DISRUPTION OF EMBRYONIC DEVELOPMENT IN CHANNEL CATFISH,

ICTALURUS PUNCTATUS, USING "STERILE-FERAL" GENE CONSTRUCTS

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DISRUPTION OF EMBRYONIC DEVELOPMENT IN CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, USING "STERILE-FERAL" GENE CONSTRUCTS

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A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama

August 8, 2005

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VITA

Christopher Michael Templeton, son of James Christopher and Sharon Rebecca (Wheelock) Templeton, was born April 15, 1976 in Kingsport, Tennessee. He graduated from Sullavin North High School, Kingsport, Tennessee in 1994. In August 1994, entered Maryville College, Maryville, TN and received the degree of Bachelor of Arts in the Biological Sciences May 1998. He married Brooke West, daughter of Bill and Suzanne West, on July 8, 2000. He and his wife had their first son, Malachi Michael Templeton on June 10, 2001. He began his graduate studies at Auburn University in the Department of Fisheries and Allied Aquacultures, January 2004, to pursue a Master of Science Degree. Their second child, Isabelle Noor Templeton, was born April 29, 2004.

THESIS ABSTRACT

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Master of Science, August 8, 2005
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103 Typed Pages

Directed by Rex A. Dunham

Channel catfish, *Ictalurus punctatus*, embryos were electroporated with a sterile feral 3 (SF3), sterile feral 4 (SF4), glutamate decarboxylase (GAD) or CAB constructs and a blank control. Doxycycline was applied to some of the SF3 and SF4 replicates, at 50, 100 and 150 ppm, from 4 hours post fertilization to first hatch. The developing embryos were observed from fertilization up through hatch. Dead and deformed embryos were removed during this period. Mortality rates were computed for different time intervals during development as well as for the entire development period. Deformity rates were also analyzed. Objectives were to determine the efficacy of the sterile feral constructs to disrupt embryonic development, the optimal concentration of doxycycline to prevent embryonic disruption and the time interval most crucial for application of doxycycline to prevent the expression of the developmental disrupter.

Embryos electroporated with the SF3 construct consistently demonstrated a higher mean mortality than that of the control groups. The overall mean mortality of the SF3 groups for all three experiments was 35.9% higher than the overall mean mortality of the three blank control groups. Similar results were obtained for the SF4 construct, whose overall mean mortality for the three experiments was 27.2% higher than that of the overall mean mortality for the three experiments with the blank control groups. The GAD construct produced an overall mean mortality that was 34.5% lower than that of the mean mortality of SF3 and 25.6% lower than that of SF4. The single mortality rate produce by the CAB construct was 82.3% lower than that of the mean mortality of SF3 and 79.9% lower than SF4. These consistently lower mortality rates produced by both CAB and GAD demonstrated that the introduction of exogenous DNA was not responsible for the larger mortality rates observed in the SF4 group and SF3 groups, but the action of the SF3 and SF4 constructs. The concentration of doxycycline most optimal for blocking the expression of the blocker in the SF3 construct was 100 ppm and 150 ppm for blocking the developmental disrupter of the SF4 construct. Some portion of the 18-61 hour post fertilization time period appears to be the time interval in which it is most crucial to apply doxycycline to prevent embryonic disruption.

ACKNOWLEDGEMENTS

The author would like to thank Fajin Wang for the many hours of assistance that he gave during a large portion of this study. I would like to thank Yi Hahn for the assistance he provided in the statistical analyses. The Aqua Bounty Technologies Inc. team also deserves recognition for conducting the PCR analyses of the eggs and embryos. I would like to thank Dr. Cova Arias, Assistant Professor, Auburn University, and Curtis Phillipson, Aqua Bounty Technologies Inc., who were responsible for growing the gene constructs used in this study. I would like to thank Vance Trudeau, University of Ottawa, for providing the GAD construct used in this study. I would also like to thank Ron Thresher and CSIRO for the Sterile Feral constructs used in this study. Also, I would like to thank Aqua Bounty Technologies Inc. for their support that provided my research assistantship throughout the entirety of this study.

Lastly, the author would like to thank his wonderful wife for her continued help. The parents and sister of the author have also been a source of encouragement and help throughout this process.

Style manual or journal used: Aquaculture

Computer software used: Windows Microsoft Word 2002, Windows Microsoft Excel 2002, SAS version 9.1.

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INTRODUCTION

Genetically Modified Organisms (GMOs) are no longer a novelty for the agricultural sector of the United States or the World. However, their application to commercial agriculture continues to be an issue of major concern. A GMO or transgenic organism is one that has been transformed by the insertion of one or more transgenes (http://www.fao.org/biotech/find-formalpha-n.asp). Transgenes are specific pieces of foreign DNA from another species or endogenous gene sequences that are re-inserted into the organism (Dunham, 2004).

Palmiter *et al*. (1982) are credited with the creation of the first transgenic animals. They demonstrated that increased growth could be achieved through insertion of growth hormone genes in mice. This motivated researchers in the field of aquaculture to apply these findings to fish. Progress in transgenic fish research was rapid from 1985 to 1987 with the creation of transgenic goldfish, *Carassius auratus,* in China, transgenic medaka, *Oryzias latipes,* in Japan, transgenic rainbow trout, *Oncorhynchus mykiss*, in France, and transgenic channel catfish, *Ictalurus punctatus,* in the U.S. (Dunham, 2004). There have been gene transfer studies conducted in over 35 species of fish, and half of these are significant for aquaculture (Zbikowska, 2003).

These advances have the potential to enable aquaculture to meet the growing demands of an increasing world population in the area of food production as well as the potential to impact the maintenance and improvement of the biodiversity of natural fisheries along with other natural resources (Melamed *et al.*, 2002; Dunham,

2004). In addition to these benefits, there is the potential to use transgenic fish in the area of biomedical research, and for monitoring potentially harmful chemicals in aquatic environments (Zbikowska, 2003). Major advances in the field of genetic improvement in aquaculture, via gene transfer have been for the traits, increased growth rate (Devlin *et al*., 2004), feed conversion efficiency (Cook *et al*., 2000), disease resistance (Sarmasik *et al*., 2002; Dunham *et al.,* 2002a), tolerance of low water quality (Dunham *et al*. 2002b), cold tolerance (Wang *et al*., 1995), body shape (Dunham *et al*. 2002b), dress-out percentage (Dunham *et al*. 2002b), and carcass quality (Dunham *et al*. 2002b).

Currently, no aquatic GMOs are commercially grown for human consumption (FAO/WHO, 2004). Research populations and commercial brood stocks are maintained, but not marketed. Transgenic ornamental fish, such as the GloFish™, have been sold to the public in the U.S. and Asia (Dunham, 2003). The extent to which GMOs are incorporated into commercial aquaculture will primarily depend upon the risk they pose to wild populations of conspecifics and to natural ecosystems, as well as the ability to confine these transgenic aquatic organisms (Zbikowska, 2003). Government regulations currently prohibit application of transgenic aquatic organisms except for the small ornamental fish (Dunham, 2004).

The prohibition placed on commercially growing transgenic fish is mostly attributable to the assumption that these transgenic fish could have superior fitness traits as compared to those of naturally occurring populations and cause demise to biodiversity or aquatic ecosystems (Hallerman *et al.*, 1993). The fitness traits, reproductive performance, foraging ability, swimming ability and predator avoidance, are key factors in determining the ability of transgenic fish to out compete conspecifics. Models evaluating the genetic risk of transgenic fish have been

developed by Hedrick (2001) and Muir and Howard (1999; 2001). Each of these models demonstrated the ability of transgenic fish to negatively impact conspecifics, but these models have weaknesses. These include utilization of an artificial environment, assumption of size related mating preferences, which do not exist for many species of fish, assumption of no genotype-environment interactions, no foraging and no predation (Dunham, 2004).

In most actual measurements of transgenic fish, their fitness is lower than that of wildtype individuals (Hedrick, 2001; Dunham, 2004). This is supported by studies that demonstrate transgenic fish have equal or lower rates of sexual reproduction and sexual maturity (Dunham *et al.*, 1992; Chatakondi, 1995), equal foraging abilities (Chitmanat, 1996), inferior predator avoidance (Dunham, 1995) and equal or inferior swimming ability (Farrell, 1997). Based upon the majority of the environmental risk data gathered on transgenic fish, in the event of an escape, ecological impacts should be minimal because of the overall reduced fitness of transgenic fish (Dunham and Devlin, 1998). However, this alone will not be enough to change current prohibition of transgenic fish from commercial application. Both physical and biological confinement, including genetic sterilization practices, will likely be necessary to ensure the absence of negative environmental impacts and government approval for application of these fish.

One method to ensure protection against negative environmental impacts is physical confinement. Physical confinement cannot guarantee the complete elimination of escapees. The continuous application of chlorine, rotenone or other approved fish toxins to a facility's discharge is chemical confinement. Apart from the obvious environmental impact of continuous application of these chemicals, this method is also unable to guarantee complete confinement. Biological confinement

such as removal of escapees via predators is another alternative. Each method of confinement discussed fails to provide a guarantee against environmental impact and also fails to address the issue of feral populations. Sterilization is the key to reducing the impact that transgenic and exotic species could exert on ecosystems.

Potentially, sterilization can be accomplished through surgery, immunology, radiation or with chemicals. Sterilization can also be accomplished genetically through hybridization, ploidy manipulation or transgenesis. The high labor costs and possibility for mortality make surgical sterilization only feasible for small numbers of valuable fish. Another pitfall is gonadal regeneration. Underwood *et al.* (1986) have demonstrated that fish have the ability to regenerate functional testes and ovaries. Ictalurid catfish (Bart, 1994) and Nile tilapia (Akhtar, 1984) have demonstrated the ability to regenerate whole or partial gonads following complete gonadectomies. The combination of its inability to guarantee permanent sterility and high labor costs make surgical sterilization of transgenics and exotics on a commercial level unfeasible. Sterilization via immunology and irradiation have also failed to provide permanent sterilization (Dunham, 2004). These methods, although potentially useful in specific situations and on a small-scale basis, all fall short of the need for a commercial scale, permanent, 100% effective sterilization.

Chemical sterilization has demonstrated some potential and needs further investigation, but to date has proven to be at best a temporary solution (Stanley, 1979). Chemicals that interfere with the production, release or utilization of sex hormones that interrupt mating behavior or cause sex reversal have all been used. Those that serve as hormone antagonists have demonstrated the most potential. Methallibure, cyproterone, trenbolone acetate, γ-aminobutyric acid (GABA) and recently bisazir are all hormone antagonists that have demonstrated varying levels of

efficacy (Dunham, 2004). A recent study by Young *et al.* (2004) employed sterile male sea lampreys, *Petromyzon marinus*, created using bisazir, in a study that evaluated this chemical's effect on production of sex steroids in this species. The study demonstrated the absence of endocrine disruption in this species when treated with bisazir. More studies are needed to further evaluate chemical sterilization's efficacy, effects on treated species, food safety issues and other important topics before chemicals such as bisazir could be commercially applied for sterilization purposes.

Genetic approaches show tremendous potential to supply a 100% effective and reversible method of sterilization. Monosex populations produced through hormonal sex reversal offer one potential method of limiting reproduction within a transgenic population (Pandian, 2003). This approach is only feasible when working with exotic species and not with conspecifics that are present in the watershed. Even if working with exotics, the escape of both sexes from a monosex/monogenetic culture unit can still have a short-term impact lasting up to two generations.

Genetic sterilization via polyploidy is another option for the production of genetically sterilized fish stocks. Although triploid mammals and birds are not viable, triploid fish are not only viable, but are also usually sterile (Chourrout *et al.*, 1986). Triploid fish also have the potential for increased growth (Chourrout *et al.*, 1986), increased carcass yield and increased survival (Dunham, 1996).

Triploid induction via heat shock, cold shock, pressure shock, or through application of chemicals has been the method of choice, but does not always provide 100% efficacy (Abdul-Razak *et al*., 1999; Dunham, 2004). Triploidy is not commercially practical for all fish because it can lead to slower growth in some species (Dunham, 2004).

Cotter *et al.* (2000) demonstrated the reduced ability of triploid Atlantic salmon to compete with naturally occurring conspecifics. Triploidy also decreases growth to the point that it negates some of the gain due to the growth hormone gene insertion (Abdul Razak *et al.,* 1999; Devlin *et al.,* 2004). Another problem with sterility via polyploidy is the necessity of fertile diploid brood stock, so environmental risk cannot be completely eliminated.

To date, the only genetic sterilization method that has the potential to produce 100% effective sterilization with the potential for reversibility is transgenic sterilization. Transgenic approaches are the best option for genetic sterilization and for confinement. Gene constructs need to be developed that have the capability for sterilization or lethality that can be temporarily reversed as needed to allow culture of these fish.

To date use of fish that are transgenics, domestics, interspecific hybrids, and exotic species is hindered by community and scientific concerns over the impact these fish could have or in some cases have had on wild stocks if introduced into natural environments. Reversible transgenic sterilization is the ultimate answer to virtually eliminate these risk issues for both aquaculture and recreational fishing. If successful, this technology could be applied in many situations. Sterilization has the potential to increase yields with precocious species, such as tilapia. Exotic species that were transgenically sterilized would be unable to establish feral populations. Sterile interspecific hybrids would be unable to backcross with parental species (Curtis *et al.,* 1987). Also, declining or decimated natural stocks could be supplemented without concern of genetic introgression from the introduced conspecifics since they were transgenically sterilized (Dunham, 2004). Lastly, the ability to transgenically sterilize fish transgenic for other constructs would eliminate all most all environmental risks

encountered when applying these fish to commercial aquaculture. Impacts of non-transgenic aquacultured fish on wild fish stocks, such as the common controversy with aquacultured salmon impacting natural populations could be greatly minimized with this technology. This technology has the potential to even benefit recreational fishing by incorporating exotics and hybrids that were successfully sterilized.

The Commonwealth Scientific Industrial Research Organization (CSIRO), Australia, has iniated research on transgenic sterilization and developed a method termed "sterile feral." The method employs the insertion of a gene that can reversibly interrupt gamete and/or embryonic development (Dunham, 2004). CSIRO has identified genes in zebrafish, *Danio rerio*, and Pacific oysters, *Crassostrea gigas,* that are essential to and active only during embryonic development and/or gametogenesis (Thresher *et al.*, 2001). In its most basic form, the construct contains a blocker to embryonic development and/or gametogenesis and a genetic switch to control it. The sterile feral constructs are made of a species-specific promoter coupled with a repressible element, which drives expression of a blocker gene. For construction of sterile feral 3 (SF3) and sterile feral 4 (SF4), a developmental stage-specific promoter was coupled to components derived from the commercially available Tet-Off controllable expression system marketed by CLONTECH (http://clontech.com/, Protocol # PT3001-1, version # PR95962). The blocker can be inactivated in captivity by signalling for the promoter to allow production of the repressor protein, which prevents transcription of the blocker and subsequent knockout (Figure 1). The promoter can be triggered to produce the repressor protein through the incorporation of doxycycline or tetracycline into the diet or through soluble form (Dunham, 2004).

The basis of this technology is the Tet-Off and Tet-On system developed by BD™ Biosciences. In its most basic form, the tetracycline repressor (*tet*R) binds to its

operators within the promoter region of the Tn*10*-specified tetracycline-resistance operon of *E. coli* and prevents transcription when tetracycline is absent from its environment. In the presence of tetracycline, the *tet*R does not bind to its operators and thus allows for transcription (Gossen and Bujard, 1992). The *tet*R is bound to the activating domain of the virion protein 16 (VP16) of herpes simplex virus (HSV). The resulting combination is referred to as a "tetracycline-controlled transactivator" (tTA) (Gossen and Bujard, 1992). This transactivator used to regulate the targeted gene is attached to a cytomegalovirus (CMV) promoter. In the Tet-Off system, the tTA transactivator binds to the Tet Response Element (TRE) contained within the CMV promoter and activates transcription when tetracycline is absent. In the Tet-On system, which employs the rtTa transactivator, the Tet repressor protein with four amino acid changes, also binds to the TRE and induces transcription in the presence of tetracycline (Figure 2). Gossen and Bujard (1992) studied this system and its be control over a luciferase gene. They found that the luciferase activity could regulated to over five orders of magnitude depending upon the concentration of the antibiotic in the culture medium. The Tet-On and Tet-Off systems have the capacity to be controlled at varying levels, thus it is not a choice between fully on or off. Yin and Schimke (1995) found that predictable levels of gene expression could be produced based upon the amount of tetracycline in the culture medium.

In the case of the SF3 or SF4 constructs, doxycycline or tetracycline act upon the tetracycline-responsive transcriptional activator protein (tTA), which is a fusion of TetR and VP16 from the pTet-Off regulatory plasmid (Thresher *et al.*, 2001). A zebrafish promoter drives expression of the tTA, and tTA regulates the tetracycline (Tet)-responsive human cytomegalovirus promoter (*P*hCMV*-1), which controls expression of the blocker gene. *P*hCMV*-1 contains the tetracycline responsive

Figure 1. Flow chart of the steps of the Sterile Feral mechanism.

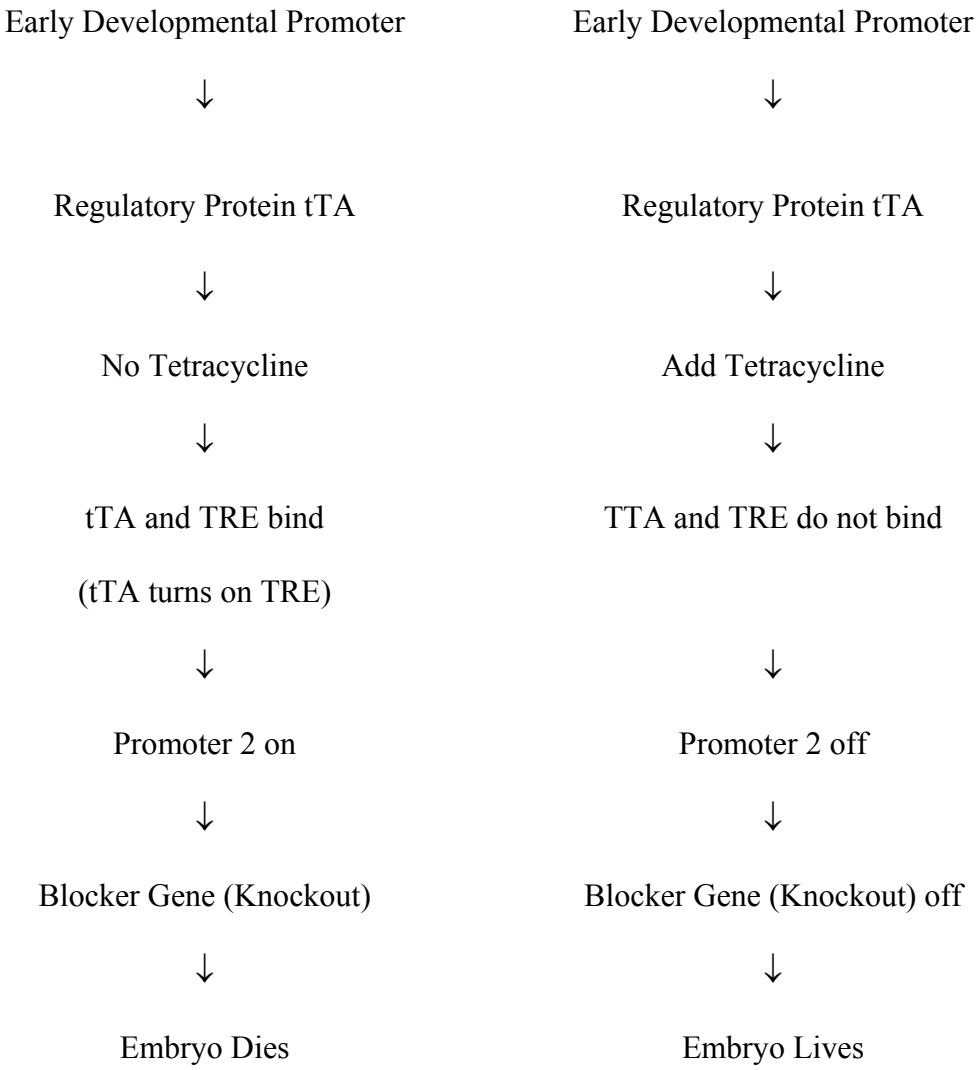
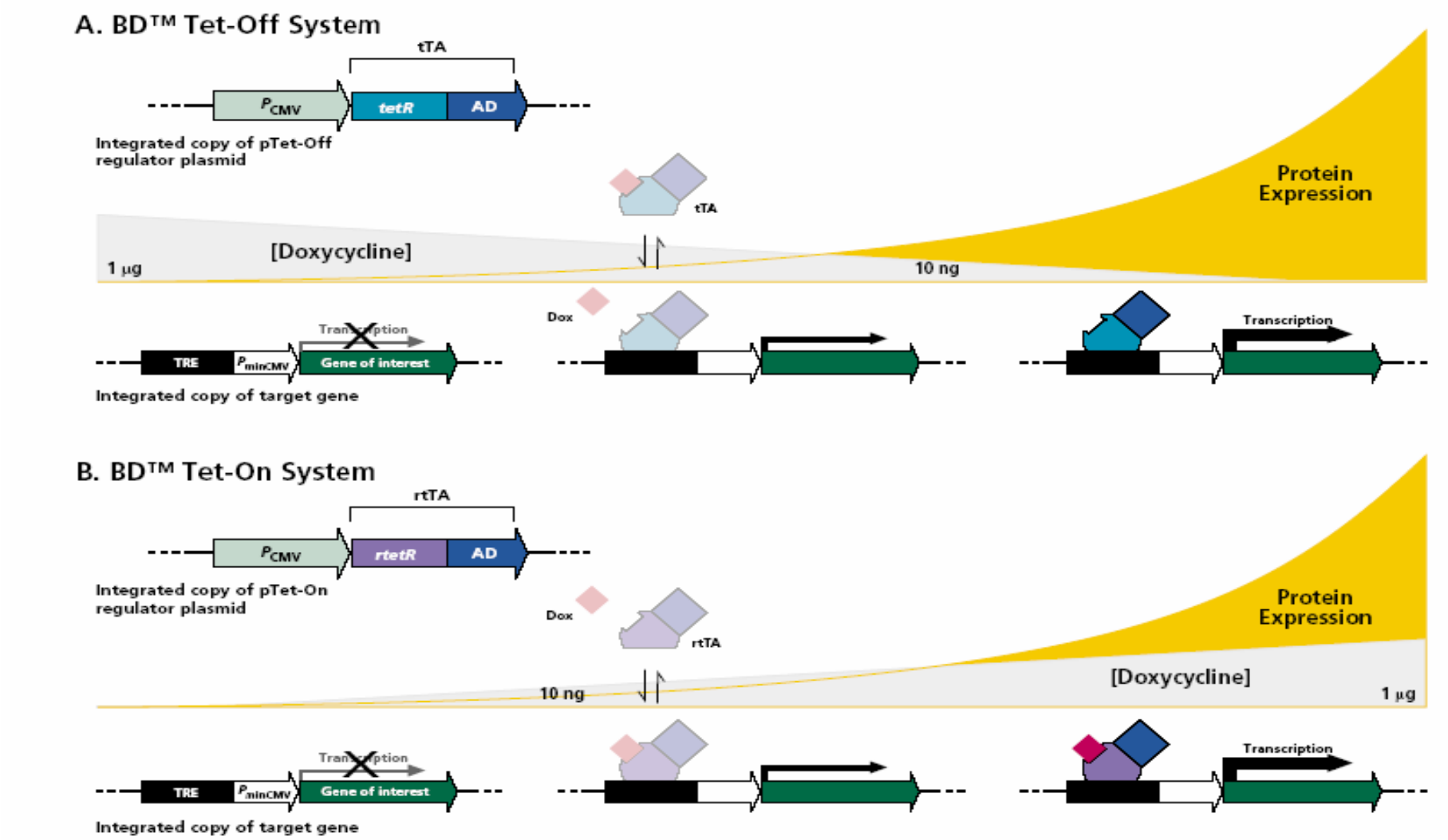


Figure 2. The BD™ Tet-Off and Tet-On Systems (Taken from BD™ Tet-Off and Tet-On Systems by BD Biosciences)



element (TRE), which consists of seven copies of the tet operator sequence (*tetO*) located just upstream of the minimal CMV promoter (*P*minCMV). *P*hCMV*-1 does not function in the absence of binding of transactivator protein to the *tetO.* The tetracycline-sensitive element is described by Gossen and Bujard (1992) for Tet-Off and Tet-On is described by Gossen *et al.* (1995) and Kistner *et al.* (1996). In the Tet-Off system, addition of tetracycline (Tet) or doxycycline (Dox; a Tet derivative) prevents the binding of tTA to the Tet-responsive element. Expression of the blocker gene is controlled by TRE/CMV, and is repressed until tetracycline is removed from the incubation water. In the absence of tetracycline or doxycycline, the blocker gene is transcribed if the developmental promoter continues to express tTA, thus killing the developing embryo.

If successful, this system would prevent any escapees from reproducing or producing viable offspring thus preventing the establishment of feral populations among native wild stocks. Reproduction would only be possible under appropriate culture conditions. A repressor compound is necessary at an embryonic stage in the hatchery to allow the organism to live (Dunham, 2004). This technology has its beginnings in a procedure that is used to genetically sterilize transgenic plants. Much controversy was raised over the use of this technology because it forced farmers to depend upon the vendor for seed supply, thus leading to its abandonment, at least for now (Niiler, 1999). The application of principles taken from this technology and applied to aquatic organisms appears to be very promising and more acceptable because of the increased mobility and risk of transgenic fish compared to transgenic plants.

Our objective was to evaluate this technology's potential for application in channel catfish. The channel catfish, *Ictalurus punctatus,* is the primary cultured fish

for food production in the U.S. (Engle, 2003). The catfish industry in the United States boasts the most successful aquaculture business to date. Catfish sales to processors for 2004 was estimated at 286 thousand to 288 thousand metric tons for 2004, at an average of 1.50 to 1.56 dollars per kilogram (Harvey, 2004). The industry is currently struggling to keep pace with the increasing cost of inputs and competition with inexpensive imported fish. Also, efficiency of production, profitability and sustainability need improvement.

Transgenic channel catfish have been developed that demonstrate increased growth rates and increased disease resistance (Dunham *et al.*, 1992; Dunham *et al.*, 2002c). The possible risks and concerns rising from the use of these transgenic aquatic animals has prevented their application to date. Transgenic sterilization, if successful, would facilitate approval of this technology for the catfish industry and others.

The primary objective of this research was to evaluate the sterile feral technology to transgenically sterilize channel catfish, *Ictalurus punctatus*, possessing transgenes, and to temporarily reverse the sterility as needed to produce sterile progeny for production. Specific objectives were to: 1) determine the ability of the SF3 and SF4 gene constructs to interrupt embryonic development, 2) to determine the concentration of doxycycline necessary to counteract the SF3 and SF4 constructs thus allowing for normal development and 3) to determine the period of development at which SF3 and SF4 disrupts development.

MATERIALS AND METHODS

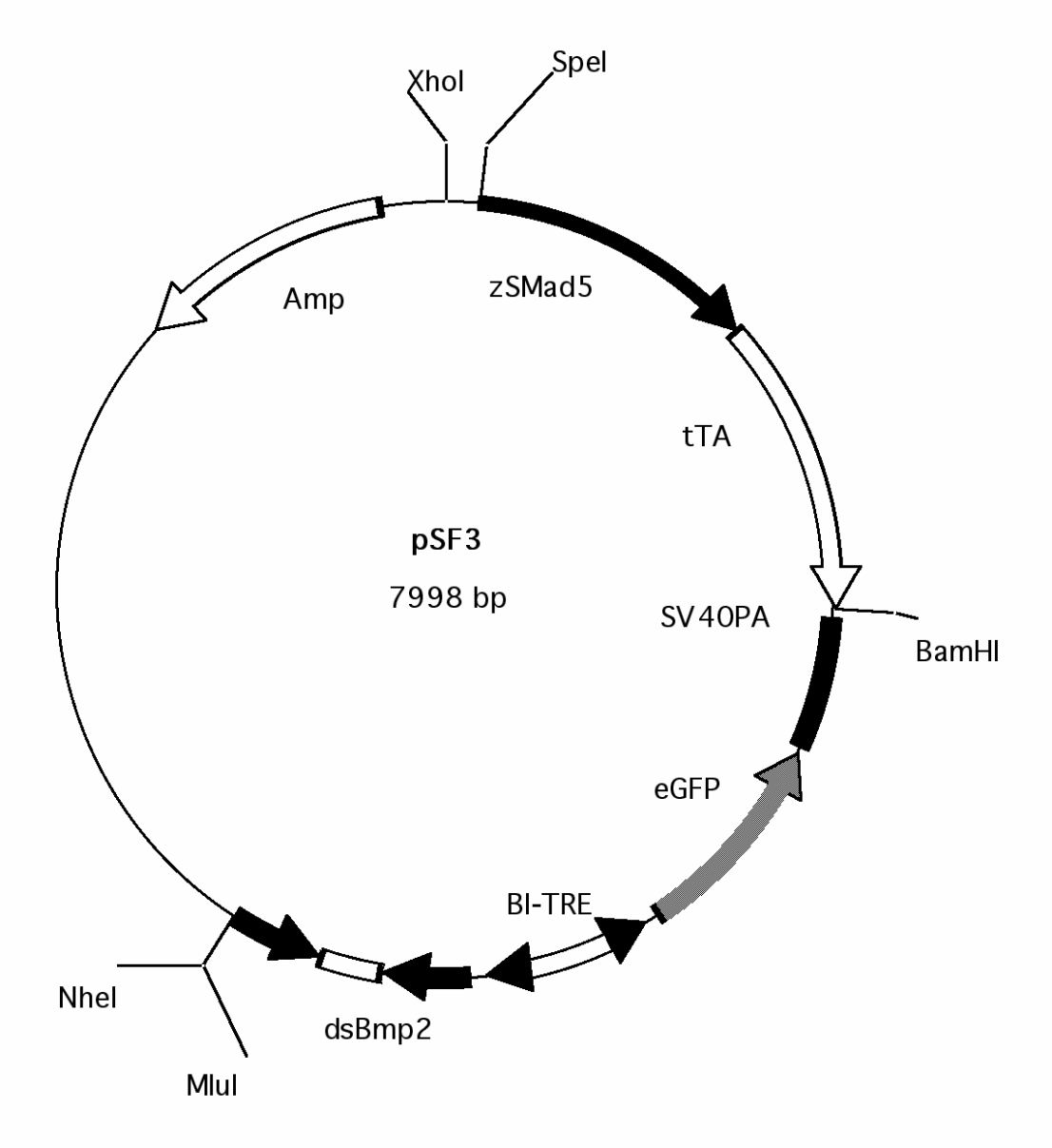
Sterile Feral Constructs

Sterile Feral gene constructs (SF3 and SF4) were obtained from CSIRO. For construction of SF3 and SF4, a developmental stage-specific promoter was coupled to components derived from the commercially available Tet-Off controllable expression system marketed by CLONTECH (http://clontech.com/, Protocol # PT3001-1, version # PR95962).

SF3 Construct

This construct contains the zebrafish zSMad5 promoter whose expression drives the tTA (Figure 3). Mutations in the zebrafish SMad5 gene result in a dorsalized mutation designated somitabun and the dorsalised mutant phenotype has been shown to be rescued by injection of SMad5 mRNA at the single cell stage. The next component is the SV40PA enhancer, which is taken from a eukaryotic cell virus. This enhancer acts on the zSMad5 promoter to increase the level of transcription (Kriegler, 1990). The next sequence is the enhanced green fluorescent protein (EGFP) coding region. The next section of the plasmid contains a bi-directional tet-responsive element (BI-TRE), which contains seven copies of the 42 bp tet operator sequence (tetO). When tetracycline is present, the tTA does not bind to the TRE which blocks gene expression from the TRE. The next sequence in the construct is a single tet responsive double stranded RNA blocker construct under the regulation of zBMP2 promoter. The dsBmp2 gene serves to block expression of the zSMAD5

Figure 3. Sterile Feral 3 Construct. (Thresher *et al.*, 2001)



promoter. The plasmid was linearized by Aqua Bounty Technologies Inc. using the Xhol restriction enzyme. The entire length of the construct is 7998 bp (Thresher *et al.*, 2001).

SF4 Construct

The only difference between the SF3 plasmid and SF4 plasmid is that the dsBMP2 construct was replaced with a pBI-bmp2-Sense construct that served as a blocker on the zSMAD5 promoter (Figure 4). This plasmid was also linearized using the Xhol restriction enzyme. The entire length of the construct is 8611 bp. (Thresher *et al.*, 2001).

Other Constructs

Glutamate Decarboxylase (GAD) Construct

The GAD construct is made up of the GAD65 gene (Accession number AF045594) put into the BsrGI, which is a isoschizomer of *Kpnl*, site of pFV-3 Cat (Figure 5). The GAD65 gene was obtained from the University of Ottawa and the carp ß-actin promoter was obtained from Auburn University. Aqua Bounty Technologies Inc. fused these two components to make the GAD construct used in this study. The GAD65 gene is 2,653 bp and the pFV-3 Cat carp ß-actin promoter is 6,782 bp.

CAB Construct

The pPCab-IfS plasmid contains the GnRHsense (long fragment) under the control of the B-actin promoter pPCab-IfA (Figure 6). This construct was employed in the first experiment. The entire length of both plasmids was 8546 bp.

Brood Stock

Potentially transgenic brood stock were used for this research. Brood stock were obtained from the Genetics Research Station of Auburn University in Auburn,

Figure 4. Sterile Feral 4 construct (Thresher *et al.*, 2001).

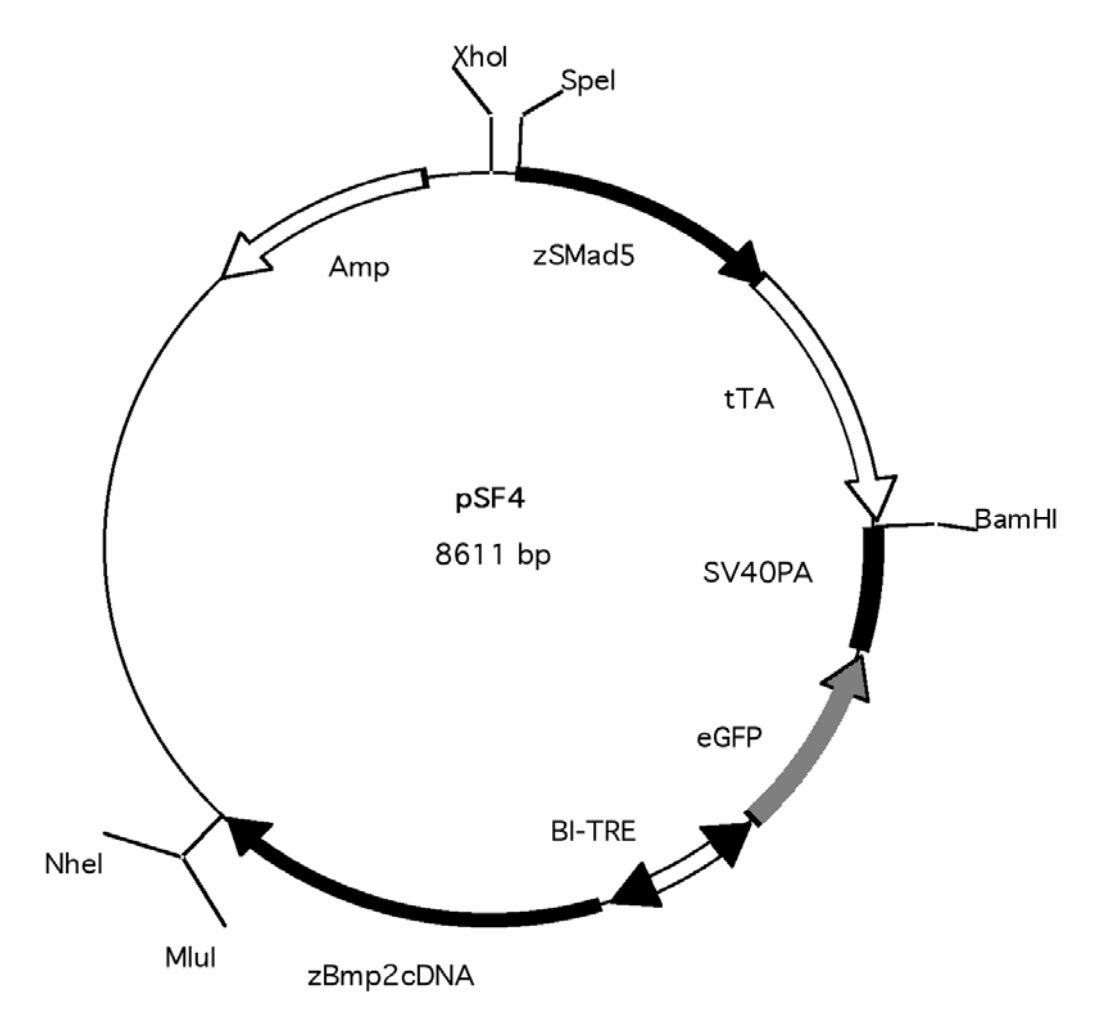


Figure 5. Glutamate decarboxylase construct (Caldovic *et. al*, 1995).

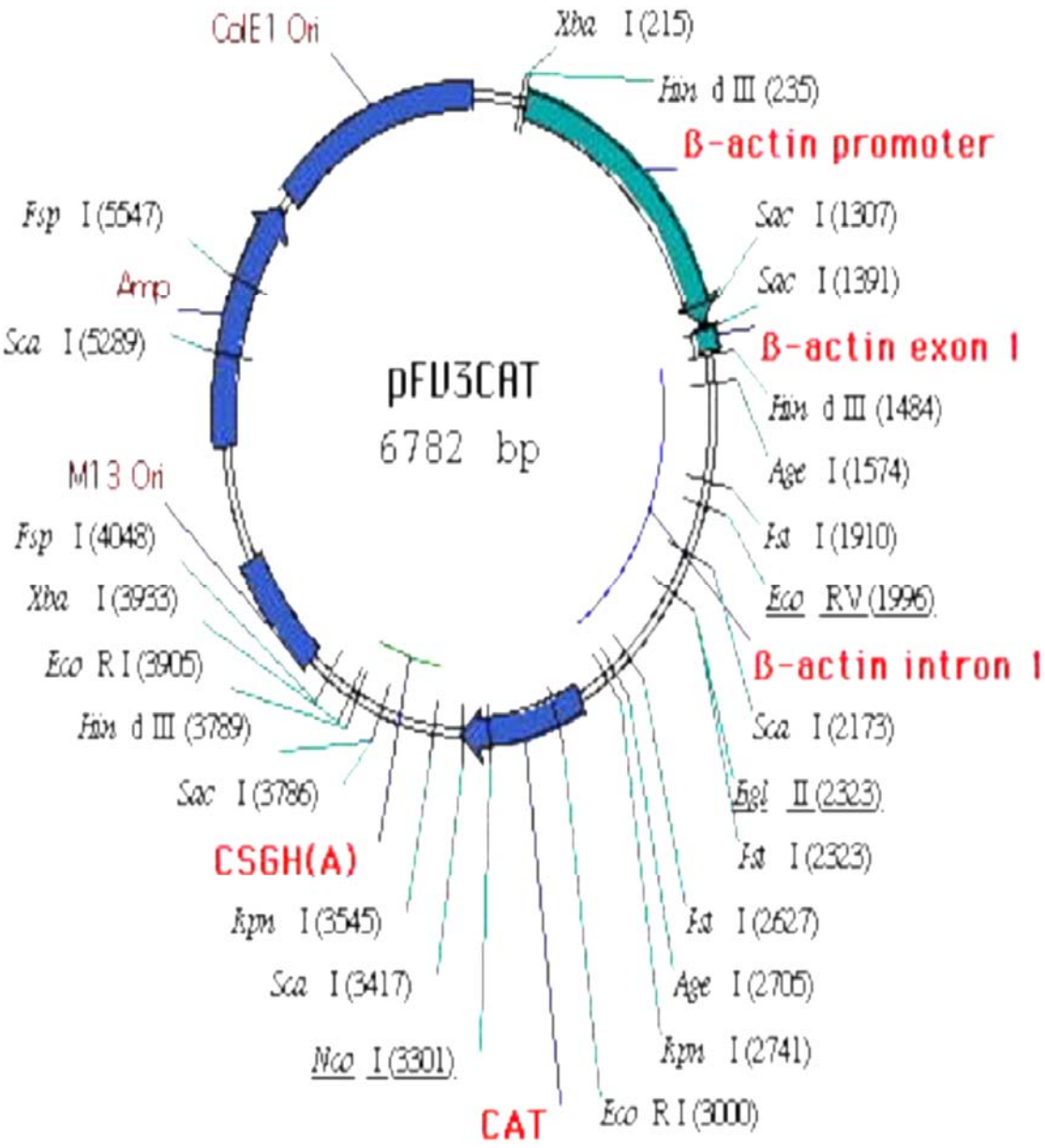
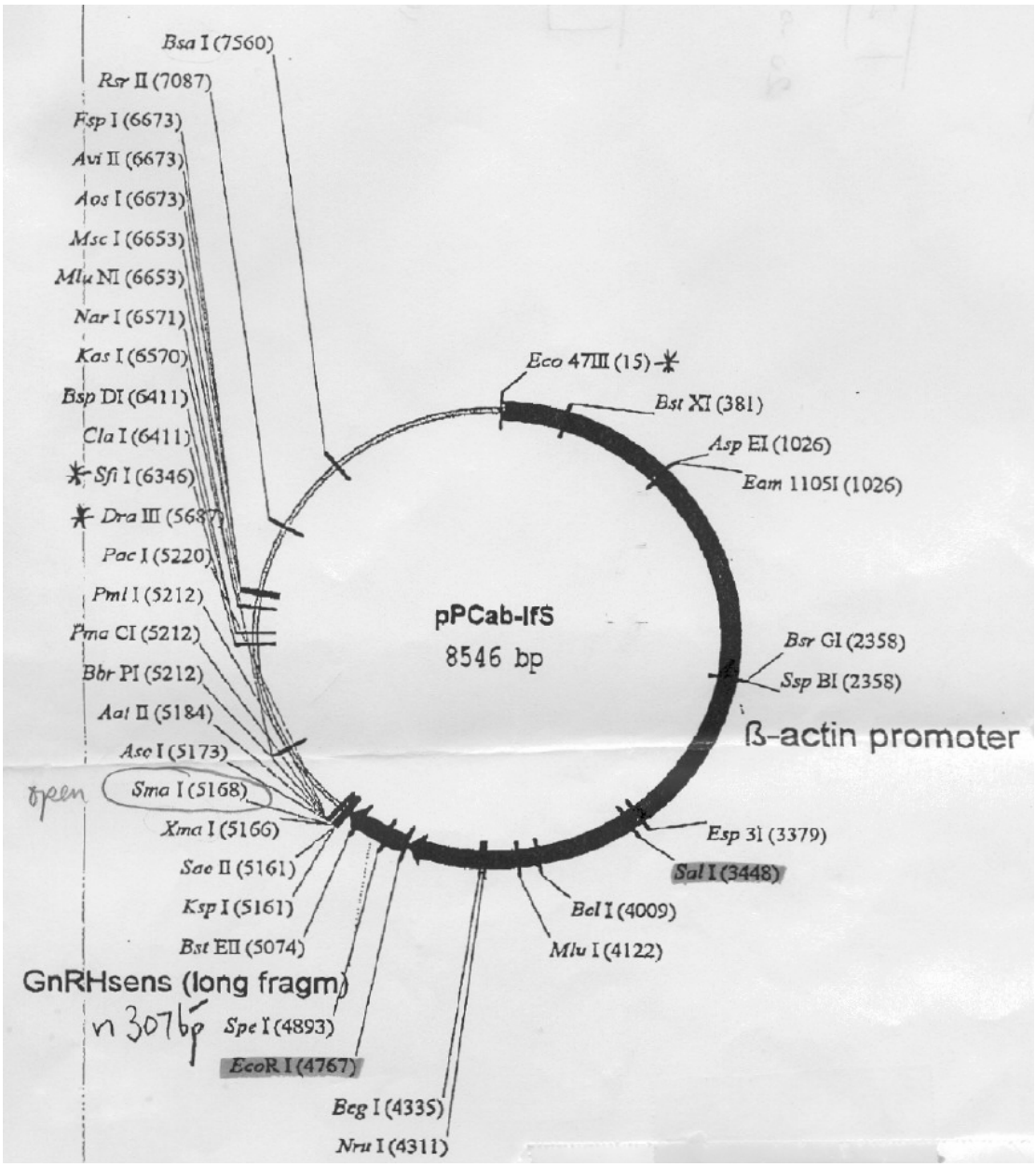


Figure 6. CAB construct.



AL, USA. The fish were collected from two separate ponds, G-32 and G-39. The brood stock collected from pond G-32 were potentially transgenic for rainbow trout metallothionein promoter coupled with channel catfish growth hormone gene. The brood stock collected from G-39 were potentially transgenic for an all fish promoter coupled with the cecropin B gene. Two separate spawnings were conducted. Procedures were the same each time except as noted. For the first round spawning, 6 females and 4 males were collected from pond G-32. Seven females and 4 males were collected from pond G-39. The second round of spawning used 12 females from pond G-32 and 12 males from G-39.

Spawning

The fish were separated by sex and held in separate flow-through tanks. They were then weighed and the females were placed in individual mesh bags. The females received two doses of luteinizing hormone releasing hormone (LHRHa). The first injection was with a concentration of 200 μg/ml at a rate of 0.1 ml/kg (20 μg/kg). The second injection had a concentration of 1000 μg/ml also at a rate of 0.1 ml/kg (100 μg/kg). The injections were given approximately 12 hours apart and at a water temperature of 25-27°C. Testes of four G-32 males and four G-39 males were harvested. The testes were harvested and placed in two separate clean sealed bags for use upon arrival of the eggs. The first eggs were released by a G-39 female approximately 35 hours post second injection. Eggs from two females from each pond were randomly separated into multiple greased pie pans to prepare for fertilization. Testes from four G-32 and four G-39 males were manually ruptured to release milt over the eggs. Water was applied at 0036 in order to stimulate fertilization of these eggs.

The second spawning began approximately twenty days after the first round began. G-32 females were crossed with G-39 males. The females received LHRHa implants at a dosage rate of 75 µg/kg. Eggs from five G-32 females were randomly fertilized with twelve males from G-39.

Gene transfer

Fertilized eggs were loaded into 50-mm plastic petri dishes for electroporation. Random samples of eggs were taken from multiple pie pans and placed in each petri dish. Approximately 2 ml of each genetic construct media (50 µg/ml) was placed in the dish containing the eggs. The first trial had six replicates of the two sterile feral constructs and three replicates of GAD and CAB. There were also 3 electroporated blank control replicates. The eggs were electroporated at 0106, 60-100 minutes post-fertilization (Dunham et al., 1992; Powers et al., 1992). Electroporation was performed with the Baekon 2000 macromolecule transfer system. Its parameters were set at 6 kV, 2^7 pulses, 0.8 sec burst, 4 cycles, 160 µsec (Powers et al., 1992). The electrode was situated 1-2 mm above the egg layer in each dish. The eggs were then incubated in the DNA solution or nanopure water for 10 minutes before transferring to prepared incubation containers. Each incubation container was initially filled with 4 L of 100% Holtzfretter's solution. Three out the six sterile feral constructs, both SF3 and SF4, were incubated in 100% Holtzfretter's solution treated with doxycycline (100 mg/ml). The doxycycline rate was increased to 150 mg/L approximately 60 hours post fertilization.

The second experimental trial of catfish followed the same electroporation protocol, except for the amount of time the eggs remained in the DNA solution. Post electroporation, the eggs remained covered in the petri dish for 30 minutes before being divided into multiple incubation containers. The electroporated eggs were

divided into six replicates for the sterile feral construct groups and three for the GAD and a electroporated blank control group. For the purpose of studying the amount of antibiotic necessary to repress the blocker, three different rates of doxycycline were used in the second experiment. In three of the six sterile feral replicates of both SF3 and SF4, from both the first and second batch, one replicate was incubated in 50 mg/L, 100 mg/L, and 150 mg/L. Thus, for the second experiment there were six SF3 replicates, three treated with one level of the doxycycline concentration and three without doxycycline. The same protocol was followed for the SF4 construct. There was also three replicates of the GAD construct. A third experiment was conducted using only the sterile feral constructs and a electroporated blank control group. The sterile feral constructs were replicated three times each while a the electroporated blank control was replicated three times (Table 1). All treatments were initially incubated in 4 L of 100% Holzfretter's solution.

Incubation and Hatching

After the electroporation procedure was completed, the eggs from each genetic group were equally distributed to the incubation containers. Each container was initially filled with 4 L of 100% Holzfretter's solution. The incubation solution was maintained as 100% Holzfretter's until 48 hours post fertilization, at which time it was reduced by 25% each 24 hour period dechlorinated city water until it was replaced with 100% dechlorinated city water. Doxycycline treatment was terminated around 24 hours prior to hatch. Each replicate was sampled at approximately 24-hour intervals. Each sampling involved a random sample of eggs or the complete number of eggs. Eggs were observed under a dissecting microscope. Dead and deformed eggs or embryos were removed and frozen for future PCR analysis. Normal fry were acclimated and transferred, 3 days post-hatch, to a recirculating system located at the

Table 1. Treatments and number of replicates employed in each experiment.

Treatment	Experiment 1	Experiment 2	Experiment 3
SF3	3	3	3
SF4	3	3	3
SF3 Dox 50	-	2	-
SF3 Dox 100	3	2	-
SF3 Dox 150	-	2	-
SF4 Dox 50	-	2	-
SF4 Dox 100	3	2	-
SF4 Dox 150	-	2	-
Control	3	3	3
CAB	3	-	-
GAD	3	3	-

Genetics Research Station, Auburn University, Auburn, AL. Fish in each replicate were observed again 1 month post-hatch for deformities. Fish were enumerated and weighed.

The second round of catfish also had two replicates each of varying concentrations of doxycycline, 50 mg/L, 100 mg/L, and 150 mg/L. The sampling intervals were slightly longer due to the increased number of replicates, and were maintained at intervals ranging from 6-48 hours.

Egg and Embryo Observations

As soon as the eggs were transferred to the incubation containers they were observed and inspected for death and deformity. The observations were made with a dissecting microscope and lamp as well as gross observations. Initially, dead eggs, white or opaque in color, were removed and frozen. The second experiment was began with a total fertilized egg count to establish a baseline on total fertilized eggs in each replicate. Fertilization was based upon the observation, starting at 2 hours post fertilization, of a rounded blastodisc, which was moderately raised from the surface of the yolk (Mansueti and Hardy, Jr., 1967). Throughout the incubation phase, dead or deformed were removed and frozen for future PCR analysis. Embryos with any structural deformities, most notably in the posterior region, were removed and frozen. Embryos or fry that were significantly behind in development as compared to others in the group or completely arrested were also removed and frozen.

Water Analysis

An alcohol thermometer was used to monitor temperature throughout the experiment. Multi-test strips from Mardel were used to monitor overall water quality parameters, such as total hardness, total alkalinity, nitrates, nitrites and pH. The pH was also monitored through pH strips. A liquid ammonia test kit, Aquarium

Pharmaceuticals, Inc., was used to monitor total ammonia levels in the incubation tubs.

PCR Analysis

DNA anaylysis was conducted at Aqua Bounty Technologies Inc. (San Diego). DNA was extracted from about 50 milligrams of catfish fin or up to 4 embryos using the DNAzol reagent. Samples were resuspended in 8 mM NaOH and further diluted 5 fold with H_2O. To check the integrity of the DNA the actin gene from catfish was used as a standard. Specific primers against the actin gene were designed. The primers are called CFaActin-3461F/3551R and have the sequences 5´-TACTCCGTTTGGATCGGTGG-3´ and 5´-GACCGGCCTCGTCGTAGTC-3´ respectively.

Samples were run in duplicate on the Corbett Research RG-3000 real time PCR system using Applied Biosciences SyBR Green master mix. Transgenic fish or embryos were determined by using one of two sets of primers one for the Bi-TRE promoter region and the other from the tTA gene. The primers are as follows: Bi-Tre-114R - 5´-GACCTATATAAGCAGAGCTCGTTTAGTG-3´, Bi-Tre-14F- 5´-GGCTGGATCGGTCCCG-3´ and SF3-4 tTA-493F -5´-CTGATAGTATGCCGCCATTATTACG-3´, SF3-4 tTA-593R – 5´-TCCGCATATGATCAATTCAAGG-3´. Each sample was run in duplicate and the melt curves were analyzed for positives.

Data Analysis

Chi-square tests were performed on cumulative mortalities, timeline mortalities and cumulative deformities to calculate significance in comparing the sterile feral constructs with various controls and doxycycline treated groups.

RESULTS

Mortality rates (dead and deformed dead)

SF constructs versus controls

For experiment one, the mean mortality percent for both SF3 (79.2%) and SF4 (70.6%) were greater ($P < 0.05$) than that of the electroporated control group (28.1%) (Table 2) (Figure 7). The mean mortality percent was higher ($P < 0.05$) in the SF3 group as compared to SF4. Mean mortality for both SF3 and SF4 were greater ($P < 0.05$) than both CAB (13.9%) and GAD (34.5%) DNA control groups (Table 2). A higher ($P < 0.05$) mean mortality percentage was demonstrated by the GAD group as compared to CAB.

Mean mortality percentages in experiment two for SF3 (70.1%) and SF4 (59.2%) were greater ($P < 0.05$) than the electroporated control group (27.5%) (Table 2) (Figure 8). The SF3 group in this experiment had a mean mortality that was greater ($P < 0.05$) than that of the SF4 group. The mean mortality percentages for both SF3 and SF4 were greater ($P < 0.05$) than the GAD (53.5%) DNA control group (Table 2).

In experiment three, mean mortality for both SF3 (85.5%) and SF4 (84.0%) were greater ($P < 0.05$) than the electroporated control group (69.4%) (Table 2) (Figure 9). The mean mortality was not different ($P > 0.05$) in SF4 as compared to SF3 (Table 2).

Table 2. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF3 and SF4) gene constructs, glutamate decarboxylase (GAD), CAB and a electroporated control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

	Mean Mortality (%)			
Treatment	**Experiment 1[a]**	**Experiment 2 [a]**	**Experiment 3 [a]**	**Grand Mean**
SF3	79.2	70.1	85.5	78.5[1]
SF4	70.6	59.2	84.0	69.6[2]
Control	28.1	27.5	69.4	50.3[3]
GAD	34.5	53.5	-	51.4[3]
CAB	13.9	-	-	13.9[4]

[a] – SF3 was greater than SF4 ($P < 0.05$); SF3 and SF4 were greater than control, GAD and CAB ($P < 0.05$), chi-square.

[1 2 3 4] – Mean percentages with different letters within the column are different ($P < 0.05$), chi-square.

Figure 7. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF3 and SF4) gene constructs and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

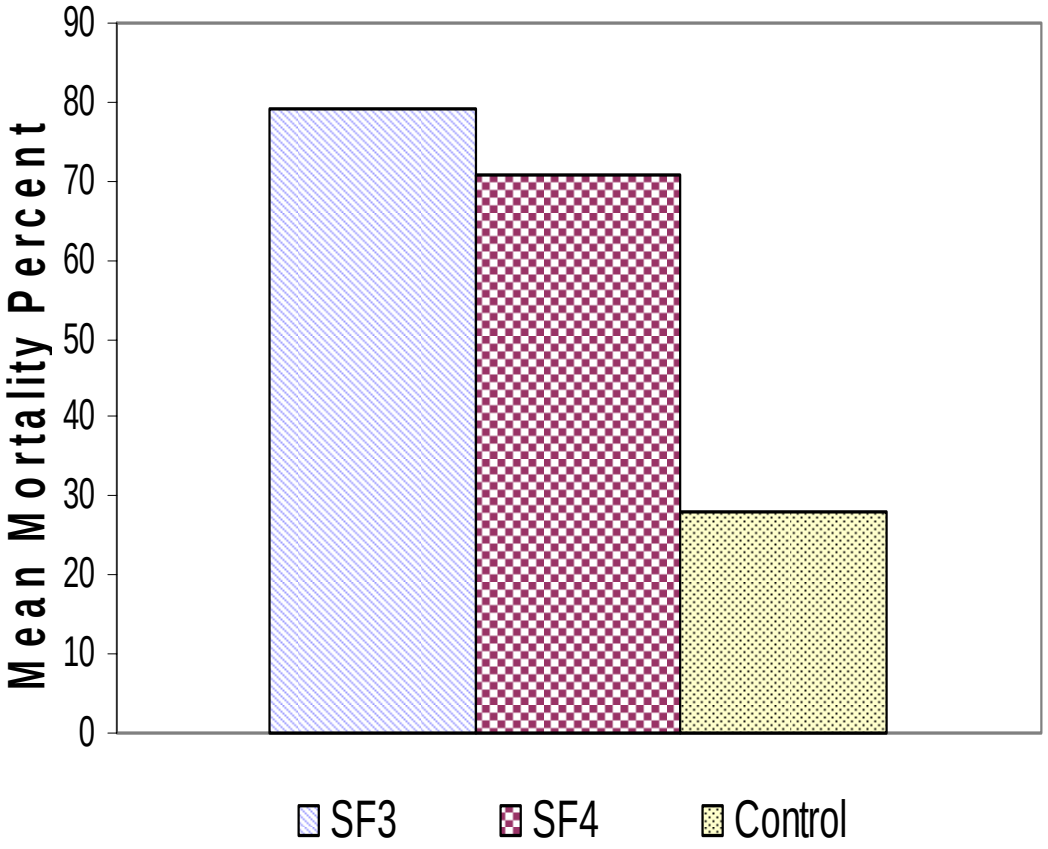


Figure 8. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF3 and SF4) gene constructs and a electroporated control in experiment two. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization up to 137 hours post fertilization.

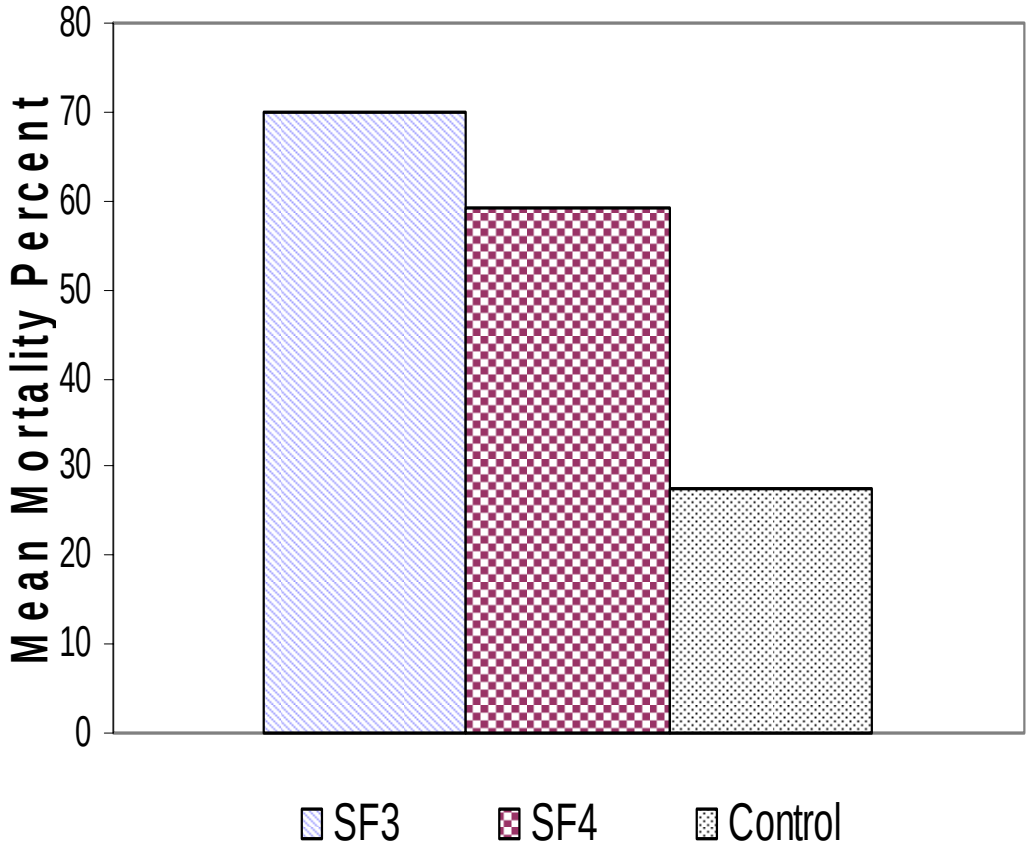
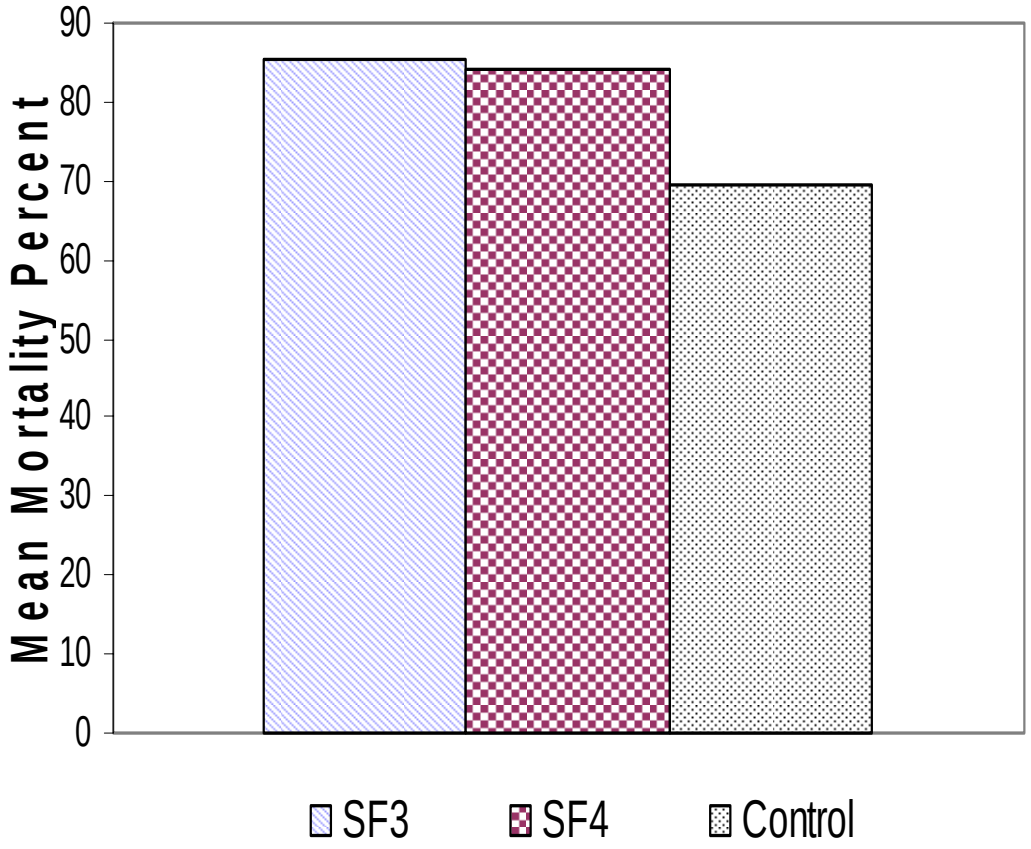


Figure 9. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF3 and SF4) gene constructs and a electroporated control in experiment three. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization up to 137 hours post fertilization.



Effect of doxycycline

The first channel catfish experiment produced a mean mortality percentage for SF3 (79.2%) that was higher ($P < 0.05$) than the SF3 doxycycline 100 group (47.3%) (Table 3) (Figure 10). Thus, the SF3 doxycycline 100 group reduced the relative mean mortality percentage of SF3, which was 179% higher than the electroporated control, to a 68.3% increase. The mean mortality percentage was higher ($P < 0.05$) in the SF3 doxycycline group as compared to the non-electroporated control (28.1%) (Table 3).

In the second experiment, a mean mortality percentage for SF3 (70.1%) was greater ($P < 0.05$) than both SF3 doxycycline 50 (56.4%), and 100 (55.6%). It was less ($P < 0.05$) than that of SF3 doxycycline 150 (77.5%) (Table 3) (Figure 11). Therefore, the SF3 doxycycline 50 group reduced the relative mean mortality percentage for SF3, 154.9%, to 105.1%. The SF3 doxycycline 100 group reduced it to 102.2% and lastly, the SF3 doxycycline 150 group produced a 181.8% increase over that of the electroporated control group. Mean mortalities were higher ($P < 0.05$) in all SF3 doxycycline groups as compared to the electroporated control group (27.5%). The mean mortality percentages were not different ($P > 0.05$) in the SF3 doxycycline 50 and 100 groups. SF3 doxycycline 150 had a higher ($P < 0.05$) mean mortality percentage than both SF3 doxycycline 50 and 100 (Table 3).

In the first experiment, the mean mortality percentage for SF4 (70.6%) was not different ($P > 0.05$) than the SF4 doxycycline 100 group (63.0%) (Table 4) (Figure 12). SF4 had a higher ($P < 0.05$) mean mortality as compared to the electroporated control group (28.1%). The mean mortality percentage of SF4 relative to that of the electroporated control was 151.2% higher and was reduced to 124.2% by the SF4 doxycycline 100 group.

Table 3. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3) gene construct, and treated with three different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post-fertilization to first hatch and a electroporated control group. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

	Mean Mortality (%)			
Treatment	**Experiment 1**	**Experiment 2**	**Experiment 3**	**Grand Mean**
SF3	79.2[a]	70.1[b]	85.5[a]	78.5[a]
SF3 Dox 50 ppm	-	56.4[c]	-	56.4[b]
SF3 Dox 100 ppm	47.3[b]	55.6 [c]	-	52.9[b]
SF3 Dox 150 ppm	-	77.5 [a]	-	77.5[a]
Control	28.1[c]	27.5[d]	69.4[c]	50.3[b]

[a b c] - mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 10. Mean mortality of channel catfish, *Ictalurus punctatus*, in experiment one. Embryos were electroporated with the Sterile Feral (SF3) gene construct, and treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch and a electroporated control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

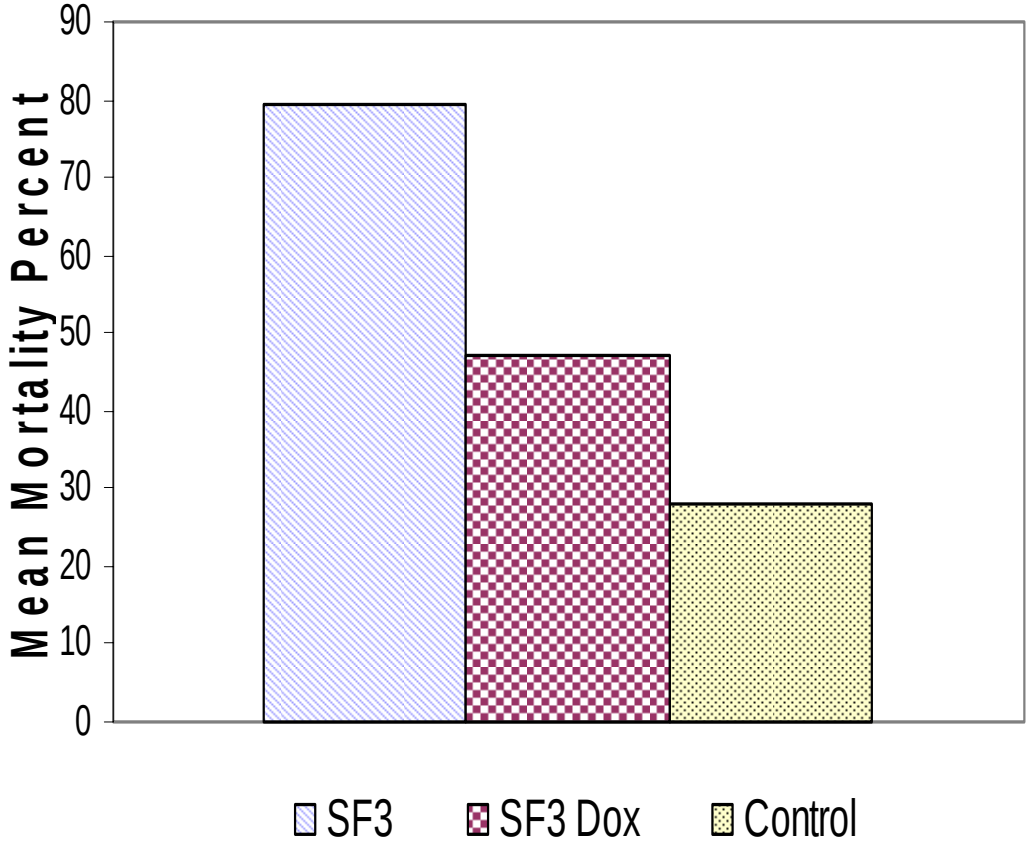


Figure 11. Mean mortality of channel catfish, *Ictalurus punctatus*, in experiment two. Embryos were electroporated with the Sterile Feral (SF3) gene construct, and treated with three concentrations of doxycycline (50, 100, and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization up to 137 hours post fertilization.

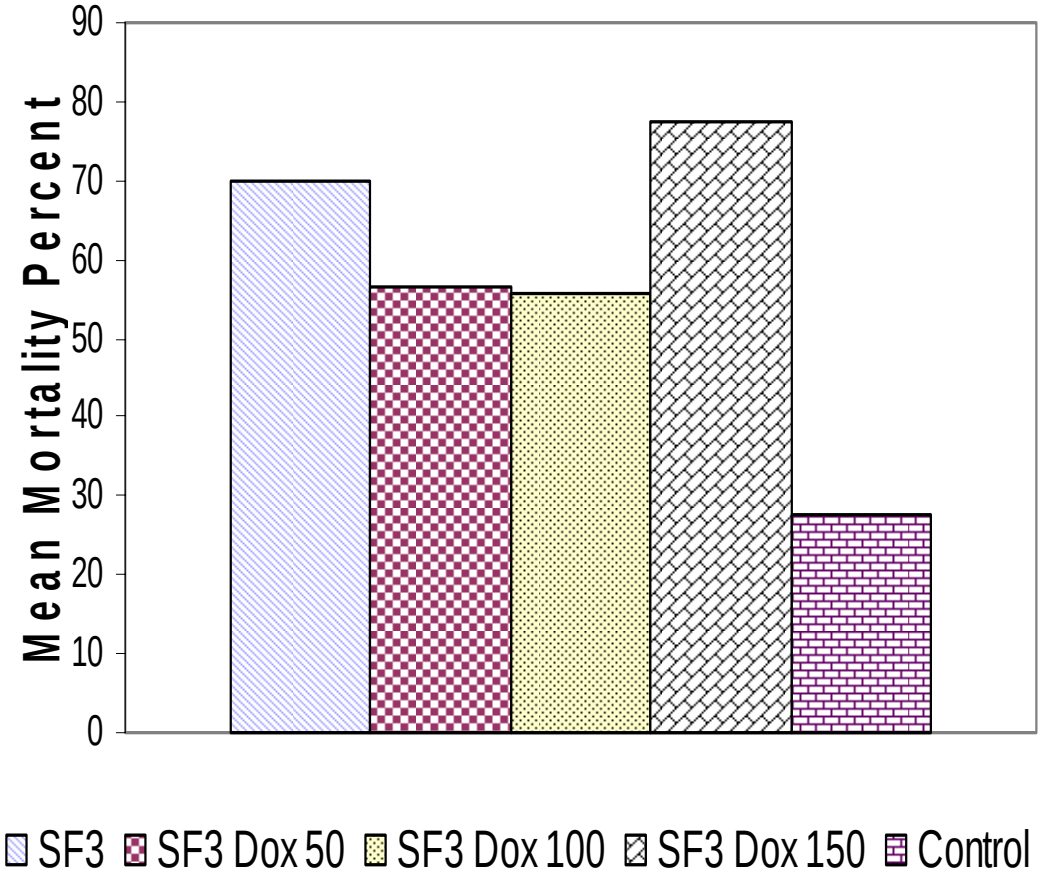
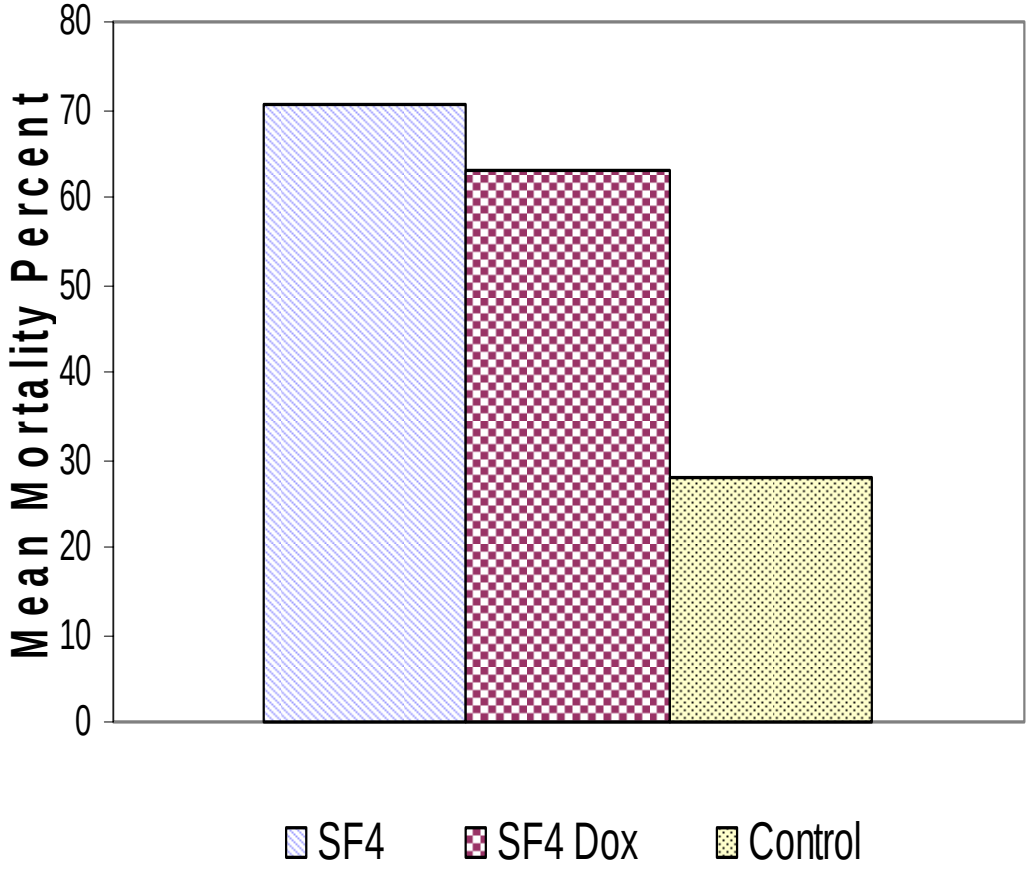


Table 4. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF4) gene construct, and treated with three different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

	Mean Mortality (%)			
Treatment	**Experiment 1**	**Experiment 2**	**Experiment 3**	**Grand Mean**
SF4	70.6[a]	59.2[a]	84.0[a]	69.1[a]
SF4 Dox 50 ppm	-	64.2[a]	-	64.2[b]
SF4 Dox 100 ppm	63.0[a]	62.3[a]	-	62.6[b]
SF4 Dox 150 ppm	-	52.8[b]	-	52.8[c]
Control	28.1[b]	27.5[c]	69.4[b]	50.3[c]

[a b c] - mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 12. Mean mortality of channel catfish, *Ictalurus punctatus*, in experiment one. Embryos were electroporated with the Sterile Feral (SF4) gene construct, and treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch and a electroporated control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.



Mean mortality percentages for the second experiment for SF4 (59.2%) were not different ($P > 0.05$) than that of SF4 doxycycline 50 (64.2%) and SF4 doxycycline 100 (62.3%) (Table 4) (Figure 13). SF4 did have a greater ($P < 0.0125$) mean mortality percentage than SF4 doxycycline 150 (52.8%) (Table 4). The SF4 doxycycline 150 group was the only SF4 doxycycline group in this experiment that demonstrated a reduction in the relative mean mortality percent of SF4. SF4 doxycycline 150 reduced the relative mean mortality percent of SF4, 115.3%, to 92.0%. Mean mortality percentages were higher ($P < 0.05$) in all SF4 doxycycline groups as compared to the electroporated control group (27.5%) (Table 4). SF4 also had a higher ($P < 0.05$) mean mortality percentage than the electroporated control group. Mean mortality percentages were not different ($P > 0.05$) in the SF4 doxycycline 50 and 100 groups. SF4 doxycycline 100 generated a higher ($P < 0.05$) mortality than SF4 doxycycline 150. SF4 doxycycline 50 also had a higher ($P < 0.05$) mean mortality percentage than that of SF4 doxycycline 150 (Table 4).

Mortality over time

SF3-SF4-control

In the first experiment, there was no difference ($P > 0.05$) in the mean mortality percentages among SF3 (2.1%), electroporated control (3.5%) and SF4 (2.8%) for the 0-14 hour time period (Table 5) (Figure 14). There were no mortalities observed during the 14-31 hour time period in the same experiment. During the 31-61 hour time period, mean mortality percentages for both SF3 (73.5%) and SF4 (62.4%) were greater ($P < 0.05$) than the control group (16.3%) (Table 5). Also during this same time period, SF3 produced a greater ($P < 0.05$) mean mortality percentage than did the SF4 group (Table 5). No mortalities were observed during the 61-85 hour time period. For the 85-109 hour interval, SF3 (2.7%) had a mean

Figure 13. Mean mortality of channel catfish, *Ictalurus punctatus*, in experiment two. Embryos were electroporated with the Sterile Feral (SF4) gene construct, and treated with three concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

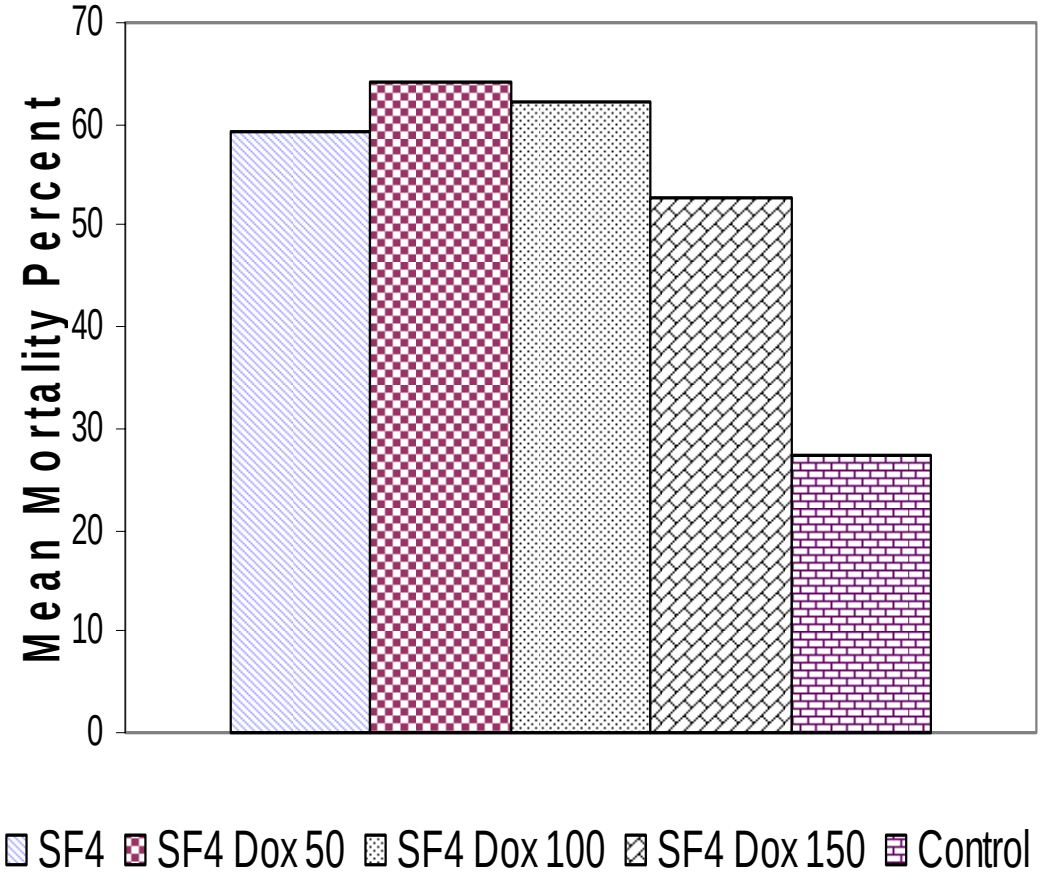
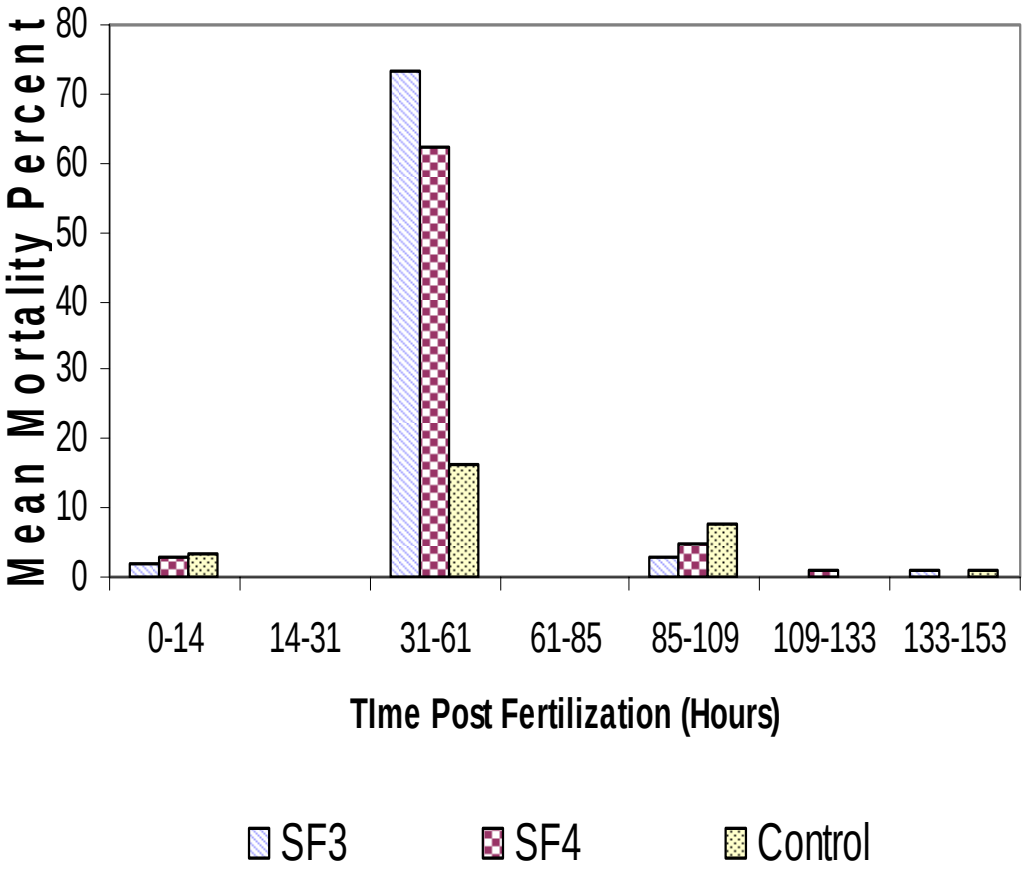


Table 5. Mean mortality at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, and a electroporated blank control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.

	Mortality (%) in Hour Intervals						
Treatment	**0-14**	**14-31**	**31-61**	**61-85**	**85-109**	**109-133**	**133-153**
SF3	2.1[a]	0.0[a]	73.5[a]	0.0[a]	2.7[b]	0.0[a]	0.9[a]
SF4	2.8[a]	0.0[a]	62.4[b]	0.0[a]	4.7[a,b]	0.8[a]	0.0[a]
Control	3.5[a]	0.0[a]	16.3[c]	0.0[a]	7.5[a]	0.0[a]	0.8[a]

[a b c] – mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 14. Mean mortality at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.



mortality percentage that was lower ($P < 0.05$) than the electroporated control group (7.5%) (Table 5). For the same time period, the mean mortalities were not different ($P < 0.05$) between SF4 (4.7%) and the electroporated control group or between SF4 and SF3 (Table 5). During the 109-133 time period, there was no difference ($P > 0.05$) among the treatments. For the final time interval, 133-153 hours, of this experiment, there were also no difference ($P < 0.05$) observed among any of the treatments (Table 5).

During the 0-18 hour time interval in the second experiment, the SF3 (5.8%) group produced a higher ($P < 0.05$) mean mortality percentage than that of the electroporated control group (2.5%) (Table 6) (Figure 15). There was no difference ($P > 0.05$) between SF4 (1.3%) and the electroporated control group during this time period. Both SF3 (57.5%) and SF4 (54.5%) had higher ($P < 0.05$) mean mortality percentages than the electroporated control group (22.7%) during the next time period of 18-59 hours (Table 6). The mean mortality percentage of SF3 was also greater ($P < 0.05$) than SF4 during this same time period.

The mean mortality percentage during the 59-96 hour time interval for the SF3 (4.4%) group was greater ($P < 0.05$) than that of the electroporated control (2.0%) group and the SF4 (2.3%) group (Table 6). During this same time period, SF4 did not produce a mean mortality percentage that was greater than the electroporated control group ($P > 0.05$) (Table 6).

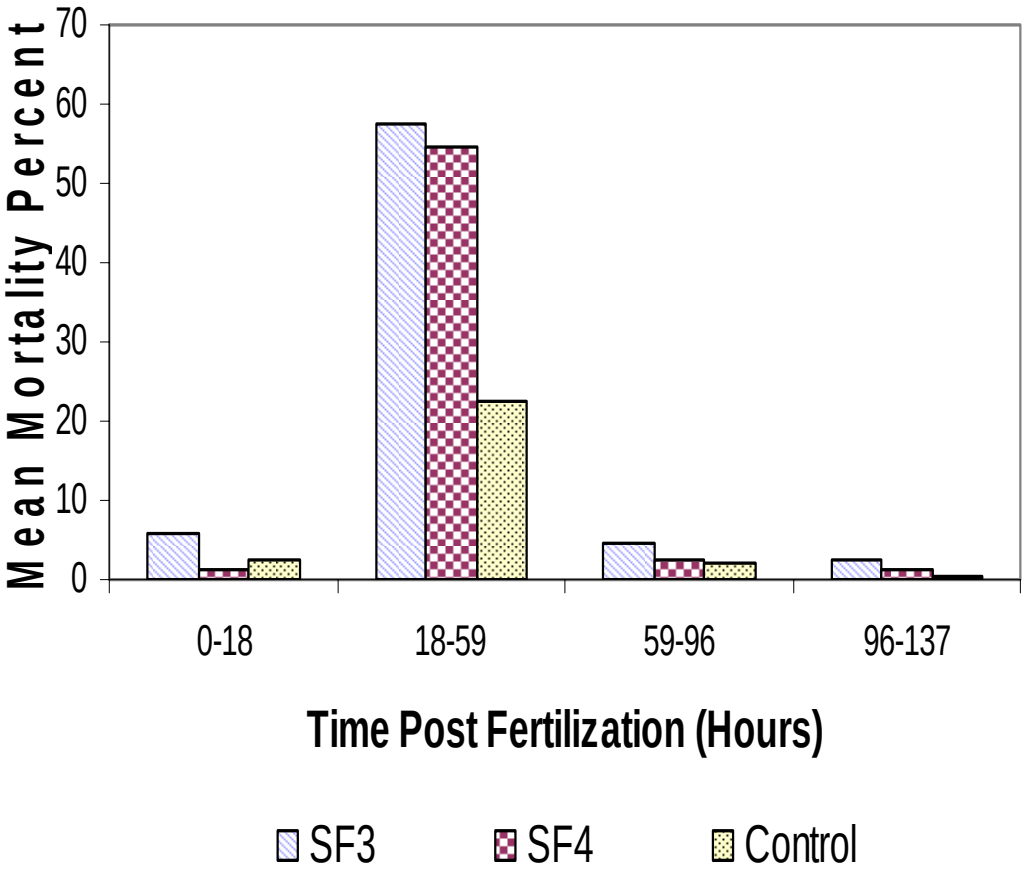
For the final time interval, 96-137 hours, SF3 (2.4%) produced a mean mortality percentage that was greater ($P < 0.05$) than both the electroporated control group (0.3%) and SF4 (1.1%) (Table 6). SF4 did not produce a mean mortality percentage that was greater ($P < 0.05$) than the electroporated control group (Table 6).

Table 6. Mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, and a electroporated control in experiment two. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization to 137 hours post fertilization.

	Mean Mortality (%) in Hour Intervals			
Treatment	**0-18**	**18-59**	**59-96**	**96-137**
SF3	5.8[a]	57.5[a]	4.4[a]	2.4[a]
SF4	1.3[b]	54.5[a]	2.3[b]	1.1[b]
Control	2.5[b]	22.7[b]	2.0[b]	0.3[b]

[a b c] –mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 15. Mean mortality at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, and a electroporated blank control in experiment two. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization to 137 hours post fertilization.



During the 0-18 hour time period for the third experiment the mean mortality percentage for the electroporated control group (10.6%) was greater ($P < 0.05$) than that of SF3 (3.5%) but not different ($P > 0.05$) than that of SF4 (9.7%) (Table 7) (Figure 16). SF4 had a higher ($P < 0.05$) mean mortality percentage than the SF3 group during this same time period (Table 7). The SF3 (73.9%) group produced a greater ($P < 0.05$) mean mortality percentage than did the electroporated control group (54.2%) but was not different ($P > 0.05$) than the SF4 (71.0%) group during the 18-59 hour time interval (Table 7). In the same time period, SF4 had a higher ($P < 0 .05$) mean mortality percentage as compared to the electroporated control group (Table 7). The mean mortality percentage during the 59-96 hour time interval for SF4 (1.8%) was less than ($P < 0.05$) that of both SF3 (5.9%) and the electroporated control group (4.2%) (Table 7). There was no difference ($P > 0.05$) between SF3 and the electroporated control group during the same time period (Table 7). Next, during the 96-137 hour time period, SF3 (2.2%) produced a mean mortality percentage that was greater ($P < 0.05$) than that of the electroporated control group (0.4%) but not greater ($P > 0.05$) than that of SF4 (1.5%) (Table 7). SF4 did have a higher ($P < 0.05$) mean mortality percentage as compared to the electroporated control group in this final time period of 96-137 hours (Table 7).

SF3-SF4-DNA Control

During the 0-14 hour time interval for the first experiment, there was no difference ($P > 0.05$) between the mean mortality percentages of SF3 (2.1%), SF4 (2.8%), CAB (1.1%), or GAD (0.0%) (Table 8) (Figure 17). There were no differences in any of the comparisons made between the treatments.

There were no mortalities for any of the groups during the 14-31 hour time interval. During the 31-61 hour time period, the mean mortality percentage of SF3

Table 7. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, and a electroporated blank control in experiment three. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization to 137 hours post fertilization.

	Mean Mortality (%) in Hourly Intervals			
Treatment	**0-18**	**18-59**	**59-96**	**96-137**
SF3	3.5[b]	73.9[a]	5.9[a]	2.2[a]
SF4	9.7[a]	71.0[a]	1.8[b]	1.5[a]
Control	10.6[a]	54.2[b]	4.2[a]	0.4[b]

[a b c] – mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 16. Mean mortality at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, and a electroporated control in experiment three. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization to 137 hours post fertilization.

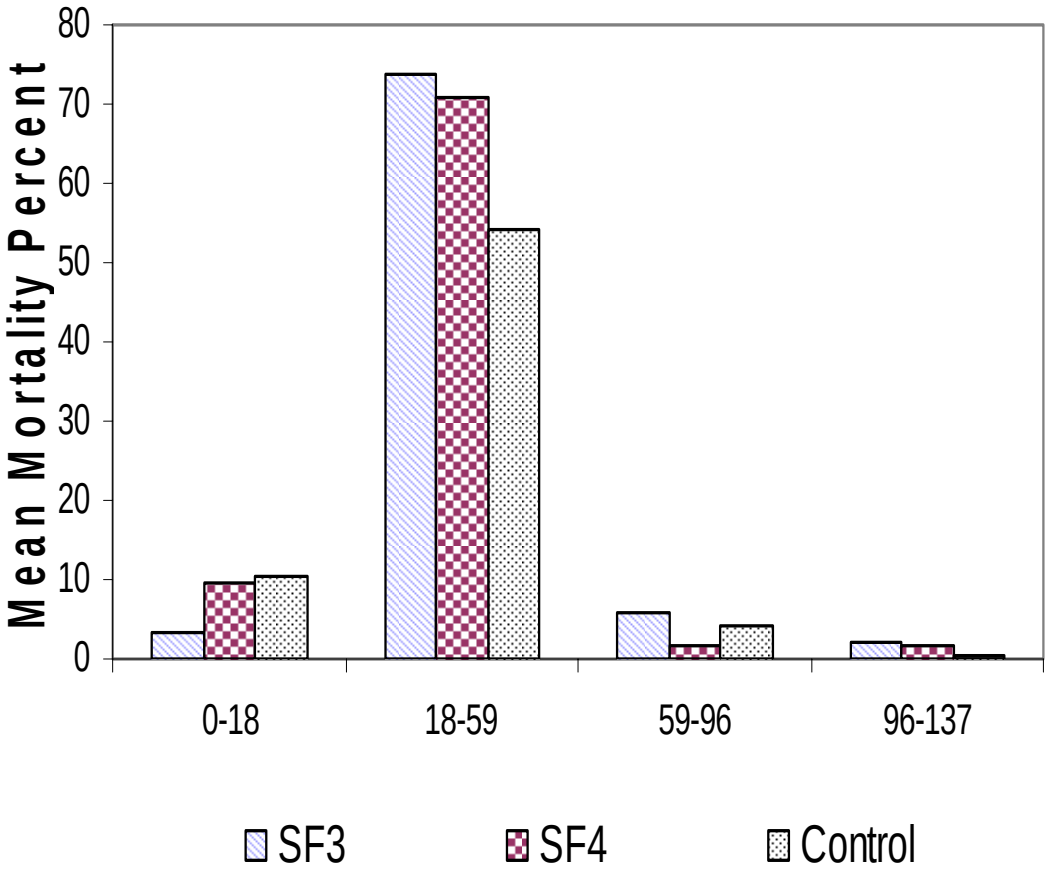
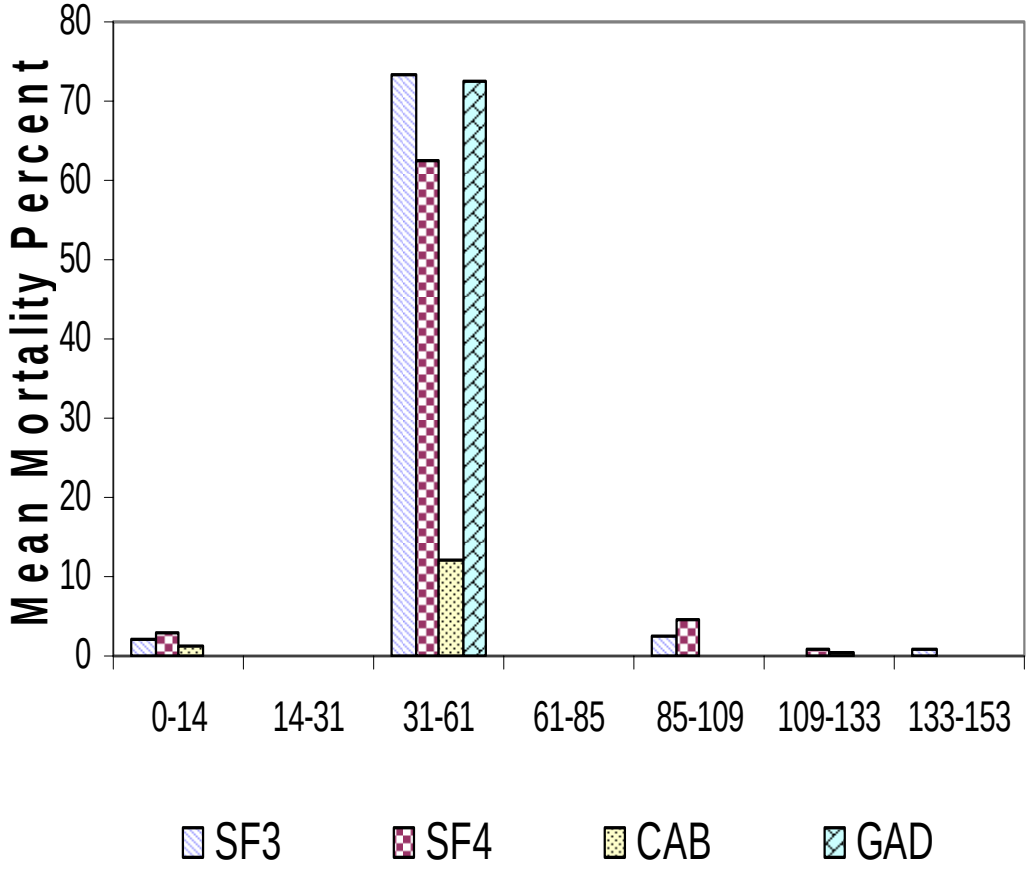


Table 8. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, glutamate decarboxylase (GAD) and CAB in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.

	Mean Mortality (%) in Hourly Intervals						
Treatment	**0-14**	**14-31**	**31-61**	**61-85**	**85-109**	**109-133**	**133-153**
SF3	2.1[a]	0.0[a]	73.5[a]	0.0[a]	2.7[ab]	0.0[a]	0.9[a]
SF4	2.8[a]	0.0[a]	62.4[b]	0.0 [a]	P4.7[b]	0.8[a]	0.0[a]
CAB	1.1[a]	0.0[a]	12.2[c]	0.0 [a]	P0.0[c]	0.4[a]	0.0[a]
GAD	0.0[a]	0.0[a]	72.3[ab]	0.0 [a]	P0.0[ac]	P0.0[a]	P0.0[a]

[a b c] – mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 17. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs, glutamate decarboxylase (GAD) and CAB in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.



(73.5%) was greater ($P < 0.05$) than SF4 (62.4%) and CAB (12.2%) but not different ($P > 0.05$) from the mean mortality percentage of GAD (72.3%) (Table 8). In the same time period, SF4 had a mean mortality percentage that was greater ($P < 0.05$) than CAB but not different ($P > 0.05$) as compared to GAD (Table 8). GAD had a mean mortality percentage that was greater ($P < 0.05$) than that of CAB (Table 8). There were no mortalities observed during the 61-85 hour time period for any of the groups in this experiment.

There was no difference ($P > 0.05$) between the mean mortality percentages of SF3 (2.7%) and SF4 (4.7%) during the 85-109 hour time interval (Table 8). During this same time period, SF3 produced a mean mortality percentage that was greater ($P < 0.05$) than that of CAB (0.0%) but was not different ($P > 0.05$) as compared to that of GAD (0.0%) (Table 8). The mean mortality percentage of SF4 was greater ($P < 0.05$) than both CAB and GAD (Table 8). For the 85-109 hour time period, there was no difference between the mean mortality percentages of CAB and GAD for neither group had mortalities recorded for this period.

During the 109-133 hour time interval, there were no differences ($P > 0.05$) in the comparison of the mean mortality percentage of SF4 (0.8%), SF3 (0.0%), CAB (0.4%) and GAD (0.0%) (Table 8). There were also no difference ($P > 0.05$) among any of the treatments in the last period of this experiment, 133-153 hours (Table 8).

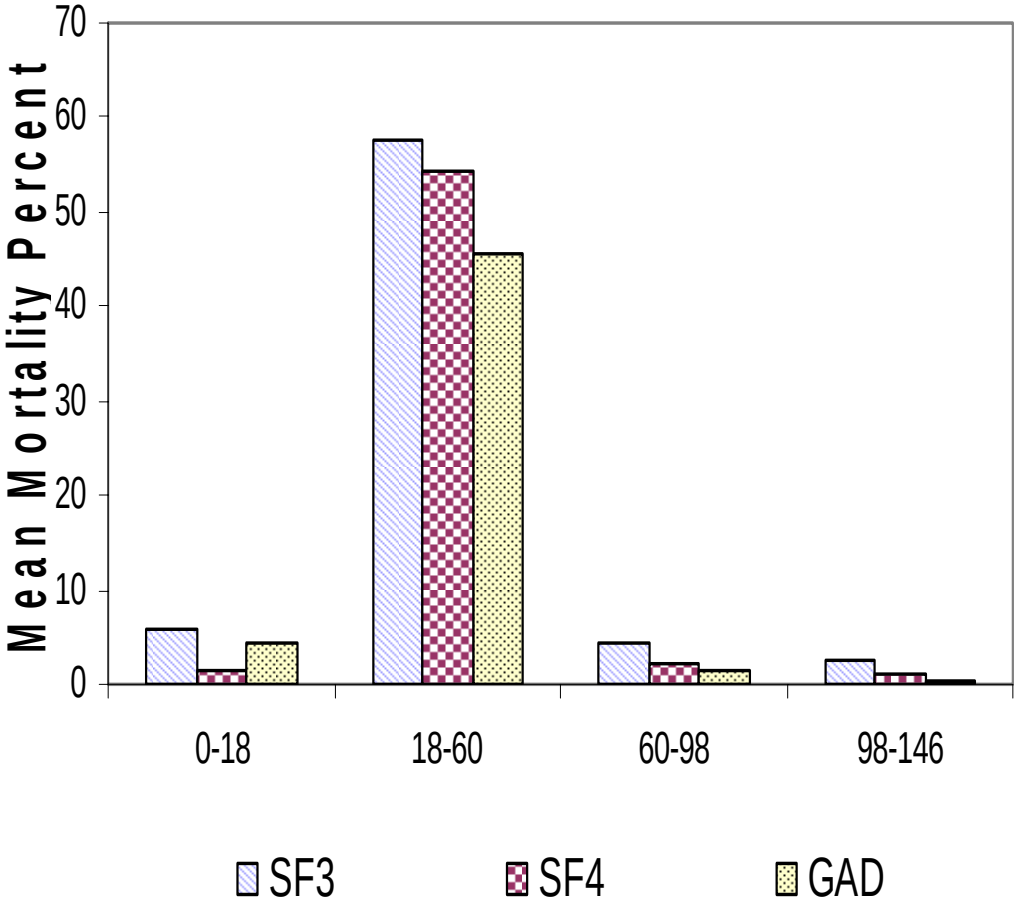
In the second experiment, during the 0-18 hour time period, SF3 (5.8%) produced a mean mortality percentage that was not different ($P > 0.05$) as compared to GAD (4.2%) but was greater ($P < 0.05$) than SF4 (1.3%) (Table 9) (Figure 18). GAD had a higher ($P < 0.05$) mean mortality percentage during this period than SF4 (Table 9).

Table 9. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs and glutamate decarboxylase (GAD) in experiment two. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization to 146 hours post fertilization.

	Mean Mortality (%) in Hourly Intervals			
Treatment	**0-18**	**18-60**	**60-98**	**98-146**
SF3	5.8[a]	57.5[a]	4.4[a]	2.4[a]
SF4	1.3[b]	54.5[a]	2.3[b]	1.1[b]
GAD	4.2[a]	45.4[b]	1.4[b]	0.3[c]

[a b c] – mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 18. Comparison of the mean mortality percentages at various time intervals (hours)for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 and SF4) gene constructs and glutamate decarboxylase (GAD) in experiment two. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization to 146 hours post fertilization.



The mean mortality percentage for SF3 (57.5%) was greater ($P < 0.05$) as compared to GAD (45.4%) but not different ($P > 0.05$) as compared to SF4 (54.5%) during the 18-60 hour time period (Table 9). In this same time period, SF4 also had a higher ($P < 0.05$) mean mortality percentage than GAD (Table 9). The mean mortality percentage for SF3 (4.4%) during the 60-98 hour time interval was greater ($P < 0.05$) than both SF4 (2.3%) and GAD (1.4%) (Table 9). During this same period, SF4 had a mean mortality that was not different ($P > 0.05$) from GAD (Table 9). During the final time period, 98-146 hours, SF3 (2.4%) produced a higher ($P < 0.05$) mean mortality percentage as compared to GAD (0.3%) and SF4 (1.1%) (Table 9). SF4 also produced a mean mortality percentage that was higher ($P < 0.0441$) than GAD (Table 9).

SF constructs and the effects of doxycyline

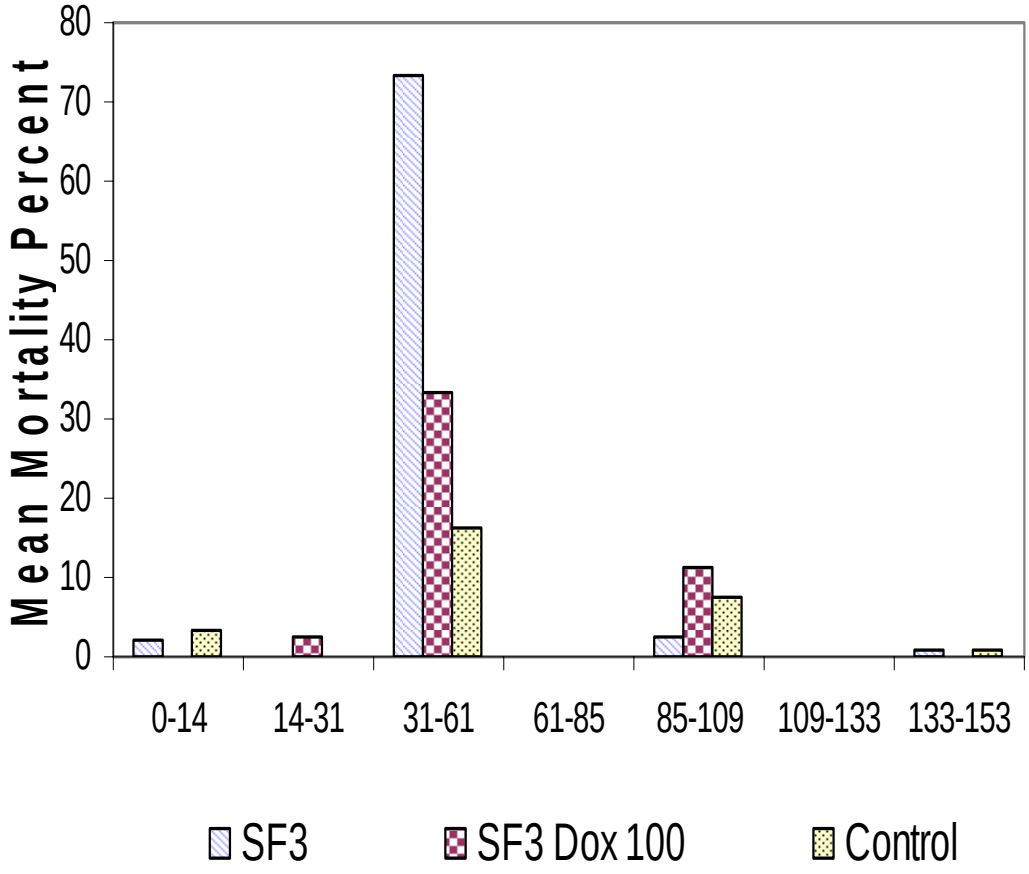
During the first experiment, the group SF3 (2.1%) exhibited a mean mortality percentage that was greater ($P < 0.05$) than SF3 doxycycline (0.0%) but not greater ($P < 0.05$) than the electroporated control group (3.5%) during the 0 to 14 hour time period (Table 10) (Figure 19). The electroporated control group had a mean mortality for this same time interval that was greater ($P < 0.05$) than SF3 doxycycline 100 (Table 10). For the 14 to 31 hour time period, SF3 doxycycline 100 (2.5%) had a higher ($P < 0.05$) mean mortality percentage as compared to both SF3 (0.0%) and the electroporated control group (0.0%) (Table 10). The mean mortality percentages for the SF3 group (73.5%) were greater ($P < 0.05$) compared to both the SF3 doxycycline 100 group (33.3%) and the control group (16.3%) during the 34 to 61 hour time interval (Table 10). SF3 doxycycline 100 had a higher ($P < 0.05$) mean mortality percentage than the electroporated control group (Table 10).

Table 10. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3) gene construct, and treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch and a electroporated blank control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.

	Mean Mortality (%) in Hourly Intervals						
Treatment	**0-14**	**14-31**	**31-61**	**61-85**	**85-109**	**109-133**	**133-153**
SF3	2.1[a]	0.0[b]	73.5[a]	0.0[a]	2.7[b]	0.0[a]	0.9[a]
SF3 Dox 100 ppm	0.0[b]	2.5[a]	33.3[b]	0.0[a]	11.4[a]	0.0[a]	0.0[a]
Control	3.5[a]	0.0[b]	16.3[c]	0.0[a]	7.5[a]	0.0[a]	0.8[a]

[a b c] - percentages with different letters within columns signifies a difference ($P < 0.05$), chi-square test.

Figure 19. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3) gene construct, and treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.



There were no mortalities observed during the next time period, which was 61 to 85 hours (Table 10). SF3 doxycycline 100 (11.4%) produced a higher ($P < 0.05$) mean mortality percentage than did SF3 (2.7%) during the 85 to 109 hour time interval (Table 10). During this same time period, the mean mortality percentage of the electroporated control group (7.5%) was higher ($P < 0.05$) than that of SF3 but not different ($P > 0.05$) than that of the SF3 doxycycline 100 group (Table 10).

For the 109 to 133 time interval, no mortalities were observed for any of the groups. There was no difference ($P > 0.05$) between SF3 (0.9%) and SF3 doxycycline 100 (0.0%), SF3 and the electroporated control group (0.8%) or between SF3 doxycycline 100 and the control group (Table 10).

For the second experiment within the time interval of 0 to 12 hours, SF3 (5.8%) produced a mean mortality percentage that was not different ($P > 0.05$) than that observed for SF3 doxycycline 50 (3.2%), SF3 doxycycline 100 (3.7%) or SF3 doxycycline 150 (4.9%) (Table 11) (Figure 20). There was no difference ($P > 0.05$) between the mean mortality percentage of the control group (2.5%) and SF3 doxycycline 50, SF3 doxycycline 100, but the mortalities of the electroporated control group were lower ($P < 0.05$) than that of SF3 doxycycline 150 (Table 11). The mean mortality percentage of SF3 was higher ($P < 0.05$) than that of the electroporated control group (Table 11). SF3 doxycycline 50 produced a mean mortality percentage that was not different ($P > 0.05$) from that of SF doxycycline 100 or of SF3 doxycycline 150 (Table 12). Lastly, the mean mortality percentage of SF3 doxycycline 100 as compared to SF3 doxycycline 150 was not different ($P > 0.05$) (Table 11).

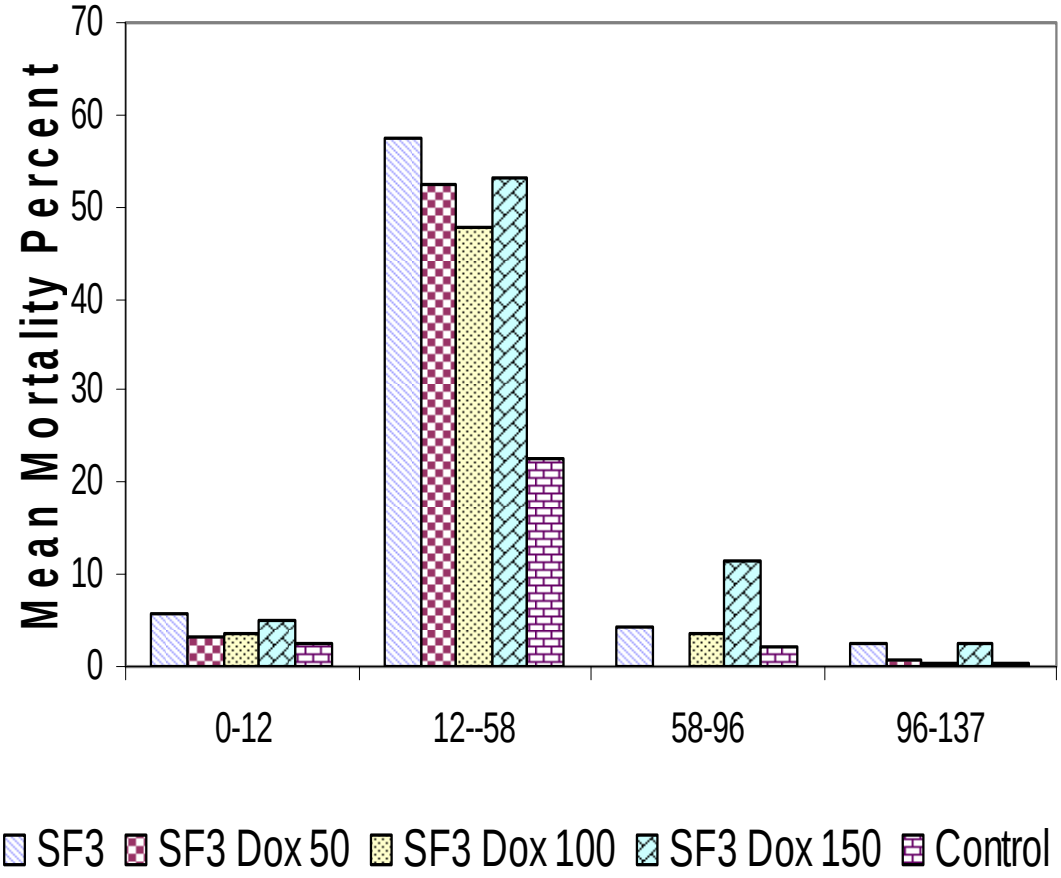
For the next time period, 12 to 58 hours, SF3 (57.5%) exhibited a mean mortality percentage that was not different ($P > 0.05$) than that of SF3 doxycycline 50

Table 11. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3) gene construct, and treated with different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control in experiment two. Dead eggs and embryos were identified and removed beginning from 12 hours post fertilization to 137 hours post fertilization.

	Mean Mortality (%) in Hourly Intervals			
Treatment	**0-12**	**12-58**	**58-96**	**96-137**
SF3	5.8[a]	57.5[a]	4.4[b]	2.4[a]
SF3 Dox 50 ppm	3.2[ab]	52.5[a]	0.0[d]	0.7[ab]
SF3 Dox 100 ppm	3.7[ab]	47.7[b]	3.7[bd]	0.5[b]
SF3 Dox 150 ppm	4.9[a]	53.1[ab]	11.4[a]	2.5[a]
Control	2.5[b]	22.7[c]	2.0[c]	0.3[b]

[a b c d] - percentages with different letters within columns signifies a difference ($P < 0.05$), chi-square test.

Figure 20. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3) gene construct, and treated with three different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control in experiment two. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.



(52.5%) and SF3 doxycycline 150 (53.1%) (Table 11). There was a difference ($P < 0.05$) between the mean mortality percentages of SF3 and SF3 doxycycline 100 (47.7%). The electroporated control group (22.7%) had a lower ($P < 0.05$) mean mortality percentage than that of SF3 doxycycline 50 and 100 (Table 11). There was no difference ($P > 0.05$) between the mean mortality percentage of the electroporated control group and SF3 doxycycline 150 (Table 11). During the same time period, SF3 had a higher ($P < 0.05$) mean mortality percentage as compared to the electroporated control group. SF3 doxycycline 50 had a higher ($P < 0.05$) mean mortality percentage than that of SF3 doxycycline 100 but was not different ($P > 0.05$) from that of SF3 doxycycline 150. SF3 doxycycline 100 had a higher ($P < 0.05$) mean mortality percentage as compared to that of SF3 doxycycline 150 (Table 11).

During the 58 to 96 hour time interval, SF3 (4.4%) had a mean mortality percentage that was higher ($P < 0.05$) than that of SF3 doxycycline 50 (0.0%), but not different ($P > 0.05$) from that of SF3 doxycycline 100 (3.7%) and lower ($P < 0.05$) than that of SF3 doxycycline 150 (11.4%) (Table 11). The mean mortality of the electroporated control group (2.0%) was greater ($P < 0.05$) than that of SF3 doxycycline 50, not different ($P > 0.05$) than that of SF3 doxycycline 100 and less ($P < 0.05$) than that of SF3 doxycycline 150 (Table 12). SF3 had a mean mortality that was higher ($P < 0.05$) than that of the electroporated control group. SF3 doxycycline 50 had no mortalities for this time period and thus had a lower ($P < 0.05$) mean mortality percentage as compared to SF3 doxycycline 100 and SF3 doxycycline 150. During this same time period, SF3 doxycycline 150 had a higher ($P < 0.05$) mean mortality percentage than SF3 doxycycline 100 (Table 11).

During the last time interval, 96 to 137 hours, the mean mortality percentage of SF3 (2.4%) was not different ($P > 0.05$) from SF3 doxycycline 50 (0.7%) or SF3

doxycycline 150 (2.5%) but was higher ($P < 0.05$) than SF3 doxycycline 100 (0.5%) (Table 11). The electroporated control group (0.3%) had a mean mortality percentage that was not different ($P > 0.05$) from SF3 doxycycline 50 or from SF3 doxycycline 100 but was lower ($P < 0.05$) than that of SF3 doxycycline 150 (Table 11). SF3 produced a mean mortality percentage that was higher ($P < 0.05$) than that of the electroporated control group. There was no difference ($P > 0.05$) between the mean mortality percentages of SF3 doxycycline 50 and 100 or between SF3 doxycycline 50 and 150 (Table 11). There was a difference ($P < 0.05$) between the mean mortality percentages of SF3 doxycycline 100 and 150 (Table 11).

SF4-Dox Comparison

The initial time period for the first experiment with channel catfish was 0 to 14 hours. During this interval, the mortality pattern was atypical, for there were no treatments that experienced more than 3.5% mortality (Table 12) (Figure 21). The next time interval was 14 to 31 hours, and mortality rates were no higher than 0.3% for any treatment (Table 12). Mortalities peaked during the 31 to 61 hour time period. SF4 (62.4%) had a mean mortality rate that was greater ($P < 0.05$) than that of SF4 doxycycline 100 (42.0 %) and the electroporated control group (16.3%) (Table 12). The mortality rate of the SF4 doxycycline 100 group was greater ($P < 0.05$) than that of the electroporated control group (Table 12).

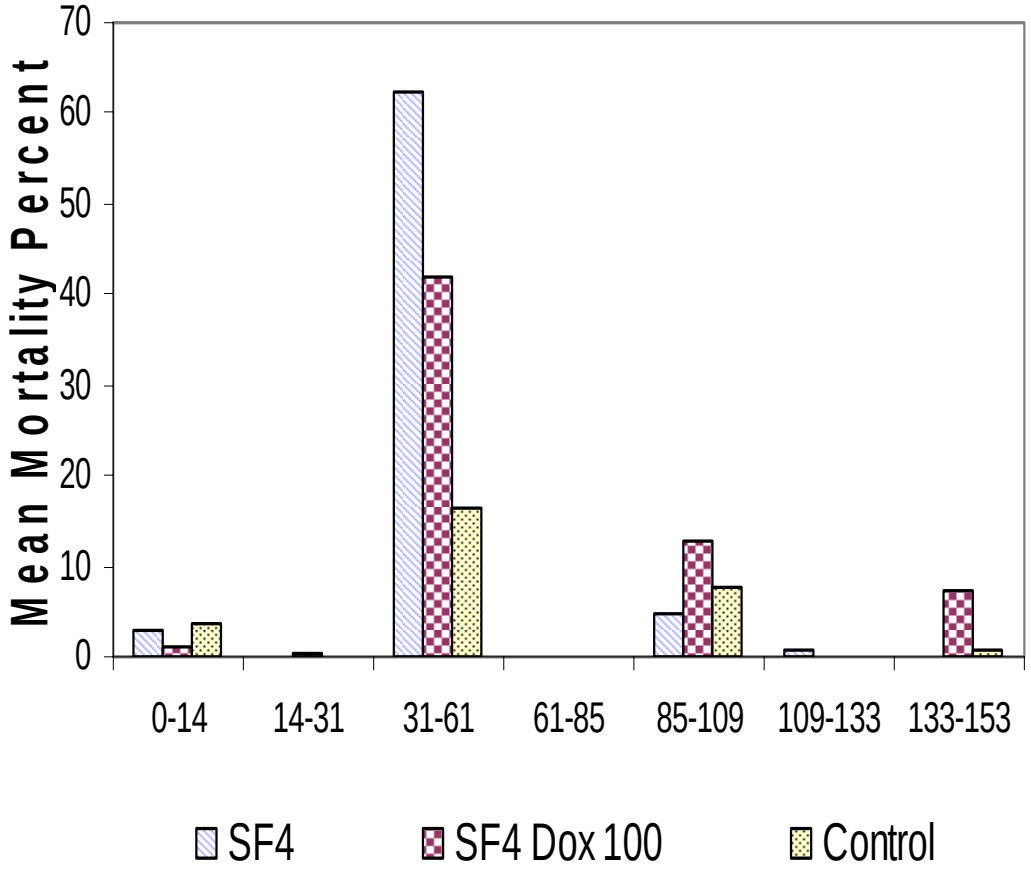
The time interval 61-85 hours had no mortalities observed. During the next time period, 85 to 109 hours, the mean mortality percentage of SF4 doxycycline 100 (12.6%) was higher ($P < 0.05$) than that of SF4 (4.7%) and the electroporated control group (7.5%) (Table 12). There was no difference ($P > 0.05$) between the mean mortality percentages of SF4 and the electroporated control group (Table 12). For the 109 to 133 hour time period, SF4 (0.8%) produced a mean mortality percentage that

Table 12. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF4) gene construct, and treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.

	Mean Mortality (%) in Hourly Intervals						
Treatment	**0-14**	**14-31**	**31-61**	**61-85**	**85-109**	**109-133**	**133-153**
SF4	2.8^{ab}	0.0^{a}	62.4^{a}	0.0^{a}	4.7^{c}	0.8^{a}	0.0^{b}
SF4 Dox 100 ppm	1.0^{b}	0.3^{a}	42.0^{b}	0.0^{a}	12.6^{a}	0.0^{a}	7.2^{a}
Control	3.5^{a}	0.0^{a}	16.3^{c}	0.0^{a}	7.5^{b}	0.0^{a}	0.8^{b}

$^{a\ b\ c}$ – mean percentages with different letters within columns are different ($P < 0.05$), chi-square test.

Figure 21. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF4) gene construct, and treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization to 153 hours post fertilization.



was not greater ($P > 0.05$) than that of SF4 doxycycline 100 (0.0%) or of the control group (0.0%) (Table 12). SF4 doxycycline 100 (7.2%) had a mean mortality greater ($P < 0.05$) than that of both SF4 (0.0%) and the electroporated control group (0.8%) during the final time interval, 133 to 153 hours (Table 12).

In the 0 to 24 hour time period for the second experiment, the SF4 (1.3%) group had a lower ($P < 0.05$) mean mortality percentage as compared to that of SF4 doxycycline 50 (10.2%), 100 (5.3%) and 150 (4.8%) (Table 13) (Figure 22). There was no difference ($P > 0.05$) between the mean mortality percentages of SF4 and the control group (2.5%) (Table 13). The electroporated control group had a lower ($P < 0.05$) mean mortality percentage as compared to SF4 doxycycline 50 and 100 and also lower ($P < 0.05$) than that of SF4 doxycycline 150 (Table 13).

Mortality peaked at 24 to 60 hours. SF4 doxycycline 50 (39.0%) and 150 (46.1%) had lower ($P < 0.05$) mean mortalities as compared to SF4 (Table 13). There was no difference ($P > 0.05$) between the mean mortality of SF4 and SF4 doxycycline 100 (52.2%) (Table 13). The SF4 group had higher ($P < 0.05$) mortality than the electroporated control group (22.7%) (Table 13). The mean mortality of the control group was less ($P < 0.05$) than all doxycycline treatments (Table 13).

In the 60 to 98 hour time period, SF4 (2.3%) had higher ($P < 0.05$) mean mortality than both SF4 doxycycline 50 (0.0%) and 150 (0.0%) (Table 13). There was no difference ($P > 0.05$) between the mean mortality percentages of SF4 and the electroporated control group (2.0%) (Table 13).

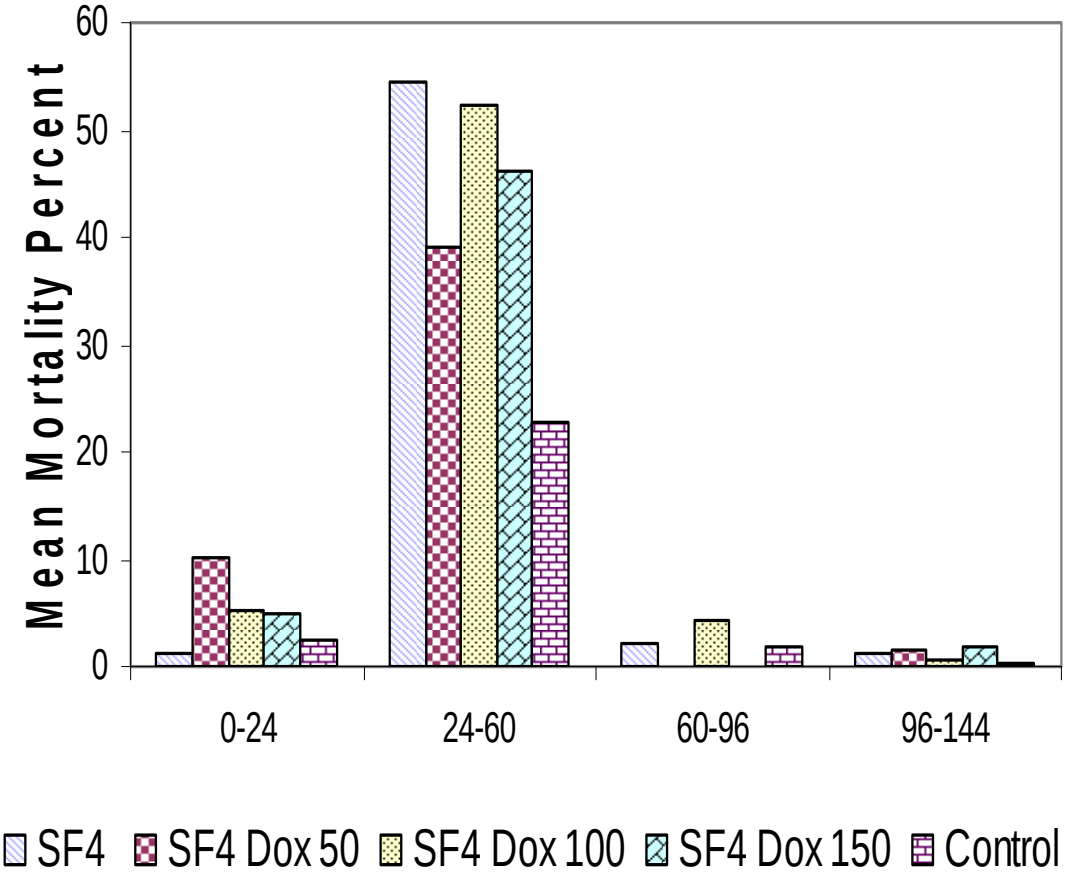
The final time interval for the second experiment with channel catfish was 96 to 144 hours. During the 96 to 144 hour period, there was no difference ($P > 0.05$) between the mean mortality percentages of SF4 (1.1%) and SF4 doxycycline 50

Table 13. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF4) gene construct, and treated with different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control in experiment two. Dead eggs and embryos were identified and removed beginning from 24 hours post fertilization to 144 hours post fertilization.

	Mean Mortality (%) in Hourly Intervals			
Treatment	**0-24**	**24-60**	**60-96**	**96-144**
SF4	1.3^{c}	54.5^{a}	2.3^{b}	1.1^{ac}
SF4 Dox 50 ppm	10.2^{a}	39.0^{c}	0.0^{c}	$1.5^{a,b}$
SF4 Dox 100 ppm	5.3^{b}	52.2^{a}	4.3^{a}	0.5^{bcd}
SF4 Dox 150 ppm	4.8^{b}	46.1^{b}	0.0^{c}	1.9^{a}
Control	2.5^{c}	22.7^{d}	2.0^{b}	0.3^{c}

$^{a\ b\ c\ d}$ – mean percentages with different letters within columns are different ($P < 0.05$), chi-square test.

Figure 22. Comparison of the mean mortality percentages at various time intervals (hours) for channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF4) gene construct, and treated with three different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control in experiment two. Dead eggs and embryos were identified and removed beginning from 18 hours post fertilization to 137 hours post fertilization.



(1.5%) or between SF4 and SF4 doxycycline 100 (0.5%) (Table 13). Mortality for all groups was 1.9% or less.

Deformities

SF constructs versus controls

The predominant deformity observed was that of scoliosis or lordosis. Embryos were also observed that had enlarged cardiac sacs and hearts and also with other varieties of posterior curvatures or abnormally formed tails. During the first experiment, mean deformity percentages for SF3 (3.7%) were greater ($P < 0.05$) than that of the control group (0.3%) (Table 14). No difference ($P > 0.05$) was observed in the deformity rates when compared between SF4 (1.5%) and control or SF4 compared to SF3 (Table 14). In the second experiment, SF3 (1.3%) had a higher ($P < 0.05$) mean deformity percentage than the electroporated control group (0.1%) (Table 14). There was not a difference ($P > 0.05$) between SF4 (0.5%) and electroporated control or between SF3 and SF4 (Table 14). In the third experiment, there were no differences ($P > 0.05$) between the mean deformity percentages of SF3 (0.1%) and SF4 (0.1%) as compared to the electroporated control group (0.0%) (Table 14). Again, there was no difference ($P < 0.05$) between the mean deformity percentages of SF3 and SF4 (Table 14).

In the first experiment, SF3 had a higher ($P < 0.05$) mean deformity percentage than both CAB (0.0%) and GAD (0.0%) (Table 14). The mean deformity percentage of SF4 was higher ($P < 0.05$) than CAB but not different ($P > 0.05$) than GAD (Table 14). In the second experiment, deformity percentages were not different ($P > 0.05$) between SF3 and GAD (0.0%) or between SF4 and GAD (Table 14).

SF constructs versus doxycycline

In the first experiment, there was no difference ($P > 0.05$) between the mean

Table 14. Mean deformity percentages of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF3 and SF4) gene constructs, a electroporated control, glutamate decarboxylase (GAD) and CAB. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

	Mean Deformity (%)		
Treatment	**Experiment 1[a]**	**Experiment 2[b]**	**Experiment 3**
SF3	3.7	1.3	0.1
SF4	1.5	0.5	0.1
Control	0.3	0.1	0.0
GAD	0.0	0.0	-
CAB	0.0	-	-

[a] – SF3 was greater than control, GAB and GAD ($P < 0.05$), chi-square; SF4 was greater than GAD and CAB ($P < 0.05$), chi-square.
[b] – SF3 was greater than control ($P < 0.05$), chi-square.

deformity percentages of SF3 (3.7%) as compared to SF3 doxycycline 100 (3.4%) (Table 15). SF3 and SF3 doxycycline 100 did have higher ($P < 0.05$) mean deformity percentages than that of the mean deformity of the control (0.3%) (Table 15). The mean deformity of SF3 for the second experiment (1.3%) was greater ($P < 0.05$) than the deformity rates of SF3 doxycycline 50 (0.0%), 100 (0.0%) 150 (0.0%) and control (0.1%) (Table 15). There was no difference ($P > 0.05$) between the mean deformity percentages of the electroporated control group (0.1%) as compared to that of SF3 doxycycline 50, 100 and 150 (Table 15).

SF4-SF4 doxycycline-control

There was no difference ($P > 0.05$) between the mean deformity percentages of SF4 (1.5%) and SF4 doxycycline 100 (2.7%) or between SF4 and the electroporated control group (0.3 %) in the first experiment (Table 16). SF4 doxycycline 100 did have a higher ($P < 0.05$) mean deformity percentage as compared to that of the electroporated control group (Table 16). There was no difference ($P > 0.05$) between the mean deformity percentages of SF4 (0.5%) and SF4 doxycycline 50 (0.0%), 100 (0.0%) and 150 (0.0%) in the second experiment (Table 16). There was also no difference ($P > 0.05$) between the mean deformity percentages of the control group (0.1%) as compared to SF4 doxycycline 50, 100 and 150 (Table 16).

Table 15. Mean deformity percentages of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF3) gene construct, and treated with different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

	Mean Deformity (%)		
Treatment	**Experiment 1**	**Experiment 2**	**Experiment 3**
SF3	3.7[a]	1.3[a]	0.1[a]
SF3 DOX 50 ppm	-	0.0[b]	-
SF3 DOX 100 ppm	3.4[a]	0.0[b]	-
SF3 DOX 150 ppm	-	0.0[b]	-
Control	0.3[b]	0.1[b]	0.0[a]

[a b c] – mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Table 16. Mean deformity percentages of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF4) gene construct, and treated with different concentrations of doxycycline (50, 100 and 150 ppm) from 4 hours post fertilization to first hatch and a electroporated control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

	Mean Deformity (%)		
Treatment	**Experiment 1**	**Experiment 2**	**Experiment 3**
SF4	1.5[ab]	0.5[a]	0.1[a]
SF4 DOX 50 ppm	-	0.0[a]	-
SF4 DOX 100 ppm	2.7[a]	0.0[a]	-
SF4 DOX 150 ppm	-	0.0[a]	-
Control	0.3[b]	0.1[a]	0.0[a]

[a b c] – mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

DISCUSSION

Disrupting embryonic development

The ability to interrupt embryonic development is contingent upon manipulating the expression of essential genes that are active only during embryogenesis. An approach to achieve embryonic gene disruption, or knockout, is the use of mRNA specifically targeted for interference of gene function. Mechanisms used to achieve mRNA knockout include expression of ribozymes, antisense mRNA, and double stranded mRNA (dsRNA) have all succeeded in several organisms (Thresher *et al.,* 2001, Dunham 2004). The sterile feral constructs investigated in this study employ one of these mechanisms and an additional one. The SF3 construct uses dsRNA to achieve gene disruption, and the SF4 construct uses sense cDNA (Thresher *et al.*, 2001).

The Sterile Feral gene constructs, SF3 and SF4, were successful in their ability to interrupt embryonic development in the channel catfish, *Ictalurus punctatus*. The mean mortality percent of the SF3 construct (78.5 %) from the three experiments conducted with the channel catfish was 56.1 % higher than the mean mortality percent of the electroporated control group (50.3%). Likewise the SF4 construct caused a mean mortality percent (69.1 %) that was 37.4 % higher than that of the electroporated control group (50.3%). Thus, both SF3 and SF4 demonstrated the potential to disrupt embryonic development at a level that was greater than the

electroporated control group showing that electroporation alone was not responsible for the increased mortality rate.

These differences would have been even greater except that the third experiment had a relatively high mortality rate in the electroporated blank control group as compared to the two other experiments. The ability of the sterile feral constructs to disrupt embryonic development in the channel catfish was further confirmed by again causing mortality rates that were significantly greater than that of embryos electroporated with the glutamate decarboxylase construct (51.4%) (GAD). Thus, SF3 demonstrated a 52.7 % increase and SF4 a 37.4 % increase as compared to the mean mortality percent of GAD. The mortality of SF3 was over 400% greater and SF4 was just under 400% greater than another electroporated control containing the CAB construct (13.9%), which was used in the first experiment. This demonstrates that it was not simply the action of electroporation or the introduction of exogenous DNA that caused the higher mortality rates in the SF3 and SF4 groups.

Through PCR analysis, dead and deformed embryos were confirmed to contain SF3 and SF4 constructs. Based upon the PCR analysis approximately 100% of the embryos sampled that were treated with the SF3 or SF4 constructs were found to contain the bi-directional TRE element or the tTA element. This demonstrates the efficacy of the electroporation in introducing the exogenous DNA into to the embryos.

In terms of percent mortality, the mortality rates in this study were greater in magnitude but correlate with similar studies conducted on other organisms. Thresher *et al.*, 2001 used the same dsRNA blocker (SF3) and 27 of 190 (14.2%) 1-4 cell stage zebrafish, *Danio rerio*, embryos died after being injected with this dsRNA blocker. Four of 51 control embryos that were injected died (0.1%). In this same study, the

SF-4 construct injected killed 141 of 234 zebra fish embryos within 24 hours post-injection (60.3%). Sixty-one of these individuals that were injected with the SF4 construct were positive for the EGFP, which was a component of SF-4, and 33 individuals were deformed. Fifty-six of 118 control embryos died (47.5%), and only 3 were deformed (Thresher *et al.*, 2001).

Thresher *et al.*, 2001 also used three variants of two Hox genes to disrupt larval development in Pacific oysters. Sense, anti-sense and double stranded RNA were increasingly effective in that order for disrupting development of Pacific oysters. The dsRNA specific to the HoxCG1 gene was the most effective, and almost 80% of treated embryos failed to reach the trocophore stage (Thresher *et al.*, 2001). In this same study, transfected oyster embryos with the pHSP-oHoxDS/BH plasmid and 29% of the embryos experienced arrested development with no heat shock and more than 65% had arrested development with heat shock. In this same experiment only 5% of control oyster embryos (not heat shocked) and 4% (heat shocked) had arrested development (Thresher *et al.*, 2001).

The disruption mechanism for the SF3 construct is based on double stranded mRNA (dsRNA), which has been shown to be superior in disrupting normal gene expression as compared to single stranded RNA (Fire *et al.*, 1998). In the first (79.2% vs. 70.6%) and second (70.1% vs. 59.2%) experiment with channel catfish, SF3 demonstrated a higher efficacy for disrupting embryonic development than SF4.

Deformed individuals are another indicator of disruption of embryonic development. The efficacy of the SF3 construct is further substantiated by Thresher *et al.* (2001), which obtained forty-three deformed (22.6%) of the 190 zebrafish embryos injected with the zBMP2-dsRNA construct, which is the same disruption mechanism used for the SF3 construct. In this same experiment, the sham injected

control embryos with buffer had 0 out of 37 embryos deformed and the uninjected controls had 1 out of 174 that was deformed. These results correlate with the amount of deformities observed in the current study. Although, no deformity rate in any treatment in any experiment was greater than 3.7%. Even with this depressed deformity rate, the deformity rate for the sterile feral constructs was consistently greater than that of the electroporated control, GAD and CAB construct in the first two experiments. It was only in the third experiment that the deformity rate for GAD surpassed that of both sterile feral constructs.

Doxycycline concentration

Overall, channel catfish electroporated with the SF3 construct had a higher percent mortality than the same group treated with doxycycline during incubation from 4 hours post hatch to first hatch. The concentration of 150 ppm doxycycline used with channel catfish electroporated with the SF3 construct was less effective in reducing mean mortality percentages than both the 50 and 100 ppm concentrations. The relative percent mortality (56.1%) for the SF3 construct was reduced to only 12.1% by the 50 ppm concentrations and to 5.2% by the 100 ppm doxycycline concentration. The doxycycline concentration of 150 ppm had a 54.1% relative percent mortality, which was almost equal to that of the SF3 group. Thresher *et al.*, (2001) also noted higher mortalities with the SF3 construct when treated with concentrations in excess of 100 ppm. Thresher *et al.* (2001) injected 52 zebrafish embryos with the SF3 construct and treated them with 125 ppm doxycycline and the results were that 25 (48.1%) died by 24 hours post hatch. In the same experiment, he injected 60 zebrafish embryos with the SF3 construct and did not treat them with doxycycline, and the results were that 22 (36.7%) died by 24 hours post hatch (Thresher *et al.* 2001). In the current study, the 100 ppm concentration of

doxycycline produced the lowest mortality rates with the SF3 construct. The relative effectiveness of the doxycycline treatments in the current study were higher than that of Thresher *et al.* (2001).

The results with doxycycline were not as consistent with the SF4 construct as compared to the SF3 construct. The first experiment demonstrated that 100 ppm doxycycline (63.0%) counteracted the SF4 construct (70.6%) by producing a lower mean mortality percentage. In the second experiment, a lower mean mortality percentage occurred in the SF4 group (59.2%) as compared to the 50 (64.2%) and 100 (62.3%) ppm doxycycline groups, but SF4 had a higher mean mortality percentage than the 150 ppm (52.8%) doxycycline group. In the third experiment, the mean mortality percentage of all three doxycycline groups (64.2%, 62.3% and 52.8%) were lower than that of the SF4 group (84.0%). These results, which demonstrated that the 150 ppm doxycycline concentration to have the lowest mean mortality percent, contradict the previous results making the same comparisons with the mean mortality percent of the SF3 construct. The possibility exists that different genes require different doxycycline treatments.

The ability of the Tet-Off™ system, which is the basis of doxycycline mediated control, to control transgene expression was also demonstrated in experiments with the Pacific oyster, *Crassostrea gigas,* (Thresher *et al.*, 2001). These experiments showed that transfected Pacific oyster embryos had expression levels equal to that of non-transfected embryos when they were treated with doxycycline (Thresher *et al.*, 2001). A second experiment in the same study with the same species also confirmed the ability of doxycycline to prevent the expression of dsRNA, for the doxycycline treated groups had lower fluorescence than the two non-doxycycline treated groups (Thresher *et al*, 2001). Percent survival for control groups were 77%

and 71% while doxycycline treated groups were 95% and 92%, demonstrating the ability of doxycycline to suppress expression of the blocker thus allowing for a higher survival rate.

Critical period of development

For the first experiment with channel catfish, the 31-61 hour post fertilization time period, had the highest rate of mortality when treated with SF3 or SF4. For both the second and third experiment, the 18-59 hour post fertilization time period produced the highest mortality rates. These varying results were due to the frequency of sampling and seems to indicate the sensitive time period is more accurately represented by the 18-61 hour post fertilization time period. More detailed experimentation is needed to determine if the critical development time that is sensitive to the SF-3 and SF-4 constructs is narrower than the 18-61 hour time period. Further investigation needs to be made into this 18-61 hour time period by making more frequent observations within this interval in order to more narrowly define the most critical time.

Conclusion

The current study demonstrates the potential of both sterile feral constructs to disrupt embryonic development. SF3 produced a mean mortality percent that was 56.1% higher than that of the electroporated control group. The SF4 construct also showed potential by causing a 37.4% increase in the mean mortality percent relative to that of the electroporated control group. In terms of effectiveness, the SF3 construct demonstrated consistently higher mortality rates. The ability of doxycycline to reduce expression of the blocker, thus allowing for normal development and survival, was also demonstrated. In the study of doxycycline, the 100 ppm was the optimal concentration level to counteract SF3. Overall, 150 ppm was the most

effective treatment to prevent expression of SF4. The crucial time period for the action of the sterile feral constructs appears to be somewhere between 18 and 61 hours post fertilization but further investigation also needs to be made in order to narrow this interval. However, the application of the doxycycline would need to precede this significantly in order to counteract the disruption of the SMad5 gene, which was shown to be active as soon as the single cell stage in zebrafish (Thresher *et al.*, 2001).

Further study is also needed into methods of applying the doxycycline in terms of commercial reproducibility and environmental sustainability. The ability of the channel catfish to pass the constructs on to future offspring needs to be evaluated and the efficacy of these constructs in a transgenic fish needs to be determined. The goal of achieving an effective and reversible sterilization technique appears feasible based on our initial results.

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APPENDIX A

Holzfretter's Solution

3.5 g NaCl

0.05g KCl

0.1g CaCl

0.2g $NaHCO_3$

Dissolve ingredients in 1L dd H_2O

APPENDIX B

PAB construct

This construct employs antisense mRNA salmon type GnRH to disrupt the production of the neuromediator, GnRH, which is essential to proper gonadal development (Zohar and Mylonas, 2001) (Figure 23). The first section of this construct (figure 3) is the salmon sGnRH Pab promoter. The next sequence is the SD/SA, which is an acceptor signal fused to the promoter. This section is followed by the GnRH antisense gene which is ultimately responsible for the disruption of GnRH production and/or utilization (Uzbekova *et al.*, 2000). The final section of this construct is the polyA SV40 which terminates transcription. The total size of this construct is 3,066 bp.

PH3 Construct

This construct employs antisense mRNA salmon type GnRH in order to disrupt the production of the neuromediator, GnRH, which is essential to proper gonadal development (Zohar and Mylonas, 2001).

Figure 23. Pab-sGNRH-antisense construct. (taken from Uzbekova *et al.*, 2000).

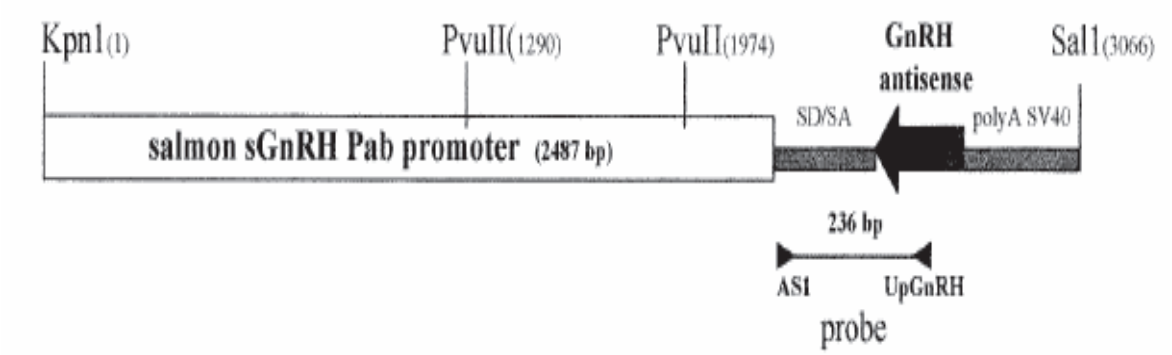

Table 17. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with Sterile Feral (SF3 and SF4) gene constructs, PH3, PAB and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

Mean Mortality (%)	
Treatment	**Experiment 1**
SF3	79.2[a]
SF4	70.6[b]
Control	28.1[c]
PAB	31.9[c]
PH3	31.2[c]

[a,b,c] - mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 24. Mean mortality of channel catfish, *Ictalurus punctatus*, in experiment one. Embryos were electroporated with the Sterile Feral (SF3 & SF4) gene constructs, PAB, PH3 and a electroporated blank control. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

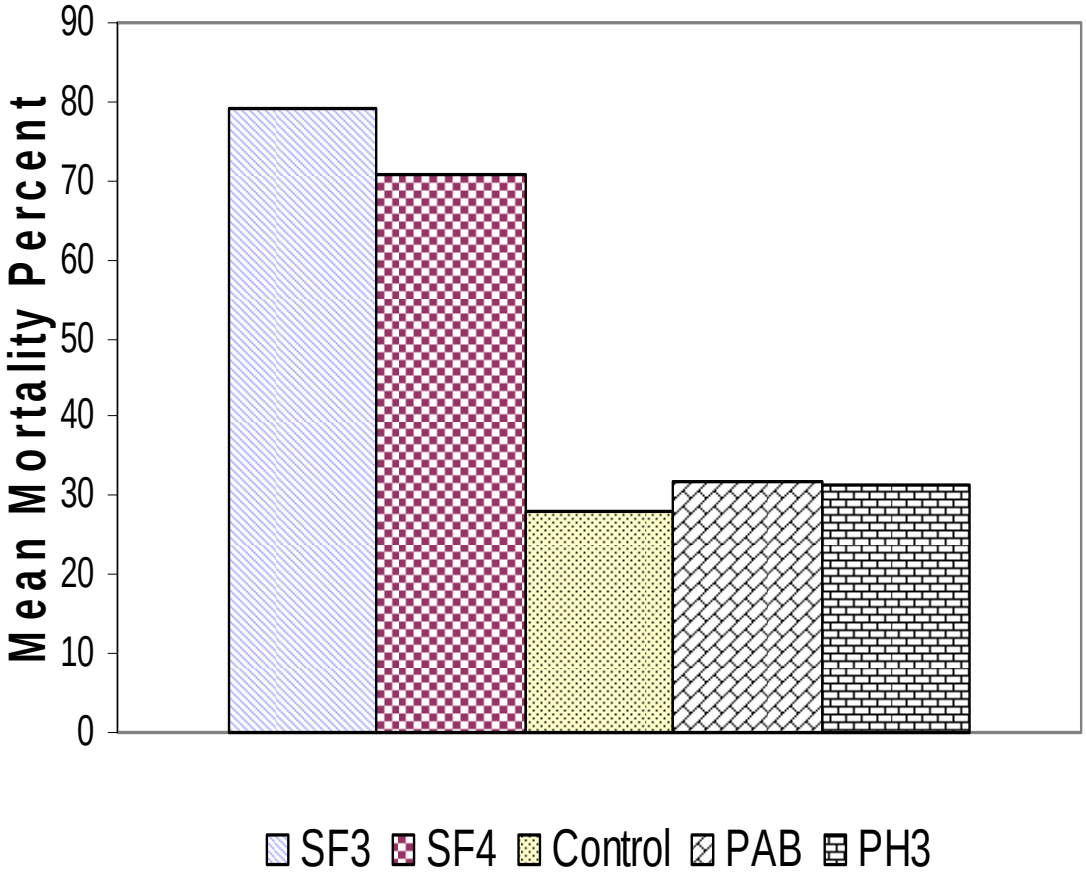

Table 18. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 & SF4) gene constructs treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch, PAB, PH3 and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.

	Mean Mortality (%)
Treatment	**Experiment 1**
SF3 Dox 100	47.3[b]
SF4 Dox 100	63.0[a]
Control	28.1[c]
PAB	31.9[c]
PH3	31.2[c]

[a,b,c] - mean percentages with different letters within columns are different ($P < 0.05$), chi-square.

Figure 25. Mean mortality of channel catfish, *Ictalurus punctatus*, embryos electroporated with the Sterile Feral (SF3 & SF4) gene constructs treated with doxycycline (100 ppm) from 4 hours post fertilization to first hatch, PAB, PH3 and a electroporated control in experiment one. Dead eggs and embryos were identified and removed beginning from 14 hours post fertilization up to 153 hours post fertilization.