years, and may result in increased susceptibility to other diseases. Our goal was to introduce a gene into channel catfish that enhances resistance to diseases caused by a variety of pathogens.

More than 50 antibacterial peptides or polypeptides have been isolated from the blood of immune-challenged insects and from some vertebrates. Twenty antibacterial or lytic peptides are classified into two major groups. These are the cecropins, which attack and kill Gram-negative and Gram-positive bacteria (Boman et al. 1991), and the defensins, which kill Gram-positive bacteria (Cociancich et al. 1993) (Table 1-2). Genes encoding cecropins and defensins are not expressed prior to bacterial challenge. Following challenge with bacteria, antibacterial activity peaks within a few hours, and plateaus and recedes after one to several days (Cociancich et al. 1994).

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Table 1-1. Selected references of transgenic research in fishes. Author(s), year of publication, species, promoter, gene, and gene transfer (transfection) method.

Citation	Common name (scientific name)	Promoter[1]	Gene[2]	Transfection method	Comments
Ozato et al. 1986	Medaka (Oryzias latipes)	cCrys	cCrys	microinjection	Expression of a chicken promoter and gene in a fish.
Dunham et al. 1987	Channel catfish (Ictalurus punctatus)	mMT	hGH	microinjection	~20% tested positive for presence of the hGH gene.
		mMT			
Guyomard et al. 1988	Rainbow trout (Oncorhynchus mykiss)		hGH	microinjection	Reported stable incorporation, but no expression.
Penman et al. 1990	Rainbow trout	mMT	rGH	microinjection	15 to 18% positive for rGH gene.
Hayat et al. 1991	Channel catfish Common carp (Cyprinus carpio)	mMT RSV RSV RSV	hGH rtGH csGH rtVit.	microinjection	Incorporation rates of ~15% for channel catfish embryos, and ~11% for common carp embryos.
Winkler et al. 1991	Medaka	HSVtk hMT X47 SV40 RSV	CAT CAT CAT lacZ lacZ	microinjection	Expression of reporter genes in ~95% of larvae tested at 1 week. Expression ceased after 2-4 weeks. Incorporation of DNA detected in 1 of 25 adults tested.
Shears et al. 1991	Atlantic salmon (Salmo salar)	wfAFP	wfAFP	microinjection	AFP gene detected in 3% of 2-year-old adults. 40% of the transgenics expressed AFP.

(Table 1-1 cont'd)

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Citation	Common name (scientific name)	Promoter[1]	Gene[2]	Transfection method	Comments
Müller et al. 1992	Common carp African catfish (Clarias gariepinus) Tilapia (Oreochromis niloticus)	RSV HSVtk	lacZ Tn5	electroporation of sperm	3 to 4% of embryos and larvae positive for presence of foreign DNA.
Xie et al. 1993	Loach (Misgurnus anguillicaudatus) Crucian carp (Carassius auratus)	mMT	hGH	electroporation of fertilized eggs	hGH gene detected in 62% of loach, and 57% of carp.
Zhao et al. 1993	Zebrafish (Brachydanio rerio)	RSV	csGH	electroporation of embryos	csGH detected in 70% of embryos tested.
Szelei et al. 1994	African catfish	MuLV RSV mMT SV40	CAT neo^r mMTI TNF	lipofection of fertilized eggs	Expression detected in > 80% of 2-day-old larvae. Expression dropped to < 27% after 3 weeks.
Sin et al. 1994	Chinook salmon (Oncorhynchus tshawytscha)	RSV	lacZ	electroporation of sperm	A high of 85% positive for presence of lacZ gene detected in 12-week-old fry.

[1] Abbreviations: mMT, mouse metallothionein promoter; RSV, Rous sarcoma virus promoter; HSVtk, thymidine kinase promoter from herpes simplex virus; hMT, human metallothionein; X47, metal responsive promoter from the platyfish Xiphophorus maculatus; SV40, simian virus 40; wfAFP, winter flounder Pseudopleuronectes americanus antifreeze protein; MuLV, Friend murine leukemia virus long terminal repeat; opAFP, ocean pout Macrozoarces americanus antifreeze protein.

[2] Abbreviations: hGH, human growth hormone; lacZ, Escherichia coli ß-galactosidase; rGH, rat growth hormone; rtGH, rainbow trout growth hormone; rtVit., rainbow trout vitellogenin; CAT, chloramphenicol acetyltransferase; wfAFP, winter flounder antifreeze protein; Tn5, kanamycin phosphotransferase; neo^r, aminoglycoside-phosphotransferase; mMTI, mouse metallothionein I; TNF, human tumor necrosis factor.

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Table 1-2. Antibacterial peptides of insects and vertebrates. Name,size in kiloDaltons (kDa), target cells, and mode of action (if known). (Cociancich et al. 1994).

Name	Size (kDa)	Target[a]	Mode of action (if known)
Cecropins			
Insect cecropins	4	G+ and G-	Bactericidal, form anion-selective voltage-dependent channels in artificial membranes.
Mammalian cecropin (pig)	4	G+ and G-	
Defensins			
Insect defensins	4-5	G+	Bactericidal, form voltage dependent channels in cytoplasmic membrane of Micrococcus luteus.
Mammalian defensins	4	G+, G- and viruses	Bactericidal, form voltage dependent channels in planar lipid bilayers.
Proline-rich peptides			
Apidaecins	2	G-	Bacteriostatic
Abaecins	4	G+ and G-	Bactericidal
Drosocin	2.5	G-	Bactericidal
Bactenecin	1.5	G+ and G-	Bactericidal, active on cytoplasmic membrane.
Bac5 and Bac7	5-7	G- and Viruses	Bactericidal or bacteriostatic, permeabilize inner and outer membrane.
Glycine-rich peptides			
Attacins	20	G-	Bacteriostatic, active on outer membrane.
Sarcotoxins	24	G-	Bactericidal
Coleoptericin	8	G-	Bactericidal
Diptericin	9	G-	Bactericidal
Hynenoptaecin	10	G+ and G-	Bactericidal
Magainins	2.5	G+ and G-	Bactericidal and bacteriostatic, form anion-selective voltage-dependent channels.

[a] G+, Gram positive bacteria; G-, Gram negative bacteria.

The complete mechanism of killing of cells by the lytic peptides has not been fully elucidated. However, in experiments with artificial membranes, cecropins have been shown to have channel-forming properties that permeabilize the lipid bilayer (Christensen et al. 1988). The gene used in this project was the cecropin B gene of the giant silkworm moth Hyalophora cecropia (Xanthopoulos et al. 1987), which is under the control of an acute phase response promoter (APRP) also from H. cecropia. The lytic peptide product of the cecropin B gene provides potent antibacterial action against a wide variety of bacterial fish pathogens including E. ictaluri (Kelly et al. 1994).

In organisms as diverse as Drosophila and man, the APR is an inducible mechanism that allows various cells to respond to injury or infection by triggering release or production of non-specific defensive proteins such as the lytic peptides (Hurt et al. 1994; Reichhart et al. 1992). Successful integration of the cecropin B gene under the control of the APR promoter would bestow upon the transgenic host a greater resistance to bacterial infection (Figure 1-1). The term integration as used in this dissertation refers to the stable and irreversible insertion of the transgene into the genome of a target cell or cells as described by Kleckner et al. (1991) for general insertion.

Researchers have used various techniques to introduce foreign DNA into fish eggs or embryos. Until recently, the most widely used technique was microinjection (Ozato et al. 1986). Millions of copies of insertion vector bearing the transgene were injected into an egg or developing embryo, and low rates of incorporation were observed (Maclean and Penman 1990). Microinjection is time-consuming, however, and requires a high degree of skill. A relatively new technique, electroporation, utilizes brief electrical pulses to deliver DNA into an egg or developing embryo (Müller et al. 1993). Hundreds or thousands of eggs can be treated simultaneously with electroporation. The effect on embryo viability of electroporation of catfish eggs has not received much attention (Dunham et al. 1987; Hayat et al. 1991).

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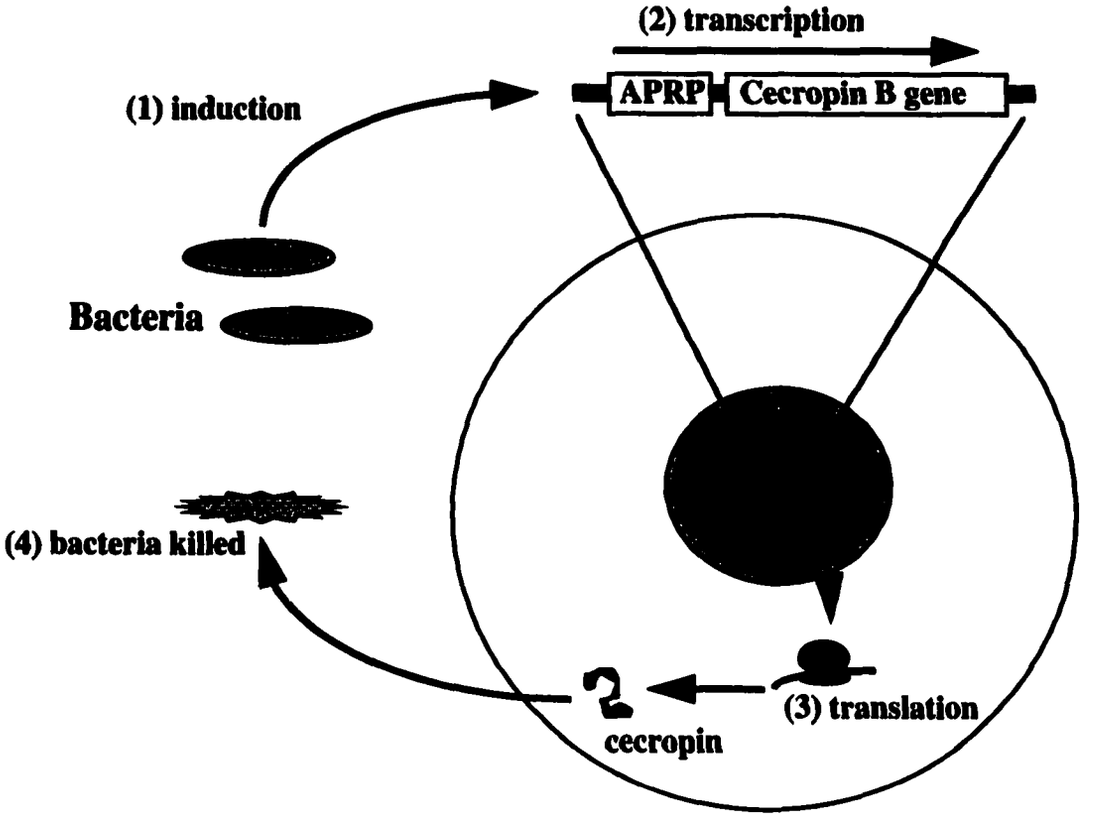

Figure 1-1. Model for inducible function of the cecropin gene in a generic host cell: (1) The presence of bacteria induces the acute phase response promoter (APRP) to trigger transcription of the cecropin B gene in the nucleus; (2) The mRNA transcript is translated to protein in the cytoplasm; (3) cecropin lytic peptide is secreted from the cell, and (4) kills the invading bacteria. When bacteria are no longer present the system is deactivated.

A screening method must be used to determine electroporation parameters required to produce transgenic fish. The polymerase chain reaction (PCR) can be used to screen the DNA of an organism for a specific gene (Hew 1995). The use of PCR requires blood or some other tissue from which DNA can be extracted and requires that the animal be grown to a size large enough to survive sampling or the animal must be sacrificed. For research purposes, sacrifice of a portion of a treatment group for screening can provide valuable information on percentage of fish carrying the transgene. The remainder can be screened after they have grown to a larger size (Maclean and Penman 1990). The PCR indicates only presence or absence of the foreign gene in the sample tested. For an animal to be able to pass the new gene on to its progeny, the gene must be stably incorporated into the germ cells of the animal (Cloud 1990). It is possible to produce animals that are transgenic in one tissue but not in others. These partially transgenic animals are referred to as mosaics or chimaeras (Vick et al. 1993). Mating of a transgenic animal with a non-transgenic animal followed by progeny testing is commonly used to determine if the gene has been incorporated into the germ cell genome.

The objectives of this study were to: (1) determine if catfish from Lake Maurepas, Louisiana could be used as a model for genetic research in channel catfush; (2) develop techniques for the collection of unfertilized channel catfish eggs for electroporation; (3) determine the effect of electroporation of eggs on the resulting embryos, and (4) develop screening methods of embryos to determine the percentage of channel catfish carrying the transgene. For consistency, all chapters of this dissertation have been prepared in the format of the Journal of the World Aquaculture Society. The CBE Style Manual (Council of Biology Editors 1994) was consulted for format matters not specifically covered by the World Aquaculture Society guidelines.

Chapter 2, "A Precocious Wild Catfish Population with Potential as a Genetic Research Model for Channel Catfish," addresses the potential for use of catfish from Lake Maurepas and Lac Des Allemands as research models, because they mature at an early age

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and small size, and spawn over an extended period in comparison to commercial strains or other wild populations of channel catfish. Cytogenetic, molecular, and morphological approaches were used to verify the taxonomic status of these populations to ensure that research with these catfish would be applicable to commercial strains of channel catfish. This chapter represents a synthesis of data collected by different researchers in our lab. Dr. Terrence Tiersch provided data on genome size collected by flow cytometry. Dr. Quiyang Zhang provided guidance and information on cytogenetics and prepared the chromosome spreads and karyotypes described in the chapter. Jeffrey Ratcliff provided assistance in preparing Figure 2-1, and in measurement of chromosomes staining positively for nucleolus organizer regions (NOR).

Chapter 3, "Artificial Spawning of Channel Catfish Ictalurus punctatus as Male-Female Pairs or All-Female Groups in a Recirculating System," deals specifically with methods of artificial spawning of channel catfish to obtain unfertilized eggs. Established artificial spawning methods for channel catfish require use of the aquarium method and male-female pairing which is time-consuming, complicated, and expensive. This chapter documents collection of unfertilized eggs (for artificial fertilization) from channel catfish females without use of pairing with males. Besides being a useful technique for establishment of breeding programs, this method has direct application in production of transgenic, polyploid, androgenetic, gynogenetic, and hybrid fish. All these techniques require unfertilized eggs and would be aided by a method which makes availability of eggs more reliable.

Chapter 4, "Effect of Electroporation on Fertilization, Hatch Rate, and Survival of Channel Catfish Eggs, Embryos, and Fry," addresses whether the process of electroporation has detrimental effects on eggs, embryos, or fry. There are several techniques available for the production of transgenic organisms. However, electroporation is an attractive option when working with fish because it allows synchronous treatment of hundreds or thousands of eggs.

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Chapter 5, "Isolation of Experimentally Treated Fish, and Screening of Channel Catfish for Transgenic Status," describes a system for the isolation of potentially transgenic treatment groups and a method to screen groups of embryos or fry for presence of the transgene. Inexpensive culture units were described to isolate the groups of fish. The screening method used was PCR. This method uses small nucleotide sequences called primers to selectively amplify a gene of interest from a DNA sample. The screening procedure described can be used during development of transfection techniques for optimization of electroporation parameters.

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CHAPTER 2
A Precocious Wild Catfish Population with Potential as a Genetic Research Model for Channel Catfish

Introduction

One measure of success in the production of transgenic fish is acquisition of transgenic progeny (Hew et al. 1995). It is possible to produce an organism that is transgenic in one or more tissues (e.g., blood cells) but not transgenic in others (e.g., gonads). Such organisms are referred to as mosaics or chimeras (Akella and Lurquin 1993; Lin et al. 1992). For a transgenic fish to produce transgenic progeny, it must be germ-line transformed (the transgene is integrated into the genome of germ cells). Progeny testing is the best way to determine if a fish that tests positive for the presence of a transgene is actually germ-line transformed (Hew et al. 1995). Therefore, to develop techniques required to produce transgenic individuals, most basic research has been carried out in model fish with short generation times. Applying these techniques to commercially important species has been hampered by relatively long generation times and large size. There are unique catfish populations in southeastern Louisiana which may avoid these problems and serve as a genetic model for channel catfish.

Two lakes in southeastern Louisiana, Lake Maurepas (LM) and Lac Des Allemands (LDA), contain populations of catfish that mature at an early age (< 2 yr) and at a small size (< 240 mm). These populations have been presumptively identified as channel catfish Ictalurus punctatus. However, because about half of the LM and LDA catfish reach sexual maturity as early as age I+ (second summer of growth) and at sizes as small as 170 mm total length (TL) (M. McElroy, Louisiana Department of Wildlife and Fisheries, personal communication 1995), these populations have caused debate over whether they are channel catfish, a subspecies, or a hybrid between channel catfish and another ictalurid.

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Hypotheses regarding these populations can be separated into two groups. Taxonomic hypotheses are based on the possibility that the LM and LDA catfish are not channel catfish, or are hybrids, and therefore would have a: 1) chromosome number; 2) genome size (amount of DNA per nucleus), or 3) meristic profile different from that expected for channel catfish. For example, LM or LDA catfish could be hybrids between channel catfish and another ictalurid common to LM or LDA, such as the black bullhead catfish Ameiurus melas. Genetic hypotheses include divergence from normal channel catfish such that the LM and LDA catfish are: 4) polyploid, or 5) aneuploid. These last two hypotheses would be expected if these populations underwent duplication of chromosome sets (i.e., triploidy or tetraploidy) or if DNA content was altered by extra or missing chromosomes, chromosome arms, or DNA content. While these hypotheses could result from different mechanisms, there is overlap in the available methods used to test them. For example, cytogenetics can be used to determine number of chromosomes (hypothesis 1), number of chromosome sets (hypothesis 4), and number of chromosome arms (hypothesis 5). Flow cytometry can be used to determine genome size (hypothesis 2) and to test for variations in cellular DNA content (hypothesis 5). Determination of the location of nucleolar organizer regions (NORs), the indicators of active ribosomal RNA synthesis on chromosomes, has been used to differentiate species (Amemiya and Gold 1988). In this study, the technique of NOR staining (Howell and Black 1980) was applied to LM catfish, LM catfish x channel catfish (LSU population) crossbreeds and blue catfish x channel catfish hybrid chromosomes. Nucleotide sequencing of the fourth exon (CH4) of the immunoglobulin M heavy chain constant region gene of the channel catfish (Wilson et al. 1990) was carried out on channel catfish, LM catfish, blue catfish, and black bullhead catfish.

The objectives of this study were to: 1) document spawning of LM catfish outside the typical channel catfish spawning season; 2) develop a meristic and external morphology table for ictalurid catfishes common to LM and LDA; 3) compare genome size of LM and

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LDA catfish to a commercial strain of channel catfish; 4) compare karyotypes from LM catfish and channel catfish (female) x LM (male) crosses to the standardized channel catfish karyotype, and 5) compare DNA sequence data from LM catfish and other ictalurids.

Materials and Methods

Spawning of Lake Maurepas Catfish

Two hoop nets (sized to catch fish < 280 mm) were baited with soy bean meal and placed along the northern shore of Lake Maurepas on two separate occasions (August 22 and 29, 1995). Nets were left for 2 d after which they were hauled up for harvest. Approximately 50 catfish were collected on the first trip and 40 on the second trip. All catfish were taken to the Ben Hur Aquaculture Research Laboratory and placed in recirculating systems. Females that appeared ripe were injected with the synthetic luteinizing-hormone releasing-hormone D-Ala^{6}DesGly^{10} LH-RH-ethylamide (LH-RHa, Peninsula Laboratories, Belmont, California) at a dosage of 50 µg/kg of body weight (Busch and Steeby 1990) and paired with a male of similar size in a 40-L spawning tank. A 20-cm length of 10-cm diameter PVC pipe (schedule 20) with one end capped was placed in each tank to serve as a spawning container. Development to the neurulation stage (~24 h at 28 C) was used as the criteria for fertilization success because unfertilized eggs can develop to the gastrula stage (Withler 1980).

Meristics and Morphology

A meristic and external morpology data table was prepared for ictalurid fishes common to southern Louisiana to differentiate LM and LDA catfish from other species or possible hybrids that might exist in the lake. Meristic and morphometric factors considered were length, weight, number of barbels, body coloration, anal fin ray count, adipose-fin shape, presence or absence of spots, and caudal fin shape. Values for the table were taken from published sources (Audubon Society, 1992; Douglas, 1974).

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Estimation of Genome Size by Flow Cytometry

Blood samples were collected in acid-citrate-dextrose (ACD) solution (Becton-Dickinson vacutainer 4606) from 15 Kansas strain channel catfish (obtained from the USDA-ARS, Catfish Genetics Research Laboratory, Stoneville, Mississippi), 36 catfish collected from Lake Maurepas, and 10 catfish collected from Lac Des Allemands and refrigerated until analysis. Blood cells of the catfish under study were suspended with those of domestic chicken Gallus gallus as a mixture in 0.5 ml of lysis-staining buffer containing 25 µg buffered RNase, 0.1% sodium citrate, 0.1% Triton X100, and 25 µg propidium iodide (Tiersch et al. 1990). DNA content of the cells was estimated with a PROFILE flow cytometer (Coulter Electronics, Hialeah, Florida) with the argon-ion laser operated at a wavelength of 488 nm. Fluorescence values of at least 40,000 propidium-iodide-stained nuclei were digitized individually and used to calculate DNA content in relation to a value of 7.0 pg DNA assigned for fresh human (male) leukocytes. In each test the value of the internal reference was canceled during the calculation of DNA content, according to the formula: nuclear DNA (pg) = 7.0 x C/R x R/H, where C is the fluorescence value for the nuclei of catfish, R is the fluorescence value for the nuclei of the chicken internal reference, and H is the fluorescence value for the nuclei of human blood cells. Genome size values were compared by one-way Analysis of Variance (ANOVA, Data Desk, version 4.2, Data Description, Inc. Ithaca, New York) with $P < 0.05$ chosen as the level of statistical significance.

Karyotyping and NOR Staining

Karyotypes were prepared from cultured leukocytes (Zhang and Tiersch 1995) of LM catfish, channel catfish (a research population maintained at LSU) x LM catfish, and channel catfish x blue catfish Ictalurus furcatus hybrids with the methods of Zhang (1996). The leukocytes were isolated from whole blood by gradient centrifugation on ficoll hypaque, and were cultured in RPMI-1640 medium (Sigma Chemicals, St. Louis, Missouri) with the addition of concanavalin A (10 µg/mL) to stimulate mitosis.

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The RPMI-1640 medium was diluted (to 270 mOsmol/kg) and supplemented specifically for culture of channel catfish leukocytes as described in Miller and Clem (1988). Chromosomes were arrested at metaphase by addition of colchicine (0.5 μg/mL). Procedures for hypotonic treatment and cold fixation were based on the methods of LeGrande (1981).

The karyotyping process was aided by use of the Optimas® (Bioscan, Inc., Edmonds, Washington) and Kary® (Pro Data, Inc., Oslo, Norway) computer software packages. The chromosomes were sorted by relative size (percent of total complement length or %TCL) and centromeric index (CI), and divided into groups. Size determination was based on the formula: TCL (%) = (length of the chromosome pair/total complement length) x 100. Where TCL is the total length of all chromosomes in the spread. The CI was determined by using the following equation: CI (%) = (short arm length/total chromosome length) x 100. Chromosomes were classified as telocentric (CI = 0-12.5%), subtelocentric (CI = 12.5-25%), submetacentric (CI = 25-37.5%), or metacentric (CI = 37.5-50%), following the method of Levan et al. (1964).

Staining of nucleolar organizing regions (NORs) was based on the method of Howell and Black (1980). Slides were covered with a solution of 30% silver nitrate and 1.5% gelatin and incubated at 50 C for 8 to 10 min. The NOR-bearing chromosomes from five channel catfish x LM catfish, and five channel catfish x blue catfish hybrids were measured, and the CI was determined as previously described. The Student's t-Test was used to determine differences between CI values at the 0.05 level of significance.

DNA Sequencing

Blood samples were collected in ACD solution from two LM catfish, two blue catfish, and one black bullhead catfish. Genomic DNA was extracted from whole blood by using the QIAamp blood and tissue kit (Qiagen Inc., Chatsworth, California). Primers designed to target the channel catfish CH4 gene were synthesized at the LSU Gene Probe and Expression Laboratory (LSU School of Veterinary Medicine). The primer DNA

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sequences were: TCCCCAAGGTTTAC TTGCTCGCTCC (Designated CH4-1) and CGATGGATCTGGATATGTGGCGCAC (CH4-2). These primers were designed to yield a 303 base pair (bp) fragment from channel catfish genomic DNA. The primer set was used for polymerase chain reaction (PCR) analysis of genomic DNA. Each PCR reaction contained 0.2 μM of CH4-1 and CH4-2, 10 μM of each deoxy-nucleotriphosphate (dNTP) (G, A, T, and C), 1.5 mM $MgCl_2$, 1% DMSO, 2.5 units of AmpliTaq DNA polymerase (Roche Molecular Systems, Inc., Branchburg, New Jersey), 1x AmpliTaq buffer (supplied as a 10-x concentrate with AmpliTaq DNA polymerase), 3 μL of sample DNA (template), and sufficient sterile distilled water to bring reaction volume to 100 μL (Table F-3). The reaction conditions were 95 C for 5 min to denature the template DNA, followed by 30 cycles of: 95 C for 30 sec (DNA denaturation step), 52 C for 30 sec (primer annealing step), and 72 C for 1 min (primer extension step) (Table F-4). After PCR, samples were electrophoresed at 8.0 V/cm in a 2% agarose gel for 1.5 h to determine relative size and number of bands amplified by the CH4 primers for each sample.

For DNA sequencing, purity and concentration of DNA in PCR products were estimated using a GeneQuant RNA/DNA calculator (Model 80-2104-98, Pharmacia Biotech, Cambridge, England). The CH4-1 primer was used with the Ready Reaction Kit (Perkin Elmer, Foster City, California) (Table F-4) to prepare the PCR products for sequencing in an ABI Prism 310 Genetic Analyzer (Perkin Elmer, Foster City, California). A 245-bp channel catfish sequence corresponding to base pair 33 to 278 of the 303 bp sequence amplified from channel catfish (LSU population) genomic DNA by the CH4 primers was available to use as a reference sequence. The reference sequence was verified by alignment to the complete sequence of the channel catfish immunoglobulin heavy chain gene described by Wilson et al. (1990). Sequences were aligned and analyzed with the Sequence Navigator® software package (ABI Inc., San

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Diego, California) on a Power Macintosh 6100 computer (Apple Computer, Cupertino, California).

Results

Spawning of Lake Maurepas Catfish

A total of five apparently ripe females (265 ± 62 g, 189 ± 176 TL) were selected from the two harvests (Table 2-1).

Table 2-1. Weight and total length of ripe Lake Maurepas catfish collected outside of the normal spawning season for southern Louisiana.

LM Female	Weight (g)	Total length (mm)	Date injected[a]
1	114	240	27 August, 1995 4 September, 1995
2	503	375	27 August, 1995
3	99	228	27 August, 1995 4 September, 1995
4	111	240	not injected
5	119	243	1 September, 1995

[a] Injected with 50 µg LHRHa/kg of body weight

Female two was paired with a smaller LM male (179 g, 280 mm TL). The pair spawned ~46 h after the female was injected with hormone. Eggs were removed from the tank and placed in an incubation trough. Fertilization from this spawn was 80%. The other two pairs from the first collection trip entered the spawning containers and exhibited spawning behavior, but had not produced eggs 8 d after the first hormone injection. These two females were reinjected with an additional 50 µg LHRHa/kg of body weight.

Female four (Figure 2-1) was not injected because she escaped from the tank and was found dead. Her gonadosomatic index (GSI), (gonad weight/body weight) x 100, was determined as 21%. Female one and her smaller mate (84 g, 230 mm) (Figure 2-2) spawned 6 d after the second hormone injection. Fertilization of this spawn was 75%.

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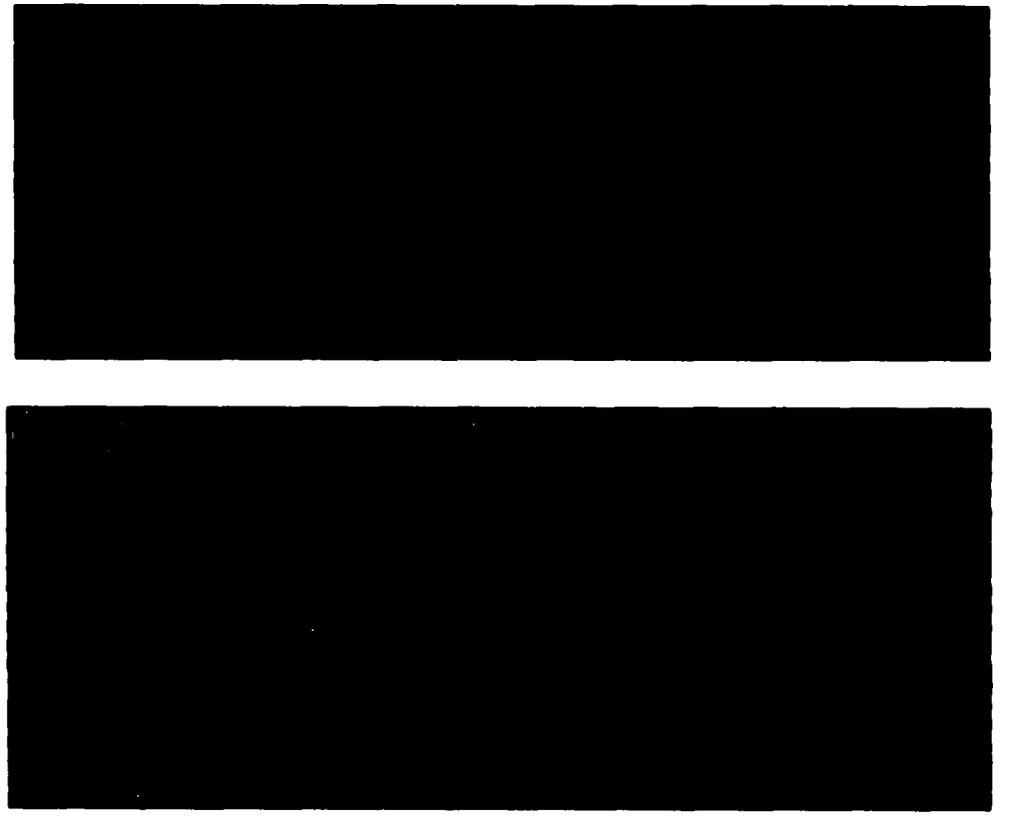

Figure 2-1. a) Lake Maurepas catfish female (111 g, 240 mm total length) with incision in abdomen to show ovaries. b) Ovaries were removed for determination of gonadosomatic index (gonad weight/body weight x 100) which was 21%.

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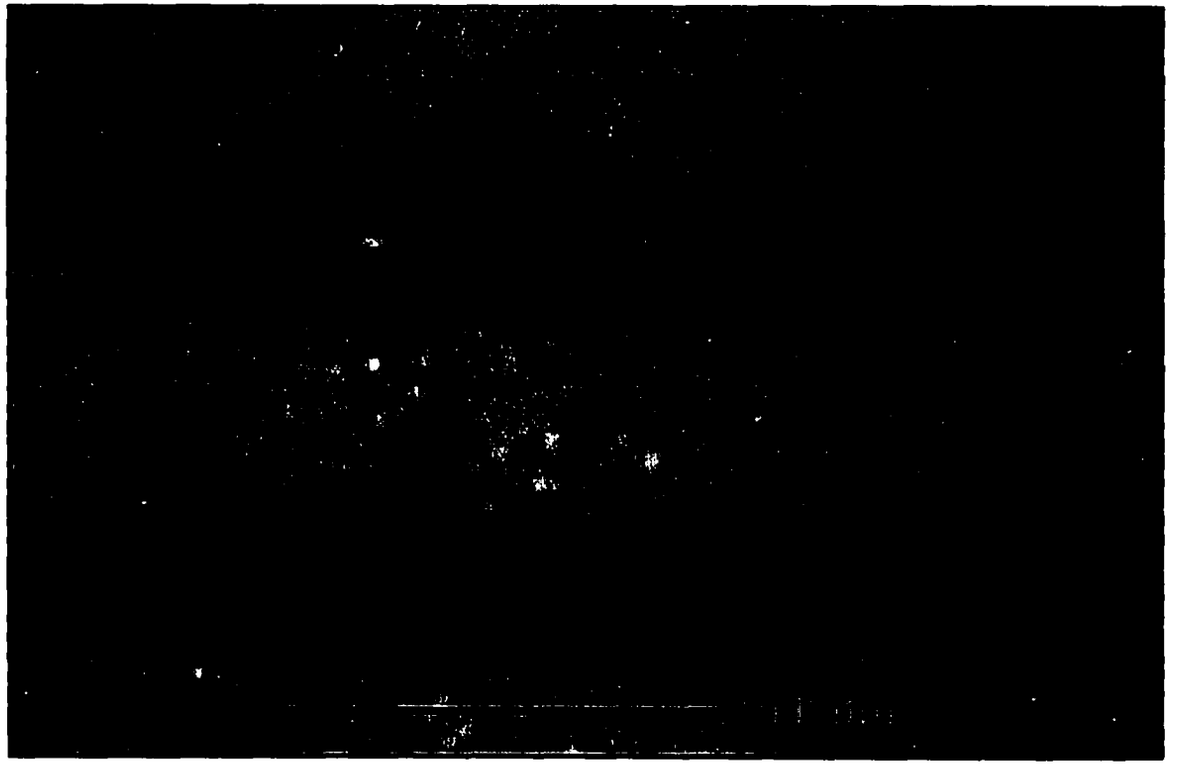

Figure 2-2. Lake Maurepas toy broodfish female (114 g, 240 mm total length, top) and male (84 g, 230 mm) photographed after spawning.

Meristics and Morphology

The meristic and morphometric analysis (Table 2-2) differentiated all species by coloration and caudal fin shape except the blue catfish and channel catfish. The blue catfish can usually be differentiated from channel catfish by three characteristics: 1) absence of spots in blue catfish (note: some mature channel catfish do not have spots); 2) presence of a straight anal fin margin in blue catfish (that of the channel catfish is rounded), and 3) presence of 30 to 36 anal fin rays in blue catfish (with 24 to 31 rays in channel catfish).

Estimation of Genome Size by Flow Cytometry

The grand mean of all values of genome size in this study (N = 61) was 2.11 ± 0.01 (mean ± SD) pg DNA. Genome size was not significantly different ($F_{60} = 3.26$, $P = 0.046$) among the populations tested (Table 2-3). The range between the lowest (2.08 pg) and highest (2.14 pg) values was 0.06 pg, or 2.84% of the mean.

Karyotyping and NOR Staining

The LM catfish x channel catfish karyotype did not differ from the standardized karyotype for channel catfish detailed in Zhang (1996). The LM catfish x channel catfish karyotype was chosen for this study because it offers an intrinsic control for differential chromosome preparation (i.e., varied exposure to reagents) that was not possible with separate karyotypes. As with the standardized channel catfish karyotype, the LM catfish x channel catfish chromosome pairs could be divided into eight groups: A, two large metacentric; B, three large subtelocentric; C, three medium metacentric; D, five medium submetacentric; E, five medium subtelocentric; F, two telocentric; G, five small metacentric, and H, four small submetacentric chromosomes for a total of 29 chromosome pairs (Figure 2-3). Pure LM catfish karyotypes were not different from the LM catfish x channel catfish or the pure channel catfish karyotype, and in every case the NOR-bearing chromosome set was the same (data not shown).

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Table 2-2. Meristic and external morphology table for ictalurid catfishes common to southern Louisiana.

Species	Max. length (cm)	Max. weight (Kg)	Barbels	Coloration dorsal/lateral/ventral	Anal fin rays	Anal fin shape	Adipose fin morphology[1]	Spots	Caudal fin morphology
Channel catfish *Ictalurus punctatus*	120	26.3	4 pairs	blue gray/lt. blue-silver/white	24-31	rounded	free	yes	deeply forked
Blue catfish *Ictalurus furcatus*	110	45.4	4 pairs	blue-slate/lighter/ white	30-36	straight	free	none	deeply forked
Flathead catfish *Pylodictis olivaris*	140	41.4	4 pairs	olive-yellow to light brown/yellow/yellow	14-17	rounded	free (elongated)	none	weakly notched
Black bullhead *Ameirus melas*	43	1.2	4 pairs	olive-black/yellow to black/yellow	17-21	rounded	free	none	slightly notched
Yellow bullhead *Ameirus natalis*	46	1.4	4 pairs	olive brown/yellow-brown/yellow	24-27	rounded	free	none	slightly notched
Black madtom *Noturus funebris*	9	NA	4 pairs	dark gray - black/dark gray - black/whitish	NA	rounded	adnate	none	truncate

[1]Adipose fin morphology: free, adipose fin free and flap-like at its posterior end, completely separated from the caudal fin; adnate, adipose fin continuous with caudal fin.

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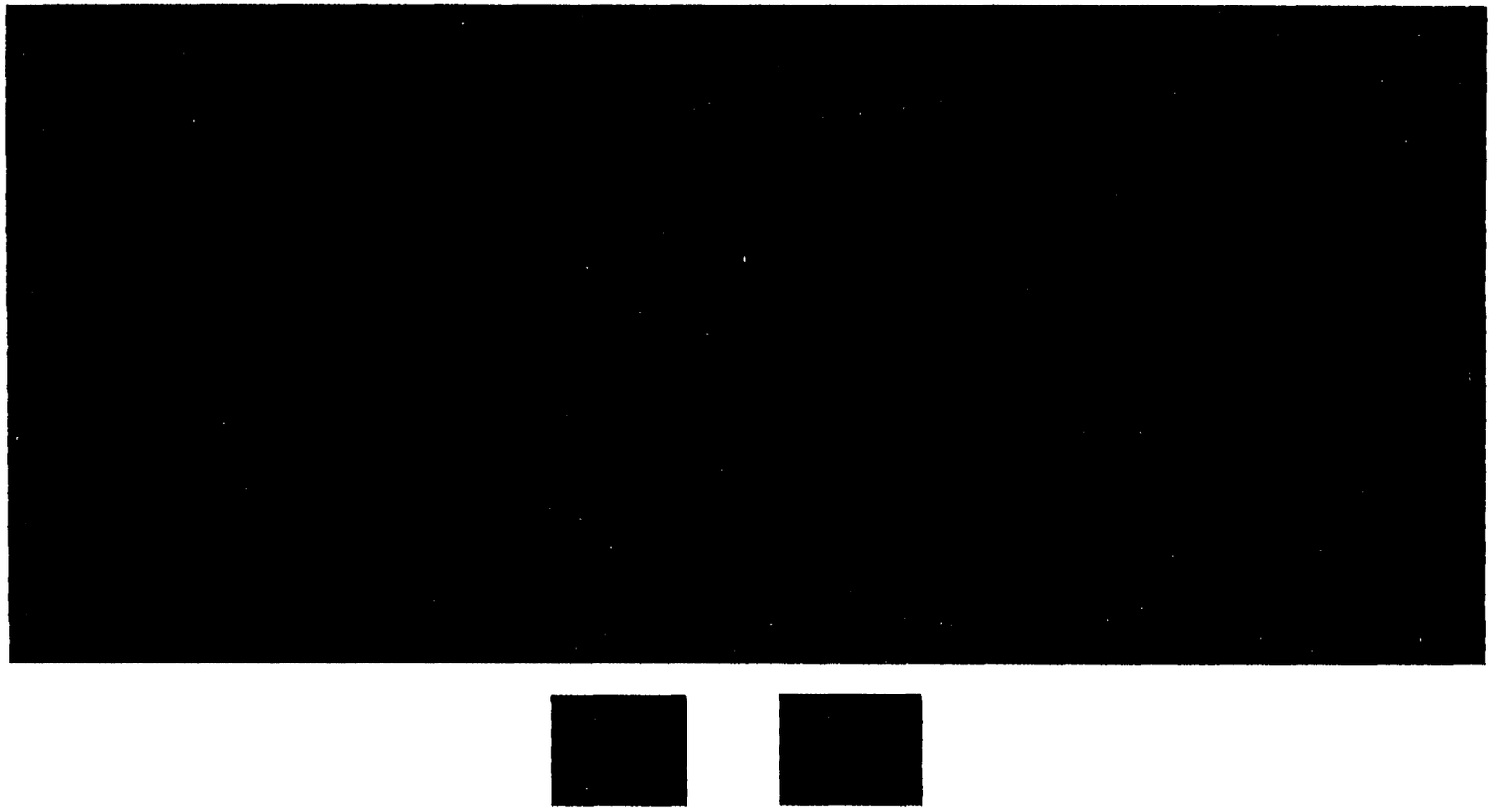

Figure 2-3. Karyotype of channel catfish x Lake Maurepas catfish. The chromosomes were grouped as: A, large metacentric; B, large subtelocentric; C, medium metacentric; D, medium submetacentric; E, medium subtelocentric; F, telocentric; G, small metacentric, and H, small submetacentric. Chromosomes staining positive for nucleolar organizer regions (NOR) from channel catfish x Lake Maurepas catfish (a) and channel catfish x blue catfish hybrid (b).

Table 2-3. Comparison of genome sizes for Kansas strain and Lake Maurepas and Lac Des Allemands populations of channel catfish.

Strain/Population	N	minimum-maximum (range)	Mean ± SD	% variation (range/mean)x100
Kansas	15	2.10-2.12 (0.02)	2.11 ± 0.01	1.14
Lake Maurepas	36	2.09-2.13 (0.04)	2.11 ± 0.01	1.99
Lac Des Allemands	10	2.10-2.14 (0.04)	2.12 ± 0.01	1.98

With the method of chromosome arrangement detailed in Zhang (1996), the NOR-bearing chromosome set is designated as D-11 for the channel catfish, LM catfish and the LM catfish x channel catfish cross. In the channel catfish x blue catfish hybrid, the NOR-bearing chromosomes appeared to stain differentially with one always staining darker than the other. The CI for NOR-bearing chromosomes from channel catfish x blue catfish hybrids (38.3 ± 8.4 %, mean ± SD; n = 10) did not differ (P = 0.42) for NOR-bearing chromosomes from LM catfish x channel catfish (35.7% ± 5.2; n = 10). However, upon further analysis of the channel catfish x blue catfish hybrid NOR-bearing chromosomes it was discovered that all of the darker-stained chromosomes were metacentric and all of the lighter-stained chromosomes were submetacentric. The darker NOR-stained chromosomes had a CI of 45.2 ± 4.5% (n = 5) while the lighter had a CI of 31.4 ± 4.2% (n = 5). There were no apparent morphological differences between the two NOR-bearing chromosomes in the LM catfish x channel catfish karyotypes by which the chromosomes could be classified into separate groups.

DNA Sequencing

A single band of the expected size (303 bp) resulting from PCR with the CH4 primers was observed for each sample (Figure 2-4). Because samples from all species tested had only one band amplified by the CH4 primers, sequencing could be carried out directly on the PCR products. Approximately 270 readable bases were returned from the automated sequencer for each sample, thus alignment with the 245-bp channel catfish reference was possible for all samples. The nucleotide sequences of the two LM catfish sequences were

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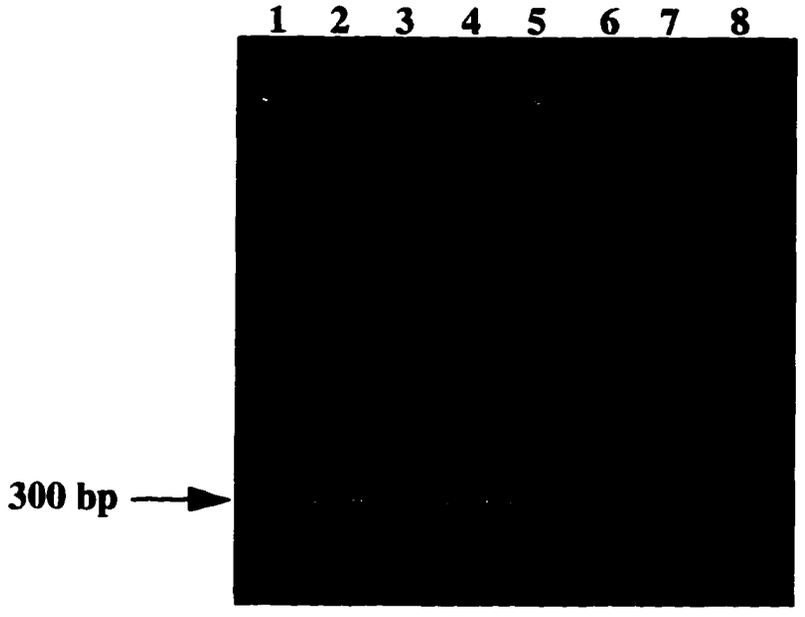

Figure 2-4. Size of fragments amplified by the PCR with CH4 primers. Samples were electrophoresed at 8 v/cm in a 2% agarose gel. Lane 1, 100 base pair DNA marker; lane 2, channel catfish; lane 3, Lake Maurepas catfish #1; lane 4, Lake Maurepas catfish #2; lane 5, blue catfish #1; lane 6, blue catfish #2; lane 7, black bullhead catfish, and lane 8, negative control (no template DNA).

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in 100% consensus with each other, the 245-bp reference sequence for channel catfish, and the published sequence for the gene (Wilson et al. 1990). The sequences for the two blue catfish were in 100% consensus with each other. The black bullhead catfish sequence was different from the channel catfish and blue catfish sequences. While the channel catfish and blue catfish sequences were different only by a single base at two locations (base pair 103 and 204, Table 2-4). A list of restriction enzyme sites was generated for each sequence with the PC Gene software package (Intelligenetics Inc., Mountainview, California).

Table 2-4. Comparison of nucleotide sequence data from three ictalurid species corresponding to base pairs 33 through 278 of a 303 bp fragment of the channel catfish immunoglobulin M heavy chain gene amplified by PCR. The ambiguity sequence highlights base differences among the species with the symbol "*". Where a consensus (majority) could not be reached among the sequences, a "k" was inserted in the consensus sequence.

Sequence	10	20	30	40
1) Channel catfish	GCTCTGGTGA	ATCAGTGACC	CTGACTTGCT	ATGTTAAAGA
2) LM catfish 1	GCTCTGGTGA	ATCAGTGACC	CTGACTTGCT	ATGTTAAAGA
3) LM catfish 2	GCTCTGGTGA	ATCAGTGACC	CTGACTTGCT	ATGTTAAAGA
4) Blue catfish 1	GCTCTGGTGA	ATCAGTGACC	CTGACTTGCT	ATGTTAAAGA
5) Blue catfish 2	GCTCTGGTGA	ATCAGTGACC	CTGACTTGCT	ATGTTAAAGA
6) Black bullhead	GCCCTGGTGA	ATCAGTGACC	CTGACTTGCT	ATGTTAAAGA
7) Ambiguity	--*-------	----------	----------	----------
8) Consensus	GCTCTGGTGA	ATCAGTGACC	CTGACTTGCT	ATGTTAAAGA
	50	60	70	80
1) Channel catfish	CTTCTACCCT	AAGGAGGTGG	CTGTGTCTTG	GCTTGTTAAC
2) LM catfish 1	CTTCTACCCT	AAGGAGGTGG	CTGTGTCTTG	GCTTGTTAAC
3) LM catfish 2	CTTCTACCCT	AAGGAGGTGG	CTGTGTCTTG	GCTTGTTAAC
4) Blue catfish 1	CTTCTACCCT	AAGGAGGTGG	CTGTGTCTTG	GCTTGTTAAC
5) Blue catfish 2	CTTCTACCCT	AAGGAGGTGG	CTGTGTCTTG	GCTTGTTAAC
6) Black bullhead	ATTCTACCCT	AAGGAGGTGG	CTGTGTCTTG	GCTTGTTGAC
7) Ambiguity	*---------	----------	----------	-------*--
8) Consensus	CTTCTACCCT	AAGGAGGTGG	CTGTGTCTTG	GCTTGTTAAC
	90	100	110	120
1) Channel catfish	GATAAACAAG	TGGAAGAAGT	GGTCGGCTAT	GAGCAGAACA
2) LM catfish 1	GATAAACAAG	TGGAAGAAGT	GGTCGGCTAT	GAGCAGAACA
3) LM catfish 2	GATAAACAAG	TGGAAGAAGT	GGTCGGCTAT	GAGCAGAACA
4) Blue catfish 1	GATAAACAAG	TGGAAGAAGT	GGGCGGCTAT	GAGCAGAACA
5) Blue catfish 2	GATAAACAAG	TGGAAGAAGT	GGGCGGCTAT	GAGCAGAACA
6) Black bullhead	GATAAACAAG	TGGAAGAAGT	GGGCGGCTAT	GAGCAGCACA
7) Ambiguity	----------	----------	--*-------	------*---
8) Consensus	GATAAACAAG	TGGAAGAAGT	GGkCGGCTAT	GAGCAGAACA

(Table 2-4 cont'd)

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Sequence	130	140	150	160
1) Channel catfish	CCACTGCAGT	TATCGACAGA	AACAACCTCT	TTTCAGTGTA
2) LM catfish 1	CCACTGCAGT	TATCGACAGA	AACAACCTCT	TTTCAGTGTA
3) LM catfish 2	CCACTGCAGT	TATCGACAGA	AACAACCTCT	TTTCAGTGTA
4) Blue catfish 1	CCACTGCAGT	TATCGACAGA	AACAACCTCT	TTTCAGTGTA
5) Blue catfish 2	CCACTGCAGT	TATCGACAGA	AACAACCTCT	TTTCAGTGTA
6) Black bullhead	CCACTGCGGT	TATCGACAGA	AACAACCTCT	TTTCAGTGTA
7) Ambiguity	-------*--	----------	----------	----------
8) Consensus	CCACTGCAGT	TATCGACAGA	AACAACCTCT	TTTCAGTGTA
	170	180	190	200
1) Channel catfish	CAGCCAGCTG	ATTATCAAAA	CTGCAGACTG	GAACAGTGGC
2) LM catfish 1	CAGCCAGCTG	ATTATCAAAA	CTGCAGACTG	GAACAGTGGC
3) LM catfish 2	CAGCCAGCTG	ATTATCAAAA	CTGCAGACTG	GAACAGTGGC
4) Blue catfish 1	CAGCCAGCTG	ATTATCAAAA	CTGCAGACTG	GAACAGTGGC
5) Blue catfish 2	CAGCCAGCTG	ATTATCAAAA	CTGCAGACTG	GAACAGTGGC
6) Black bullhead	CAGCCAGCTG	ATTATCAAAA	CTACACAATG	GAACAATGGC
7) Ambiguity	----------	----------	--*--*-*--	-----*----
8) Consensus	CAGCCAGCTG	ATTATCAAAA	CTGCAGACTG	GAACAGTGGC
	210	220	230	240
1) Channel catfish	AGTGTGTTCA	GCTGCCTGGT	TTATCATGAG	TCCATCAAGG
2) LM catfish 1	AGTGTGTTCA	GCTGCCTGGT	TTATCATGAG	TCCATCAAGG
3) LM catfish 2	AGTGTGTTCA	GCTGCCTGGT	TTATCATGAG	TCCATCAAGG
4) Blue catfish 1	AGTTTGTTCA	GCTGCCTGGT	TTATCATGAG	TCCATCAAGG
5) Blue catfish 2	AGTTTGTTCA	GCTGCCTGGT	TTATCATGAG	TCCATCAAGG
6) Black bullhead	AGTGTGTTCA	GCTGCCTGGT	TTATCATGAG	TCCATCAAGG
7) Ambiguity	---*------	----------	----------	----------
8) Consensus	AGTGTGTTCA	GCTGCCTGGT	TTATCATGAG	TCCATCAAGG
	245			
1) Channel catfish	ACTGT			
2) LM catfish 1	ACTGT			
3) LM catfish 2	ACTGT			
4) Blue catfish 1	ACTGT			
5) Blue catfish 2	ACTGT			
6) Black bullhead	ACTGT			
7) Ambiguity	-----			
8) Consensus	ACTGT			

Discussion

The early age and small size at sexual maturity of the LM and LDA catfish populations has been previously studied. Zeringue et al. (1988) studied the populations of channel catfish in Lake Maurepas, Lac Des Allemands, and a control population in Flat Lake (Atchafalaya Basin) to determine if the LM and LDA catfish grew slower than channel catfish in other bodies of water due to stunting. Stunted growth in fish is defined

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as individuals or populations that are well below the potential growth rate for a species (Burrough and Kennedy 1979). In addition, stunted fish are sexually mature at normal age, but are short for their age (Woodhead 1978). Stunting can be caused by overcrowding or by competition for forage. Dietary analyses offered no evidence to indicate stunting or overcrowding in any of the three lakes. A study of growth of channel catfish from eight Louisiana lakes found that channel catfish from Lake Maurepas were substantially different from the other populations in terms of growth and length-weight relationships (Zeringue 1989). In a 3-year study, channel catfish from Lake Maurepas and Lac Des Allemands were sampled to determine if age structure, growth rate, and length at maturity indicated stunting in these populations (McElroy et al. 1990). It was concluded that mean total length (TL) at age 2 and beyond compared favorably with commercially fished populations in other areas of the lower Mississippi River drainage and that fish from Lake Maurepas achieved higher percentages of sexual maturity at small size classes than did fish in Lac Des Allemands. The 100% level of sexual maturity was reached by age 2+ fish in Lake Maurepas (280-290 mm TL), one year earlier than in Lac Des Allemands (360-379 mm TL). Spawning at small size and late in the year in comparison to other wild populations was documented during this study by collection of ripe fish from Lake Maurepas in late August and successful spawning of these fish in captivity during the months of August and September.

The spawning of a LM female catfish (119 g, 243 mm TL) and LM male catfish (September 1995) resulted in production of viable fry and demonstrates that these fish can be collected and induced to spawn in captivity at a small size. The small size at sexual maturity and late spawning is of use for genetic research because commercial strains of channel catfish have a generation time of 3-to-4 years and mature at ~1.5 kg and > 500 mm TL (Busch 1985). Artificial spawning of large fish is made difficult and expensive by the size requirements for even a basic hatchery. Use of the smaller LM channel catfish would allow researchers to gather more basic data in the same space.

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Channel catfish were distinguished easily from all other large ictalurids in Lake Maurepas and Lac Des Allemands, except possibly the blue catfish, with external morphological characteristics and meristic data. Meristics may be useful in identifying some ictalurid hybrids such as the intergeneric hybrid channel catfish x black bullhead catfish which has meristic characteristics intermediate to those of the parents (Goudie et al. 1993). However, meristics may fail to identify some ictalurid hybrids such as the interspecific hybrid channel catfish x blue catfish and its reciprocal cross because paternal dominance for some characteristics (external appearance, anal fin shape, and anal-fin rays) has been documented in these hybrids (Dunham et al. 1982).

The genome size determined for LM channel catfish in this study agrees closely with that reported previously by Tiersch et al. (1990) for populations of channel catfish. Tiersch and Goudie (1993) studied other ictalurids and reported genome sizes for: blue catfish, flathead catfish Pylodictus olivaris, and black bullhead catfish. In the same study they reported genome sizes for: channel x blue catfish, channel x black bullhead catfish, and channel catfish x flathead catfish hybrids. The genome sizes for all hybrids were exactly intermediate to those of the parental species. Genome size, therefore, can be predicted for ictalurid hybrids by dividing the sum of the genome values for the parental species by two.

Channel catfish, blue catfish, and channel catfish x blue catfish hybrids all possess 58 chromosomes, and their karyotypes are indistinguishable from one another (LeGrande et al. 1984). The black bullhead catfish has 60 chromosomes and the channel catfish x black bullhead catfish hybrid has 59 chromosomes and thus would be identified by chromosome number alone (Zhang and Tiersch in review). The location of the NOR-bearing chromosome pair for blue catfish is unpublished. The data presented in this study show that the NOR-bearing chromosomes of the channel catfish x blue catfish hybrid are of different types (submetacentric and metacentric). Because the NOR-bearing chromosomes of channel catfish are known to be submetacentric (Figure 2-3), it can be

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deduced that the NOR-bearing chromosomes from the blue catfish belong to a different chromosome pair. Therefore, NOR-staining would allow identification of either parental strain or their hybrid because the hybrid would inherit one NOR-bearing chromosome from one parent and one from the other.

The CH4 sequence data for the LM catfish and channel catfish were the same. The base pair difference (bp 103) between channel catfish and blue catfish (Table 2-4) would allow differentiation of the two species by restriction enzyme digest. A restriction enzyme recognizes and cuts DNA at a specific nucleotide sequence. The restriction enzyme Fnu4HI (New England Biolabs, Boston, Massachusetts), recognizes the five nucleotide sequence or site GCNGC (where N is any nucleotide: A, G, T, or C) and cuts after the first C. A site recognized and cut by Fnu4HI is present once in the channel catfish CH4 sequence (GCTGC, bp 211 to 215), while such a site occurs twice in the blue catfish sequence (GCGGC, bp 102 to 106; GCTGC, bp 211 to 215) and three times in the black bullhead catfish sequence (GCGGC, bp 102 to 106; GCAGC, bp 112 to 116, and GCTGC, bp 211 to 215). Successful digestion of the CH4 fragment with this enzyme would yield two fragments from the channel catfish, three fragments from the blue catfish and four fragments from the black bullhead CH4 sequence. This method could also be useful for identification of hybrids because a restriction digest of PCR products amplified from genomic DNA extracted from an ictalurid hybrid would yield a mixture of fragments representative of both parents.

This study presents a synthesis of morphologic, cytogenetic, molecular, and biological evidence that the population of catfish in Lake Maurepas is a normal population of channel catfish in all respects other than maturing at an early age and small size, and spawning over a broader time period when compared to other populations of channel catfish in southern Louisiana. The Lake Maurepas channel catfish thus extends the time that spawning experiments can be carried out. After the domestic (LSU) population has completed spawning for the year, around late June to early July, ripe Lake Maurepas

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broodstock can be collected and spawned. Supplementing broodstock with fish from Lake Maurepas extends spawning of catfish to 5 months rather than 5 to 6 weeks with our domesticated population. Unlike genetic experiments with an unrelated model fish, techniques developed using LM channel catfish will be more applicable to domestic channel catfish strains. Small size and early maturity are the greatest reasons to use LM channel catfish as a genetic model for channel catfish. However, these traits are not commercially desireable. Therefore, techniques developed with the LM channel catfish would have to be re-applied to commercial domesticated populations. One option would be to produce crossbreeds between LM channel catfish and a fast-growing strain of channel catfish such as the Kansas strain. The Kansas strain grows faster than other domesticated strains of channel catfish, but requires 5-to-6 years to mature. The product of an LM channel catfish x Kansas channel catfish cross might be a fast-growing fish that matures at a reasonable time.

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CHAPTER 3
Artificial Spawning of Channel Catfish Ictalurus punctatus as Male-Female Pairs or All-Female Groups in a Recirculating System.

Introduction

Channel catfish Ictalurus punctatus have been cultured for more than 70 years (Huner and Dupree 1984) for food production and recreational purposes. There are three recognized methods for spawning channel catfish: 1) natural (pond) spawning, 2) pen spawning, and 3) aquarium or artificial spawning (Busch 1985). The channel catfish industry relies exclusively on pond spawning to supply fry and fingerlings for grow-out. Farmers provide containers (e.g.; milk cans, wooden boxes, metal drums) in ponds where spawning is desired (Steeby 1987). The containers simulate natural sites required by channel catfish for spawning (Clapp 1929). As in natural spawning, the male chooses a spawning site, attracts a female, and the pair spawns until completion of egg laying by the female. The eggs form a mass held together by a proteinaceous matrix (Ringle et al. 1992). The male guards the egg mass by driving other fish away, including the female, once spawning is completed.

Pen spawning involves wire mesh enclosures in ponds. The enclosures are often 120 cm x 180 cm minimum, with sides extending $\geq$ 30 cm above water level. The pens allow pairing and control of timing of spawning (Huner and Dupree 1984). A brood pair is selected, and the female can be injected with hormone to induce ovulation. The female is removed from the pen after spawning, and egg masses can be collected and placed in a hatchery or left with the male to hatch in the pond (Huner and Dupree 1984).

Aquarium spawning allows control over pairing and time of spawning, and allows collection of unfertilized eggs from females. This offers benefits including crosses with more than one male, production of hybrids, and manipulation of ploidy. The aquarium method is currently used only in research because it is time-consuming, complicated, and relatively expensive. Channel catfish have been spawned in aquaria as small as

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38-L; however, 120 to 200-L aquaria or tanks are recommended (Tucker and Robinson 1990). In this method, a brood pair is selected and the female is injected with a suitable hormone to induce ovulation. Occasionally male channel catfish are also injected to induce or increase spermiation. The pair is allowed to begin spawning in the tank before eggs are hand-stripped and artificially fertilized. Because sperm cannot be stripped from male channel catfish (Bart and Dunham 1990) testes are removed surgically and crushed in buffer (about 10 to 20 mL/g of testis) to produce a sperm suspension (Tiersch et al. 1994). The buffer solution must have an osmotic pressure of more than 275 mosmol/kg to ensure that sperm are not activated prior to fertilization (Bates et al. 1996).

Production of polyploid and hybrid fish requires collection of unfertilized eggs. In addition, unfertilized eggs are desirable for production of transgenic channel catfish because they are not cohesive until after fertiliztion, easing handling and counting. The greatest disadvantages of the aquarium spawning method are that it is difficult to predict when fish will spawn or how many injections will be required, necessitating constant monitoring of broodfish. Another method of artificial spawning, hormonal induction of grouped (unpaired) females, has been used in fishes (Piper et al. 1983) including other species of catfish. In this method, several females are injected with hormone and grouped in a common tank until ovulation occurs. The Indian catfish Heteropneustes fossilis has been spawned using this method within 14 to 18 h after injection of salmon gonadotropin-releasing hormone analog (Gn-RHa; 25 µg/Kg of body weight) (Alok et al. 1993). The Asian catfish Clarias macrocephalus has also been spawned in groups by hormonal induction, with eggs remaining viable for as long as 10 h after ovulation when hand-stripped (Mollah and Tan 1983). Although previously studied (R. Dunham, personal communication 1996), there are no published reports of this technique with channel catfish.

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Most accounts of induced spawning of channel catfish report as an endpoint the percentage of fish that produce egg masses (spawning success) (Busch and Steeby 1990). However, production of an egg mass by itself does not mean that the eggs are viable. Percent fertilization was used as an indicator of egg quality in this study.

Genetic improvement of channel catfish and commercial production of catfish hybrids would be aided by a hormonal induction technique that could reliably trigger synchronized ovulation of high-quality eggs from multiple females (Nwadukwe 1995). In this study, the aquarium method was used during the 1994 and 1995 spawning seasons to produce eggs for genetic experiments. In 1996, approximately half of our female brood fish were spawned by the aquarium method and half were spawned by grouping of unpaired females. Our objectives were to compare: 1) spawning success (percent of females producing eggs); 2) latency (time between hormone injection and spawning or stripping of eggs); and 3) percent fertilization for the 3 years of the study and between paired and grouped experiments from the 1996 season. In addition to the main objectives, percent fertilization data were plotted against time and temperature to make inferences about spawning success at different stages (early, middle, late) of the spawning season. Problems encountered with spawning fish in recirculating systems were identified and discussed.

Materials and Methods

Fish Collection and Hormone Injection

Pond temperature was measured 4 to 5 days per week from a reference 0.1 hectare earthen pond located at the LSU Ben Hur Aquaculture Research Laboratory (ARL). On days that pond temperature was not measured, temperature was estimated from ambient air temperature. The estimates were based on comparison of daily temperature data taken at the Ben Hur farm and supplied by the Southern Regional Climate Center (Louisiana State University, Baton Rouge, Louisiana). Comparison revealed that pond temperatures for the 1995 spawning season (mid-May to mid-June) were always 2 to 6

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degrees (average of 4.5 C) lower than the daily high temperature. Therefore, a correction factor of 4.5 C was subtracted from the daily high temperature for days when pond temperature was not measured. In spring, when pond temperatures stablized above 21 C for at least 3 d, mature ($\geq$ 3 yr old) broodfish were collected. Males were selected based on secondary sexual characteristics (Tucker and Robinson 1990), and females were selected if they possessed a soft distended abdomen and red swollen urogenital area. Broodfish were moved to the hatchery in a hauling tank within 30 min of capture. Fish were segregated by sex and placed in a recirculating system maintained at a temperature of 23 to 28 C for temporary holding. All fish were anesthetized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington) weighed, and measured before pairing or grouping in tanks for spawning. Females were injected intramuscularly (inferior to the dorsal fin) with synthetic luteinizing-hormone releasing-hormone (LH-RHa, Peninsula Laboratories, Belmont, California) at a dosage of 50 or 100 µg/kg of body weight (Busch and Steeby 1990). Males were not injected in this study. Broodfish of similar size were paired to limit injury from aggressive spawning behavior.

Spawning Systems

Paired spawning trials were conducted in a system consisting of eight, rectangular, 120-L fiberglass tanks. Each tank was constructed with a plexiglass viewing window to allow monitoring of broodfish for spawning behavior and egg release. The system was equipped with a 0.30-m^3 upwelling biofilter (Armant Aquaculture Inc., Vacherie, Louisiana) for function as a recirculating system. The system could be operated as a flow through system with dechlorinated Baton Rouge city water when water quality variables were outside of the desired range (described below).

Grouped female trials took place in a system consisting of four, round, fiberglass tanks (three 1,000-L and one 2,500-L), equipped with a 0.60-m^3 upwelling biofilter (Water Garden Gems Inc., Marion, Texas). Females were grouped in one of the three

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1,000-L tanks after injection. Desired water quality parameters were: $\leq$ 0.5 ppm ammonia nitrogen (NH_3-N), $\leq$ 0.5 ppm nitrite nitrogen (NO_2-N), 100 to 400 ppm alkalinity as $CaCO_3$, 50 to 100 ppm hardness as $CaCO_3$, and 7.5 to 8.0 pH. Water quality was monitored daily with a freshwater test kit (Model No. FF-1, Hach Co., Loveland, Colorado).

Sperm Storage Solutions

Mature channel catfish males were killed by overdose with MS-222, and testes were removed surgically. Testes were dissociated to yield a homogenate of tissue and sperm cells which was filtered through a 100-μ tissue sieve. The sperm were suspended in Hanks' balanced salt solution (HBSS) at a ratio of 20 ml of HBSS for each g of testis (Christensen and Tiersch 1996). Solutions were stored at 4 C, and sperm quality was evaluated by motility estimates: 2 μL of sperm suspension were diluted with 20 μL of de-ionized water to activate the sperm cells (Bates et al. 1996). Percent motility was estimated using darkfield microscopy (200-x magnification) and suspensions with motility $\geq$ 25% (typically >75%) were retained for use in artificial fertilization. Sperm suspensions were monitored daily and were discarded when estimated motility was < 25%, or when bacterial contamination was evident (Jenkins and Tiersch, in press).

Hand-stripping of Eggs

When a female was spawning readily (frequent release of eggs), it was removed from the tank, anesthetized with MS-222, and dried thoroughly with paper towels to ensure that eggs would not come in contact with water. The female was cradled in one arm while sufficient pressure was applied to the abdominal area to strip eggs (Tiersch et al. 1994).

Grouped females were checked for ovulation by netting and applying gentle pressure to the abdominal and urogenital region; if eggs were released, the female was removed for stripping. Eggs were stripped into food-grade plastic bowls that contained HBSS and were coated with silicone grease (Dow Corning, Midland, Michigan) to prevent

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eggs from adhering to the bowl (Goudie et al. 1992). When stripping was complete, blood clots (if present) were removed by pipetting and the eggs were poured into a graduated conical flask to measure volume.

Artificial Fertilization

Eggs were divided into ~10-mL aliquots (200 to 300 eggs) for fertilization. The eggs were placed into 400-mL plastic beakers (Tri-pour, Oxford Labware, St. Louis, Missouri) coated with silicone grease, and excess HBSS was decanted. For fertilization, 0.5 mL of sperm suspension and 50 mL of tank water were added to activate the gametes and the beaker was swirled to facilitate mixing. An additional 75 mL of water was added to the beaker after 2 to 3 min to facilitate water hardening and adhesion of eggs. After fertilization, the eggs formed gelatinous masses which were transferred to screened containers and placed in a hatching trough for incubation. Females were considered successful spawners if they produced eggs that appeared normal in color (yellow) and size (about 5 mm) (Markmann and Doroshov 1983) and formed a single cohesive mass following fertilization. Development to the neurulation stage (~24h at 28 C) was used as the criteria for fertilization success because unfertilized eggs can develop to the gastrula stage (Withler 1980).

1994 Spawning Season

During the 1994 spawning season (May 16 to June 18) mature broodfish were selected from earthen ponds at the LSU ARL. Males used in 1994 weighed 2.8 ± 1.1 kg (mean ± SD) and females weighed 2.9 ± 1.0 kg (n = 36 for each sex). Females were injected with 100 µg LH-RHa/kg of body weight at the time of pairing.

1995 Spawning Season

During the 1995 spawning season (May 12 to June 17) two groups of channel catfish were spawned: LSU channel catfish and wild channel catfish collected from Lake Maurepas (Manchac, Louisiana) that were held in earthen ponds at the ARL for 1 to 2 yr. Also, an attempt was made to spawn wild Lake Maurepas (LM) channel catfish.

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Forty-four pairs of LSU channel catfish were collected for spawning; the males weighed 2.7 ± 0.8 kg and the females weighed 2.4 ± 0.9 kg. Six pairs of pond-held LM channel catfish were used for spawning; the males weighed 1.0 ± 0.3 kg and the females weighed 0.8 ± 0.4 kg. Four pairs of newly captured LM channel catfish were used for spawning; the males weighed 0.1 ± 0.1 kg and the females weighed 0.2 ± 0.2 kg. All females were injected with a priming dose of 100 µg LH-RHa/kg body weight at the time of pairing, followed by a resolving dose of 50 µg LH-RHa/kg body weight 24 h later.

1996 Spawning Season

During the 1996 spawning season (April 17 to July 15) 27 sets of LSU channel catfish were paired for spawning; males weighed 2.3 ± 0.6 kg (n = 25) and females weighed 2.4 ± 0.5 kg (n = 27, two males were mated twice). Females were injected with a single dose of 100 µg LH-RHa/kg of body weight at the time of pairing. Females used in grouped spawning experiments weighed 2.6 ± 1.1 kg (n = 26). These females were injected with a single dose of 100 µg LHRHa/kg of body weight and placed in tanks in groups of two to seven.

Statistical Analysis

Spawning success data for paired spawning in 1994, 1995 and 1996 were tested by chi-square analysis using a 3 x 2 contingency table, and differences between the paired and grouped data (1996 only) were tested using a 2 x 2 contingency table. For paired females that were hand-stripped, latency (time in h between hormone injection and ovulation) and percent fertilization were analyzed with a multivariate analysis of variance (MANOVA) with year as the factor and latency and percent fertilization as dependent variables. The Fisher's protected least significant difference (LSD) test was used for means separation. For 1996 paired and grouped females that were hand-stripped, latency and percent fertilization were compared with a t-Test. Percent fertilization values were arcsine-square-root-transformed for analysis. The level of

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statistical significance was set at $P < 0.05$ for all analyses. Analyses were performed with the Data Desk satistical analysis program (version 4.2, Data Description, Inc. Ithaca, New York).

Results

1994 Spawning Season

Of the 36 pairs, 13 (36%) successfully spawned. Three pairs spawned completely in their spawning tanks, and these females were not available for stripping. The latency period (mean ± SD) for the females that were hand-stripped was 113 ± 69 h (minimum = 36 h; maximum = 200 h; n = 10). Fertilization of hand-stripped eggs was attempted within 108 min after stripping (mean ± SD = 66 ± 24 min; minimum = 42). Fertilization percentages were 16 ± 26 (minimum = 0; maximum = 80; n = 10). Pond water temperature dropped below 21 C, the minimum temperature for final gonadal maturation, several times during the spawning season (Figure 3-1a). Water temperature in the hatchery spawning system ranged from 23 to 28 C. Water quality was maintained within desired parameters throughout the season.

1995 Spawning Season

During the spawning season, we experienced high mortality of female broodfish. The proportion of females that died (24/44) was significantly higher (P = 0.0001) than the number of males that died (4/44) as determined by chi-square analysis with a 2x2 contingency table. The cause of mortality appeared to be related to water quality problems. The spawning system biofilter was not functioning adequately during the spawning season which resulted in periodic instances of high ammonia (1 to 5 ppm) and nitrite (1 to 3 ppm) in the system. This required switching the system to flow through (dechlorinated Baton Rouge city water) several times each day for about three weeks. Because Baton Rouge city water has a low hardness (~16 ppm hardness as $CaCO_3$) tanks were equipped with a drip system to deliver $CaCl_2$ to increase hardness and NaCl to reduce stress. Although formal necropsies were not performed on dead fish, ovaries

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were examined. Females that died were close to ovulation or had ovulated (eggs were 4 to 5 mm in diameter and yellow in color) before dying . Females that died were eliminated from statistical analysis. Of the 20 remaining pairs of LSU channel catfish, seven (35%) spawned successfully. Of the six pairs of pond-held LM channel catfish, three (50%) spawned successfully. Two of these pairs spawned completely in the tanks and the females were not stripped. Of the four pairs of LM channel catfish captured during spawning season, two (50%) spawned successfully. These pairs spawned completely in the tanks and females were not stripped. The average latency period for the stripped females was 109 ± 57 h (minimum = 28; maximum = 162 h; n = 8). Fertilization of hand-stripped eggs was attempted within 108 min after stripping (mean ± SD = 66 ± 24 min; minimum = 42). Fertilization rates for the artificially fertilized eggs were 72 ± 26% (minimum = 20%; maximum = 100%; n = 8). Pond water temperature remained above 21 C during the spawning season (Figure 3-1b). Water temperature in the hatchery spawning system ranged from 26 to 30 C.

1996 Spawning Season

Of the 27 pairs of LSU channel catfish, 11 (41%) spawned successfully. The average latency period for paired females was 44 ± 8 h (minimum = 32h; maximum = 51 h). Fertilization of hand-stripped eggs was attempted within 90 min after stripping (mean ± SD = 40 ± 19 min; minimum = 18). Fertilization rates for eggs from paired females were 43 ± 37% (minimum = 0; maximum = 95%).

Of the 26 grouped (unpaired) females, 15 (58%) were successfully stripped of eggs. The latency period for grouped females was 50 ± 9 h (minimum = 38 h; maximum = 70 h). Fertilization of hand-stripped eggs was attempted within 66 min after stripping (mean ± SD = 38 ± 17 min; minimum = 18 min). Fertilization rates were 16 ± 20% (minimum = 0%; maximum = 60%). Pond water temperature remained above 21 C throughout the spawning season (Figure 3-1c and d).

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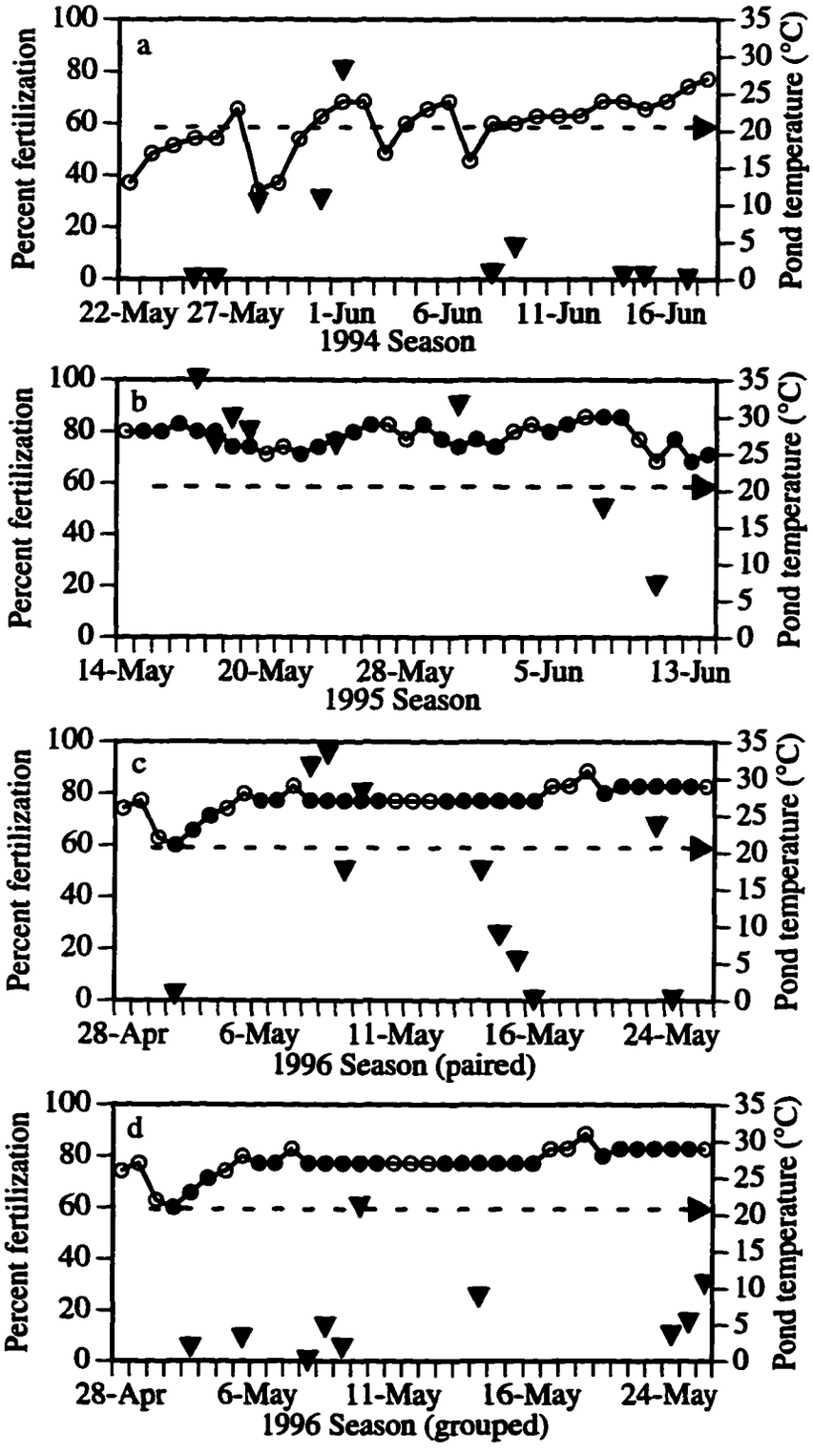

Figure 3-1. Percent fertilization of channel catfish eggs (triangles) and average pond temperature (solid line) during the spawning seasons of 1994 (a), 1995 (b), paired 1996 (c), and grouped 1996 (d). Closed circles represent actual pond temperatures; open circles represent estimated pond temperatures based on air temperatures. The dashed reference line represents the minimum temperature (21 C) for final gonadal maturation and spawning of channel catfish.

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Water temperature in the hatchery spawning system ranged from 26 to 33 C. Water quality was maintained within desired parameters throughout the season.

Statistical Analysis

Analysis by chi-square did not detect differences ($P = 0.17$) in the proportion of successful spawns in the three paired (male-female pairs) data sets (1994, 1995, and 1996) (Table 3-1).

Table 3-1. Spawning success of channel catfish females spawned in male-female pairs over a 3-year period.

Result	1994[a]	1995[a]	1996 paired[a]	Total
Did not spawn	23	18	16	57
Spawned	13	12	11	36
Total	36	30	27	93

[a] No significant diffences ($P = 0.17$) were detected in the proportion of spawns by chi-square analysis in a 3 x 2 contingency table.

The proportion of successful spawns in the paired 1996 data set was not significantly different ($P = 0.64$) from that of the grouped 1996 data set (Table 3-2).

Table 3-2. Comparison of spawning success of 1996 channel catfish females spawned in male-female pairs or in all-female groups.

Result	1996 Paired[a]	1996 Grouped[a]	Total
Did not spawn	16	11	27
Spawned	11	15	26
Total	27	26	53

[a] No significant difference was detected in the proportion of spawns as detected by chi-square analysis in a 2 x 2 contingency table.

The latency period for fish spawned in 1994 was not significantly different ($P = 0.88$) from that of fish spawned in 1995, however, the latency period for 1996 females was significantly shorter than those of 1994 ($P = 0.004$) and 1995 ($P = 0.01$). The latency

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period for grouped 1996 females was not significantly different ($P = 0.10$) from that of paired 1996 females (Table 3-3). Percent fertilization for eggs stripped in 1994 was significantly lower than that of eggs stripped in 1995 ($P = 0.0005$), but not significantly different than that of eggs stripped from 1996 females ($P = 0.05$). Percent fertilization was not significantly different ($P = 0.05$) for eggs stripped from 1995 and 1996 paired females. Percent fertilization was significantly different ($P = 0.02$) for eggs stripped from paired 1996 and grouped 1996 females (Table 3-3).

Table 3-3. Summary for females spawned during the 1994, 1995, and 1996 spawning seasons. Number injected is the total number of females injected with synthetic luteinizing-hormone releasing-hormone (LHRHa). Number spawned is the number of fish that produced eggs. Number stripped is the number of fish that were stripped by hand. Latency is the time in hours between injection with LHRHa and observed release of eggs. Percent fertilization is the percent of eggs that reached the neurulation stage.

Year	Number injected	Number spawned	Number stripped	Latency (h) (mean ± SD)[1]	Percent fertilization (mean ± SD)[1]
1994 paired	36	13	10	113 ± 69^{a}	16 ± 26^{a}
1995 paired	30	12	8	109 ± 57^{a}	72 ± 25^{b}
1996 paired[2]	27	11	11	$^{x}44 \pm 8^{b}$	$^{x}43 \pm 20^{b}$
1996 grouped[2]	26	15	15	$^{x}50 \pm 9$	$^{y}16 \pm 37$
Total	143	51	44	---	---

[1]Means for paired spawns in a column followed by a common lowercase superscript letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference (LSD) means separation test.

[2]1996 means preceded by a common superscript lowercase letter were not significantly different ($P > 0.05$) as determined by Student's t-Test.

Discussion

The variation in spawning success, latency and percent fertilization that we observed is probably due to female variation in reproductive readiness, differential response to the hormone used, stage of spawning season (early, middle, late) when the fish were injected, and possible water quality problems. Water quality was an issue particularly

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during the 1995 spawning season. Ammonia and nitrite in the spawning system accumulated to levels that were sublethal to the males but were apparently lethal to the ovulating females resulting in a selective loss of the females most likely to provide eggs. In flow through systems, clean water is added to the culture system at a constant rate. Waste build up is not a factor because it is discharged continuously from the system. The process of ovulation combined with high nitrite levels may have resulted in brown blood disease or methemoglobinemia (Bowser et al. 1983; Wise et al. 1988). Possible reasons for the poor water quality experienced during the 1995 season were: (1) Insufficient time allowed for the filter to be colonized by nitrifying bacteria prior to stocking with broodfish; (2) change in temperature from spring to summer could have impacted bacteria in the biofilter; (3) adding large load of fish at one time (~48 kg when stocked), and (4) regurgitation of stomach contents upon stocking into spawning system. To reduce water quality problems in recirculating spawning systems we recommend: (1) maintain a biological load on the filter year-round; (2) maintain a constant temperature in the hatchery; (3) gradually increase biomass before stocking, and (4) purge fish prior to stocking.

Nutritional state and health of individual females also affects reproductive performance, egg quality, and larval survival. Burton (1994) found in winter flounder Pleuronectes americanus that an increase in non-reproductive females resulted from reduced rations during any part of the first half of the normal 6-month feeding cycle. When channel catfish are produced using the natural or pond method, only those fish physiologically prepared to spawn will enter spawning containers (Huner and Dupree 1984). However, in artificial spawning, fish are selected if they appear ready to spawn. Because broodfish are commonly returned to ponds after spawning in the hatchery and are not seen again until the following spawning season, their health and nutritional state can go unmonitored. Therefore, it is possible that some broodfish have not fed well during the year or have been diseased during important parts of the feeding cycle.

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Accordingly, broodstock will be at different states of reproductive readiness when harvested.

Another important factor in reproductive readiness of channel catfish is temperature. Water temperatures must remain at or above 21 C long enough for females to undergo final gonadal maturation. If water temperature drops below 21 C or rises much above 30 C, egg quality will be reduced (Tucker and Robinson 1990) and some channel catfish may not spawn (Huner and Dupree 1984). Although not tested statistically, fish collected more than 3 weeks after pond temperatures stabilized above 21 C seemed to be more likely to produce eggs of low quality. Kelly and Kohler (1996) were able to spawn small numbers of channel catfish out of the normal season by holding them at cool temperatures (~17 C). A modification of this technique might be used to spawn broodfish in groups over an extended period.

A variety of hormones have been used to induce final maturation and ovulation of eggs in channel catfish. Pituitary glands of common carp Cyprinus carpio or, more commonly, carp pituitary extract (CPE) are available in powdered form and are delivered in a dosage of 4.5 mg of pituitary/kg of body weight at 24-h intervals for up to 10 d (usually requiring three to four injections). Human chorionic gonadotropin (HCG) can be used at a dosage of 1760 IU/kg of body weight in one or more injections administered until ovulation occurs (Busch and Steeby 1990). LH-RHa and Gn-RHa have gained popularity as reliable means to induce ovulation in teleost fishes. Commonly, one or two injections of 50 to 100 μg LH-RHa/Kg of body weight are required for channel catfish (Busch and Steeby 1990). Hormone induced ovulation followed by stripping of eggs is widely used in many species of finfish. However, uncertainty surrounds the proper protocol to reliably induce ovulation in channel catfish (Busch and Steeby 1990), and to properly time the stripping of eggs to prevent under-ripeness or over-ripeness (Dunham 1993).

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Some species of teleost fishes have a long period of time between ovulation and over-ripening of eggs. Ovulated eggs retained in vivo in rainbow trout Oncorhynchus mykiss reared at temperatures of 10 C or less, remained viable for 1 to 2 weeks (Sakai et al. 1975). Fertilizability of eggs of the Asian catfish Clarius macrocephalus was not significantly affected until 12 h post ovulation (Mollah and Tan 1983). However, in cyprinids, the time between ovulation and over-ripening at a rearing temperature of 20 C can be as short as 30 min (Horvath 1978), and in striped bass Morone saxatilis, eggs can be unfertilizable in as little as 15 min post-ovulation (Rottman et al. 1991). The time between ovulation and over-ripening of eggs of channel catfish is undocumented. However, Dunham (1993) reported multiple hand-strippings of eggs over a 10-h period from a single channel catfish female induced to ovulate with CPE. Although 10 h elapsed from the first stripping to last, fertilization rates of 90 to 100% were reported.

Egg quality may vary throughout the spawning season. During the 3 years of this study, channel catfish (LSU population) were spawned as early as 1 May and as late as 17 June. However, the period of greatest likelihood of inducing ovulation and stripping eggs of high quality was a 4-week period from early May to early June. Ninety three percent of paired strip-spawns obtained in this period exhibited percent fertilization greater than or equal to 50%. In addition, 68% of the grouped female spawns occurred within this same period (Figure 3-2). Therefore, to make best use of broodstock and time, effort should be concentrated in the early part of the spawning season (May in southern Louisiana). Successful production of desired numbers of fish early in the season would avoid problems associated with the warmer temperatures of late season (e.g. fungus).

Artificial spawning of grouped channel catfish females without pairing with males appears to be a viable technique if further research can optimize hormone dose and the timing required to consistently strip high-quality unfertilized eggs. If the channel

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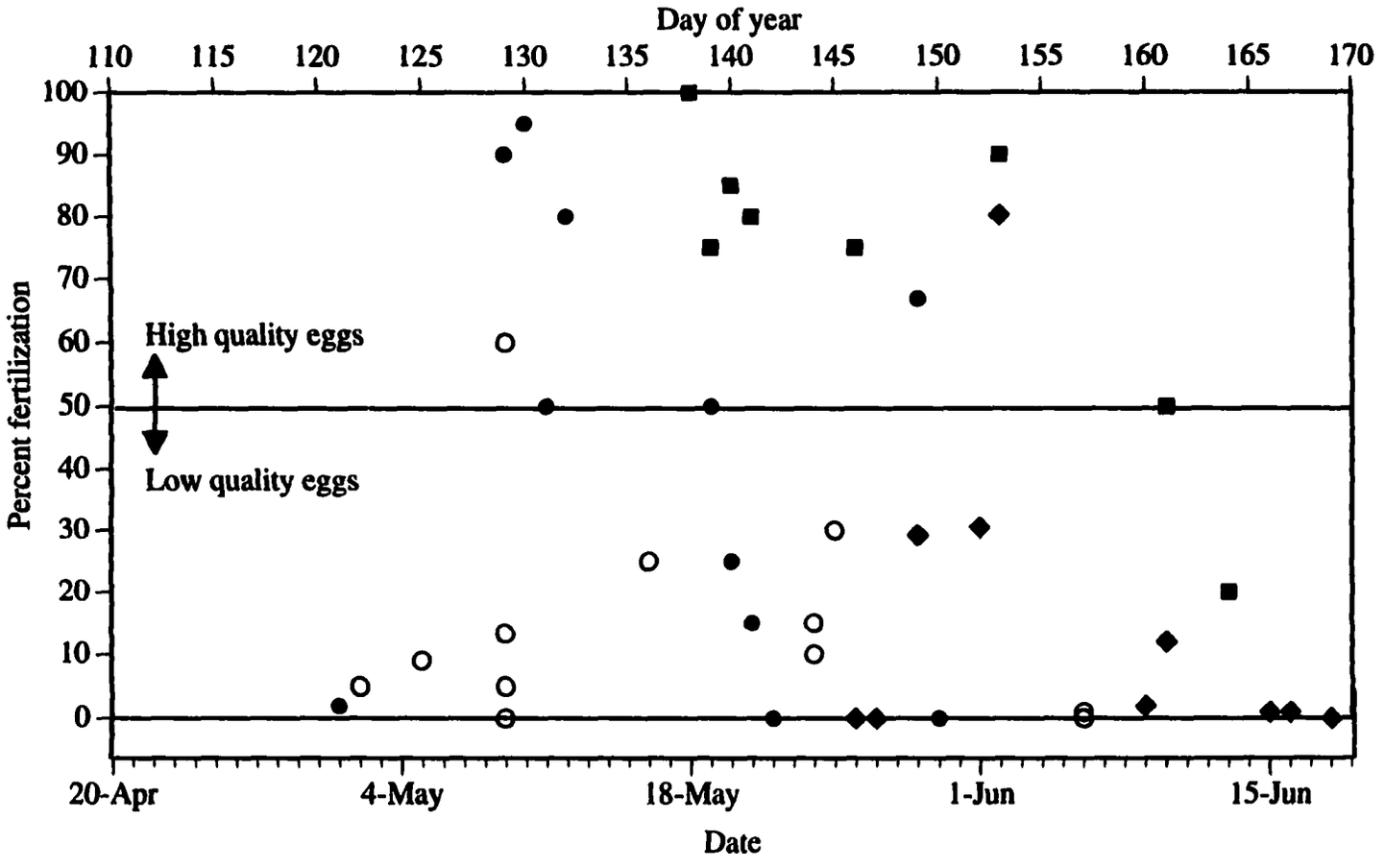

Figure 3-2. Overlay of 3 yr of percent fertilization data: diamond, 1994; square, 1995; closed circle, paired 1996; and open circle, grouped 1996. Time is shown as day of the year (upper horizontal axis) and date (lower horizontal axis).

catfish industry is to realize the degree of genetic improvement enjoyed by the cattle, pork, and poultry industries, artificial spawning must be developed into a reliable tool for use at the commercial level.

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CHAPTER 4
Effect of Electroporation on Fertilization, Hatch Rate, and Survival of Channel Catfish Eggs, Embryos, and Fry

Introduction

Transgenic organisms have a foreign gene incorporated in their genome, potentially bestowing some new or improved trait (Cloud 1990). Transgenic techniques have been used to produce organisms that have faster growth rates (Dunham et al. 1987), greater cold tolerance (Shears et al. 1991), or increased disease resistance (Abel et al. 1986). Techniques used for the production of transgenic organisms include retroviral transfection (Lin et al. 1994); particle bombardment (Bower and Birch 1992); microinjection (Inoue 1992); lipofection (Szelei et al. 1994), and electroporation (Sin et al. 1993).

Retroviral transfection is limited by the relatively short length of the foreign gene that can be carried by viral-based vectors and the requirement that the target cell be susceptible to infection by the vector. Particle bombardment, the penetration of cell walls with accelerated microscopic metal particles that have been coated with DNA, is commonly used to transfect plant cells that are difficult to transfect with other methods (Gasser and Fraley 1992). Microinjection, the injection of new genetic material into the cytoplasm of a target cell is laborious, tedious and requires a high degree of skill (Cloud 1990). Lipofection, the use of lipid-DNA vesicles to deliver foreign DNA into a target cell (Felgner and Rungold 1989) shows promise as a means to transfect cells in vivo (e.g. gametes) of adult animals (Etches et al. 1993). Electroporation, the opening of transient pores in cell membranes caused by brief application of elctrical current, has been used to transfect prokaryotic cells (Cutrin et al. 1994), cell lines (Fitzgerald et al. 1994; Kluppel et al. 1991), invertebrate embryos (Powers et al. 1995), vertebrate embryos (Mueller et al. 1993), and gametes (Sin et al. 1993; Inoue et al. 1992). This technique has been used to produce transgenic fish and has been reviewed by Houbedine and Chourrout (1991) and Pandian and Marian (1994).

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Electroporation is often used in fish instead of microinjection because electroporation allows simultaneous transfection of hundreds or thousands of eggs. Electroporation uses an electrical field to open pores in the target cell through which foreign DNA apparently diffuses into the cell. Electroporation parameters (e.g. voltage, capacitance, resistance) will vary for each cell type and can be optimized for increased efficiency (Kingston 1992).

The gene used in this study was the cecropin B gene, of the giant silkworm moth Hyalophora cecropia (Xanthopoulos et al. 1988), which is under the control of an acute phase response (APR) promoter also from H. cecropia. The APR promoter is an inducible mechanism that triggers release or production of defensive substances in response to injury or infection (Hurt et al. 1994; Reichhart et al. 1992). Cecropin, the product of the cecropin B gene, is a member of a large family of lytic peptides produced by insects that attack and damage membranes of Gram-negative and Gram-positive bacteria (Boman et al. 1991). In the insect, the cecropin gene remains silent until an injury resulting in infection by bacteria or fungi triggers expression. Cecropin is detectable in hemolymph within 30 min of injury and its levels peak between 12 and 48 h later at which time transcription ceases (Hoffmann 1995). The cecropin lytic peptide has been shown experimentally to be effective against a wide variety of bacterial fish pathogens including Edwardsiella ictaluri (Kelly et al. 1993) which causes enteric septicemia of catfish (ESC) (Hawke 1979).

Integration is defined as the stable and irreversible insertion of the transgene (minus the delivery vector) into the genome of a target cell (Kleckner et al. 1991). Although rates of integration of foreign DNA into the fish genome have been reported, little attention has been given to the effect of these methods on viability of embryos following electroporation of eggs. Survival rates following microinjection of DNA into fish eggs have been studied for channel catfish, Ictalurus punctatus and common carp, Cyprinus carpio (Hayat et al. 1991). Hayat et al. reported survival of 64% in channel catfish

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embryos microinjected at the 1-cell stage and 27% for those injected at the 2-cell stage of development. Powers et al. (1992) reported a survival rate of 5% for channel catfish hatched from eggs that had been electroporated or microinjected. Embryo cells of most higher animals are totipotent (possess the potential to become a complete organism) until beyond the 8-cell stage of embryogenesis (Hafez 1993). Beyond this stage of embryogenesis, cells differentiate and start on a path of specialization. If electroporation can successfully deliver the transgene into eggs prior to fertilization, and if integration occurs at the 1-cell stage or before the embryonic cells differentiate, a greater number of tissues should maintain the transgene. The goal of this study was to determine the effect of electroporation of DNA into unfertilized channel catfish eggs on fertilization rate, hatching rate, and survival of embryos and fry.

Materials and Methods

Electroporation and Transfection Vector

All electroporation was carried out with a Bio-Rad gene pulser (Model No. 165-2076, Hercules, California) and pulse controller (Model No. 165-2098). The electroporation apparatus allowed adjustment of voltage, capacitance, resistance, and number of electrical pulses applied. Eggs were electroporated in either of two identical 6-cm^3 chambers constructed of clear plexiglass with aluminum electrodes on opposing walls (Figure 4-1). The two chambers were used to ensure that the DNA construct would not come into contact with eggs in control treatments. One chamber was used for controls only and the other was used when the treatment called for the presence of the transgene in the electroporation buffer.

The transfection vector for the cecropin gene was based upon the plasmid, pCEP90 (Cooper 1993) (Figure D-1). In this vector the cecropin gene is contained within a transposable element activated by isopropyl-ß-D-thiogalactopyranoside (IPTG) (Kleckner et al. 1991). The vector used for the 1994 spawning season, TXI (Figure D-2), was a modification of pCEP90. In the TXI construct, the cecropin gene was contained within

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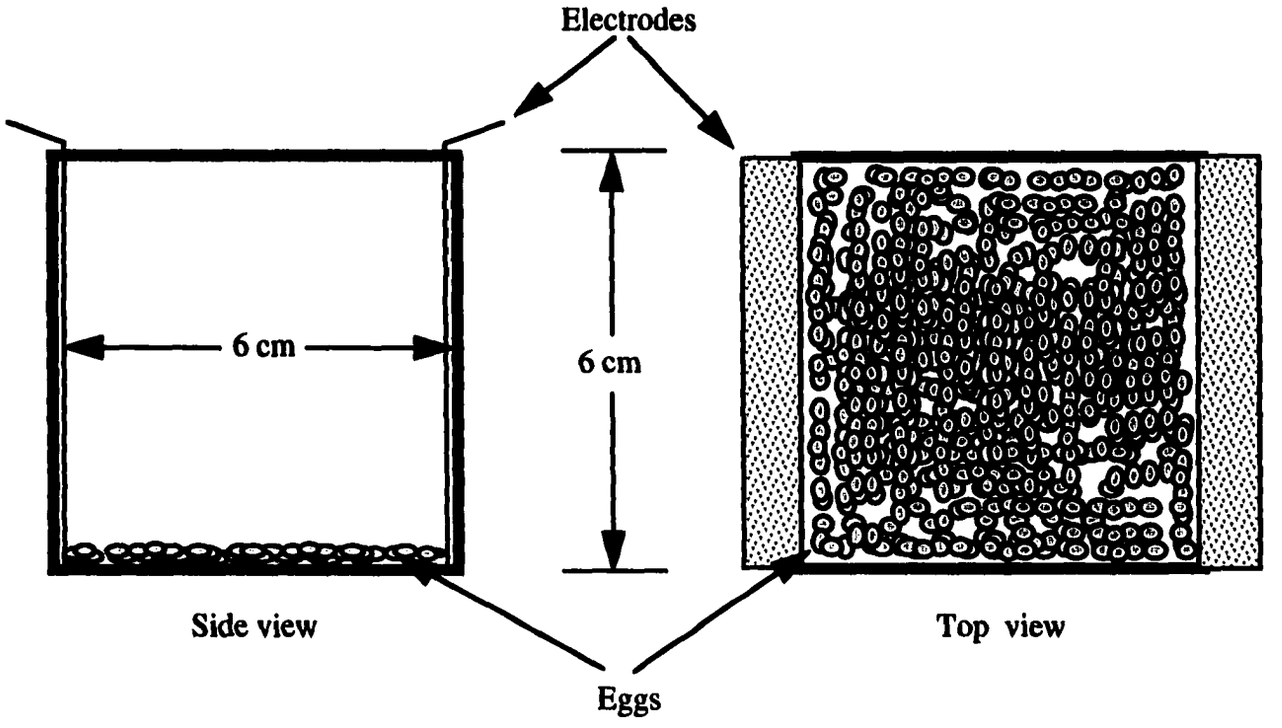

Figure 4-1. Diagram of chambers constructed for the electroporation of fish eggs. Electrodes were connected to a Bio-Rad gene pulser with wire leads and spring-loaded electrical clips.

the same transposable element, however, the vector was changed to a pUC19 based vector. This new configuration allowed higher copy numbers of the plasmid to be produced during amplification in the bacterial hosts.

The delivery vector used for the 1995 and 1996 spawning seasons was further modified. The latest vector, pPC6 (Figure D-3), differs from TXI in that two non-coding regions (~2 kilobases each) flanking the cecropin promoter and gene were removed to shorten the insertion fragment by ~4 kb. Larger transposons have lower transformation efficiencies, such that for every additional kilobase of transposon length the frequency of successful transfection decreases by ~40% (Kleckner et al. 1991). All vectors included the ß-lactamase gene for ampicillin resistance which allowed selective culture and amplification of bacteria containing the plasmid vector (those bacteria without the plasmid succumbed to the antibiotic). Plasmid DNA was extracted from the amplified bacterial cultures and purified with Qiagen Maxi-columns (Qiagen Inc., Chatsworth, California). Purified plasmid DNA was cut by a restriction enzyme that resulted in a purified linear DNA construct (The ampicillin resistance gene was inactivated when the plasmid was linearized. (Figure D-3).

The plasmid DNA was suspended at a concentration of 100 µg/mL in Hanks' balanced salt solution (HBSS)(Tiersch et al. 1994). Eggs were electroporated in HBSS containing 500 µL of suspended DNA contruct and 100 µL of IPTG (200 µg/µL). Data for this study were collected over three consecutive channel catfish spawning seasons (1994, 1995 and 1996).

1994 Spawning Season

Unfertilized eggs were collected from nine female channel catfish that had been paired with males. Electroporation parameters were: 125, 142, 158, or 175 volts/cm^2; capacitance was set at 0.25 microFarads (µF), and resistance was set at 200 Ohms (Ω). Electroporation can be applied in single or multiple pulses and treatment groups were pulsed one, three, or six times. Twenty mL of eggs from each spawn were preserved in

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5% formalin for counting at a later time. Initial egg numbers were estimated for treatment groups from the number of eggs in the preserved samples.

1995 Spawning Season

Unfertilized eggs were collected from six female channel catfish that had been paired with males. Electroporation parameters were: 83, 125, or 167 volts/cm^2; capacitance was set at 0.25 microFarads (μF), resistance was set at 200 Ohms (Ω). Treatment groups were pulsed one, two, or three times. Prior to fertilization, eggs for each treatment were placed on a fluorescent light box (Model E2, Laboratory Supplies Company, Inc., Hicksville, New York). An acrylic sheet (such as the type used on overhead projectors) was used to protect the surface of the light box. A photograph was taken of the eggs with a digital camera (Fotoman, Logitech Inc., Fremont, California). Later, image analysis (Bates and Tiersch 1997) was performed on a Macintosh LCIII computer using the public domain NIH Image program (version 1.57, written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov).

1996 Spawning Season

For the 1996 season, three balanced replicated electroporation experiments were carried out. Unfertilized eggs were collected from three channel catfish females. Each experiment consisted of four treatment groups with three replicates of ~900 eggs in each group. The eggs for treatment group A were fertilized and recieved no electroporation (fertilization controls), eggs for treatment group B were electroporated without the DNA construct (electroporation controls), eggs for treatment group C were electroporated with the DNA construct, and eggs for treatment group D were electroporated with the DNA construct and IPTG. Electroporation parameters used in 1996 were selected based on information from the previous 2 yr: voltage = 125 volts/cm^2, capacitance = 0.25 μF, resistance = 200 Ω, and eggs were pulsed twice. This design allowed direct evaluation

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of the effects of electroporation of eggs on embryos and fry without variation in the electroporation parameters.

Artificial Fertilization

Male channel catfish were killed by overdose of tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington) and testes were removed. Sperm solutions were prepared in HBSS following the methods of Tiersch et al. (1994). Following electroporation, eggs were placed into 400-mL plastic beakers (Tri-pour, Oxford Labware, St. Louis, Missouri) coated with silicone grease, and excess HBSS was decanted. For fertilization, 0.5 mL of sperm suspension and 50 mL of fresh water from the culture tanks were added to activate the gametes and the beaker was swirled to mix. An additional 75 mL of fresh water was added to the beaker after 2 to 3 min to facilitate water hardening and cohesion of eggs. After fertilization, the eggs formed gelatinous masses which were transferred to screened containers (described below) and placed in a hatching trough for incubation.

Egg Incubation and Fish Culture

Fertilization percentage was estimated 30 to 36 h after fertilization. To aid estimation of fertilization, eggs were separated from the egg mass by dissolving the glycoprotein matrix with a 1.5% sodium sulfite solution (Isaac and Fries 1991) during the 1994 season and part of the 1995 season.

Embryos were considered fertilized when the neurulation stage of embryogensis was observed (~24 h after fertilization assuming an incubation temperature of 28 C). Egg quality varied from female-to-female, and spawns with fertilization rates < 10% in control treatments were excluded from the analysis. Hatching rate was estimated when viable sac-fry were clustered at the bottom of the culture tank (~6 to 7 d after fertilization). Fertilization and hatching rates were each recorded as percentages of the original egg number.

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For the 1994 and 1995 spawning seasons, eggs were incubated and hatched in screened PVC cups (Figure 4-2). The cups were constructed from 8.25-cm diameter schedule 20 PVC pipe. The pipe was ~15 cm long and had four 5-cm diameter holes drilled through the sides. One end and all side holes were covered with plastic window screen permanently fastened by clear hot melt glue (Thermogrip, Black and Decker Inc., Hunt Valley, Maryland). After the fertilized eggs were poured in the cup, another piece of window screen (held with a rubber band) was used to cover the top. The cups allowed isolation of individual treatment groups in a single hatching tank. Following hatch, fry were transferred from the screened cups to another culture system which consisted of 100 culture units (2-L working volume each), five 40-L tanks, and an upwelling biofilter (Bates and Tiersch 1995).

For the 1996 spawning season, the smaller culture units used in the previous years were replaced with 25 larger culture units (20-L working volume in each). Fertilized egg masses were held in baskets constructed from 5-mm mesh plastic screen placed within the 20-L tanks. Following hatch, the baskets were removed after the fry dropped through the mesh basket. Swim-up fry (fry that had digested the yolk sac and begun to feed) were provided a diet of 41% protein fry feed (fry I, Delta Western, Greenville, Mississippi) three times daily.

Statistical Analysis

For the 1994 and 1995 data, percent fertilization and percent hatch values were arcsine-square-root-transformed and analyzed with a multi-variate analysis of variance (MANOVA) with fertilization and hatch rate as response variables and voltage, number of pulses, presence or absence of DNA, and presence or absence of IPTG as factors. Fisher's least significant difference (LSD) test with the level of significance set at 0.05 was used for means separation.

For the 1996 data, percent fertilization, hatch rate, and survival values were arc-sine-square-root transformed and analyzed with a multivariate analysis of variance

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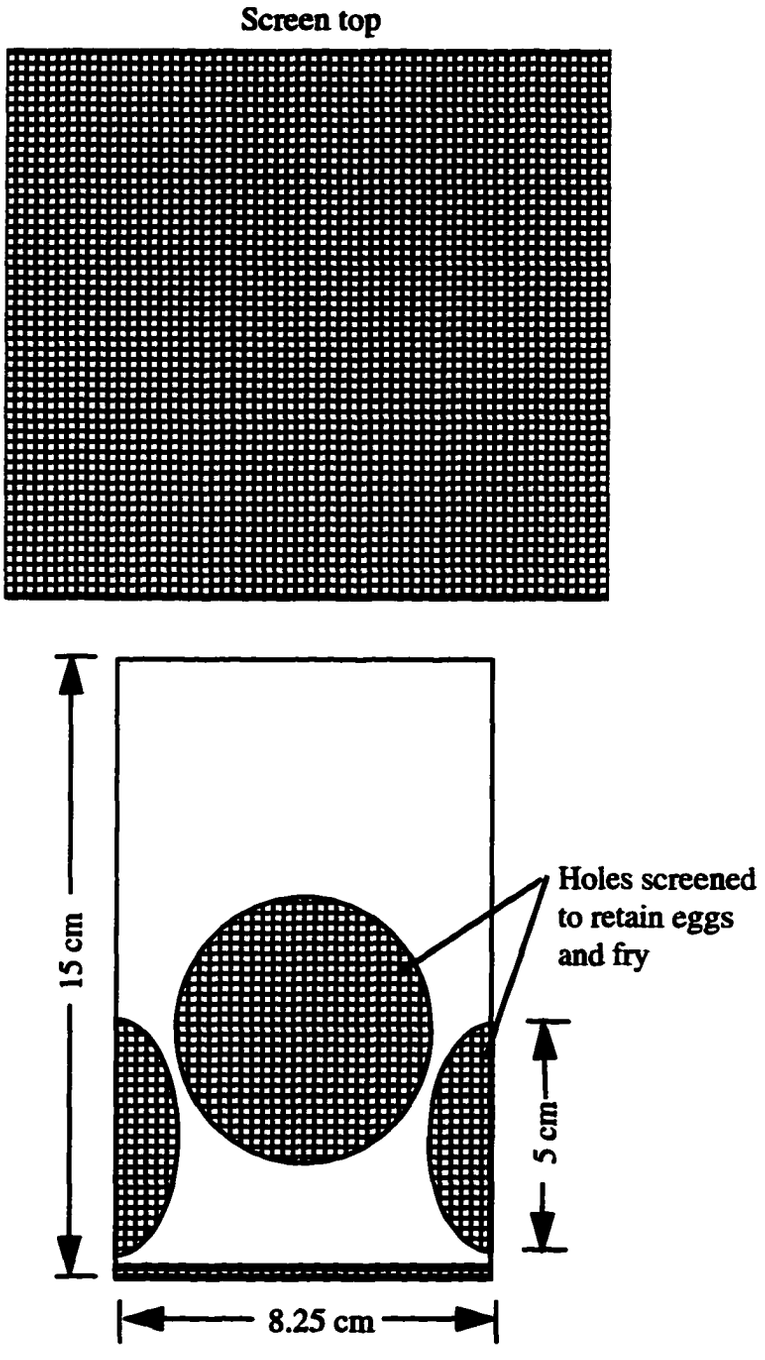

Figure 4-2. Diagram of screened egg isolation cups constructed from PVC pipe and nylon window screen. Screen top is fastened with a rubber band.

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(MANOVA) with fertilization, hatch rate, and survival as response variables and treatment group and spawn as factors. Percent fertilization and hatching rate were determined as described above. Survival was determined at 2 weeks after fertilization as the number of fish still alive in a treatment group and was expressed as a percentage of the original egg number. Fisher's least significant difference (LSD) test with the level of significance set at 0.05 was used for means separation. All statistical comparisons were made within years and analyses were performed with the Data Desk statistical software package (version 4.2, Data Description, Inc., Ithaca, New York).

Results

1994 Spawning Season

No interactions were detected among any of the factors for the effect of voltage on percent fertilization (Table C-1) or on hatching rate (Table C-4). Therefore all factors will be presented independently. For eggs electroporated during the 1994 spawning season, the effect of voltage level on fertilization was significant ($P = 0.03$). Fertilization rate did not decline significantly until voltage levels of 233 volts/cm^2 or higher were used. However, voltage had no effect ($P = 0.86$) on hatch rate (Table 4-1).

Table 4-1. Effect of voltage level on fertilization and hatch rate (percentage of original egg number) for electroporated channel catfish eggs during the 1994 spawning season.

Voltage (V/cm^2)	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
0	4	38.0 ± 29.5^a	15.8 ± 10.5^a
125	6	64.1 ± 27.6^a	28.4 ± 23.0^a
142	7	45.4 ± 27.6^a	20.2 ± 23.3^a
158	7	53.8 ± 26.8^a	25.1 ± 24.9^a
175	5	40.2 ± 22.6^a	17.5 ± 17.8^a
233	6	8.9 ± 3.6^b	8.9 ± 3.6^a
289	6	8.7 ± 6.1^b	8.7 ± 6.1^a

[1]Means sharing a lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

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The number of pulses applied during the electroporation of eggs had no effect on fertilization ($P = 0.43$) or hatch rate ($P = 0.90$) (Table 4-2).

Table 4-2. Effect of number of pulses on fertilization and hatch rate (percentage of original egg number) for electroporated channel catfish eggs during the 1994 spawning season.

Pulses	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
0	2	20.6 ± 12.2[a]	13.0 ± 1.4[a]
1	31	46.2 ± 28.2[a]	21.0 ± 20.2[a]
3	4	11.4 ± 6.6[a]	11.4 ± 6.6[a]
6	4	5.0 ± 2.0[a]	5.0 ± 2.0[a]

[1]Means sharing a lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

The presence of the DNA construct in the electroporation buffer during the electroporation of eggs had no effect on fertilization ($P = 0.70$) or hatch rate ($P = 0.45$) (Table 4-3).

Table 4-3. Effect of presence or absence of DNA construct on fertilization rate and hatch rate (percent of original egg number) for channel catfish eggs electroporated during the 1994 spawning season.

DNA	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
Absent	23	35.4 ± 28.6[a]	15.9 ± 15.7[a]
Present	18	40.2 ± 30.5[a]	20.9 ± 21.5[a]

[1]Means sharing a lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

The presence of IPTG in the electroporation buffer during the electroporation of eggs also had no effect on fertilization ($P = 0.76$) or hatch rate ($P = 0.61$) (Table 4-4).

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Table 4-4. Effect of presence or absence of isopropyl-ß-D-thiogalactopyranoside (IPTG) on fertilization rate and hatch rate (percent of original egg number) for channel catfish eggs electroporated during the 1994 spawning season.

IPTG	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
Absent	11	19.3 ± 22.3[a]	11.2 ± 8.0[a]
Present	30	44.2 ± 28.8[a]	20.7 ± 20.5[a]

[1]Means sharing a lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

1995 Spawning Season

No interactions were detected among any of the factors for the effect of voltage on percent fertilization (Table C-6) or hatching rate (Table C-8). Therefore all factors will be presented independently. For eggs electroporated during the 1995 spawning season, the effect of voltage level on fertilization was significant ($P \leq 0.0001$). The non-electroporated eggs had the highest fertilization rates while the eggs electroporated at 167 V/cm^2 had the lowest fertilization. The percent fertilization of eggs electroporated at 83 and 125 V/cm^2 were not significantly different, but were different from the control and eggs electroporated at 167 V/cm^2. However, as in the 1994 experiments, voltage had no effect ($P = 0.42$) on hatch rate (Table 4-5).

Table 4-5. Effect of voltage level on fertilization and hatch rate (percentage of original egg number) for electroporated channel catfish eggs during the 1995 spawning season.

Voltage (v/cm^2)	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
0	7	85.4 ± 9.7[a]	8.7 ± 11.4[a]
83	15	71.7 ± 21.1[b]	5.9 ± 6.4[a]
125	8	76.9 ± 13.1[b]	6.3 ± 5.2[a]
167	15	44.0 ± 32.0[c]	5.8 ± 8.6[a]

[1]Means sharing a lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

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The number of pulses applied had a significant effect on fertilization ($P = 0.0002$), but not on hatch rate ($P = 0.24$) (Table 4-6).

Table 4-6. Effect of number of pulses on fertilization and hatch rate (percentage of original egg number) for electroporated channel catfish eggs during the 1995 spawning season.

Pulses	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
0	7	85.4 ± 9.7[a]	8.7 ± 11.4[a]
1	28	71.1 ± 21.9[b]	7.2 ± 7.7[a]
2	4	45.0 ± 34.9[bc]	3.3 ± 3.2[a]
3	6	30.0 ± 26.7[c]	1.8 ± 1.5[a]

[1]Means sharing a common lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

The presence of the DNA construct in the electroporation buffer had a significant effect on fertilization ($P = 0.02$), but not on hatch rate ($P = 0.15$) (Table 4-7).

Table 4-7. Effect of the presence or absence of DNA on fertilization and hatch rate (percent of original egg number) for channel catfish eggs electroporated during the 1995 spawning season.

DNA	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
Absent	20	63.3 ± 28.8[a]	4.3 ± 4.8[a]
Present	25	67.3 ± 27.2[b]	8.1 ± 9.2[a]

[1]Means sharing a common lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

The presence of IPTG had a significant effect on fertilization ($P = 0.04$), but not on hatch rate ($P = 0.82$) (Table 4-8).

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Table 4-8. Effect of the presence or absence of IPTG on fertilization and hatch rate (percent of original egg number) for channel catfish eggs electroporated during the 1995 spawning season.

IPTG	n	Fertilization (Mean ± SD)[1]	Hatch (Mean ± SD)[1]
Absent	14	78.1 ± 18.0[a]	7.1 ± 9.2[a]
Present	31	59.8 ± 29.6[b]	6.0 ± 7.1[a]

[1]Means sharing a common lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

1996 Spawning Season

The effect of treatment group on fertilization was significant ($P \leq 0.0001$). The non-electroporated treatment group had a higher fertilization rate than the electroporated treatment groups which were not significantly different from one another. The effect of treatment group on hatch rate was highly significant ($P \leq 0.0001$). The non-electroporated treatment group had a lower hatch rate than did the electroporated treatment groups which were not different from one another. The effect of treatment group on 2-week survival was not significant ($P = 0.32$) (Table 4-9).

Table 4-9. Effect of treatment group on mean fertilization, hatch rate, and survival at 2 weeks for channel catfish eggs electroporated during the 1996 spawning season. Fertilization, hatch rate, and 2-week survival values are given as percentages of original egg numbers (Mean ± S.D.)[1]

Group	Treatment description	n	Fertilization	Hatch rate	2-week survival
A	no electroporation	9	56.7 ± 34.6[a]	14.11 ± 12.8[a]	10.47 ± 10.9[a]
B	electroporation only	9	42.8 ± 31.9[b]	23.2 ± 13.9[b]	14.9 ± 8.5[a]
C	electroporation + DNA	9	44.9 ± 31.9[b]	16.7 ± 13.4[b]	9.5 ± 7.4[a]
D	electroporation + DNA + IPTG	9	40.44 ± 30.9[b]	21.2 ± 19.1[b]	12.0 ± 9.3[a]

[1]Means in a column sharing a common lowercase letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

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The effect of spawn on fertilization was highly significant ($P < 0.0001$). All three spawns were significantly different from one another. A significant interaction of spawn by treatment group ($P = 0.02$) was detected for fertilization and for hatch rate ($P = 0.01$). Most of the variation was due to the low fertilization values observed for the second spawn (Table 4-10).

Table 4-10. Effect of spawn on fertilization, hatch rate, and survival.

Spawn	n	Fertilization (%) (Mean ± SD)[1]	Hatch rate (%) (Mean ± SD)[1]	Survival (%) (Mean ± SD)[1]
1	12	75.8 ± 12.0^{a}	30.6 ± 11.1^{a}	14.8 ± 6.3^{a}
2	12	5.7 ± 5.5^{b}	3.4 ± 4.3^{b}	2.6 ± 3.6^{b}
3	12	57.0 ± 10.8^{c}	22.6 ± 11.4^{a}	17.7 ± 7.8^{a}

[1]Means in a column sharing a common lowercase superscript letter were not significantly different ($P > 0.05$) as determined by a Fisher's least significant difference means separation test.

Discussion

Electroporation has become an accepted method to produce transgenic fish because it is efficient on a wide variety of cell types and it is rapid and simple when compared to microinjection (Kingston 1993). Regardless of transfection efficiency, if electroporation decreases the viability of the resulting animal, it might not be deemed an acceptable technique for the production of transgenic animals. The results from the 1994 and 1995 spawning season showed significant effects for voltage on fertilization. However, there was no difference found in either year for hatch rate. The 1996 results suggest that electroporation significantly reduced fertilization rates. However, the total number of embryos surviving to time of hatch, and surviving for 2 weeks was not reduced by electroporation.

Incubation of eggs and culture of fry of channel catfish is usually carried out in flow-through systems (Huner and Dupree 1984, Goudie et al. 1993). In these systems, accumulation of waste products is not a concern because contaminated water is replaced

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by a constant flow of clean water. Recirculating systems may not adequately remove toxic compounds or may fail due to a variety of reasons (Mayo 1991). While the use of recirculating systems in this study could have had an effect on incubation of eggs and culture of fry, these closed systems offer enhanced security against escape of transgenic fish not available with open flow-through systems (ABRAC 1995).

To collect data on fertilization rate, hatching rate, and survival for embryos and fry hatched from electroporated eggs, treatment groups must be isolated. However, survival and hatch rates may be reduced by the culture methods required to maintain separation of treatment groups. This seems to be the case particularly in 1994 and 1995 when eggs were incubated in small screened cups (Figure 4-2). Although no direct comparison can be made among years, hatch and survival rates appeared to be better in 1996 when eggs were hatched and fry were held in larger culture tanks. In addition, there is some indication that transgenic treatment of channel catfish eggs and embryos results in relatively low survival when compared to other species. For example, microinjection of channel catfish eggs resulted in survival rates of 10-20% according to Dunham et al. (1987). In the same study, survival rates of goldfish Carassius auratus were 50-70%. Powers et al. (1992) reported survival of 5% when channel catfish eggs were electroporated or microinjected. Survival in this study averaged ~9 to 15% and was comparable to the levels reported for channel catfish by Dunham et al. (1987). In addition, hatching rate and survival could have been affected by treatment with sodium sulfite to remove the glycoprotein matrix which holds the egg mass together. Ringle et al. (1992) and Weirich and Tiersch (In press) noted early hatching of embryos from eggs treated with sodium sulfite. It is possible in this study that the chorion was weakened by exposure to sodium sulfite, leading to premature rupture and hatching of the embryo leading to high mortalities. For this reason, use of sodium sulfite was suspended during the 1995 spawning season.

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Hayat et al. (1991) demonstrated a decrease in survival of channel catfish from 65% to 27% when eggs were microinjected at the 1-cell or 2-cell stage of embryogenesis. In the time that it takes to microinject one egg, electroporation can synchronously treat hundreds or thousands, making electroporation the method of choice for large-scale production of transgenic fish. Large-scale production would allow additional research on transgene stability of integration, inducibility of expression, and inheritance by progeny.

Genetic improvement by transgenic techniques offers the potential to improve a trait faster than would be possible with conventional breeding techniques or to confer a new trait on cultured fish. Transgenic fish could either be used as broodfish using conventional spawning techniques or sperm from transgenic males could be cryopreserved and sent to producers for artificial fertilization of eggs from their female broodfish.

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CHAPTER 5
Isolation of Experimentally Treated Fish, and Screening of Channel Catfish for Transgenic Status

Introduction

Experiments to optimize techniques for the production of genetically modified organisms (GMOs) require multiple treatments with replication and often require maintenance of early life stages in separate containers. The expense of building multiple-tank systems with commercially available containers (e.g., glass aquaria) can be difficult to justify, especially if the system will be in use for only part of the year. Culture systems constructed from disposable beverage bottles are inexpensive and can be tailored to a variety of applications. For example, beverage bottles have been modified for use as hatching jars (Rottman and Shireman 1988) and disposable test chambers (Goodfellow et al. 1985). The system described in this study used 3-L clear, plastic beverage bottles or 20-L plastic buckets as culture units to hold channel catfish Ictalurus punctatus. The bottles were single-piece, self-standing, with five molded feet in the base. Some bottling companies use a two-piece, round-bottom bottle with a removable plastic base. Either 3-L bottle is suitable for this application. The buckets were commercially available 20-L (~5 U.S. gallon) food-grade, white plastic buckets that were purchased new. The bottles and buckets were inexpensive, easy to modify, and sturdy.

To determine transfection efficiency (percentage of fish in a treatment that carry the transgene) with a particular procedure, it is often necessary to screen for presence of the transgene soon after fertilization. This requires sacrifice of a portion of the treatment group. The remainder of the treatment group should, if possible, be isolated from other groups until screening has been completed. The polymerase chain reaction (PCR) can be used to screen for genes of interest. Examples where PCR has been successfully used for genetic screening include sexing of bovine embryos (Saberivand and Outteridge 1996), identification of genetic disorders in humans (Binder et al. 1995), and the

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identification of the presence of a transgene in fish (Hew et al. 1995). A positive PCR test indicates only that the gene of interest is present in the sample. It does not prove that the gene is integrated or that it is integrated in the proper orientation to be transcribed. A new gene is integrated when it is inserted into the genome of the host and is replicated with the host DNA. But, regardless of integration or orientation of the transgene, a high percentage of individuals carrying the gene indicates that the parameters used for electroporation were at least successful in getting the gene into the cytoplasm of the target cells. In this study, potentially transgenic channel catfish Ictalurus punctatus were produced by electroporation of unfertilized eggs followed by artificial fertilization. The transgene used was the cecropin B gene from the giant silkworm moth Hyalophora cecropia. The goals of this study were to demonstrate: (1) a cost-effective system to isolate genetic treatments, and (2) screening of potentially transgenic embryos by PCR.

Materials and Methods

Construction of Culture Units

All pipe and fittings used for construction of culture units were of schedule 40 polyvinyl chloride (PVC) unless noted otherwise. The final components for the smaller culture vessels are shown in Figure 5-1. For construction, the neck of a 3-L plastic bottle was removed (2.54 cm from the top) with a bench-mounted radial saw and the base was removed (5 cm from the bottom) with a utility knife. A 2.54-cm x 1.27-cm female, normal pipe thread (NPT) reducer bushing was inserted into the bottle so that the 1.27-cm fitting was inside the bottle and the 2.54-cm fitting projected through the neck. Placement of the bushing in the bottle neck immediately after sawing allowed contraction of the cooling plastic to produce a tight fit around the bushing. Clear silicone sealant (Dow Corning Inc., Midland Michigan) was spread around the 2.54-cm fitting already in the bottle and a 2.54-cm coupler was forced onto the bushing in the bottle. Clear silicone sealant was spread around the inside of the bottle neck to completely seal between the bottle and the bushing.

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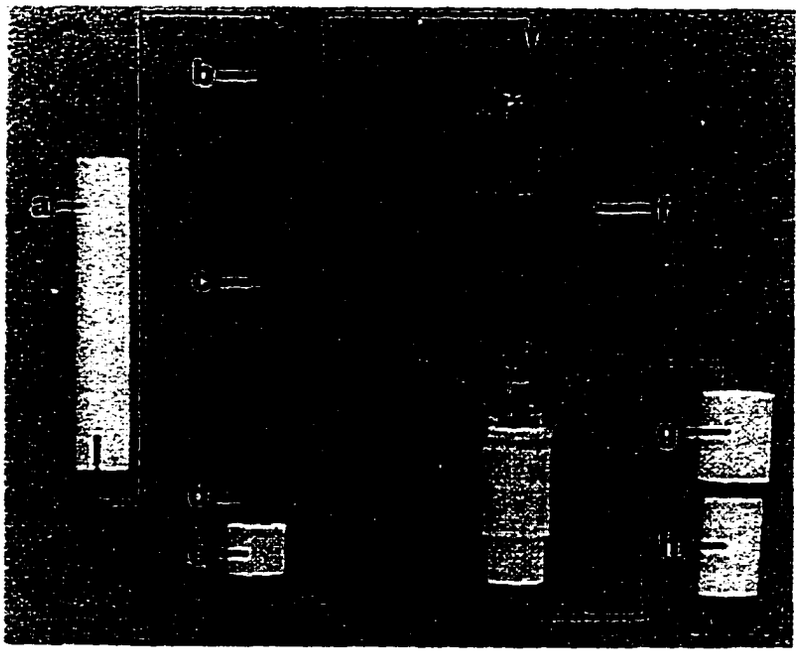

Figure 5-1. Components and assembled culture unit based on 3-L disposable beverage containers. (a) 21.5-cm length of 2.54-cm PVC pipe with slits; (b) removable drain screen; (c) 19-cm length of 1.27-cm clear rigid tubing; (d) 1.27-cm normal pipe thread x 0.95-cm barbed polycarbonate fitting; (e) 2.54-cm x 1.27-cm female normal pipe thread reducer bushing; (f) assembled culture unit; (g) 2.54-cm coupler; (h) 7.62-cm length of 2.54-cm PVC pipe.

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A 1.27-cm male NPT x 0.95-cm barbed polycarbonate plastic fitting (Aquatic Ecosystems Inc., Apopka, Florida) was placed into the 1.27-cm hole in the bushing. A 19-cm length of 1.27-cm clear rigid tubing was attached over the barbed end of the polycarbonate plastic fitting and sealed with clear silicone sealant. A 7.62-cm length of 2.54-cm diameter pipe was fitted into the 2.54-cm coupler on the bottom of the culture unit to drain water from the bottle. A venturi drain was constructed by placing a 21.5-cm length of 2.54-cm diameter pipe with four vertical slits (2.54 cm high x 0.95 cm wide) cut in the bottom to allow water to drain from the bottom of the culture bottle. Construction of tops for the culture units depended on the type of bottle used. For the one-piece bottle, (the type used for this system) two beverage bottles were used to build a single culture unit. The base was removed (5.5 cm from the bottom) from a second bottle, and a 2.54-cm hole saw was used to drill a hole in one of the five molded feet. This hole allowed feeding of fish without the removal of the top. A 0.95-cm drill bit was used to drill holes for air and water lines in two other feet. This modified bottom was placed on top of the culture unit. If the two-piece bottles were available, the removable plastic base when modified as described above, served as a top for the culture bottle.

The final components for the larger culture vessels are shown in Figure 5-2. For construction, a 2.54-cm hole saw was used to drill a hole in the center of the bottom of each bucket. A 4-cm length of 2.54-cm PVC pipe was placed in the hole in the bucket. Two 2.54-cm couplers were forced onto the 2.54-cm pipe in the bottom of the bucket so that one was on the inside and the other was on the outside. Clear silicone sealant was spread around each coupler. A 20-cm length of 2.54-cm pipe was placed in the coupler inside the bucket (inner standpipe) and a 4-cm length was placed in the coupler outside the bucket (drain). An outer standpipe was constructed from a 25-cm length of 5-cm diameter PVC pipe with four 1.25-cm holes drilled around the base to allow water to drain from the bottom of the bucket. Removable screens were made for all culture vessels by cutting plastic window screen to a size that wrapped around the inner

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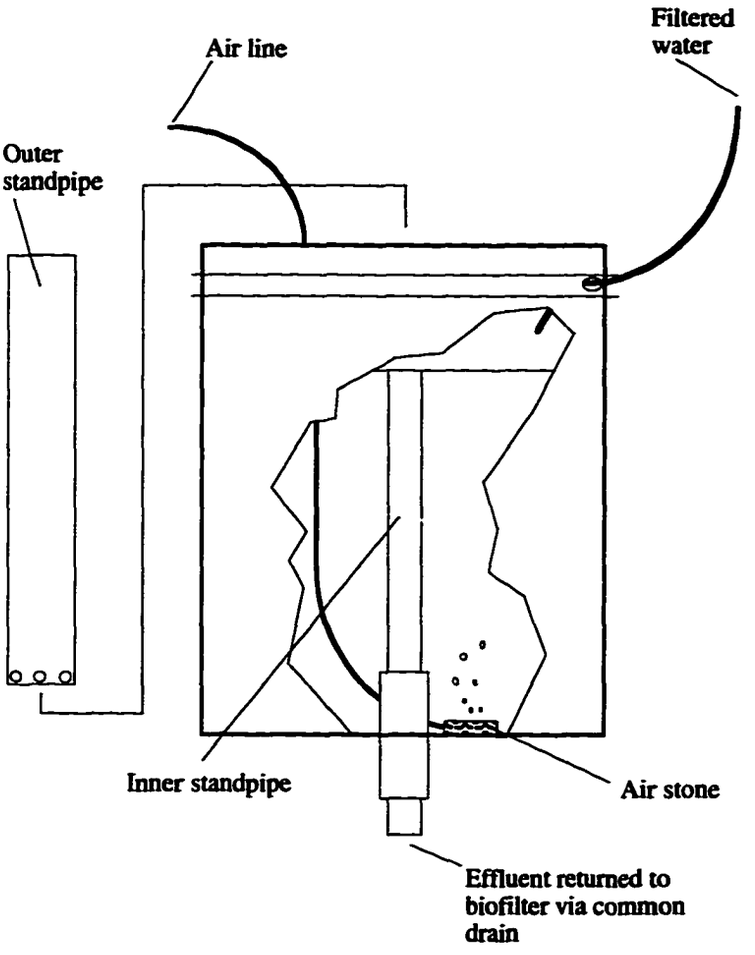

Figure 5-2. Diagram of 20-L bucket modified for use as an egg incubation and fry culture container.

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standpipe. The screen was sealed with clear hot melt glue (Thermogrip, Black and Decker Inc., Hunt Valley, Maryland) along the side and the top.

System Description

The culture units were placed on a two-tier wood frame with flat PVC sheeting (12 mm thick) used as the shelf surface. The system held either two rows of 25 small units per tier, 12 of the larger units per tier, or a combination of the two sizes (Figure 5-3). The discharge pipe from each unit was mounted to fit through a 3.5-cm hole in the shelf and the overflow drained through 3.8-cm holes drilled in a pipe (7.62 cm in diameter) mounted below each row of bottles. There was no permanent attachment between the culture units and the drain pipe. The two upper-tier drain pipes were plumbed downward through 90° elbow fittings to connect with the lower-tier drain pipes by "T" fittings. The two lower-tier drain pipes were plumbed downward with one 90° elbow fitting and one "T" fitting to lead into a single 7.62-cm line leading to the floor. The drain line was routed into another 90° elbow, and a 7.62-cm x 5.08-cm reducer bushing was used to connect to a 5.08-cm pipe leading to a 100-L plastic sump with a 5.08-cm bulkhead fitting. Five 40-L, circular, plastic tanks were connected in line between the filter and the culture unit rack (not shown). The 40-L tanks were included to increase total volume of the system, to allow operation of the filter when bottles were not in use, and for temporary storage of fish as treatments were pooled following screening. A 0.5-hp centrifugal pump (Little Giant Pump Co. Inc., Tulsa, Oklahoma) was plumbed to the sump tank with a 2.54-cm bulkhead fitting and 2.54 cm pipe for the intake. Water was pumped into a 0.03-m^3 upwelling biofilter (Armant Aquaculture Inc., Vacherie, Louisiana)(Malone et al. 1993) through 1.27-cm diameter pipe. Filtered water exited the biofilter through a 2.54-cm diameter manifold that delivered water to the upper and lower tiers of the system. A valved return line allowed shunting of some the filtered water back to the sump tank to reduce pressure in the water delivery manifold if needed. Twenty-five 1.27-cm diameter holes were drilled and tapped for NPT fittings in each tier

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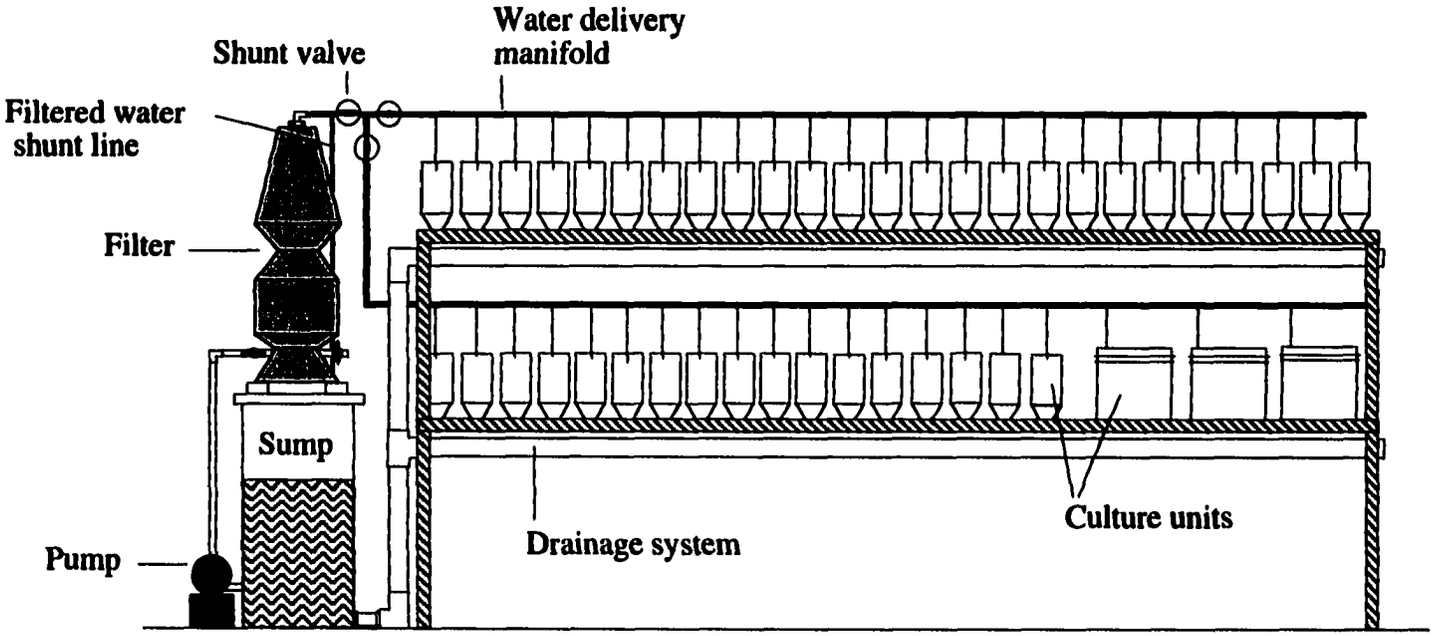

Figure 5-3. Diagram of egg incubation and fry culture system (40-L tanks not shown).

of the water delivery manifold. A 1.27-cm NPT male x 0.95-cm barbed polycarbonate plastic fitting (Aquatic Ecosystems Inc.) was screwed into each tapped hole.

A tubing assembly was prepared for each fitting in the water manifold. The assembly consisted of one 20-cm length of clear 0.95-cm aquarium tubing and two 12-cm lengths of 0.95-cm tubing joined by a plastic "Y" fitting. The 20-cm tubing was attached to the plastic fitting in the water manifold. This assembly allowed two units to receive water from each outlet in the delivery manifold. A Dura-clamp tubing flow valve (Aquatic Ecosystems Inc.) was placed on each water delivery tube. Air was supplied by a 1-hp Sweetwater regenerative air blower (Aquatic Ecosystems Inc.) already in place for aeration of existing culture systems. An air-delivery manifold, similar in construction to the water manifold, supplied air to the two tiers (not shown). Each air manifold was drilled and tapped for twenty-five, 0.635-cm plastic needle valves (Aquatic Ecosystems Inc.). An aquarium airstone in each unit was connected to the air manifold with a tubing assembly constructed as described for the water manifold.

System Operation

Water flow rate to the culture units was adjusted by changing the overall flow through the system with the return line shunt valve, or by adjusting the individual tubing valve at each unit. The biofilter was backwashed every other day, solids were cleaned from standpipe screens, and fish were transferred to clean culture units when sides became fouled with surface-growing organisms. Culture units were cleaned with a stiff-bristled round brush and rinsed with fresh water. Eggs were incubated in separate containers and fry were transferred following hatch to the 3-L containers. The 20-L containers doubled as egg incubation tanks (with the addition of removable plastic baskets to hold egg masses) and as fry holding tanks. Outer standpipes were not placed in the culture units until about 1 week after hatching of fry.

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Collection of Eggs for Electroporation

Five mature channel catfish females were injected with 100 µg leuteinizing hormone-releasing hormone analog (LH-RHa)/kg of body weight and were placed in a 1,000-L tank with four other hormone-treated females. After ~38 h, ovulation was indicated by eggs flowing from the urogenital pore of one female. The female was anesthetized with tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington) and dried thoroughly with paper towels to ensure that water would not contact the eggs. The female was cradled in one arm while sufficient pressure was applied to the abdominal area to strip eggs. Eggs were collected into food-grade plastic bowls that containing Hanks' balanced salt solution (HBSS; 300 mOsmol/kg) (Tiersch et al. 1994) and coated with silicone grease (Dow Corning, Midland, Michigan) to prevent eggs from adhering to the bowl.

Electroporation

A Bio-Rad gene pulser (Model 165-2076, Hercules, California) and pulse controller (Model 165-2098) were connected to electrodes on two opposing walls of a 6-cm^3 chamber constructed of clear plexiglass. Approximately 900 eggs were placed in the chamber with sufficient HBSS to cover the eggs. The transfection vector used in this study, pPC6 (Figure D-3), carried the cecropin B gene. The plasmid DNA was suspended at a concentration of 100 µg/mL in Hanks' balanced salt solution (HBSS, 300 mOsmol/kg). Eggs were electroporated in HBSS containing 500 µl of suspended DNA contruct (100 µg/mL) and 100 µL of isopropyl-ß-D-thiogalactopyranoside (IPTG) (200 µg/µL). Electroporation parameters were: two pulses of 125 volts/cm^2, capacitance of 0.25 µF, and resistance of 200 Ω.

Fertilization

The eggs were placed into a 400-mL plastic beaker (Tri-pour, Oxford Labware, St. Louis, Missouri) that had been coated with silicone grease, and excess HBSS was decanted. For fertilization, 1 mL of sperm suspension was added to the eggs, and 50 mL

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of fresh water (from the culture system) were added to activate the gametes and the beaker was swirled to facilitate mixing. An additional 75 mL of fresh water was added to the beaker after 2 to 3 min to facilitate water hardening and cohesion of eggs. After fertilization, the eggs formed gelatinous masses which were transferred to mesh baskets and placed in an incubation tank. Another ~900 eggs were fertilized without any further treatment to serve as a control group.

Sample Collection and DNA Extraction

Embryos were collected ~96 h after fertilization. Part of the egg mass was placed in a solution of 1.5% sodium sulfite to dissolve the gelatinous matrix (Isaac and Fries 1991). Individual embryos were transferred with disposable plastic pipets and placed into the wells of a 96-well plate. Embryos from control treatments were collected and placed in a separate 96-well plate. The plates were wrapped in aluminum foil and placed into labeled freezer bags for storage at -20 C until screening could be carried out.

The embryos were transferred to individually labelled 1.8-mL centrifuge tubes. A commercial kit (QIAmp blood and tissue kit, Qiagen Inc., Chatsworth, California) was used to extract genomic DNA from a total of 34 potentially transgenic embryos and from 10 control embryos.

Screening

Screening for the presence of the cecropin gene was carried out using PCR (Table E-3). Primers designed to target an 846 base pair (bp) fragment of the cecropin gene were synthesized at the LSU Gene Probe and Expression Laboratory (LSU Veterinary Medical School). The primer DNA sequences were: AGACTTGACTCCGCTGCATAAGTG (designated Vec-1) and TACCGTTTCTGATGTTGCGACC (Vec-2). The primer set was used for PCR analysis of the genomic DNA samples extracted from the embryos. Each PCR reaction contained 0.20 µM of Vec-1 and Vec-2, 10 µM of each dNTP (G, A, T, and C), 1.5 mM $MgCl_2$, 1% DMSO, 2.5 units of Taq DNA polymerase, 1x Taq buffer (supplied as a 10x concentrate with Taq DNA polymerase), 3 µL of sample DNA

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(template), and sufficient sterile distilled water to bring the reaction volume to 100 µL (Table E-3). The reaction conditions were 95 C for 5 min to denature the template DNA, followed by 30 cycles of: 95 C for 30 sec (DNA denaturation step), 59 C for 30 sec (primer annealing step), and 72 C for 1 min (primer extension step) (Table E-4). The PCR was carried out in a programmable thermal cycler (Model PTC-100, MJ Research, Watertown, Massachusetts). After PCR, samples were electrophoresed at 8.0 V/cm in a 2% agarose gel for 1.5 h to determine relative size of bands amplified by the Vec primers for each sample. The gel was stained with ethidium bromide and destained in distilled water.

Results and Discussion

Multiple glass aquaria systems designed for isolation of groups or families during genetic experiments have been described (Rottman and Campton 1989). However, readily available alternatives to all-glass aquaria offer a cost-saving approach (Goodfellow et al. 1985; Rottmann and Shireman 1988). The system described in this study allowed isolation of treatment groups until sampling. The 20-L culture vessels had the added utility of being able to serve as egg incubation tanks and fry holding tanks. Individual culture vessels could be easily removed from the system because they were not attached to the drain.

Of the 34 potentially transgenic embryos screened, bands of the expected 846 bp size were seen in 16 (47%). No bands were seen in any of the lanes corresponding to control fish (Figure 5-4). Additional screening will be required to determine if these results are consistent with other groups of eggs treated in the same manner. Hayat et al. (1991) microinjected a variety of growth hormone genes and promoters into fertilized eggs of common carp Cyprinus carpio and channel catfish. The fertilized eggs were microinjected at the 1-, 2-, or 4-cell stages of development. The highest integration rate reported following Southern blot analysis was 11.5% for common carp injected at the 2-cell stage, and 15.2% for channel catfish injected at the 1-cell stage.

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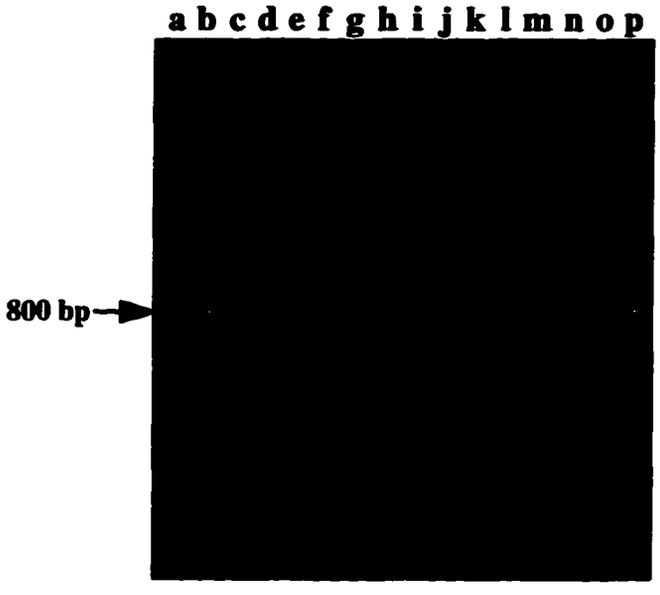

Figure 5-4. Agarose gel used to determine size of fragments amplified by PCR with Vec primers. Samples were electrophoresed at 8 v/cm in a 2% agarose gel. Lane "a" was loaded with a DNA marker (100-base pair ladder). Lanes "b"-"k" correspond to potentially transgenic embryos. Lane "l" is a negative control lane (no DNA loaded). Lanes "m" and "n" correspond to control embryos. Lane "o" was loaded with DNA marker. Lane "p" was loaded with the transfection vector pPC6 as a positive control. Five lanes ("b", "c", "d", "e", and "j") contain the expected 846-base pair band for the cecropin fragment.

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Age of fish at screening was not reported. Dunham et al. (1987) reported integration rates of 10-20% for channel catfish hatched from eggs that were microinjected with the metallothionein-human growth hormone fusion gene. Powers et al. (1992) compared electroporation and microinjection as methods for transferring rainbow trout growth hormone linked to the Rous sarcoma virus promoter into eggs of channel catfish. Treatment groups that were electroporated ranged from 0 to 100% transgenic with a mean of 65%. Treatment groups that were microinjected ranged from 0-33% transgenic.

All of these studies used Southern blotting (Maniatis et al. 1982) or an initial screening with PCR followed by Southern blotting to verify integration of the foreign gene with genomic DNA in the sample. In Southern blotting, genomic DNA is electrophoresed, denatured to yield single stranded DNA, and transferred or blotted from agarose to a nitrocellulose or nylon filter. The filter or blot is then incubated in a buffer commonly containing a radioactively labeled DNA probe which has a nucleotide sequence complementary to that of the gene of interest. Where the probe encounters a complementary strand of DNA (in this case the gene of interest) hybridization produces a radioactive band. If the transgene is integrated with genomic DNA it will have migrated with larger molecular weight DNA fragments during electrophoresis (Perbal 1988). If the DNA has remained as a discrete unincorporated fragment it will migrate with smaller molecular weight fragments or will run off the end of the gel during electrophoresis.

The PCR is a useful screening technique in that it indicates presence of the gene of interest in a sample, but gives no indication of integration or functionality. To determine integration, PCR must be coupled with another technique to provide definitive proof of transgenic status. Southern blots have been the most common method used to verify incorporation. However, methods that will offer more information about transgenic status are being developed. For example, the method of in-situ PCR (ISPCR) has the potential to show integration, function, and location of a transgene. This method uses labelled primers to detect a particular gene on tissue sections, in cell nuclei, or on

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chromosomes (Zhang 1996). Therefore, ISPCR can potentially be used to show integration, identify transfected tissues, and determine number of copies and site of transgene integration on chromosomes.

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CHAPTER 6
Summary and Conclusions

The objectives of this dissertation project were to determine if catfish from Lake Maurepas, Louisiana, could be used as a model fish for genetic research; develop techniques for the collection of unfertilized eggs for electroporation; determine the effect of electroporation of eggs on the resulting embryos, and develop isolation and screening methods for channel catfish. Before work could begin on any of these objectives, a laboratory suitable for working with transgenic fish had to be constructed.

All transgenic research was carried out in a 100 m^2 indoor facility which first went into operation shortly before the 1994 spawning season. The laboratory housed eight independent closed recirculating systems (Figure 6-1). System "A" (detailed in chapter 5) was designed for maintenance of channel catfish fry in separate treatment groups and included five 40-L round tanks and a two-tiered rack that could be fitted with either one hundred 2-L, or twenty four 20-L culture vessels (Bates and Tiersch 1995). Systems "B" and "C" were designed for holding fingerlings and each had three 1,000-L round, fiberglass tanks. System "D" had two 1,000-L and two 400-L round, fiberglass tanks. Systems "E" and "F" were designed for holding larger fish and each had a single 2,500-L round, fiberglass tank. System "G" was designed for work with shellfish and includes five 400-L round, fiberglass culture tanks. System "H" was designed as a multiple-use culture system and included a two-tiered rack with five 60-L glass aquaria on each tier. All systems were equipped with ultraviolet (UV) filters placed after the biological filter for control of pathogenic organisms (and larvae of shellfish in system "H"). All systems featured magnetically driven pumps and bead-filter biofiltration. All culture tanks utilized a center inner standpipe surrounded by an outer standpipe to pull water from the bottom of the tank for self-cleaning. All inner standpipes were screened to exclude passage of the smallest organism in the tank. There were no direct plumbing connections from any of the culture systems with the drainage system in the building. Sump tank tops were

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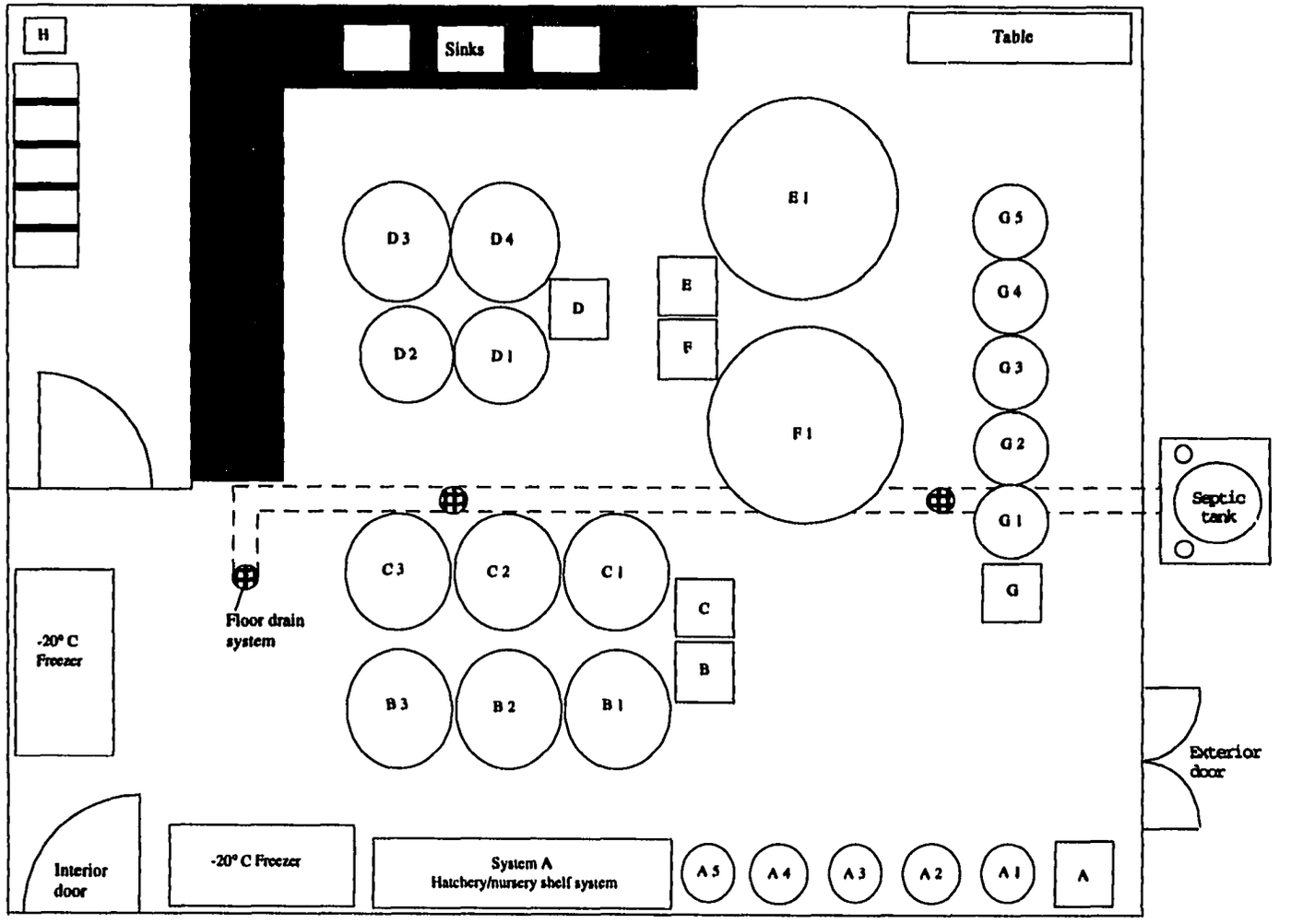

Figure 6-1. Floorplan of the Transgenic Laboratory (Room 142 of Aquaculture Research Laboratory, Ben Hur Aquaculture Research Farm, Baton Rouge, Louisiana). Squares containing uppercase letters represent the biofilters and sumps for each recirculating system. Location of floordrains and septic tank are also indicated.

screened to prevent escape of fish from that possible route. The systems were designed so that if the standpipe was removed, the water level in the culture tanks would not go below the level of the top of the sump. In addition, overflow holes were drilled in the lip of the sump tank (opposite the side of the pump) to ensure that water did not flow onto the pump. This allowed water changes to be made through the sump without the need to remove the standpipes from tanks occupied by fish. The facility is equipped with an emergency generator which supplies power to vital systems (recirculating systems) in the event of power failure. In addition all wet laboratories are equipped with chlorinated and dechlorinated water sources.

In 1995, a set of voluntary guidleines was released by the Agricultural Biotechnology Research Advisory Committee Working Group on Aquatic Biotechnology and Environmental Safety was released by the USDA. The guidelines were adopted for the management of the Transgenic Laboratory and an operational plan was developed (Appendix E).

The study on the early maturing catfish population from Lake Maurepas (LM) served to pull together different types of information from several sources to test the stated hypotheses. These dealt with testing whether the LM populations were correctly identified as channel catfish or if they should be considered as a distinct subspecies or possibly a hybrid between channel catfish and another ictalurid.

Analysis of meristic characteristics showed no external differences between LM channel catfish when compared to reference meristics for channel catfish. In addition, no indication of hybridization was observed from this analysis. Flow cytometry showed that the genome size of LM and Lac Des Allemands (LDA) channel catfish was not different from that of the commercial Kansas strain of channel catfish. This verified that the LM and LDA channel catfish had not undergone divergence that altered the number of chromosome sets or the cellular DNA content.

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Karyotyping indicated that the LM channel catfish, LM channel catfish x channel catfish (LSU population), and channel catfish karyotypes were indistinguishable from one another.

The technique of nucleolus organizer region (NOR) staining of chromosomes yielded identical results for LM channel catfish, LM channel catfish x channel catfish (LSU population) crossbreed, and channel catfish (LSU population). For all, the NOR-bearing chromosome pairs were submetacentric chromosomes. The karyotype of channel catfish x blue catfish Ictalurus furcatus hybrid was indistinguishable from those of the LM channel catfish, LM channel catfish x channel catfish crossbreed, and channel catfish in chromosome number. However, in the chromosome spread of the channel catfish x blue catfish hybrid, it was found that one of the NOR-bearing chromosomes was a submetacentric (as in the channel catfish) while the other was a metacentric. This difference would allow differentiation of channel catfish, blue catfish, and their hybrids.

Nucleotide sequence analysis was performed on a 245 base pair fragment of the IgH gene amplified by the polymerase chain reaction (PCR). This gene (which encodes fourth exon of the immunoglobulin heavy chain constant region) was sequenced and described by Wilson et al. (1990). A channel catfish (LSU population) IgH gene sequence which was verified by alignment with the published sequence was used as a reference. No sequence differences were found among the LM channel catfish, channel catfish reference, and published sequence. Two single nucleotide differences were found between the channel catfish and blue catfish sequences. Several nucleotide sequence differences were found between the channel catfish, blue catfish, and black bullhead catfish Ameirus melas. These differences appear sufficient to differentiate the three species and their hybrids by restriction enzyme digest. Successful spawning of LM channel catfish in late August and September documented that these fish spawn at a small size and outside of the normal spawning season for this species in southern Louisiana (late April to mid June).

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All channel catfish spawning described in this dissertation was performed in closed recirculating systems. During the 1994 and 1995 spawning seasons, channel catfish females were injected with synthetic luteinizing-hormone releasing-hormone and paired with males for spawning. This method required constant monitoring to obtain ovulating females for stripping of eggs. This method is expensive, unreliable and time-consuming. During the 1996 spawning season, half of the females were spawned as male-female pairs and the rest were spawned in groups of females. The grouped females were injected with the same dose of hormone as the paired females and were checked for ovulation starting ~36 h after injection. The fish were netted and gentle pressure was applied to their abdominal area. If a female did not release eggs, it was moved to another tank. Ovulating fish were removed from the tank and stripped. Spawning success (the percentage of females that produced eggs) for the grouped females (58%) was not different ($P = 0.64$) from that of the paired females (41%). However, fertilization for the eggs of the grouped females (mean $\pm$ SD, $16 \pm 20\%$) was significantly lower ($P = 0.02$) than that for eggs of the paired females ($43 \pm 37\%$). We suspect that the eggs were overripe, because they were stripped too late. The only published account of this type of spawning with catfishes was with the Indian catfish Heteropneustes fossilis (Alok et al. 1993) and the Asian catfish Clarias macrocephalus (Mollah and Tan 1983). In these species, eggs are not stripped until they flow from the urogenital pore when little or no pressure is applied to the abdomen. We hypothesize that waiting until eggs flow from the urogenital pore of channel catfish with little or no pressure will yield poor quality eggs.

If proper timing can be determined to reliably strip high quality channel catfish eggs, grouped spawning has potential for the production of transgenic fish and hybrids such as the channel catfish x blue catfish hybrid. One of the limiting factors in production of this hybrid in numbers large enough for commercial production is the lack of a reliable technique to obtain unfertilized channel catfish eggs.

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Assuming unfertilized eggs can be obtained for the production of transgenic channel catfish, the next step is to deliver the transgene into the egg. Electroporation is a popular technique because many eggs can be treated synchronously. The data presented in this study indicate that although electroporation of eggs has an effect on fertilization rate, this effect is small in relation to the large number of eggs that can be treated at once (Powers 1995). We have produced transgenic fish over the last four spawning seasons (1993 to 1996) with this technique. However, more research needs to be done on screening and characterization of our transgenic population. In addition, other techniques such as lipofection (the use of lipid-DNA complexes to deliver foreign DNA into a target cell) should be tested and evaluated (Felgner and Rungold 1989). Lipofection has the potential to transfect the gametes of fully mature animals which can then be spawned normally to produce potentially transgenic offspring (Etches et al. 1993).

A screening method utilizing the polymerase chain reaction (PCR) was described in Chapter 5. This technique detected the cecropin gene in 47% of the embryos tested. A positive PCR reaction indicated only that the cecropin gene was present in the sample tested. It did not demonstrate that the gene was integrated into the genome of the particular catfish or if the gene was functional. More work needs to be done to determine if the transgene is functional.

Fish that test positive for the presence of the transgene should be challenged with bacteria or bacterial cell components to elicit expression of the gene. The technique reverse transcriptase PCR (RT-PCR) utilizes the enzyme reverse transcriptase which is isolated from retro-viruses (Zhang and McCabe 1992). Reverse transcriptase produces a complementary DNA copy from a messenger RNA (mRNA) transcript. With RT-PCR a positive result is obtained if the mRNA transcript from the gene of interest is present in the sample. The mRNA transcript will be present if the gene is integrated and being transcribed. Even if the transgene is integrated correctly and being transcribed into mRNA, a mature functional protein may not be translated or processed correctly to be

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secreted into the circulatory system where it could be of use. Hew et al. (1995) successfully transferred the winter flounder Pleuronectes americanus antifreeze protein (AFP) gene to Atlantic salmon Salmo salar. They found that only 40% of the transgenic salmon produced AFP at detectable levels in the blood. Upon further investigation the protein was identified as proAFP rather than the AFP. Now that successful delivery of the cecropin gene into channel catfish eggs has been accomplished, as evidenced by positive PCR results on tested embryos, the focus of future research should be to verify integration and function of the transgene.

The work described in this dissertation project could not have been done without the ability to collect unfertilized eggs. This need for eggs encouraged experimentation and deviation from conventional methods for the collection of unfertilized eggs from channel catfish (Huner and Dupree 1984). Further improvement of grouped female spawning will have applications besides the production of transgenic fish. Pond spawning, the method used by the commercial catfish industry to produce channel catfish has not changed for at least 70 years (Clapp 1929). While effective for the production of large numbers of fish, pond spawning is not a good method for genetic improvement. The major livestock industries have made great strides in genetic improvement because of selection programs made possible by artificial insemination. There is great potential for genetic improvement of commercial traits in channel catfish (Smitherman 1978).

The technology for refrigerated storage and cryopreservation of channel catfish sperm is available for application within the industry. Artificial spawning, the collection of eggs from a female followed by fertilization with sperm from one or more males, is a powerful method for genetic improvement of fish. However, the limiting factor is the production of high-quality unfertilized channel catfish eggs. This shortcoming must be overcome if the catfish industry is to achieve the level of genetic improvement realized by the major livestock industries.

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Selective breeding programs for channel catfish could follow any of three models; extensive, intensive, or shared risk. In the extensive model, each farm would practice artificial spawning in an attempt to improve important traits in their fish. In the intensive model, fry would be produced by artificial spawning at specialized commercial hatcheries and distributed to customer farms. In the shared risk model, based on the breeders cooperative theme, the industry would fund research and production efforts at specialized cooperative hatcheries and would recieve the resulting fry. Adoption of one of these models by the commercial catfish industry would be a first step in improving fry and fingerling production techniques that have remained essentially unchanged for 70 years.

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APPENDIX A
Raw Data

Note: All data included in this appendix has been archived on a "ZIP" disk labeled "Production of transgenic channel catfish data." The disk is in the posession of Dr. Terrence R. Tiersch, room #204, Louisiana State University Aquaculture Research Laboratory, 2410 Ben Hur Road, Baton Rouge, Louisiana, 70816.

Table A-1. Raw genome size data (picograms of DNA per nucleus) for Lake Maurepas channel catfish (n = 36), Lac Des Allemands channel catfish (n = 10), and Kansas strain channel catfish (n = 15).

Lake Maurepas	Lac Des Allemands	Kansas
2.107	2.144	2.115
2.117	2.102	2.112
2.099	2.121	2.104
2.102	2.115	2.104
2.124	2.119	2.121
2.119	2.128	2.116
2.091	2.115	2.097
2.126	2.119	2.107
2.095	2.110	2.118
2.122	2.105	2.107
2.108		2.102
2.112		2.112
2.106		2.114
2.086		2.101
2.107		2.100
2.108		
2.117		
2.116		
2.100		
2.115		
2.125		
2.123		
2.114		
2.120		
2.120		
2.109		
2.109		
2.111		
2.120		
2.098		
2.095		
2.097		
2.096		
2.091		
2.078		
2.086		

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Table A-2. Total length, long-arm length, and short-arm length of chromosomes staining positive for nucleolus organizer regions (NORs) from karyotypes of five channel catfish x blue catfish hybrids. Chromosome 1 of each pair was stained darker than its homolog.

NOR Spread	Chromosome	Total length	Long-arm length	Short-arm length
1	1	1.4007	0.7709	0.6297
1	2	1.4506	1.0542	0.3963
2	1	0.9609	0.4980	0.4629
2	2	1.0683	0.7085	0.3597
3	1	0.7221	0.4460	0.2760
3	2	0.9040	0.6442	0.2598
4	1	0.9016	0.4980	0.4035
4	2	0.8435	0.5957	0.2477
5	1	0.5650	0.2825	0.2825
5	2	0.8750	0.5451	0.3294

Table A-3. Total length, long-arm length, and short-arm length of chromosomes staining positive for nucleolus organizer regions (NORs) from karyotypes of five channel catfish x Lake Maurepas channel catfish crossbreeds. Gross morphological differences were not observed, thus chromosome designations as 1 or 2 in each pair was arbitrarily assigned.

NOR Spread	Chromosome	Total length	Long-arm length	Short-arm length
1	1	0.6633	0.3995	0.2638
1	2	0.5922	0.3306	0.2616
2	1	0.9765	0.6433	0.3332
2	2	0.9855	0.6612	0.3243
3	1	0.9542	0.5957	0.3585
3	2	0.9740	0.6677	0.3062
4	1	0.8824	0.6205	0.2618
4	2	0.7589	0.5256	0.2332
5	1	0.8818	0.5018	0.3799
5	2	0.8013	0.5337	0.2675

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Table A-4. Percent fertilization, percent hatch, voltage, number of pulses, and presence or absence of transfection vector (DNA) and isopropyl-ß-D-thiogalactopyranoside (IPTG) in treatments for the 1994 channel catfish spawning season.

Spawn	Fertilization (%)	Hatch (%)	Voltage (V/cm^2)	Pulses	DNA	IPTG
1	0	0	0	0	Absent	Absent
1	0	0	0	0	Present	Absent
1	0	0	122	1	Present	Present
1	0	0	122	1	Absent	Present
1	0	0	122	1	Present	Absent
1	0	0	178	1	Present	Present
1	0	0	178	1	Absent	Present
1	0	0	178	1	Present	Absent
1	0	0	233	1	Present	Present
1	0	0	233	1	Absent	Present
1	0	0	233	1	Present	Absent
1	0	0	289	1	Present	Present
1	0	0	289	1	Absent	Present
1	0	0	289	1	Present	Absent
1	0	0	289	1	Absent	Absent
1	0	0	556	1	Absent	Absent
1	0	0	833	1	Absent	Absent
1	0	0	1,111	1	Absent	Absent
2	0	0	0	1	Absent	Absent
2	0	0	117	1	Absent	Absent
2	0	0	133	1	Absent	Absent
2	0	0	150	1	Absent	Absent
2	0	0	167	1	Absent	Absent
2	0	0	183	1	Absent	Absent
3	29	14	0	0	Absent	Absent
3	86	46	125	1	Absent	Present
3	43	6	125	1	Present	Present
3	36	1	142	1	Absent	Present
3	46	19	142	1	Present	Present
3	90	53	158	1	Absent	Present
3	93	67	158	1	Present	Present
3	13	2	175	1	Absent	Present
3	57	19	175	1	Present	Present
4	31	7	0	1	Absent	Absent
4	44	11	125	1	Absent	Present
4	32	6	125	1	Present	Present
4	44	13	142	1	Absent	Present
4	21	2	142	1	Present	Present
4	23	11	158	1	Absent	Present
4	40	24	158	1	Present	Present
4	30	9	175	1	Absent	Present
4	32	10	175	1	Present	Present
5	80	31	0	1	Absent	Absent
5	84	46	125	1	Absent	Present
5	96	55	125	1	Present	Present

(Table A-4 cont'd).

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Spawn	Fertilization (%)	Hatch out (%)	Voltage (V/cm^2)	Pulses	DNA	IPTG
5	72	42	142	1	Absent	Present
5	89	62	142	1	Present	Present
5	39	8	158	1	Absent	Present
5	51	11	158	1	Present	Present
5	41	3	158	1	Absent	Present
5	69	48	175	1	Present	Present
5	10	2	142	1	Present	Present
6	2	1	0	0	Absent	Absent
6	2	1	122	1	Absent	Absent
6	1	0	122	1	Present	Present
6	1	0	178	1	Absent	Absent
6	1	1	178	1	Present	Present
6	1	0	233	1	Absent	Absent
6	0	0	233	1	Present	Present
6	0	0	289	1	Absent	Absent
6	0	0	289	1	Present	Present
7	12	12	0	0	Absent	Absent
7	9	9	233	1	Absent	Absent
7	11	11	233	1	Present	Present
7	12	12	233	3	Absent	Absent
7	12	12	233	3	Present	Present
7	3	3	233	6	Absent	Absent
7	7	7	233	6	Present	Present
7	7	7	288	1	Absent	Absent
7	13	13	288	1	Present	Present
7	19	19	288	3	Absent	Absent
7	3	3	288	3	Present	Present
7	7	7	288	6	Absent	Absent
7	4	4	288	6	Absent	Absent
8	1	1	0	0	Absent	Absent
8	0	0	233	1	Absent	Absent
8	1	1	233	1	Present	Present
8	0	0	233	3	Absent	Absent
8	1	1	233	3	Present	Present
8	0	0	233	6	Absent	Absent
8	8	8	233	6	Present	Present
8	0	0	288	1	Absent	Absent
8	0	0	288	1	Present	Present
8	0	0	288	3	Absent	Absent
8	0	0	288	3	Present	Present
8	0	0	288	6	Absent	Absent
8	0	0	288	6	Absent	Absent
9	1	1	0	0	Absent	Absent
9	0	0	233	1	Absent	Absent
9	1	1	233	1	Present	Present
9	1	1	233	3	Absent	Absent
9	0	0	233	3	Present	Present
9	0	0	233	6	Absent	Absent

(Table A-4. cont'd).

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Spawn	Fertilization (%)	Hatch out (%)	Voltage (V/cm^2)	Pulses	DNA	IPTG
9	0	0	233	6	Present	Present
9	5	5	288	1	Absent	Absent
9	0	0	288	1	Present	Present
9	0	0	288	3	Absent	Absent
9	0	0	288	3	Present	Present
9	0	0	288	6	Absent	Absent
9	0	0	288	6	Absent	Absent
10	0	0	0	0	Absent	Absent
10	0	0	122	1	Absent	Absent
10	0	0	178	1	Absent	Absent
10	0	0	233	1	Absent	Absent
10	0	0	288	1	Absent	Absent
10	0	0	344	1	Absent	Absent
10	0	0	400	1	Absent	Absent
10	0	0	456	1	Absent	Absent
10	0	0	511	1	Absent	Absent
10	0	0	567	1	Absent	Absent
10	0	0	622	1	Absent	Absent
10	0	0	678	1	Absent	Absent
10	0	0	733	1	Absent	Absent

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Table A-5. Percent fertilization, percent hatch, voltage, number of pulses, and presence or absence of transfection vector (DNA) and isopropyl-ß-D-thiogalactopyranoside (IPTG) in treatments for the 1995 channel catfish spawning season.

Spawn	Fertilization (%)	Hatch out (%)	Voltage (V/cm^2)	Pulses	DNA	IPTG
1	100	1	0	0	Absent	Absent
1	98	31	0	0	Present	Absent
1	85	18	83	1	Present	Present
1	90	0	83	1	Absent	Absent
1	90	10	125	1	Present	Present
1	90	15	125	1	Absent	Absent
1	90	23	167	1	Present	Present
1	85	16	167	1	Absent	Absent
2	75	2	0	0	Absent	Absent
2	80	17	0	0	Present	Absent
2	70	3	83	1	Present	Present
2	75	1	83	1	Absent	Absent
2	65	1	125	1	Present	Present
2	70	2	125	1	Absent	Absent
2	55	26	167	1	Present	Present
2	55	5	167	1	Absent	Absent
3	85	4	0	0	Absent	Absent
3	90	1	83	1	Present	Present
3	85	8	125	1	Present	Present
3	90	0	167	1	Present	Present
4	80	2	0	0	Absent	Absent
4	85	0	83	1	Present	Present
4	90	11	125	1	Present	Present
4	90	0	167	1	Present	Present
5	75	13	83	1	Present	Present
5	75	12	83	1	Absent	Present
5	65	3	83	3	Present	Present
5	65	5	125	1	Absent	Present
5	60	1	125	1	Present	Present
5	50	1	167	1	Absent	Present
5	35	2	167	1	Present	Present
5	30	0	167	3	Present	Absent
6	90	19	83	1	Present	Present
6	60	4	83	1	Absent	Present
6	70	6	83	2	Present	Present
6	80	7	83	2	Absent	Present
6	60	3	83	3	Present	Present
6	5	0	83	3	Absent	Present
6	10	3	167	1	Present	Present
6	20	4	167	1	Absent	Present
6	15	1	167	2	Present	Present
6	15	0	167	2	Absent	Present
6	10	4	167	3	Present	Present
6	10	2	167	3	Absent	Present
6	80	4	0	0	Absent	Absent

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Table A-6. Spawn, treatment group, percent fertilization, percent hatch, and 2-week percent survival for the 1996 channel catfish spawning season.

Spawn	Treatment group	Fertilization (%)	Hatch (%)	Two-week survival (%)
1	A	90	11	3
1	A	90	18	12
1	A	90	23	22
1	B	80	30	14
1	B	75	28	12
1	B	80	32	17
1	C	75	32	13
1	C	60	21	6
1	C	70	41	22
1	D	75	50	20
1	D	50	40	14
1	D	75	41	22
2	A	20	3	2
2	A	10	0	0
2	A	10	2	1
2	B	5	0	0
2	B	5	8	8
2	B	5	11	11
2	C	5	3	2
2	C	2	11	5
2	C	2	0	0
2	D	1	0	0
2	D	2	0	0
2	D	1	2	1
3	A	70	36	28
3	A	75	6	3
3	A	55	28	22
3	B	50	39	28
3	B	40	39	22
3	B	45	22	22
3	C	70	19	17
3	C	60	7	7
3	C	60	17	14
3	D	50	25	22
3	D	50	19	17
3	D	60	14	11

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Table A-7. Percent fertilization, voltage, number of pulses, and presence or absence of transfection vector (DNA) and isopropyl-ß-thiogalactopyranoside (IPTG) for the 1996 channel catfish spawning season for production spawns.

Spawn[a]	Fertilization (%)	Voltage (V/cm^2)	Pulses	DNA	IPTG
P1	95	125	2	Present	Present
P1	95	125	2	Present	Present
P1	95	125	2	Present	Present
P1	95	125	2	Present	Present
P1	95	125	2	Present	Present
P2	0	750	2	Present	Present
P2	0	750	2	Present	Present
P2	0	750	2	Present	Present
P2	0	750	2	Present	Present
P2	0	750	2	Present	Present
P3	5	0	0	Absent	Absent
P3	5	0	0	Absent	Absent
P3	5	750	2	Present	Present
P3	5	750	2	Present	Present
P3	5	750	2	Present	Present
P3	5	750	2	Present	Present
P4	50	0	0	Absent	Absent
P4	50	750	2	Present	Present
P4	50	750	2	Present	Present
P5	60	0	0	Absent	Absent
P5	60	750	2	Present	Present
P5	60	750	2	Present	Present
P6	0	0	0	Absent	Absent
P6	0	0	0	Absent	Absent
P6	0	750	2	Present	Present
P6	0	750	2	Present	Present
P6	0	750	2	Present	Present
P6	0	750	2	Present	Present
P7	25	0	0	Absent	Absent
P7	25	0	0	Absent	Absent
P7	20	750	2	Present	Present
P7	20	750	2	Present	Present
P7	25	750	2	Present	Present
P8	15	0	0	Absent	Absent
P8	15	0	0	Absent	Absent
P8	10	750	2	Present	Present
P8	10	750	2	Present	Present
P8	10	750	2	Present	Present
P9	0	0	0	Absent	Absent
P9	0	0	0	Absent	Absent
P9	0	750	2	Present	Present
P9	0	750	2	Present	Present
P9	0	750	2	Present	Present
P10	2[b]	0	0	Absent	Absent
P10	2[b]	0	0	Absent	Absent
P10	2[b]	0	0	Absent	Absent
P10	2[b]	0	0	Absent	Absent

(Table A-7 cont'd).

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Spawn	Fertilization (%)	Voltage (V/cm^2)	Pulses	DNA	IPTG
P11	10*	0	0	Absent	Absent
P12	15*	0	0	Absent	Absent
P12	10*	0	0	Absent	Absent
P12	10*	0	0	Absent	Absent
P13	1	0	0	Absent	Absent
P13	1	750	2	Present	Present
P13	1	750	2	Present	Present

[a] P, Production spawn

[b] Fertilized with cryopreserved sperm.

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APPENDIX B
Successful Spawns

Table B-1. Successful spawning pairs from the 1994 channel catfish spawning season, time of synthetic luteinizing-hormone releasing-hormone (LHRHa) injection, time of spawn, and latency period between hormone injection and spawning.

Female			Male					Latency	
Fish No.	Weight (Kg)	Total Length (mm)	Fish No.	Weight (Kg)	Total Length (mm)	Time of injection[d]	Time of spawn	Days	Hours
CCF94F9	1.43	510	CCF96M6	1.83	542	5/23/94 1700	5/25/94 2030	2.2	51.5
CCF94F14	•	•	CCF96M18	•	•	5/23/94 1700	5/26/94 0300	2.4	58.0
CCF94F11	2.37	600	CCF96M11	2.40	610	5/23/94 1700	5/28/94 0347	4.5	106.8
CCF94F16[b]	2.00	•	CCF96M12	1.55	530	5/23/94 1700	5/31/94 2400	7.3	175.0
CCF94F15[b]	1.55	•	CCF96M20	1.99	600	5/23/94 1700	6/1/94 0110	8.3	200.2
CCF94F20[ac]	3.37	635	•	•	•	5/31/94 1430	6/6/94 0210	5.5	131.7
CCF94F25	2.97	640	CCF96M33	2.86	664	5/31/94 1430	6/8/94 1800	8.2	195.5
CCF94F24	3.59	630	CCF96M33	2.86	664	6/6/94 1530	6/9/94 2145	3.3	78.3
CCF94F26[a]	3.76	630	CCF94M32	3.74	690	6/6/94 1530	6/11/94 2045	5.2	125.3
CCF94F32	3.74	690	CCF94M31	3.14	650	6/6/94 1530	6/14/94 1100	7.8	187.5
CCF94F34	3.75	640	CCF96M33	2.86	664	6/13/94 1755	6/15/94 1200	1.8	42.1
CCF94F35	2.33	595	CCF94M45	1.94	590	6/15/94 1315	6/17/94 0130	1.5	36.3
CCF96F23[a]	2.14	590	CCF94M30	2.66	610	6/6/94 1530	6/18/94 1000	11.8	282.5

Abbreviations used in table: CCF, LSU select channel catfish; F, female; M, male.

[a] Eggs not used in electroporation experiments

[b] Female was injected a second time (5/30/94 1700)

[c] Female was injected a second time (6/5/94 1140)

[d] All injections were 100 µg LHRHa/Kg of body weight

• Data not available

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Table B-2. Successful spawning pairs from the 1995 channel catfish spawning season, time of synthetic luteinizing-hormone releasing-hormone (LHRHa) injection, time of spawn, and latency period between hormone injection and spawning.

Female			Male					Latency	
Fish No.	Weight (Kg)	Total Length (mm)	Fish No.	Weight (Kg)	Total Length (mm)	Time of injection	Time of spawn	Days	Hours
CCF95F1	2.20	572	CCF95M4	2.70	598	5/12/95 1400	5/18/95 2215	6.3	152.3
CCF95F6	2.80	632	CCF95M1	2.70	590	5/12/95 1400	5/18/95 2315	6.4	153.3
CCF95F8	2.50	618	CCF95M3	2.50	560	5/12/95 1400	5/19/95 0600	6.7	160.0
CCF95F3	2.00	564	CCF95M8	2.60	618	5/12/95 1400	5/19/95 0745	6.7	161.8
CCF95F12	2.20	590	CCF95M17	2.60	610	5/23/95 1540	5/24/95 2000	1.2	28.3
CCF95F27	2.80	525	CCF95M27	4.00	650	5/30/95 1500	5/31/95 2221	1.3	31.4
CCF95F34	1.50	510	•	•	•	6/3/95 1645	6/8/95 0205	4.4	105.3
LM95F3	1.40	490	LM95M3	1.10	440	6/7/95 2055	6/11/95 0720	3.4	82.4
LM95F5	0.37	325	LM95M4	0.65	385	6/10/95 2050	6/17/95 1125	2.9	68.9
LM95F4[a]	0.80	400	CCF95M30	1.70	520	6/14/95 1430	6/17/95 1300	2.9	70.5
WLM95F2[a]	0.50	375	WLM95M2	0.18	280	8/27/95 1955	8/29/95 1810	1.9	46.3
WLM95F1[a]	0.11	240	WLM95M1	0.08	230	8/29/95 2015	9/10/95 1130	11.7	279.3

Abbreviations used in table: CCF, LSU select channel catfish; F, female; M, male; LM, pond held (1-2 years) Lake Maurepas channel catfish; WLM, wild Lake Maurepas channel catfish.

[a] Eggs not used in electroporation experiments

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Table B-3. Successful spawning pairs from the 1996 channel catfish spawning season, time of synthetic luteinizing-hormone releasing-hormone (LHRHa) injection, time of spawn, and latency period between hormone injection and spawning.

Female			Male					Latency	
Fish No.	Weight (Kg)	Total Length (mm)	Fish No.	Weight (Kg)	Total Length (mm)	Time of injection	Time of spawn	Days	Hours
CCF96F2	2.1	572	CCF96M1	2.6	660	4/29/96 1225	5/1/96 1540	2.1	51.3
CCF96F14	2.4	559	CCF96M9	2.7	597	5/7/96 1120	5/9/96 0350	1.7	40.5
CCF96F17	2.7	610	CCF96M5	3.1	610	5/7/96 1255	5/9/96 0500	1.7	40.1
CCF96F12	2.5	584	CCF96M4	2.7	597	5/7/96 1055	5/9/96 1340	2.1	50.8
CCF96F15[a]	2.6	610	CCF96M6	2.8	584	5/7/96 1130	5/9/96 2040	2.4	57.2
CCF96F33A[a]	2.1	572	CCF96M20	2.0	610	5/14/96 1303	5/16/96 0100	1.5	36.0
CCF96F27A	2.9	686	CCF96M16	2.7	635	5/14/96 1235	5/16/96 0223	1.6	37.8
CCF96F32A	2.2	584	CCF96M21	2.0	597	5/14/96 1258	5/16/96 1235	2.0	47.6
CCF96F29A[a]	3.0	673	CCF96M17	2.7	635	5/14/96 1245	5/16/96 1640	2.2	51.9
CCF96F43[a]	2.5	597	CCF96M33	2.5	610	5/21/96 1508	5/23/96 0300	1.5	35.9
CCF96F44	2.3	570	CCF96M39	2.4	600	5/22/96 1520	5/23/96 2250	1.3	31.5
CCF96F45[a]	1.9	530	CCF96M41	2.4	630	5/22/96 1532	5/24/96 1055	1.8	43.4

Abbreviations used in table: CCF, LSU select channel catfish; F, female; M, male; LM, pond held (1-2 years) Lake Maurepas channel catfish; WLM, wild Lake Maurepas channel catfish.

[a] Eggs not used in electroporation experiments

• Data not available

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Table B-4. Successfully stripped females not paired with a male from the 1996 channel catfish spawning season, time of synthetic luteinizing-hormone releasing-hormone (LHRHa) injection, time of stripping, and latency period between hormone injection and stripping.

Female					Latency	
Fish No.	Weight (Kg)	Total Length (mm)	Time of injection	Time stripped	Days	Hours
CCF96F7	1.90	572	4/29/96 1230	5/2/96 1015	2.9	69.8
CCF96F4	1.90	546	5/2/96 1450	5/5/96 1100	2.8	68.2
CCF96F20	3.10	635	5/7/96 1410	5/9/96 0938	1.8	43.5
CCF96F21	2.10	546	5/7/96 1423	5/9/96 1040	1.9	44.3
CCF96F23	1.70	508	5/7/96 1439	5/9/96 1230	1.9	45.9
CCF96F24	3.00	610	5/7/96 1445	5/9/96 1452	2.0	48.1
CCF96F26	2.10	559	5/7/96 1500	5/9/96 1530	2.0	48.5
CCF96F38A	2.90	660	5/14/96 1334	5/16/96 1440	2.1	49.1
CCF96F49	2.00	545	5/22/96 1615	5/24/96 1455	1.9	46.7
CCF96F48	2.30	560	5/22/96 1610	5/24/96 1650	2.0	48.7
CCF96F46[a]	3.00	620	5/22/96 1600	5/25/96 0915	2.7	65.3
CCF96F57	6.50	780	6/4/96 1350	6/6/96 1133	1.9	45.7
CCF96F55[a]	3.30	640	6/4/96 1335	6/6/96 1230	2.0	46.9
CCF96F56[a]	3.30	660	6/4/96 1340	6/6/96 1300	2.0	47.3
CCF96F63	0.53	410	6/29/96 1815	7/1/96 0800	1.6	37.8

Abbreviations used in table: CCF, LSU select channel catfish; F, female; M, male; LM, pond held (1-2 years) Lake Maurepas channel catfish; WLM, wild Lake Maurepas channel catfish.

[a] Eggs used in electroporation experiments.

• Data not available.

APPENDIX C
Data Analysis

Table C-1. Analysis of Variance (ANOVA) table for channel catfish genome size.

Source	df	Sums of squares	Mean square	F-ratio	P-value
Model	1	245.220	245.220	2450128	≤ 0.0001
Population	2	0.000529	0.000265	2.6443	0.0806
Error	52	0.005204	0.000100		
Total	54	0.005734			

Table C-2. Summary statistics for genome size of channel catfish populations.

Population	Mean	Std. dev.	n	Range
Kansas	2.109	0.007	15	0.024
Maurepas	2.111	0.010	30	0.040
Des Allemands	2.118	0.012	10	0.042

Table C-3. Two sample t-Test comparing the centromeric index (CI) of nucleolus organizer region (NOR) stained chromosomes from channel catfish x blue catfish hybrids to those from channel catfish x Lake Maurepas channel catfish crossbreeds ($P = 0.4184$, $n = 5$).

	CI% (Mean ± Std. dev.)
Channel catfish x blue catfish hybrids	38.3 ± 8.4%
Channel catfish x Lake Maurepas crossbreed	35.7 ± 5.2%

Table C-4. Two sample t-Test comparing the centromeric index (CI) of chromosome 1 to the CI of chromosome 2 from nucleolus organizer region (NOR) stained karyotypes of five channel catfish x blue catfish hybrids. The darker stained chromosome in each karyotype was designated as number 1 ($P = 0.0015$, $n = 5$).

Chromosome	CI% (Mean ± Std. dev.)
1	45.2 ± 4.5%
2	31.6 ± 4.2%

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Multiple analysis of variance (Manova) performed on arcsine-square-root-transformed 1994 spawning season data

Table C-5. Analysis of variation table for effect of electropoartion on fertilization of eggs during the 1994 channel catfish spawning season.

Source	df	Sums of squares	Mean square	F-ratio	P-value
Model	1	14.0631	14.0631	321.74	≤ 0.0001
Voltage	6	0.698837	0.116473	2.6647	0.0349
Pulse	3	0.125684	0.041895	0.95847	0.4255
DNA	1	0.006621	0.006621	0.15148	0.7000
IPTG	1	0.004108	0.004108	0.09399	0.7614
Error	29	1.26759	0.043710		
Total	40	3.13567			

Table C-6. Summary statistics for effect of voltage on fertilization of eggs during the 1994 channel catfish spawning season.

Voltage (V/cm^2)	Mean	n	Std. dev.
0	0.60343090	4	0.26002935
125	0.84251016	6	0.23411704
142	0.67057334	7	0.24810194
158	0.75503103	7	0.22863488
175	0.62669798	5	0.20242557
233	0.29158761	6	0.07047422
289	0.28019688	6	0.10154341

Table C-7. Means separation: Fisher's LSD for the effect of voltage on fertilization of eggs during the 1994 channel catfish spawning season.

Voltage	Difference	Std. error	P-value
125 - 0	0.111824	0.2175	0.611084
142 - 0	-0.062460	0.2140	0.772474
142 - 125	-0.174285	0.1165	0.145363
158 - 0	0.026693	0.2164	0.902671
158 - 125	-0.085131	0.1165	0.470694
158 - 142	0.089153	0.1124	0.434122
175 - 0	-0.107275	0.2194	0.628533
175 - 125	-0.219099	0.1269	0.094837
175 - 142	-0.044814	0.1224	0.717018
175 - 158	-0.133968	0.1233	0.286090
233 - 0	-0.433128	0.2049	0.043291
233 - 125	-0.544952	0.1627	0.002263
233 - 142	-0.370667	0.1580	0.026039
233 - 158	-0.459821	0.1612	0.007922
233 - 175	-0.325853	0.1652	0.058191
289 - 0	-0.446877	0.1982	0.031852
289 - 125	-0.558701	0.1709	0.002783
289 - 142	-0.384417	0.1664	0.028224
289 - 158	-0.473570	0.1695	0.009122
289 - 175	-0.339602	0.1733	0.059705
289 - 233	-0.013749	0.1226	0.911462

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Table C-8. Analysis of variation table for hatch rate of eggs electroporated during the 1994 channel catfish spawning season.

Source	df	Sums of squares	Mean square	F-ratio	P-value
Model	1	6.02954	6.02954	126.56	≤ 0.0001
Voltage	6	0.118682	0.019780	0.41519	0.8628
Pulse	3	0.028190	0.009397	0.19724	0.8974
DNA	1	0.027737	0.027737	0.58221	0.4516
IPTG	1	0.012320	0.012320	0.25860	0.6149
Error	29	1.38159	0.047641		
Total	40	1.66310			

Multiple analysis of variance (Manova) performed on arcsine-square-root-transformed 1995 spawning season data

Table C-9. Analysis of variation table for effect of electroporation on fertilization of eggs during the 1995 channel catfish spawning season.

Source	df	Sums of squares	Mean square	F-ratio	P-value
Model	1	31.9076	31.9076	1095.8	≤ 0.0001
Voltage	3	1.01392	0.337975	11.607	≤ 0.0001
Pulse	2	0.642086	0.321043	11.025	0.0002
DNA	1	0.161330	0.161330	5.5404	0.0240
IPTG	1	0.129135	0.129135	4.4348	0.0421
Error	37	1.07740	0.029119		
Total	44	2.87599			

Table C-10. Summary statistics for effect of voltage on fertilization of eggs during the 1995 channel catfish spawning season.

Voltage (V/cm2)	mean	n	Std. dev.
0	1.035	7	0.126
83	0.890	15	0.204
125	0.943	8	0.112
167	0.650	15	0.291

Table C-11. Means separation: Fisher's LSD for the effect of voltage on fertilization of eggs during the 1995 channel catfish spawning season.

Voltage (V/cm2)	Difference	Std. error	P-value
83 - 0	-0.875314	0.2460	0.001043
125 - 0	-0.940757	0.2589	0.000843
125 - 83	-0.065443	0.0782	0.408324
167 - 0	-1.12600	0.2451	0.000049
167 - 83	-0.250687	0.0625	0.000285
167 - 125	-0.185244	0.0779	0.022725

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Table C-12. Summary statistics for effect of pulse on fertilization of eggs during the 1995 channel catfish spawning season.

Pulses	Mean	n	Std. dev.
0	1.035	7	0.126
1	0.892	28	0.193
2	0.655	4	0.310
3	0.509	6	0.266

Table C-13. Means separation: Fisher's LSD for the effect of pulse on fertilization of eggs during the 1995 channel catfish spawning season.

Pulses	Difference	Std. error	P-value
1 - 0	0.930583	0.2393	0.000405
2 - 0	0.761817	0.2755	0.008818
2 - 1	-0.168766	0.0955	0.085366
3 - 0	0.564133	0.1691	0.001939
3 - 1	-0.366450	0.0795	0.000047
3 - 2	-0.197684	0.1119	0.085599

Table C-14. Summary statistics for effect of DNA on fertilization rate of eggs during the 1995 channel catfish spawning season.

DNA	Mean	n	Std. dev.
no	0.821	20	0.273
yes	0.859	25	0.245

Table C-15. Means separation: Fishers' LSD for effect of DNA on fertilization rate of eggs during the 1995 channel catfish spawning season.

DNA present	Difference	Std. error	P-value
yes - no	0.138283	0.0587	0.024004

Table C-16. Summary statistics for effect of IPTG on fertilization rate of eggs during the 1995 channel catfish spawning season.

IPTG present	Mean	n	Std. dev.
no	0.963	14	0.168
yes	0.787	31	0.271

Table C-17. Means separation: Fisher's LSD for effect of IPTG on fertiliation rate of eggs during the 1995 channel catfish spawning season.

IPTG present	Difference	Std. error	P-value
yes - no	-0.170426	0.0809	0.042060

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Table C-18. Analysis of variation for hatching rate (no interactions)

Source	df	Sums of squares	Mean square	F-ratio	Prob
Model	1	1.92548	1.92548	87.775	≤ 0.0001
Voltage	3	0.063027	0.021009	0.95772	0.4229
Pulse	2	0.066060	0.033030	1.5057	0.2351
DNA	1	0.045379	0.045379	2.0687	0.1588
IPTG	1	0.001109	0.001109	0.05054	0.8234
Error	37	0.811648	0.021936		
Total	44	0.954054			

Multiple analysis of variance (Manova) performed on arcsine-square-root-transformed 1996 spawning season data

Table C-19. Analysis of variation table for fertilization of eggs during the 1996 channel catfish spawning season.

Source	df	Sums of squares	Mean square	F-ratio	P-value
Model	1	14.8717	14.8717	4650.4	≤ 0.0001
Spawn	2	3.40833	1.70417	532.90	≤ 0.0001
Treatment Group	3	0.172490	0.057497	17.979	≤ 0.0001
Spawn*Trt. Group	6	0.061975	0.010329	3.2300	0.0180
Error	24	0.076750	0.003198		
Total	35	3.70955			

Table C-20. Summary statistics for effect of treatment group on fertilization of eggs during the 1996 channel catfish spawning season.

Treatment groups	Mean	n	Std. dev.
A	0.758	9	0.316
B	0.618	9	0.319
C	0.622	9	0.343
D	0.573	9	0.353

Table C-21. Means separation: Fisher's LSD for effect of treatment group on fertilization of eggs during the 1996 channel catfish spawning season.

Treatment groups	Difference	Std. Error	P-value
B - A	-0.139422	0.0267	0.000023
C - A	-0.136199	0.0267	0.000032
C - B	0.003223	0.0267	0.904761
D - A	-0.184963	0.0267	0.000000
D - B	-0.045540	0.0267	0.100480
D - C	-0.048764	0.0267	0.079816

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Table C-22. Summary statistics for effect of spawn on fertilization of eggs during the 1996 channel catfish spawning season.

Spawn	Mean	n	Std. dev.
1	0.933	12	0.101
2	0.217	12	0.104
3	0.779	12	0.085

Table C-23. Means separation: Fishers' LSD for effect of spawn on fertilization of eggs during the 1996 channel catfish spawning season.

Spawns	Difference	Std. error	P-value
2 - 1	-0.715821	0.0231	0.000000
3 - 1	-0.153603	0.0231	0.000000
3 - 2	0.562218	0.0231	0.000000

Table C-24. Summary statistics for effect of spawn*treatment group on fertilization of eggs during the 1996 channel catfish spawning season.

Spawn	Treatment group	Mean	Std. dev.	n
1	A	1.0581916	0	3
1	B	0.94894210	0.02428266	3
1	C	0.86788490	0.06036632	3
1	D	0.85513570	0.11391213	3
2	A	0.36057133	0.07634695	3
2	B	0.22365340	0	3
2	C	0.16883520	0.04747395	3
2	D	0.11380923	0.02391691	3
3	A	0.85486580	0.08196729	3
3	B	0.68276647	0.04105449	3
3	C	0.82831233	0.04525352	3
3	D	0.74979593	0.04537044	3

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Table C-25. Means separation: Fisher's LSD for effect of spawn*treatment group interaction on fertilization rate of eggs during the 1996 channel catfish spawning season.

Spawn*trt. group	Difference	Std. error	P-value
2,A - 1,A	-0.697620	0.0462	0.000000
3,A - 1,A	-0.203326	0.0462	0.000189
3,A - 2,A	0.494294	0.0462	0.000000
1,B - 1,A	-0.109249	0.0462	0.026393
1,B - 2,A	0.588371	0.0462	0.000000
1,B - 3,A	0.094076	0.0462	0.052772
2,B - 1,A	-0.834538	0.0462	0.000000
2,B - 2,A	-0.136918	0.0462	0.006738
2,B - 3,A	-0.631212	0.0462	0.000000
2,B - 1,B	-0.725289	0.0462	0.000000
3,B - 1,A	-0.375425	0.0462	0.000000
3,B - 2,A	0.322195	0.0462	0.000000
3,B - 3,A	-0.172099	0.0462	0.001046
3,B - 1,B	-0.266176	0.0462	0.000006
3,B - 2,B	0.459113	0.0462	0.000000
1,C - 1,A	-0.190307	0.0462	0.000387
1,C - 2,A	0.507314	0.0462	0.000000
1,C - 3,A	0.013019	0.0462	0.780388
1,C - 1,B	-0.081057	0.0462	0.091929
1,C - 2,B	0.644232	0.0462	0.000000
1,C - 3,B	0.185118	0.0462	0.000515
2,C - 1,A	-0.889356	0.0462	0.000000
2,C - 2,A	-0.191736	0.0462	0.000358
2,C - 3,A	-0.686031	0.0462	0.000000
2,C - 1,B	-0.780107	0.0462	0.000000
2,C - 2,B	-0.054818	0.0462	0.246752
2,C - 3,B	-0.513931	0.0462	0.000000
2,C - 1,C	-0.699050	0.0462	0.000000
3,C - 1,A	-0.229879	0.0462	0.000044
3,C - 2,A	0.467741	0.0462	0.000000
3,C - 3,A	-0.026553	0.0462	0.570584
3,C - 1,B	-0.120630	0.0462	0.015264
3,C - 2,B	0.604659	0.0462	0.000000
3,C - 3,B	0.145546	0.0462	0.004311
3,C - 1,C	-0.039573	0.0462	0.399893
3,C - 2,C	0.0659477	0.0462	0.000000
1,D - 1,A	-0.203056	0.0462	0.000192
1,D - 2,A	0.494564	0.0462	0.000000
1,D - 3,A	0.000270	0.0462	0.995384
1,D - 1,B	-0.093806	0.0462	0.053404
1,D - 2,B	0.631482	0.0462	0.000000
1,D - 3,B	0.172369	0.0462	0.001031
1,D - 1,C	-0.012749	0.0462	0.784821
1,D - 2,C	0.686300	0.0462	0.566701
2,D - 1,A	-0.944382	0.0462	0.000000
2,D - 2,A	-0.246762	0.0462	0.000017
2,D - 3,A	-0.741057	0.0462	0.000000

(Table C-25 cont'd).

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Spawn*trt. group	Difference	Std. error	P-value
2,D - 1,B	-0.835133	0.0462	0.000000
2,D - 2,B	-0.109844	0.0462	0.025662
2,D - 3,B	-0.568957	0.0462	0.000000
2,D - 1,C	-0.754076	0.0462	0.000000
2,D - 2,C	-0.055026	0.0462	0.245016
2,D - 3,C	-0.714503	0.0462	0.000000
2,D - 1,D	-0.741326	0.0462	0.000000
3,D - 1,A	-0.308396	0.0462	0.000000
3,D - 2,A	0.389225	0.0462	0.000000
3,D - 3,A	-0.105070	0.0462	0.032088
3,D - 1,B	-0.199146	0.0462	0.000238
3,D - 2,B	0.526143	0.0462	0.000000
3,D - 3,B	0.067029	0.0462	0.159531
3,D - 1,C	-0.118089	0.0462	0.017281
3,D - 2,C	0.580961	0.0462	0.000000
3,D - 3,C	-0.078516	0.0462	0.101964
3,D - 1,D	-0.105340	0.0462	0.031688
3,D - 2,D	0.635987	0.0462	0.000000

Table C-26. Analysis of variance for hatch rate of eggs electroporated during the 1996 channel catfish spawning season.

Source	df	Sums of squares	Mean square	F-ratio	P-value
Model	1	5.26968	5.26968	433.54	≤ 0.0001
Spawn	2	1.13328	0.566642	46.618	≤ 0.0001
Treatment group	3	0.065914	0.021971	1.8076	0.1727
Spawn*trt group	6	0.143664	0.023944	1.9699	0.1101
Error	24	0.291718	0.012155		
Total	35	1.63458			

Table C-27. Summary statistics for effect of treatment group on hatch rate of eggs electroporated during the 1996 channel catfish spawning season.

Treatment group	Mean	n	Std. dev.
A	0.327	9	0.202
B	0.446	9	0.206
C	0.369	9	0.194
D	0.389	9	0.274

Table C-28. Means separation: Fisher's LSD for treatment group on hatch rate of eggs electroporated during the 1996 channel catfish spawning season.

Treatment groups	Difference	Std. error	P-value
B - A	0.119017	0.0520	0.031110
C - A	0.042613	0.0520	0.420327
C - B	-0.076403	0.0520	0.154528
D - A	0.062121	0.0520	0.243658
D - B	-0.056896	0.0520	0.284497
D - C	0.019508	0.0520	0.710700

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Table C-29. Summary statistics for effect of spawn on hatch rate of eggs electroporated during the 1996 channel catfish spawning season.

Spawn	Means	n	Std. dev.
1	0.549	12	0.109
2	0.137	12	0.128
3	0.462	12	0.131

Table C-30. Means separation: Fisher's LSD for spawn on hatch rate of eggs electroporated during the 1996 channel catfish spawning season.

Spawns	Difference	Std. error	P-value
2 - 1	-0.412469	0.0450	0.000000
3 - 1	-0.087649	0.0450	0.063284
3 - 2	0.324820	0.0450	0.000000

Table C-31. Analysis of Variance For: 2-week survival for fry from the 1996 channel catfish spawning season.

Source	df	Sums of squares	Mean square	F-ratio	P-value
Model	1	3.26401	3.26401	286.85	≤ 0.0001
Spawn	2	0.613692	0.306846	26.967	≤ 0.0001
Treatment group	3	0.041776	0.013925	1.2238	0.3227
Spawn*trt. group	6	0.047847	0.007974	0.70082	0.6517
Error	24	0.273090	0.011379		
Total	35	0.976404			

Table C-32. Summary statistics for effect of treatment group on 2-week survival for fry from the 1996 channel catfish spawning season.

Treatment group	Mean	n	Std. dev.
A	0.271	9	0.191
B	0.358	9	0.156
C	0.280	9	0.139
D	0.296	9	0.192

Table C-33. Means separation: Fisher's LSD for Treatment group on 2-week survival for fry from the 1996 channel catfish spawning season.

Treatment groups	Difference	Std. error	P-value
B - A	0.087437	0.0503	0.094882
C - A	0.009550	0.0503	0.850975
C - B	-0.077887	0.0503	0.134493
D - A	0.024945	0.0503	0.624356
D - B	-0.062491	0.0503	0.225970
D - C	0.015395	0.0503	0.762123

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Table C-34. Summary statistics for the effect of spawn on 2-week survival for fry from the 1996 channel catfish spawning season.

Experiment	Mean	n	Std. dev.
1	0.376	12	0.092
2	0.118	12	0.114
3	0.410	12	0.107

Table C-35. Means separation: Fisher's LSD for spawn on 2-week survival for fry from the 1996 channel catfish spawning season.

Spawns	Difference	Std. error	P-value
2 - 1	-0.258190	0.0435	0.000004
3 - 1	0.034351	0.0435	0.437947
3 - 2	0.292542	0.0435	0.000000

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APPENDIX D
Plasmid Maps

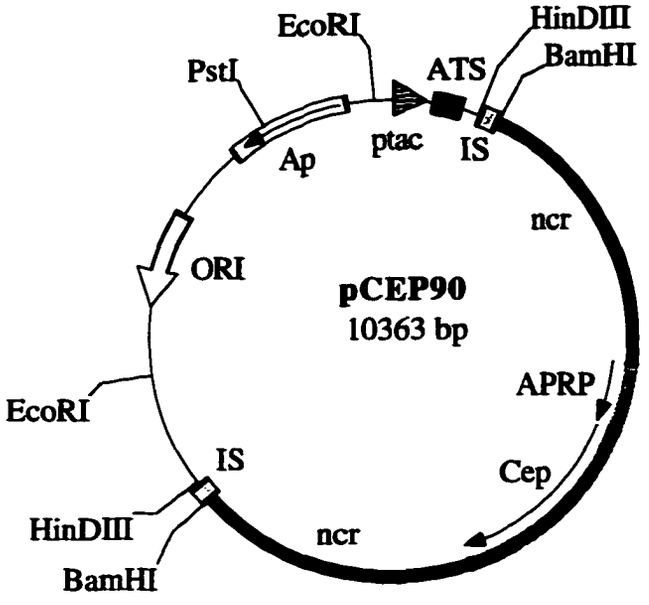

Figure D-1. Plasmid map of the pCEP90 transfection vector. Abbreviations: ORI, bacterial origin of replication; Ap, ampicillin resistance gene; ptac, inducible promoter; ATS, transposase gene; IS, insertion sequence; ncr, non-coding regions; APRP, cecropin acute phase response promoter; cep, cecropin gene. Restriction sites shown are recognized and cut by the endonucleases PstI, HinDIII, EcoRI, and BamHI.

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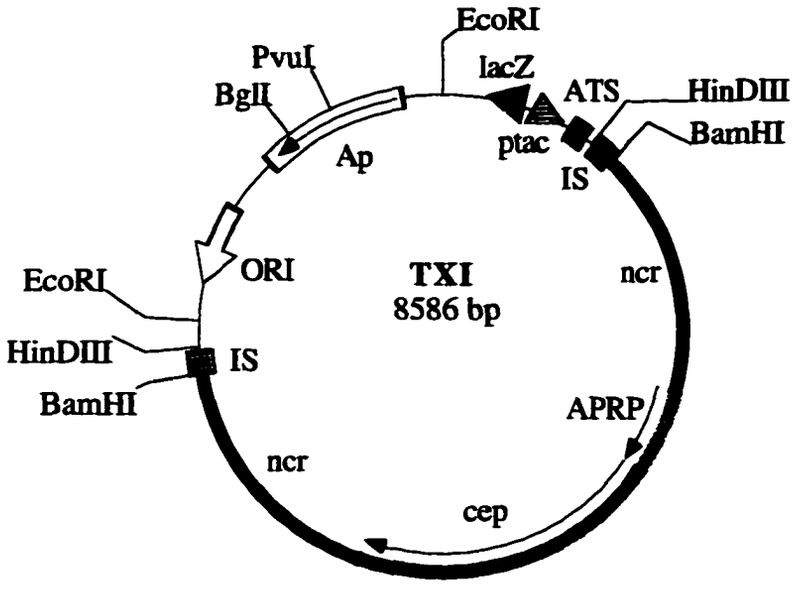

Figure D-2. Plasmid map of the TXI transfection vector. Abbreviations: ORI, bacterial origin of replication; Ap, ampicillin resistance gene; lacZ, lactose Z gene; ptac, inducible promoter; ATS, transposase gene; IS, insertion sequence; ncr, non-coding regions; APRP, cecropin acute phase response promoter; cep, cecropin gene. Restriction sites shown are recognized and cut by the endonucleases PstI, HinDIII, BglI and PvuI.

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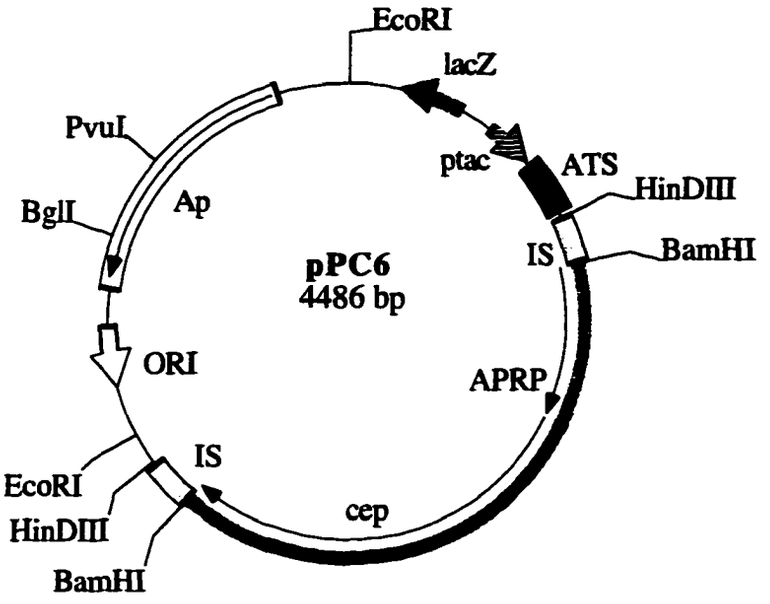

Figure D-3. Plasmid map of the pPC6 transfection vector. Abbreviations: ORI, bacterial origin of replication; Ap, ampicillin resistance gene; lacZ, lactose Z gene; ptac, inducible promoter; ATS, transposase gene; IS, insertion sequence; APRP, cecropin acute phase response promoter; cep, cecropin gene. Restriction sites shown are recognized and cut by the endonucleases Bgl I, PvuI, EcoRI, HinDIII, and BamHI.

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Appendix E: Compliance with guidelines and performance standards distributed by USDA Agriculture Biotechnology Research Advisory Commmittee (ABRAC)

Worksheet Accompanying Performance Standards for Safely Conducting Research with Genetically Modified Finfish and Shellfish

These Performance Standards are voluntary guidelines intended to aid researchers and institutions in assessing the genetic and ecological effects of research activities involving genetically modified fish, crustaceans, and molluscs, and in determining appropriate procedures and safeguards so that the research can be conducted without causing adverse impacts on the environment. This worksheet is designed to assist researchers and reviewers in evaluating the project.

****

Name of the Principal Investigator(s):

Answer: Dr. Terrence R. Tiersch

Dr. Richard K. Cooper

****

Description of the proposed project:

Answer: Production of transgenic channel catfish

****

List names, addresses, telephone numbers, and area of expertise of the experts you contacted for substantial advice in assessing effects of a proposed experiment and in designing adequate safety measures.

Answer:

LSU Animal Care and Use Committee
Dr. William J. Todd, Chairman
Professor, Department of Veterinary Science
Louisiana State University

Phone: (504) 388-5423

LSU Biosafety Committee
Dr. Konstantin G. Kousoulas, Chairman
Associate Professor,
Department of Veterinary Microbiology and Parasitology
Louisiana State University

Phone: (504) 346-3310
Email: vtgusk@lsuvax.sncc.lsu.edu

****

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Please enter address information for contacting the researcher(s):

Answer:

Terrence R. Tiersch
School of Forestry, Wildlife, and Fisheries,
Louisiana Agricultural Experiment Station,
Louisiana State University Agricultural Center,
Baton Rouge, Louisiana 70803

Phone: (504) 765-2848
Fax: (504) 765-2877
Email: tteirsch@agctr.lsu.edu

Signature of researcher 5/13/97 Date

Richard K. Cooper
Veterinary Science
Louisiana Agricultural Experiment Station,
Louisiana State University Agricultural Center,
Baton Rouge, Louisiana 70803

Phone: (504) 388-5421
Fax: (504) 388-4890
Email: rcooper@lsuvm.sncc.lsu.edu

Signature of researcher 5/14/97 Date

****

---------- PART 1. ----------

Risk Assessment Documentation for Worksheet TGENCAT

(Section I)
Are the research organisms finfish, crustaceans, or molluscs?

Answer: Yes

****

(Section I)
Do the research organisms have a non-dioecious form of reproduction?

Answer: No

****

(Section I)
Are the organisms modified solely by intraspecific selective breeding or captive breeding? l plan was developed (Appendix Enswer: No

****

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(Section I)
Are the organisms modified solely by interspecific hybridization or by selective breeding of an interspecific hybrid?

Answer: No

****

(Section I)
Survival and Reproduction Assessment Necessary.

Considerations will include:
1) Deliberate Gene Changes;
2) Deliberate Chromosomal Manipulations;
3) Interspecific Hybridization

****

Attach a written description of any identified risks. (See Operational Plan attached)

****

(Section II.A)
II.A Survival and Reproduction Assessment - Deliberate Gene Changes

Does the GMO result from deliberate changes of genes?

Answer: Yes

****

(Section II.A)
If containment is removed, does the GMO have direct access to (a) suitable natural ecosystem(s)?

Direct access is possible through natural waterbodies and human-created physical pathways, including navigation canals and interbasin transfers (e.g., irrigation, municipal water supply, etc.) See table in Supporting text.

Answer: Yes

****

(Section II.A)
Is/Are the accessible ecosystem(s) isolated from other aquatic ecosystem(s) and of low enough concern that killing of all fish/shellfish in the event of a GMO escape would be possible and practical?

Answer: No

****

Attach a written description of any identified risks. (See Operational Plan attached)

****

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(Section II.A.1)

II.A.1 Impact of Deliberate Gene Changes
This section is designed to assess organisms bearing a deliberate gene change and possibly bearing one or more additional genetic modifications.

Is the only gene change a gene deletion and/or an addition of a marker sequence, neither of which has any of the phenotypic effects listed in Table 1? (View Supporting text for Table 1)

Answer: No

****

(Section II.A.1)

Do(es) the accessible ecosystem(s) contain conspecifics, or other closely related species with which the GMO could interbreed?

Answer: Yes

****

(Section II.A.1)

Are the GMOs permanently sterile?

Answer: No

****

(Section II.A.1)

Is/Are the natural population(s) with which the GMO could interbreed threatened, endangered, or of special concern? (see Supporting text)

(If YES, one option is to move to a site where no protected species are present. However, if this is considered, other topics in the Standards must be addressed. To explore the implications of site relocation, answer NO here and continue.)

Answer: No

****

(Section II.A.1)

Immediate potential for introgression.

Accidentally escaped GMOs may establish a viable population of GMOs with immediate potential for gene introgression into natural populations.
Continue to Ecosystems Effects Assessment.

****

Attach a written description of any identified risks. (See Operational Plan attached)

****

(Section IV.A)

IV.A Ecosystem Effects - Deliberate Gene Changes
These GMOs:
-are NOT permanently sterile
-do have potential for interbreeding because of presence of conspecifics and/or closely

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related species in the accessible ecosystem(s). None of these species are protected.
Does the gene modification produce intentional or unintentional changes in one or more phenotypic traits listed in the accompanying Table? (See Supporting text)

Answer: Yes

****

Attach a written description of any identified risks. (See Operational Plan attached)

****

(Section IV.A.1)

****

(Section IV.A.1)

These GMOs:
- are NOT permanently sterile
- have potential for interbreeding with conspecifics and/or closely related species in the accessible ecosystem. None of these species are protected.
- have gene change(s) resulting in changes in one or more of the traits listed in accompanying Table. (See Supporting text)

****

(Section IV.A.1)

Can you estimate the reproductive potential of escaped GMOs in the accessible ecosystem?

Yes = reproductive potential estimated;
No = estimation not possible

Answer: No

****

(Section IV.A.1)

Lack of necessary information or methods prevents reliable estimation.

Continue to Risk Management - Insufficient Information

****

(Section VI.B)

Conclusion - Insufficient Information

The Standards indicate there is not enough information to evaluate risk. The precautionary approach used herein requires that in the absence of information to evaluate risk, the goal of risk management must be no/negligible accidental escapees.

****

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(Section VI.B)
Unable to Estimate/Evaluate Ecosystem Effects

*No/negligible accidental escape
These GMOs are NOT sterile. Conspecifics or closely related species ARE present in the accessible ecosystem(s), but none are protected species. Because the GMOS have an unfamiliar overall phenotype, unknown reproductive potential or unknown fitness, no determination can be made of their impact on the structure or processes of the accessible ecosystem(s).

****

Consult the Risk Management Recommendations on the following menu for information concerning project siting and the design of barriers.

****
(Section VI)

- Select sufficient barriers listed in Supporting text above to assure that accidental escapees are fewer than the acceptable number for your research project.

- Ensure that your project meets requirements for security, alarms, Operational Plan and inspection, as explained in the text of the Recommendations.
Consult the Supporting Text now.

****

---------- PART 2. ----------

Risk Management Documentation for Worksheet TGENCAT

As part of compliance with the Standards, the researcher must describe and provide the rationale for risk management measures.

Questions on the major points explained in the Risk Management Recommendations follow.

Researchers and reviewers should refer to the Library while answering the questions. Risk management documentation should fully respond to these major points.

****

You were directed to Section VI.B Risk Management - Insufficient Information to assess risks. What measures do you plan to adopt to effectively confine the proposed experiment?

Attach a written description of the risk management measures you plan to implement. Be certain to address the topics listed in the Risk Management Documentation section below.

Answer: See attached documentation (See Operational Plan attached)

****

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Are you working with a non-indigenous species?

Answer: No

****

1. Explain how the siting and structures of the project prevent accidental releases during natural flooding or other natural disasters.

Answer: Although the facility faces possible flooding risk, the scale of experimentation is small enough for adequate response to natural disaster. Because the organisms are maintained inside an aquaculture laboratory and in recirculating systems with no direct plumbing connection to the building plumbing, maintenance is simplified.

****

Project Siting

1a. If project involves placement of GMOs in uncovered outside tanks or ponds, is there the potential for sudden high winds to wash organisms into a natural water body (accessible ecosystem) via water spray or waves?

Answer: No

****

Design of Barriers

The standards identify four types of barriers: (1) physical or chemical; (2) mechanical; (3) biological; and (4) scale of experiment as a barrier.

2. Was the project site chosen because the surrounding accessible ecosystems are lethal to all life stages of the GMO?

Answer: No

****

3. If the project's GMO could escape through the path (aquatic or non-aquatic) listed below, describe the arrangement and types of barriers to escape; a diagram of the layout of barriers at the site may be useful. Ignore the listed path only if escape is clearly precluded.

a. Influent/makeup water

Answer: All GMO's are held in closed recirculating systems. All makeup water is added to a system sump tank by garden hose.

****

3. If the project's GMO could escape through the path (aquatic or non-aquatic) listed below, describe the arrangement and types of barriers to escape; a diagram of the layout of barriers at the site may be useful. Ignore the listed path only if escape is clearly precluded.

b. Effluent and drawdown water

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Note: if discharge to sanitary sewer is used as one barrier against accidental escape of GMOs in effluent, at least one additional barrier is necessary.

Answer: All biofilter effluent is discharged into an intermediate holding tank which is dosed with chlorine bleach. When sumps or tanks are drained, netting is placed on drains to capture any fish that might escape.

****

3. If the project's GMO could escape through the path (aquatic or non-aquatic) listed below, describe the arrangement and types of barriers to escape; a diagram of the layout of barriers at the site may be useful. Ignore the listed path only if escape is clearly precluded.

c. Waste slurries

Answer: Waste slurries are treated with chlorine bleach before discharge over a gravel substrate.

****

3. If the project's GMO could escape through the path (aquatic or non-aquatic) listed below, describe the arrangement and types of barriers to escape; a diagram of the layout of barriers at the site may be useful. Ignore the listed path only if escape is clearly precluded.

d. Disposal of Experimental Animals

Answer: Animals that die are maintained in a -20° C freezer. Further disposal is by incineration at the LSU School of Veterinary Medicine.

****

3. If the project's GMO could escape through the path (aquatic or non-aquatic) listed below, describe the arrangement and types of barriers to escape; a diagram of the layout of barriers at the site may be useful. Ignore the listed path only if escape is clearly precluded.

e. Aerosols (applies only to shellfish with small larvae)

Answer: Not applicable

****

3. If the project's GMO could escape through the path (aquatic or non-aquatic) listed below, describe the arrangement and types of barriers to escape; a diagram of the layout of barriers at the site may be useful. Ignore the listed path only if escape is clearly precluded.

f. Equipment cleaning and storage

Answer: Equipment used in the transgenic laboratory (e.g., nets, buckets, etc.) is maintained in the laboratory and not used for any other projects.

****

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4. Have you identified additional potential escape paths?

Answer: Yes

****

Briefly describe each potential path.

Answer: Fish may be transported to a secured disease challenge laborartory at the LSU School of Veterinary Medicine.

****

6. Describe how the types and numbers of barriers in series are sufficient to achieve the "acceptable number of accidental escapees" specified in the Risk Management portion of the Performance Standards.

Consult the Library above for Risk Management Recommendations.

Answer:
1. Fish are in closed recirculating systems.
2. Laboratory is in an enclosed secure site.
3. Access to the laboratory is controlled.
4. All effluent is discharged into a holding tank that is treated with chlorine bleach.
5. All standpipes are screened to prevent escape from tanks into system pipes and sump.
6. Floor drains are screened to prevent escape.
7. The entire facility is enclosed and equipped with electronic security gates.
8. Scale of experiment is small enough to allow rapid response to emergency situations.
9. Tops of sump tanks are covered with screens.

****

Special Concerns

7. If biological barriers are used for a given escape path, does the path have at least one other type of barrier? (Because of their variable efficacy, biological barriers cannot comprise an entire set of barriers.)

Answer: Not applicable

****

8. If scale is used as a barrier, are you certain the GMO is not a self-fertilizing hermaphrodite or true parthenogen?

Answer: Yes

****

Attach supporting evidence. (See Operational plan).

****

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Security

9a. Describe the security measures implemented to:

Control normal movement of authorized personnel.

Answer:
1. Doors remain locked outside normal operating hours and during most of regular operating hours.
2. Access is limited to authorized personnel.
3. Key availability is limited to principal investigators, specific graduate assistants and research associates.

****

9b. Describe the security measures implemented to:

Prevent unauthorized access to the site.

Answer:
1. Signs are posted for authorized access only.
2. Doors remain locked outside of normal operating hours.
3. Electronic access to facility required after normal operating hours.
4. Key availability is limited.

****

9c. Describe the security measures implemented to:

Eliminate access for predators who could potentially carry animals off-site (applies only to outdoor projects).

Answer: Not applicable

****

Alarms

10. Describe and justify the adequacy of the entire set of installed alarms. Be sure to address the following:

a. Have you installed a water level alarm (required for all projects)?

Answer: No

Note: All culture systems in transgenic laboratory are closed recirculating systems. In case of system shut down, water level will be maintained by standpipes in each tank. If a standpipe is removed water level will be maintained by the height of the system sump tank. In the case of standpipe removal, excess water is filtered through screens mounted on the top of the sump tanks.

****

Alarms

b. Do all installed alarms have backup power?

Answer: No

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****

Alarms

c. Describe the plan for notifying designated personnel.

Note: Building is on automatic backup generator power. If power is interrupted building maintenance personnel are notified.

****

Operational Plan

11. Attach the written Operational Plan. A required component is:

a. Training.

Answer: See Operational Plan.

****

Operational Plan

11. Attach the written Operational Plan. A required component is:

b. Traffic Control.

Answer: See Operational Plan.

****

Operational Plan

11. Attach the written Operational Plan. A required component is:

c. Record Keeping.

Answer: See Operational Plan.

****

Operational Plan

11. Attach the written Operational Plan. A required component is:

d. Emergency Response Plan.

Answer: See Operational Plan.

****

Review and Inspection

12. Has your institutional biosafety committee, biosafety officer, or other appropriate expert reviewed and approved the proposed project and its risk management measures?

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Answer: Yes

****

Have you notified federal, state, and local agencies having jurisdiction over any aspects of your proposed project?

Answer: Not applicable

****

Please list all required permits and authorizations and check the appropriate line regarding status of your application, as below:

Permit/Authorization:

Answer:

LSU Animal Care and Use Committee
Permit/Authorization: approved

LSU Biosafety Committee
Permit/Authorization: approved

****

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---------- APPENDIX ----------

(May be deleted if not applicable)

Table 1

Classes, examples, and possible ecological effects of phenotypic changes in genetically modified fish, crustaceans, and molluscs. For projects involving GMOs expressing one or more of these phenotypic changes,continue assessment (proceed to the appropriate step in the Flowcharts) in order to reach a defensible decision about safety or risk.

Class	Phenotypic Change	Ecological Effect
Metabolism	- Growth rate - Energy metabolism - Food Utilization	- Shift to different prey size - Alter nutrient and energy flows
Tolerance of Physical Factors	- Temperature - Salinity - pH - Pressure	- Shift preferred habitats - Alter geographic range
Behavior	- Reproduction - Territoriality - Migration - Chemosensory (including pheromones, allelochemicals) - Swimming/navigation	- Alter life history patterns - Alter population dynamics - Alter species interactions
Resource or Substrate Use	- Food utilization - Alter food webs	- Release from ecological limits
Population Regulating Factors	- **Novel disease resistance** - Reduced predation/ parasitism - Habitat preference	- Alter population and community dynamics - Release from ecological limits
Reproduction	- Mode - Age at maturation and duration - Fecundity - Sterility	- Alter population and community dynamics - Interfere with reproduction of related organisms
Morphology	- Shape and size - Color - Fin/appendage form	- Alter species interactions
Life History	- Embryonic and larval development - Metamorphosis - Life span	- Alter life history patterns - Alter population and community dynamics

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Table 2: Direct Access to Natural Ecosystems

Direct access is possible through natural waterbodies and human-created physical pathways, including navigation canals and interbasin water transfers (e.g., irrigation, municipal water supply, etc.)

EXAMPLES OF SUSPECTED GENERIC PATHWAYS INVOLVED IN THE UNINTENTIONAL INTRODUCTION OF NONINDIGENOUS AQUATIC SPECIES

Shipping

- o Ballast water and sediments
- o Anchor chains and chain lockers
- o Sanitary water
- o Hull surfaces
- o Bilge water and sediments
- o Propeller-shaft housing
- o Trash/refuse/garbage

Floating Oil/Gas Drilling Platforms
Recreational Boating

- o Hull surfaces
- o Waste sanitary water
- o Bait wells
- o Bilge water and sediments
- o Motors
- o Associated tools and equipment

Media (e.g., water, seaweed, soil, etc.), Containers and Equipment Used to Transport Live Organisms

- o Aquarium fish, plants, etc.
- o Bait
- o Aquaculture fish, shellfish, plants, etc.
- o Fishery management (e.g., fish stocking)
- o Research specimens
- o Ornamental, other plants
- o Pathogens in target animals

Fresh or Frozen Seafood Transport and Disposal

Human Created Water Connections

- o Navigation canals (e.g., Erie and Welland Canals)
- o Interbasin water transfers (e.g., for irrigation, municipal/industrial water supply, etc.) (Meador 1992)

Natural Pathways

- o Waterfowl and other water birds
- o Hybrid backcrosses

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Operational Plan

Transgenic Laboratoty Culture Systems

All production of transgenic fish is carried out at the LSU Aquaculture Research Farm (ARF). The ARF is accessed by two roads from Ben Hur road (Figure E-1). Each road has an electronic security gate which requires a card with a magnetic strip for access. One gate is open to public access during normal operating hours (6:45 AM to 4:00 PM; Monday through Friday). The gates are closed at all other times including holidays. There is no other access to vehicles by any other route and the entire ARF is enclosed within a fence. The Ben Hur Aquaculture Research Laboratory (Figure E-2), a 1,860-m^2 multi-disciplinary aquaculture resarch laboratory, located on the grounds of the ARF houses the transgenic laboratory (Figure E-3). Access to the laboratory is through an interior and exterior doors. Keys are issued only to authorized personnel. The transgenic laboratory is a 100-m^2 indoor wet laboratory that houses eight independent closed recirculating systems. System "A" was designed for maintenance of channel catfish fry in separate treatment groups and includes five 40-L round tanks and a two-tiered shelf system that can be fitted with either one hundred 2-L, or twenty four 20-L culture vessels. Systems "B" and "C" were designed for holding fingerlings and each has three 1,000-L round, fiberglass tanks. System "D" has two 1,000-L and two 400-L round, fiberglass tanks. Systems "E" and "F" were designed for holding larger fish and each has a single 2,500-L round, fiberglass tank. System "G" was designed for work with shellfish and includes five 400-L round, fiberglass culture tanks. System "H" was designed as a multiple-use culture system and includes a two-tiered rack with five 60-L glass aquaria on each tier. All systems were equipped with ultraviolet (UV) filters placed after the biological filter for control of pathogenic organisms. All systems feature magnetically driven pumps (Model 5-MD-SC, 0.09-Kw, Little Giant Pump Co. Inc., Tulsa, Oklahoma), except for system "A" which uses a solid shaft centrifugal pump (Model 1P788, 0.37-Kw, Dayton Electric Manufacturing Co., Niles, Illinois) and bead

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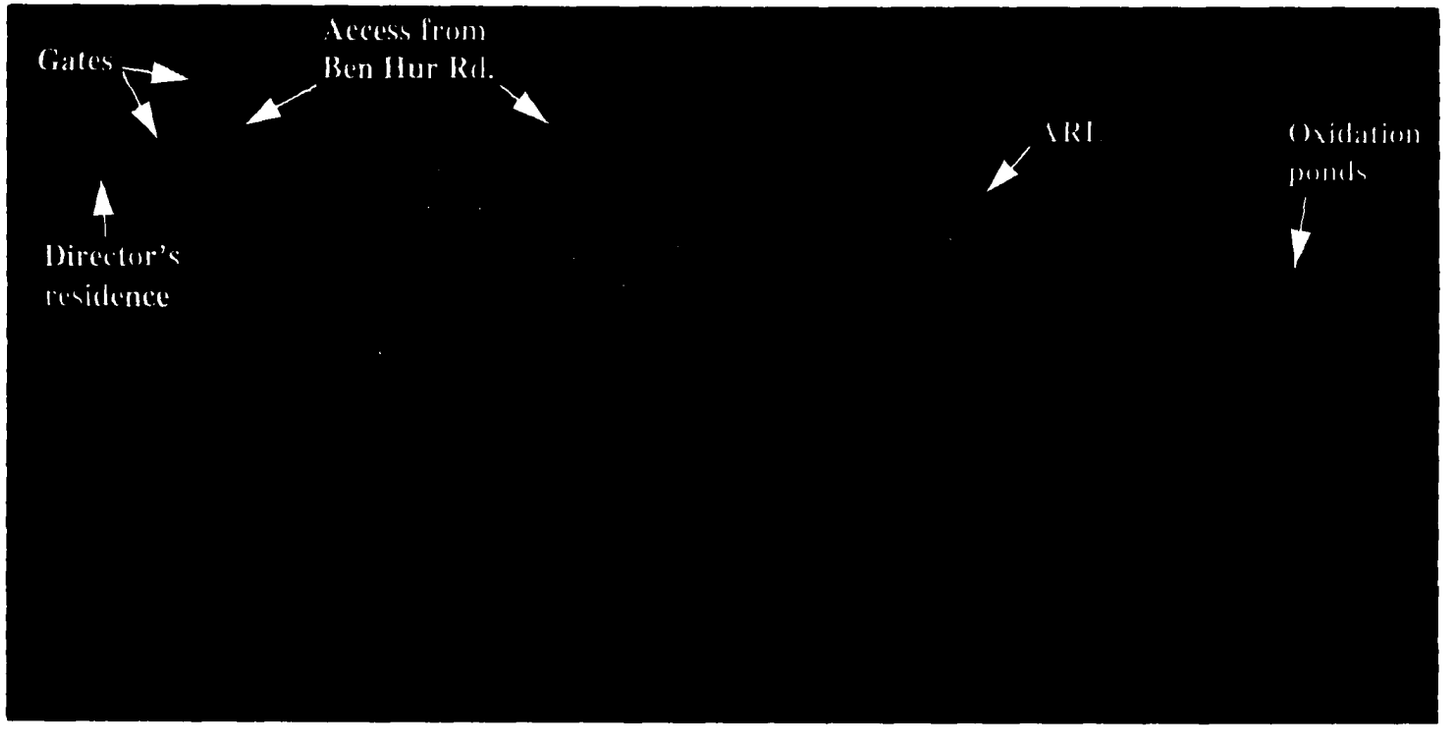

Figure E-1. Aerial view of the Ben Hur Aquaculture Research Facility (ARF) detailing electronic gates on each of the two access roads, location of the Aquaculture Research Laboratory (ARL), and oxidation ponds.

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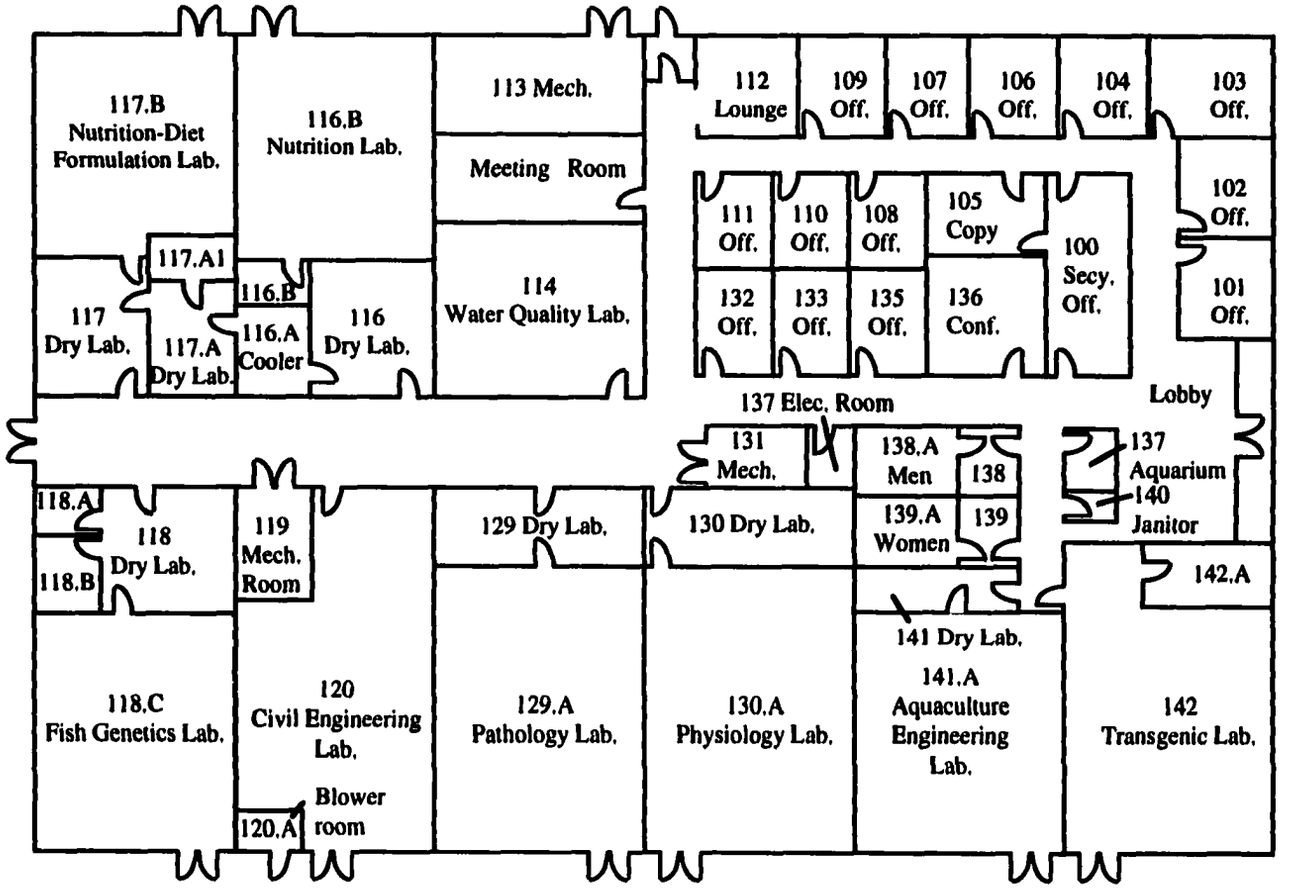

Figure E-2. Floor plan of the Ben Hur Aquaculture Research Facility (ARF) showing location of the transgenic laboratory (Room 142, lower right).

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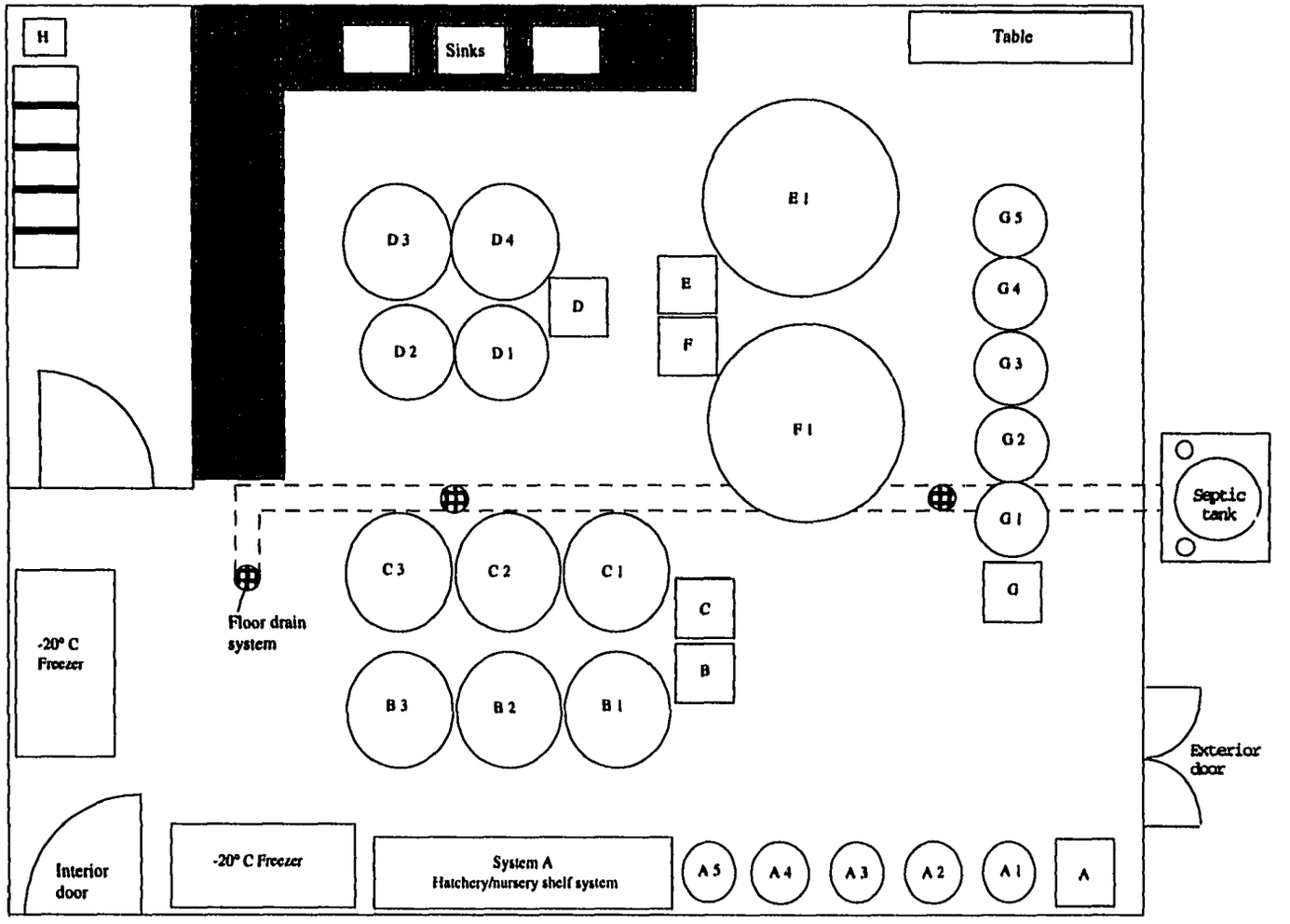

Figure E-3. Floor plan of the Transgenic Laboratory detailing layout of recirculating systems used to maintain transgenic channel catfish and the floor drain system.

filter biofiltration. All culture tanks utilize a center inner standpipe surrounded by an outer standpipe to force water up from the bottom of the tank for self-cleaning. All inner standpipes are screened to exclude passage of the smallest organism in the tank (Figure E-4). If a stand pipe is removed, the culture tank water level will be maintained at the level of the top of the sump. Water that might overflow the sump must pass through a screen placed in the lip of the sump tank before flowing out of the overflow relief holes located opposite the pump. There are no direct plumbing connections from any of the culture systems to the drainage system of the building. There are four floor drains in the transgenic laboratory. One has been covered with a solid plate. The other three are plumbed to a septic tank located behind the laboratory. From the septic tank plumbing leads through a grinding pump (Model SGV301-754, Peabody Barnes, Inc. Mansfield, Ohio) and finally to oxidation ponds (Figure E-5). The SGV301-754 pump uses a two-stage cutting and grinding mechanism constructed of 440C stainless steel. The slurry produced by the pump is transferred to oxidation pond #1 by a 5-cm diameter pipe. If the water level of pond #1 exceeds the level of the standpipe, water flows into pond #3 when the water level exceeds the standpipe height of pond #2. In the event of a major flood, water from pond #3 would drain into storm ditches. (Note: as of the last update of this Operational Plan, May 12, 1997, pond #3 has never exceeded the overflow level).

Training

All project personnel are trained in the operation and maintenance of all culture systems, and in security procedures for the transgenic laboratory. Personnel responsible for tank cleaning are trained to always have screens on tank standpipes to preclude escape of fish from tanks. Backwash from biofilters is disposed of by pouring over a gravel substrate.

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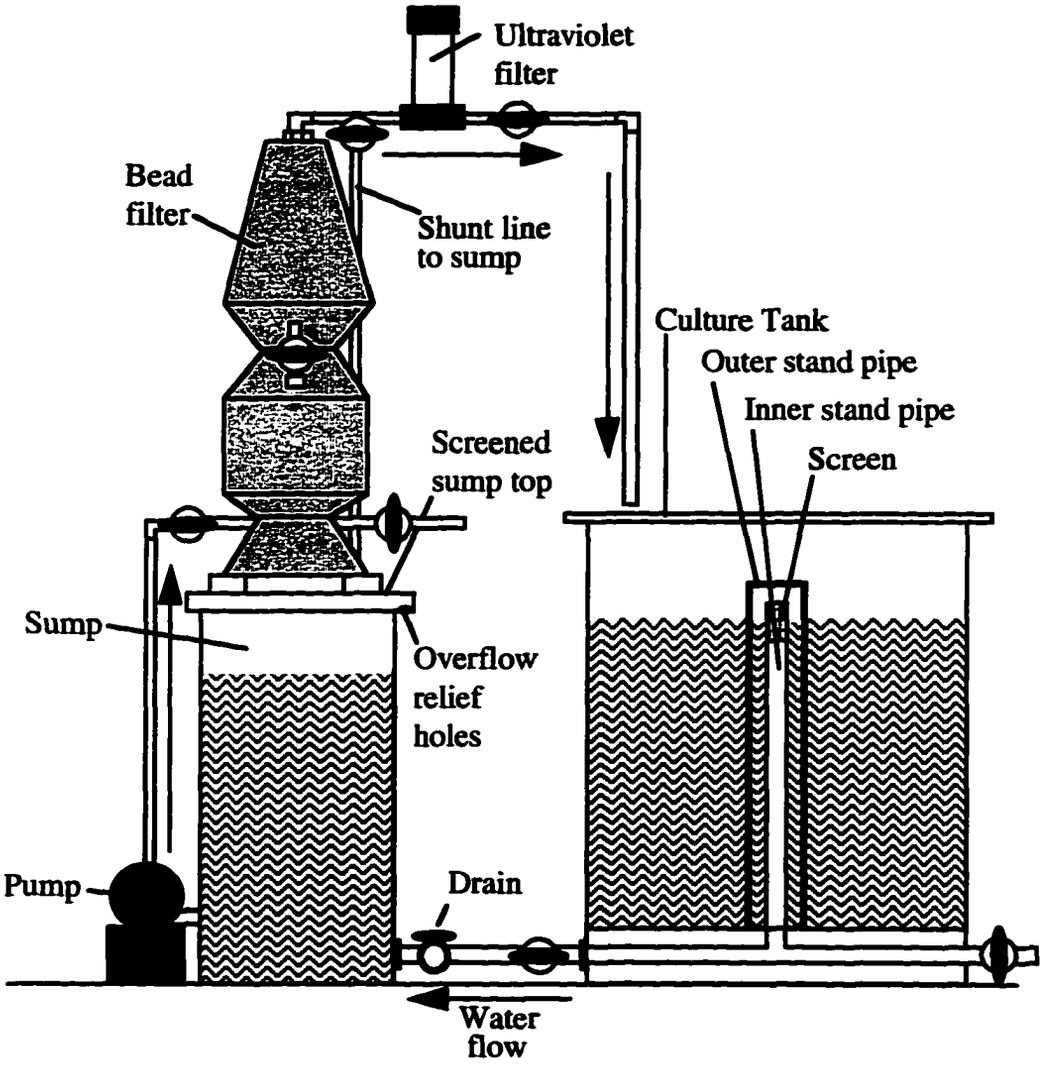

Figure E-4. Diagram of sump, filter, and culture tank system layout as used in the transgenic laboratory. The sump, culture tank, and outer standpipe are shown in cross-section.

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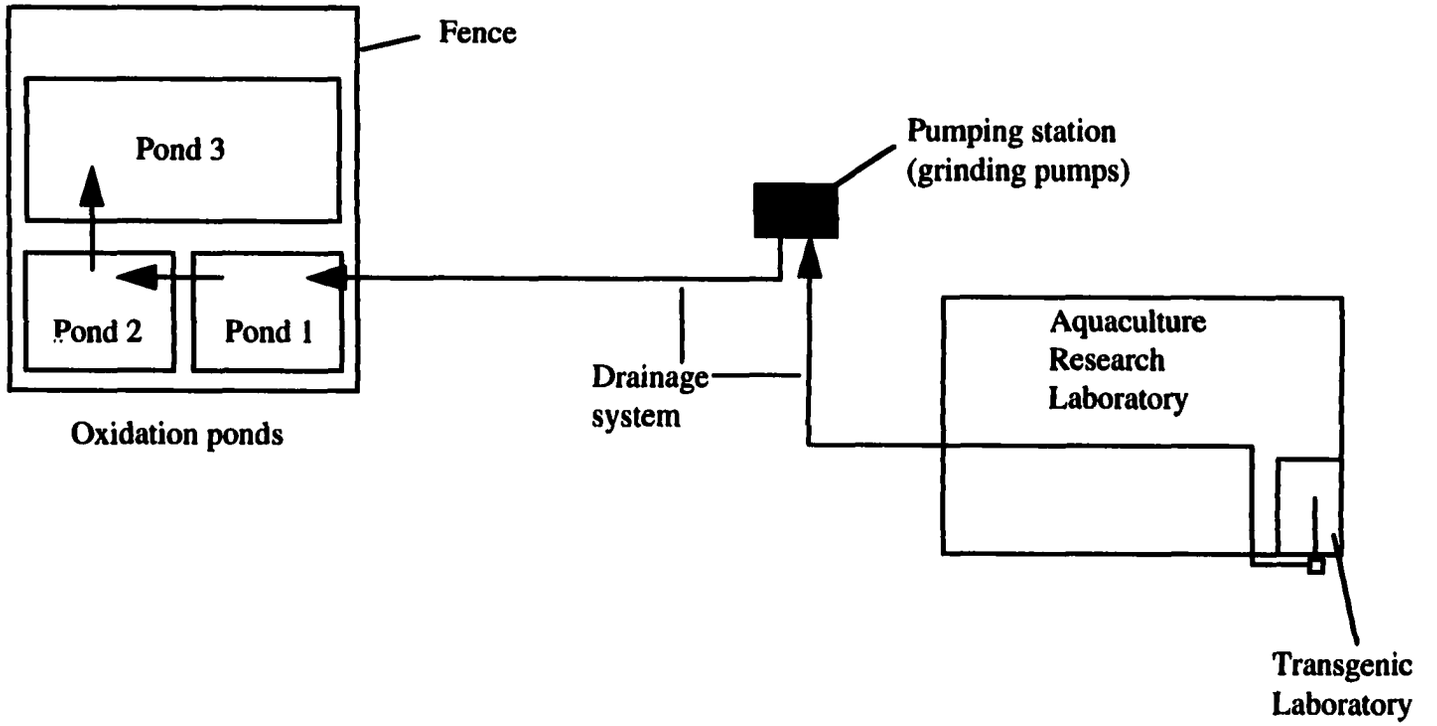

Figure E-5. Diagram detailing flow of waste water (arrows) from the Transgenic Laboratory through the grinding pump (pumping station) and finally to the oxidation ponds.

Traffic Control

Access to the transgenic laboratory is restricted to faculty, staff, and students involved in the project. Doors are locked when project personnel are not present. All visitors are required to sign in and out to keep a record of entries into the laboratory. Equipment specifically designated for use in the transgenic laboratory is not taken from the room for any other use. Dead organisms are stored frozen in -20° C freezers, located in the laboratory. Final disposal of organisms is by incineration at the LSU School of Veterinary Medicine.

Record Keeping

Records are maintained of: (1) numbers of fish in each tank; (2) movements of experimental fish to new tanks, and (3) people entering or leaving the transgenic laboratory. Fish are tagged (when large enough) with passive integrated transponder (PIT) tags. The tags register a unique nine-digit code for each fish, allowing tracking and characterization of individual status within a group. All tanks are labeled with water proof tags.

Emergency Response Plan

Water from the transgenic laboratory drains through a grinding pump to three oxidation ponds on the grounds of the LSU Aquaculture Research Facility. In the highly unlikely event that eggs or fry should survive the grinding pump and reach the oxidation ponds the ponds will be treated with rotenone.

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Responsible parties

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Notification in case of loss of confinement

LSU Animal Care and Use Committee

LSU Biosafety Committee

Mitigation or recovery plan

In the highly unlikely event that eggs or fry should survive the grinding pump and reach the oxidation ponds the ponds will be treated with rotenone.

Movement to safe site or destruction of animals

Other than transport of animals to the LSU School of Veterinary Medicine for disease challenge experiments, it is our policy to not remove live transgenic organisms from the transgenic laboratory. Therefore, in the event of an emergency or disaster (hurricane, flood, etc.) organisms that cannot be contained will be killed with rotenone or bleach.

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APPENDIX F
Standard Operating Procedures

SOP-1. Hanks' balanced salt solution

Hanks' balanced salt solution (HBSS) is a multi-purpose buffer used throughout the studies reported in this dissertation for storing sperm and holding eggs until ready for fertilization, and as a medium for electroporation of eggs. All ingredients are reagent grade chemicals purchased from Sigma Chemicals, St. Louis, Missouri.

Table F-1. Hanks' balanced salt solution ingredients.

Ingredient	g/L	Molarity
NaCl	8.0	0.14
KCl	0.4	0.005
$CaCl_2 \cdot 2H_2O$	0.16	0.001
$MgSO_4 \cdot 7H_2O$	0.20	0.001
Na_2HPO_4	0.06	0.0004
KH_2PO_4	0.06	0.0004
$NaHCO_3$	0.35	0.004
$C_6H_{12}O_6$ (glucose)	1.00	0.006

Note: For storage of channel catfish sperm or for holding stripped channel catfish eggs, the osmotic pressure of HBSS must be above 275 mOsmol/Kg to ensure that activation does not occur.

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SOP-2. Polymerase Chain Reaction (PCR)

Table F-2. Ingredients for 100-µL PCR reaction.

Ingredient	Concentration	Volume per reaction
Template DNA	~ 0.5 µg	Volume depends on template DNA concentration
Primer 1	20 µM	1 µL
Primer 2	20 µM	1 µL
dNTP mix[a]	1 mM each	8 µL
$MgCl_2$	25 mM	6 µL
10x PCR buffer[b]	NA	10 µL
DMSO	100%	1 µL
Taq DNA Polymerase	5 units/µL	0.5 µL
Sterile distilled water	NA	Volume required to bring total reaction volume to 100 µL

[a] deoxy nucleotriphosphate mix: 10 µL of each dNTP (G,A,T,C) and 60 µl of sterile distilled water.
[b] 10x PCR II (PE) buffer (supplied with Taq DNA polymerase).

Table F-3. PCR primers.

Vec primers: primer set specific for an 850 base pair sequence of the cecropin gene.	
Vec_1:	5'- AGACTTGACTCCGCTGCATAAGTG -3'
Vec_2:	5'- TACCGTTTCTGATGTTGCGACC -3'

CH4 primers: designed to target a 303 base pair sequence on the exon of the fourth heavy chain (Ch4) of the Ig H gene	
$CH4_1$:	5'- TCCCCAAGGTTTACTTGCTCGCTCC -3'
$CH4_2$:	5'- CGATGGATCTGGATATGTGGCGCAC -3'

Table F-4. Parameters for PCR with Vec and CH4 primer sets.

Step	Activity	Temperature (°C)	Time
1	Hot start	95	5 min
2[b]	Denature	95	30 sec
3[b]	Anneal	primer dependent[a]	30 sec
4[b]	Extend	72	1 min
5	Hold	4	indefinite

[a] Anealling temperature was 59° C for Vec primers and 52° C for CH4 primers.
[b] Steps 2 through 4 were repeated 30 times (30 cycles) before proceeding to step 5.

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SOP-3. Bubble-washed bead-filter maintenance

Bead filters must be backflushed on a regular basis to remove solids and bacterial biofloc from the bead bed to assure adequate nitrification takes place in the filter. The filter is backwashed by turning off the pump and opening and closing valves in a particular order. The sump tank should also be flushed on a regular basis to remove solids from the system.

Backwashing (Figure F-1)

Starting with the filter in operation.

1) Disconnect pump from power source

2) Close valve #1

3) Close valve #5

4) Open valve #2 (run to waste receptacle)

5) Open valve #3 (allow water to drain completely from filter)

6) Close valves #2 and #3

7) Open valve #4

8) Open valve #1 and restore power to pump (allow several gallons to run into sump to allow re-seating of beads and clearing of water).

9) Open valve #5 and close valve #4.

The filter is back in operation.

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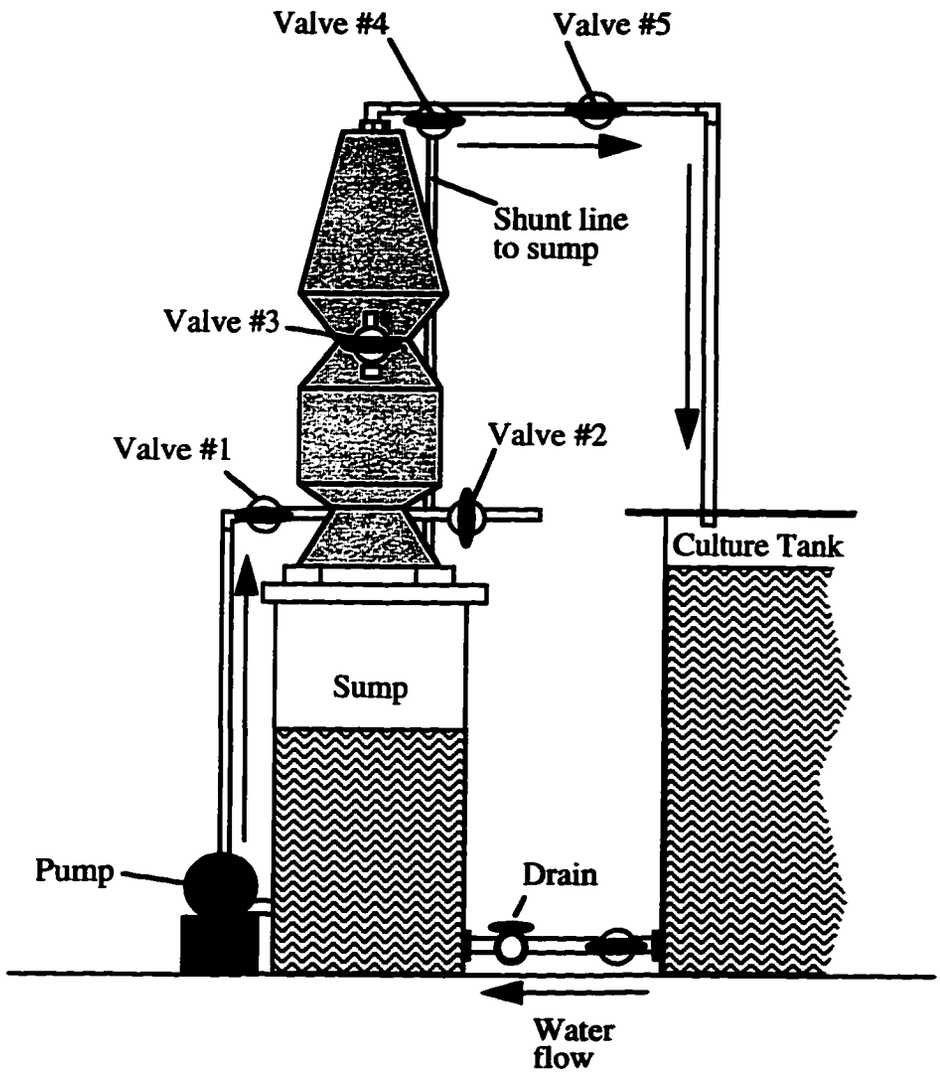

Figure F-1. Diagram of 0.03-m^3 bubble-washed bead-filter and sump tank as used in the Transgenic Laboratory.

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SOP-4. Pond seining.

1) Disconnect power at power box before entering the pond (Figure F-2).

2) Remove aerator(s) from pond.

3) Align seine at end of pond with drain pipe.

4) Connect seine to towing vehicles (tractor or truck) on each side of pond. Begin seining with seine inside of drain pipe if possible (to avoid snagging seine on pipe).

5) Have someone stand on the lead line ("ride the line") on the bottom of the slope of each bank. This will force the lead line to remain taut against the pond bottom.

4) Proceed forward until towing vehicle #1 reaches the power box. Pass the line around the power box and water inlet. Re-attach to vehicle.

5) Towing vehicle #1 turns right, vehicle #2 proceeds forward. Both ends of the seine can then be connected to vehicle #1 or, if the seine is too heavy, both vehicles can pull the seine together.

6) Pool the fish in the net being very careful to keep the lead line on the pond bottom until it is possible to pull the load from the pond.

7) Quickly place the fish into oxygenated or aerated hauling tanks.

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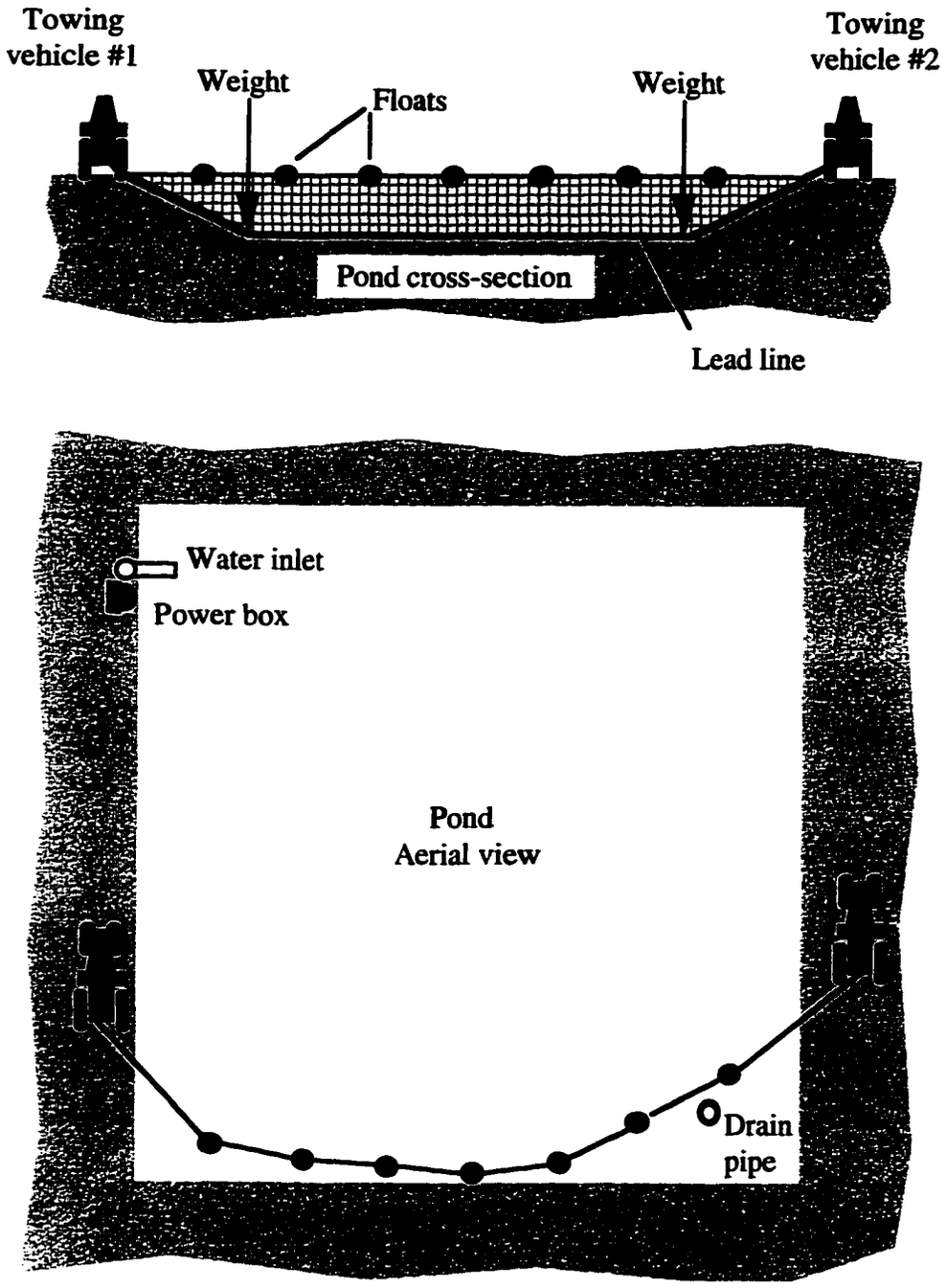

Figure F-2. Pond seining cross section and aerial view.

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SOP-5. Hydration of synthetic leuteinizing-hormone releasing-hormone.

Table F-5. Preparation of leuteinizing-hormone releasing-hormone analog and injection of female channel catfish to induce ovulation

Item:	D-Ala^{6}DesGly^{10} LH-RH-ethylamide (synthetic leuteinizing hormone-releasing hormone). 5 mg lyophilized powder.
Source:	Peninsula Laboratories, Belmont, California.
Reconstitution:	Add 10 mL deionized water to yield an LH-RHa concentration of 500 µg/mL.
Injection:	Inject females intramuscularly (i.m.) with 0.1 mL/kg of body weight to administer a dose of 50 µg/kg of body weight.

Note: Rehydration with 5 mL of water results in a final concentration of 1,000 µg/mL. This reduction from 10 to 5 mL for hydration allows injection a smaller volume. For example, injecting 0.1 mL/kg of body weight will administer a dose of 100 µg/kg of body weight.

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SOP-6. Paired spawning method

For paired spawning females and males are weighed (kg), measured (total length in mm), and a blood sample is collected from the caudal vein. Females are injected with 100 μg LHRHa/Kg of body weight (See SOP-5).

1) Anesthatize fish with MS-222.

2) Process females first and place them into windowed spawning tanks or aquaria.

3) After processing, place males with similar sized females. Never put a very large fish with a small fish. Spawning behavior can be extremely aggressive.

4) Fish may spawn any at any time following stocking into the tanks. However, it is unlikely that they will spawn sooner than 36 to 48 hours after injection of the female with hormone.

5) Watch for and record spawning behavior. When fish are head-to-tail they are displaying spawning behavior. When the female has laid 200 to 300 eggs it is time to remove her for stripping (See SOP-8).

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SOP-7. Grouped female spawning

Grouped spawning offers advantages over paired spawning, however, it is not a proven method to collect high quality eggs. The main advantage is that spawning of many females can be attempted simultaneously. Processing females for grouped spawning also includes weighing (kg), measuring (total length in mm), and blood sample collection from the caudal vein. Females are injected with 100 µg LHRHa/Kg of body weight (See SOP-5). This method requires a system with at least 2 tanks.

1) Anesthetize females with MS-222.

2) Process females and place 8 to 12 females in a 1,000 L tank.

3) Fish should be checked ~24 to 30 hours after injection with hormone.

4) Remove females one at a time, apply pressure to the abdominal region. If eggs are not released, place female in another tank and continue checking. If eggs are released, strip female (SOP-8).

5) Continue to check females every 1-2 hours for ovulation.

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SOP-8. Stripping eggs from an ovulating female channel catfish.

1) Identify a female that is either laying or releasing eggs (record time).

2) Anesthetize with MS-222.

3) Remove from anesthetic, and dry female thoroughly with paper towels.

4) Carefully strip eggs, beginning at the area local to the oviduct and slowly proceeding to the abdominal area. Strip eggs into a food-grade plastic bowl (tupperware, rubber maid) that has been coated with silicon vacuum grease (record time stripping was started).

5) Add enough Hanks' balanced salt solution (SOP-1) to cover eggs. Have an assistant swirl the egg collection bowl during stripping. Continue stripping until sure you have most of the eggs. (record time finished stripping).

6) Clean any blood clots or clumped eggs from the stripped eggs. Measure the volume stripped.

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SOP-9. Electroporation of channel catfish eggs.

The electroporation protocol described here assumes the operator has a Bio-Rad gene pulser (Model 165-2076, Hercules, California) and pulse controller (Model 165-2098) connected to electrodes on two opposing walls of a 6-cm^3 chamber constructed of clear plexiglass (Figure F-3).

1) Apply a thin coat of silicone vacuum grease (Dow Corning, Midland, Michigan) to the electroporation chamber.

2) Set voltage, capacitance, and resistance to desired parameters.

3) Place eggs (up to ~900 eggs) in chamber.

4) Add sufficient Hanks' balanced salt solution (HBSS) to cover eggs.

5) Add 500 µL of DNA construct (suspended in 300 mOsmol/kg HBSS at a concentration of 100 µg/mL).

6) Add 100 µL of isopropyl-ß-D-thiogalactopyranoside (IPTG, 200 µg/µL).

7) Swirl chamber to mix thoroughly.

8) Depress both red bottons to release electrical pulse, hold buttons down until the machine beeps.

9) Transfer eggs to a greased container for fertilization (see SOP-10).

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SOP-10. Artificial fertilization of channel catfish eggs.

1) Remove testis from mature channel catfish. Crush anterior testis (white part) in 20 mL of Hanks' balanced salt solution (HBSS, SOP-1) for each g of testis weight in a ziploc bag. Strain crushed testis through a fine screen or tissue sieve.

2) Estimate percent motility of sperm. Add 1 µL of sperm suspension to microscope slide and 20 µL of distilled water. Observe using 100-x magnification with a darkfield microscope. Record motility.

3) Decant excess HBSS from stripped eggs.

4) Add 0.5 to 1.0 mL of sperm suspension (volume is adjusted up or down depending on motility of sperm).

5) Add a volume of water equal to the volume of eggs in cup. Swirl to mix gametes.

6) Add another volume of water to facilitate water hardening and cohesion of eggs.

7) Check eggs every few minutes until an egg mass is formed. Record time until eggs clumped. Place eggs in plastic mesh basket in hatching tank or trough with highly aerated, high quality water maintained at 26 to 28° C.

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SOP-11. Anti-fungal treatment of eggs.

Eggs should be dipped daily in an iodine solution to inhibit growth of fungus. Eggs can be dipped immediately after fertilization and until reaching the eyed-stage.

1) Prepare a 1:100 solution of iodine (Argentyne) : water from the culture system in a food-grade container large enough to hold several egg baskets. Fill a similar-sized container with water from the same system.

2) Aerate the solution and place the egg baskets in the container.

3) Let eggs soak for 10 minutes.

4) Rinse eggs in the water-filled container to remove excess iodine.

5) Return eggs to the culture system.

6) Dip eggs daily until the eyed-stage is reached (around 5 days at 26°C).

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VITA

Mark Bates was born in Morgan City, Louisiana, on April 13, 1964. He is the youngest of three boys. During a large part of his childhood, Mark and his family lived overseas because his father was an executive with an international oil exploration company. The family lived in Singapore, Scotland, and Africa, until his father suddenly became ill. The family returned to the United States and settled briefly in Coral Gables, Florida, where his father underwent treatment for lung cancer. Following his father's death, the family returned to Louisiana and eventually settled in Lafayette, Louisiana. Mark attended Comeaux High School and the University of Southwestern Louisiana. He took time away from school to join the U. S. Naval Reserve and trained in San Diego, California, as a Hospital Corpsman and then at Camp Pendleton, California, as a Field Medical Technician. He returned to school and earned his bachelor of science in Microbiology in 1990. He began graduate school at Texas Tech University in Lubbock, Texas, as a student in the Texas Cooperative Fish and Wildlife Research Unit. For his thesis project, he grew the blue-green alga Spirulina platensis for evaluation as a protein source in fish feeds. In 1992, shortly before completing his master of science degree in Wildlife Sciences, Mark persuaded Karen Manuel to marry him. They both lived and worked in Lubbock, Texas, for one year before he continued his education at Louisiana State University in the School of Forestry, Wildlife, and Fisheries. They have one child, Amanda, who was born in Baton Rouge on January 6, 1995. He is currently a candidate for the Doctor of Philosophy degree in Wildlife and Fisheries Science.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Mark C. Bates

Major Field: Wildlife and Fisheries Science

Title of Dissertation: Production of Transgenic Channel Catfish

Approved:

Terence Tiersch
Major Professor and Chairman

John W. Larkin
Dean of the Graduate School

EXAMINING COMMITTEE:

James Avault

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Date of Examination:

April 1, 1997

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