DISRUPTION OF EMBRYONIC DEVELOPMENT IN COMMON CARP, *CYPRINUS CARPIO*, AND CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, VIA KNOCK DOWN OF BMP2 GENE FOR REPRESSIBLE TRANSGENIC STERILIZATION

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DISSERTATION ABSTRACT

DISRUPTION OF EMBRYONIC DEVELOPMENT IN COMMON CARP*, CYPRINUS CARPIO*, AND CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, VIA KNOCK DOWN OF BMP2 GENE FOR REPRESSIBLE TRANSGENIC STERILIZATION

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The primary objective of this research was to evaluate the sterile feral (SF) technology to accomplish reversible transgenic sterilization in fish. Secondary objectives were to 1) determine the ability of SF3 and SF4 gene constructs to interrupt embryonic development, 2) determine the optimum concentration of doxycycline to prevent the interruption of embryonic development, and 3) determine the shortest appropriate time period to apply doxycycline.

Transgenic common carp, *Cyprinus carpio*, and channel catfish, *Ictalurus punctatus*, embryos were produced from P1 putative transgenic common carp and channel catfish parents that had been electoporated with SF3 or SF4 constructs (modified Tet-off system for knock down of the BMP2 gene). Fifty, 100 or 150 ppm of doxycycline (dox) was applied to some families and replicates of F1 and F2 embryos at different

times, 15-20 hours, 20-25 hours, 25-30 hours, 0-30 hours, 30-35 hours, 35-40 hours, 0-60 hours, 30-60 hours after fertilization and 0hr – first hatch to rescue embryonic development.

There were 2 SF3 families and 4 SF4 families of F1 common carp produced, however, only dox treatment of SF4B3M2 and SF4B4M2 families significantly rescued the transgenic embryos ($P < 0.05$, χ^2). Nine F2 SF3 families and 12 F2 SF4 families were produced by mating F1 male with 2 putative females of each gene construct. No difference in hatchability between dox and no-dox treatments was found ($P>0.05$).

Dox treatment of both SF3 and SF4 families significantly increased the hatch and rescued the transgenic channel catfish embryos ($P< 0.01$, χ^2). Dox treatment of SF3 families and Sf4 families which had 0-30, 0-60 and 30-60 treatments increased the hatch and rescued the transgenic embryos (highest percent was in 30-60 treatment for SF3, and in the 0-30 treatment for SF4) ($P< 0.05$, χ^2). Data from SF3 families suggested that 30-35 treatment increased hatch and rescued transgenic embryos ($P<0.01$, χ^2).

The data suggests that the SF approach of embryonic disruption and transgenic sterilization has potential for 100% effectiveness, especially in channel catfish. The F1 and in some cases the F2 generation was reached with promising results for some families. Selection of the correct families and producing the next generation to increase copy number (reaching homozygosity) could make this system completely effective. However, juvenile mortality and decreased growth rate in some families may indicate that the first generation tet-off system may be too leaky and may need to be replaced to obtain optimum results.

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INTRODUCTION

The world population is increasing, as is demand for food, protein and seafood. Aquaculture, the fastest growing food production sector (Jia et al., 2001), has the potential to help address the world's growing food supply demand.

Common carp is a frequently grown species, and from a global perspective, is the most important cultured species in the world. European countries and China are major producing countries (FAO, c2004-2008). This fish was likely the first fish aquacultured by the Romans and Chinese more than 2000 years ago (Dunham et al., 2001).

In 2006, there was a large trade deficit, 7 $ million, (U.S. Census Bureau, 2008) for fisheries products in the US, thus aquaculture production could have a major impact on the US economy. Catfish farming is the largest aquaculture industry in United States. Sustainability needs to be improved for catfish farming, and profitability and competitiveness are threatened by the recent establishment of catfish imports from Southeast Asia and other species. Transgenic technology has high potential to increase production and profit to catfish industry, but this technology can not be applied if public concern about escape of these fish is not overcome. Transgenic sterilization is an option to solve this problem.

Genetic technologies can greatly contribute to aquaculture production and efficiency assisting in providing sufficient food supply. Resource utilization can be greatly improved and impediments to sustainability such as slow growth of fish, inefficient feed conversion, heavy mortality from disease and the associated use of

chemicals, loss of fish from low oxygen levels, inefficient harvest, poor reproduction, inefficient use of land space and processing loss can be diminished by utilizing genetically improved fish. Genetic enhancement of farmed fish had advanced to the point that it is now impacting aquaculture worldwide, however, potential maximum improvement in overall performance is not close to being achieved (Dunham et al., 2001).

To maximize fish product from aquaculture, various genetic improvement techniques have been applied to aquaculture, such as domestication selection, interspecific hybridization, sex reversal and breeding, and transgenesis (Dunham, 2004). Every technology has some drawback, and genetic improvement technology is not an exception. A major public concern is the potential adverse effects that escaped, farmed, domesticated fish, and especially transgenic fish, might have on ecosystems, the natural environment and native gene pools (Hackett, 2002; Kapuscinski and Patronski, 2005). Transgenic fish containing growth hormone and/or disease resistance gene have been produced for several fish species, and have been shown to significantly improve production (Dunham, 2003, 2004). Application of transgenic fish has not occurred due to public and government concerns regarding environmental risk and possible negative impact on natural populations.

To prevent ecological risk and environmental impact of aquacultured fish, domesticated, interspecific hybrids, exotics or transgenics, several approaches are possible. These include; physical confinement, mechanical sterilization, chemical sterilization, monosex populations, and genetic manipulation (Dunham 1990, 1996; NRC, 2004; Kapuscinski and Patronski, 2005). Various genetic, chemical, mechanical

sterilization approaches are temporary, have inherent disadvantages or are not feasible on a commercial scale in fish (Dunham 1990, 1996). Overall, genetic manipulation seems to be the strongest approach to reduce ecological risk and environmental impact.

One alternative for genetic sterilization is triploid induction. This method involves application of either hydrostatic pressure, temperature, or chemical shock to fertilized eggs to induce the retention of second polar body resulting in an embryo containing 2 sets of chromosomes from the female parent and the third set from male (NRC, 2004). The extra set of chromosomes may cause problems in the paring of homologous chromosomes during cell division disrupting the normal development of gametes (Benfey, 1999). Even though triploid sterilization has several advantages such as low costs and rapid implementation, the induction technology does not always result in 100 % triploidy, it is not feasible on commercial scale for all species, there is uncertainty regarding to reproductive behavior exhibited by sterilized adults (Kapuscinski and Patronski, 2005) and the technology requires fertile diploid adults, preventing total elimination of risk. Additionally, in some species triploidy decreases performance for traits, such as growth and low oxygen tolerance negating some of the improvement from the primary breeding program (Dunham, 2004).

Sex reversal and breeding to produce monosex, monogenotypic populations, is another genetic approach that had potential to control reproduction of transgenic, domestic and exotic species populations (Pandian, 2003). Even though technologies for producing monosex populations already exist, the application of this technology is only feasible when applied with exotic species which are not already present in the watershed, and in species where this methodology is 100% effective.

Repressible transgenic sterilization based on antisense and RNA interference (RNAi) approaches has the greatest potential for effective environmental protection from farmed or exotic fish. Theoretically, RNAi based transgenic methods could be used to induce sterility in fish (Kapuscinski and Patronski, 2005). RNAi was originally discovered as an unexpected result from an experiment with the petunia flower (Napli et al, 1990). The first phenomenon was name 'co-suppression' and later renamed post-transcriptional gene silencing (PTGS) (Shrivastava and Srivastava, 2008).

Additionally, the experiment of Que et al. (1997) with the petunia flower suggested that a sense transgene can induce gene silencing if a strong promoter was used to overexpress the transgene. Lindbo et al. (1993) proposed the threshold hypothesis to explain how a sense strand transgene can induce gene silencing through their work in transgenic tobacco. When the quantity of sense strand has been hyper-elevated, then the sequences will be targeted and inactivated by cellular factor. Consequently the complex between targeted RNA sequence and cellular factor will direct cellular enzyme to degrade the RNA, resulting in its elimination of that RNA from cytoplasm.

Later, Fire et al. (1999) found that double stranded RNA (dsRNA) was substantially more effective at producing interference than either sense or antisense strand in *Caenorhabditis elegans,* and the effects of interference were evident in both injected animals and their progeny. Since then, RNAi has been studied in variety of organisms, and it is broadly accepted that dsRNA is the ultimate trigger for this process, even if not the initial trigger in cases such as sense cosuppression (Jorgensen, 2003).

In general, RNAi pathway is initiated by RNase III-like endonuclease termed Dicer, which will cleave dsRNA into 20-25 base pair pieces named small or short

interfering RNAs (siRNA). Antisense strand of siRNA is then incorporated in the RNA-induced silencing complex (RISC) leading to localization of the RISC complex through perfect sequence alignment of the two RNA molecules and induces degradation of that mRNA by argonaute, catalytic component of RISC complex (Matzke et. al., 2003; Caplen, 2005; Shrivastava and Srivastava, 2008). The short fragments are known as siRNA when they were derived from exogenous sources, and miRNA when produced from a RNA-coding gene in the cell's own genome. Both are involved in the RNAi pathway (Caplen, 2005; Shrivastava and Srivastava, 2008).

The evaluation of an RNAi technology, termed sterile feral (SF), for the repressible transgenic sterilization of fish and shellfish has been initiated by the Commonwealth Scientific Industrial Research Organization (CSIRO) (Thresher et al., 2001; Kapuscinski and Patronski, 2005). This approach interrupts embryonic development by using mRNA specifically targeted for interference of gene function. Thresher et al. (2001) identified genes in zebrafish, *Danio rerio*, which are essential for embryonic development and activated in a short period of time only during embryonic development. SF constructs were made to disrupt embryonic development using RNAi and overexpression approaches.

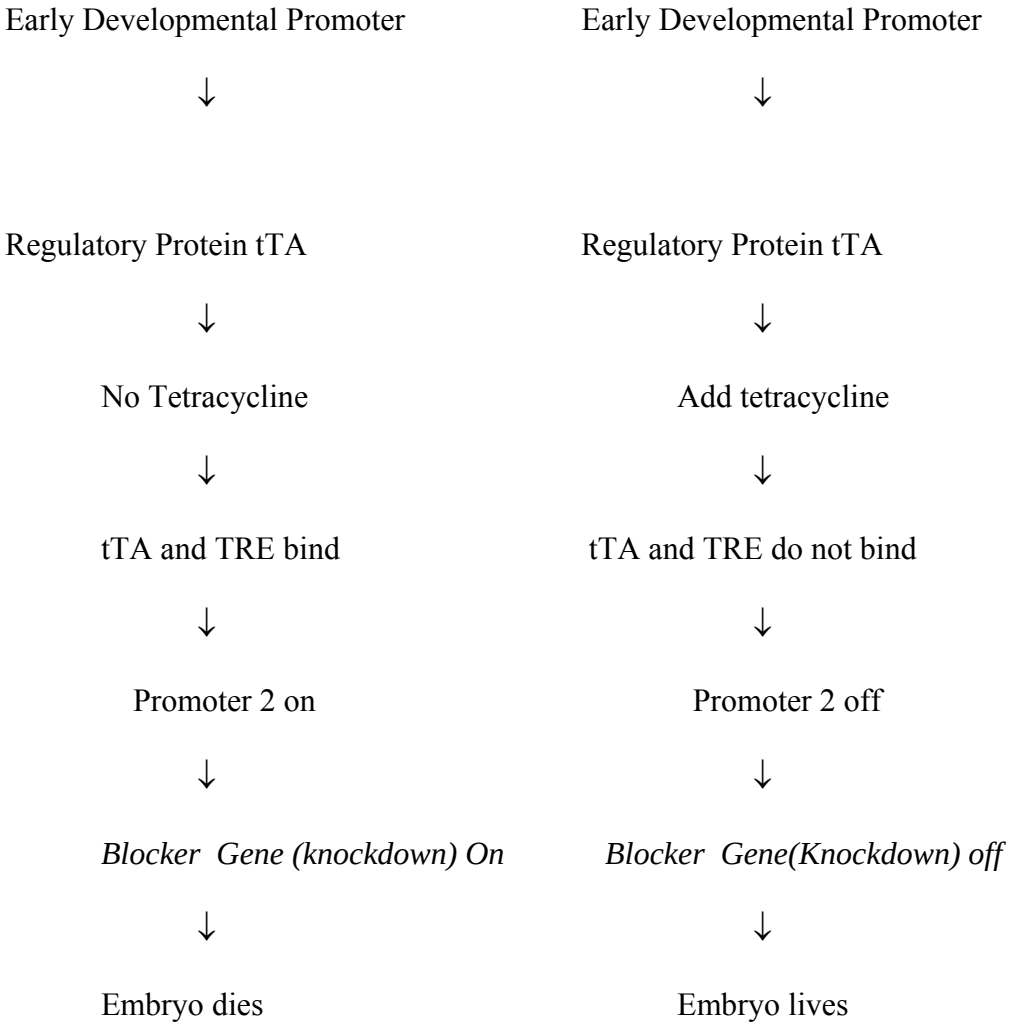
SF constructs are Tet-off based systems (Tet-off systems, http://clontech.com/, Protocol # PT3001-1, version # PR95962) containing 2 different promoters which drive different target genes. The first promoter, zSmad5, is coupled to tetracycline transactivator protein (tTA). The second promoter is a tetracycline regulated promoter (TRE), which initiates the expression of a blocker gene, antisense RNA, double-strand RNA(dsRNA), or sense RNA. The zSmad5 promoter that initiates tTA expression has a

narrow spatial and temporal window of activity. Thus, addition of a specific repressor molecule to the water in the hatchery is only required for a brief period to repress transcription (Thresher et al., 2001). Doxycycline (dox) or tetracycline functions as the repressor molecule, and both can be easily administered in water to allow embryonic development when desired.

Outside of this temporal window, even in the absence of the repressor molecule, the promoter is inactive and the blocker gene is not transcribed. This permits hatchery reared offspring to survive once placed into the farm environment for growing. The promoter expresses in the absence of the repressor molecule in any offspring that are produced outside of the hatchery resulting in the death of the offspring. The active promoter transcribes the blocker sequence that leads to disruption of critical gene function and eventual mortality. The blocker gene functions as a dominant allele, and thus, escapees cannot produce viable offspring even if they interbreed with wild type fish. The sterile feral mechanism is illustrated in Figure 1.

Specifically, the Tet-off system was developed by Gossen and Bujard, (1992), and has two major components. First component is tetracycline controlled transactivator (tTA) which was created by fusing *tet*R (tetracycline repressor) with viron protein 16 (VP16). The second component is the tetracycline regulated promoter (TRE) which was created by placing seven repeats of tet operator (*tetO*) elements upstream of minimal human CMV promoter. In SF constructs, tTA was coupled with zSmad5 promoter, and TRE promoter was coupled with blocker genes, zBmp2dsRNA or zBmp2cDNA. Theoretically, when zSmad5 activates, it will initiate transcription of the tTA protein.

Figure 1. Pathway for the repressible Sterile Feral mechanism.



In the absence of tetracycline or its derivatives, the *tetR* moiety of tTA binds with high affinity and specificity to *tetO*. At this point, the VP16 moiety of tTA strongly induces transactivation of the target gene (zBmp2dsRNA or zBmp2cDNA) by promoting the assembly of a transcriptional initiation complex (Agha-Mohammadi et al., 2004). Theoretically, after the target mRNA was knocked down, by theory, the embryo could not produce enough Bmp2 protein to normally develop ventro-dorsal muscle, leading to the deformation and/or the death of embryo. To block the initiation of second promoter, doxycycline (Dox) or its derivative, was applied to prevent the binding between Bi-TRE and tTA. When tetracycline is present, binding of tetracycline to tTA leads to a conformational change in *tetR* domain and loss of transactivation (Gossen and Bujard, 1992), and thus, bi-TRE cannot initiate the blocker gene expression. The embryo is rescued when Dox is applied.

zSmad5 is a gene that produces Smad proteins which might be involved in signaling process of members of transforming growth factor beta (TBG β) superfamily (Hild et al, 1999). The members of TBG β genes in zebrafish, Bmp2/4, is crucial for dorsal – ventral formation during early development.(Dosch et al, 1997). The mutation of Bmp2, known as *swirl* in zebrafish, can cause the defective dorsal – ventral phenotype during early development the same as the phenotypic mutation of zSmad5 gene (Kishimoto et al., 1997; Hild et al., 1999). Injection of wild-type zBmp2 mRNA leads to a complete rescue of the *swirl* mutant phenotype (Kishimoto et al., 1997). Both zSmad5 and zBmp2 genes were thought to express during the early development stage and have no function during adult stage.

The overall objective of this research was to evaluate the sterile feral technology to accomplish reversible transgenic sterilization in common carp and channel catfish Specific objectives were to 1) determine the ability of SF3 and SF4 gene constructs to interrupt embryonic development, 2) determine the optimum concentration of doxycycline to prevent the interruption of embryonic development, and 3) determine the shortest appropriate time period to apply doxycycline.

MATERIALS AND METHODS

Sterile Feral Constructs

Both sterile feral constructs, SF3 and SF4, were obtained from Commonwealth Scientific Industrial Research Organization (CSIRO). A map of sterile feral constructs is shown in Figures 2 and 3.

SF3 construct

The major components of the SF3 constructs are the zSMad5 promoter, tetracycline transactivator (tTA), SV40PA, green fluorescent reporter gene (eGFGP), bi-directional tet-responssive promoter (Bi-TRE), and zBmp2dsRNA blocker gene. zSMad5 drives the expression of the tTA gene. Then tTA binds to Bi-TRE promoter to initiate the expression zBmp2dsRNA. The entire length of SF3 construct is 7998 bp (Thresher *et al*., 2001).

SF4 construct

All components of SF4 construct are the same as SF3 except zBmp2dsRNA was replaced with zBmp2cDNA to serve as blocker gene. The entire length of SF4 construct is 8611 bp (Thresher et al., 2001). For both SF3 and SF4, target mRNA for knock down was Bmp2 mRNA, .

The zSmad5 promoter was used SF3 and SF4 constructs because of the potential shortcoming of Bmp2 promoter in combination with a tet-responsive element to effectively block its own transcript. Also, Smad5 was employed to overcome a potential

Figure 2. Map of the SF3 construct.

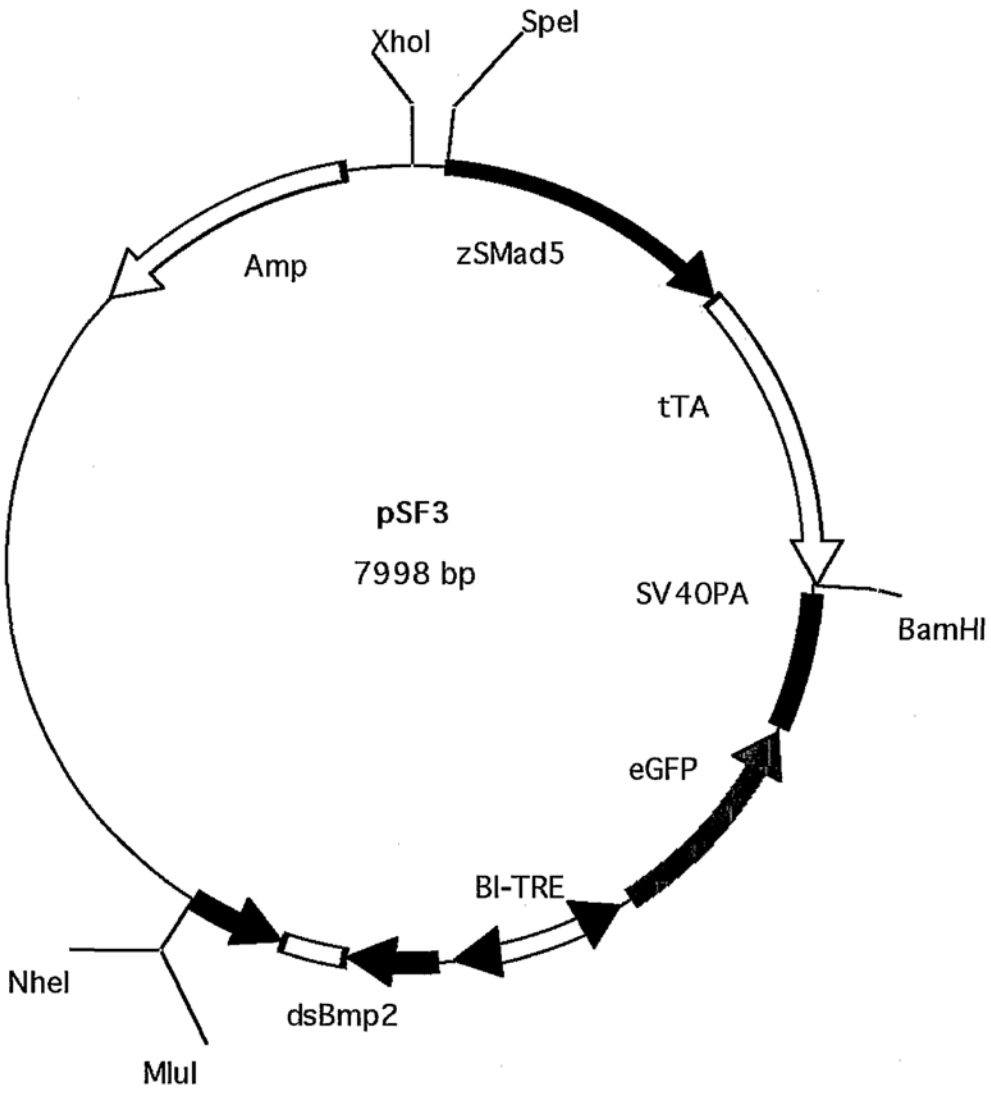
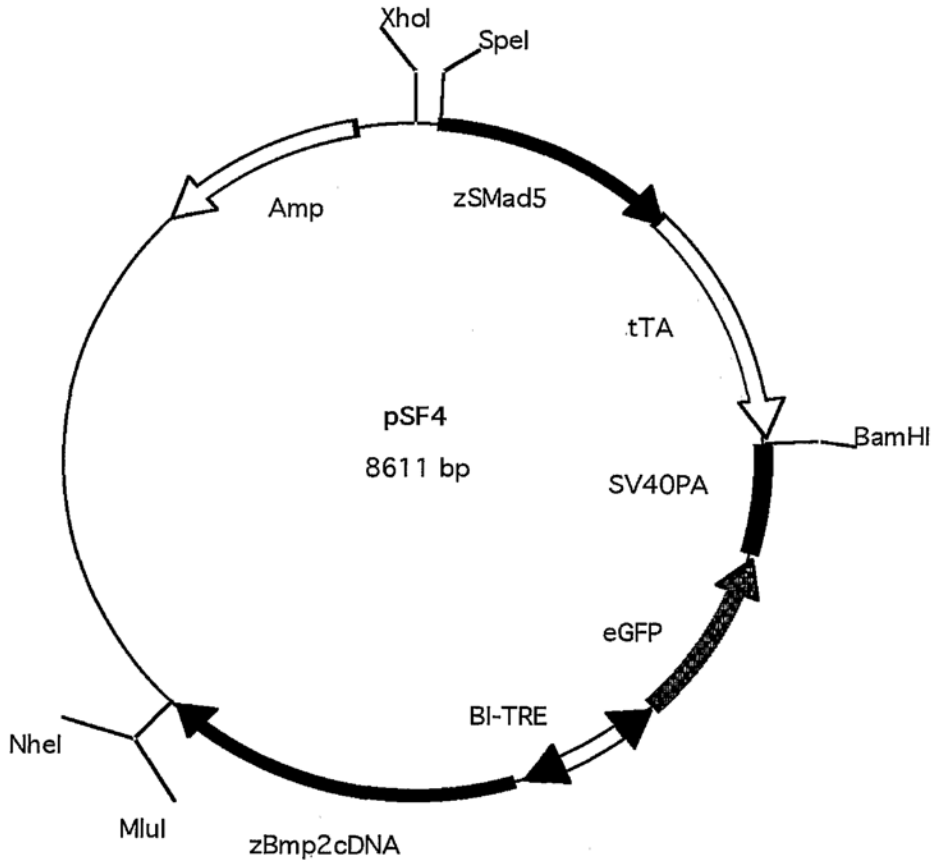


Figure 3. Map of the SF4 construct.



temporal delays associated with the Bmp2 promoter to ensure that knock down occurred before any BMP2 proteins could be produced.

Commom carp

Putative P1 transgenic common carp, produced via electroporation of SF3 and SF4 constructs as described by Templeton (2005), were raised in a 0.1 ha earthen pond. When fish reached 2 years of age, gravid males and females were selected and moved into an indoor hatchery, and held in 500 liter fiberglass tanks until spawning. Females were injected with carp pituitary extract (CPE) solution to induce spawning. Females received the first injection of 0.3 mg/kg of CPE solution, and then were injected with a second dose of 3.3 mg/kg 12 hours later. After 200 degree hours from the first injection, fish were monitored for any signs of ovulation. When fish started dropping eggs, they were anesthetized in 200 ppm of tricaine methanesulfonate (MS-222). About 15-20 grams of egg were taken from each female and fertilized with sperm from putative P1 males as single pair matings. Two families for the SF3 construct, and 4 families for the SF4 construct were produced.

Two putative SF3 females were crossed with 6 different transgenic males which came from two F_1 transgenic families (SF3B2M2 and SF3B1M1) to produce F_2 embryos. F_2 SF4 families were produced by mating 2 putative SF4 females with 11 different transgenic males which came from SF4B1M1 and SF4B4M2 F_1 transgenic families. Nine families of F_2 SF3 construct and 12 families of F_2 SF4 construct were produced.

Treatment and egg incubation

Fifty to 100 eggs were incubated in a plastic container containing 6 L of 100% Holzfretter's solution (appendix A). Embryos from each family were treated with 100

ppm of doxycycline (Dox), or 100 % Holzfretter's solution only, two replicates per treatment. The water was changed at 24 hour intervals, dead embryos from each replicate were removed, placed in microcentrifuge tubes and frozen for future PCR analysis every day until 15 days after hatch. Dox concentration was kept at 100 ppm until hatching. Holtzfretter's solution was kept at 100% for the first 2 days, at which time it was reduced by 25% daily until hatching. On the 15th day after hatching, fry were moved in to a recirculating system. Fish were separated by family and treatment, and raised in the recirculating system for about 10 months. After that, fish were then weighed and fin samples taken for PCR analysis. A second trial was conducted by adding 150 ppm treatment of dox, and keeping the other treatments and incubation procedure the same as the first trial. Two families of SF3 construct were produced.

F2 embryos were treated and incubated in the same manner as F1 embryos. One replication of a 50 ppm dox treatment was added for each F2 family.

Channel catfish

Putative P1 channel catfish produced via electroporation of SF3 and SF4 constructs as described by Templeton (2005) were raised in 0.1 ha earthen ponds. Ripe males and gravid females, 2 – 3 years of age, were selected and kept in an indoor hatchery for spawning. Females were implanted with 100 µg/kg of luteinizing hormone releasing hormone analogue (LHRHa) implants. Females were kept in spawning bags until they started dropping a few eggs, and then the ovulating females were anesthetized with 200 ppm tricaine methanessufonate (MS 222). Putative transgenic males were sacrificed, and testes extracted for preparing sperm solution. Eggs were taken from each female by stripping and were fertilized with putative male sperm. Twenty- three SF3

families and 9 SF4 families were produced from putative P1 transgenic males and females in this trial.

Treatment and egg incubation

First trial, 100 to 150 eggs from each of 32 families were incubated in a plastic container containing 6 L of 100% Holtzfretter's solution (appendix A). Embryos from each family were treated with 100 ppm of doxycycline (Dox), or 100 % Holtzfretter's solution only, two replicates per treatment. The water was changed at 24 hour intervals, dead embryos from each replicate were removed, placed in microcentrifuge tubes and frozen for future PCR analysis every day until 15 days after hatch. Dox concentration was kept at 100 ppm until hatching. Holtzfretter's solution was kept at 100% for the first 2 days, at which time it was reduced by 25% daily until hatching. On the 15th day after hatching, fry were moved in to a recirculating system. Fish were separated by family and treatment, and raised in the recirculating system for about 10 months. After that, fish were then weighed and fin samples taken for PCR analysis.

A second trial was conducted with about 50 eggs from each of 31 families. Fertilized eggs from each family were treated with 100 ppm of doxycycline at 0-15, 15-20, 20-25, 25-30, 30-35, 0-30,35-40, 30-45, 30-60, and 0-60 hours post fertilization, and with 0 ppm dox as a control, one replicate per treatment. Culture and sampling were as described above.

PCR Analysis

DNA analysis was conducted at Aqua Bounty Technologies Inc. (San Diego, CA) for common carp samples and the first experiment with channel catfish. DNA was extracted from eggs and fin using DNAzol reagent. DNA samples were resuspended in 8

mM NaOH and adjusted to pH 8.0 with 0.1 M HEPES. Samples were run on The Corbett Research RG-3000 real time PCR system using Applied Bioscience SyBR Green master mix. Specific primers for the carp beta actin gene were designed and used as standard (carp β actin (108 bp fragment amplified) 5'-
CAGAATTTTTTTATTTAATGAATTTGACCC,
5'- GTAATGAATCAGGCTTGCATTCC . Specific primers for tTA Tetracycling transactivator (100 bp fragment amplified) 5'- CTGATAGTATGCCGCCATTATTACG, 5'- TCCGCATATGATCAATTCAAGG
and Bi-Tre , Bi-drirectional tetracycline responsive promoter (100 bp fragment amplified): 5'- GGCTGGATCGGTCCCG , 5'- GACCTATATAAGCAGAGCTCGTTTAGTG were designed for evaluating the presence of the transgene in the progeny .

DNA analysis for the the second experiment with channel catfish was conducted at the Fish Molecular and Genetics Lab, Auburn University. Genomic DNA from egg was extracted with DNAzol reagent, and genomic DNA from fin sample was extracted with PUREGENE reagent. To determine the presence of the transgene, specific primers against zSmad5tTAgene and TREzBmp2 gene were designed. The presence of the transgene in the progeny was determined by using one of these primers. One microliter from each sample was amplified in 10 µl PCR mix. Thirty- five to forty PCR cycles were performed in a PTC-100 thermocycler (MJ Research Inc.). Each cycle contained 30 seconds denaturation at 94 °C, 30 seconds annealing at 58 °C, and 60 seconds amplification at 72 °C. Specific primers for catfish beta actin gene were designed and used as standard (110 bp fragment amplified): 5' TACTCCGTTTGGATCGGTGG, 5'- GACCGGCCTCGTCGTAGTC. Specific primers for zSmad5- tTA gene (454 bp

fragment amplified): 5’ GTGGACTTTGAGTCCGGTGT. GGCGAGTTTACGGGTTGTTA, and TRE-Bmp2 gene (220 bp fragment amplified): 5’ ATCCACGCTGTTTTGACCTC, 5’ GTCGATCTCGGGAATGAGTC were designed for evaluating the presence of the transgene in progeny.

Water Analysis

Temperature throughout the experiment was monitored using alcohol thermometers. Total hardness, total alkalinity, nitrates, nitrites and pH were monitored using multi-test strips from Mardel. A liquid ammonia test kit, Aquarium Pharmaceuticals, Inc., was used to monitor total ammonia levels in the incubation containers.

Feeding

Common carp and channel catfish fry were fed to satiation with *Artemia* nauplii 3 times per day. Fry were fed with *Artemia* until 20 days after hatching and then changed to powdered feed and pellets as size of the fish grew. Fish were then fed once a day to satiation level after they were moved to the recirculating system.

Data collection and data analysis

Number of dead embryos was recorded. Hatching percentage, percent transgenic individuals, mortality timeline, and cumulative deformities were calculated. Final body weight of fish was recorded and compared between transgenic and non-transgenic individuals within families. Chi-square test was performed to find any significant relationship between number of transgenic individuals and treatments (P =0.05). A t-test or contrast comparison was performed to compare any significant difference in percent deformity, hatchability and mean final body weight within family (P=0.05).

RESULTS

Common carp

First trial

Data for first trial was shown in Table 1. Observed percent hatchability of dox treatment in both SF3B1M1 and SF3B2M2 families was lower than for no-dox treatments. In family SF3B1M1, percent hatchability of dox treatment was lower than no-dox treatment by 20.9 % while in family SF3B2M2 it was lower by 43.7 %. Percent transgenic individuals in these families also was higher in no-dox treatment. Dox treatment had no effect on rescuing transgenic embryos ($P>0.05$, χ^2)

Of the 4 families containing the SF4 gene construct (Table 1), SF4B1M1, SF4B2M1 and SF4B4M2 had the observed higher hatchability for the dox treatment compared to the no-dox treatment. In contrast, only the no-dox treatment of family SF4B3M2 that had higher observed percent hatchability than its corresponding dox treatment. The dox treatment had higher percent transgenic individuals than no-dox treatment for only SF4B3M2 and SF4B4M2 families. In these two families, dox treatment significantly rescued the transgenic embryos ($P < 0.05$, χ^2). There is no significance difference on over all mean of percent hatchability between dox and no-dox treatment ($P>0.05$).

Mean final body weight of SF3 and SF4 transgenic and non-transgenic full-siblings within treatments are shown in Table 2. Transgenic individuals in SF3B2M2

Table 1. Hatchability, percent non-transgenic and transgenic F1 common carp, *Cyprinus carpio*, containing SF3 and SF4 gene constructs incubated without doxycycline (no-dox) or with 100 ppm doxycycline (dox) solution.

Family[1]	Treatment	% hatch	Number of fish analyzed	Number transgenic	% transgenic	Number non-transgenic	% non-transgenic
SF3B1M1	dox	55.8	10	5	50.0	5	50.0
	no-dox	70.6	13	8	61.5	5	38.5
SF3B2M2	dox	25.0	40	35	87.5	5	12.5
	no-dox	44.4	25	22	88.0	3	12.0
SF4B1M1	dox	79.4	7	3	42.9	4	57.1
	no-dox	65.8	33	19	57.6	14	42.4
SF4B2M1	dox	26.8	18	0	0.0	18	100.0
	no-dox	17.2	12	7	58.3	5	41.7
SF4B3M2	dox	52.6	30	19	63.3	11	36.7
	no-dox	67.5	30	11	36.7	19	63.3
SF4B4M2	dox	41.1	72	13	18.1	59	81.9
	no-dox	24.7	66	4	6.1	62	93.9

[1]Family nomenclature; SF3B1M1 = putative SF3 transgenic female #1 crossed with putative SF3 transgenic male #1, etc.

Table 2. Mean body weight (BW) of non-transgenic and transgenic common carp, *Cyprinus carprio*, containing SF3 and SF4 gene constructs at 4 -6 months of ages when grown in 40L aquaria in a recirculating system.

Family	Group	Genotype[1]	Number of fish	BW (g)[2]
	dox	N	5	4.2 ± 0.8[a]
		T	5	8.8 ± 5.8[a]
	no-dox	N	5	7.0 ± 4.6[a]
SF3B1M1		T	8	5.2 ± 1.8 [a]
	dox	N	5	7.8 ± 4.6 [a]
		T	35	3.3 ± 1.3 [b]
	no-dox	N	4	9.8 ± 4.9[a]
SF3B2M2		T	21	6.2 ± 3.2[a]
	dox	N	4	5.7 ±2.4[a]
		T	3	13.7 ± 3.2[a]
	no-dox	N	14	4.0 ± 2.9[a]
SF4B1M1		T	19	5.4 ± 4.2[a]
	no-dox	N	5	5.0 ± 3.6[a]
SF4B2M1		T	7	4.3 ± 1.7[a]
	dox	N	11	10.5 ± 7.3[a]
		T	19	9.2 ± 7.1[a]
	no-dox	N	19	9.9 ± 5.6[a]
SF4B3M2		T	11	5.4 ± 2.5[b]
	dox	N	59	3.9 ± 1.9[a]
		T	13	3.8 ± 1.6[a]
	no-dox	N	62	3.9 ± 1.6[a]
SF4B4M2		T	4	3.0 ± 0.8[a]

[1]Genotype; N = non-transgenic, T = transgenic

[2]Mean body weights within a family and treatment with different superscripts are significantly different ($P < 0.05$, t-test).

(dox treatment) and SF4B3M2 (no-dox treatment) grew more slowly ($P<0.05$) than controls.

Survival of transgenic common carp when grown from 1 to 5 months of ages is shown in Table 3. The number of transgenic fish that had a low CT value (high copy number of the transgene) in family SF3B2M2 decreased significantly during this period ($P < 0.05$, χ^2). There was a significant positive relationship between CT value of the transgene and survival (negative relationship between copy number of transgene and survival), survival = 12.25 + 33.37 CT, r = 0.61, p = 0.01 (Figure 4).

Second trial

In a second trial, 19 families of SF3 gene construct and ten families of SF4 gene construct were produced by matings between putative SF3 and SF4 males and females. Only SF3B4M3 and SF3B8M1 had expected hatching rates , however, no transgenic individuals died in the no-dox treatment. The 150 ppm dox treatment in both families had a lethal effect on the embryos. Percent inheritance ranged from 25.0 – 48.1 % in these families.

Performance of the F_2 generation

Data for nine F_2 SF3 families produced by crossing putative P1 SF3 females with transgenic F1 SF3 males are shown in Table 4. Two putative SF3 females were crossed with 6 different transgenic males, which came from two F_1 transgenic families (SF3B2M2 and SF3B1M1) to produce F_2 embryos.

Mean percent hatchability of F2 embryos that were produced from female number 1 mated with transgenic males M1, M3 and M5 of the SF3B2M2 family were not different ($P>0.05$) between dox and no-dox treatments in each family. For the 50 ppm

Table 3. Survival of F1 transgenic common carp, *Cyprinus carpio*, containing either SF3 or SF4 gene constructs at differing CT values when grown from 1- 5 months of age in a recirculating system.

Family	treatment	Number fish when stock in re-circulating system			Number fish at 5 months of age (% survival)		
		CT value			CT value		
		30 <	30-33	33-39	30 <	30-33	33-39
SF3B2M2	dox	11	11	-	2 (18.2)	11 (100.0)	-
	no-dox	7	15	-	1 (14.3)	14 (93.3)	-
SF4B1M1	dox	-	3	-	-	3 (100.0)	-
	no-dox	5	7	-	5 (100.0)	5 (71.4)	-
SF4B2M2	dox	-	-	-	-	-	-
	no-dox	-	2	1	-	2 (100.0)	1 (100.0)
SF4B3M2	dox	10	8	-	7 (70.0)	8 (100.0)	-
	no-dox	9	3	-	0 (0.0)	1 (33.3)	-
SF4B4M2	dox	-	-	10	-	-	10 (100.0)
	no-dox	-	4	-	-	4 (100.0)	-

* Mortality was due to an undiagnosed disease with the symptom of scales falling off of the fish

Figure.4. Relationship between relative CT value and survival for F1 common carp, *Cyprinus carpio*, containing SF3 or SF4 transgenes (1 = CT $\leq$ 30, 2 = CT 30-33, 3= CT 33-39)..

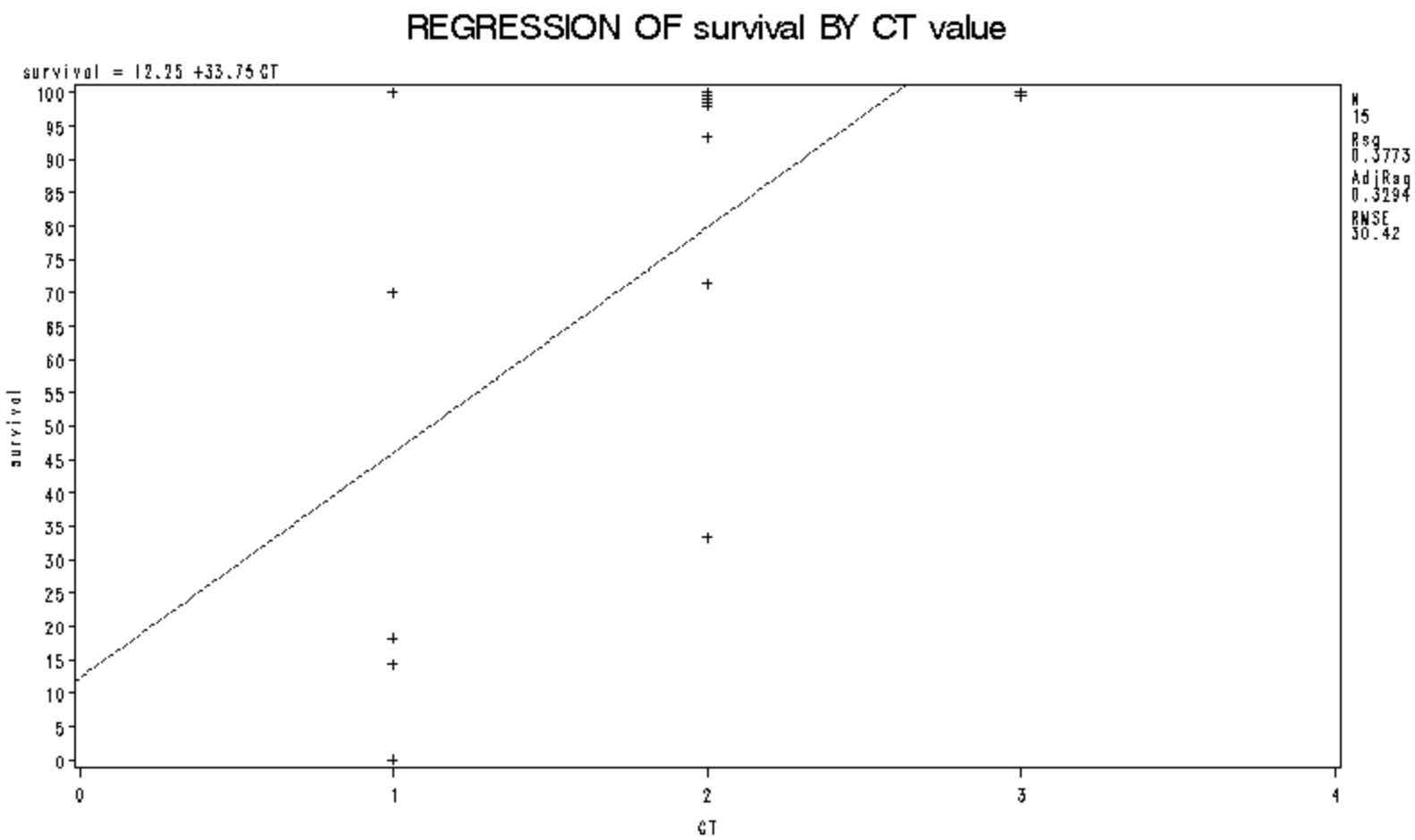


Table 4. Hatchability of F_2 commom carp, *Cyprinus carpio*, containing SF3 gene construct

F2 Family[1]	Female	Male information of F1 family[2]				Treatment [3]	% hatch (MEAN ± SD)[4]
		Family	Treatment	Bi-TRE	tTA		
						no-dox	58.8 ± 10.8[a]
						dox	41.6 ± 17.6[a]
AAF1A2M1	P1 Female # 1	SF3B2M2	dox	nd	30-35	50	56.4
						no-dox	87.5 ± 13.7[a]
						dox	52.2 ± 22.2[a]
AAF1A2M3	P1 Female # 1	SF3B2M2	dox	nd	30-35	50	84.8
						no-dox	47.3 ± 20.5[a]
						dox	32.5± 46.0[a]
AAF1A2M5	P1 Female # 1	SF3B2M2	dox	nd	30-35	50	73.7
						no-dox	50.2± 8.8[a]
						dox	19.8 ± 0.7[b]
AAF2A2M1	P1 Female # 2	SF3B2M2	dox	nd	30-35	50	32.5
						no-dox	69.4± 1.7[a]
						dox	61.9 ± 0.1[a]
AAF2A2M2	P1 Female # 2	SF3B2M2	dox	nd	30-35		
						no-dox	41.8 ± 12.2[a]
						dox	50.5 ± 6.7[a]
AAF2A2M4	P1 Female # 2	SF3B2M2	dox	nd	30-35	50	51.1
						no-dox	92.6 ± 3.1[a]
						dox	94.7 ± 2.9[a]
AAF1A5M3	P1 Female # 1	SF3B2M2	no-dox	30-33	nd	50	95.2
						no-dox	57.7 ± 12.6[a]
						dox	81.1 ± 20.9[a]
AAF2A5M2	P1 Female # 2	SF3B2M2	no-dox	30-33	nd	50	65.8
						no-dox	79.7 ± 0.5[a]
						dox	84.2± 1.2[b]
AAF1A6M1	P1 Female # 1	SF3B1M1	no-dox	nd	<31	50	69.9

[1]Family nomenclature; AAF1A2M1 = putative SF3 transgenic female# 1 crossed with group 2 putative SF3 transgenic male # 1, etc.
[2]Male information; Treatment , dox = when male was an embryo, it was treated with 100 ppm doxycycline, no-dox = embryo was incubated in no doxycycline solution, Bi-TRE = CT (cycle threshold) value for PCR for bidirection-tetracycline responsive element promoter, tTA = CT (cycle threshold) value for PCR for tetracycline transactivator gene, nd = not detected.
[3]Embryo treatment; no-dox = embryo was incubated without doxycycline solution, dox = embryo was treated with 100 ppm doxycycline, 50 = embryo was incubated in 50 ppm doxycycline solution.
[4]Mean percent hatchability within families that have different superscripts are significantly different ($P < 0.05$, t-test)

doxycycline treatment of these families, percent hatchability ranged from 56.4 to 84.9 %

Hatchability of F2 embryos produced from a second P1 female crossed with transgenic males M1, M2 and M5 of SF3B2M2 family are shown in Table 4. There was no difference in hatchability between dox and no-dox treatments of family AAF2A2M4, but there were differences ($P<0.05$) for mean percent hatchability of the other families AAF2A2M1 and AAF2A2M2, with no-dox treatment having a higher percent hatchability than dox treatment. Percent hatchability of 50 ppm doxycycline treatment from these families ranged from 32.5 to 51.1 %.

AAF1A5M3 and AAF2A5M2 were the other F2 families for which the male came from F1 family SF3B2M2. The mean hatchability between dox and no-dox treatment of each family was not different ($P>0.05$) even though observed means were higher in the dox treatment.

AAF1A6M1 was the only family among SF3 F2 families for which the transgenic F1 male came from a different family. The observed mean hatchability for the dox treatment of this family was significant higher than that for the no-dox treatment ($P <0.05$). Percent hatchability of 50 ppm doxycycline treatment from this family was 69.8 %, lower than that for the 100 ppm dox and no-dox treatment. There were no significant difference between over all mean of no-dox, dox and 50 ppm treatment of F2 SF3 families ($P>0.05$).

There were 12 F_2 SF4 families produced by crossing 2 putative transgenic females with 12 males from 2 different F_1 families (Table 5). BBF1B1M1 and BBF2B1M2 were 2 families from transgenic males from the same family. The observed

Table 5. Hatchability of F_2 commom carp, *Cyprinus carpio*, containing SF4 gene construct.

F2 Family[1]	Female	Male information of F1 family[2]				Treatment[3]	% hatch (MEAN ± SD)[4]
		Family	Treatment	Bi-TRE	tTA		
BBF1B1M1	P1 Female # 1	SF4 B1M1	dox	31-34	31-33	no-dox dox 50	46.7+ 31.0[a] 62.9 +14.6[a] 75.1
BBF2B1M2	P1 Female # 2	SF4 B1M1	dox	31-34	31-33	no-dox dox 50	60.9 66.9 +17.6 78.5
BBF1B2M1	P1 Female # 1	SF4 B1M1	no-dox	nd	30-33sin	no-dox dox 50	39.2 + 0.4[a] 32.1 + 17.0[a] 57.9
BBF1B4M1	P1 Female # 1	SF4 B1M1	no-dox	nd	40	no-dox dox 50	30.9 + 23.4[a] 52.2+20.5[a] 35.3
BBF2B4M2	P1 Female # 2	SF4 B1M1	no-dox	nd	40	no-dox dox 50	53.9+ 16.7[a] 49.6+ 1.1[a] 65.2
BBF1B8M1	P1 Female # 1	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	50.1 + 20.7[a] 59.6 + 2.9[a] 53.9
BBF1B8M3	P1 Female # 1	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	40.8+ 13.5[a] 21.8+22.1[a] 0.0

Table 5 (continued).

F2 Family[1]	Female	Male information of F1 family[2]				Treatment[3]	% hatch (MEAN ± SD)[4]
		Family	Doxy	Bi-TRE	tTA		
BBF2B8M2	P1 Female # 2	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	55.4+13.8[a] 31.7+6.8[a] 46.62
BBF2B8M4	P1 Female # 2	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox	17.9+ 4.7[a] 19.4+27.4[a]
BBF2B8M5	P1 Female # 2	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	11.0 + 0.6 58.59 34.89
BBF1B13M1	P1 Female # 1	SF4B2M2	no-dox	30-35sin	40	no-dox dox 50	71.01 67.52 47.29
BBF2B13M2	P1 Female # 2	SF4B2M2	no-dox	30-35sin	40	no-dox dox 50	15.08 30.35 42.62

[1]Family nomenclature; BBF1B1M1 = putative SF4 transgenic female# 1 crossed with group 1 putative SF4 transgenic male # 1, etc. .

[2]Male information; Treatment , dox = when male was an embryo, it was treated with 100 ppm doxycycline, no-dox = embryo was incubated in no doxycycline solution, Bi-TRE = CT (cycle threshold) value for PCR for bidirection-tetracycline responsive element promoter, tTA = CT (cycle threshold) value for PCR for tetracycline transactivator gene, nd = not detected.

[3]Embryo treatment; no-dox = embryo was incubated without doxycycline solution, dox = embryo was treated with 100 ppm doxycycline, 50 = embryo was incubated in 50 ppm doxycycline solution.

[4]Mean percent hatchability within families that have different superscripts are significantly different ($P < 0.05$, t-test)

percent hatchability of the dox treatment of these two families was higher than the corresponding no-dox treatment, but there was no significant difference (P>0.05). BBF1B2M1 was the other family derived from a transgenic male from SF4B1M1, but this male was not treated with dox during the embryonic stage. Mean percent hatchability of the dox treatment was not different (P>0.05) than the no-dox treatment. There were 2 families, BBF1B4M1 and BBF2B4M1, derived from the crossing between 2 non-transgenic F_1 males of SF4B1M1 with 2 different putative transgenic females. Observed mean percent hatch of the dox treatment was higher than no-dox treatment in BBF1B4M1, but not in BBF2B4M2. There was no difference (P>0.05) on mean percent hatch between dox and no-dox treatment in BBF1B4M1and BBF2B4M2.

BBF1B8M1 and BBF1B8M3, produced from 2 different males of F1 SF4B4M2 mated with putative transgenic SF4 female 1, had mean percent hatchability of dox and no-dox treatments with observed results in opposite directions (Table 5). However, there was no difference (P>0.05) on mean percent hatch between dox and no-dox treatment in BBF1B8M1 and BBF1B8M3 families. BBF2B8M2, BBF2B8M4, and BBF2B8M5 were produced from putative transgenic female 2 mated with 3 different transgenic males from family F_1 SF4B4M2. Percent hatchability of these families ranged from 11.0 to 55.4 % in the no-dox treatment, and 19.4 to 58.6 in the dox treatment. Percent hatchability of the 50 ppm doxycycline treatment from these families ranged from 34.9 to 46.4 %.

Families BBF1B13M1 and BBF2B13M2 were produced from 2 putative transgenic females crossed with 2 different males from family SF4B4M2. Percent hatchability varied between families and treatment. No-dox treatment of BBF1B13M1 had higher percent hatchability than dox treatment, and the opposite result was found in

BBF2B13M1. There were no significant differences between over all mean of no-dox, dox and 50 ppm treatment of F2 SF4 families (P>0.05).

Deformities

Percent deformity of F1 transgenic common carp is shown in Table 6. Percent deformity for SF3 and SF4 families varied from 0.0 – 50.0 %. Percent deformity of F2 common carp containing SF3 and SF4 gene constructs is shown in Tables 8 and 9. There were no differences (P >0.05) among 50, dox and no-dox treatments in both SF3 and SF4 families even though observed mean percent deformity was higher in dox treatment for both constructs. The deformities observed are illustrated in Fig. 5., and these deformities were not present in non-transgenic control common carp.

Channel catfish

First trial

Data from the first trial are summarized in Table 9. Six of 23 SF3 families and 2 of 9 SF4families exhibited expected results. For SF3 construct, percent hatchability ranged from 0 to 16.8% in control groups, with the lowest hatch in family SF3B2M5 (0 %) and highest in family SF3B4NM1 (16.8%). For SF3 families , doxycycline (dox) treatment group, percent hatchability was lowest in family SF3B2M5 (13.0 %), and , in control group from both families, while percent hatchability in dox treated group was 68.3 % and 14.9% in SF4B1M1 and SF4B1M2, respectively. Overall mean percent hatchability of SF3 dox treatment was higher than no-dox treatment (P<0.05).

Table 6. Percent deformity of F1 common carp, *Cyprinus carpio*, containing SF3 and SF4 gene constructs incubated without doxycycline (no-dox), with 100 ppm doxycycline (dox) solution or with 150 ppm doxycycline (150)

	% Deformity *		
Family	no-dox	dox	150
SF3B4M3	0.0	20.0	-
SF3B5M5	-	50.0	-
SF3B8M1	-	3.0	-
SF4B3M5	-	2.1	-
SF4B3M6	0.0	4.6	-
SF4B3M8	-	4.8	-
SF4B3M9	-	0.0	76.67

* Percent deformity = (# deform fry/ # hatched fry) x100

Table 7. Percent deformity of F2 common carp, *Cyprinus carpio*, containing SF3 gene construct.

		Male information of F1 family[2]					
F2 Family[1]	Female	Family	Doxy	Bi-TRE	tTA	Doxy treatment [3]	% deformity
AAF1A2M1	P1 Female # 1	SF3B2M2	dox	nd	30-35	no-dox dox 50	2.1 9.1 4.6
AAF1A2M3	P1 Female # 1	SF3B2M2	dox	nd	30-35	no-dox dox 50	1.5 0.6 1.4
AAF1A2M5	P1 Female # 1	SF3B2M2	dox	nd	30-35	no-dox dox 50	3.5 1.2 1.0
AAF2A2M1	P1 Female # 2	SF3B2M2	dox	nd	30-35	no-dox dox 50	6.3 46.3 14.8
AAF2A2M2	P1 Female # 2	SF3B2M2	dox	nd	30-35	no-dox dox 50	5.2 9.0 -
AAF2A2M4	P1 Female # 2	SF3B2M2	dox	nd	30-35	no-dox dox 50	5.1 6.6 7.3
AAF1A5M3	P1 Female # 1	SF3B2M2	no-dox	30-33	nd	no-dox dox 50	1.2 2.9 4.3
AAF2A5M2	P1 Female # 2	SF3B2M2	no-dox	30-33	nd	no-dox dox 50	3.4 6.9 3.8
AAF1A6M1	P1 Female # 1	SF3B1M1	no-dox	nd	<31	no-dox dox 50	0.7 11.8 1.0

[1]Family nomenclature; AAF1A2M1 = putative SF3 transgenic female# 1 crossed with group 2 putative SF3 transgenic male # 1, etc.
[2]Male information; Treatment , dox = when male was an embryo, it was treated with 100 ppm doxycycline, no-dox = embryo was incubated in no doxycycline solution, Bi-TRE = CT (cycle threshold) value for PCR for bidirection-tetracycline responsive element promoter, tTA = CT (cycle threshold) value for PCR for tetracycline transactivator gene, nd = not detected.
[3]Embryo treatment; no-dox = embryo was incubated without doxycycline solution, dox = embryo was treated with 100 ppm doxycycline, 50 = embryo was incubated in 50 ppm doxycycline solution.

Table 8. Percent deformity of F2 common carp, *Cyprinus carpio,* containing SF4 gene construct.

F2 Family[1]	Female	Male information of F1 family[2]					
		Family	Doxy	Bi-TRE	tTA	Treatment[3]	% deformity
BBF1B1M1	P1 Female # 1	SF4 B1M1	dox	31-34	31-33	no-dox dox 50	14.2 12.9 23.8
BBF2B1M2	P1 Female # 2	SF4 B1M1	dox	31-34	31-33	no-dox dox 50	16.2 10.8 14.8
BBF1B2M1	P1 Female # 1	SF4 B1M1	no-dox	nd	30-33sin	no-dox dox 50	7.1 20.2 6.1
BBF1B4M1	P1 Female # 1	SF4 B1M1	no-dox	nd	40	no-dox dox 50	12.3 12.6 18.6
BBF2B4M2	P1 Female # 2	SF4 B1M1	no-dox	nd	40	no-dox dox 50	31.8 19 11.5
BBF1B8M1	P1 Female # 1	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	47.1 17.1 0
BBF1B8M3	P1 Female # 1	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	11.5 0 10.3

Table 8. (continued).

F2 Family[1]	Female	Male information of F1 family[2]					
		Family	Doxy	Bi-TRE	tTA	Treatment[3]	% deformity
BBF2B8M2	P1 Female # 2	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	16.3 31 18.2
BBF2B8M4	P1 Female # 2	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	0 21.6 10
BBF2B8M5	P1 Female # 2	SF4B2M2	dox	31-36sin	32-39sin	no-dox dox 50	9.5 5.8 0
BBF1B13M1	P1 Female # 1	SF4B2M2	no-dox	30-35sin	40	no-dox dox 50	8.3 0 9.1
BBF2B13M2	P1 Female # 2	SF4B2M2	no-dox	30-35sin	40	no-dox dox 50	0 25 25

[1]Family nomenclature; BBF1B1M1 = putative SF4 transgenic female# 1 crossed with group 1 putative SF4 transgenic male # 1, etc. .

[2]Male information; Treatment , dox = when male was an embryo, it was treated with 100 ppm doxycycline, no-dox = embryo was incubated in no doxycycline solution, Bi-TRE = CT (cycle threshold) value for PCR for bidirection-tetracycline responsive element promoter, tTA = CT (cycle threshold) value for PCR for tetracycline transactivator gene, nd = not detected.

[3]Embryo treatment; no-dox = embryo was incubated without doxycycline solution, dox = embryo was treated with 100 ppm doxycycline, 50 = embryo was incubated in 50 ppm doxycycline solution.

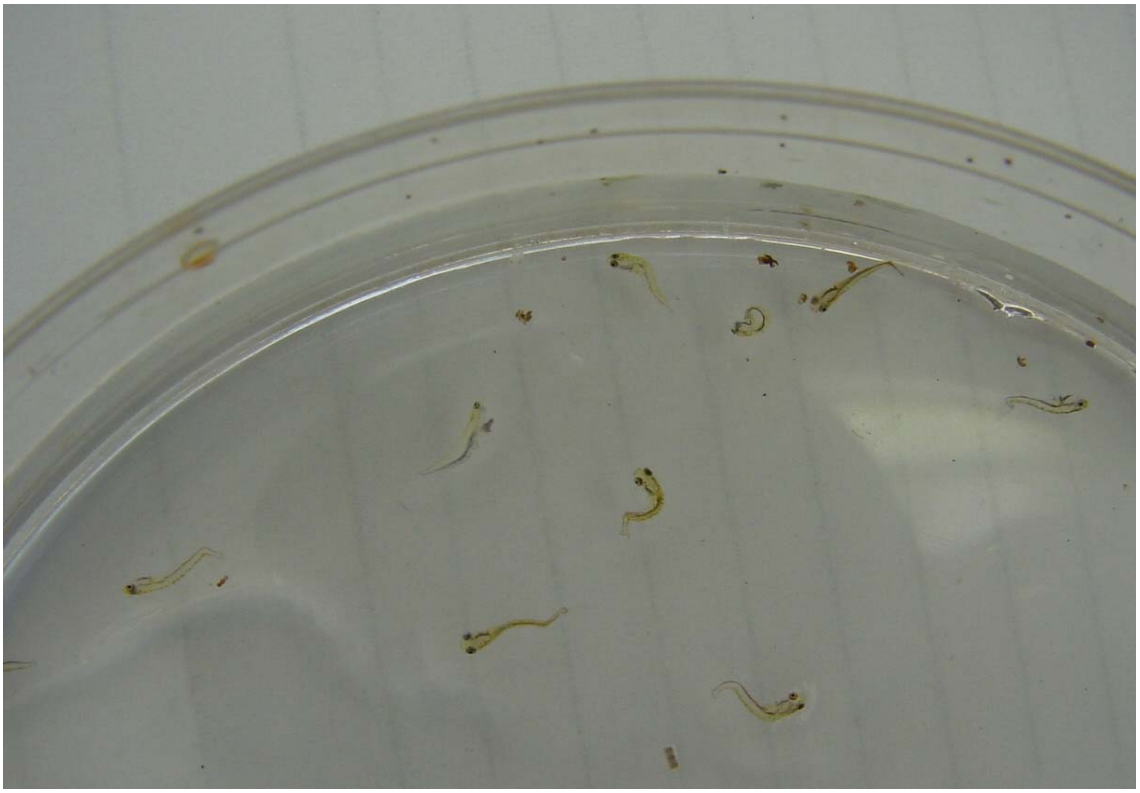


Figure.5 Deformed common carp, *Cyprinus caprio*.

Table 9. Hatchability, percent inheritance of the transgene, percent mortality of embryos, and percent hatching of F1 transgenic channel catfish *Ictalurus punctatus,* containing SF3 and SF4 gene constructs.

Family[1]	Treatment[2]	Number of individuals genotype	% hatch of the treatment	% embryo mortality [3]		% hatch		% inheritance[4]
				Non-transgenic	Transgenic	Non-transgenic	Transgenic	
SF3B2M1	no-dox	379	4.2	94.2(260)	100(103)	5.8(16)	0.0(0)	25.0
	Dox	381	78.2	16.9.0(50)	37.9(33)	83.1(244)	62.1(54)	
	no-dox	304	0.0	100.0(67)	100(327)	0.0(0)	0.0(0)	56.1
SF3B2M3	Dox	261	13.0	94.1(208)	55(22)	5.9(13)	45(18)	
	no-dox	377	1.6	97.4(226)	96.6(140)	2.6(6)	3.4(5)	27.2
SF3B2M5	Dox	327	14.6	85.5(248)	68(34)	14.5(42)	32(16)	
	no-dox	343	16.8	63.9(76)	90.1(201)	36.1(43)	10.3(23)	40.6
SF3B4NM1	Dox	327	17.5	88.9(248)	43.8(21)	11.1(31)	56.3(27)	
	no-dox	286	2.9	63.2(24)	98.4(244)	36.8(14)	1.6(4)	64.9
SF3B5M4	Dox	138	50.4	43.2(48)	59.3(16)	56.8(63)	40.7(11)	
	no-dox	278	14.6	41.3(19)	92.7(215)	58.7(27)	7.3(17)	59.6
SF3B5NM2	Dox	163	60.3	39.8(53)	28.1(9)	60.2(80)	71.9(23)	
	no-dox	408	0.0	100.0(18)	100.0(390)	0.0(0)	0.0(0)	61.9
SF4B1M1	Dox	289	68.3	26.2(65)	61.0(25)	73.8(183)	39.0(16)	
	no-dox	271	0.0	100.0(31)	100.0(240)	0.0(0)	0.0(0)	47.2
SF4B1M2	Dox	315	14.9	72.0(198)	21.2(7)	28.0(77)	78.8(33)	

*1 Family nomenclature; SF3B3M1= putative SF3 transgenicfemale #3 crossed with putative SF3 transgenic male #1, etc.
*2 No-dox = embryos were incubated without doxycycline, dox = embryos were incubated in 100 ppm doxycycline solution.
*3 % mortality and % after hatch; number in parentheses represents total number in each category
*4 % inheritance; = total positive individuals in each family divided by total number of individuals analyzed X 100.

Mean percentage mortality of transgenic embryos in control SF3 group was 100 % in SF3B2M1 (Fig. 6) and SF3B2M3 (Fig. 7) families. The remaining control families in the SF3 group, had mean mortality of transgenic embryos from 90.1 % to 98.4 %. Dox treated SF3 transgenic embryos had mean mortality percentage of 28.1 % (SF3B5NM2, Fig. 8) to 68.0% (SF3B2M5, Fig. 9). Percent hatching of transgenic embryos in the dox treated group was higher than transgenic individuals in the control group. The mean percent hatching of transgenic embryos ranged from 32 to 71.9 % in the dox treated group and 0 to 10.3 % in the no-dox group (Table 9, Figs. 6-11). Dox treatment significantly increased the hatch, and rescued the transgenic embryos ($P< 0.01$, χ^2).

In SF4 group, the mean mortality percentage of transgenic embryos was 100% in both families of the control group. In contrast to mean mortality of the control group, mean percent hatching of positive transgenic embryos in the dox treated group was 39.0 and 78.8 % in SF4B1M1 (Fig.12) and SF4B1M2 (Fig.13) families, respectively, which was higher than the control group. Dox treatment significantly increased the hatch and rescued the transgenic embryos ($P< 0.01$, χ^2).

Percent cumulative mortality of embryos from SF3 and SF4 families are shown in figures 6-13. Cumulative mortality of control transgenic embryos, sharply increased during day 1 to day 2 in every family of SF3 construct, while cumulative mortality of control transgenic embryos sharply increased during day 2 to day 3 for SF4 families.

Percent inheritance in SF3 families ranged from 25 % in SF3B2M1 to 64.9 % in SF3B5M4. In SF4 families, percent inheritance was 47.2 % and 61.9% in SF4B1M2 and SF4B1M1, respectively.

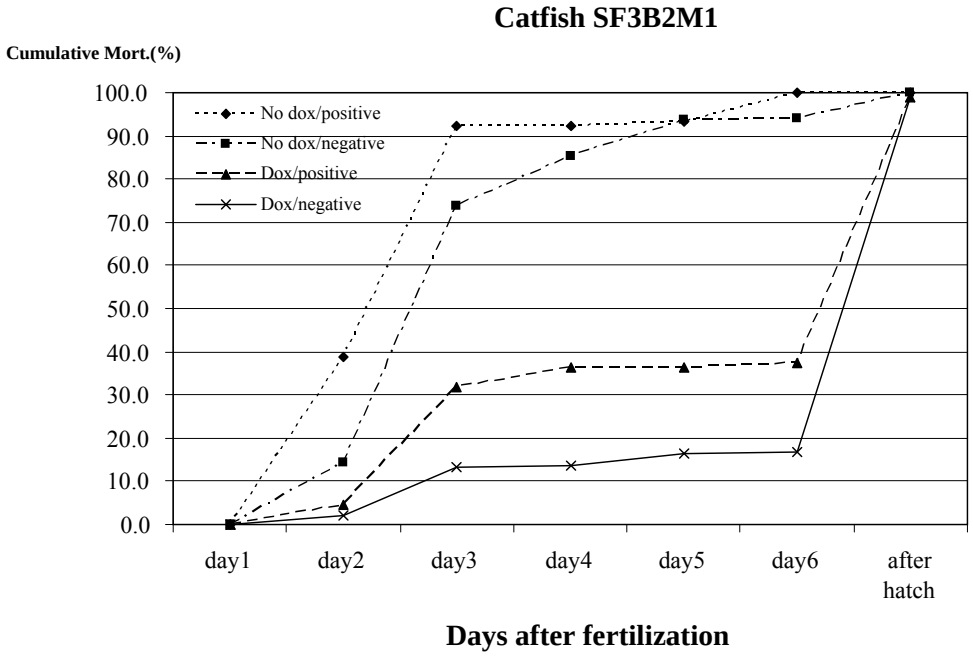


Figure 6. Cumulative mortality of channel catfish, *Ictalurus punctatus*, embryos from family SF3B2M1.

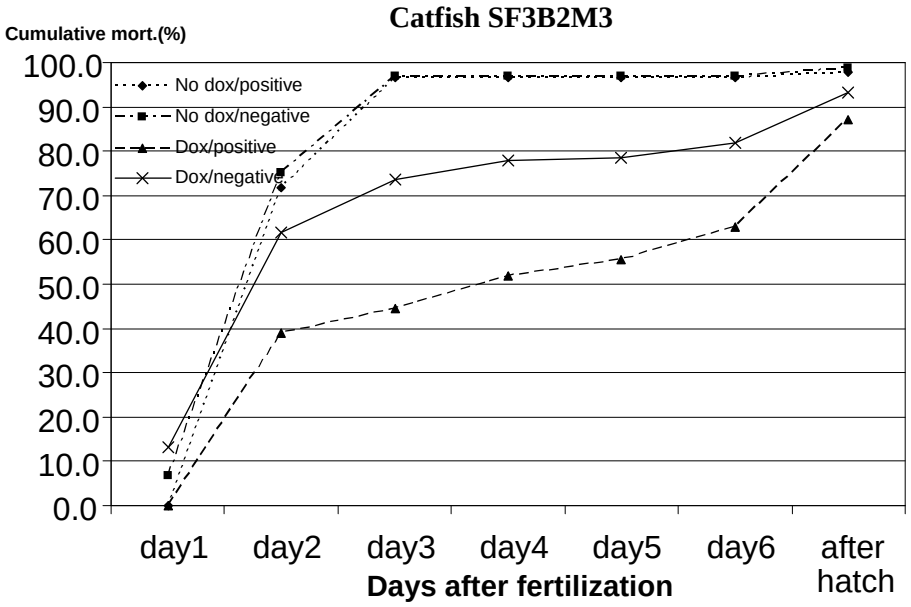


Figure 7. Cumulative mortality of channel catfish, *Ictalurus punctatus,* embryos from family SF3B2M3.

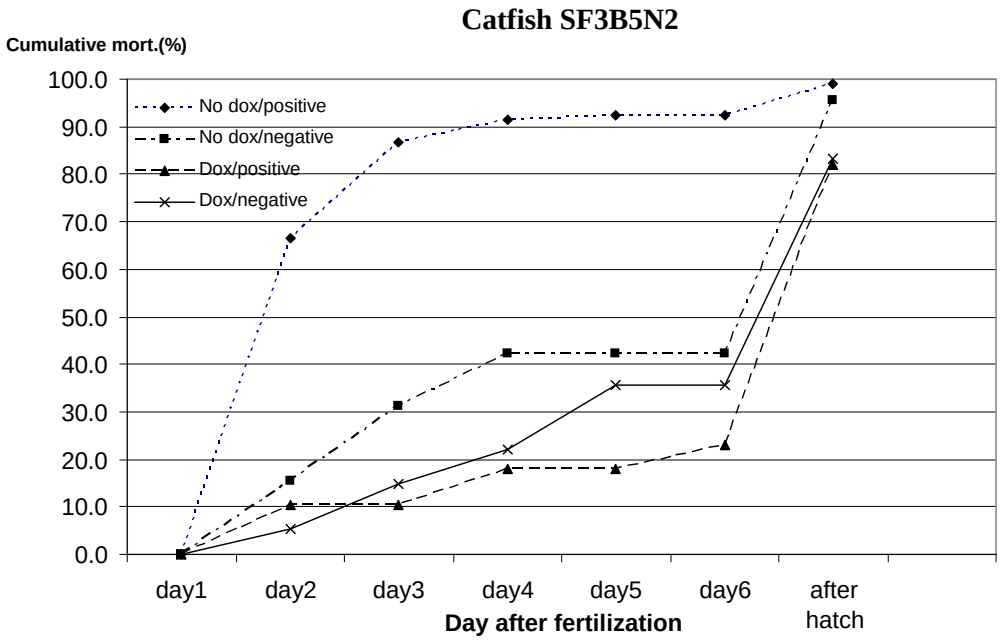


Figure 8. Cumulative mortality of channel catfish, *Ictalurus punctatus*, embryos from family SF3B5NM2.

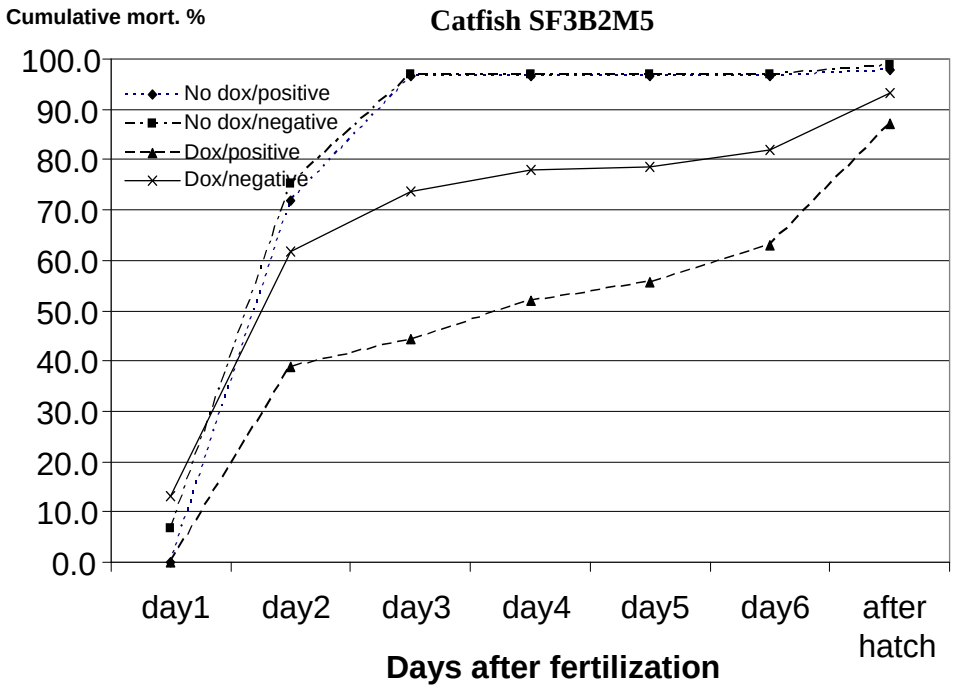


Figure 9. Cumulative mortality of channel catfish, *Ictalurus punctatus*, embryos from family SF3B2M5

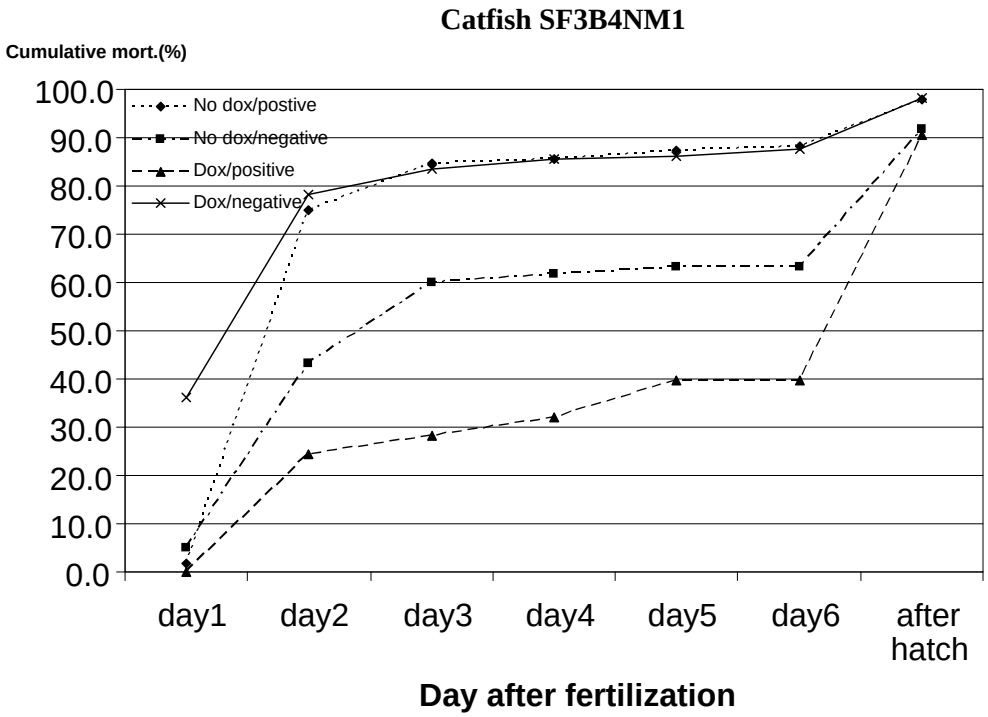


Figure 10.Cumulative mortality of channel catfish, *Ictalurus punctatus,* embryos from family SF3B4NM1.

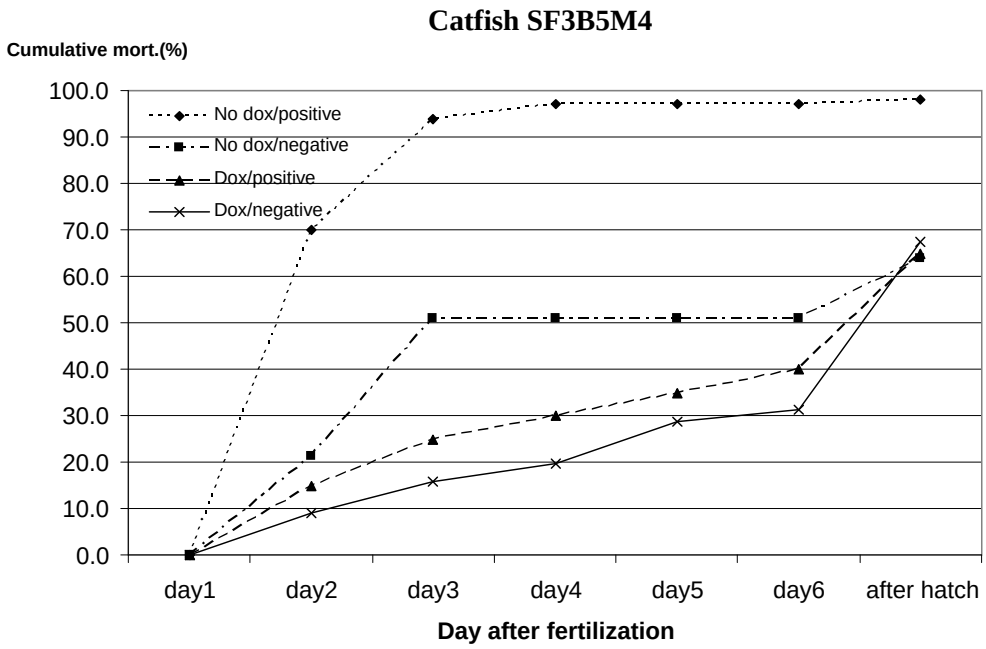


Figure 11. Cumulative mortality of channel catfish, *Ictalurus punctatus*, embryos from family SF3B5M4.

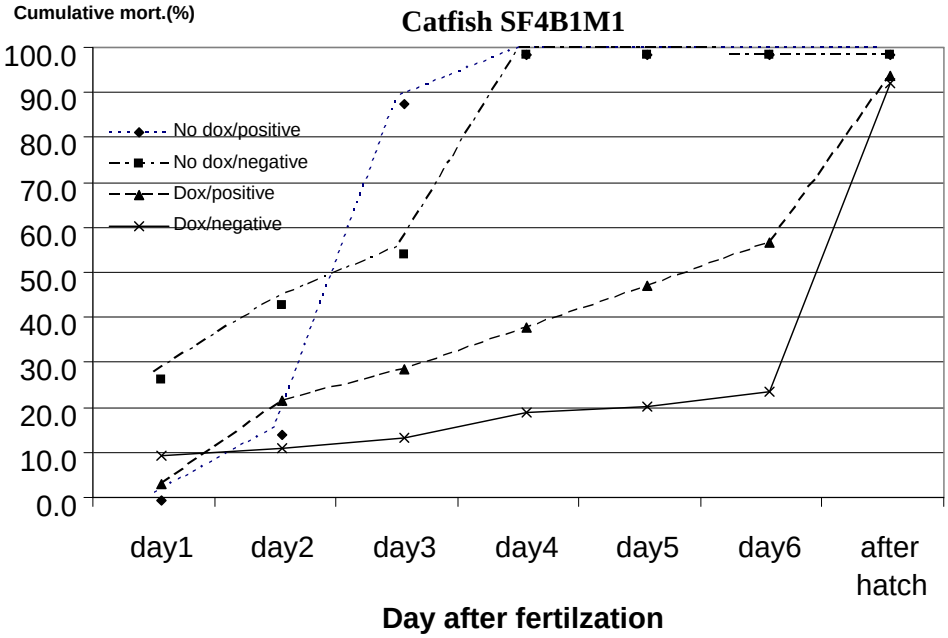


Figure 12. Cumulative mortality of channel catfish, *Ictalurus punctatus*, embryos from family SF4B1M1.

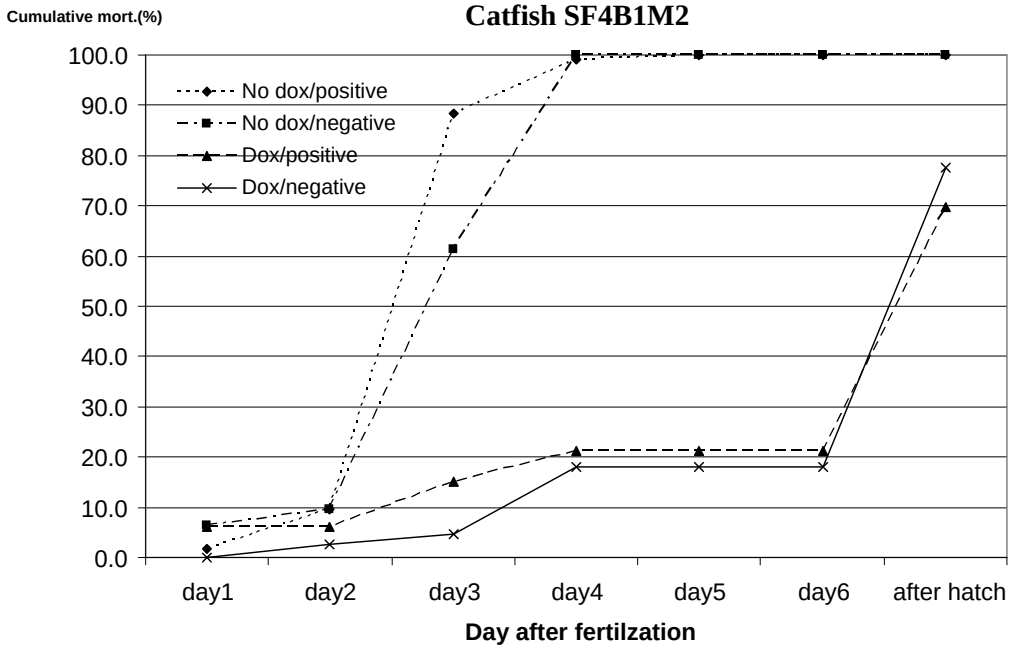


Figure 13. Cumulative mortality of channel catfish, *Ictalurus punctatus*, embryos from family SF4B1M2.

Data on deformity percentage are shown in Table 10. Observed mean deformity of dox treatment was lower than no-dox treatment. The highest % deformity was in family SF3B2M5 (33.3%), while the lowest was in family SF3B5M4 (1.1%). There was no significant difference between the overall mean of dox and no-dox treatment (P >0.05).

Second trial

Data for second trial are summarized in Tables 11, and 12. Five of 9 SF3 families and 2 of 8 SF4 families which had no-dox, 0-30, 0-60 and 30-60 treatments showed expected results. For no-dox, 0-15, 15-30, and 30-45 treatments, only 1 of 3 families from both SF3 and SF4 families showed expected results. For no-dox, 15-20, 20-25, 25-30, 30-35, and 35-40 treatment, 3 of 5 SF3 families and 1 of 3 SF4 families showed expected results. In the SF3 no-dox treatment, 9 families total, percent hatchability ranged from 3.8 to 61.8 %. The highest hatch was in SF3B7M4 (61.8%) and the lowest was in SF3B24M24 (3.8%). In SF4 no- dox treatment (data shown in table 12) percent hatchability was highest in SF4B1M1 (61.8 %) and lowest in SF4B20M20 (3.6%). Percent hatchability of dox treatment varied among family and treatments. Percent inheritance ranged from 17.7 to 63.3 % in SF3 families, and from 24-55.5 % in SF4 families in trial 2.

Percent mortality and percent hatching of transgenic embryos from this trial are shown in tables 11 and 12. In SF3 families (total 5 families) which had no-dox, 0-30, 0-60 and 30-60 treatments, no-dox treatment of family SF3B7M4 and SF3B11M8 had higher % embryo mortality than 0-30, 0-60 and 30-60 treatments. Percent hatching of transgenic embryos in these families varied among families and treatments. Dox

Table 10. Percent deformity of channel catfish, *Ictalurus punctatus*, F1 fry containing SF3 and SF4 transgenes.

	% Deformity	
Family[1]	no-dox[2]	dox
SF3B2M5	33.3	2.4
SF3B4NM1	9.7	6.5
SF3B5M4	7.7	1.1
SF3B5NM2	3.1	2.4

[1] Family nomenclature; SF3B3M1= putative SF3 transgenic female #3 crossed with putative SF3 transgenic male #1, etc.
[2] no-dox = embryos were incubated without doxycycline, dox = embryos were incubated in 100 ppm doxycycline solution

Table 11. Hatchability, percent inheritance of the transgene, percent mortality of embryos, and percent hatching of F1 transgenic channel catfish *Ictalurus punctatus*, containing SF3 gene constructs.

Family[1]	Treatment[2]	Initial egg number	% hatch	Total individuals genotyped	% embryo mortality [3]		% hatch		% inheritance[4]
					Non-transgenic	Transgenic	Non-transgenic	Transgenic	
SF3 B4M1	no-dox	42	30.9	41	8.3(1)	58.1 (18)	91.7(11)	41.9(11)	63.6
	0-30	47	19.2	42	71.4(10)	67.9 (19)	28.6(4)	32.1 (9)	
	0-60	29	41.4	19	80(8)	44.4 (4)	20(2)	55.6 (5)	
	30-60	53	64.2	36	33.3(5)	38.1(8)	66.7(10)	61.9(13)	
SF3 B5M2	no-dox	42	54.8	36	65(13)	68.8(11)	35(7)	31.2 (5)	46.9
	0-30	38	65.8	31	52.6(10)	25(3)	47.4(9)	75(9)	
	0-60	39	61.5	27	38.9(7)	69.2 (9)	61.1(11)	30.8(4)	
	30-60	37	75.7	30	27.3(3)	31.6(6)	72.7(8)	68.4 (13)	
SF3 B6M3	no-dox	32	31.2	33	56.5(13)	80(8)	43.5(10)	20(2)	34.8
	0-30	35	57.1	19	90.9(10)	62.5 (5)	9.1(1)	37.5(3)	
	0-60	40	55.0	27	35.7(5)	76.9(10)	64.3(9)	23.1(3)	
	30-60	60	51.7	44	42.9(12)	81.2(13)	57.1(16)	18.8(3)	
SF3 B7M4	no-dox	34	61.8	26	38.9(7)	62.5(5)	61.1(11)	37.5(3)	52.8
	0-30	49	86.1	34	26.7(4)	36.8(7)	73.3(11)	63.2(12)	
	0-60	49	74.1	39	10(2)	26.3(5)	90(18)	73.7(14)	
	30-60	40	55.6	26	16.7(1)	40(8)	83.3(5)	60(12)	
SF3 B11M8	no-dox	36	58.3	19	23.1(3)	16.7(1)	76.9(10)	83.3(5)	40.7
	0-30	44	91.9	24	6.3(1)	12.5(1)	93.7(15)	87.5(7)	
	0-60	48	80.9	22	13.3(2)	0(0)	83.7(13)	100(6)	
	30-60	40	80.7	21	20(2)	9.1(1)	80(8)	90.9(10)	

Table 11 . (continued).

Family[1]	Treatment[2]	Initial egg number	% hatch	Total individuals genotyped	% embryo mortality [3]		% hatch		% inheritance[4]
					Non-transgenic	Transgenic	Non-transgenic	Transgenic	
SF3 B13M9	no-dox	32	13.8	16	84.6(11)	100(6)	0(0)	0(0)	24.7
	0-15	30	15.0	18	88.2(15)	100(1)	11.8(2)	0 (0)	
	15-30	37	24.0	23	88.9(16)	60(3)	11. 1(2)	40 (2)	
	30-45	33	27.0	19	66.7(8)	57.1 (4)	33.3(4)	42.9(3)	
SF3 B20M20	no-dox	29	13.8	11	88.9(8)	100(2)	11.1(1)	0(0)	34.2
	15-20	25	12.0	11	42.9(3)	25 (1)	57.1(4)	75(3)	
	20-25	40	30.0	14	75(6)	50(3)	25(2)	50(3)	
	25-30	33	27.8	14	75(6)	33.3(2)	25 (2)	66.7(4)	
	30-35	38	60.5	13	44.4(4)	0(0)	55.6(5)	100 (3)	
	35-40	29	37.9	11	57.1(4)	75 (3)	42.9(3)	25(1)	
SF3 B21M21	no-dox	39	10.3	8	66.7(4)	100(2)	33.3(2)	0(0)	17.7
	15-20	36	0.0	10	100(6)	100 (4)	0(0)	0(0)	
	20-25	40	42.5	17	33.3(4)	40 (2)	66.7(8)	60(3)	
	25-30	47	25.5	18	55.6(10)	0(0)	44.4(8)	0(0)	
	30-35	43	58.1	20	27.8(5)	0(0)	72.2(13)	100 (2)	
	35-40	34	2.9	9	100(5)	75(3)	0(0)	25 (1)	
SF3 B24M24	no-dox	26	3.8	11	100 (11)	0(0)	0(0)	0(0)	22.4
	15-20	29	41.4	20	46.7(7)	0(0)	53.3(8)	100(5)	
	20-25	31	32.3	17	64.3(9)	33.3(1)	35.7(5)	66.7(2)	
	25-30	28	32.1	17	46.8(7)	0(0)	53.3(8)	100(2)	
	30-35	45	42.2	28	47.1(8)	18.2(2)	52.9(9)	81.8(9)	
	35-40	44	38.6	23	27.8(5)	40(2)	72.2(13)	60(3)	

[1]Family nomenclature; SF3B4M1= putative SF3 transgenicfemale #4 crossed with putative SF3 transgenic male #1, etc.
[2] no-dox = embryos were incubated without doxycycline, 0-30 = embryos were incubated in 100 ppm doxycycline solution at zero to 30 hours after fertilized, 0-60 = embryos were incubated in 100 ppm doxycycline solution at zero to 60 hours after fertilized, 30-60 = embryos were incubated in 100 ppm doxycycline solution at 30 to 60 hours after fertilized, 0-15 = embryos were incubated in 100 ppm doxycycline solution at zero to 15 hours after fertilized, 15-30 = embryos were incubated in 100 ppm doxycycline solution at 15 to 30 hours after fertilized, 30-45= embryos were incubated in 100 ppm doxycycline solution at 30 to 45 hours after fertilized, 15-20= embryos were incubated in 100 ppm doxycycline solution at 15 to 20 hours after fertilized, 20-25 = embryos were incubated in 100 ppm doxycycline solution at zero to 30 hours after fertilized, 25-30 = embryos were incubated in 100 ppm doxycycline solution at 25 to 30 hours after fertilized, 30-35= embryos were incubated in 100 ppm doxycycline solution at 30 to 35 hours after fertilized, 35-40 = embryos were incubated in 100 ppm doxycycline solution at 35 to 40 hours after fertilized.
[3] % mortality and % hatch; number in parentheses represents total number in each category
[4] % inheritance; = total positive individuals in each family divided by total number of individuals analyzed X 100.

Table 12. Hatchability, percent inheritance of the transgene, percent mortality of embryos, and percent hatching of F1 transgenic channel catfish *Ictalurus punctatus,* containing SF4 gene constructs.

Family[1]	Treatment[2]	Initial egg number	% hatch	Total individuals genotyped	% embryo mortality [3]		% hatch		% inheritance[4]
					Non-transgenic	Transgenic	Non-transgenic	Transgenic	
	no-dox	34	61.8	33	30(4)	45(9)	70(9)	55 (11)	54.4
	0-30	32	86.1	30	33.3(2)	33.3(7)	66.7(7)	65 (14)	
SF4	0-60	27	74.1	22	63.6(7)	9.1(1)	36.4(4)	90.9(10)	
B1M1	30-60	36	55.6	31	47.6(10)	60(6)	52.4(11)	40(4)	
	no-dox	42	30.9	18	0(0)	28.6(4)	100(4)	71.4(10)	55.5
	0-30	33	21.2	13	42.9(3)	16.7(1)	57.1(4)	83.3(5)	
SF4	0-60	32	62.5	16	33.3(4)	25(1)	66.7(8)	75(3)	
B7M7	30-60	44	81.8	15	20(1)	0(0)	80(4)	100(11)	
	no-dox	43	4.0	35	89.7(26)	83.3(5)	10.3(3)	16.7 (1)	24
	0-15	50	7.0	18	100(11)	100 (7)	0(0)	0(0)	
SF4	15-30	48	25.0	33	100(23)	80 (8)	0(0	20(2))	
B9M8	30-45	39	20.0	35	82.8(24)	66.7(4)	17.2 (5)	33.3(2)	
	no-dox	28	3.6	7	100(5)	50 (1)	0(0)	50 (1)	27.6
	15-20	32	43.8	7	66.7(6)	0(0)	33.3(0)	1001)	
	20-25	32	40.6	8	50 (3)	0(0)	50(3)	100 (2)	
	25-30	26	46.2	8	25(1)	50(2)	75(3)	50(2)	
SF4	30-35	39	43.6	11	66.7(4)	60 (3)	33.3(2)	40 (2)	
B20M20	35-40	32	68.8	14	33.3(4)	50(1)	66.7(8)	50 (1)	

[1] Family nomenclature; SF3B4M1= putative SF3 transgenicfemale #4 crossed with putative SF3 transgenic male #1, etc.
[2] no-dox = embryos were incubated without doxycycline, 0-30 = embryos were incubated in 100 ppm doxycycline solution at zero to 30 hours after fertilized, 0-60 = embryos were incubated in 100 ppm doxycycline solution at zero to 60 hours after fertilized, 30-60 = embryos were incubated in 100 ppm doxycycline solution at 30 to 60 hours after fertilized, 0-15 = embryos were incubated in 100 ppm doxycycline solution at zero to 15 hours after fertilized, 15-30 = embryos were incubated in 100 ppm doxycycline solution at 15 to 30 hours after fertilized, 30-45= embryos were incubated in 100 ppm doxycycline solution at 30 to 45 hours after fertilized, 15-20= embryos were incubated in 100 ppm doxycycline solution at 15 to 20 hours after fertilized, 20-25 = embryos were incubated in 100 ppm doxycycline solution at zero to 30 hours after fertilized, 25-30 = embryos were incubated in 100 ppm doxycycline solution at 25 to 30 hours after fertilized, 30-35= embryos were incubated in 100 ppm doxycycline solution at 30 to 35 hours after fertilized, 35-40 = embryos were incubated in 100 ppm doxycycline solution at 35 to 40 hours after fertilized.
[3] % mortality and % after hatch; number in parentheses represents total number in each category
[4] % inheritance; = total positive individuals in each family divided by total number of individuals analyzed X 100.

treatment (0-30, 0-60 and 30-60) significantly increased the hatch and rescued the transgenic embryos (highest percent in 30-60 treatment) ($P< 0.05$, χ^2).

For SF4 families, there were 2 families that had no-dox, 0-30, 0-60, and 30-60 treatments (Table 11). No-dox treatment family SF4B7M7 had higher % embryo mortality than dox treatments (0-30, 0-60 and 30-60). Dox treatment of SF4B1M1 and SF4B7M7 significantly increased the hatch and rescued the transgenic embryos (highest percent in 0-30 treatment) ($P< 0.05$, χ^2).

Families SF3B13M9 and SF4B9M8 had no-dox, 0-15, 15-30 and 30-45 in both families treatments (Tables 11 and 12). SF3B13M9 had 100 % embryo mortality in both no-dox and 0-15 treatments, while SF4B9M8 had 100 % embryo mortality in the 0-15 treatment only. There was no effect of treatment on hatch rate ($P> 0.05$, χ^2).in these two families.

There were three SF3 families that had no-dox, 15-20, 20-25, 25-30, 30-35 and 35-40 treatments. Among these, SF3B20M20 and SF3B21M21 had 100 % mortality in no-dox treatment, while SF3B24M24 had a high percentage of hatched embryos (60-100%) in dox treatments when compared to the other 2 families. Dox treatment significantly increased the hatch and rescued the transgenic embryos (highest percent in 30-35 treatment) ($P<0.01$, χ^2).

SF4B20M20 was the only SF4 family that had no-dox, 15-20, 20-25, 25-30, 30-35, and 35-40 treatments. Two treatments, 15-20 and 20-25, had 100 % hatched embryos, but with low numbers individuals (Table 13). There was no effect of treatment on hatch rate ($P> 0.05$, χ^2).

Growth data was available for 7 families. In only one family was a significant difference in body weight found ($P<0.05$). Final body weight of non-transgenic full-siblings was higher than their transgenic full siblings, but $N = 6$ was low.

Table 13. Mean body weight of non-transgenic and transgenic full-sibling channel catfish, *Ictalurus punctatus*, containing SF3 gene construct at 10 months of ages when grown in 100 L aquaria in a recirculating system .

Family	Number of fish	Genotype[1]	Body weight (g) (mean ± sd)[2]
SF3B4M1	7	N	111.4 ± 36.4[a]
	4	T	114.2 ± 32.2[a]
SF3B5M2	7	N	79.1 ± 31.1[a]
	4	T	80.3 ± 18.1[a]
SF3B6M3	5	N	143.4 ± 63.7[a]
	2	T	85.2 ± 18.2[a]
SF3B7M4	18	N	40.4 ± 15.2[a]
	23	T	47.5 ± 15.3[a]
SF3B11M8	29	N	32.3 ± 10.4[a]
	23	T	28.8 ± 8.2[a]
SF3B13M9	6	N	136.0± 36.2[a]
	6	T	90.6 ± 11.4[b]
SF3B24M24	30	N	39.9 + 13.8[a]
	17	T	47.4 + 13.5[a]

[1] N = non-transgenic, T= transgenic.

[2] Mean body weights within families that have different superscript are significantly different (P <0.05, t-test),

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DISCUSSION

Common carp

Based on hatching rates, none of the F1 SF3 transgenic families of common carp exhibited clear signs of embryonic disruption. In 2 of 4 F1 SF4 common carp families, treatment with dox increased the number of transgenic individuals that hatched and presumably repressed the expression of the knockouts. However, SF4, the cDNA of BMP2 was not totally effective in killing all transgenic individuals so the modified tet-off system was not totally effective in interrupting embryonic development.

The percent inheritance of the transgene in the F1 generation ranged form 12.3 to 87.7 %, which varied among families and constructs. The variation of inheritance of the transgene especially on F1 generation was an expected result for transgenic fish due to mosaicism and possible multiple integration events, which have been reported in several species (Stuart *et al*. 1988, Zhang *et al*. 1990, Gross *et al*. 1992, Uzbekova *et al*. 2000).

Variable results were observed for 9 F2 SF3 common carp families. One family had hatch rates that were consistent with dox treatments rescuing at least a portion of transgenic individuals. None of no-dox treatments for any of the families had 100 % embryo mortality. The data from both F1 and F2 generations would indicate that this construct was not 100% effective in interrupting embryonic development.

Results for F2 transgenic common carp families were more promising for SF4. Four of 12 families exhibited hatching rates consistent with dox treatments rescuing transgenic individuals from mortality. Two of these families had high mortality in the no

dox treatment providing evidence that the modified tet-off system may be operating with high effectiveness, however, not with 100% effectiveness.

Aspects of the PCR analysis may explain some of the variation from one family to the next. The amplification pattern indicated several of the families may have integrated a partial gene construct. The two families exhibiting the best and expected result likely had the entire trangene integrated based on the PCR analysis. Obviously, if key parts of the tet-off system were lost, the knockout function and/or the repressibility of the system would be lost.

Transgenic individuals from two F1 common carp SF3 families had decreased growth rates, and these same families experienced high mortality of higher copy number individuals. This pleiotropic effect has multiple possible explanations. Disruption of BMP2 could have negative direct effects on other traits or could attack indirectly on the function of other key genes and pathways resulting in adverse effects.

BMP2 does have functions in adult fish. BMP2 probably has a role in fin regeneration in zebrafish (Schebesta *et al*. 2006), and is expressed in calcified tissues bone, caudal fin and scales and in liver in adult *Sparus auratus* (Rafael *et al*. 2006). Disruption of BMP2 beyond embryonic development could also be damaging. The TRE promoter used in the first generation tet-off system utilized in our experiments can be leaky (Agha-Mohammadi *et al*. 2004). If tet-off system is not completely shut down, the continued expression of the knockdown constructs could disrupt BMP2 expression leading to slower growth, juvenile mortality and other adverse effects. This could also explain the negative results for SF3. If all of the high copy number fish were dieing even after dox treatments, all families that could have responded properly to the system were

eliminated. Those that remained alive would not be expressing strongly enough to give the expected results.

Another potential problem with common carp and the tet-off system may be their responsiveness to dox. The hatching results sometimes indicated that 100 ppm dox may be either too high and might be killing embryos or too low, and therefore, not repressing SF3 or SF4. However, 150 ppm was an overdose resulting in little or no hatch. The 50ppm dose appeared to be variable in its efficacy. Common carp families appear to be variable in their response to dox treatments in the tet-off system making proper dosing difficult.

Seven of 9 F2 SF3 families and 4 of 12 F2 SF4 families had higher % deformity in dox treatment than no-dox treatment. This could indicate that dox treatment can cause the deformity or that the 100 ppm dox treatment could not totally repress the function of BMP2 gene, and cause a partial disruption of doso-ventral development resulting in deformity. The reason the no dox treatment would have a lower percent deformity would be because their was no repression at all a greater percent of the embryos dies leaving fewer partially repressed individuals alive, but deformed. Non-transgenic families had no deformities, thus the SF constructs were causing at least a percentage of the deformities. PCR data on these embryos would be required for a full explanation.

Channel catfish

Five F1 SF3 channel catfish families were evaluated using the constant dox treatment during incubation (Table 9). The repressible sterility system worked with high effectiveness in 3 of 5 families as all but 1-10% of the transgenic individuals died in the absence of dox during incubation. In the case of two families, all untreated transgenic

individuals died, and high percentages of transgenic individuals were rescued with dox, apparently, indicating the system was working with near 100% effectiveness. However, in these two cases, large numbers of untreated controls also died, perhaps because of water quality issues related to the massive mortality of the transgenic embryos, even though key water quality measures were taken regularly, water was changed once-twice daily and no water quality problems were detected. Therefore, there is the possibility that a portion of the transgenic channel catfish embryos also died from environmental causes rather than solely from the expression of SF3.

An alternative, explanation is that high mortality of the no-dox treatment was due to bacterial contamination, and transgenic individuals were dieing from pathogens rather than gene knock down. However, it is believed that bacteria is not a major problem for catfish egg mortality. This premise needs further testing based on the results observed in this experiment.

When the data from all 5 families are examined, it is apparent that SF3 transgenic individuals die at high rates when incubated without dox, and high percentages of these individuals are rescued when incubated in dox. In particular, treated and untreated, transgenic and non-transgenic individuals of channel catfish family SF3B5M4 responded in the predicted manner almost perfectly, providing strong evidence for proof of principle. Family SF3B5NM2 (Figure7.) also provided strong evidence that repressible sterility was effective as untreated transgenic embryos died rapidly, and the 3 other treatments had high survival until the last day of incubation before hatch and suddenly died of unexplained causes.

Deformity data also supports this conclusion. The frequency of deformity is surviving SF3 progeny was consistently higher in the no dox treatment compared to the dox treatment as expected.

Two SF4 transgenic families also resulted in 100% mortality of transgenic individuals when incubated without dox, and high percentages of transgenic progeny were rescued with dox. Again, exact determination of 100% effectiveness was not possible because of high mortality of non-transgenic siblings in the no dox treatment. However, the potential of the SF4, overexpression of BMP cDNA, to accomplish repressible transgenic sterilization with the tet-off approach is apparent. Families, SF3B2M1, SF3B2M3, SF4B1M1 and SF4B1M2 had 0 % transgenic individuals hatching in the no-dox treatment, indicating potential 100 % effectiveness of SF3 and SF4 gene constructs on interrupting embryonic development. Dox treatment can rescue catfish embryos in both SF3 and SF4 constructs. The tet-off system can working properly, blocking the expression of zBmp2 gene, and zBmp2 gene can interrupt catfish embryonic development leading to the high mortality of embryo in the no-dox treatment.

The SF3 and SF4 repressible systems could become 100% effective by selecting for individuals whose progeny and genotype responded completely in the expected manner to the gene construct and the dox. Selection of the correct families and producing the next generation to increase copy number (reaching homozygosity) could make this system completely effective. An alternative to achieve 100% effectiveness might be to alter the transgene to increase expression or to mate individuals that would result in increased copy number and perhaps increased copy number. It may be necessary to stack more than one repressible transgenic system to guarantee 100% effectiveness.

Mortality of trangenic embryos in each family show a sharp increase (no-dox treatment) during the 2nd to 3rd day of embryonic development. Apparently, sometime just before 2nd and 3rd day post fertilization is a critical time period for BMP2 expression and dorsal-ventral development for channel catfish embryos. This is in close agreement with the data of Templeton (2005) who observed mortality in non-transgenic channel catfish embryo 18-61 hour post fertilization after electroporation with SF3 and SF4 gene constructs.

The repressible transgenic sterilization approach utilizing the modified tet-off approach to disrupt BMP2 resulting in embryonic death appears to have potential for 100% effectiveness in channel catfish. A potential drawback to this system is the necessity of doxycycline. The small scale studies reported here were conducted statically. Commercial scale catfish hatcheries rely on large volumes of flow-through water as well as hatcheries for most cultured fish. If this procedure were scaled up, the cost of the dox would be quite high. Additionally, this procedure would not be environmentally friendly as it would release significant quantities of antibiotic into the environment. These problems could be solved if less expensive forms of tetracycline were used to reduce cost assuming they were able to turn the tet system off, and alternative forms of tetracycline should be evaluated. If the treatment duration of dox were sufficiently reduced, cost would be reduced, and treatment of waste antibiotic would become more manageable.

Additional trials attempted to find optimum incubation time to rescue the embryos while reducing the length of use and the amount of doxycycline utilized. Reducing the treatment duration of the dox from 144hr to 30-60 hr was promising for the repressible system in channel catfish. In 4 of 5 families, the SF3 transgene resulted in

high mortality rates, and the transgene was ineffective in the 5th family. However, the transgene was not as effective in embryonic disruption as in the first 5 families evaluated resulting in 20-38% hatch compared to the 0-10% hatch in the first families. However, treating the embryos with dox from 0-30, 30-60 or 0-60hr after fertilization significantly repressed the transgene expression and doubled hatch. The critical period for dox treatment is 30hr or less during the appropriate developmental time.

Data on SF4 was more limited, 2 families, but supported the results found for SF4. It was unclear from the SF4 data if the treatment duration could be reduced to 30 or 60hr, but considering both sets of data a treatment of 60hr compared to 30 hr is probably unnecessary.

One family of SF3 and one family of SF4 were evaluated for 15hr treatments to further refine definition of the critical period for expression of BMP2. Treatment from 0-15hr after fertilization was ineffective for rescuing development of transgenic channel catfish embryos. The critical period for BMP2 gene expression in channel catfish appears to be sometime between 15 and 60hr after fertilization, but does not need to function during this entire period. Based on this set of experiments, although limited to observations on only 2 families, treatment of dox 30-45 hr after fertilization appeared to give the most consistent repression of the SF system.

Five hour treatment intervals were then examined to try to further reduce the window of treatment needed to counteract the effects of SF3 and SF4 gene constructs on embryonic development. SF3 and SF4 were highly effective in causing embryo mortality in the families evaluated in this set of experiments. The 5 hr intervals examined were between 15 and 40 hr after fertilization. Three SF3 families were evaluated and minimal

data was available from one SF4 family. All 5 treatments repressed SF3 and SF4 expression to some degree. Treaments 15-20 hr and 35-40 hr after fertilization gave the most variable results, and must be on the periphery of the critical window for BMP2 expression. Treatments 20-25,25-30 and 30-35 hr after fertilization yielded the highest hatching rates of transgenic embryos with 30-35hr after fertilization having the highest rescue of embryos. Further experimentation is needed to determine if this duration of treatment can be further reduced for SF3 and SF4 transgenic channel catfish while enabling repression of the transgene an normal development of the embryos. If the data is confirmed, BMP2 expression is most critical at 30-35hr after fertilization for proper embryonic development, but expression for adequate periods near that timing can allow normal development and survival of some if not all developing embryos.

Conclusions

Our data and that of Thresher et al. (2001) suggests that the SF approach of embryonic disruption and transgenic sterilization has potential for 100% effectiveness. The F1 and in some cases the F2 generation was reached with promising results for some families. Selection of the correct families and producing the next generation to increase copy number (reaching homozygosity) could make this system completely effective. Doxycycline is the drawback to this system. The initial small scale studies were conducted statically. Commercial scale catfish hatcheries rely on large volumes of flow-through water as well as hatcheries for most cultured fish. If this procedure were scaled up, the cost of the dox would be quite high. Additionally, this procedure would not be environmentally friendly as it would release significant quantities of antibiotic into the environment or would require major steps to prevent environmental contamination.

Strategies need to be developed that would target genes using tet-off or alternative repressible systems that would further decrease the use of doxycycline.

Knock out constructs designed to disrupt, BMP2, and thus embryonic development in zebrafish, were able to knock out embryonic development in both common carp and channel catfish. Apparently, this gene is sufficiently conserved to allow developmental studies and knock out of this gene across widely divergent families of fish. It was in some ways surprising that the current experiment was successful as the transgenes were designed specifically for zebrafish. Another surprising result was that the effectiveness of the zebrafish knockouts of BMP2 were slightly more effective in channel catfish than common carp which is the opposite of what is expected as phylogenetically zebrafish and common carp are more closely related to each other than to channel catfish (Wulliman, 1998). Alternatively, the knockout of BMP2 was too effective for common carp and coupled with an inadequate tet-off system, the most promising transgenic individuals or families were eliminated from the population.

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APPENDIX A

Holzfretter's Solution

3.5 g NaCl

0.05 g KCl

0.1g CaCl

0.2g NaHCO3

Dissolve ingredients in 1 L dd H_2O