Alternative Approaches for Repressible Transgenic Sterilization of Channel Catfish (*Ictalurus punctatus*)

by

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Abstract

Transgenic sterilization has the potential for accomplishing 100% reproductive confinement to avoid genetic communication of transgenic or domestic genotypes with wild populations. Two strategies of transgenic sterilization were developed and tested in channel catfish (*Ictalurus punctatus*).

For the first strategy, three sterilization constructs (Nanos-nanos, Nanos-dnd, Dazl-vasa) were introduced into channel catfish embryos to overexpress the Bax gene specifically in the germ line to ablate the germ cells, leading to infertility. Four-year-old Nanos-nanos, Nanos-dnd, or Dazl-vasa construct exposed males, but not females, had significantly lower ($p < 0.05$) gonadosomatic index (GSI) than the control, and lower ($p < 0.05$) percentages (54.2%, 44.4%, 56.7%, respectively) of individuals with a gonad development score ≤ 2 (maximum = 5) than the control (0.0%). Mosaicism of transgene integration was widely observed in the P1 fish. Unexpectedly, doxycycline treatment did not effectively suppress expression of the transgene, indicating efficient reversible transgenic sterilization will likely not be possible for this system. This study demonstrated that the overexpression of Bax gene could lead to the death of germ cells in the male fish with high efficacy, and could be useful to produce sterile fish when repressible control is not needed.

The second strategy aimed to disrupt the reproductive endocrine regulation of channel catfish by overexpression of glutamic acid decarboxylase (GAD) to increase γ-aminobutyric acid (GABA) level, thereby producing infertile fish. Repressed reproductive performance was

observed in all generations of GAD transgenic fish examined, as revealed by less mature fish in the F1 generation at the age of 5, fewer fish spawned naturally in the F1 (6 and 9-year-old) and F2 (3-year-old) generations, and lower serum GnRH levels in the 1-year-old F2 fish. Interestingly, male transgenic fish showed lower ($p < 0.05$) serum levels of GnRH and testosterone than control fish at the age of 4, indicating a different regulation mode of GABA on GnRH in adult males than females as transgenic and control females were not different. Overexpression of GAD could repress the reproductive performance of channel catfish, hormone therapy could sometimes restore fertility, but further research is needed to make this approach 100% effective.

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Abstract

Transgenic technology has shown remarkable potential for improving the production of aquaculture animals by enhancing their growth, body composition, disease and extreme environment tolerance. Sterilization is the ultimate approach to avoid the environmental risk that transgenic animals may pose, as other physical or chemical methods cannot guarantee 100% confinement, whereas, transgenic approaches have that potential on a large-scale. In this study, three transgenic sterilization constructs (Nanos-nanos, Nanos-dnd, Dazl-vasa) were developed to make sterile channel catfish, *Ictalurus punctatus,* which is one of the most important aquaculture fish species in the USA, through the overexpression of the pro-apoptosis gene Bax specifically in the germ cells. In general, all three constructs showed potential and similar efficacy for sterilizing male channel catfish but were not very efficient for females in the P1 generation, which might be due to higher tolerance of female germ cells against apoptosis promoted by Bax overexpression. The mean gonadosomatic index (GSI) of the 4-year-old fish was not different ($p = 0.11$) between females exposed to the constructs and control fish, while it was significantly lower ($p < 0.0001$) in construct exposed males in comparison to the control. There was no significant difference in the percentages of fish spawned at three years of age between the three construct treated groups except that the Dazl-vasa females had a lower ($p = 0.049$) percentage than the Nanos-nanos fish (10.5% and 37.9%, respectively). The 4-year-old Nano-nanos,

Nanos-dnd, Dazl-vasa males had 54.2%, 44.4% and 56.7% of the individuals with a gonad development score $\leq$ 2 (maximum = 5), respectively. These were significantly more ($p < 0.05$) with poor gonadal development than that in the control group which was 0.0%. The same percentages for the 4-year-old female Nanos-nanos, Nanos-dnd, and Dazl-vasa fish (27.1%, 34.0% and 36.0% respectively) were higher than the control fish (8.3%), but were not statistically different at the 95% confidence level. Mosaicism of transgene integration was observed among different tissues, as well within the same tissue of the P1 fish. The transgene was successfully transmitted to the next generation through the female, but not male germ cells. Doxycycline treatment during embryogenesis did not turn off the expression of the transgene effectively as expected, and was detrimental as it caused lower survival of fry in the construct treated groups. Although the results indicate that the potential for efficient reversible transgenic sterilization will not be possible for this system, this study demonstrated that the overexpression of Bax gene could lead to the death of germ cells in the male fish with high efficacy, and it could be useful to produce sterile fish when repressible control is not needed.

Introduction

With the increase in global population, the demand for seafood as an excellent resource of protein will keep rising. In the past decade, the global production of seafood by aquaculture has doubled and now accounts for more than half of the total global harvest as that by capture has remained the same or even slightly reduced [1]. With the stagnating of capture fisheries, the vast majority of the increased demand for seafood will be fulfilled by aquaculture. However, due to the increasing production cost and the environmental pollution issues associated with aquaculture, aquaculture sustainability, optimizations and efficiency are needed. Genetic

engineering technology has shown great potential for the improvement of fish production for traits such as growth, color, disease resistance, extreme environment tolerance, body composition and even pharmaceutical proteins production [2–4], which cannot be achieved or achieved as effectively by traditional breeding methods. However, regulatory approval is needed for commercial application of transgenic fish due to food safety and environment concerns [3]. Reliable and effective confinement of transgenic fish is essential for the government approval and public acceptance. Among the currently employed containment strategies, physical and physicochemical containments are thought to be useful and necessary as the first line of defense, but cannot provide absolute confinement because of potential mechanical breakdown, human error or theft [5]. Biological containment such as monosex, triploidy or a combination of these two has shown efficacy for some species [6–8]. However, these methods are not commercially feasible for all species or circumstances. Triploid induction does not always result in 100% efficacy, is not practical for all species and the effectiveness varies from species to species. Moreover, in some cases, triploidy can negatively affect the performance of the organism [5,9–11].

Sterilization by transgenic approaches targeting reproductive hormone genes or early embryonic development is thought to be the only option that has the potential of producing 100% effective, permanent and large-scale sterilization [2,5]. Disruption of the gonadal development through the deactivation of reproduction related hormones has been a major area of research interest. However, some reproductive hormones have other biological functions in addition to reproduction regulation, which may lead to other unwanted pleiotropic effects when disrupted [12–14].

The interruption of the primordial germ cells (PGCs) development by transgenesis is another option which could result in the sterilization goal. The primordial germ cells (PGCs) are a line of cells singled out from the somatic cells during the early stage of embryonic development and that can give rise to the gametes. In most organisms, PGCs are formed in several different positions, which are far away from the location that the gonad will develop. With the guidance of directional cues such as SDF-1 [15,16] and Six4 [17], PGCs can migrate to the destination where they interact with somatic gonadal cells and differentiate into germ cells. The specification of PGCs in fish is directed by maternally derived cytoplasmic components, also known as germ plasm [18–20]. Germ plasm is a region in the cytoplasm of egg cells, which contains determinants that are essential for germ cell differentiation and is asymmetrically allocated to the PGCs during the early development of embryos [21,22].

Among those PGCs specification determinants that have been found in various animals, the vasa gene is the most universal and common and has been found to be essential for germ cell development [23]. The vasa gene was firstly identified in *Drosophila* as a maternally derived factor essential for germ cell specification [24] and was found to be continuously expressed in the germ line [25,26]. During embryogenesis, vasa mRNA and protein derived from germ plasm of the egg is specifically allocated to the PGCs and play key roles in the migration of PGCs. Vasa belongs to the DEAD-box protein family, which is involved in a broad range of molecular events related to duplex RNA. A variety of methods have been used to generate vasa-null animals, and disruption of PGCs migration or germ cell deficiency was observed in *Drosophila, Caenorhabditis Elegans* and mouse [27–30]. 3’ UTR of the vasa gene is essential for the specific localization of the vasa mRNA into the PGCs. This mechanism has been widely used for the

visualization of PGCs and research on embryo development. When the 3' UTR of the vasa gene was fused to the 3' of fluorescence protein gene and the recombinant plasmid transferred into the embryo, the fluorescence protein was specifically expressed in the PGCs [31]. Besides the maternally derived transcript, zygotic transcription of vasa was also observed in several organisms and is believed to be initiated from the onset of gastrulation in *Drosophila* [32–34]. Braat et al. (2001) deduced that the failure of preventing PGC formation by using vasa morphant to abolish vasa protein in zebrafish was due to the restoration of vasa protein from zygotic expression after four dpf [35].

The nanos gene is another PGC marker gene which was first found to be required for abdomen formation as a maternally derived factor in *Drosophila* [36]. Further study has shown it is essential for germ line development in diverse organisms and the function for nanos proteins is conserved among invertebrates and vertebrates regardless of their different germ cell specification mechanisms [37,38]. Nanos is an RNA binding protein encoded by the nanos gene and contains a conserved zinc-finger motif. According to the types of motifs, three nanos homologs have been identified [39]. These nanos homologs are expressed preferentially in the PGCs, germ line stem cells or multipotent stem cells and have different functions during different life stages [40]. In *Drosophila*, maternally supplied nanos was required for survival of PGCs in the embryo, while in adults, nanos was required for the continued production of oocytes by maintaining germline stem cells self-renewal [41]. Similarly, in zebrafish, nanos1 was necessary for PGC survival during embryogenesis, and to maintain oocyte production in the adult [42]. Nanos was shown to function as a translational repressor in the germ line stem cells to suppress its somatic cell fates, and thus maintain the germline [37,38,43]. The loss-of-function

experiments in *Xenopus* have shown that nanos1 is required for PGC preservation. Knockdown of this gene led to significant decrease in PGCs and reduction of germ cell production in the gonads [44]. As a maternally derived mRNA, nanos mRNA and protein are produced in the ovarian nurse cells around the oocyte and are transferred into the oocyte during the late stage of oogenesis, subsequently inherited to the embryo after fertilization [45–47]. In addition to the maternal inheritance, zygotic nanos transcription was also found in leech [48]. The 3' UTR of nanos mRNA is responsible for its specific localization to the germ plasm during oogenesis and to the PGCs during embryogenesis. Because of this, 3' UTR of nanos is widely used for the location and visualization of PGCs [20,49–52].

DAZ-like (dazl) is another PGC marker gene encoding protein and mRNA essential for the migration and survival of PGCs, as well for gametogenesis both in male and female [53–55]. Dazl is an RNA binding protein that can regulate the translation of some key transcripts by binding to the 3' UTR of the mRNA [56,57]. In the embryo of medaka, maternally derived dazl protein was abundant enough for supporting the normal formation of PGCs when the protein translation from mRNA was blocked by two antisense morpholino oligos. However, abolishing dazl protein by antibody successfully interrupted PGC formation, while the somatic tissue was not affected [58]. In *Xenopus*, dazl protein expression was detected in all the spermatogenic cells at different stages except for the spermatozoa in the testes, and in the ovary, a strong signal was captured in oogonia, previtellogenic oocytes as well as in the growing oocytes. In the embryo, the signal of dazl protein persists until gastrulation and drastically declined thereafter, with the disappearance of mRNA at the same time. The signal was redetected in PGCs already migrated to the genital ridge and was enhanced with the proliferation of PGCs, indicating the

maternal derivation and zygotic expression of dazl during the early and late embryogenesis respectively [59].

Unlike vasa and nanos, dead end (dnd) is a germ plasm component only found in vertebrate so far [60]. Knockdown of dnd has been used to produce sterile salmon for germ cell transplantation [61]. In zebrafish, the migration of PGCs was blocked after knockdown of the dnd gene and this lead to the death of PGCs [60], and in another study, dnd expression was ablated by its antisense morpholino oligonucleotides (MO), resulting in smaller or the absence of gonads [62]. In zebrafish, dnd gene encodes an RNA binding protein, which possesses ATPase activity and contains an RNA-binding motif that is found in a large variety of RNA binding proteins. Dnd regulates germ line gene expression (eg., nanos) by binding to the 3' UTR of the mRNA, thus relieving the repression effect of miRNA [63,64]. The expression of dnd was germ line specific during embryogenesis, but sex dependent in adult organisms. In *Xenopus*, the expression of dnd was only found in the ovary [65], but in mouse, it is exclusively expressed in the germ cells of testis [66]. In turbot, dnd was expressed in the germ lines of both sexes, but more abundantly in the gonad of females than males before sex maturation [67]. The expression of dnd occurred in both sexes, but restricted to germ cells in medaka [68]. The cis-acting element at the 3' of the dnd mRNA is believed to be essential for the PGC specific localization and expression during embryogenesis [67, 71].

Apoptosis is not just an immune response of an organism to outside stimulation or pathogen invasion, but also a normal process occurs during development and is essential for homeostasis of cell populations. There are two main apoptotic pathways regulating this process: the extrinsic

death receptor pathway and the intrinsic mitochondrial pathway [70]. Bax, also named bcl-2-like protein 4, is a protein encoded by the Bax gene, which plays a key role in the mitochondrial apoptotic pathway. As a pro-apoptotic regulator, Bax works with the other Bcl-2 gene family members to regulate apoptosis of the cell [40, 41]. The mitochondrial pathway is initiated by the activation of pro-apoptotic BH3-only proteins as a result of responding to the upstream cellular stress signals [73]. Once activated, BH3-only proteins bind to the pro-apoptotic subfamily proteins (Bax, Bak) to activate these proteins directly or indirectly to bind to the pro-survival Bcl-2-like proteins, which form a heterodimer with Bax and restrain its activity under normal condition [74,75]. The activated Bax proteins undergo oligomerization and their relocation to the mitochondrial membrane alters the permeability of the mitochondrial membrane, leading to the release of cytochrome c, caspase activation, and subsequent cell death [76–78]. Numerous studies have proved that the activation [79] or overexpression [80–84] of Bax is related to or can lead to increased apoptosis. The majority of zebrafish bcl-2 family members have similar functions to the corresponding mammalian homologs [85]. Research on zebrafish has shown that the ratio of pro-apoptotic protein Bax to pro-survival protein Bcl-2 was decisive for the determination of cell destiny [86]. Both the induction of the pro-survival Bcl-2 family member and the knockdown of the Bax were sufficient to counteract the apoptosis induced by irradiation [85]. Based on the above evidence, it is reasonable to propose that the overexpression of the Bax gene in catfish PGCs will result in the death of these cells, thus leading to the infertility of catfish.

The tet-off system is a gene expression controlling system first developed by Hermann Bujard and Manfred Gossen in 1992 [87]. The tet-off system consists of three major

components: the tetracycline transactivator (tTA) protein, the tetracycline response element (TRE) and tetracycline. The tTA protein is a recombinant of the tetracycline reprosessor, TetR protein, with the activation domain of the VP16 protein, which is an essential transcriptional activation domain from herpes simplex virus (HSV). TRE is composed of several TetO operators and a downstream minimal promoter usually the CMV promoter. The tTA protein activates the downstream promoter by binding to the TetO operator, thus turn on the expression of the gene of interest (GOI). tTA can be deactivated by binding with tetracycline or its derivatives such as doxycycline (dox), so the expression of GOI will be turned off when tetracycline is added. Based on this pioneering system, a variety of modified tet-off systems have been developed and widely used for controlling the expression of exogenous genes [88–91].

The catfish industry was the largest aquaculture industry in the US, accounting for approximately 70% of the aquaculture production in 2000 (USDA 2000). Catfish production dropped to 138 million kg in 2011 (NASS 2012), while it was 350 million kg in 2000, because of increasing costs of production, the fast-growing production of other competitive substitutes such as tilapia, and the competition with cheap imported fish from other countries. Transgenic technologies have been used for the genetic improvement of catfish performance with considerable success. The growth rate of channel catfish, *Ictalurus punctatus*, was improved by 33% by transferring salmonid growth hormone gene [92], and bacterial disease resistance has also been significantly improved by cecropin gene transfer [93]. However, these advances cannot be utilized without highly effective confinement.

The objective of this research was to develop a strategy to reversibly sterilize fish, specifically channel catfish, by overexpression of the pro-apoptosis gene Bax driven by the tet-off system in the PGCs during embryogenesis. The long term goal is application of this technology in transgenic fish to restore the fertility of potential brood stock with dox treatment, while production fish and progeny of brood stock that might escape into the environment will be sterile, eliminating long-term impact on the natural ecosystem.

Methods and materials

Construction of the transgene plasmids: Nanos-nanos, Nanos-dead end, Dazl-vasa

The constructs were designed and fused by AquaBounty Technologies (Maynard, MA). Briefly, all the constructs were based on the tet-off-vector backbone. Each component of the PGC marker genes and the pro-apoptosis gene Bax were cloned from zebrafish and then recombined with the tet-off vector. PGCs marker gene (nanos, dazl) promoters were fused into the vector to drive the expression of the tTA protein, which is a transcription factor that can bind to the Bi-TRE and turn on the expression of a downstream pro-apoptosis gene, Bax, in the absence of dox. The 3' UTR of PGC marker genes (nanos, dnd and vasa) were added to the 3' of Bax gene to make this gene specifically expressed in the germ line cells in concert with the PGC specific initiation promoters (Fig. 1-1, 1-2, 1-3).

Plasmid preparation

The plasmids were transformed into the One Shot® Top 10 Chemically Competent *E.coli* cells (Invitrogen, Grand Island, NY) following the manufacturer's instruction. After the incubation of the transformed cells, 25 µl or 50 µl of the culture were spread on LB agar plates

containing 100 μg/ml ampicillin. A single colony was picked from the plate and inoculated into 500 ml LB containing 100 μg/ml ampicillin and cultured at 37 ^{o}C overnight (12h~16h). Plasmids were then extracted from the culture using IsoPure Plasmid Maxi II Prep Kit (Denville, Holliston, MA).

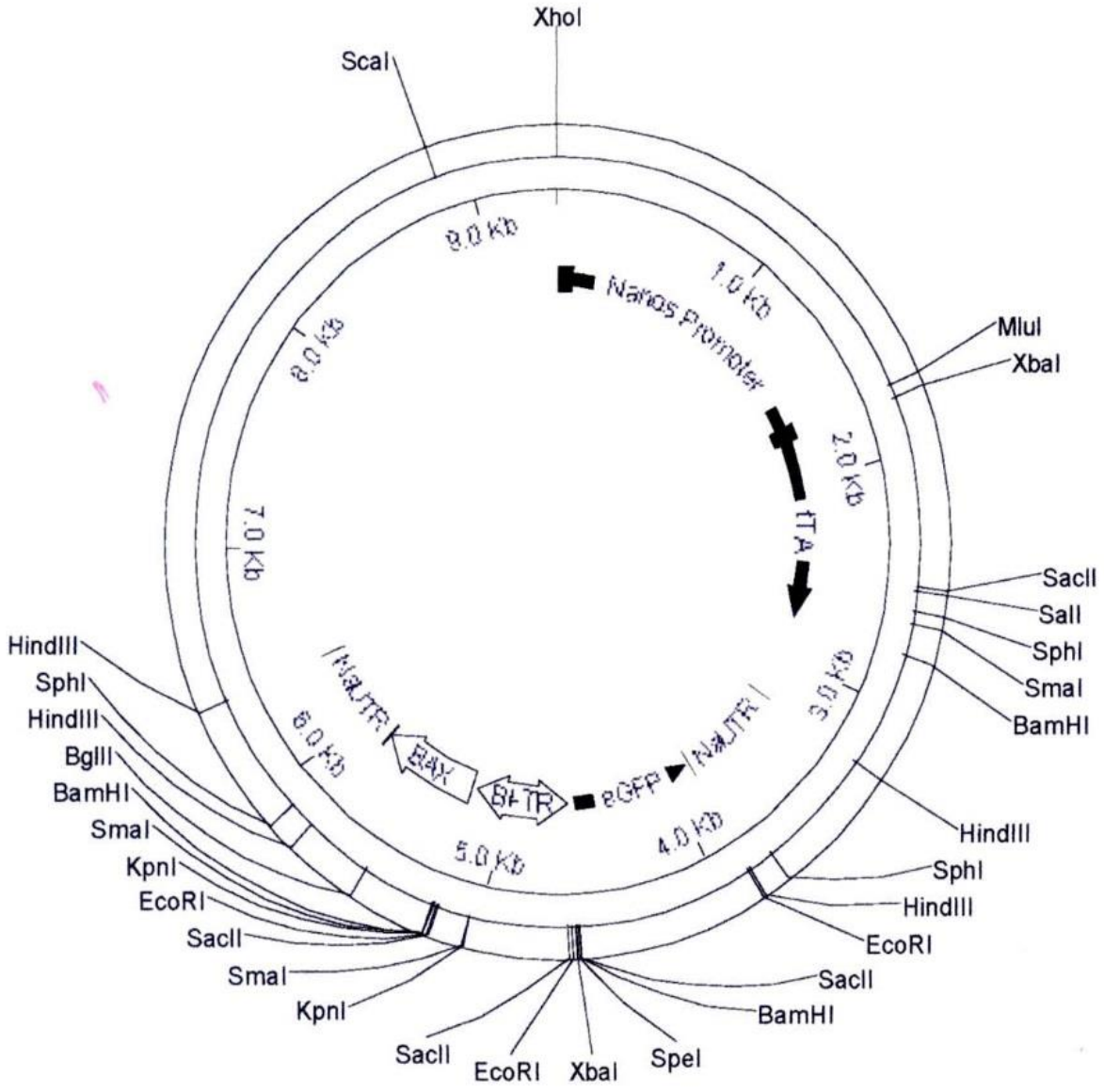

Fig. 1-1 Nanos-nanos sterilization construct map. Nanos gene promoter was used to drive the expression of tetracycline transactivator (tTA). Bax gene was driven by the binal tetracycline response element (Bi-TRE) and followed by the 3' UTR of nanos gene to make the allocation of Bax mRNA primordial germ cell (PGC) specific. Bax gene, nanos promoter and 3' UTR were from zebrafish, *Danio rerio*. NaUTR: nanos 3' UTR.

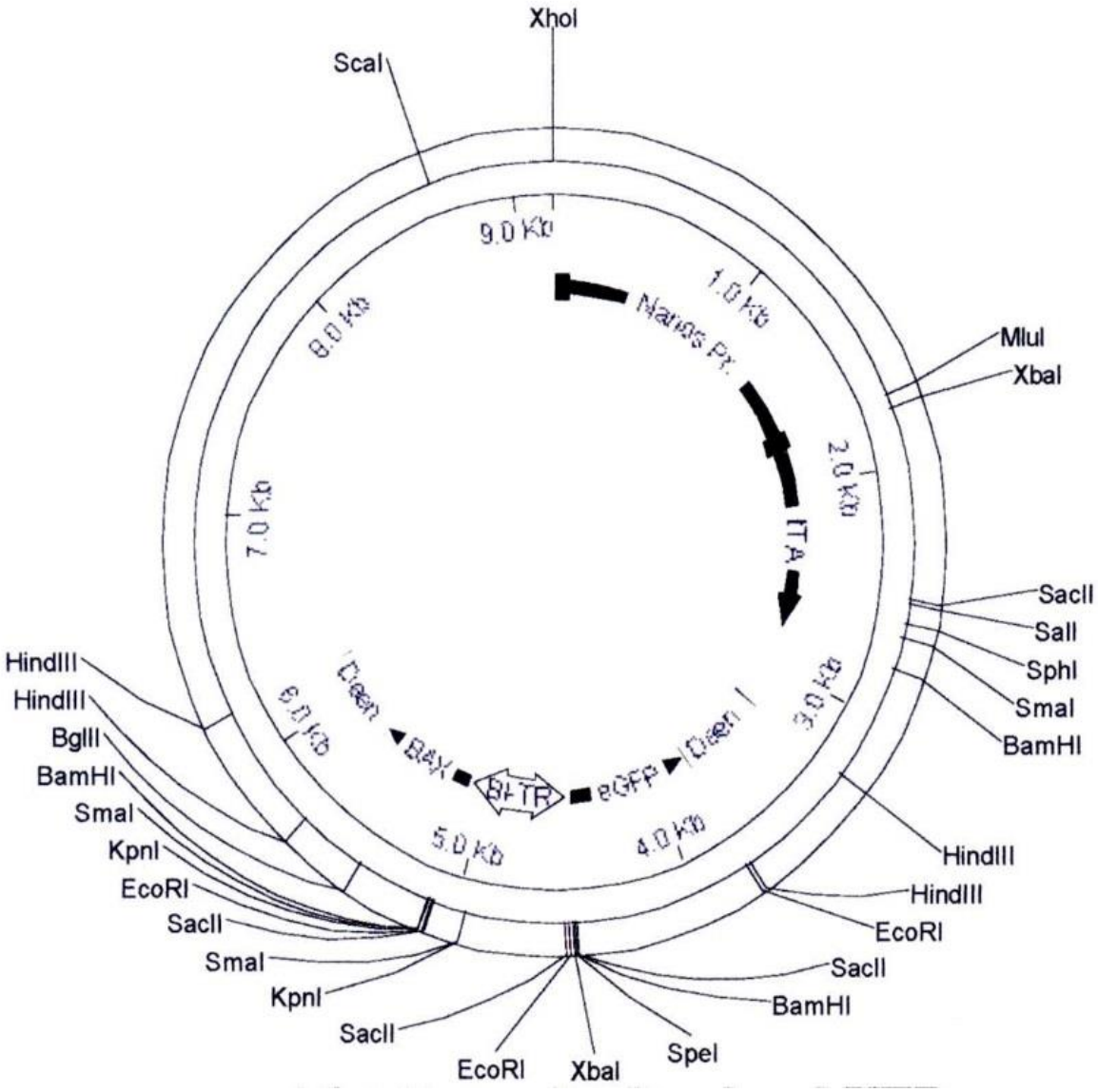

Fig. 1-2 Nanos-dead end (Nanos-dnd) sterilization construct map. Nanos gene promoter was used to drive the expression of tetracycline transactivator (tTA). Bax gene was driven by the binal tetracycline response element (Bi-TRE) and followed by the 3' UTR of dead end gene to make the allocation of Bax mRNA primordial germ cell (PGC) specific. Bax gene, nanos promoter and dead end 3' UTR were from zebrafish, *Danio rerio*. Nanos Pr.: nanos promoter; Deen: dead end 3' UTR.

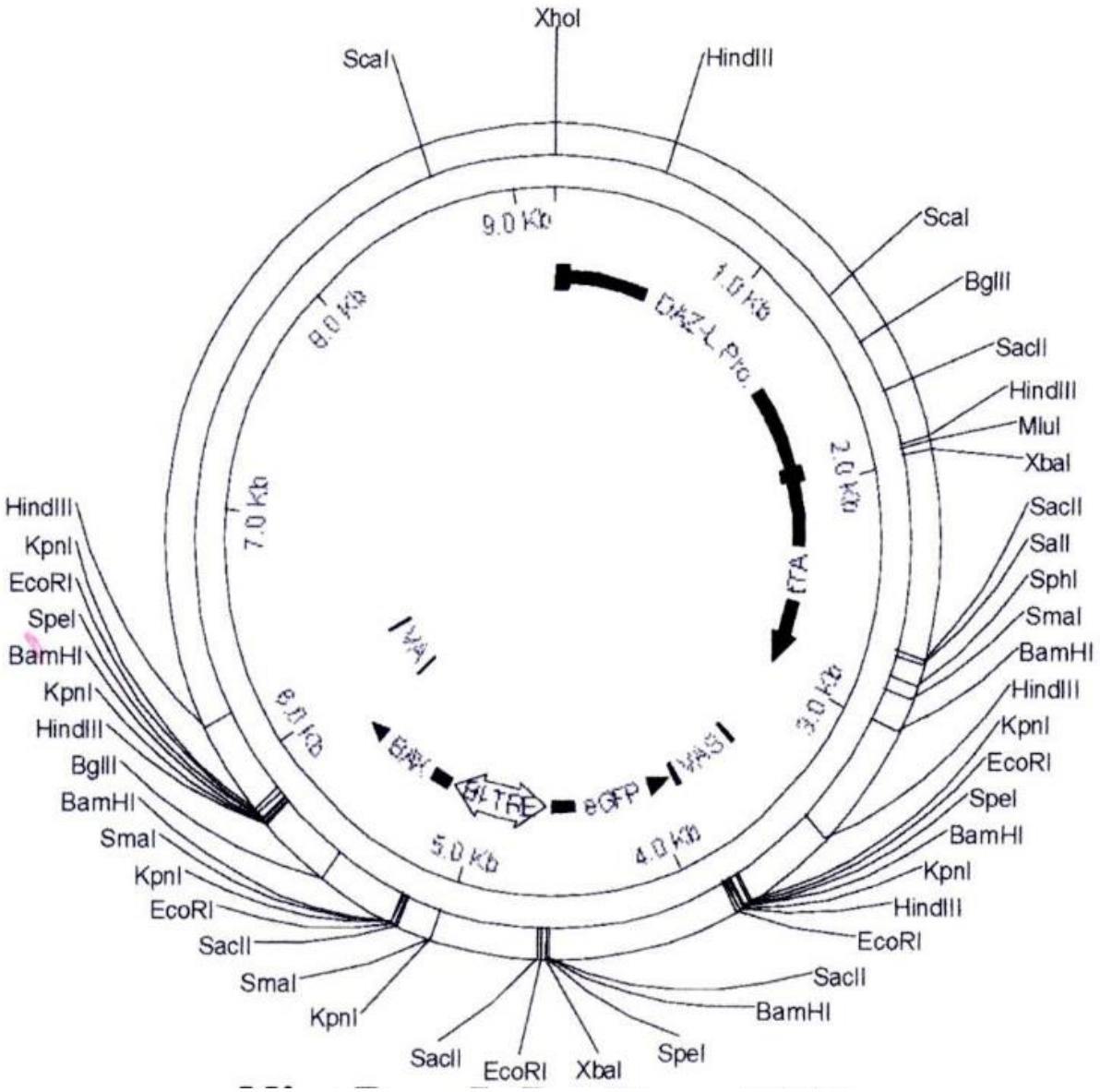

Fig. 1-3 Dazl-vasa sterilization construct map. Dazl gene promoter was used to drive the expression of tetracycline transactivator (tTA). Bax gene was driven by the binal tetracycline response element (Bi-TRE) and followed by the 3' UTR of vasa gene to make the allocation of Bax mRNA primordial germ cell (PGC) specific. Bax gene, dazl gene promoter and vasa gene 3' UTR were from zebrafish, *Danio rerio*. DAZ-L Pro.: dazl promoter; VA: vasa gene 3' UTR.

Quality and quantity were checked with a Nanodrop 2000 (Thermo Scientific, Wilmington, DE) and by electrophoresis. Half of the extracted plasmids were linearized with the restriction enzyme XhoI following the manufacturer's instructions. At the end of the digestion, the reaction was inactivated, and linearized plasmids were purified by the phenol chloroform ethanol extraction method. The success of digestion was examined by electrophoresis and the concentration was re-determined with a Nanodrop 2000. Both the linearized and circular plasmids were diluted to a final concentration of 50 μg/ml with TE buffer (5 mM Tris-HCl, 0.5M EDTA, pH = 8.0) for electroporation.

Artificial spawning of brood stock/gamete preparation

Channel catfish broodstocks were obtained from the ponds at the Catfish Genetics Research Unit, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, AL, USA. Females and males with well-developed, secondary sex characters (well-rounded and distended abdomen for the females and muscular head and elongated urinogenital papillae for the males) were chosen for artificial spawning. Females were implanted with 90 μg luteinizing hormone releasing hormone analogue (LHRHa) per kilogram of the body weight, placed into a spawning bag and kept in a flow-through tank with continuous aeration. The fish were first checked for egg ovulation once 1,040 degree hours post-hormone injection were reached and then checked every 4h thereafter. Once ovulation was observed (more than 10 eggs on the spawning bag), the females were anesthetized with 100 ppm buffered tricaine methane sulfonate (MS 222), and the remaining eggs were obtained by hand stripping. The males were sacrificed a few hours before the expected time of ovulation and sperm were squeezed from the testes through a fine mesh and

into 10 ml of 0.9% saline per gram of testes. The sperm solution and stripped eggs were mixed, and pond water added to activate fertilization.

Introduction of transgene by electroporation

Twenty min after the fertilization, 100 to 200 fertilized eggs were transferred to a 7 ml petri dish and 3 ml of the plasmid solution was added into it. After 10 min of incubation, the eggs were electroporated with a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, California, USA). Parameters were set at 6 kV, 27 pulses, 0.8 sec burst, 4 cycles and 160 µsec. A control group electroporated with TE buffer only was also included.

Embryo treatment and incubation

Electroporated embryos for each construct were divided into two groups and transferred to 10 L tubs with 5.0 L Holtfreter's solution. One of the groups was treated with 100 ppm dox to allow the production of fertile individuals by preventing the expression of Bax. The embryos were gently agitated with compressed air delivered through an airstone. Holtfreter's solution was changed every 12 h and dead embryos were counted and removed before each solution change. Once the embryos hatched, the fry were transferred to fry baskets and temporally reared in a flow-through tank with pond water in the greenhouse. A 50% protein powdered fry starter was used for fry and # 0, 1, 2, or 3 pellet feed with 36 to 48% protein content were used to feed small fingerlings as they grew. Once they grew large enough (around 100 g), the fingerlings were anesthetized with 100 ppm buffered MS 222, heat branded, evenly divided for each group and stocked into two 404.7 square meter ponds (G30 and G36) with 1 meter depth water, for a density of approximately 0.7 fish per cubic meter water. Fish were fed ad-libitum with

commercial adult fish feed containing 32 to 36% protein once per day and 7 days per week. Feeding rates were reduced to once every other day when water temperature was under 15 ℃.

Sample collection, DNA extraction and transgenic identification with PCR and sequencing

Anal fin and/or gonad samples were taken from the P1 fish and barble samples were taken from the F1 fish for DNA extraction. Samples were digested in cell lysis buffer with 100 μg/ml proteinase K. After full digestion, genomic DNA was isolated from the solution by protein precipitation, DNA precipitation by isopropanol, washing and then precipitation of DNA by ethanol [94]. DNA was dissolved in DNase free ddH_2O after air drying and kept in a refrigerator overnight to allow complete rehydration. Quality and quantity of extracted DNA were measured on a Nanodrop 2000 and integrity checked by electrophoresis.

Primers targeting the boundary area of the PGC marker gene promoter (nanos and dazl) and the tTA sequence, and primers flanking the Bax gene and the PGC marker gene 3' UTR region (nanos, dnd, vasa), were designed for the screening of potential transgenic fish using the Primer premier 5.0 software. The primers given by the software were checked for quality parameters such as GC content, primer dimer, hairpins, 3' end stability and melting temperature with Oligo Analyzer 3.1. They were also blasted against the channel catfish genome from the National Center for Biotechnology Information (NCBI) database to ensure their specificity. At least 4 pairs of primers were designed and tested. The best pair with the highest specificity and efficiency was used for the screening of the transgene for each construct (Table 1-1). Plasmids used as the positive control template for primer test were diluted to approximately 0.05ng/μl with water containing genomic DNA from non-transgenic control fish. Ten negative control PCR

reactions in which genomic DNA from ten normal channel catfish was used as the template were conducted to check the specificity of primers. The PCR reaction was accomplished in a 15 μl volume mix with the following components: 10 x buffer 1.5 μl, 2.5 mM dNTP 1.5 μl, 50 mM $MgCl_2$ 0.5 μl, 10mM forward primer 0.75 μl, 10mM reverse primer 0.75 μl, Taq polymerase 0.4 U, template genomic DNA 200 ng, and addition of ddH_2O to 15 μl. The PCR program was as follows with slight modifications on the annealing temperature (Ta) and elongation time for each primer pair: initial denaturation for 5min at 95℃; followed by 39 cycles of 95 ℃ for 30s, 59 ℃ for 30s and 72℃ for 45s; and a final elongation for 5min at 72℃. PCR results were checked by electrophoresis on 1% agarose gel.

In addition to PCR, the insertion of the transgene was further confirmed by sequencing. Briefly, the band at the correct size was cut out and purified with the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. The purified product was sent to the Auburn University Genomics and Sequencing Lab for sequencing. The sequence was confirmed by aligning to the plasmid sequence.

Spawning and gonad development evaluation of the P1 fish

When the P1 fish reached three years of age, the fish in one pond (G36) were harvested during spawning season and maturity evaluated by a scoring system in which each fish was given a score from 1 to 5 according to the secondary sex characters. All fish were examined and scored by the same well-trained examiner to make sure the score was given to each fish with the same standard. The examiner knew which fish belong to the same group but did not know which group it is. Score 5 was the highest and was characterized by a well-rounded and distended abdomen,

open red genitalia for the females and a very muscular head and elongated urinogenital papillae for the males; score 4 was characterized by rounded and distended abdomen, open red genitalia for the females and a muscular head and elongated urinogenital papillae for the males, score 3 was a fish with rounded abdomen, open light red genitalia for the females and a median muscular head and elongated urinogenital papillae for the males; score 2 means a fish with slightly rounded abdomen, not swollen genitalia for the females and a normal head and slightly elongated urinogenital papillae for the males; score 1 was the lowest and was assigned to fish with a flat abdomen, not swollen pale genitalia for the females and a normal head and small, soft urinogenital papillae for the males.

A fish with a score of 3-5 was gravid and spawnable. All the fish with a score ≥ 2 were used for hormone (LHRHa) induced spawning as described above. The fish spawned, and those fish not spawnable were stocked back into the pond. The weight of testes and eggs of spawned females was recorded. The eggs from each female were divided into at least two portions and fertilized with the sperm from males with the same construct to produce F1 progeny. The embryos of each family were divided into two groups: one group was hatched in Holtfreter's solution and treated with 100 ppm dox as described before; the other group was incubated in hatching baskets in an aerated flow-through hatching trough with a paddlewheel and water change at least twice per hour without dox. pH was maintained at 7.0 to 7.6, and DO was higher than 7 mg/L.

Table 1-1 Primers used for the Nanos-nanos, Nanos-dnd, Dazl-vasa transgenic fish identification. Two pairs of primers targeting the promoter (nanos, dazl)–tTA region and the Bax-3' UTR (nanos, dnd, vasa) regions respectively were designed and used for confirmation of the integration of these two critical parts required for the proper function of the transgenic constructs for channel catfish (*Ictalurus punctatus*). The forward primers were designed within the nanos, dazl promoter or Bax gene region, and the reverse primers were designed within the tTA or the nanos, dnd, vasa 3' UTR region.

Primer name	Sequence(5' – 3')	Region	Product size(bp)	Ta (°C)	Elongation time (s)
Nanos-tTA F	GGACTTGGCATTTCTCGTGAC	Nanos promoter	283	57	30
Nanos-tTA R	GGGCGAGTTTACGGGTTGTT	tTA			
Bax-nanos F	AGTCGGAGTTTTCCTCGCTG	Bax	336	58	30
Bax-nanos R	TGTTTTTGAGTGCGGTTGCG	Nanos 3' UTR			
Bax-dnd F	AGGGTGGATGGGACGGAATC	Bax	477	57	40
Bax-dnd R	AACGCAAACCAAAGTGAAGTCG	dnd 3' UTR			
Dazl-tTA F	CGCGGTCGATTATGTGCATC	Dazl promoter	360	57	35
Dazl-tTA R	CCTAGCTTCTGGGCGAGTTT	tTA			
Bax-vasa F	GGGACGGAATCCGCAGTTAT	Bax	406	55	35
Bax-vasa R	TGTGCTCCAGTCCAAACGAG	vasa			

At the age of 4, all the fish were harvested and gonad development evaluated again during the spawning season. Fish were given a score according to their secondary sex characters as described above and then euthanized with 300 ppm buffered MS 222. Fish were dissected to check the gonad development, and a score from 0 to 5 was given according to the size of the gonad, the amount of gametes in the gonad, and the quality of gametes. All fish were examined and scored by the same well-trained examiner to make sure the score was given to each fish with the same standard. The examiner knew which fish belong to the same group but did not know which group it is. Score 5 was the highest and was characterized by a large gonad fully filled with white sperm for the males and yellow eggs for the females; score 4 was characterized by a large size of testis with most of the tubules filled with white sperm for the males and a large size of ovary filled with a moderate quantity of yellow eggs for the females; score 3 was characterized by a medium size of testis with less than half of the tubules filled with white sperm and a medium size of ovary filled with yellowish eggs for the females; score 2 was characterized by a medium size of testis with no white sperm that can be observed by naked eye for the males and a small size of ovary filled with mostly pale immature eggs; score 1 was characterized by small size of testis with no white sperm that can be observed by naked eye for the males and a small size of ovary filled with transparent eggs; score 0 was the lowest and characterized by no or just a tiny strip of testis for the males and a very small size of ovary with no eggs. Gonadal weight was recorded and the gonadosomatic index (GSI) used to evaluate the development of gonad. GSI was calculated as follow:

$$GSI = \frac{\text{Weight of gonad}}{\text{Weight of body}} \text{ X } 100$$

Fin and gonad samples were taken from each fish for DNA analysis.

Sex ratio check of F1 families

At 8 months of age (weight: 5 - 15 g), the F1 fish were euthanized with 300ppm MS 222 and then dissected to check their apparent sex. Female fish have two transparent long oval shaped ovaries, while male fish have no visually apparent testes at this stage. The genetic sex of fish was determined by PCR using a sex-linked marker (AUEST0678) [95], females should have one band with a size of 212bp and males have an additional band at 205bp.

Statistical analysis

All the data analysis was conducted using a SAS program (edition 9.4). Log rank test was used to compare the P1 embryo survival curves. Fisher's exact test was used for analysis of fry mortality, percentages of spawned fish, and transgenic percentage data. ANOVA (one-way, two-way, N-way) was used to analyze the effect of construct, dox treatment and body weight on the gonad development (score and GSI). Tukey's test was used for multiple comparisons between each group once a significant factor was confirmed by ANOVA analysis. Exact binomial test was used to check if the sex ratio of the F1 populations equals to 1:1. Significance for all tests was set at $p < 0.05$.

Results and discussion

Mortality (hatch) of the P1 embryos and survival of the hatched fry seven days post-hatch

The survival probability of embryos decreased rapidly for all treatments during 1.5 to 3.5 dpf, indicating this is the most vulnerable stage of channel catfish embryo development. This result is consistent with the findings of Brian and Terry, 2001 [96]. Proper egg care is critical during this time. In fact, it is not surprising to see higher mortality during this period as this is the time of

channel catfish organogenesis and many essential organs such as the brain, heart and vascular system are formed [97].

When not treated with dox, the survival curves of the embryos with the Nanos-nanos, Nanos-dnd, Dazl-vasa transferred had no significant difference between each other as well as with the TE buffer control embryos (Fig. 1-4). When treated with dox, there was no difference in the survival curves between the three construct groups ($p > 0.05$). While the survival curve of the TE control group was significantly under ($p < 0.0001$) the curves of the other three groups (Fig. 1-5), indicating the TE control embryos had a lower possibility of survival than the transgene exposed embryos when treated with dox. The comparison of survival between the dox treated and not treated embryos showed that survival probability of the TE control embryos was lowered by the treatment of dox. While the Nanos-nanos and Dazl-vasa embryo survival was not affected by dox treatment, the survival of the Nanos-dnd embryos increased slightly when treated with dox (Fig. 1-6). In summary, the dox treatment had no negative and even slightly positive impact on the survival of the three constructs transferred embryos, but negatively affected the survival of the TE control embryos.

Dox is an antibiotic belonging to the tetracycline class and is used for the treatment of bacterial or protozoal infection [98–100]. In addition to its therapeutic role, dox is routinely used in research because of its role as the regulating compound for the tet-off or tet-on system. Even though it has been used in transgenic research for a long time and proven highly efficient in transcriptional regulation [101], the tet systems are now being questioned because experiments may be confounded by tetracycline treatment due to its impact on mitochondrial function and the

basal gene expression of the organism [102,103]. Tetracycline has also been reported to have an adverse effect on leech embryo development [104], impaired the fetal development of mice [105], and delayed hatch of channel catfish eggs [106]. On the other hand, because of its ability to reduce the risk of bacterial infection, dox has been used successfully to increase the survival of fish embryos, especially for those vulnerable to disease after electroporation or microinjection [106]. These contradictions on embryo survival are likely dose dependent. In the current experiment, the dox concentration required for controlling gene expression was 100ppm, which is ten times the concentration usually used for improving survival. The phenomenon of increased embryo mortality after dox treatment in the TE control group, but not in the constructs exposed groups might be explained by the consumption of dox partially by the tet-off system in the construct transferred groups, giving these a lower effective dose.

Dox treatment during the embryo stage before hatch resulted in higher ($p < 0.05$) mortality of hatched fry through 7 days post hatch (dph) than for untreated controls in the sterilization construct exposed groups. A similar result was observed for the dox treated TE control groups, though not statistically significant ($p > 0.05$). There was no significant ($p > 0.05$) difference in the fry mortality between the constructs transferred groups and TE control group, for both those treated or not treated with dox during embryogenesis (Fig. 1-7). The purpose of dox treatment in this experiment was to produce fertile transgenic brood stock for producing sterile transgenic offspring, and perpetuating the transgenic lines. The drawback of the dox treatment lowering fry survival could be counteracted by producing more embryos for brood stock or preferably, developing a more efficient tet-off system, requiring less dox for regulation of expression.

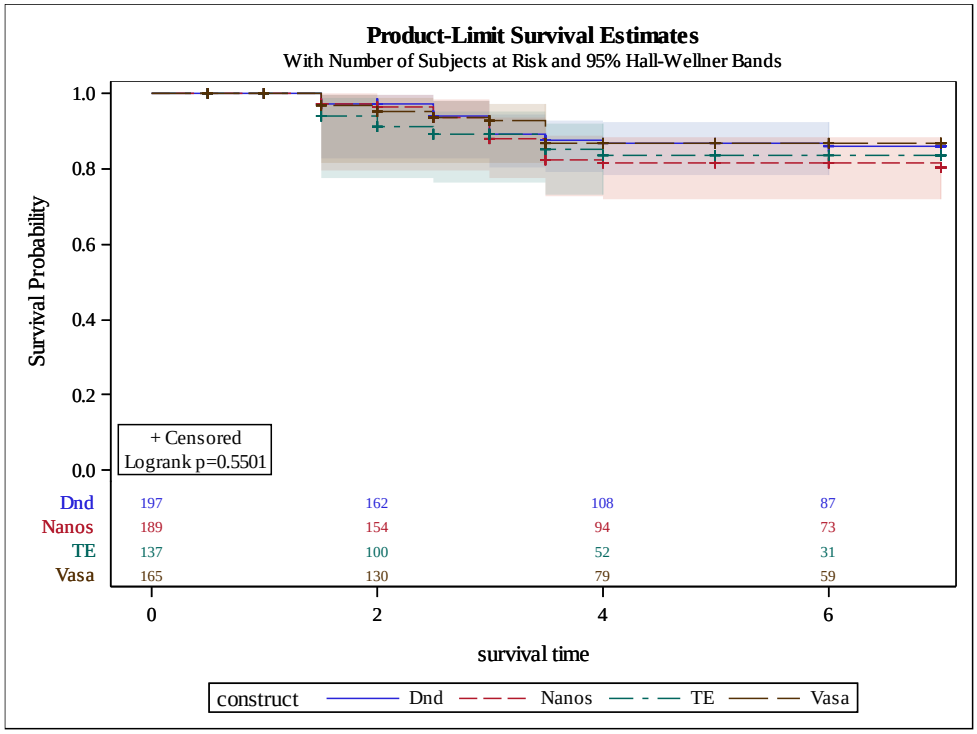

Fig. 1-4 Kaplan-Meier survival curves for the sterilization constructs (Nanos-nanos, Nanos-dnd, Dazl-vasa) transferred or TE buffer exposed control channel catfish (*Ictalurus punctatus*) embryos without doxycycline treatment. Logrank test was used to compare the survival curves. There was no significant difference between the curves of each group ($p > 0.05$). Dnd: Nanos-dnd construct; Nanos: Nanos-nanos construct; Vasa: Dazl-vasa construct; TE: TE buffer.

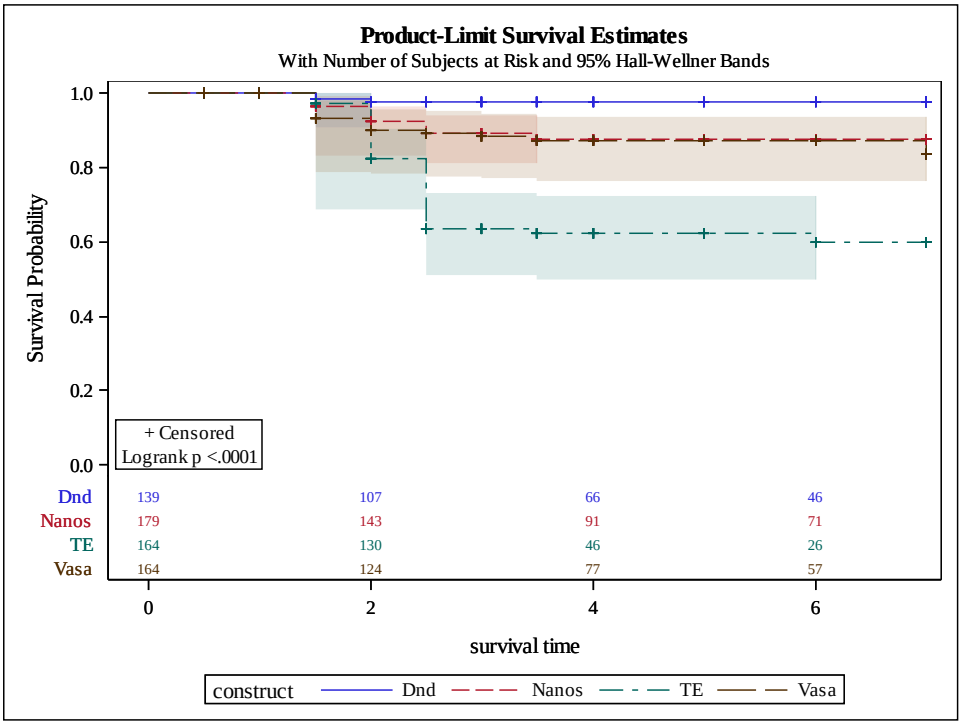

Fig. 1-5 Kaplan-Meier survival curves for the sterilization constructs transferred (Nanos-nanos, Nanos-dnd, Dazl-vasa) or TE buffer control channel catfish (*Ictalurus punctatus*) embryos treated with 100 ppm doxycycline. The survival curve of the TE buffer control embryos is significantly under the other three curves of embryos exposed to sterilization constructs ($p < 0.05$), which means lower survival probability of the embryos in the control fish. Dnd: Nanos-dnd construct; Nanos: Nanos-nanos construct; Vasa: Dazl-vasa construct; TE: TE buffer.

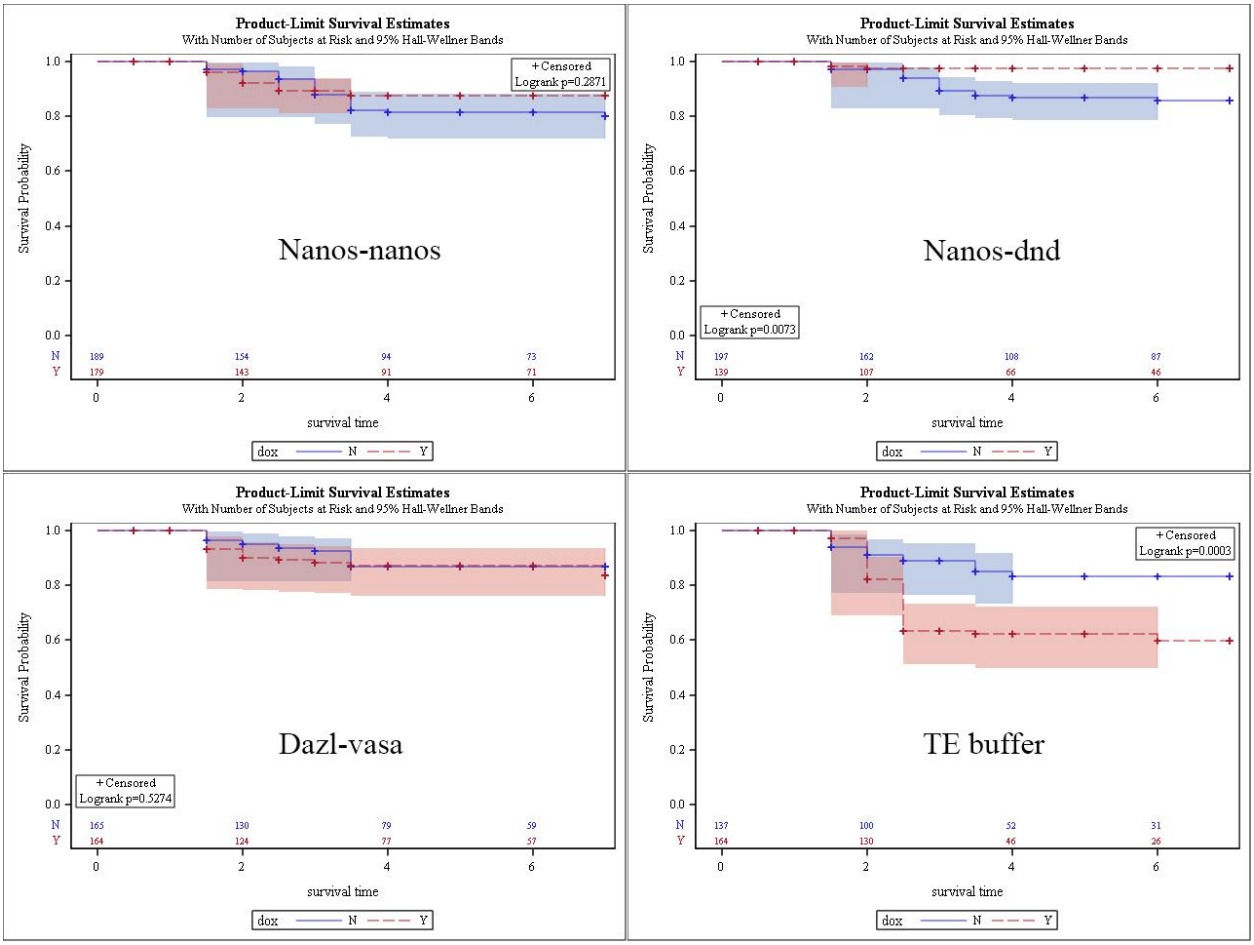

Fig. 1-6 Kaplan-Meier survival curves of channel catfish (*Ictalurus punctatus*) embryos treated or not treated with 100 ppm doxycycline (dox) within each sterilization construct (Nanos-nanos, Nanos-dnd, Dazl-vasa) transferred group and the TE buffer control group. Logrank test was used to compare the survival curves. No significant difference in the survival was observed between the dox treated or not treated fish in the Nanos-nanos and Dazl-vasa groups. The survival was slightly increased by dox treatment in the Nanos-dnd group (p = 0.007), while the TE buffer control embryos had decreased survival when treated with dox (p = 0.0003). N: not treated with dox; Y: treated with dox.

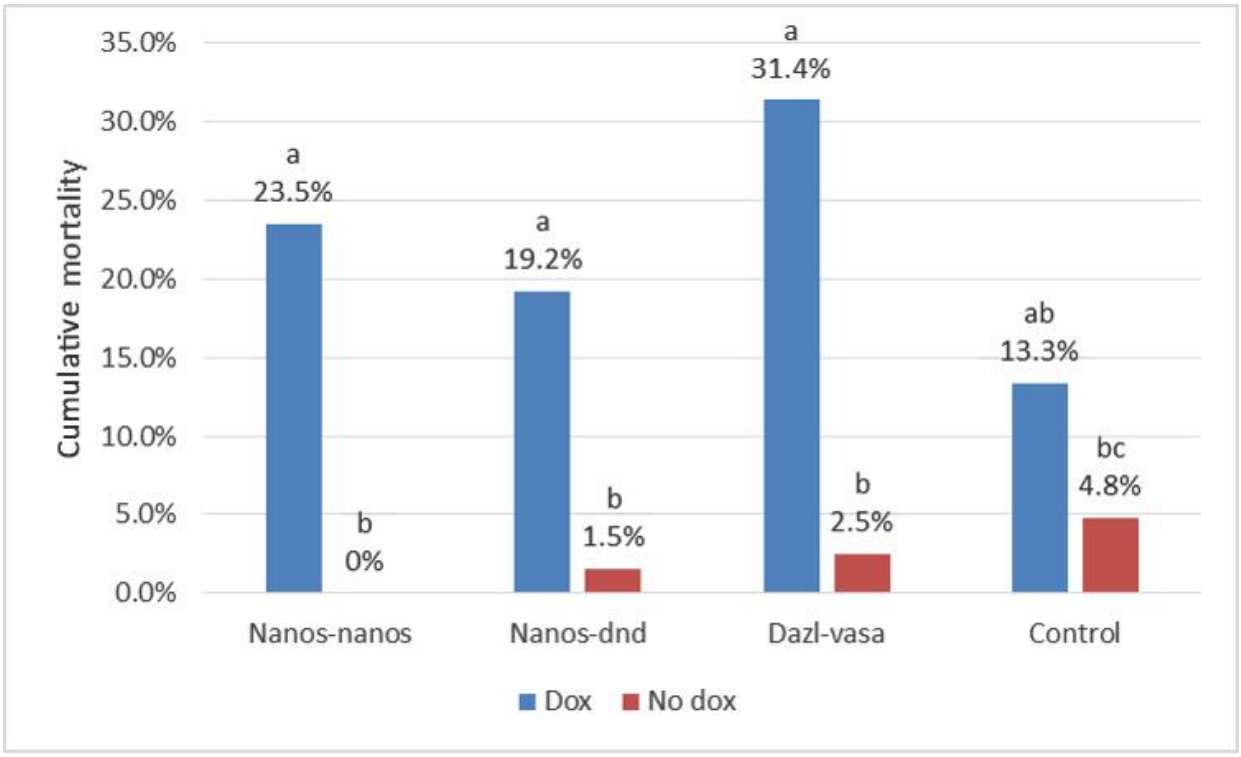

Fig. 1-7 Cumulative mortality of channel catfish (*Ictalurus punctatus*) fry through 7 days post-hatch. Fry were hatched from embryos exposed to Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct or TE buffer (control) via electroporation at 1 cell stage, and treated or not treated with 100ppm doxycycline (dox) during embryogenesis. Fisher's exact test was used to test the difference of mortality among the transgene exposed and control groups when treated or not treated with dox, and between the dox treated and not treated groups within each construct or control group. There was no significant difference in fry mortality among the transgene exposed and control group, when treated or not treated with dox. The dox treated fish had higher mortality than the fish did not receive dox treatment in the transgene exposed groups, but the difference was not significant ($p > 0.05$) in the control group. Dox/No dox: treated/not treated with 100 ppm dox during embryogenesis. Percentages followed with the same letter were not significantly different ($p > 0.05$).

Spawning and gonad development evaluation of the P1 fish

The dox treated Dazl-vasa fish were lost to predation, and only the untreated fish for this construct were available for evaluation. There was no significant difference among the three constructs (p = 0.49) for sexual maturity at 3 years of age and the dox treatment did not affect the sexual maturity of channel catfish (p = 0.35). The body weight was highly correlated with the sexual maturity score (R = 0.58, $p < 0.001$). In all the three construct transferred groups, there was no significant ($p > 0.05$) difference between the fish transferred with circular or linearized plasmids within the female population, but in the Nanos-nanos and Dazl-vasa groups, the males transferred with circular plasmid had higher sexual maturity score than the ones transferred with linearized plasmid (Table 1-2). With respect to the percentages of spawned fish, no significant difference was found between the dox treated and untreated fish in both the males and females for any construct group. Among the constructs, there was no significant difference among the subgroups divided by sex and treatment, except that the Nanos-nanos and Nanos-dnd untreated females had a higher percentage of fish spawned than the Dazl-vasa untreated female (p = 0.049 and 0.064 respectively). When the spawning data for female and male were combined, only the Nanos-nanos fish showed higher (p = 0.012) percentage of fish spawned than the Dazl-vasa fish (Table 1-3).

Gonad development evaluation at 4-years of age showed that the external maturity score which was given according to the secondary sex characters was highly correlated with the internal gonad development score (R = 0.85, $p < 0.0001$), indicating the evaluation of maturity utilizing the secondary sex characters at the age of 3 was valid.

Table 1-2 Means with standard deviation of the sexual maturity score of the 3-year-old female and male channel catfish (*Ictalurus punctatus*). Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline during embryogenesis. All fish were evenly distributed and grown in two replicate ponds at the same density. Maturity scores were compared between the fish transferred with circular or linear plasmid within the same construct and sex group. Two-way ANOVA using body weight as a covariance was used to test the significance of difference. The Nanos-dnd and Dazl-vasa males transferred with circular plasmid had significantly (p = 0.0089 and 0.008, respectively) higher maturity score than their counterparts transferred with linear plasmid. Means in the same row followed by different letters are significantly different ($p < 0.05$). F: female; M: male.

Construct	Sex	Mean score	
		Circular	Linear
Nanos-nanos	F	2.96 ± 1.49	3.00 ± 0.94
	M	2.44 ± 1.21	2.03 ± 1.29
Nanos-dnd	F	3.31 ± 1.03	1.89 ± 1.08
	M	3.15 ± 1.39[a]	1.78 ± 0.94[b]
Dazl-vasa	F	3.66 ± 0.98	2.50 ± 1.29
	M	2.00 ± 0.71[a]	1.00 ± 0.00[b]

Table 1-3 Percentages of channel catfish (*Ictalurus punctatus*) spawned at the age of 3. Fish were exposed to Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation, and treated or not treated with doxycycline (dox) during embryogenesis. All fish were evenly distributed and grown in two replicate ponds (G30, G36) at the same density. The fish in G36 were evaluated for sexual maturity at the age of 3 and those potential spawnable fish were subjected to a induce spawning trial. The numbers in the bracket refer to number of fish spawned/ number of total fish evaluated for each group. Fisher's exact test was used for statistical analysis. There was no significant ($p > 0.05$) difference between the dox treated or untreated fish within the same construct and sex. The Nanos-nanos and Nanos-dnd untreated female fish had significantly ($p = 0.049$) and marginally significantly ($p = 0.064$) higher percentage of fish spawned, respectively, than the Dazl-vasa female fish. When female and male fish data were combined, the Nanos-nanos untreated fish had significantly ($p = 0.012$) higher percentage of fish spawned than the Dazl-vasa fish. Percentages in the same column followed by different letters (lowercase and uppercase letters for separate sex and pooled sex, respectively) are significantly different ($p < 0.05$). F: female; M: male.

Construct	Sex	Percentage of fish spawned		
		Treated	Untreated	Combined
Nanos-nanos	F	16.7% (2/12)	37.9% (11/29)[a]	31.7% (13/41)
	M	25.0% (5/20)	33.3% (7/21)	29.3% (12/41)
	Combined	21.9% (7/32)	36.0% (18/50)[A]	30.5% (25/82)
Nanos-dnd	F	26.3% (5/19)	50.0% (3/6)	32.0% (8/25)
	M	21.7% (5/23)	20.0% (2/10)	21.2% (7/33)
	Combined	23.8% (10/42)	31.3% (5/16)	25.9% (15/58)

	F	-	10.5% (2/19)[b]	-
Dazl-vasa	M	-	12.5% (2/16)	-
	Combined	-	11.4% (4/35)[B]	-

There was no significant difference in the gonad development (as determined by GSI) of fish in the two replicate ponds (p = 0.79, ANOVA). Thus, data from these two ponds were pooled. There was no significant difference in the GSI of fish among the different construct transferred groups. Within each construct, no effect of dox treatment (Table 1-4) and plasmid structure (circular or linear) (Table 1-5) on GSI was observed. When compared to the control fish, the mean GSI of the Nanos-nanos, Nanos-dnd and Dazl-vasa male fish were significantly lower (p < 0.05), while the mean GSI of the females was not significantly different (p = 0.65) from that of control fish (Table 1-4). The fish with a gonad development score equal or less than 2 were thought to be infertile., although the observed percentage of fish with gonad development score equal or lower than 2 in the constructs transferred groups was higher than in the control group for the female channel catfish, the difference was not statistically significant (p = 0.3). For the male fish, none of the 13 non-transgenic control fish had a gonad development score equal or lower than 2, while all the construct transferred groups had near 50% of fish with a low score, which is significantly higher than the control (p < 0.05). There was no significant difference (p > 0.05) among the different construct transferred fish within the same sex (Table 1-6). Since body weight was a key element related to gonad development, the mean body weight was compared between the construct exposed groups and control fish. There was no significant difference (p > 0.05) in the mean body weight among the different construct exposed female fish, but they were all lower (p < 0.05) than the mean body weight of the control fish. For male fish, there was no significant difference in body weight between the construct transferred fish and control fish (Table 1-7).

Table 1-4 Means with standard deviation of gonadosomatic index (GSI) of channel catfish (*Ictalurus punctatus*) at 4-years of age. Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline (dox) during embryogenesis. Control fish were exposed to TE buffer and did not receive dox treatment. All fish were evenly distributed and grown in two replicate ponds at the same density. GSI = weight of gonad*100/weight of body. Two-way ANOVA with the body weight as a covariance was used to test the difference among different groups and Tukey's test was used for multiple comparison. No significant ($p > 0.05$) difference was observed in GSI of fish treated or not treated with dox within the same construct and sex. There was no significant ($p > 0.05$) difference in the mean GSI among the female transgenic constructs exposed fish and control fish, while the transgenic constructs transferred male fish had significantly lower mean GSI when compared to the control. Different letters following the means indicate a significant difference ($p < 0.05$).

Construct	Sex	Mean GSI		
		Dox treated	Not treated	Combined
Nanos-nanos	F	5.40 ± 4.34	6.08 ± 3.92	5.86 ± 4.04
	M	0.09 ± 0.08	0.19 ± 0.14	0.15 ± 0.13[a]
Nanos-dnd	F	4.97 ± 3.61	5.86 ± 4.48	5.29 ± 3.92
	M	0.15 ± 0.13	0.18 ± 0.14	0.16 ± 0.13[a]
Dazl-vasa	F	-	4.97 ± 3.94	4.97 ± 3.94
	M	-	0.12 ± 0.11	0.12 ± 0.11[a]
Control	F	-		6.74 ± 2.56
	M	-		0.35 ± 0.11[b]

Table 1-5 Means with standard deviation of gonadosomatic index (GSI) for the 4-year-old channel catfish (*Ictalurus punctatus*) which had been exposed to circular or linear plasmids. Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline during embryogenesis. All fish were evenly distributed and grown in two replicate ponds at the same density. GSI = weight of gonad*100/weight of body. Two-way ANOVA with the body weight as a covariance was used to test the difference of GSI among the fish exposed to circular or linear plasmid within each construct and sex. There was no significant ($p > 0.05$) difference in the mean GSI between the fish transferred with circular or linear plasmid for both females and males except that female and male fish treated with linear Dazl-vasa plasmid had marginally significant lower GSI than their counterparts treated with circular plasmid ($p = 0.087$ and 0.056, respectively).

Construct	Sex	Mean GSI	
		Circular	Linear
Nanos-nanos	F	6.78 ± 4.56	4.96 ± 3.28
	M	0.15 ± 0.13	0.15 ± 0.14
Nanos-dnd	F	6.75 ± 4.09	4.17 ± 3.45
	M	0.15 ± 0.14	0.17 ± 0.13
Dazl-vasa	F	6.92 ± 3.90	2.53 ± 2.38
	M	0.22 ± 0.12	0.07 ± 0.08

Table 1-6 Percentages of P1 channel catfish (*Ictalurus punctatus*) with gonad development score equal or lower than 2 at the age of 4. Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline (dox) during embryogenesis. Control fish were exposed to TE buffer and did not receive dox treatment. All fish were evenly distributed and grown in two replicate ponds at the same density. A score from 0 (worst) to 5 (best) was given to the fish according to the size of gonad, amount of gametes in the gonad, and the quality of gametes. Fisher's exact test was used to test the difference among different groups. There was no significant difference in the percentage among the female construct exposed fish and control fish ($p > 0.05$). There was no significant ($p > 0.05$) difference in the percentage among the male fish exposed to different constructs, but it was higher ($p < 0.05$) in the constructs exposed fish when compared to the control male. Percentages in the same column followed by different letters are significantly different ($p < 0.05$).

Construct	Female			Male		
	Total N	N ≤ 2	% ≤ 2	Total N	N ≤ 2	% ≤ 2
Control	12	1	8.3	13	0	0.0[a]
Nanos-nanos	59	16	27.1	48	26	54.2[b]
Nanos-dnd	50	17	34.0	45	20	44.4[b]
Dazl-vasa	25	9	36.0	30	17	56.7[b]

Table 1-7 Mean body weight with standard deviation of the 4-year-old channel catfish (*Ictalurus punctatus*). Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline (dox) during embryogenesis. Control fish were exposed to TE buffer and did not receive dox treatment. All fish were evenly distributed and grown in two replicate ponds at the same density. One-way ANOVA was used to test the difference among differenct groups within the female or male population. The female fish transferred with the three constructs had significantly lower mean body weight than the control ($p < 0.05$). There was no significant difference between the construct exposed and control males. Means followed by different letters are significantly different ($p < 0.05$).

Construct	Mean body weight (kg)	
	Female	Male
Nanos-nanos	0.68 ± 0.32^{a}	0.99 ± 0.64
Nanos-dnd	0.68 ± 0.39^{a}	1.00 ± 0.61
Dazl-vasa	0.63 ± 0.28^{a}	0.89 ± 0.43
Control	1.33 ± 0.42^{b}	1.26 ± 0.73

Most (34 out of 42) of the female fish with a low gonad development score (≤ 2) had a low body weight (< 0.5 kg), with only a few females that were large (body weight ≥ 0.5 kg) but had little gonadal development in the Nanos-nanos and Dazl-vasa groups (6 and 2, respectively). In the male transgene exposed populations, large fish (body weight ≥ 0.5 kg) with under developed gonads (score ≤ 2) were more frequently (65.1%) observed (Fig. 1-8~1-10, and Appendix 1).

Both the sexual maturity assessment at three years of age and the gonad development evaluation of the fish at four years of age showed that the dox treatment during the embryogenesis did not make a difference on the gonad development, indicating the tet-off system used in this experiment did not work well for regulating the expression of the Bax gene. The low efficiency of turning off the transgene expression could be due to leakage of the tet-off system, improper dosage or timing of dox treatment, or a combination of these reasons. The leakage of the tet-on/off system had been reported widely and was thought to be the major disadvantage of this system [107–111]. These constructs were produced before the availability of tight TRE. In addition to the design (the structure of the tTA and TRE) of the tet system, the leakiness can be affected by the strain and species of the organism operating, the site of integration, the tetracycline analogue used for induction, and the promoter driving the tTA expression [111,112]. If leakage of the constructs occurred in the current experiment, it would have partially contributed to failure of repression of the transgene expression by dox treatment. The constructs used in this experiment had been tested on zebrafish and were proven to efficiently drive or repress the expression of GFP through the treatment of 100 ppm dox. However, the efficiency and leakiness of the constructs, as well the optimal dosage of dox treatment for turning off the transgene need to be re-evaluated on channel catfish as they have different size eggs and the

transgenic constructs were designed based on the gene sequences of zebrafish. However, earlier studies with a similar tet-off system were successful in channel catfish (Dunham et al., unpublished). Thus, some environmental or genetic effect or the interaction between dox and the Bax may be responsible for these variable results.

Regardless, when the embryos are treated with dox to produce fertile broodstock, or not treated with dox to sterilize the fish, the first step of the proper function of the constructs is the expression of tTA driven by the nanos or dazl promoter. The nature of regulation characteristics of these two genes determines the timing and efficacy of tTA expression. Evidence of zygotic expression of the nanos gene during embryogenesis is rare. The only reference indicating the zygotic expression of the nanos gene was found in leech, which has a distant genetic relationship with channel catfish and a different pattern of gonad development, is hermaphroditic and has no obvious segregation of PGC precursors in early development [48]. The nanos1 mRNA in zebrafish PGCs gradually decreased over time and was not detectable by 5 dpf [113], indicating the nanos mRNA needed for embryogenesis might be all maternally derived. Otherwise there should be a rise of nanos mRNA at some time point when zygotic expression starts.

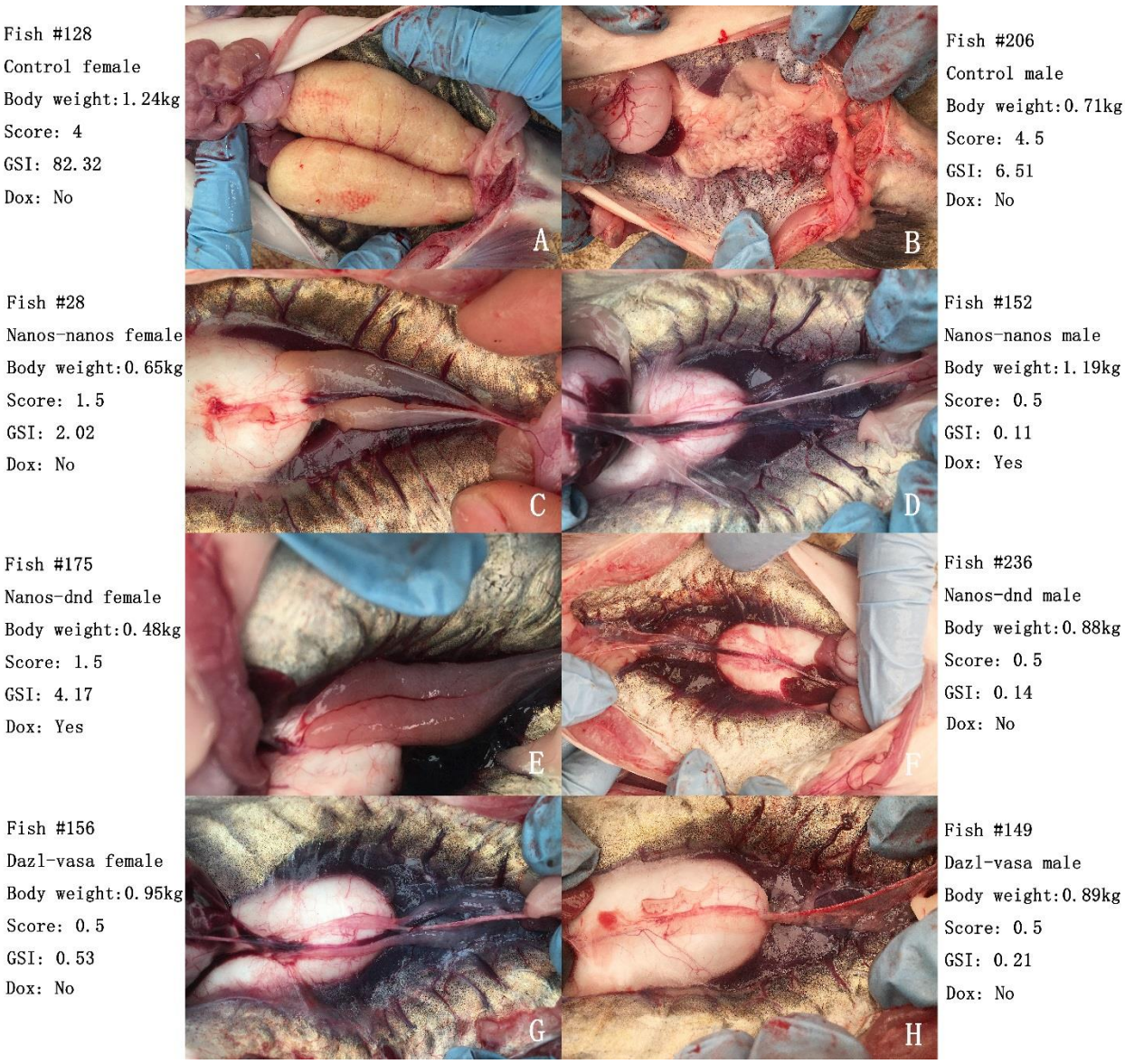

Fig. 1-8 Gonad morphology of the 4-year-old Nanos-nanos, Nanos-dnd, or Dazl-vasa construct exposed channel catfish (*Ictalurus punctatus*) with underdeveloped gonad in comparison to the normally developed control fish. Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline (dox) during embryogenesis. Control fish were exposed to TE buffer and did not receive dox treatment. All fish were evenly distributed and grown in two replicate ponds at the same density. The left panel shows the female fish and the right panel shows the male fish.

A, B: control fish; C, D: Nanos-nanos fish; E, F: Nanos-dnd fish; G, H: Dazl-vasa fish; Score: A score from 0 (worst) to 5 (best) given to the fish according to the size of gonad, amount of gametes in the gonad, and the quality of gametes; GSI: gonadosomatic index = weight of gonad*100/weight of body.

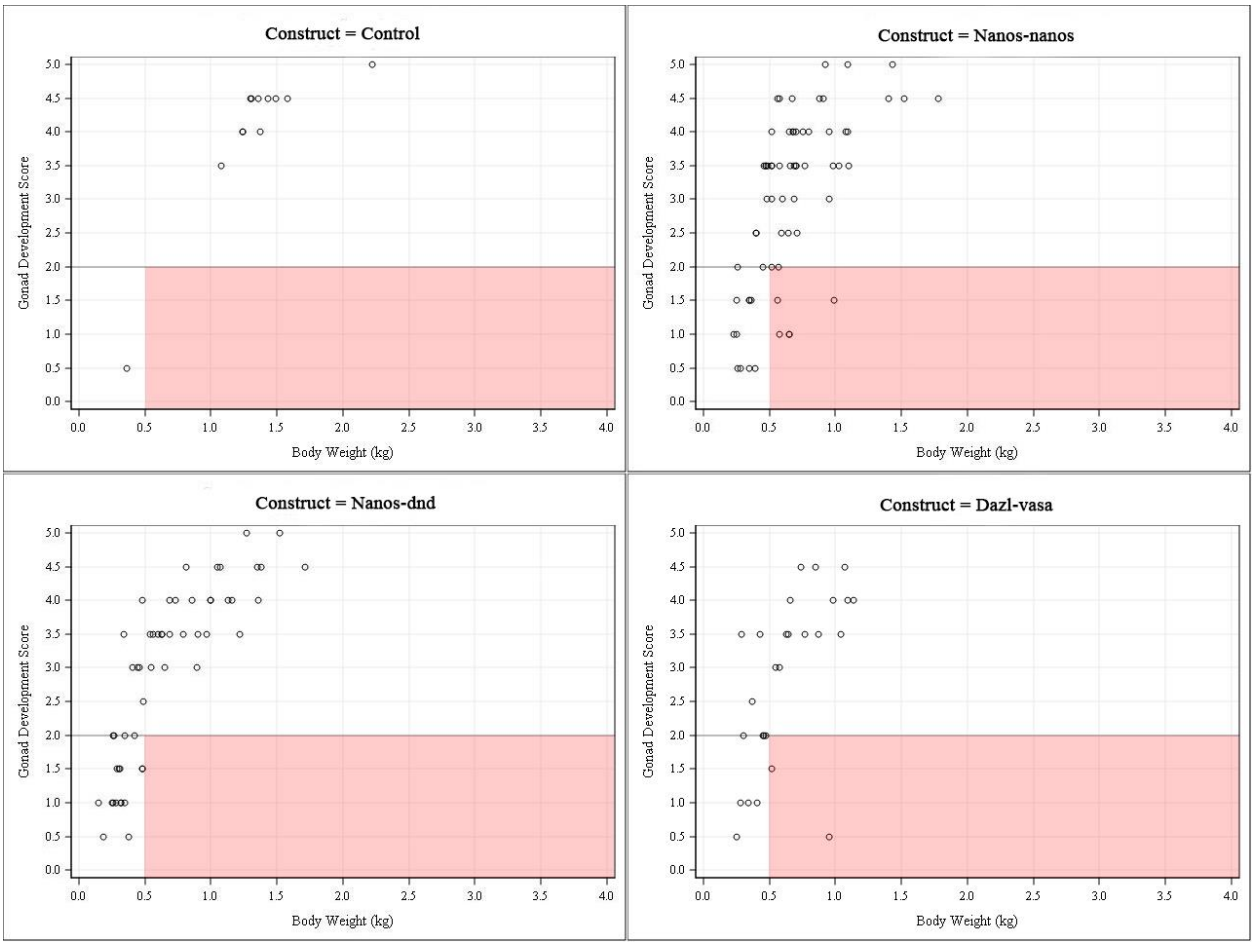

Fig. 1-9 Gonad development score by body weight for the 4-year-old female channel catfish (*Ictalurus punctatus*). Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline (dox) during embryogenesis. Control fish were exposed to TE buffer and did not receive dox treatment. All fish were evenly distributed and grown in two replicate ponds at the same density. A gonad development score from 0 (worst) to 5 (best) was given to the fish according to the size of gonad, amount of gametes in the gonad, and the quality of gametes. Highlighted area shows the range in which the body weight is higher than 0.5kg and the gonad development score is smaller than 2. No control fish was located in this area, and a few individuals in the Nanos-nanos and Dazl-vasa groups were distributed in this area.

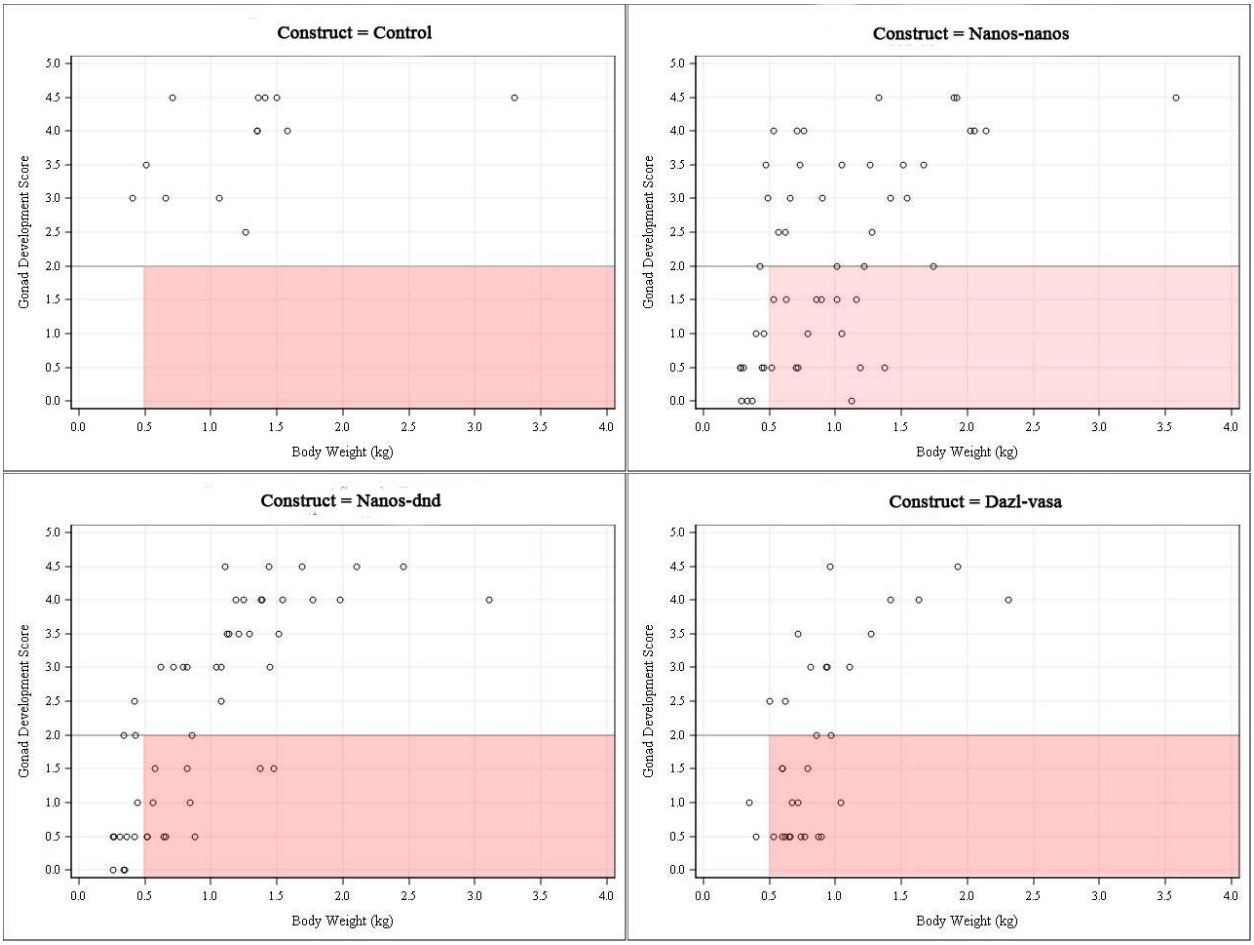

Fig. 1-10 Gonad development score by body weight for the 4-year-old male channel catfish (*Ictalurus punctatus*). Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline (dox) during embryogenesis. Control fish were exposed to TE buffer and did not receive dox treatment. All fish were evenly distributed and grown in two replicate ponds at the same density. A gonad development score from 0 (worst) to 5 (best) was given to the fish according to the size of gonad, amount of gametes in the gonad, and the quality of gametes. Highlighted area shows the range in which the body weight is higher than 0.5kg and the gonad development score is smaller than 2. No fish from the control group was located in this area,

while a remarkable number of fish from the sterilization construct exposed groups were distributed in this area.

Since only the untreated fish were available in the Dazl-vasa group, it was not possible to compare between the treated and untreated groups. Though not always statistically significant at the 95% confidence level, the spawning percentages of the 3-year-old untreated Dazl-vasa fish were only 10.5% and 12.5% for the female and male respectively, which were lower than the Nanos-nanos and Nanos-dnd groups, which were dox treated or not treated (Table 1-3). Since the body weight is an important factor that can affect the fecundity and maturity of catfish, we compared the body weight of the Dazl-vasa fish with that of the Nano-nanos and Nanos-dnd fish. No significant difference was observed, indicating the difference resulted from the different efficiency of the constructs. In *Xenopus*, maternally derived dazl disappeared after gastrulation, and zygotic expression of dazl was thought to be initiated in the later embryogenesis stage when PGCs have migrated to the genital ridge [58,59]. The relatively early zygotic activation of the dazl promoter may explain the lower spawning rate of the untreated Dazl-vasa fish. The effectiveness of the tet-off controlling system needs to be further evaluated by comparison with the treated group. It is also not possible to evaluate the effectiveness of the tet-off system on the F1 generation as the large amount of maternally derived Bax protein and mRNA will mask the effect of the zygotic expression which is the part could be controlled by dox treatment during embryo stage. The expression of the transgene could be transient without integrating into the genome or stable expression after integrating into the genome. The expression of the transgene could be from the plasmids in the cytoplasm as episomes, as well as from the transgene integrated into the genome. The cytoplasm located plasmids expression should be short-term and gradually decreased over time because of degradation and reduced amount of plasmid in each cell due to cell proliferation. The later zygotic expression of the Bax gene may be sufficient for ablating PGCs if the functional sequences (the sequences driving tTA production and the Bi-

TRE-Bax part) are integrated into the genome, but there should only be a small proportion of fish ablated in this case, given the low integration rate of the transgene observed in other species and the existence of mosaicism [114,115].

Transgene screening of the P1 and F1 fish

The transgene was identified by PCR and confirmed by sequencing (Fig. 1-11~1-19). The results showed that the transgene construct was not always integrated into the genome as a whole, and some area of the plasmid seems to have a higher chance of recombination with the genomic DNA, as revealed by the different percentages of positive samples by the primers designed from two different regions of the constructs. Different constructs also had variable integration rate (Table 1-8). For the Nanos-nanos construct exposed P1 females, 33 of 44 (75%) fin samples were recognized as positive by at least one of the primer pairs designed from the nanos promoter-tTA and Bax gene-nanos 3' UTR regions, respectively. Among these fish, 19 fish (43.18%) had only the nanos promoter-tTA fragment integrated and 5 fish (11.36%) had only the Bax gene-nanos 3' UTR part integrated, 9 fish (20.45%) had both parts integrated. In the male fin samples, 11 of 20 (55%) had the nanos promoter-tTA fragment integrated and 1 fish (5%) had the Bax gene-nanos 3' UTR part integrated, none of these fish had both parts integrated. In the testis samples, 15 of 36 (41.67%) had the nanos promoter-tTA fragment integrated and 3 fish (8.33%) had the Bax gene-nanos 3' UTR part integrated, none of these fish had both parts integrated.

In the Nanos-dnd construct exposed P1 fish population, 21 of 28 (75%) female fish had at least part of the plasmid integrated into the genome in the fin. Among these fish, 14 fish (50%) had

only the nanos promoter-tTA fragment integrated and 1 fish (3.57%) had only the Bax gene-dnd 3' UTR part integrated, 6 (21.43%) had both parts integrated. In the fin samples of males, 13 of 23 (56.52%) were recognized as positive by at least one of the two primer pairs. Among them, 8 samples (34.78%) were only nanos-tTA positive, 2 samples (8.69%) were Bax-dnd positive, and 3 samples (13.04%) had both parts integrated. In the testis samples, 13 of 39 (33.33%) were recognized as positive by at least one of the two primer pairs. Among them, 8 samples (20.51%) were only nanos-tTA positive, 2 samples (5.13%) were Bax-dnd positive, and 4 samples (10.26%) had both parts integrated.

In the Dazl-vasa group, 5 of 17 (29.41%) females had at least part of the plasmid integrated into the genome in fin samples. Among these fish, 2 fish (11.76%) had only the Dazl promoter-tTA fragment integrated and 2 (11.76%) fish had only the Bax gene-vasa 3' UTR part integrated, 1 (5.88%) had both parts integrated. For the male fish, none of the fins of 14 fish had any part of the plasmid integrated. In the testis samples, 2 of 16 (12.5%) were Dazl-tTA positive, none were Bax-vasa positive.

There was no transgene identified from the egg samples, likely due to DNA isolation problems associated with the yolk. However, we were able to evaluate the patterns of transgenic plasmid integration into the female germ cells by analyzing the transgenic patterns of its F1 populations.

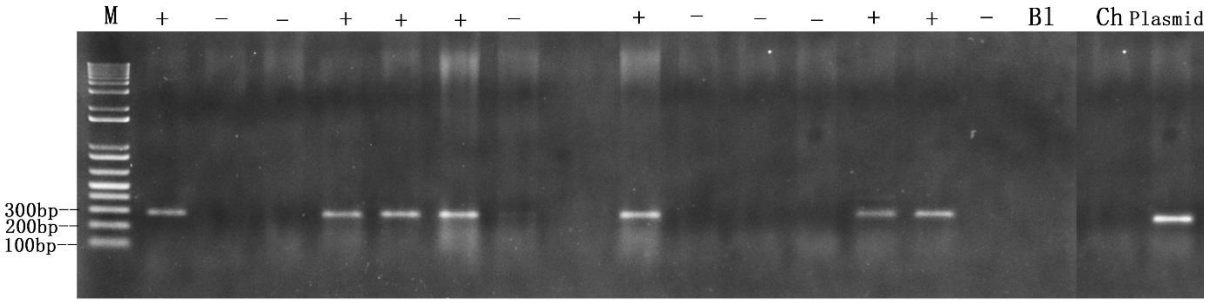

Fig. 1-11 Identification of Nanos-nanos transgenic channel catfish (*Ictalurus punctatus*) with primers targeting the Nanos promoter-tTA region. Forward and reverse primers were designed from the nanos promoter and tTA sequence region, respectively. The size of target amplicon is 283bp. M: DNA ladder; +: transgene positive; -: transgene negative; Ch: Normal channel catfish control, Bl: blank control; Plasmid: positive control using plasmid as template.

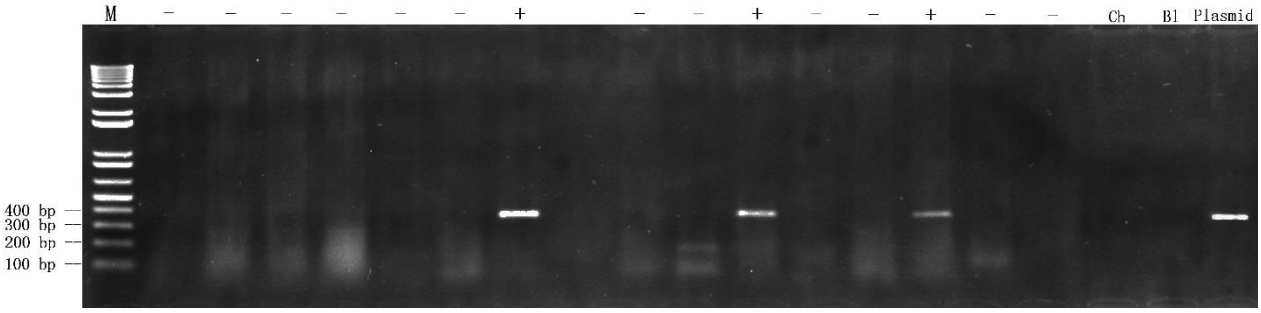

Fig. 1-12 Identification of Nanos-nanos transgenic channel catfish (*Ictalurus punctatus*) with primers targeting the Bax gene-nanos 3' UTR region. Forward and reverse primers were designed from the Bax gene sequence and nanos 3' UTR region, respectively. The size of target amplicon is 336bp. M: DNA ladder; +: transgene positive; -: transgene negative; Ch: Normal channel catfish control, Bl: blank control; Plasmid: positive control using plasmid as template.

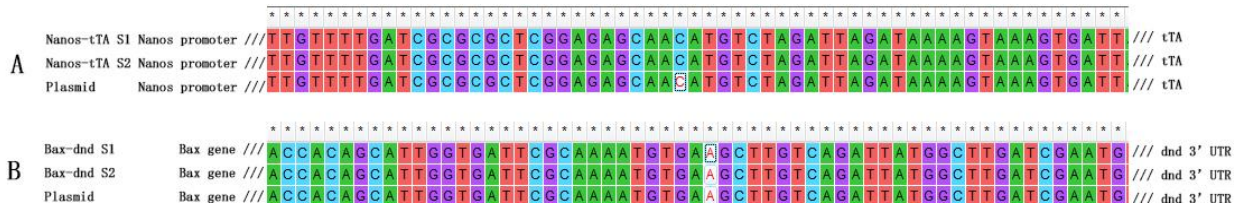

Fig. 1-13 Alignment of PCR product sequences with the corresponding plasmid sequences for the Nanos-nanos positive channel catfish (*Ictalurus punctatus*). A: PCR amplicons by the primer Nanos-tTA. B: PCR amplicons by the primer Bax-nanos. Two samples (S1, S2) for each primer pair were sequenced. The sequencing results confirmed that the amplicons amplified were the target fragment of the designed transgene specific primers.

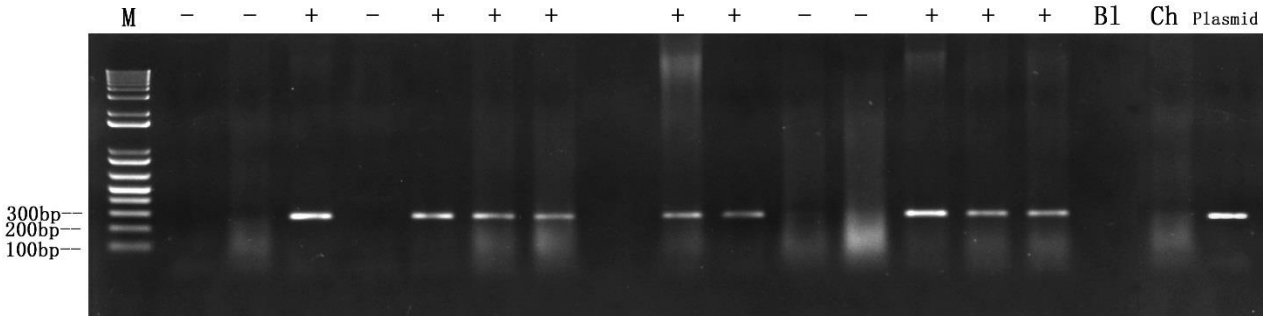

Fig. 1-14 Identification of Nanos-dnd transgenic channel catfish (*Ictalurus punctatus*) with primers targeting the Nanos promoter-tTA region. Forward and reverse primers were designed from the nanos promoter and tTA sequence region, respectively. The size of target amplicon is 283bp. M: DNA ladder; +: transgene positive; -: transgene negative; Ch: Normal channel catfish control, Bl: blank control; Plasmid: positive control using plasmid as template.

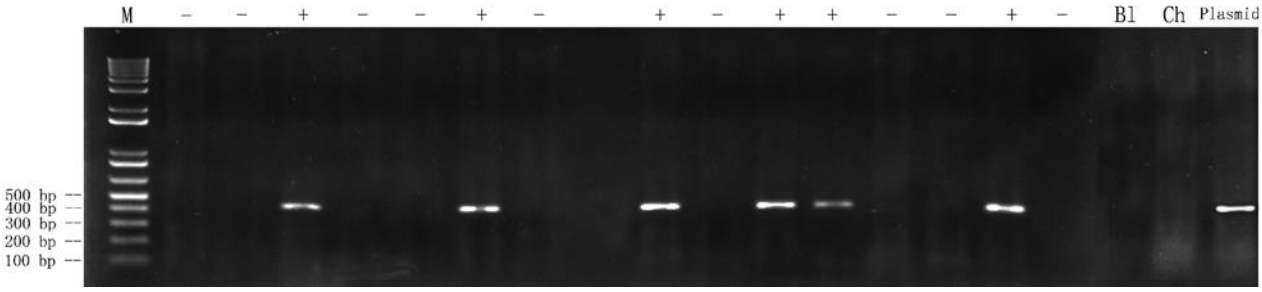

Fig. 1-15 Identification of Nanos-dnd transgenic channel catfish (*Ictalurus punctatus*) with primers targeting the Bax gene-dnd 3' UTR region. Forward and reverse primers were designed from the Bax gene sequence and dnd 3 'UTR region, respectively. The size of target amplicon is 477bp. M: DNA ladder; +: transgene positive; -: transgene negative; Ch: Normal channel catfish control, Bl: blank control; Plasmid: positive control using plasmid as template.

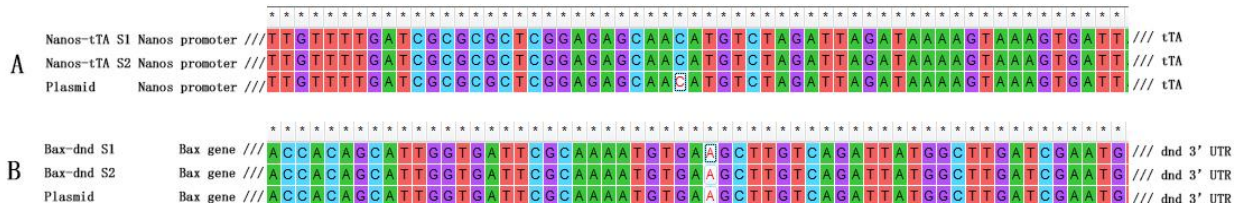

Fig. 1-16 Alignment of PCR product sequences with the corresponding plasmid sequences for the Nanos-dnd positive channel catfish (*Ictalurus punctatus*). A: PCR amplicons by the primer Nanos-tTA. B: PCR amplicons by the primer Bax-dnd. Two samples (S1, S2) for each primer pair were sequenced. The sequencing results confirmed that the amplicons amplified were the target fragment of the designed transgene specific primers.

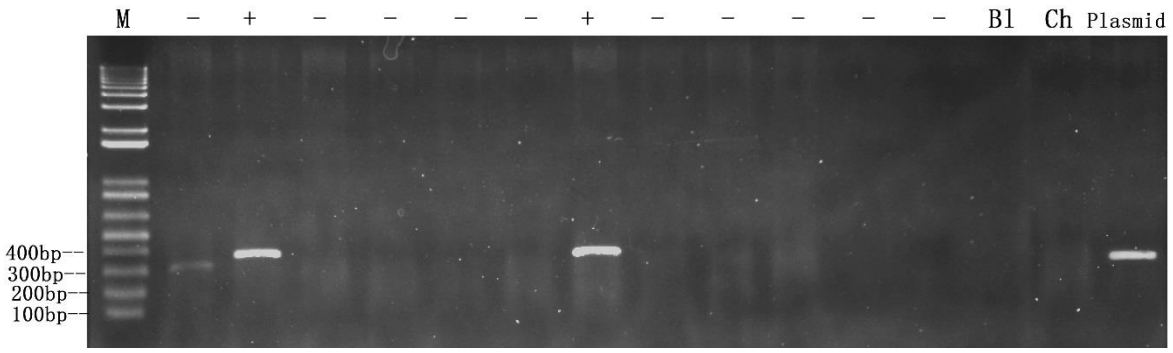

Fig. 1-17 Identification of Dazl-vasa transgenic channel catfish (*Ictalurus punctatus*) with primers targeting the Dazl promoter-tTA region. Forward and reverse primers were designed from the dazl promoter and tTA sequence region, respectively. The size of target amplicon is 360bp. M: DNA ladder; +: transgene positive; -: transgene negative; Ch: Normal channel catfish control, Bl: blank control; Plasmid: positive control using plasmid as template.

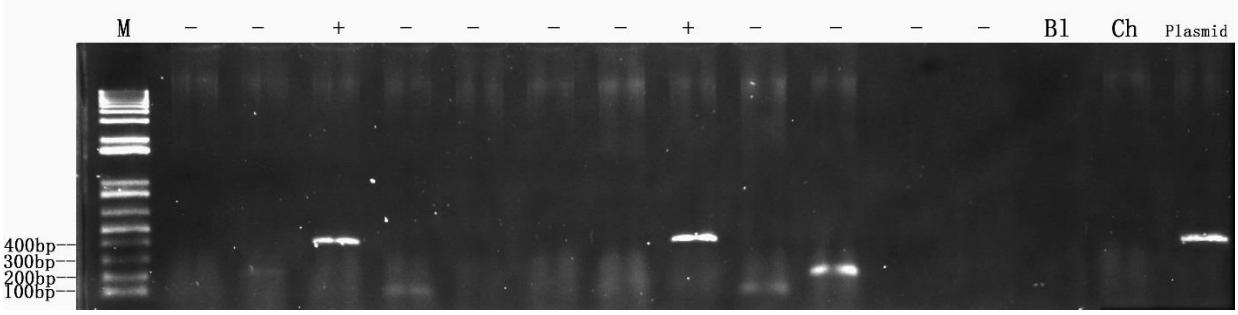

Fig. 1-18 Identification of Dazl-vasa transgenic channel catfish (*Ictalurus punctatus*) with primers targeting the Bax gene-vasa 3' UTR region. Forward and reverse primers were designed from the Bax gene sequence and vasa 3' UTR region, respectively. The size of target amplicon is 406bp. M: DNA ladder; +: transgene positive; -: transgene negative; Ch: Normal channel catfish control, Bl: blank control; Plasmid: positive control using plasmid as template.

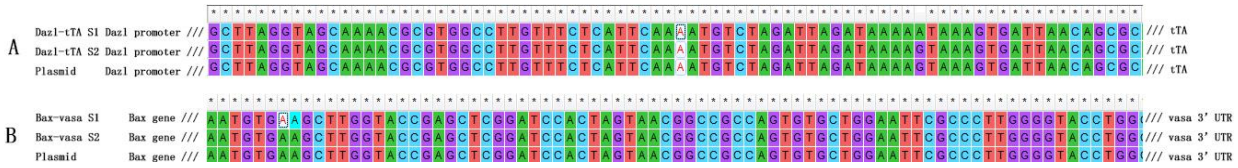

Fig. 1-19 Alignment of PCR product sequences with the corresponding plasmid sequences for the Dazl-vasa positive channel catfish (*Ictalurus punctatus*). A: PCR amplicons by the primer Dazl-tTA. B: PCR amplicons by the primer Bax-vasa. Two samples (S1, S2) for each primer pair were sequenced. The sequencing results confirmed that the amplicons amplified were the target fragment of the designed transgene specific primers.

Table 1-8 Summary of transgene positive percentages in the P1 generation channel catfish (*Ictalurus punctatus*) transferred with Nanos-nanos, Nanos-dnd, or Dazl-vasa constructs at 1 cell stage via electroporation. PCR was conducted to identify the transgene in the genomic DNA extracted from different tissues, using primers targeting the promoter-tTA and Bax-3' UTR regions. The numbers in the bracket refer to number of positive samples/ number of total samples checked for each group. Fin and testis: identified as positive both in the fin and testis of the same fish.

Construct	Sample	Promoter-tTA	Bax-3' UTR	Both primers
Nanos-nanso	Female(fin)	63.6% (28/44)	31.8% (14/44)	20.5% (9/44)
	Male(fin)	55.0% (11/20)	5.0% (1/20)	0.0% (0/20)
	Male(testis)	41.7% (15/36)	8.3% (3/36)	0.0% (0/36)
	Male (fin & testis)	20.0% (4/20)	0.0% (0/20)	0.0% (0/20)
Nanos-dnd	Female(fin)	71.4% (20/28)	25.0% (7/28)	21.4% (6/28)
	Male(fin)	47.8% (11/23)	21.7% (5/23)	13.0% (3/23)
	Male(testis)	30.8% (12/39)	15.4% (6/39)	10.3% (4/39)
	Male (fin & testis)	4.4% (1/23)	0.0% (0/23)	0.0% (0/23)
Dazl-vasa	Female(fin)	17.6% (3/17)	17.6% (3/17)	5.9% (1/17)
	Male(fin)	0.0% (0/14)	0.0% (0/14)	0.0% (0/14)
	Male(testis)	12.5% (2/16)	0.0% (0/16)	0.0% (0/16)
	Male (fin & testis)	0.0% (0/14)	0.0% (0/14)	0.0% (0/14)

There were a total of 15, 12 and 4 F1families generated from the P1 fish exposed to the Nanos-nanos, Nanos-dnd or Dazl-vasa construct, respectively. The positive percentages of the F1 progeny possessing at least part of the transgene fragment ranged from 0% to 90% among different families, while the percentage of fish with both the two parts (promoter-tTA and Bax-3' UTR) required for proper function of the transgene was generally lower, ranged from 0% to 60% (Table 1-9). Theoretically, highest transgene transmission rate in the F1 progeny would be 100% if the transgenic parent is homozygous or has insertion sites on multiple chromosomes, and 50% if heterozygous when paired with a wild type mate. However, the observed rates are usually lower than these numbers because of mosaicism in founder transgenic animals, which is universal [115–118]. Because of the late integration of the transgene into the genome, the transgene will likely to only be carried by some tissues or by some fraction of the cells in the same tissue. In this experiment, since P1 females were mated with partially transgenic (carry only the promoter-tTA region of the transgene) or non-transgenic males to produce the F1 progeny, in some cases the transgene transmission of the female fish was evaluated based on the Bax-3' UTR region. All fertile females identified transgenic by PCR screening of fin samples transmitted the transgene to the next generation, but at different efficiencies, indicating varying degree of germline mosaicism among each P1 individual. The half-sib families from the same dam had similar percentages of transgenic individuals. Though some F1 populations derived from females, which were identified as negative by PCR of the fin tissue with a certain primer pair, were 100% negative, a considerable portion of these families had positive offspring recognized by the same primer, illustrating the tissue mosaicism. These results confirmed

previous research that indicated that mosaicism exists among different tissues, and also within the same tissue of the P1 fish.

Early identification of the transgenic fish is important as keeping large numbers of individuals, which may contain only a small portion of transgenic individuals, is costly and labor consuming, as well as inefficient in regards to spawning experimental fish. Early sampling of gonads from P1 fish for transgene identification has inherent survival risks. Because of these reasons, fin, barbel or other easy biopsy and low-risk sampling tissues are usually used to identify potential transgenic fish for generating next generation. The high mosaic rates observed in this experiment suggest that, at least in some cases, prediction of the transgene in the gonad by DNA analysis on other tissues needs to be used with caution and an evaluation of mosaicism is suggested.

Both nanos and dazl proteins were reported to be expressed not only in the PGCs during embryogenesis, but also in the gonads of adult female and male and play important roles in the germ cell maintenance and gametogenesis in fish and mouse [40–42,59,119–123]. Thus the Bax gene construct, if successfully integrated into the genomes, is supposed to be expressed in the germ cells during the gametogenesis and should lead to the killing of these cells. The proper function of the constructs requires both the promoter-tTA and Bax-3' UTR to be integrated into the genome.

In 9 of the 15 F1 families that were derived from 12 females mated with non-transgenic males, individuals with both of the two crucial parts integrated were observed (Fig. 1-9), indicating the complete integration of the Bax constructs did not lead to apoptosis of the female germ cells. In

contrast, only 4 out of the 91 testis samples tested were identified as positive by both primers. Assuming the chance of transgene integrating into the germ cell genome is equal between the female and male fish, the different positive rates of female and male gonad might be due to the different tolerance of female and male germ cells to apoptosis. Male germ cells might be more sensitive to the overexpression of the Bax gene. This is in agreement with results we observed previously that 4-year-old males transferred with sterilization construct had significantly smaller GSI (Table 1-4) and lower percentage of fish with a gonad development score ≤ 2 than the control fish, while no significant difference was observed in the females (Table 1-6, Fig. 1-9, 1-10).

The programmed apoptosis of germ cells is a common phenomenon in the developing gonad and is essential for the homeostasis of the size and quantity of each type of cells. The bcl-2 protein family members were thought to be the key players in this process [124]. For most mammals, which have only a few eggs ovulated during each menstrual cycle, the regulation of apoptosis is mainly reached through the expression of the pro-apoptosis and anti-apoptosis proteins. The change of the ratio between pro-survival and pro-apoptosis proteins had been widely observed in oocytes undergoing apoptosis [125–128]. However, anti-apoptosis mechanisms also exist in normal oocytes which are chosen to survive during some developing stages [129]. Notably, a natural apoptosis suppression mechanism highlighted with markedly expressed pro-apoptosis gene Bcl-2 and weak expression of the pro-apoptosis gene Bax was found in the ovary of South American plains viscacha *Lagostomus maximus*, which is a mammalian species, but ovulates 400~800 eggs during each cycle [130].

Table 1-9 Summary of sex ratio and percentages of positive fish in the F1 channel catfish (*Ictalurus punctatus*) families. F1 families were generated from mating of female and male P1 fish treated with the same sterilization construct (Nanos-nanos, Nanos-dnd, or Dazl-vasa). In some cases, eggs from one female were fertilized with sperm from several males or vice versa to make half-sib populations. Families were named by their parents. For instance, F18B4: female #18 mated with male B4. Transgene was identified by two pairs of primers targeting the promoter-tTA and Bax-3' UTR region, respectively. * indicates a family derived from a non-transgenic sire; ** indicates a sex ratio significantly different from 1:1.

Construct	Family	% Promoter-tTA positive	% Bax-3' UTR positive	% Both positive	Sex ratio (M/F)
Nanos-nanos	F18B4*	90	20	10	2.2**
	F52B11	60	20	20	0.85
	F47B5	30	20	10	1.25
	F31B1*	30	50	20	1
	F45B9*	30	10	0	0.84
	F28A3	20	30	10	1.07
	F32B14*	70	10	0	1.15
	F53B5	80	20	20	1
	F27B11	40	0	0	1.2
	F13A3	30	0	0	1
	F13A13	50	0	0	1.7
	F19B4*	20	40	10	1.64
	F19B5	30	40	10	-
	F48A3	43.94	19.7	4.54	-
	F48A13	85.71	17.14	17.14	-
Nanos-dnd	F23B3*	80	10	10	1.26
	F7A1	80	40	30	1.25
	F7A7*	60	40	30	1.12
	F12A9	20	20	10	3**
	F12A8	40	20	10	2
	F10A1	60	60	40	0.7
	F8A1	60	30	10	1.08
	F8A7*	70	30	30	1
	F2A9	80	30	20	0.69
	F25B8*	60	50	30	1.25

	F57B6*	72.73	63.64	54.54	1.33
	F57B10*	90	70	60	1.16
Dazl-vasa	F21B6*	20	20	0	0.92
	F21B16*	10	0	0	1.12
	F36B15*	20	0	0	0.75
	F36B16*	20	10	0	1

Similarly, apoptosis of germ cells in fish at certain stages is a necessary process for homeostatic maintenance of the reproductive system [131–133], and multiple mechanisms are involved in suppressing the apoptosis of oocytes [134]. Even though there is no reference indicating if there is apoptosis suppression strategy similar to *L. maximus* in fish, it has been proven that the oocyte is more resistant to apoptosis than many other types of cells and the overexpression of Bax alone is not enough to accelerate the apoptosis of the oocyte. During development of male germ cells, apoptosis is also a common way of eliminating deficient cells and the frequency of apoptosis in male germ cells is even higher than in the female. The different regulation of apoptosis was thought to be due to the variant surrounding gonadal environments [124,135,136]. Also, cytochrome c as a downstream inducer of the mitochondrial apoptosis pathway, is three to five times more effective in spermatozoa than in somatic cells [137]. Therefore, the significantly lower transgenic rate observed in the male testis samples was likely due to the apoptosis of the male germ cells, which had the transgene integrated. This is in agreement with the gonad development evaluation data, as the transgene constructs exposed male fish had significantly lower ($p < 0.05$) mean gonad development score than control fish, while there was no significant ($p > 0.05$) difference between the transgene construct exposed and control female fish.

Sex ratio of the F1 families.

Most of the F1 families had a sex ratio close to 1:1 (Table 1-9). Interestingly, two families had biased sex ratios with more males than females. One is the Nanos-nanos family F18B4, which had a significant male skewed sex ratio of 2.2:1 ($p = 0.05$). The other was the Nanos-dnd family F12A9, for which 24 out of the 32 fish were male, which is significantly deviated to the theoretic 50% male percentage ($p = 0.007$). It has been demonstrated that a minimum threshold number of

PGCs is required for keeping the female status of zebrafish, and the ablation of PGCs could lead to the transformation of female to male [138,139]. Germ cell dependent sex differentiation was also observed in medaka and tilapia [140,141]. Genetic sex verification using the sex-associated marker showed there was no sex reversed individuals in the male-skewed population (Fig. 1-20). Thus, the extra males observed were not sex reversed due to PGCs apoptosis, assuming that the sex marker is universal. The skewed sex ratio may be a result from sex-biased (female-biased in this case) mortality or genetic parental effects on sex ratio as these phenomena have been observed in other species[142,143].

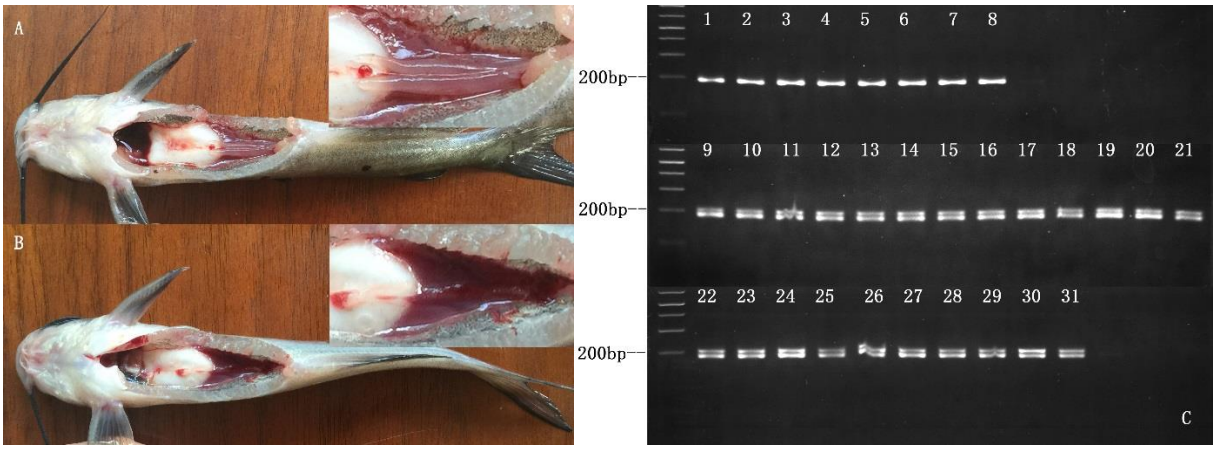

Fig. 1-20 Identification of the phenotypic and genetic sex of the F1 channel catfish (*Ictalurus punctatus*) individuals from families with male skewed sex ratio. F1 families were generated from mating female and male P1 fish treated with the same sterilization construct (Nanos-nanos, Nanos-dnd, or Dazl-vasa). A fish with clear ovary was identified as female phenotype (A) and a fish with no clear gonad was identified as male phenotype (B). Genetic sex was identified by PCR using a sex-associated marker for channel catfish, genetic female has one band with a size of 212bp, and male has an additional band at 205bp. The phenotypic sex of the tested individuals were all consistent with their genetic sex. Samples 1 - 8 in picture C were the fish with a male phenotype, and 9 - 31 were fish with a female phenotype.

Conclusion

The overexpression of the pro-apoptosis gene Bax, specifically in the germ cells using the Nanos-nanos, Nanos-dnd, Dazl-vasa constructs led to sterilization of channel catfish and the transgene was able to be transmitted to the next generation through female gamete. However, two major flaws existed in this system making utilization of this technology limited. Firstly, the overexpression of Bax gene could not or had low efficacy for sterilizing female fish in the P1 generation, likely due to the higher tolerance of female germ cells to apoptosis. However, its effectiveness on the F1 fish remained to be evaluated as if the undifferentiated PGC is sensitive to the overexpression of Bax gene, both male and female fish will be sterile. Secondly, dox treatment during embryogenesis did not turn off the expression of the transgene as expected, which might be due to the continuous expression of the Bax gene during gametogenesis. If true, continuous dox delivery probably through feeding will be needed to make the tet-off gene expression switch work, while this is unwanted because of the consequent problems of food safety and environmental pollution. In conclusion, this technology is potentially useful for generating sterile male fish in the P1 generation and probably all PGCs ablated male and female sterile fish in the F1 generation as well, but the repression of this process using dox treatment is not feasible for producing food fish.

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Chapter 2 Reversible Sterilization of Channel Catfish (*Ictalurus punctatus*) by Overexpression of Glutamic Acid Decarboxylase (GAD) Gene

Abstract

As the key regulator at the top of the hypothalamus-pituitary-gonad (HPG) axis, gonadotrophin-releasing hormone (GnRH) is a master player in the endocrine regulation of fish reproduction. Gama-aminobutyric acid (GABA) has been proven to not only mediate migration of GnRH neuron from the olfactory placode to the hypothalamus during embryogenesis, but also is involved in the regulation of GnRH production, luteinizing hormone (LH) secretion and sex steroid feedback. To develop a transgenic sterilization technology that can be used for avoiding unwanted genetic communication of the gene engineered aquaculture animals or domestic populations with the wild populations, and at the same time keep the capacity of reproductive restoration, we sought to disrupt the normal regulation of GABA by overexpression of glutamic acid decarboxylase (GAD), which could convert glutamic acid to GABA. Three generations of GAD transgenic fish were produced. Repressed reproductive performance was observed in all generations, but was not always statistically significant. Only 5.4% of the F1 transgenic fish with a sexual maturity score ≥ 4 (maximum = 5) at the age of 5, which was lower ($p = 0.07$) than the percentage in control group (16.8%). In spawning trials conducted at the age of 6 and 9, F1 transgenic fish had 45.5% and 20.0% of fish spawned naturally, which were lower ($p = 0.09$ and 0.12, respectively) than the percentages in control fish (83.3% and 66.7%, respectively). Four of

6 pairs of the putative infertile 6-year-old fish spawned after LHRHa therapy. Similar results were observed in 3-year-old F2 fish, with a lower percentage (20.0%) of transgenic fish spawned, but the mean was not (p = 0.12) different from the control (66.7%). The 1-year-old transgenic fish had lower GnRH levels (9.23 ± 2.49 and 8.14 ± 2.21 ng/ml for the female and male, respectively) in serum than control fish (11.04 ± 4.06 and 9.03 ± 2.36 ng/ml for the female and male, respectively), but were not significantly different from the control (p = 0.15 and 0.27 for the female and male, respectively). There was no significant difference in estradiol levels of the female transgenic and non-transgenic fish both in the 1 and 4-year-old F2 generation. Again, no significant difference (p = 0.9069) of GnRH level was detected between the female transgenic and non-transgenic F2 fish at 4 years of age. However, 4-year-old F2 generation male transgenic fish had significantly (p = 0.0028 and 0.0018, respectively for GnRH and testosterone) lower levels of GnRH (1.02 ± 0.31 ng/ml) and testosterone (288.84 ± 62.62 pg/ml) than control fish (2.34 ± 1.22 ng/ml and 699.12 ± 211.90 pg/ml, respectively for GnRH and testosterone), indicating a different regulation mode of GABA on GnRH in males than females. In conclusion, overexpression of GAD repressed the reproductive performance of channel catfish, but was not 100% effective in sterilizing channel catfish.

Introduction

Similar to other vertebrates, fish reproduction is mainly regulated by reproductive hormones such as gonadotrophin-releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), estrogen and androgen through the hypothalamus-pituitary-gonad (HPG) axis [1,2]. GnRH secreted by the hypothalamus induces the release of gonadotropins, LH

and FSH, from the pituitary, which in turn bind to their receptors in the gonads and result in the secretion of sex steroid hormones [1].

Sex steroid hormones are the ultimate effectors that directly act on the gonad and regulate its development [3]. The development of germ cells from the undifferentiated primordial germ cells to mature gametes is mainly divided into two major steps: the growing phase in which the germ cells go through proliferation, growth and differentiation, and the final maturation phase in which oocytes and spermatozoa mature and are ready for release. Renewal of spermatogonia is regulated by estradiol-17β (E2), which is converted from androgen in the Sertoli cells and induces the downstream synthesis of the factors responsible for spermatogonial renewal. The major androgen in the teleost, 11-ketotestosterone (11-KT), is the key regulator of spermatogenesis, is expressed by the Leydig cells and is involved in the initiation of spermatogonial proliferation toward meiosis [4]. The final step of sperm maturation is regulated by 17α,20β-dihydroxy-4-pregnen-3-one (DHP), a hormone secreted by the germ cells themselves in a paracrine or autocrine manner, which enables the mobility of spermatozoa through increasing the pH of the seminal plasma [1,5].

During oogenesis, oogonia proliferation is stimulated by E2, which is converted from the thecal cell supplied androgen under the catalyzation of P450 aromatase in the granulosa cells. DHP is thought to be the hormone inducing initiation of the first meiosis of oogonia. The oogonia arrest at the prophase of the first meiosis for a long time and undergo vitellogenesis during this process. E2 is the main hormone stimulating the synthesis and transport of the egg yolk precursor, vitellogenins, chorionic proteins and chorion genins from the liver to the developing oocyte.

Before ovulation, the oocytes go through a final maturation process in which meiosis resumes. During this stage, maturation-inducing hormones (MIHs) such as DHP and 17α-20β, 21 trihydroxy-4-pregnen-3-one (20β-S) secreted by the granulosa cells bind to their receptors on the oocyte, and thus activate the maturation-promoting factor (MPF). The germinal vesicle (GV) then moves toward the animal pole, and its membrane disintegrates. The first meiotic division ends with discharge of the first polar body. At the end of the maturation process, the follicular layers collapse and ovulation of mature ovum occurs [6].

Mainly through the regulation of sex steroid hormones synthesis and secretion by binding to their receptors in the gonad, FSH and LH play important roles in control of gonad development. LH and FSH are both heterodimeric glycoproteins containing two subunits: α and β subunits. LH and FSH share the same α subunit but contain different β subunits, LHβ and FSHβ respectively. It is thought that FSH plays a more important role during early gonadal development stages, while LH is responsible for the final stages of germ cell maturation and discharge of mature gametes [7,8].

Unlike in mammals, the biological activities of FSH and LH in fish are less clearly separated. Both FSH and LH can regulate the steroidogenesis of Leydig cells, while Sertoli cell functions are mainly regulated by FSH in fish [9]. FSH is thought to be the regulator of Sertoli cell proliferation and testicular growth in African catfish (*Clarias gariepinus*) as the changes of FSH and FSHR expression in the testis led to corresponding changes in Sertoli cell numbers [1]. In rainbow trout (*Oncorhynchus mykiss*), incubation of testis explants with FSH resulted in expression of many genes related to the onset of spermatogenesis [10]. The surge of LH in

plasma has been observed during the final gamete maturation stage in many species, and the increase of LH induces a remarkable increase of the testicular steroids such as 11-KT and DHP or 20β-S [9,11].

In females, both FSH and LH could stimulate the production of E2 by theca cells and granulosa cells, but FSH dominantly controls the early development of the gonad with its relatively high expression and the low level of LH [6,12]. FSH plays a key role in the onset of puberty as revealed by the delay of puberty onset in the FSH mutant zebrafish [13] and the increased expression of FSH receptor (FSHR) in follicles entering into the secondary growth stage [14]. During the final stage of oocyte growth, an acute increase in plasma LH level occurs and initiates the process of ovarian follicle maturation by binding to its receptor on granulosa cells, which induces the production of mullerian inhibiting hormone (MIH) [1,6,15]. The LH-deficient female zebrafish had normal gonad growth, but were not able to spawn [6].

As the regulator at the top of the HPG axis, GnRH plays a central role in the regulation of reproduction in fish. GnRH is secreted by the hypothalamus and delivered to the adenohypophysis through hypothalamic nerve fiber branchs to regulate the synthesis and release of the gonadotropins, FSH and LH from the pituitary, thereby regulating the production of steroids [16,17]. In some species (mostly freshwater fish), the hypothalamus also produces dopamine, which works as an inhibitory factor to reversely regulate the production of gonadotropins [18–20]. In the process of reproductive hormone coordination through the HPG axis, a mechanism of feedback regulation also exists to achieve precise control of reproductive activities. Steroids produced by the gonads can feed back to the hypothalamus or the pituitary,

thus indirectly or directly regulating the production and release of FSH and LH [21]. For most of gonadal development, only negative feedback exists, but positive feedback also operates during the early gonadal development phase, and before ovulation, positive feedback of estradiol occurs to facilitate the surge event of GnRH and LH [22–24].

Fifteen GnRH forms have been identified in vertebrates and 11 GnRHs identified from fish [25]. In every fish species studied, there is a minimum of two, and a maximum of three isoforms of GnRH coexisting in the central nerve system (CNS) [26]. The neurons producing different GnRH isoforms have distinct locations in the CNS, and according to their locations, GnRH neurons are classified into three types: the GnRH1 neuron which is located in the hypothalamus, the GnRH2 neuron located in the midbrain, and the GnRH3 neuron located in the telencephalon [16,27]. GnRH1 and GnRH3 are collectively called forebrain GnRH. GnRH1, which is secreted by the hypothalamus and released to the anterior pituitary via a direct neural connection in fish, is thought to modulate the production and release of GtH, while GnRH2 and GnRH3 are involved in the mediation of reproductive behavior[27]. During embryogenesis, forebrain GnRH neurons and midbrain GnRH neurons migrate from their origins, the olfactory placode and the mesencephalic region of the neural tube, respectively, to their ultimate locations where they function to synthesize the corresponding forms of GnRH, thereby regulate the reproduction [27–29]. The development of the GnRH neuron system in fish is similar to that of mammals and birds [30]. The migration of the GnRH1 neuron is the most widely studied. After the initiation from their origin, GnRH cells migrate along with a subset of vomeronasal axons through the cribriform plate, then turn caudally into the developing forebrain and finally enter into the hypothalamus [31–33]. During this long journey of migration, GnRH neurons pass through

varying molecular environments. Complex mechanisms of interaction with the surrounding cells and molecular regulations are involved to ensure the precise migration of GnRH neurons in the right direction and at the proper speed.

Gamma-aminobutyric acid (GABA) is one of the important factors involved in the guidance of GnRH neuron migration [31,34,35]. GABA is best known as one of the major inhibitory neurotransmitters in the CNS. It is formed from glutamic acid through a reaction catalyzed by glutamic acid decarboxylase (GAD), mainly in neurons in the CNS [36,37]. GABA is involved in multiple biological functions such as brain development, locomotor activity, learning, reproduction and circadian rhythms [37–40]. The close spatial relationship of GABA neurons and GnRH neurons [41], and the intimately associated expression patterns of GABA and GAD in the early developing zebrafish embryo before the formation of synapses indicated that GABA has a role in the nervous system development [42,43] (Fig. 2-1).

In the mouse, GABA works synergistically with stromal derived growth factor (SDF-1) to regulate the rate of GnRH neuron migration, exerting a slowing or accelerating effect on the migration by activation of depolarizing or hyperpolarizing signaling pathways, respectively [44]. GABAergic neurons inhibit the movement of GnRH neurons via GABA receptors (A and B) in the GnRH neuron cells. A subset of GnRH neurons produces GABA during migration and function in an autocrine manner [35,45]. Also, GABA was seen in the cells surrounding the migration route of GnRH through the nasal compartment [35,46,47]. When treated with $GABA_A$ receptor agonist muscimol, the migration speed of GnRH neuron was reduced by 22%. While the speed was increased by 14% when the nasal explant was treated with the $GABA_A$ receptor

antagonist, picrotoxin [44]. In another study, GABA was also proven to participate in the migration of GnRH neuron into the CNS, as revealed by the increased and inhibited neuronal migration distance after $GABA_A$ receptor antagonists and agonist treatment, respectively [34]. Both *in vivo* and *in vitro* experiments of $GABA_A$ receptor manipulation showed that GABA plays various roles in GnRH neuron migration at different developing stages, including inhibiting GnRH neuron movement out of the nasal compartment, coordinating association of GnRH neurons with the guiding fibers, and regulating the extension of GnRH fibers toward the median eminence [48].

Transgenic and loss of function studies revealed the roles of GABA in GnRH neuron development further. Overexpression of GAD67 in the GnRH neurons disrupted the migration of GnRH neurons, resulted in fewer neurons reaching the hypothalamic-preoptic region and reduced GnRH content during the first week of postnatal life in mice. The estrous cyclicity and reproductive capacity of the transgenic females were also affected, though the onset of puberty was unaffected [49]. Knockout of GAD67 genes in mice resulted in a 90% reduction of GABA in the developing brain [50], and the decreased levels of GABA accelerated the migration speed of GnRH1 neurons [44,51].

Even though not large in number, the appropriate location of GnRH neurons is critical for the establishment and maintenance of reproduction. When GnRH neuron migration is disrupted, the synthesis and release of GnRH will be affected, leading to abnormal reproductive function and possibly infertility. Kallmann Syndrome, which is characterized by delayed or absent puberty and infertility, is a common disease caused by the incorrect migration of GnRH neurons in

humans [52]. Female mice with disrupted migration of GnRH neurons had infertility or subfertility due to abnormal LH surge [53].

In addition to its implication in the control of GnRH neuron migration, GABA has also been shown to have regulatory functions in LH release, puberty, and both the positive and negative feedback of estrogen. GABA, along with glutamate are the two principal inhibitory and excitatory neurotransmitters respectively in the adult brain. The role of the $GABA_A$ receptor experienced a transformation from depolarization to hyperpolarization during development, which means the effect of GABA on these neurons changed from excitatory to inhibitory [54]. In most neurons, this switch started after the formation of the glutamate synapse, while GnRH neurons showed delayed switch in their response to GABA until puberty onset. The excitatory or inhibitory effect of GABA on GnRH neuron activity or LH secretion seems to be species and developmental stage dependent. In mammals, GABA exerts depolarizing actions on prepubertal GnRH neurons while exclusively hyperpolarizing actions on postpubertal GnRH neurons [55]. Consistently, several studies have shown a stimulatory effect of GABA on gonadotrophin secretion in prepubertal rats and an inhibitory impact in postpubertal animals [56,57]. However, in most of the fish species studied, GABA showed no or an inhibitory effect on gonadotropin secretion at the larval stage, while an excitatory effect in the adult fish [40]. In zebrafish, the expression of GnRH3 was inhibited by GABA in larval fish, but stimulated in adult fish via the $GABA_B$ receptor signaling pathway. The expression of LH and FSH were also boosted by GABA in adult fish [58]. *In vitro* and *in vivo* studies conducted on adult female sea lampreys showed increased GnRH1 and GnRH3 concentration in the brain after the administration of GABA or its analog [59]. In adult dwarf gourami (*Trichogaster lalius*), the activation of $GABA_A$

receptor resulted in the excitation of the terminal nerve GnRH neurons [60]. In the Atlantic croaker (*Micropogonias undulatus*), the effect of GABA on LH secretion was proved to be mediated through the $GABA_A$ receptors, and the effect is stage dependent. GABA administration stimulated the expression of LH in croaker with regressed or pre-recrudescence phase gonads, while it had no significant effect on the fish in the early to middle recrudescence phase of the gonadal cycle, and inhibited LH secretion in fish with fully recrudesced gonads [61]. Similarly, GABA injection increased serum GtH levels in regressed or early maturing goldfish, but did not affect late maturing fish. The stimulatory effect of GABA on GtH was thought to be achieved indirectly through the increase of GnRH release [62]. There is also evidence that GABA stimulates LH secretion by blocking the inhibition of dopamine on LH release [63]. In rainbow trout, GtH release of immature fish was not affected by GABA injection, while plasma LH in mature females was remarkably increased after GABA injection. In spermiating males, though the basal GtH release was not affected, GABA stimulated FSH secretion and potentiated GnRH-stimulated LH release when coadministered with GnRH analog (GnRHa) [64]. Nevertheless, the inhibitory effect of GABA on mature fish LH secretion was also observed in some fish species. In the mature male carp, it was suggested that GABA exerts an inhibitory effect on GnRH-stimulated LH release, probably through the $GABA_B$ receptor [65] (Fig. 2-1).

GABA is also involved in the positive and negative feedback of estrogen both in mammals and teleost fish. Most of the time, GnRH is under the negative feedback regulation of estrogen, but during the surge event before ovulation, this feedback switched to positive to initiate the surge of GnRH and GtHs. Change of GABA transmission frequency was regulated in an estradiol-dependent manner during the shift of the feedback action, which resulted in the altering of GnRH

neuron firing activity [47]. During negative feedback, the frequency of GABAergic postsynaptic currents was low, while it was increased along with enhanced amplitude at surge onset [66]. *In vitro* study in ewe showed the stimulation of GnRH release after $GABA_A$ antagonist treatment was attenuated by oestradiol, indicating GABA plays a role in the negative feedback of estradiol on GnRH release through the $GABA_A$ receptor [67]. It was also concluded in the rat that steroid sensitive GABAergic neurons are involved in mediating the negative feedback action of androgen and estrogen to GnRH release in male and female rats [68–70]. Estradiol treatment of the female goldfish not only abolished the stimulatory effect of GABA on GtH secretion but also reduced the GABA concentration in the telencephalon [62]. In immature rainbow trout, the influence of GABA on LH release changed from no effect to stimulatory 13 days after steroid implantation [64]. GnRH neurons do not express estrogen receptors (ER) [71], but ER was found in GABAergic neurons and dopaminergic neurons [72,73], which supported the important role of GABA in the feedback regulation of estrogen on GnRH [74] (Fig. 2-1).

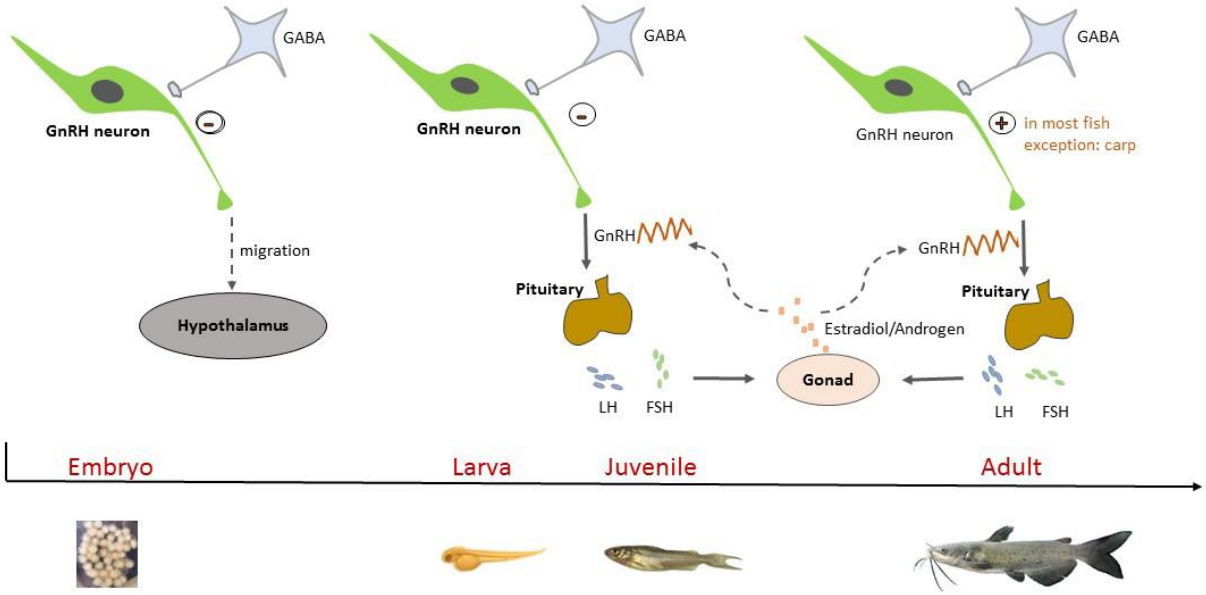

Fig. 2-1 Involvement of γ-aminobutyric acid (GABA) in the regulation of reproductive activities in teleost fish. GABA has an inhibitory effect on the migration of GnRH neurons from the olfactory placode to the hypothalamus during embryogenesis. In most of the fish species studied so far, GABA showed an inhibitory and stimulatory effect on the production of GnRH before and after puberty, respectively. GABA also plays a role in the regulation of gonadotropin secretion and positive and negative feedback of estrogen and androgen.

Two GAD genes (GAD65 and GAD67) which encode two GAD proteins with different biomass (65kDa and 67kDa) have been isolated and characterized in multiple organisms [42,75–78]. Phylogenetic analysis showed these two distinct genes resulted from duplication of an ancestral GAD gene before or during the evolution of fish [42,78]. Both genes are expressed in the early developing CNS, but they express at distinct locations and synthesize GABA for different purposes [37,76,79]. GAD65 appears to be targeted to membranes and nerve endings, and the generated GABA is more likely for vesicular release, while GAD67 is more widely distributed in cells and synthesizes cytoplasmic GABA [37].

In this study, we aimed to disrupt the normal migration of GnRH neurons and production of GnRH in channel catfish through the overexpression of goldfish GAD65 gene driven by carp beta-actin promoter. Thus, the sexual maturation of channel catfish would be interrupted, which could then be restored by hormone therapy. The possibility of using this technology together with transgenic fish production could prevent the risk of potential environmental impact from the transgenic fish since they would be unable to reproduce without hormone therapy administered by man.

Methods and materials

Construction of the GAD65 transgene construct

The transgene used in this study was constructed by AquaBounty Technologies (Maynard, MA). Briefly, goldfish GAD65 gene (Accession number: AF045594.1) fragment provided by Vance Trudeau, Ottawa University was amplified with primers containing the BsrGI site, and then inserted into pCR2.1 using the topo TA kit. The recombinant vector was then digested with BsrGI restriction enzyme, and the GAD fragment was purified with a gel purification kit.

Purified GAD fragment was fused into the KpnI site of the pFV3CAT vector, which contains the common carp β-actin promoter on the upstream (Fig. 2-2).

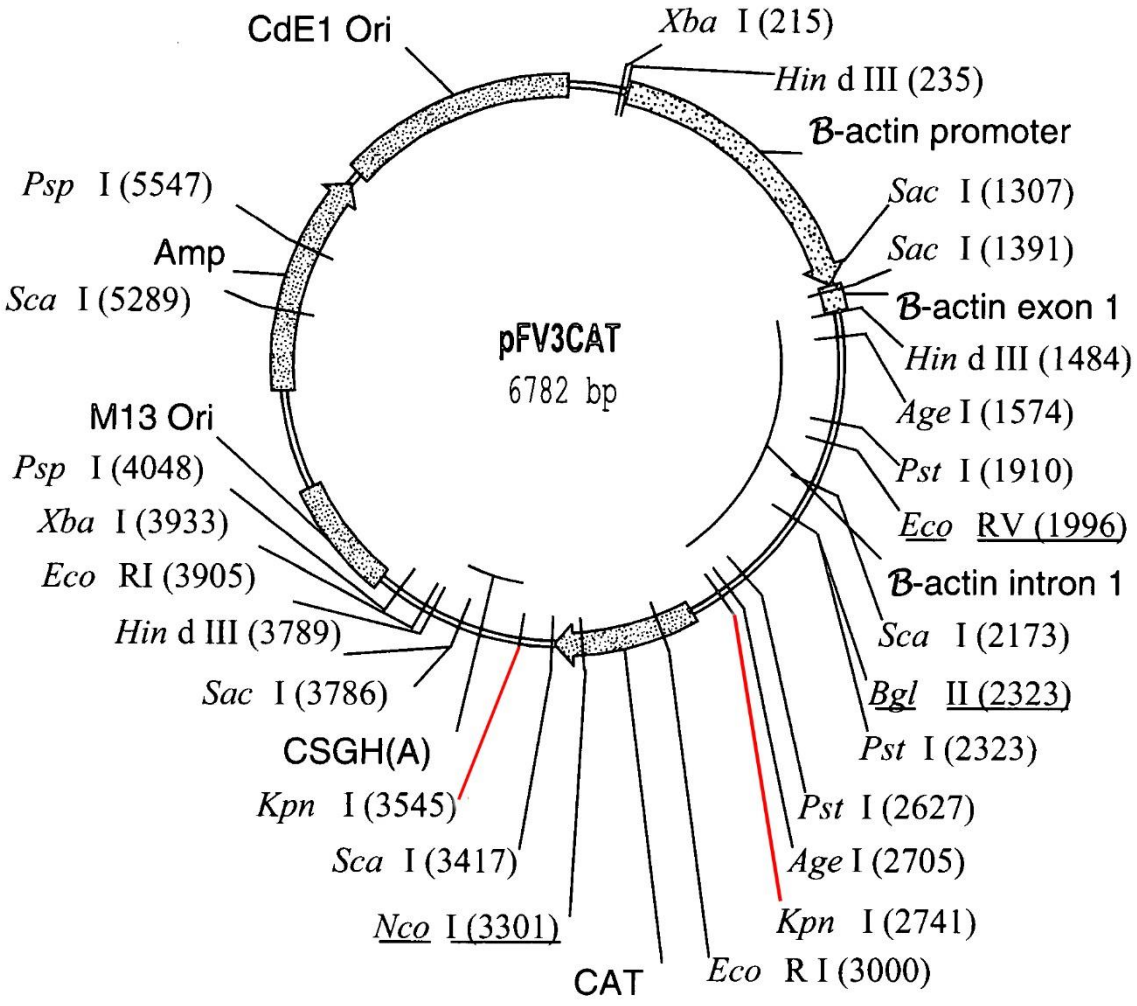

Fig. 2-2 Construction of the glutamic acid decarboxylase (GAD) transgene construct. Goldfish (*Carassius auratus*) GAD65 gene fragment with BsrGI restriction enzyme cutting sticky ends was inserted into the pFV3CAT vector by replacing the CAT fragment between the two Kpn I restriction enzyme cutting sites (shown in red lines). Carp β-actin promoter on the upstream drives the expression of GAD. Map of the pFV3CAT vector was from Perry et. al. (2004).

Plasmid preparation

The plasmids provided by AquaBounty were transformed into the One Shot® Top 10 Chemically Competent E.coli cells (Invitrogen, Grand Island, NY) following the manufacturer's instructions. Twenty-five microliters of the transformed *E. coli* culture were spread on an LB agar plate containing 100 μg/ml ampicillin. A single colony was picked and inoculated into 500 ml LB containing 100 μg/ml ampicillin and cultured at 37 ^{o}C for 16h. Plasmids were then extracted from the culture using IsoPure Plasmid Maxi II Prep Kit (Denville, Holliston, MA). Quality and quantity of the extracted plasmids were determined by a Nanodrop 2000 (Thermo Scientific, Wilmington, DE) and electrophoresis. The concentration of plasmid was adjusted to 50 ng/μl with TE buffer before the electroporation.

Introduction of GAD65 construct through electroporation

Channel catfish broodstocks were obtained from the ponds at the Catfish Genetics Research Unit, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, AL, USA. Females and males with well-developed, secondary sex characters (well-rounded and distended abdomen for the females and muscular head and elongated urinogenital papillae for the males) were chosen for artificial spawning. Females were implanted with 90 μg luteinizing hormone releasing hormone analogue (LHRHa) per kilogram of the body weight, placed into a spawning bag and kept in a flow-through tank with continuous aeration. The fish were first examined for egg ovulation once 1,040 degree hours post-hormone injection were reached and then examined every 4h thereafter. Once ovulation was observed (more than 10 eggs on the spawning bag), the females were anesthetized with 100 ppm buffered tricaine methane sulfonate (MS 222), and the remaining eggs were obtained by hand stripping. The males were sacrificed within 12 hours

before the expected time of ovulation and sperm squeezed from the testes through a fine mesh and into 10 ml of 0.9% saline per gram of testes. The sperm solution and stripped eggs were mixed, and pond water added to activate fertilization. Twenty min after fertilization, 100 to 200 fertilized eggs were transferred to a 7 ml petri dish and 3 ml of the plasmid solution was added into it. After 10 min of incubation, eggs were electroporated with a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, California, USA). Parameters were set at 6 kV, 27 pulses, 0.8 sec burst, 4 cycles and 160 μsec. A control group electroporated with TE buffer only was also included.

Embryo incubation

Electroporated embryos were incubated in 10 L tubs with 5.0 L Holtfreter's solution. The embryos were gently agitated with compressed air delivered through an airstone. Holtfreter's solution was changed every 12 h and dead embryos were counted and removed before each solution change. Once the embryos hatched, fry were transferred to fry baskets and temporally reared in a flow-through tank with pond water in the greenhouse.

Confirmation of transgene integration by PCR and sequencing

Anal fin samples were taken from the fish for DNA extraction. Samples were digested in cell lysis buffer with 100 μg/ml proteinase K. After full digestion, genomic DNA was isolated from the solution by protein precipitation, DNA precipitation by isopropanol, washing and then precipitation of DNA by ethanol [94]. DNA was dissolved in DNase free ddH_2O after air drying and kept in the 4 ℃ refrigerator overnight to allow complete rehydration. Quality and quantity of extracted DNA were measured on a Nanodrop 2000 and integrity examined by electrophoresis.

Primers targeting the boundary area of the β-actin promoter and the GAD65 gene were designed for the confirmation of the transgene integration using Primer Premier 5.0 software. The primers given by the software were checked for quality parameters such as GC content, primer dimer, hairpins, 3' end stability and melting temperature with Oligo Analyzer 3.1. They were also blasted against the channel catfish genome (IpCoco_1.2) from the National Center for Biotechnology Information (NCBI) database to ensure their specificity. Primers were first tested for their specificity and efficiency by conducting PCR reactions using the genomic DNA from normal channel catfish as the negative control template and the GAD plasmid DNA as the positive control template. The primer pair with no or minimum non-specific amplification in the negative control and highest amplifying efficiency (with the brightest band shown by gel electrophoresis) was used for the following transgene screening. The primer pair used was: forward primer sequence is 5' TTGTCTGGCACATCTGAG 3', the reverse primer sequence is 5' TACAATCACACCTGTCCAA 3'. The size of the PCR product is 274 bp. The PCR reaction was accomplished in a 15 μl volume mix with the following components: 10 x buffer 1.5 μl, 2.5 mM dNTP 1.5 μl, 50 mM $MgCl_2$ 0.5 μl, 10 mM forward primer 0.75 μl, 10 mM reverse primer 0.75 μl, Tag polymerase 0.4 U, template genomic DNA 200 ng, and adding ddH_2O to 15 μl. The PCR program was as follows: initial denaturation for 5 min at 95 ℃; followed by 39 cycles of 95 ℃ for 30s, 59 ℃ for 30 s and 72 ℃ for 30 s; and a final elongation for 5 min at 72 ℃. PCR results were checked by electrophoresis on 1% agarose gel.

In addition to PCR, insertion of the transgene was further confirmed by sequencing. Briefly, the band at the correct size was cut off and purified with the QIAquick Gel Extraction Kit (Qiagen,

Germantown, MD) following manufacturer's instruction. The purified product was sent to the Auburn University Genomics and Sequencing Lab for sequencing. The sequence was confirmed by aligning to the plasmid sequence.

Sexual maturation, fertility evaluation and hormone therapy of F1 and F2 fish

F1 generation was produced from positive P1 fish using artificial spawning as described above. Fish were harvested during the spawning season at the age of 5 for sexual maturation evaluation. Each fish was given a score from 1 to 5 according to the secondary sex characters. All fish were examined and scored by the same well-trained examiner to make sure the score was given to each fish with the same standard. The examiner knew which fish belong to the same group but did not know which group it is. Score 5 was the highest and was characterized by a well-rounded and distended abdomen, open red genitalia for the females and a very muscular head and elongated urinogenital papillae for the males; score 4 was characterized by rounded and distended abdomen, open red genitalia for the females and a muscular head and elongated urinogenital papillae for the males, score 3 was a fish with rounded abdomen, open, light red genitalia for the females and a median muscular head and elongated urinogenital papillae for the males; score 2 means a fish with slightly rounded abdomen, not swollen genitalia for the females and a normal head and slightly elongated urinogenital papillae for the males; score 1 was the lowest and was assigned to fish with a flat abdomen, not swollen pale genitalia for the females and a normal head and small, soft urinogenital papillae for the males.

During the spawning season (June) of 2013, F1 GAD transgenic fish and control fish were paired with the following combination: GAD ♀ X control ♂; control ♀ X GAD ♂; control ♀ X control

♂. Each pair was placed in an individual 120L flow-through aquarium, wrapped with black plastic film and given a photoperiod of 12h/12h. Water was aerated with compressed air delivered through an airstone to maintain the DO above 7ppm. Water quality was monitored and kept within the safe range for catfish (Nitrite = 0 ppm, Ammonia = 0 ppm, pH = 6.8~8). The fish were given 14 days to allow natural spawning. The fertility of fish was determined by the successful production of fertilized eggs. Fish that did not spawn for 14 days during a natural spawning trial were classified as putatively infertile and were given hormone therapy, receiving an LHRHa implant at the dosage of 90 μg LHRHa per kilogram fish weight to induce maturation. The implanted fish returned to the aquarium and another 7 days spawning trial was conducted.

Embryos from the GAD transgenic fish, which spawned after hormone therapy were incubated in a flow-through hatching trough. Hatched fry were cultured in flow-through tanks with pond water. Powdered 50% protein, starter feed was fed to the fry starting at approximately 3 dph, and pelleted 36 to 48% protein feed as the fingerlings grew. Once they reached 10 g, the fish were stocked into 404.7 square meter pond with 1 meter depth water at the density of approximately 0.7 fish per cubic meter water until maturation. Fish were fed ad-libitum with commercial floating catfish feed containing 32 to 36% protein once per day and 7 days per week. Feeding rates were reduced to once every other day when the water temperature was lower than 15 ℃.

During the spawning season (June) of 2016, the spawning trial was repeated with another batch of F1 generation GAD fish and the F2 progeny of a family (GAD11) derived from a GAD

transgenic dam, which spawned after hormone therapy and was paired with a non-transgenic sire.

Measuring of serum GnRH, estradiol and testosterone levels by ELISA

Blood was collected from the F2 generation GAD11 family fish at the age of 1 and 4 years in June 2014 and March 2017, respectively. Fish were anesthetized with 100 ppm buffered tricaine methane sulfonate (MS 222), and 0.5 and 1.0 ml blood samples were collected from the caudal vein of the 1 and 4-year-old fish, respectively, using a sterile syringe. Blood samples were kept at 4 ^{o}C overnight to clot and then centrifuged at 1000 x g for 15 min to isolate the serum. The serum was aliquoted and stored in a -80 ^{o}C freezer until hormone measurement. Hormone levels in the serum were measured using ELISA kits (GnRH and estradiol kits were from CUSABIO Biotech Corp., LTD, Baltimore, USA; testosterone kit was from Cloud-Clone Corp., Houston, USA) according to the manufacturer's instructions. GnRH level was measured in both sexes, estradiol was measured in the female 1-year-old fish, estradiol and testosterone levels were measured in the female and male 4-year-old fish, respectively.

Statistical analysis

All the data analysis used a SAS program (edition 9.2). Log rank test was used to compare the P1 embryo survival curves. Fisher's exact test was used to analyze the sexual maturation evaluation data and the spawning percentage data. Chi-square test was used to test the transgenic fish ratio in the F2 generation to the 50% expectation. ANOVA (one-way, two-way) was used to analyze body weight and hormone levels. Significance for all tests was set at $p < 0.1$.

Results

Mortality (hatch) of the P1 embryos

Both control and GAD construct exposed channel catfish embryos had a rapid decline of survival probability during the 1.5 to 3.5 days post fertilization (dpf) period, which is consistent with results observed previously in the Bax overexpression experiment. There was no significant difference (p = 0.94) in the survival curves of the GAD construct transferred and control embryos, indicating the introduction of GAD did not increase mortality of channel catfish embryos (Fig. 2-3).

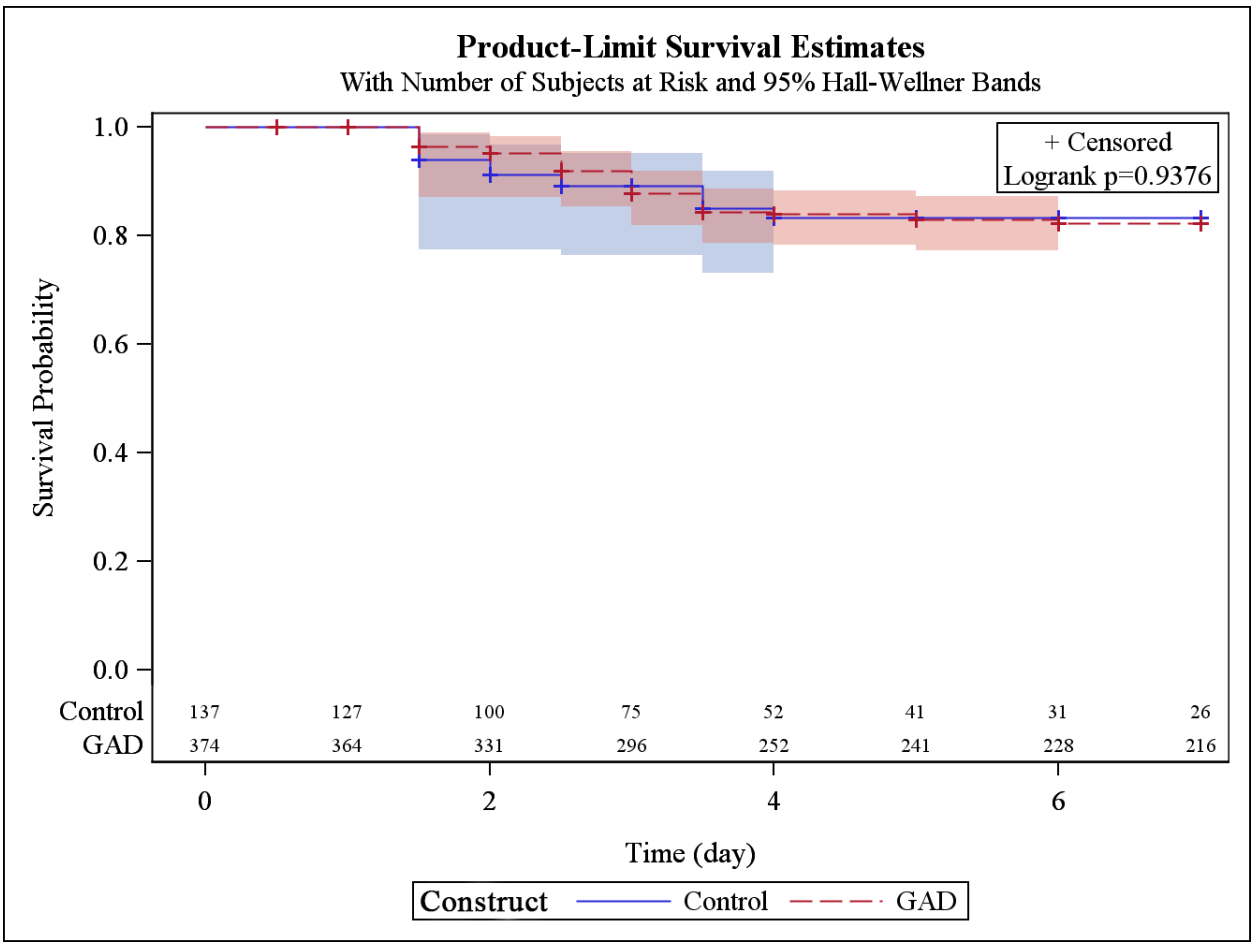

Fig. 2-3 Kaplan-Meier survival curves for the P1 channel catfish (*Ictalurus punctatus*) embryos exposed to glutamic acid decarboxylase (GAD) construct or TE buffer (control). The embryos were transferred with GAD construct or TE buffer (control) at one cell stage via electroporation, and then incubated in 10 L tubs with 5.0 L Holtfreter's solution. The embryos were gently agitated with compressed air delivered through an airstone. Holtfreter's solution was changed every 12 h, dead embryos were counted and removed before each solution change. Observation continued until all embryos hatched. There was no significant difference between the survival curves of GAD and control groups ($p > 0.1$, log rank test).

Transgenic identification of F2 progeny and comparison of growth between transgenic and non-transgenic fish

In the F2 population of the GAD11 family, 73 out of the 150 (48.7%) tested fish were transgenic identified by PCR using the transgene specific primers (Fig. 2-4). The specific amplification of the transgene in the positive samples was further confirmed by sequencing (Fig. 2-5). The transgenic ratio was consistent with the theoretical ratio of 50% when one of the parents is a homozygous transgenic fish ($p = 0.74$).

No significant ($p = 0.64$) difference was observed in the body weight of the 1-year-old full-sib transgenic (10.96 ± 4.33g) and non-transgenic (11.22 ± 2.87 g) F2 progeny in the GAD11 family.

Gravidness, fertility assessment, and hormone therapy of F1 and F2 generation fish

At the age of 5, none of the 37 F1 generation GAD transgenic female (14) or male (23) fish had a sex maturity score of 5, and only one female and one male had a score of 4 (Table 2-1). When the sexes were pooled, the percentage of GAD transgenics at a reproductive readiness of 4 or 5 was less ($p = 0.07$) than non-transgenic controls. This indicated that the sex maturation of the GAD transgenic fish was inhibited to some extent and these fish could be sterile. Three years is the age threshold of reproductive capability for most of channel catfish strains [80].

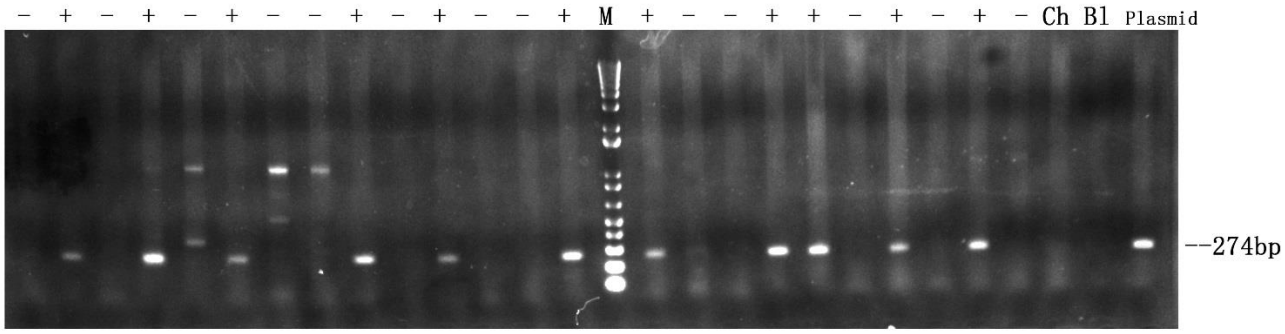

Fig. 2-4 Identification of glutamic acid decarboxylase (GAD) transgenic channel catfish (*Ictalurus punctatus*) with primers targeting the β-actin promoter-GAD gene area. Forward and reverse primers were designed from the β-actin promoter and GAD gene region, respectively. The size of target amplicon is 274 bp. -: GAD transgene negative; +: GAD transgene positive; M: DNA ladder; Ch: non-transgenic channel catfish control; Bl: blank control; Plasmid: positive control using GAD plasmid as the template for PCR.

Fig. 2-5 Alignment of PCR product sequences with the corresponding glutamic acid decarboxylase (GAD) plasmid sequence for the GAD positive channel catfish (*Ictalurus punctatus*). Sequencing results confirmed that the amplicons amplified from the positive fish samples were the target fragment of the designed transgene specific primers.

Table 2-1 Percentages of F1 generation glutamic acid decarboxylase (GAD) transgenic and non-transgenic channel catfish (*Ictalurus punctatus*) with a reproductive score of 5 (females released eggs and males were in top reproductive condition) or 4 (good reproductive condition) at the age of 5. Scores of 1-3 are low or no reproductive readiness. When sexes were pooled, GAD transgenics had lower percentages ($p = 0.07$, Fisher's exact test) of fish with a reproductive score of 4 or 5, compared to the non-transgenic controls. F: female; M: male. Percentages followed by different letters were significantly different ($p < 0.1$).

Sex	Genotype	Total number	5	4	4&5
F	Control	227	22 (9.7%)	25 (11%)	47 (20.7%)
	GAD	14	0	1 (7.1%)	1 (7.1%)
M	Control	232	10 (4.3%)	20 (8.6%)	30 (12.5%)
	GAD	23	0	1 (4.3%)	1 (4.3%)
F&M	Control	459	32 (7.0%)	45 (9.8%)	77 (16.8%)[a]
	GAD	37	0	2(5.4%)	2 (5.4%)[b]

For the F1 generation, in the spawning trial conducted during the 2013 spawning season (6-year-old), 83.3% (10 out of 12) of the control channel catfish pairs spawned naturally without LHRHa implants, while the observed mean of 45.5% (5 out of 11) was of the GAD fish pairs spawned naturally, which was significantly lower ($p = 0.09$) than the controls. Four of the 6 pairs of GAD fish that did not spawn naturally did so after induction with LHRHa (Table 2-2). These results showed that overexpression of GAD could lead to infertility of channel catfish, and the fertility could be restored by hormone therapy. In the spawning trial conducted during the spawning season of 2016 (9-year-old), the GAD transgenic fish had a lower percentage of fish spawned than controls (20% and 66.7%, respectively), but not statistically significant ($p = 0.12$; Table 2-2).

For the F2 generation, the 3-year-old GAD transgenic fish had only 20% (3 of 15) spawned naturally, which was lower than the control (66.7%) but not statistically lower ($p = 0.12$). None of the 5 pairs of infertile GAD fish, which were still in good spawning condition after the natural spawning trial and received LHRHa therapy, had the fertility restored (Table 2-3).

Table 2-2 Spawning percentages of glutamic acid decarboxylase (GAD) transgenic F1 generation channel catfish (*Ictalurus punctatus*) at 6 and 9 years of age, under natural aquarium spawning conditions and after hormone therapy. Spawning trials were conducted in 2013 (6-year-old) and 2016 (9-year-old) with two different batches of F1 generation GAD transgenic fish. GAD transgenic male or female fish were paired with a control female or male fish respectively, and each pair was kept in separate aquariums, given 14 days for natural spawning. The control group was matings of non-transgenic females and males. Hormone therapy, implantation with 90 μg LHRHa per kilogram of body weight, was conducted on the GAD transgenic fish which did not spawn naturally, but were still in good spawning condition after the 14 day spawning trial. Fish were given another 7 days to spawn after induction. In both the spawning trials of 2013 and 2016, GAD transgenic fish had lower percentages of fish spawned naturally compared to the control, but was only significant in the 2013 trial (p = 0.09 and 0.12, respectively, Fisher's exact test).

		Without implant			Implanted		
		NO.	Spawn	%	NO.	Spawn	%
2013	Control	12	10	83.3	-	-	-
	GAD	11	5	45.5	6	4	66.7
2016	Control	6	4	66.7			
	GAD	10	2	20.0	3	0	0.0

Table 2-3 Spawning percentages of the 3-year-old F2 generation glutamic acid decarboxylase (GAD) transgenic channel catfish (*Ictalurus punctatus*) under natural aquarium spawning condition and after hormone therapy. GAD transgenic male or female fish were paired with a control female or male fish respectively, and each pair was kept in separate aquariums, given 14 days for natural spawning. The control group was matings of non-transgenic females and males. Hormone therapy, implantation with 90 μg LHRHa per kilogram of body weight, was conducted on the GAD transgenic fish which did not spawn naturally, but were still in good spawning condition after the 14 day spawning trial. Fish were given another 7 days to spawn after induction. There was no significant difference in the percentage of fish naturally spawned between the GAD transgenic and control fish ($p = 0.12$, Fisher's exact test).

		Without implant			Implanted		
		NO.	Spawn	%	NO.	Spawn	%
2016	Control	6	4	66.7	-	-	-
	GAD	15	3	20.0	5	0	0.0

Hormone levels in the F2 fish

At 1 year of age (weight = 11.1 ± 3.6 g), the level of GnRH in the serum was lower (p = 0.069) in GAD transgenic fish than in non-transgenic full-siblings when sexes were pooled. The difference between the GAD and control fish was not significant within each sex (p = 0.15 and 0.27 for female and male, respectively). Female fish (9.76 ± 3.09) had significantly higher (p = 0.038) GnRH level in serum than males (8.46 ± 2.27) when fish from GAD and control group were pooled, but no significant difference between female and male fish was observed within each genotype group (p > 0.05). No genotype X sex interaction was observed (p = 0.77). The estradiol level in transgenic female fish serum was not different (p = 0.78) than that of the non-transgenic fish (Table 2-4).

For the 4-year-old fish, again, the GnRH level in the GAD transgenic fish was lower (p = 0.099) than in non-transgenic control fish when female and male data were pooled. The GAD transgenic males had significantly (p = 0.012) lower GnRH level than control male fish, but the difference was not significant (p > 0.1) between the transgenic and non-transgenic female fish. GnRH levels in different sexes were also significantly (p = 0.002) different, with the females having higher GnRH levels than the males. When split by genotype, only the GAD group the females had significantly higher (p = 0.0028) levels of GnRH than the males. Control females had higher observed GnRH levels in the serum than the males, but it was not significantly different (p = 0.39) (Table 2-5).

There was no (p = 0.9069) difference between the GAD transgenic and non-transgenic control female fish for estradiol levels. GAD transgenic male fish had significantly (p = 0.0018) lower levels of testosterone than non-transgenic males (Table 2-5).

Table 2-4 Means with standard deviation of Gonadotrophin-releasing hormone (GnRH) and estradiol levels in the serum of the 1-year-old F2 generation glutamic acid decarboxylase (GAD) transgenic and full-sib non-transgenic control channel catfish (*Ictalurus punctatus*). Means were compared by ANOVA. When sexes were pooled, GnRH levels in GAD fish was lower (p = 0.069) than in non-transgenic fish, but the difference was not significant when sexes were split (p = 0.15 and 0.27 for female and male, respectively). Female fish had a higher (p = 0.038) level of GnRH in the serum than males when data from GAD and control fish were pooled but no significant difference between female and male fish was observed within each genotype group (p > 0.1). No significant (p = 0.78) difference was observed in the estradiol levels of transgenic and non-transgenic female fish. F: female; M: male. Means in the same row followed by different letters were significantly different (p < 0.1, ANOVA).

Genotype	GnRH (ng/ml)			Estradiol (pg/ml)
	F	M	F&M	F
GAD	9.23 ± 2.49	8.14 ± 2.21	8.73 ± 2.40^{A}	501.00 ± 69.96
Control	11.04 ± 4.06	9.03 ± 2.36	9.99 ± 3.36^{B}	520.48 ± 148.31
GAD&Control	9.76 ± 3.09^{a}	8.46 ± 2.27^{b}		

Table 2-5 Means with standard deviation of gonadotrophin-releasing hormone (GnRH), estradiol and testosterone levels in the serum of the 4-year-old F2 generation glutamic acid decarboxylase (GAD) transgenic and non-transgenic channel catfish (*Ictalurus punctatus*). Means were compared by ANOVA. There was no significant (p = 0.98) difference in GnRH level between GAD transgenic and non-transgenic female fish, while male GAD transgenic fish had significantly (p = 0.012) lower GnRH levels, compared to non-transgenic control males. Female fish had higher (p = 0.0022) levels of GnRH in the serum than males when pooled transgenic and non-transgenic fish, but it was only significant in the GAD transgenic fish when with genotypes separated (p = 0.39 and 0.0028 for control and GAD fish, respectively). There was no genotype X sex interaction (p = 0.18) in GnRH level. The GAD transgenic male fish had significantly (p = 0.0018) lower levels of testosterone in the serum than non-transgenic control males. There was no significant (p = 0.9069) difference in the level of estradiol in the transgenic and non-transgenic female fish. F: female; M: male. Means in the same row or column followed by different letters (lowercase for GnRH and uppercase letters for testosterone) were significantly different.

Genotype	GnRH (ng/ml)			Estradiol (pg/ml)	Testosterone (pg/ml)
	F	M	F&M	F	M
GAD	3.05 ± 1.22^{a}	1.02 ± 0.31^{b}	2.03 ± 1.36	262.17 ± 82.25	288.84 ± 62.62^{A}
Control	3.07 ± 0.66^{a}	2.34 ± 1.22^{a}	2.58 ± 0.88	254.14 ± 71.73	699.12 ± 211.90^{B}
GAD&Control	3.05 ± 1.06^{a}	1.54 ± 1.36^{b}			

Discussion

Three generations of GAD transgenic fish were produced, and reproductive performance was repressed in all the three generations, as revealed by sexual maturation evaluation and spawning percentages. The reproductive performance of the putative infertile GAD transgenic fish was not always restored by hormone therapy. GnRH levels in the serum of 1-year-old and 4-year-old F2 generation fish showed the same trend that no significant difference exists between female transgenic and non-transgenic fish. The lower GnRH levels in the 4-year-old male fish implied a different regulation modes of GABA on GnRH in adult males than females as transgenic and control females were not different.

As one of the major inhibitory neurotransmitters in the CNS, GABA is involved in multiple biological functions [37–40,81]. A study in humans showed that oral administration of GABA could elevate serum growth hormone concentration [82]. The overexpression of the GAD gene may have pleiotropic effects in fish in addition to the desired disruption of reproduction examined in this study. However, growth of 1-year-old F2 generation full-sib GAD transgenic and non-transgenic fish was not different. Other aspects of pleiotropic effects such as disease or other stress resistance, survival and seinability should also be included in the future, as GABA has also demonstrated to be effective as a relaxant and could enhance immunity under stress conditions in human and chickens [83,84].

The roles of GABA in teleost fish reproduction discovered so far consist of guiding GnRH neurons migration during embryogenesis, regulating GnRH secretion in different life stages, as well as mediating LH secretion and estradiol feedback regulation. Overexpression of GAD gene

should lead to the increase of GABA, which in return would interfere with the normal regulation of GABA on these reproduction related biological processes, thereby disrupting the normal reproductive activities. However, studies have shown that the interruption of GnRH neuron migration or the production of GnRH may not be sufficient to cause major problems of reproduction. In GAD67 transgenic mice, the overexpression of GAD slowed the migration of GnRH neurons and resulted in increased positional diversity of these neurons, but did not block the neurons from migrating into the brain. The reproduction of the mutant mice was not totally interdicted, but was affected to a certain extent as the mutant mice had normal puberty initiation but inordinate estrous cyclicity and reduced reproductive capacity [49]. Another study in mice demonstrated that a small portion (12%) of the GnRH neuron population was sufficient for puberty onset and that dysfunction of the majority of GnRH neurons is required for disrupting fertility [53]. Similarly, mice with the $GABA_A$ receptor knocked down in GnRH neurons had normal puberty onset, cyclicity and fertility, but the negative feedback of estrogen on LH was affected with the increment of LH in the $GABA_A$ KO mice 2 weeks after ovariectomy being almost double of that in the control mice [85].

In zebrafish, the bi-allelic knocking out of the GnRH3 gene did not cause major changes in ontogeny and reproduction, the higher levels of gonadotropin genes mRNA observed in the early developing mutant fish were also adjusted to normal levels in the adults [86]. Partial ablation of GnRH3 neurons in zebrafish caused reduction of fecundity, but oocyte development was normal, and the fish were fertile, while fish with GnRH3 neurons completely ablated were infertile [87]. In our study, although not always statistically significant, GAD transgenic fish showed reduced reproductive capacity consistently in the F1 and F2 generations as compared to non-transgenic

controls. The less than 100% disruption of reproduction by GAD overexpression in this study might be due to incomplete disruption of GnRH neuron migration so that some of the neurons migrated to their destination, which could be sufficient to partially regulate the proper development of the reproduction system. If controlled properly, the incomplete disruption of GnRH neuron could be beneficial as the ultimate goal was to make the sterilization reversible so that reproduction of the transgenic fish could be restored when the production of a next generation is desired. Optimization of hormone therapy strategy is needed to increase the success of fertility restoration, as the efficiency of hormone therapy could be affected by the dosage, timing and frequency of hormone administration [15,88], and in some cases, the restoration of fertility was unsuccessful in the current study. The induction of GAD transgenic fish spawning could be different to that of the non-transgenic fish.

GABA exhibited no or inhibitory effect on the secretion of GnRH in immature fish and a stimulatory effect on the mature fish for most fish species [40]. In this study, observed GnRH levels in the 1-year-old transgenic fish serum were lower than in non-transgenic fish for both females and males, but the difference was not statistically significant. The difference was significant when sexes were pooled, but not dramatically.

In addition to the inhibitory effect of GABA on GnRH secretion, GAD transgenic fish should have less functional GnRH neurons and thereby less GnRH secreted if the migration of GnRH neurons was successfully disrupted by the overexpression of GAD, assuming other compensatory pathways do not exist in these fish. In GAD transgenic mice, GnRH in the hypothalamus was lower than the control mice, but quickly adjusted back to the normal values

by the second week after birth. Accordingly, GtH levels in the serum had the same change and were returned to similar levels as in the control mice by four weeks old [49]. Similar compensatory mechanisms might also exist in the GAD transgenic fish to adjust their hormone synthesis and secretion so that they could address the problems of insufficient GnRH neurons. In the 4-year-old fish, there was no significant difference in the GnRH and estradiol levels between the transgenic and non-transgenic female fish. However, GnRH level in the GAD transgenic males was lower than in the non-transgenic males. Coinciding with GnRH, the level of testosterone in the transgenic male fish was also considerably lower than in the control fish, a different mode of GnRH and testosterone regulation by GABA in males compared to the female channel catfish was implied. Correlation analysis showed that GnRH level was positively correlated with the level of estradiol and testosterone in the 4-year-old female ($R = 0.96$, $p = 0.0001$) and male ($R = 0.73$, $p = 0.17$) fish, respectively. While there was no correlation between the levels of GnRH and estradiol in the 1-year-old female fish ($p = 0.13$). This discrepancy of correlations between the GnRH and sex steroid levels in the 1-year-old and 4-year-old fish is likely due to the different life stages they were at, as the feedback regulation of sex steroid in fish is age, season and reproductive stage dependent [22,23,89,90].

Conclusion

Overexpression of GAD showed some potential for repressing the reproductive performance of fish. A small percentage of GAD transgenic channel catfish were fertile. The overexpression of GAD may not be 100% effective, compensatory mechanisms may exist or there may be allelic differences in the transgene insertion leading to the fertility of this small percentage of GAD individuals. These potential factors should be evaluated. Additionally, selection for individuals

that exhibit the appropriate sterile phenotype may lead to 100% effectiveness of this approach. The hormone therapy was not always effective which is not surprising since GnRH is not responsible for the last step in the gamete maturation and release. A more complex regime in GnRH administration may be needed to lead to more consistent restoration of fertility and spawning.

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Appendix 1. Supplementary information for the sexual maturity evaluation of the 4-year-old sterilization construct (Nanos-nanos, Nanos-dnd, Dazl-vasa) exposed channel catfish (*Ictalurus punctatus*). Fish were exposed to linear or circular Nanos-nanos, Nanos-dnd, Dazl-vasa sterilization construct via electroporation at 1 cell stage, and treated or not treated with doxycycline during embryogenesis. Control fish were exposed to TE buffer and did not receive dox treatment. All fish were evenly distributed and grown in two replicate ponds at the same density. A gonad development score from 0 (worst) to 5 (best) was given to the fish according to the size of gonad, amount of gametes in the gonad, and the quality of gametes. GSI: gonadosomatic index = weight of gonad*100/weight of body; F: female; M: Male.

Fish ID	Construct	Sex	Body Weight (kg)	Gonad Weight (g)	Gonad Development Score	GSI
200	Control	F	0.36	1.00	0.5	2.78
107	Control	F	1.08	44.45	3.5	41.16
14	Control	F	1.58	86.64	4.5	54.83
105	Control	F	1.37	89.81	4	65.56
18	Control	F	1.36	95.26	4.5	70.04
195	Control	F	1.24	91.50	4	73.79
227	Control	F	1.49	110.00	4.5	73.83
104	Control	F	2.22	170.55	5	76.83
266	Control	F	1.43	112.50	4.5	78.67
126	Control	F	1.24	102.06	4	82.31
146	Control	F	1.31	112.00	4.5	85.50
205	Control	F	1.3	135.00	4.5	103.85
277	Control	M	1.26	2.60	2.5	2.06
35	Control	M	3.3	7.61	4.5	2.31
242	Control	M	1.06	2.54	3	2.40
109	Control	M	1.35	4.06	4	3.01
271	Control	M	0.66	2.05	3	3.11
179	Control	M	0.41	1.38	3	3.37
25	Control	M	1.5	5.18	4.5	3.45
108	Control	M	1.58	5.68	4	3.59
208	Control	M	1.35	5.01	4	3.71
181	Control	M	0.51	1.90	3.5	3.73
4	Control	M	1.41	5.46	4.5	3.87

17	Control	M	1.36	5.94	4.5	4.37
206	Control	M	0.71	4.62	4.5	6.51
174	Nanos-dnd	F	0.32	0.55	1	1.72
163	Nanos-dnd	F	0.26	0.45	1	1.73
201	Nanos-dnd	F	0.25	0.50	1	2.00
292	Nanos-dnd	F	0.185	0.50	0.5	2.70
289	Nanos-dnd	F	0.35	1.00	1	2.86
189	Nanos-dnd	F	0.32	1.00	1	3.13
180	Nanos-dnd	F	0.29	0.93	1.5	3.21
161	Nanos-dnd	F	0.15	0.50	1	3.33
248	Nanos-dnd	F	0.3	1.00	1.5	3.33
221	Nanos-dnd	F	0.28	1.00	1	3.57
175	Nanos-dnd	F	0.48	2.00	1.5	4.17
188	Nanos-dnd	F	0.31	1.50	1.5	4.84
31	Nanos-dnd	F	0.48	3.18	1.5	6.62
247	Nanos-dnd	F	0.26	3.50	2	13.46
135	Nanos-dnd	F	0.35	6.00	2	17.14
284	Nanos-dnd	F	0.49	14.00	2.5	28.57
65	Nanos-dnd	F	0.41	14.52	3	35.40
68	Nanos-dnd	F	0.42	14.97	2	35.64
64	Nanos-dnd	F	0.55	19.96	3	36.29
102	Nanos-dnd	F	0.27	10.43	2	38.64
30	Nanos-dnd	F	0.69	29.03	3.5	42.07
47	Nanos-dnd	F	0.89	41.73	3	46.89
7	Nanos-dnd	F	0.6	28.58	3.5	47.63
9	Nanos-dnd	F	0.46	22.23	3	48.32
177	Nanos-dnd	F	0.79	41.50	3.5	52.53
147	Nanos-dnd	F	1.36	82.50	4	60.66
116	Nanos-dnd	F	1	61.69	4	61.69
251	Nanos-dnd	F	0.86	54.00	4	62.79
63	Nanos-dnd	F	0.97	62.14	3.5	64.07
125	Nanos-dnd	F	1.22	80.74	3.5	66.18
111	Nanos-dnd	F	1.13	77.57	4	68.64
143	Nanos-dnd	F	0.34	24.50	3.5	72.06
130	Nanos-dnd	F	0.65	48.08	3	73.97
139	Nanos-dnd	F	0.9	68.50	3.5	76.11
255	Nanos-dnd	F	0.63	48.00	3.5	76.19
49	Nanos-dnd	F	0.44	34.02	3	77.32
165	Nanos-dnd	F	1.71	133.50	4.5	78.07
16	Nanos-dnd	F	1.05	82.10	4.5	78.19
53	Nanos-dnd	F	1.38	112.49	4.5	81.52
158	Nanos-dnd	F	0.48	40.00	4	83.33

280	Nanos-dnd	F	0.63	53.00	3.5	84.13
212	Nanos-dnd	F	1.07	90.50	4.5	84.58
57	Nanos-dnd	F	1.35	115.21	4.5	85.34
151	Nanos-dnd	F	0.56	51.00	3.5	91.07
253	Nanos-dnd	F	1	93.00	4	93.00
170	Nanos-dnd	F	0.73	68.50	4	93.84
209	Nanos-dnd	F	1.16	111.00	4	95.69
194	Nanos-dnd	F	0.69	71.00	4	102.90
150	Nanos-dnd	F	0.81	102.50	4.5	126.54
217	Nanos-dnd	F	1.27	167.00	5	131.50
12	Nanos-dnd	M	0.34	0.03	0	0.09
236	Nanos-dnd	M	0.88	0.12	0.5	0.14
164	Nanos-dnd	M	0.35	0.05	0	0.14
51	Nanos-dnd	M	0.27	0.05	0.5	0.19
218	Nanos-dnd	M	0.66	0.13	0.5	0.20
48	Nanos-dnd	M	0.52	0.11	0.5	0.21
117	Nanos-dnd	M	0.84	0.21	1	0.25
141	Nanos-dnd	M	0.36	0.11	0.5	0.31
3	Nanos-dnd	M	1.48	0.47	1.5	0.32
38	Nanos-dnd	M	0.44	0.14	1	0.32
275	Nanos-dnd	M	0.26	0.09	0.5	0.35
231	Nanos-dnd	M	0.64	0.29	0.5	0.45
137	Nanos-dnd	M	0.42	0.21	0.5	0.50
169	Nanos-dnd	M	1.37	0.87	1.5	0.64
185	Nanos-dnd	M	0.58	0.37	1.5	0.64
8	Nanos-dnd	M	0.56	0.49	1	0.88
192	Nanos-dnd	M	0.34	0.38	2	1.12
10	Nanos-dnd	M	0.43	0.50	2	1.16
240	Nanos-dnd	M	0.82	1.06	3	1.29
115	Nanos-dnd	M	0.86	1.21	2	1.41
283	Nanos-dnd	M	1.54	2.27	4	1.47
2	Nanos-dnd	M	1.45	2.18	3	1.50
134	Nanos-dnd	M	3.11	5.00	4	1.61
67	Nanos-dnd	M	0.42	0.69	2.5	1.64
55	Nanos-dnd	M	1.51	2.63	3.5	1.74
127	Nanos-dnd	M	0.82	1.54	1.5	1.88
13	Nanos-dnd	M	1.19	2.28	4	1.92
211	Nanos-dnd	M	0.72	1.39	3	1.93
88	Nanos-dnd	M	1.08	2.09	3	1.94
254	Nanos-dnd	M	1.98	4.07	4	2.06
89	Nanos-dnd	M	2.46	5.50	4.5	2.24
121	Nanos-dnd	M	1.12	2.54	3.5	2.27

210	Nanos-dnd	M	1.14	2.59	3.5	2.27
36	Nanos-dnd	M	1.29	3.01	3.5	2.33
293	Nanos-dnd	M	1.69	4.10	4.5	2.43
106	Nanos-dnd	M	1.08	2.73	2.5	2.53
168	Nanos-dnd	M	1.04	3.00	3	2.88
95	Nanos-dnd	M	1.77	5.12	4	2.89
250	Nanos-dnd	M	1.38	4.02	4	2.91
82	Nanos-dnd	M	1.39	4.11	4	2.96
274	Nanos-dnd	M	0.62	1.84	3	2.97
235	Nanos-dnd	M	0.79	2.36	3	2.99
249	Nanos-dnd	M	1.21	4.18	3.5	3.45
265	Nanos-dnd	M	2.1	9.57	4.5	4.56
204	Nanos-dnd	M	1.44	7.42	4.5	5.15
241	Nanos-nanos	F	0.65	1.10	1	1.69
193	Nanos-nanos	F	0.58	1.00	1	1.72
281	Nanos-nanos	F	0.99	2.00	1.5	2.02
28	Nanos-nanos	F	0.65	1.36	1	2.09
183	Nanos-nanos	F	0.35	0.79	1.5	2.26
120	Nanos-nanos	F	0.28	0.91	0.5	3.24
144	Nanos-nanos	F	0.26	1.00	0.5	3.85
24	Nanos-nanos	F	0.253	1.36	1	5.38
86	Nanos-nanos	F	0.23	1.36	1	5.92
75	Nanos-nanos	F	0.56	3.63	1.5	6.48
187	Nanos-nanos	F	0.35	2.50	1.5	7.14
11	Nanos-nanos	F	0.25	2.27	1.5	9.07
191	Nanos-nanos	F	0.26	4.50	2	17.31
84	Nanos-nanos	F	0.36	6.35	1.5	17.64
77	Nanos-nanos	F	0.64	15.42	2.5	24.10
78	Nanos-nanos	F	0.59	20.41	2.5	34.60
197	Nanos-nanos	F	0.71	25.00	2.5	35.21
246	Nanos-nanos	F	0.52	18.50	3	35.58
100	Nanos-nanos	F	0.57	20.41	2	35.81
5	Nanos-nanos	F	0.47	18.60	3.5	39.57
42	Nanos-nanos	F	0.98	39.01	3.5	39.81
20	Nanos-nanos	F	1.03	41.73	3.5	40.52
87	Nanos-nanos	F	0.4	16.33	2.5	40.82
46	Nanos-nanos	F	0.6	27.22	3	45.36
70	Nanos-nanos	F	0.45	20.87	2	46.37
245	Nanos-nanos	F	0.52	25.00	3.5/1.5	48.08
184	Nanos-nanos	F	1.1	53.00	3.5	48.18
186	Nanos-nanos	F	0.58	33.50	3.5	57.76
93	Nanos-nanos	F	0.48	29.03	3	60.48

124	Nanos-nanos	F	0.95	58.51	3	61.59
118	Nanos-nanos	F	0.4	24.95	2.5	62.37
198	Nanos-nanos	F	0.66	42.00	3.5	63.64
60	Nanos-nanos	F	0.69	44.45	3	64.42
26	Nanos-nanos	F	1.78	117.94	4.5	66.26
261	Nanos-nanos	F	0.52	35.00	3.5	67.31
243	Nanos-nanos	F	0.46	33.50	3.5	72.83
66	Nanos-nanos	F	0.49	35.83	3.5	73.13
110	Nanos-nanos	F	0.77	57.61	3.5	74.81
258	Nanos-nanos	F	0.69	52.00	3.5	75.36
44	Nanos-nanos	F	0.95	76.66	4	80.69
157	Nanos-nanos	F	0.68	55.50	4	81.62
76	Nanos-nanos	F	0.65	53.98	4	83.04
256	Nanos-nanos	F	0.7	58.50	4	83.57
272	Nanos-nanos	F	0.68	57.50	4	84.56
123	Nanos-nanos	F	1.08	94.80	4	87.78
58	Nanos-nanos	F	1.4	122.93	4.5	87.80
268	Nanos-nanos	F	1.52	135.00	4.5	88.82
23	Nanos-nanos	F	0.67	61.24	4.5	91.40
74	Nanos-nanos	F	0.91	83.46	4.5	91.72
229	Nanos-nanos	F	1.09	106.50	4	97.71
101	Nanos-nanos	F	0.52	52.62	3.5	101.19
287	Nanos-nanos	F	0.7	72.50	3.5	103.57
61	Nanos-nanos	F	1.43	150.14	5	104.99
294	Nanos-nanos	F	0.52	55.00	4	105.77
98	Nanos-nanos	F	0.75	79.38	4	105.84
196	Nanos-nanos	F	0.8	86.50	4	108.13
220	Nanos-nanos	F	0.88	102.00	4.5	115.91
214	Nanos-nanos	F	0.56	65.00	4.5	116.07
224	Nanos-nanos	F	0.92	152.50	5	165.76
276	Nanos-nanos	M	1.12	0.02	0	0.02
40	Nanos-nanos	M	0.28	0.02	0.5	0.07
33	Nanos-nanos	M	0.37	0.03	0	0.08
152	Nanos-nanos	M	1.19	0.13	0.5	0.11
136	Nanos-nanos	M	0.72	0.08	0.5	0.11
173	Nanos-nanos	M	0.46	0.08	0.5	0.17
153	Nanos-nanos	M	0.52	0.11	0.5	0.21
171	Nanos-nanos	M	0.33	0.07	0	0.21
238	Nanos-nanos	M	0.79	0.19	1	0.24
148	Nanos-nanos	M	0.7	0.19	0.5	0.27
52	Nanos-nanos	M	1.01	0.32	1.5	0.32
72	Nanos-nanos	M	1.37	0.47	0.5	0.34

43	Nanos-nanos	M	1.05	0.43	1	0.41
167	Nanos-nanos	M	1.16	0.48	1.5	0.41
145	Nanos-nanos	M	0.44	0.22	0.5	0.50
190	Nanos-nanos	M	0.3	0.15	0.5	0.50
166	Nanos-nanos	M	1.74	0.93	2	0.53
263	Nanos-nanos	M	0.28	0.17	0.5	0.61
162	Nanos-nanos	M	0.46	0.32	1	0.70
286	Nanos-nanos	M	0.43	0.32	2	0.74
22	Nanos-nanos	M	0.63	0.56	1.5	0.89
80	Nanos-nanos	M	0.57	0.55	2.5	0.96
131	Nanos-nanos	M	0.4	0.41	1	1.03
1	Nanos-nanos	M	1.54	1.81	3	1.18
34	Nanos-nanos	M	0.53	0.72	1.5	1.36
79	Nanos-nanos	M	0.49	0.69	3	1.41
203	Nanos-nanos	M	1.42	2.00	3	1.41
257	Nanos-nanos	M	0.62	0.88	2.5	1.42
114	Nanos-nanos	M	0.89	1.49	1.5	1.67
178	Nanos-nanos	M	0.73	1.27	3.5	1.74
15	Nanos-nanos	M	2.02	3.72	4	1.84
278	Nanos-nanos	M	1.22	2.30	2	1.89
83	Nanos-nanos	M	1.92	4.02	4.5	2.09
202	Nanos-nanos	M	3.58	7.50	4.5	2.09
230	Nanos-nanos	M	0.9	1.89	3	2.10
159	Nanos-nanos	M	0.47	1.00	3.5	2.13
19	Nanos-nanos	M	1.26	2.77	3.5	2.20
54	Nanos-nanos	M	2.14	4.84	4	2.26
6	Nanos-nanos	M	1.67	4.43	3.5	2.65
267	Nanos-nanos	M	1.05	2.80	3.5	2.67
96	Nanos-nanos	M	1.28	3.43	2.5	2.68
225	Nanos-nanos	M	0.76	2.15	4	2.83
270	Nanos-nanos	M	1.9	5.60	4.5	2.95
85	Nanos-nanos	M	0.29	0.90	0	3.10
138	Nanos-nanos	M	1.51	5.00	3.5	3.31
213	Nanos-nanos	M	1.01	3.67	4/0.5	3.63
269	Nanos-nanos	M	0.71	2.74	4	3.86
112	Nanos-nanos	M	1.33	7.19	4.5	5.41
156	Dazl-vasa	F	0.95	0.50	0.5	0.53
160	Dazl-vasa	F	0.34	0.50	1	1.47
291	Dazl-vasa	F	0.28	0.50	1	1.79
262	Dazl-vasa	F	0.52	1.00	1.5	1.92
219	Dazl-vasa	F	0.41	1.00	1	2.44
199	Dazl-vasa	F	0.45	3.00	2	6.67

37	Dazl-vasa	F	0.47	4.99	2	10.62
288	Dazl-vasa	F	0.3	4.50	2	15.00
133	Dazl-vasa	F	0.46	10.43	2	22.68
50	Dazl-vasa	F	0.37	15.42	2.5	41.68
62	Dazl-vasa	F	0.58	27.22	3	46.92
172	Dazl-vasa	F	0.55	26.50	3	48.18
122	Dazl-vasa	F	1.04	54.89	3.5	52.77
155	Dazl-vasa	F	0.87	48.50	3.5	55.75
290	Dazl-vasa	F	0.29	16.50	3.5	56.90
99	Dazl-vasa	F	0.77	59.42	3.5	77.17
32	Dazl-vasa	F	0.43	34.47	3.5	80.17
252	Dazl-vasa	F	1.14	92.00	4	80.70
282	Dazl-vasa	F	0.63	54.00	3.5	85.71
223	Dazl-vasa	F	0.98	89.50	4	91.33
94	Dazl-vasa	F	1.07	109.32	4.5	102.17
97	Dazl-vasa	F	0.85	89.36	4.5	105.13
228	Dazl-vasa	F	0.74	78.00	4.5	105.41
285	Dazl-vasa	F	0.66	85.00	4	128.79
92	Dazl-vasa	F	0.64	340.20	3.5	531.56
91	Dazl-vasa	M	0.62	0.00	2.5	0.00
132	Dazl-vasa	M	0.4	0.04	0.5	0.10
207	Dazl-vasa	M	0.65	0.11	0.5	0.17
27	Dazl-vasa	M	0.87	0.15	0.5	0.17
142	Dazl-vasa	M	0.74	0.15	0.5	0.20
149	Dazl-vasa	M	0.89	0.19	0.5	0.21
140	Dazl-vasa	M	0.6	0.14	0.5	0.23
244	Dazl-vasa	M	0.53	0.13	0.5	0.25
154	Dazl-vasa	M	0.66	0.18	0.5	0.27
259	Dazl-vasa	M	0.62	0.17	0.5	0.27
216	Dazl-vasa	M	0.72	0.26	1	0.36
233	Dazl-vasa	M	0.35	0.13	1	0.37
73	Dazl-vasa	M	0.6	0.25	1.5	0.42
21	Dazl-vasa	M	0.67	0.33	1	0.49
273	Dazl-vasa	M	0.79	0.40	1.5	0.51
239	Dazl-vasa	M	0.86	0.77	2	0.90
81	Dazl-vasa	M	0.5	0.61	2.5	1.22
176	Dazl-vasa	M	0.6	0.85	1.5	1.42
226	Dazl-vasa	M	1.11	1.89	3	1.70
56	Dazl-vasa	M	0.81	1.42	3	1.75
113	Dazl-vasa	M	0.97	1.78	2	1.84
260	Dazl-vasa	M	0.93	1.74	3	1.87
45	Dazl-vasa	M	0.72	1.77	3.5	2.46

103	Dazl-vasa	M	1.27	3.25	3.5	2.56
59	Dazl-vasa	M	1.63	4.27	4	2.62
182	Dazl-vasa	M	0.96	2.74	4.5	2.85
237	Dazl-vasa	M	0.94	2.76	3	2.94
90	Dazl-vasa	M	2.31	7.20	4	3.12
279	Dazl-vasa	M	1.42	4.48	4	3.15
41	Dazl-vasa	M	1.93	7.95	4.5	4.12