Reproductive Confinement of Common Carp, *Cyprinus carpio*, and Channel Catfish, *Ictalurus punctatus*, via Transgenic Sterilization

by

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Abstract

Transgenic animals had shown great potential for increasing growth rate, ability to resist disease, and for use as tool to research in forward and reverse genetics. Genetically modified animals could pose ecological risk to the environment upon escapement. Modified sterile feral constructs based on sterile feral concept and technology has been applied to reproductively control the genetically modified animals in the field of aquaculture.

Constructs utilzing short hair-pin structures to produce cDNA, theoretically could control primordial germ cell (PGC) marker genes, *nanos, vasa* and *dead end (dnd*) in channel catfish, *Ictalurus punctatus*. Constructs were electroporated into embryos of common carp and channel catfish. Ideally, the embryos would undergo normal development and the gonad would form and mature in the presence of repressible compounds. However, without repressible chemicals, the gonad would not develop normally. Real-time PCR was used to test the relative temporal expression level of natural PGC marker gene expression between constructs electroporated and no constructs non-electroporated embryos (common carp) and no constructs electroporate embryos (channel catfish). Sexual maturity of electroporated and control channel catfish and common carp were evaluated.

In the case of knockdown common carp, two proteins, *nanos* and *dead end* were targeted and a third related, but off-target PGC protein, *vasa*, was assayed. The effectiveness of constructs varied depending upon the promoter. The expression levels of these proteins naturally decreased during development. In some, but not all cases, the expression of targeted mRNA was knocked down to minimal levels or degradation accelerated. Up-regulation was also observed for *dead end*, possibly due to interferon responses. In other cases, the sterilization constructs had little effect on the targeted gene,

but strongly down-regulated one or two of the alternative PGC genes examined in this study. At 19 months of age, common carp males exposed to these constructs had reduced rates of sexual maturity, but evaluation of reduction of female rates of sexual maturity were inconclusive.

In the case of channel catfish electroporated with the knockdown constructs some constructs knocked down PGC genes at certain time points. As was the case with common carp, some constructs appeared to induce sterility in catfish at 3-year of age.

Further experimentation is needed to evaluate sterility in the F1 generation. Potential pleiotropic effects of these PGC knockdown constructs should be evaluated, especially since off-target effect was observed.

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Introduction: will transgenic sterilization reduce the ecological risk?

Fisheries and aquaculture has been making great contributions to the world's well-being and prosperity, providing the population with nutritious food and animal protein. The world population is increasing year by year and will continue to grow in the near future from current projection, with an increase from 7 billion to 10.5 billion by 2050, a 50 percent increase (Population Division, 2009; World population clock, 2012). With the increasing of world population, human beings would experience food crisis. Global consumption of meat is estimated to double by 2020 and total food consumption will double by 2030 (Population Resource Center, 2012). Hardy (1999) predicted that aquaculture will have to increase by 350% to meet the shortage of 55 million t seafood products by 2025 from leveled wild catch and increasing demand. Fish provide 3.0 billion people with almost 20 percent of their intake of animal protein, and 4.3 billion people with about 15 percent of such protein (FAO, 2012). Although world food supply has grown dramatically in the past five decades, exceeding global population growth, a great and steady demand for food are much more urgent than ever before because of sluggish economic crisis, greater climate change vulnerabilities, declining domestic fishery production (10% down in the period 2000-2010) (FAO, 2012).

The United States ranks the world's second largest importer of seafood and the third in wild capture fishery landings, but is the 14th in aquaculture production (FAO, 2010). Catfish is one of the primary cultured species in the U.S., ranking sixth in the "top 10" fish and seafood consumption list for Americans (Hanson and Sites, 2010). Catfish farming accounted for over two-thirds of US aquaculture production and over half of the value in the 1980's and 1990's (ARS, Aquaculture National Program, 2008). However, catfish production has been stagnating since 2003. Catfish production in the United States peaked in 2003 when approximately 650 million pounds were processed (Hanson, 2010). For a variety of reasons, primarily the low cost of imports and the increased cost

associated with domestic production (e.g., ingredients to manufacture feed, fuel, fish disease, survival, growth conversion), production has since decreased and the amount of catfish processed in 2010 was equivalent to the amount processed in the early 1990's (approximately 470 million pounds) (Hanson, 2010). The number of U.S catfish operations and catfish production acres continues to decrease, especially in last four years. High prices for corn and soybean resulted in high catfish feed prices from 2008 to 2012. Imported catfish fillets increased by 6.3% in 2010 as substitutes to meet food needs, accounting for 57% of all U.S sale of frozen catfish fillet product. Sustainability needs to be greatly improved for catfish farming and profitability and competitiveness should be strived for regarding the deficit balance. The US catfish industry is in crisis, and undergoing a critical evolution.

Culture of carps dominates fish farming in fresh water. Common carp (*Cyprinus carpio*) is a widespread freshwater fish, and is one of the most economically important cultured species worldwide with an annual global production of 3.4 million tons (out of 24.2 million tones carp), accounting for 14% of the freshwater aquaculture production in the world (FAO, 2012). Farming of common carp is popular in many countries and represents about 64% of the farmed cyprinids in Europe (Huntingford et al. 2012). Common carp is also kept as an ornamental species. Color variants of common carp, koi, are valuable as ornamentals with more than 100 different types of coloration (Wang and Li, 2004). Although common carp have great economic importance, in many countries it is considered an invasive species. Once introduced and established, common carp are very hard to eradicate. Common carp populations have a negative effect on the environment in Australia, and the U.S government has been spent millions of dollars annually to control common carp populations. Transgenic technologies could be used to enhance common carp for aquaculture, but might also be used to help control common carp populations.

Transgenesis can be utilized as a tool in genetic and genome research, as a means to increase knowledge of gene regulation in organisms; Transgenic animals have the potential to increase economic benefit by improving growth rate, disease resistance, and feed conversion, enhancing nutritional and processing qualities, coloration, recreational

traits and other desirable traits (Dunham, 2003; Van Eenennaam, 2006). Fish lay large quantities of eggs, have external fertilization and embryonic development, a lower possibility of carrying human pathogens, and have large eggs that are readily pierced by a suitable glass needle (Liu, et al. 1990; Maclean, 1998; Van Eenennaam, 2006), making them amenable for transgenesis compared to mammals.

Transgenic fish have been produced for at least 14 species of fish, including model species, such as zebrafish and medaka, non-model species such as Atlantic salmon, coho salmon, chinook salmon, tilapia, rainbow trout, catfish, carp and northern pike (Dunham, 2003; Devlin et al 2006). Traits and phenotypes addressed include growth (growth hormone), freeze tolerance (antifreeze protein), disease resistance (cecropin and lactorferrin), carbohydrate metabolism (glucose transporter and hexokinase), lipid metabolism (D6-desaturase), phosphorus metabolism (phytase), and vitamin C metabolism (L-gulono-gamma-lactone oxidase) (Devlin et al 2006). Growth hormone is one of the most widely used transgenes. Additionally, different fluorescent proteins are widely used to study the gene interaction, protein function, embryonic development and gene expression.

In the middle 1980s'introduction of mammalian GH genes later fish GH sequences driven by non-piscine promoters resulted in 10-80% faster growth in transgenic fish compared to non-transgenic fish in aquaculture conditions (Dunham, 2003). In the 1990's , all-fish GH gene constructs were developed and used, resulting in transgenic Atlantic salmon and Nile tilapia (Du, et al 1992; Rahman and Maclean, 1999) with growth enhanced from 10% to 30X. Growth rate of transgenic Atlantic salmon increased by 400% to 600%, but body weight at maturity was slightly larger than controls. Feed input decreased 25% for these transgenic salmon, and the time to market is greatly shortened (Du et al. 1992).

Although GH genes greatly enhanced growth rate, pleiotropic effects occurred for moisture content, body composition, intestinal surface area, morphological abnormalities, gill morphology, endocrine stimulation, and deposition of cartilage. The intestinal surface area in GH transgenic Atlantic and coho salmon was enlarged 2.2X compared to that of

control salmon and growth rate was about twice that of the controls (Stevens and Devlin, 2000). Moisture content in GH transgenic Atlantic salmon was higher than normal controls (Cook et al. 2000). GH transgenic Pacific salmonids had morphological abnormalities in the head, fin, jaw and operculum when compared to controls (Devlin et al 1995). GH transgenesis had different pleiotropic effects in closely related species. GH transgenic Atlantic salmon and Pacific salmon had different gill morphology than controls. Transgenic Pacific salmon had smaller lamellar spacing than control, and transgenic Atlantic salmon had longer gill filaments than controls (Stevens and Devlin, 2000). GH transgenic salmonoids had other pleiotropic effects, such as reduced reproductive fitness (Fitzpatrick et al. 2011), reduced swim ability (Farrell, et al. 1996), reduced disease resistance (Jhingan et al. 2003), modified regulation of GH (Mori et al.1999) and the thyroid (Eales, et al. 2004). GH transgenic mature parr of Atlantic salmon (*Salmon Salar*), an alternative male reproductive phenotype (i.e. precocially mature and mature only in freshwater), have showed reduced fertilization success compared with nontransgenic wild-type mature parr. However, GH transgenic anadromous males (i.e. larger, fighter males) out-performed wild counterparts. Though breeding differences among the two phenotypes of GH transgenic Atlantic salmoon existed, results demonstrated both male reproductive had the ability to pass down transgene into offspring (Moreau et al. 2011). GH gene is one of the few genes used in production transgenic fish breeding, and benefits the breeding program as a whole.

Zbikowska (2003) predicted that fish will be the first group of transgenic animals, to be applied commercially. At this time, the only commercialized transgenic fish is the GloFish, a zebrafish danio, which expresses fluorescence protein and is used for ornamental purposes. Transgenic Atlantic salmon containing a single copy of a DNA sequence coding for Chinook salmon growth hormone driven by regulatory sequences derived from Chinook salmon and the eel-like ocean pout is reported to close to be approved by the Food and Drug Administration (FDA) (Marris, 2010). Fish Information & Services (FIS) reports that the US Senate rejected an amendment demanding further study of evaluation of environmental and economic impact of transgenic AquaAdvantage® salmon on May 24, 2012 (FIS, 2012).

The first transgenic fish was produced by Zhu et al. (1985) and many researchers have developed transgenic fish with improved growth rate and disease resistance but 27 years later none of the transgenic fish have been commercialized for the food market. The commercialization of transgenic fish is likely dependent on food safety and effective containment. The food safety of a transgenic fish largely depends on the DNA insert, transgene coding sequence, gene product and insertion process and insertion mutagenesis (Berkowitz and Kryspin-Sorensen, 1994; Maclean, 2003). Public concerns include consumption of transgenes, change in the nutritional composition of foods, potential production of toxins, and activation of vial sequences and allergenicity of transgenic products (Dunham 2003).

Aquaculture activity is conducted in land-based or sea-based facilities. Sea-based facilities such as pens are more vulnerable to accidents and pose much greater risk of escapee than do land-based aquaculture facilities because of storm damage, accidental mechanical damage from boats, predator damage or human errors (Devlin and Donaldson, 1992). For example, farmed salmon escapement has been reported worldwide, including the North Atlantic, Scotland, and Norway (Bratspies, 2006). They had the ability to adapt to the local environmental condition, and in some cases, the number of escapees of Atlantic salmon outnumbered the wild population, and threaten the wild population thorough either competition or interbreeding. Evidence showed that Atlantic salmon has successfully spawned in British Columbian waters and become an invasive species (Volpe, 2000).

Fish are highly mobile and because of escapement or intentional stocking they can easily become feral, are difficult to contain and this is of greatest concern (NRC, 2002). There are no standard criteria for evaluating the potential risk of exotic introductions, models have been suggested to estimate the genetic risk of transgenic fish, including the Trojan gene effect (Muir and Howard, 1999), and a deterministic model (Hedrick, 2001).

Fitness determines the ability of a genotype, exotic species or transgenic fish to become established in the natural environment. Fitness is defined as the probability of producing off-spring and transmitting genetic information to future generations of a population.

Muir and Howard (2001) concluded that six components of an organism's life cycle are needed for a fitness evaluation, including juvenile viability, adult viability, age at sexual maturity, female fecundity (number of eggs), male fertility and mating success, which were used to group into two category if survival or reproductive capacity of transgenic differ from wild type. Dunham (2011) also indicated that predator avoidance, foraging ability and swimming ability are also important components of fitness.

The Trojan gene effect model was developed using GH and non-transgenic medaka in aquaria, and the result indicated that normal females had a mating preference for large, less fit transgenic males that could lead to extinction of a population (Muir and Howard 1999). Shortcomings of this model include no consideration for genotype-environment interactions, no predation, artificial feed (no foraging), a highly artificial environment and an atypical mating preference for a typical fish species. Large male fish do not always have mating advantage, for example, reproductive success of male Atlantic cod was determined by female size (Rakitin et al. 2001).

Similarly, Hedrick (2001) developed a deterministic model on the assumption that transgene has a male-mating advantage, but a general viability disadvantage, providing a detailed examination of mating advantage and viability disadvantage for transgenic organisms. The conclusion was that transgene increase in frequency for 66.7% of possible combinations of the possible mating and viability parameters; the transgene goes to fixation for 50% of the combinations. Increasing the frequency of the GH transgene results in the reduced viability of natural population and increased probability of extinction of conspecific natural populations.

All models are limited, and. no single model can take all factors from nature into consideration. Different research groups have different opinions on the relative fitness of transgenic, domesticated and wild types (Maclean and Laight, 2000; Hedrick, 2001; Dunham, 2003; Dunham 2011). Risk-based frameworks or platforms need to be developed to evaluate individual transgenes and transgenic species under the actual (best simulation of) ecosystem on a case-by-case basis.

Regardless of risk, several options exist for containment of transgenic fish. Simultaneous application of multiple containment strategies were recommended by the National Research Council (NRC, 2004). Wong and Van Eenennaam (2008) had reviewed and compared different containments strategies, and grouped them into different categories including physical, biological and genetic containment. Physical containment and biological containment have their internal and distinct disadvantages.

One option for genetic containment is triploid induction. Triploid induction rates vary between 10 and 100%, depending on the species, shock conditions and egg batch quality. Triploidy can decrease performance in fish (Dunham, 2004), and is not feasible on a commercial scale in the case of catfish and many other species of fish. Triploid induction reduced performance of GH transgenic fish negating about half of the enhancement from the transgenesis (Dunham, 2004; Nam et al. 2004). Triploidy also has the disadvantage that it required fertile diploid brood stock, so the possibility of escape and risk can be reduced, but not eliminated.

An alternative genetic containment is transgenic sterilization. An option is the disruption of translation of reproductive genes with antisense followed by hormone therapy when reproduction is needed. Earlier study (Uzbekova et al., 2001) was using antisense Atlantic salmon (*Salmo salar*) sGnRH cDNA, driven by the sGnRH Pab promoter in production of transgenic rainbow trout. They had positive results in their initial studies but never followed up. Also this was not the sterile feral approach, it was straight antisense followed by hormone therapy.

Hu et al. (2006) reported that a recombinant construct using carp beta –actin driving antisense Atlantic salmon GnRH gene microinjected into fertilized common carp eggs, and produced 30% undeveloped gonads in the founders. They also reported that the fertility could be restored by exogenous hormone administration. Unfortunately, 100% sterility was not produced in the transgenic common carp, hindering further application and commercialization. The system might be improved by the careful analysis of construct design, altering the dosage of external GnRH administration, and the environmental design.

Wu et al (2010) also modified similar system to control reproduction of transgenic zebrafish by microinjection. The construct was driven by an ovary-specific and a testis-specific promoter, respectively. The transgene of interest was a suicide gene, consisting of a reductase and photosensitizer, while the reductase gene was linked to a reporter gene. The novelty of this concept is that infertility is induced if transgenic fish expressing the reductase are treated with an effective amount of reductase-activated cytotoxic prodrug or if the transgenic fish expressing photosensitizer are treated with light irradiation. They had reported 100% reliable infertility in zebrafish. The author has doubt regarding how to effectively providing a transgenic male with three copies of fusion transgene because the high copy number of transgene prevents high transfection rate of the cell, and integration sites are random events. A piece sequence of Simian virus 40, a polyomavirus, was used for design of the construct, which may be disadvantage when considering commercialization.

One form of transgenic sterilization, Sterile Feral (SF) technology has shown promise for achieving transgenic reproductive confinement. Components of these constructs are fused so that a specific promoter is coupled to a repressible element that in turn drives expression of a blocker gene, antisense RNA, dsRNA, sense RNA or ribozyme to an early key developmental gene (Dunham 2004; Thresher et al. 2005; Thresher et al. 2009). This strategy has two parts: one is to suppress the expression of critical gene related to embryonic development gonad development or sexual maturity via a knockdown construct in the absence of a repressible element. The second step is to reverse the sterility of transgenic fish by administrating exogenous compound to shut off expression of the blocker gene allowing rescue of the embryos and/or to produce brood stock. This knockdown strategy could be used to generate reversibly sterile transgenic fish. Proof of principle of the sterile feral system has been demonstrated with the repression of the knockout function demonstrated in zebrafish, oysters, channel catfish and common carp for disruption of embryonic development (Thresher et al. 2005; Templeton 2005; Chaimongkol 2009; Thresher et al. 2009). This approach could be utilized to disrupt gamete production by preventing primordial germ cell (PGC) migration during embryogenesis.

PGCs migrate far from the site of developing gonads to the genital ridge where they differentiate into gametes. The decision by PGCs to develop into gametes varies and depends on the species and clues. Mammals and *Drosophila* have different mechanisms controlling the differentiation of gametes. The mechanism of fish gamete differentiation can be grouped into two different ways (Slanchev et al. 2005). Channel catfish and medaka have undifferentiated gonads, which develop into ovaries in females and testes in males. Zebrafish is representative of the second mechanism. Ovary-like structure is initially found in all embryos; subsequently this structure develops into ovaries in females, or after the death of the oocytes, into testis in males.

A number of genetic markers are associated with and expressed in PGCs such as *vasa*, *nanos*(nos), *dead end* (*dnd*), cxcr4b, and dazl (Raz, 2004). In general, the function of PGC marker genes was studied using morpholino (MO) antisense oligonucleotides by specifically inhibiting the translation of the corresponding RNA (Raz, 2003; Slanchev et al. 2005). Expression pattern, function, cell-fate maintenance, migration mechanism of PGC marker genes were reviewed (Raz, 2004; Richardson and Lehmann, 2010).

Weidinger et al. (2003) found that *dead end* protein RNA binding protein is expressed in PGCs of zebrafish throughout embryogenesis. *Dead end* expression was required for PGC migration, survival and female development. *Dnd* knockout produced all male with highly reduced fertility (Weidinger et al 2003; Slanchev et al. 2005).

Zebrafish nanos is essential for proper migration and survival of primordial germ cells (Koprunner et al. 2001). Nanos deficient PGCs migrate, but are abnormal, have premature activation of germ cell markers, exhibit abnormal morphology and express mRNA usually found in the soma. When nos-1 was knocked out, PGC migration stopped and they died. Dazl is another protein involved in PGC migration. *zDazl* and *brul* are locally expressed like *vas*, *nanos*, and *dead end* (Hashimoto et al. 2004).

Vasa was discovered in zebrafish (Yoon et al. 1997). *Vasa* differs from other PGC markers in that it is continually expressed in the germ line (Yoon et al. 1997; Koprunner et al. 2001; Weidinger et al. 2003).

The expression of the PGC genes is very specific, and they do not appear to have other functions. This is another advantage for application in transgenic sterilization as pleiotropic effects would be predicted to be minimal when multiple functions and interactions do not exist. However, pleiotropic effects are possible, and should be examined in transgenically sterilized fish. Pleiotropic effects have been primarily studied for GH transgenic fish. The list of observed pleiotropic effects in various species of fish include changes in feed conversion efficiency, feed consumption, behavior, survival, disease resistance, tolerance of low oxygen, respiration, swimming ability, predator avoidance, body shape, body composition, dressout %, organelle number, gill morphology, gene expression, IGF level, reproduction and intestinal length (Dunham 2008).

Our long-term goal is to accomplish reversible transgenic sterility in fish, specifically catfish and carp, and to reproductively confine and prevent the establishment of transgenic or domestic genotypes in the natural environment. Our specific objectives are to prevent primordial germ cell (PGC) migration and gamete formation rendering the carp and catfish sterile, and to reverse this process in a subset of embryos to produce fertile brood stock that generate sterile offspring.

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II Expression and Knockdown of Primordial Germ Cell Genes, *Vasa*, *Nanos* and *Dead end* in Common Carp, *Cyprinus carpio,* Embryos for Transgenic Sterilization and Reduced Sexual Maturity

Abstract:

Common carp, *Cyprinus carpio,* is the most widely grown aquaculture species worldwide. Four types of knock down constructs designed to transgenically sterilize fish were electroporated into common carp embryos to knockdown or eliminate expression of primordial germ cell (PGC) proteins, *nanos* and *dead end*. Embryos from different developmental stages were collected and tested using quantitative realtime PCR. REST program was used to analyze gene expression among control embryos and embryos exposed to these constructs during development. Two proteins, *nanos* and *dead end* were targeted, each specific for a set of constructs, and a third related, but non-targeted PGC protein, *vasa,* was assayed. The effectiveness of constructs varied depending upon the promoter. The expression levels of these proteins naturally decreased during development. In some, but not all cases, the expression of targeted mRNA was knocked down to minimal levels or degradation was accelerated. Up-regulation was also observed for *dead end*, possibly due to interferon responses. In other cases, the sterilization constructs had little effect on the targeted gene, but strongly down-regulated one or two of the alternative PGC genes examined in this study. At 19 months of age, common carp males exposed to these constructs had reduced rates of sexual maturity; however, reduction of female rates of sexual maturity was not conclusively shown. These constructs appear promising for the potential transgenic sterilization of common carp. This accomplishes the first step for transgenic sterilization of transgenic fish harboring other transgenes or other fish of concern for preventing environmental effects of aquacultured fish.

Keyword: Transgenic sterilization, Common carp, Real-time PCR

1. Introduction

Common carp (*Cyprinus carpio*), is a widespread freshwater fish, and is one of the most economically important cultured species worldwide with an annual global production of 3.4 million tons, accounting 14% of the freshwater aquaculture production in the world (FAO, 2007). Common carp is also selected and kept as ornamental species. One of the color variants of common carp, koi, is valuable ornamental species with more than 100 different types of coloration (Wang and Li, 2004). Common carp is used as model species in the fields of immunology, developmental biology and others (Ji et al. 2012). Common carp have reproductive traits and early sexual maturity that may make them excellent, large, realistic, aquaculture model species for other warm water aquaculture species with long generation intervals such as channel catfish, *Ictalurus punctatus.* Although common carp have great economic importance, in many countries it is considered an invasive species. Once introduced and established, common carp are very hard to eradicate. Common carp populations have a negative effect on the environment in Australia, and the U.S government has been spent millions of dollars annually to control common carp populations. Transgenic technologies could be used to enhance common carp for aquaculture, but might also be used to help control common carp populations.

Although transgenic technologies have shown promise for helping to supply the world populations with food, public fear exists that transgenic animals would pose risk to the environment and ecology upon release or escape. Two major categories of containment are physical and genetic confinement. Physical control has inherent risk, and cannot guarantee the establishment of transgenic lines in the natural environment (Wong and Eenennaam, 2008). Genetic control could include triploidy, however, triploidy requires existence of fertile, diploid transgenic brood stock, which opens the possibility of escapement and establishment of transgenic fish. Additionally, triploidy can decrease performance in fish (Lilyestrom et al. 1999; Dunham 2004, 2011) and is not feasible on a

commercial scale in the case of catfish and many other species of fish. Triploid induction reduced performance of GH transgenic fish negating about half of the enhancement from the transgenesis (Dunham 2004, Nam et al. 2004). Transgenic sterilization might obviate these problems, and could address other environmental impact issues with interspecific hybrid fish, exotic species and domestic fish.

One transgenic sterilization technology that has been evaluated is Sterile Feral (SF) technology, a modified tet-off system (Thresher, et al 2005, 2009). The SF system comprises: a trigger molecule and construct. The construct is made of four main parts: first nucleic acid encoding a promoter, closely linked to the second nucleic acid encoding the tetracycline transcriptional activator protein (tTA), the third nucleic acid encoding a promoter called tet repressible element (TRE) and coupled to and tightly regulated a minimal promoter region, the fourth nucleic acid encoding a blocker molecule which could be antisense RNA, sense RNA, double-stranded RNA, or ribozyme. The SF system works as follows: the first promoter drives the expression of tTA in a spatio-temporal way; in the absence of trigger molecule (such as doxycycline), the tTA binds to TRE, thus activating the minimal promoter, eventually resulting in the transcription of the blocker gene and the death of embryos; in the presence of trigger molecule, the tTA binds to trigger molecule, which prevents the TRE, minimal promoter and block gene from working, the embryos develop normally.

Expression of double stranded RNA, RNA interference (RNAi) technology is one potential mechanism used in SF technology. When sense and antisense molecules linked by loop sequence bind each other and form a double-stranded RNA, they can silence genes after undergoing procession in the cell (McManus and Sharp, 2002; Bantounas, et al, 2004; Wong and Van Eenennaam 2008). Several important components are involved in the procession, which include Dicer, recognizing and cleaving double stranded long RNA into fragments; another protein complex, called RNA-induced silencing complex

(RISC), bind these fragments and unwind the duplex fragment into single strands. Sense strand of the siRNA is removed, but antisense remains bound to the RISC complex to detect mRNA molecules and guide their cleavage. The process would result in RNA degradation with siRNA, translational inhibition with microRNAs and probably target chromatin remodeling with the outcome of gene silencing.

Another mechanism that can be used in SF technology is overexpression of cDNA, sense RNA, and this approach has disrupted BMP-2 function in zebrafish, common carp and channel catfish (Thresher et al. 2009). Gene overexpression with multiple-copy plasmid libraries has successfully suppressed a temperature-sensitive cdc24 mutation, leading to discovery of four such genes (Bender and Pringle, 1989). Overexpression could complement loss-of-function screens, and has dominant effects (Prelich, 2012).

SF technology has been demonstrated with repression of the knockout function shown in zebrafish, oysters, channel catfish and common carp (Thresher et al. 2009). Although doxycycline (dox), used as the repressor molecule, can be easily administered in water to allow embryonic development, discharge of dox into the environment would be public concern and application of such system would be of great cost if scaled up. These problems might be solved by knocking out reproductive genes rather than embryonic development genes or by developing systems that are not dependent on dox. In both cases, targets could be primordial germ cell (PGC) genes responsible for PGC migration, colonization of the genital ridge and ultimately gamete formation. Candidate genes include *nanos, vasa* and *dead end*.

Vasa gene is a germ cell marker in zebrafish, trout, tilapia and medaka and from gibel carp, *Carassius auratus gibelio* (Xu et al., 2005). *Vasa* gene was only expressed in the gonads, rather than brain, liver, spleen, kidney, muscle and heart in gibel carp. *Vasa* is expressed continuously in the germ line (Yoon, et al. 1997; Koprunner et al. 2001; Weidinger, et al 2003). Satio et al. (2006) concluded that *vasa* sequence does not work across many species and may have evolved to have different function in different species, while *nanos* has a more constant function across species.

Zebrafish *nanos* gene is essential for PGC formation, proper migration and survival (Koprunner, et al 2001). *Nanos* deficient PGCs migrate, but they exhibit abnormal morphology, and express mRNA in the soma. When *nanos* was knocked out, the PGC migration stopped and the PGCs eventually died.

Dead end (dnd) is another important gene for migration and survival of PGCs. Knockdown of *dnd* confined the PGCs to the deep blastoderm in zebrafish, preventing migration and leading to the death of PGCs, but did not influence the secondary effect of PGC specification because of *nos-1* and *vasa* were normally expressed till early gastrulation stages (Weiginger, et al 2003). However, expression of both *nanos-1* and *vasa* markers gradually disappeared, though *dnd* RNA expressed longer after the PGC died (Weidinger et al. 2003). Males and females have different timing of germ cell proliferation (Moringa et al. 2004). Another study demonstrated a novel function of *dnd* that zebrafish would invariably develop into sterile males in PGCs were ablated using *dnd* antisense morpholino oligonucleotide (MO). *dnd* was vital for female development (Slanchev et al. 2005), confirming the result of the *dead end* knockouts being male in zebrafish with higher sterility (Weidinger et al. 2003). Ablation of the germ line with *Dnd* (MO) resulted in smaller gonad structure in zebrafish embryos (Slanchev, et al., 2005). No gonad-like structures were found in dnd MO injected fish at 90 days post-fertilization. In zebrafish, germ cells are not required for the formation of the gonads but are essential for its differentiation and survival. Sterile zebrafish males without gonads still exhibited sexual behavior and could induce normal females to ovulate. If these embryos were treated with estrogen, they developed into sterile fish with female characteristics. The unique expression pattern of *nanos, dnd* and *vasa* make them applicable and potentially advantageous in transgenic sterilization, because pleiotropic effects would be predicted to be minimal when multiple functions and interactions do not exist.

Our objectives were to determine the normal pattern of gene expression of *nanos, vasa* and *dead end* in common carp embryos, evaluate shRNAi constructs and a cDNA construct designed for knockdown of *nanos* and *dead end* in channel catfish (theoretically, disrupting migration of the PGCs and preventing germ cell development), examine off target effects, determine the effectiveness of different promoter systems, and

determine sexual maturity in 19-month-old common carp exposed to transgenic sterilization constructs during embryonic development.

2. Materials and Methods

2.1 Construction of plasmids

All the constructs used in this experiment have the backbone of the plasmid FRMwg (GenBank: AF170915.1; Gibbs and Schmale, 2000), which has three primary elements, insulator, Ocean pout terminator and boundary element. The regulatory elements triggering the knockdown systems included Tet-off based, yeast copper control based, yeast reduced copper control based, zebrafish racemase based and zebrafish Adss based systems. These systems were modified according to the sterile feral, SF, system described by Thresher, et al (2005, 2009).

Channel catfish *nanos* gene and the channel catfish *vasa* promoter were sequenced. All construct systems included 4 alternative knockdown genes components, *nanos*1 ds-sh RNA, *nanos*2 ds-sh RNA, *nanos* cDNA and *dead end* ds-sh RNA. These were inserted between the insulater and Ocean pout terminator. *Nanos*1 and *Nanos*2 in our study were designed as short hairpin structure targeting 5' (abbreviation N1) and 3' (abbreviation N2) of *nanos* gene; *Nanos* cDNA was full-length of cDNA sequence of *nanos* of channel catfish (abbreviation cDNA). *Dead end* ds RNA was as short hairpin structure of channel catfish *dead end* gene (*dnd*) (see Fig.1.).

Channel catfish, *Ictalurus punctatus*: *dnd* shRNA designs.

ShRNA design against ***dead end*** gene of the channel catfish.

Oligo1.5′GTTCCAGAGCGTAGCGCCGCTCTACGAGTTCCGAGCGGCGCTACGCTCTGGAAC3′

Oligo2.3′CAAGGTCTCGCATCGCGGCGAGATGCTCAAGGCTCGCCGCGATGCGAGACCTTG5′

Channel catfish, *Ictalurus punctatus*: *nanos* shRNA designs.

N1: targeting 5' *nanos* gene

Oligo1.5′CTCTCTATCCCGATGACTCGCGATGGTGTTTTCGCGAGTCATCGGGATAGAGAG3′

Oligo2.3′GAGAGATAGGGCTACTGAGCGCTACCACAAAAGCGCTCAGTAGCCCTATCTCTC5′

N2: targeting 3' *nanos* gene

Oligo3.5′CCGAAAATCTGAACCCCACTCTCACACTCGCTAGAGTGGGGTTCAGATTTTCGG3′

Oligo4.3′GGCTTTTAGACTTGGGGTGAGAGTGTGAGCGATCTCACCCCAAGTCTAAAAGCC5′

Legend: Loops were in grey shade, stem sequences were underlined.

Fig. 1. Double stranded short hairpin structures dead end sh (dnd shRNA), and nanos shRNAs (5'nanos sh and 3'nanos sh) used for knockdown Primordial germ cell marker genes

The catfish nanos promoter was used to drive the tet-off system. Other promoters included adenylase promoter and racemase promoter from zebrafish, yeast (copper specific transcriptional repression) CTR3 promoter and CTR3 promoter but less sensitive. They are designated as Nanos, ADSS, RM, M and MCTR, respectively. After designing the constructs, they were synthesized at GenScript Corporation (Piscataway, New Jersey, U.S.A).

2.2 Construct preparation, extraction and linearization

Plasmids were transformed with One Shot® Top10 chemically competent *E.coli* cell, following the procedures recommended by the protocol. About 100 to 200 µl transformation mix was used to plate on the LB agar (10.0 g Tryptone, 5.0 g yeast extract, 5.0 g NaCl, 15.0 g agar in 1.0 L solution) plates. A single colony was picked up from each plate to conduct a mini-prep plasmid DNA extraction (Qiagen mini-prep). Plasmids were linearized with *SfiI* (20,000 units/ml, BioLabs) following the product's protocol with little modification. For each plasmid digestion, a mixed 50 µl reaction solution contains 1 µg of plasmid DNA, NEBuffer 4 (10×), BSA (100×) and the volume adjusted using water to 50 µl.

DNA Agarose Gel electrophoresis was used to analyze the plasmid DNA (using uncut plasmid, *SfiI* cut plasmid). Once the size of plasmid was confirmed, a maxi-prep plasmid DNA extraction was carried out using Qiagen maxi-prep kit. Plasmid DNA was linearized following the same enzyme digestion protocol. After plasmids linearization, phenol-chloroform-ethanol method was used to inactivate *SfiI* enzyme and purify the

DNA. The quantity of DNA was measured using a UV-spectrophotometer. Each plasmid from above procedure was prepared and diluted in 6.0 ml TE buffer (5 mM Tris-HCl, 0.5 M EDTA, pH=8.0) at the concentration 50 µg/ml.

2.3 Brood fish and gamete

Common carp males and females were kept in troughs at the Fish Genetics Research Unit, Auburn University. The water temperature was kept at 25 °C before the fish were induced to spawn in the early January, 2011. Brood stock carp with visible reproductive traits were induced using cap pituitary extraction (CPE). CPE were dissolved 30-45 min in the 0.85% saline before injected with priming dose of 0.4 mg/kg and followed 12 hours later with a resolving dosage of 3.6 mg/kg, respectively. After the priming injection, male common carp were placed downstream of the females, but separated by a divider.

Females ovulating eggs were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222), and were slight rinsed in the trough with genital vent covered with a finger. The anesthetized females were towel dried and eggs were hand-stripped into pans or bowl lubricated with Crisco vegetable shortening. Males were anesthetized, rinsed and dried in the same way and sperm were hand stripped into a sterile dry cup.

2.4 Gene transfer and electroporation procedures

High quality eggs with greenish and yellowish color were distributed evenly in small petri dishes. One to two drops of sperm were spread onto one thin layer egg (around 250 eggs) using 3.0 ml syringe. Pond water (pre-heated to 25 °C) was quickly added for fertilization. After 5minutes, fertilized eggs were transferred into a larger pan with enough pond water to incubate the zygotes with periodic agitation. After 20 min, the eggs were collected into the petri dishes (cuvette) for electroporation. Plasmid DNA solution was quickly added onto the fertilized eggs. Electroporation was performed with the Baekon 2000 macromolecule transfer system. Parameters were set at 6 kV, 2^7 pulses, 0.8 sec burst, 4 cycles, 160 µsec (Powers et al., 1992). Non-contact mode of electroporation with the electrode 1 to 2 mm above the buffer was applied. Once finishing

electroporation, the eggs were transferred into the Holtfreter's solution (NaCl 3.5g, $NaHCO_3$ 0.2 g, KCl 0.05 g, $MgSO_4$ 333 µl (300 g in 500 ml), $CaCl_2$ 333 µl (150 g in 500 ml), pH: 7~7.5 in 1.0 L decholorined water) in separate tubs (Armstrong, et al. 1989). Dead embryos were picked out and water was changed daily. Water temperature was kept at 25~26 °C using submersible heaters.

2.5 Embryos collection and RNA extraction

Embryos were collected at different time points, 0, 6, 12, 24, 48, and 72 hours post fertilization (hpf) for both non-transgenic embryos and potential transgenic embryos. Around 0.5 ml of embryos were collected into a 1.5 ml sterile tube and three replicates per sample were immediately submerged in liquid nitrogen. All the samples were stored at -80 °C till RNA extraction. Samples were ground using mortar and pestle in the presence liquid nitrogen.

RNA was extracted using TRIzol® Reagent (Ambio, cat #: 15596-018) and the extraction procedure followed the manufacture's recommendation. The quality and concentration of all the samples were checked by gel electrophoresis and UV-spectrophotometer. All extracted samples had an A260/280 ratio greater than 1.8, and were diluted to 500 ng/ µl.

2.6 Real-time PCR

RNAs were reverse transcribed into cDNAs by iScript Synthesis Kit (Bio-Rad, USA, cat #: 170-8891). Each of the reaction consisted of a total volume of 10 µl containing 4.0 µl iScript reaction mix, 1.0 µl iScript reverse transcriptase, 500 ng RNA template, and water to reach the 10 µl volume. The reaction followed the protocol: 5 minutes at 25 °C, 30 min at 42 °C, 5 min at 85 °C.

qRT-PCR was carried out on a C1000 Thermal Cycler (Bio-Rad, USA) by using SsoFast EvaGreen supermix kit (Bio-Rad, USA, cat: #172-5201) following the manufacturer's instructions with modification. Briefly, all qRT-PCR reactions were performed in a 10 µl total reaction volume (9.0 µl mix and 1.0 µl 200 ng/µl cDNA). The mix contained 1.0 µl of each primer (5 umol/ µl), 5.0 µl SsoFast EvaGreen supermix, 2.0 µl RNase/DNase-

free water. The same cycling conditions were used for all the tested samples: denaturation, 95 °C /30s, 40 cycles of 95 °C /5s, and 57 °C /5s followed by melting curve analysis, 5s at 65°C, then up to 95°C at a rate of 0.1 °C/s. The mRNA levels of all the samples were either normalized to the levels of 0 hpf PGC marker genes expression for non-transgenic control or to the non-transgenic control sample of the same time point for the knockdown effect measurement. Ribosome 18s mRNA was used a reference gene. Crossing-point (Ct) values were exported into Excel sheet from Bio-Rad CRX Manager (Version 1.6.541.1028, 2008). The relative expression ratio of target gene was analyzed for significance test using a randomization test in the REST (Pfaffl, et al 2002) software assuming 100% efficiencies. The results were graphed with corresponding standard errors.

2.7 Data mining and primer design

Sequence of *nanos*, *vasa*, and 18s ribosomal mRNA gene of *Cyprinus carpio* could be found in NCBI with the accession ID, 323098305, 28275371 and 29336521, respectively. However, sequence of *dead end* in *Cyprinus carpio* was unavailable up to now. Therefore, sequences of *dead end* from *Danio rerio*, *Misgurnus anguillicaudatus*, *Oncorhynchus mykiss*, and *Carassius auratus* were aligned and analyzed (Fig. 12.) using Multiple Sequence Alignment by CLUSTALW program (http://www.genome.jp/tools/clustalw/). Conserved region from the alignment was used to design *dead end* primer. Primers were designed using primer3plus (Rozen and Skaletsky, 2000. Online primer design tool). Primers were listed in (Table 1).

Table 1 QT-PCR Primers used for detection knockdown effect on PGC marker genes in common carp (*Cyprinus carpio*).

Gene name	GID	Forward primer	Reverse primer
18s	gi|29336521	CATGGCCGTTCTTAGTTGGT	CGGACATCTAAGGGCATCAC
Nanos	gi|323098305	GGAGAAAAGAGACCCGGAAC	AATCTCTTGGTGTGCGCTTT
Vasa	gi|28275371	GGGCTGCAATGTATTGTGTG	GGCACTGAACATGAGGGTTT
Dead end	gi|47086450	AAGTCAATGGGCAGAGGAAA	TCATGAGGCGAAACTCGTAA

2.8 Sexual Maturity

The electroporated common carp were kept in aquarium and fed AquaMax® Fish Diet (PMI Nutrition International, LLC, St. Louis) till 19 month old. Two indicators were used to determine sex and state of sexual maturity in the current study (Jhingran and Pullin, 1985). Male common carp have a concave and triangular pit-like genital opening, while females have a protruding, swollen genital opening which is more oval with pinkish margins. Sexually mature females have a soft, swollen abdomen, and sometimes when gentle pressure was applied against the abdomen, the vent would slightly open and ova became visible inside the genital aperture. When abdominal pressure was applied to sexually mature males milt could be extruded.

2.9 Statistical analysis

Fisher's Exact Test and Fisher's Multi-treatment Exact Test were used to compare rates of sexual maturity between and among the different treatment groups. REST© (Relative Expression Software Tool, Pfaffl, et al 2002) using Pairwise Fixed Reallocation Randomisation Test© was applied to compare the three target genes with a reference gene between the treated group and control group. Group means were used to express differences in expression between control and treated sample. Mean crossing point deviation of control and sample group was normalized by a reference transcript. Statistical significance was performed by randomization test with 2,000 randomisations/test ($P<0.05$).

3. Results

3.1 Primer specificity

The alignment of sequences of *dnd* from *Danio rerio*, *Misgurnus anguillicaudatus*, *Oncorhynchus mykiss*, and *Carassius auratus* are highly conserved (Fig.2). Although *dnd* sequence from a close family number of Cyprinidae, *Carassius auratus* was chosen, primer design was based on the highly conserved region from zebrafish. Only a one

nucleotide difference in the forward primer and a three nucleotide difference in the reverse primer were found when comparing *dnd* sequences of *C. auratus* and *Danio rerio.*

DNA agarose gel electrophoresis was used to check the primer specificity from a RT-PCR test, indicating *dnd* primer could amplify one single band at 168 bp (data not shown). Specificity of each primer set was confirmed from real-time PCR because single peaks present in the melting curve analysis indicated that PCR product was specifically amplified as a single product (data not shown).

3.2 Relative expression of the three PGC marker genes in control embryos.

Embryos underwent early gastrulation (blastopore) at 6 hpf and neurulation at 12 hpf. At 24 hpf, embryonic movement started, and the cephalic region broadened with distinct fore brain. At 48hpf, the lens formed in the eyes and the heart formed while at 72 hpf, hatching began.

Nanos and *vasa* exhibited a similar expression pattern. Both *nanos* and *vasa* were significantly up-regulated at 6 hpf (approximately 1.5X, Fig. 3.). Both *nanos* and *vasa* genes down regulated significantly and progressively from 6hpf to 72hpf (2X at 12 h, 3-5X at 24 h and 5-7X at 48 h). At each point, *vasa* gene expression was relatively lower than that of *nanos* gene except at 12 hpf. *Vasa* gene was down-regulated more than that of *nanos* from 24 hpf toward 72 hpf, and progressively so. At 72 hpf, both of *nanos* and *vasa* were expressed at their lowest level with *nanos* down-regulated about 7X and *vasa* down regulated approximately 11X.

Fig.2. CLUSTAL 2.1 multiple sequence of *dead end* genes alignment result among *Danio rerio, Misgurnus anguillicaudatus, Oncorhynchus mykiss, and Carassius auratus* (*C.auratus*).

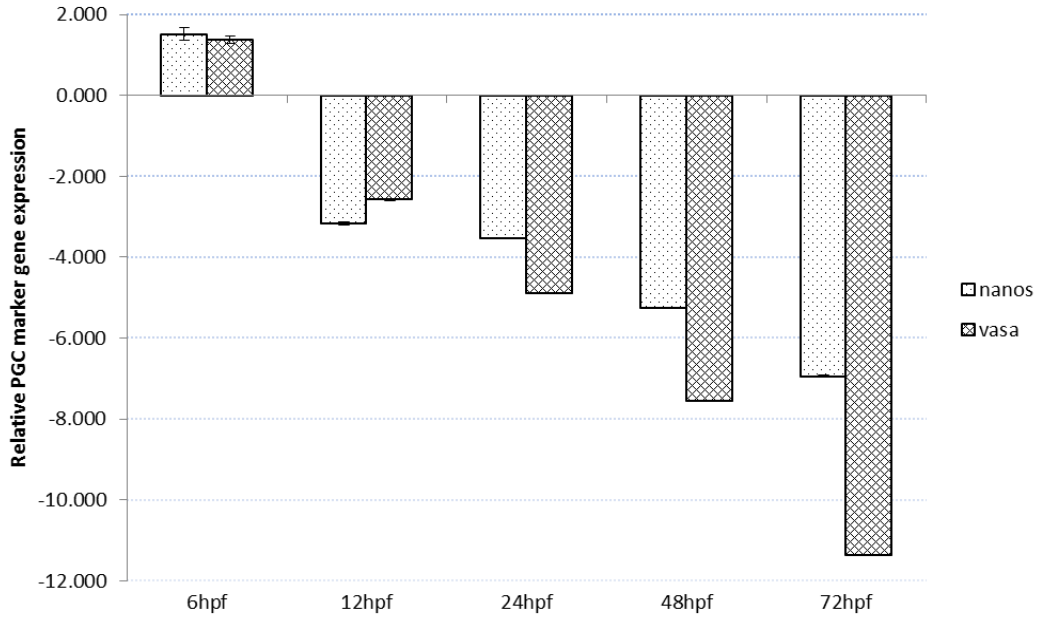

Fig. 3. Analysis of relative expression of *nanos* and *vasa* gene during embryos development using real-time PCR in non-transgenic common carp (*Cyprinus carpio*). The samples were analyzed at 6 h, 12 h, 24 h, 48 h and 72 h post fertilization, respectively. Relative *nanos* and *vasa* genes expression were expressed as fold change over control samples taken at 0h of unfertilized egg as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE; all the fold changes were significant at the level of $P< 0.05$ using Pairwise Fixed Reallocation Randomisation Test.

Dnd gene was down-regulated during early embryo development (Fig. 4). *Dnd* gene was down-regulated more dramatically at each time point compared with that of the other *nanos* and *vasa*. From 6 hpf to 72 hpf, *dnd* was continuously down regulated; 72 hpf expression showed the lowest down-regulated value (580X).

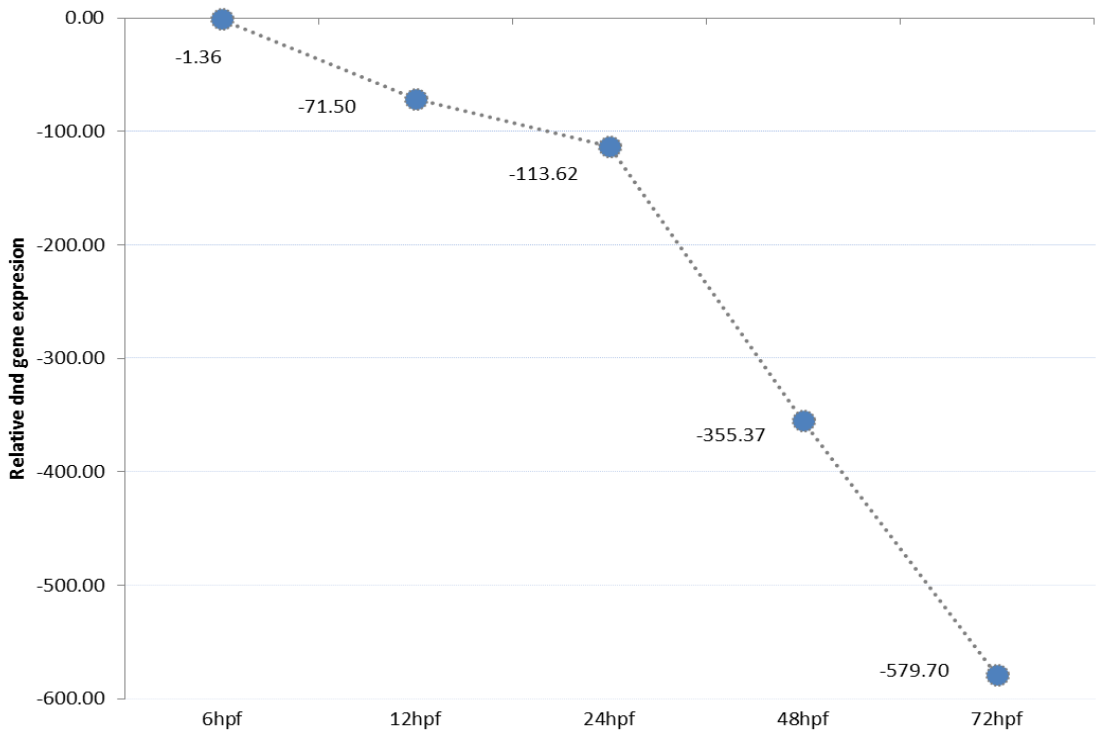

Fig.4. Analysis of expression of *dead end* gene during early embryos development using real-time PCR in non-transgenic common carp (*Cyprinus carpio*). The samples were analyzed at 6 h, 12 h, 24 h, 48 h, and 72 h post fertilization (hpf). Relative *dead end* expression was expressed as fold change over control samples taken at 0h of unfertilized egg normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. Numbers next to the dot on the line indicated relative fold change over time. Stand error were less than 0.001 except that at 6hpf which was 0.107. All the values were significant at the level of $p < 0.05$ except that of 6 hpf ($p=0.081$) using Pairwise Fixed Reallocation Randomisation Test.

3.3 Effect of promoters on each knock down construct.

3.3.1 Effects of Nanos, M, MCTR and RM promoter on knock down by *dnd* shRNAi

In general, these promoters were ineffective in knocking down the target, *dnd* (Fig.5), and had the opposite effect, up-regulation of *dnd*. At one time point, 24 h, the *nanos* driven construct did down-regulate *dnd* 4X, and at 72 h the M driven construct down-regulated *dnd* approximately 2X.

Off target knock down of *nanos* and *vasa* was prevalent with the *dnd* shRNAi construct (Fig.5). *dnd* shRNAi construct driven by promoters M, Mctr and RM knocked down *nanos* upwards to 7-22X at all-time points with MCTR appearing to be the most consistent. *Vasa* was knocked down to a lesser degree and less consistently. The

construct driven by M gave the strongest and most consistent result.

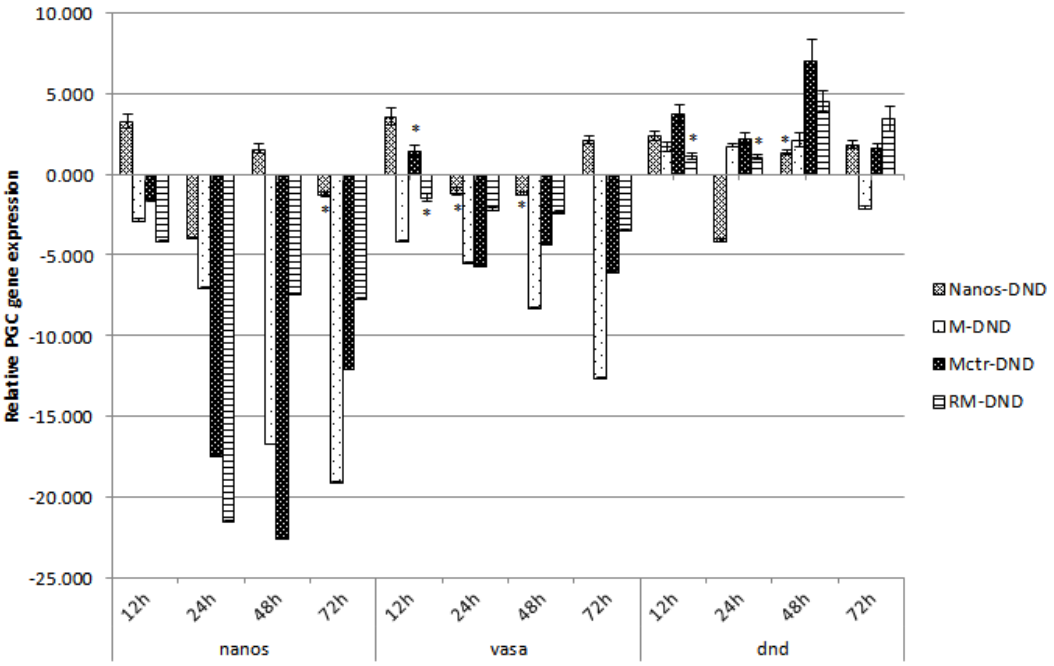

Fig.5. Effect of different promoters (channel catfish *nanos* (*nanos*), yeast CTR3 promoter M, yeast CTR3 promoter mctr (less sensitive) and zebrafish racemase (RM)) on channel catfish *dead end* (dnd) expression. Expression of PGC genes (*nanos*, *vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio*, using knockdown constructs with shRNAI targeting *dead end* gene (*DND*). Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.3.2 Effects of Nanos, Mctr and Racemase promoter on knock down by *nanos* cDNA

Nanos, mctr and rm promoter worked in concert with *nanos* cDNA to knockdown the PGC markers *nanos* and *vasa* (Fig.6). Knock down was the least at 12 hpf. Consistent with the results for the dnd shRNAi constructs, MTCR drove the strongest knock down of *nanos* and *vasa*.

Again, *dnd* was not knocked down dramatically or was up- regulated. The MCTR driven construct had strong up-regulation 25X on *dnd* at 12 h, but little or no effect during the rest of development. The RM driven construct had the most consistent up-regulation of

dnd, 4-10X at all-time points. Overall, the *nanos* driven construct did not have much effect on RNA levels of *nanos*, *vasa* and *dnd*.

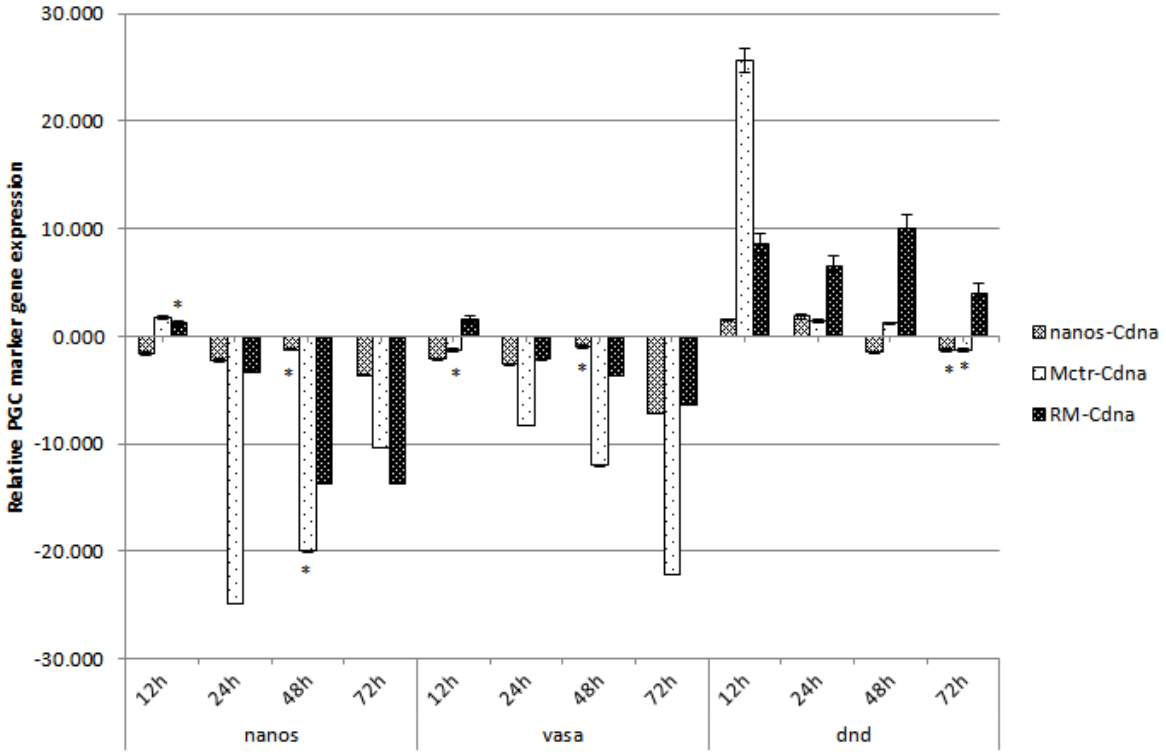

Fig.6. Effect of different promoters (channel catfish nanos (nanos), yeast CTR3 promoter mctr (less sensitive) and zebrafish racemase (RM)) on channel catfish *nanos* cDNA expression. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio,* using knockdown constructs with shRNAI targeting channel catfish *nanos* cDNA. Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.3.3 Effects of Nanos, M and Racemase promoters on knock down by *nanos* sh-5' construct

The M promoter worked best with sh5' *nanos* gene (Fig.7). It knocked down *nanos* and *vasa* at 24, 48 and 72 hours.This combination also knocked down the off-target *dnd* at 24 and 72 h, although it up-regulated *dnd* at 12 and 48 h.

The Nanos promoter with sh5' *nanos* gene also knocked down *nanos* at 24 h, 48 h and 72 h, but not as strikingly as M. This *nanos* construct did not affect *vasa* mRNA levels, but

was the only construct that up-regulated *dnd* gene at all-time points. The RM promoter construct knocked down *nanos* at 12 h, 24 h and 48 h, but not dramatically.

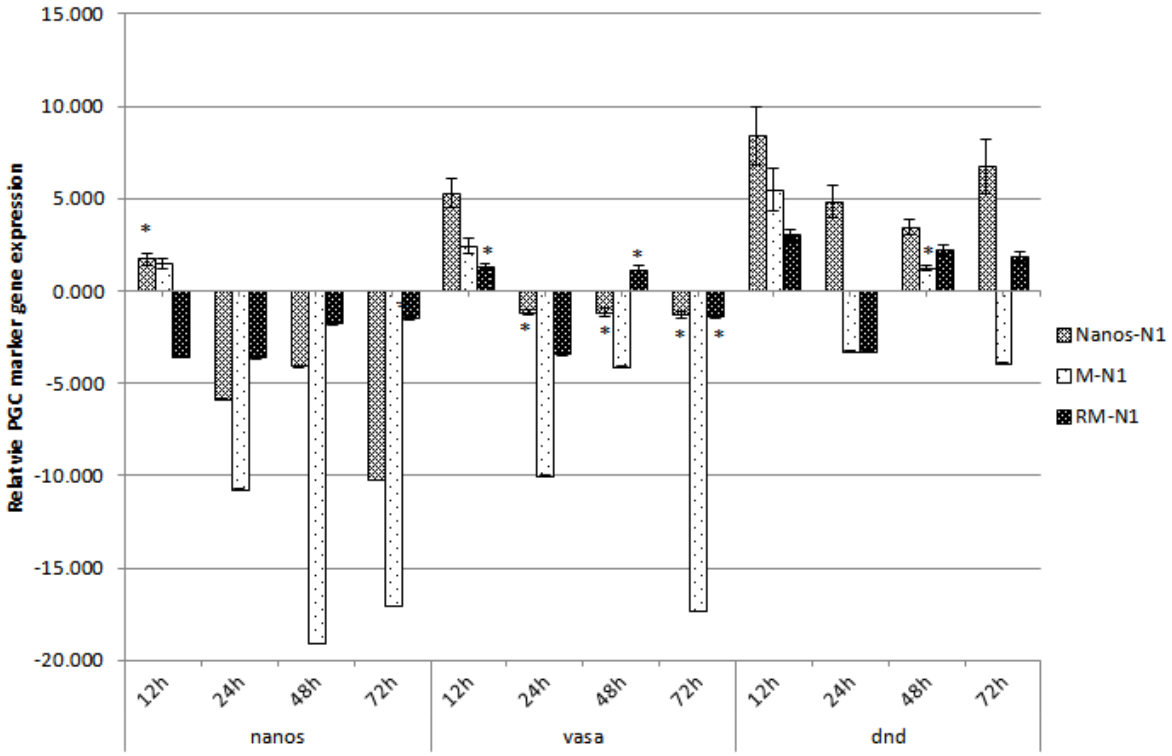

Fig.7. Effect of different promoters (channel catfish *nanos (nanos*), yeast CTR3 promoter m and zebrafish racemase (RM)) on 5' channel catfish *nanos* expression. Expression of PGC genes (*nanos*, *vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio,* using knockdown constructs with shRNAI targeting channel catfish 5' *nanos*. Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.3.4 Effects of Nanos, M, Racemase and ADSS promoter on knock down by nanos sh-3' construct

Again, the construct driven by M was the most consistent, knocking down *nanos* at all-time points (Fig.8), and it also knocked down the off-target vasa at all-time points. The ADSS driven construct was also quite effective for knocking down *nanos* from 12 hpf to 48 hpf. The construct driven by RM had strong down-regulation on *nanos* and *vasa* at hatch (72 hpf). Regulation of *dnd* was inconsistent from one time point to another with the *nanos* driven construct again up-regulating *dnd* 12-48 hpf.

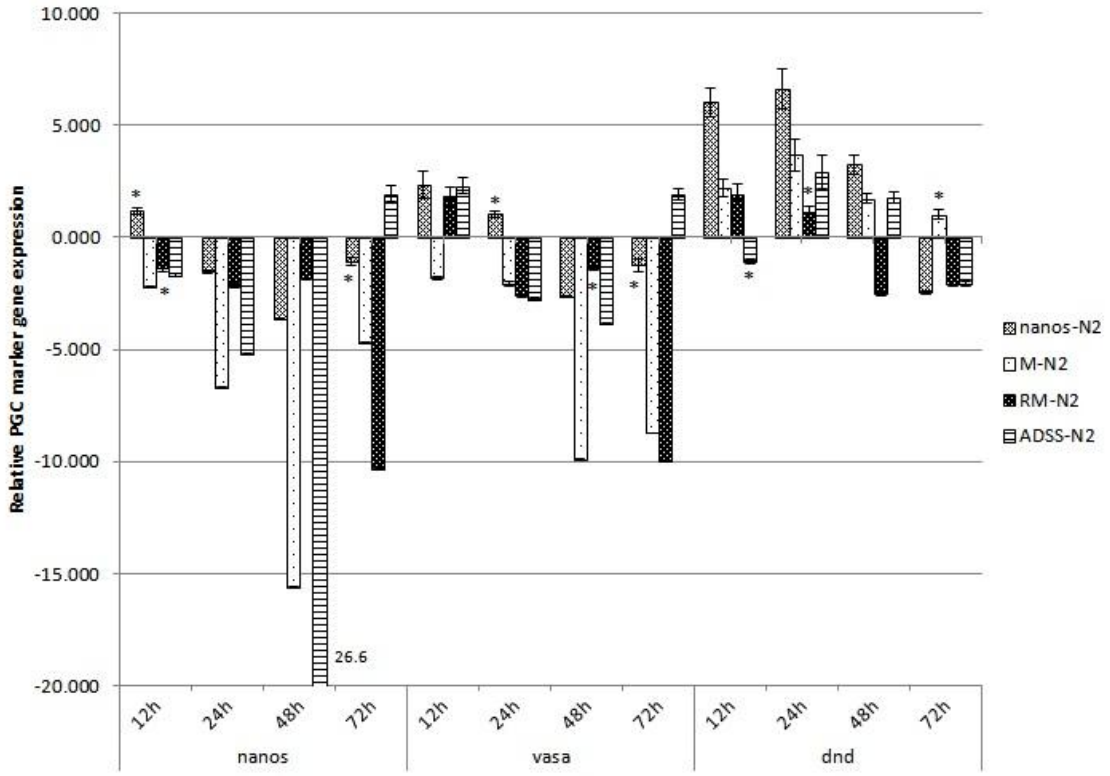

Fig.8. Effect of different promoters (channel catfish nanos (nanos), yeast CTR3 promoter m and zebrafish racemase (RM)) on 3' channel catfish *nanos* expression. Expression of PGC genes (*nanos*, *vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio*, using knockdown constructs with shRNAI targeting channel catfish 3' *nanos*. Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.4 Effects of genes in nanos constructs on PGC marker genes

Nanos

In general, constructs driven by the catfish *nanos* promoter down-regulated their target except when the target was *dnd* (Fig.9). Nanos-cDNA was the only construct that down-regulated *nanos* at all-time points. Nanos-N5'had the strongest down-regulation 4-10X during the period 24 hpf-72 hpf.

These constructs had sporadic effects on the off-target *vasa*. However, Nanos-cDNA was again the only construct to down-regulate at all-time points and most strongly. In general,

this set of constructs up-regulated *dnd*, and the shRNAi constructs had the greatest up-regulation.

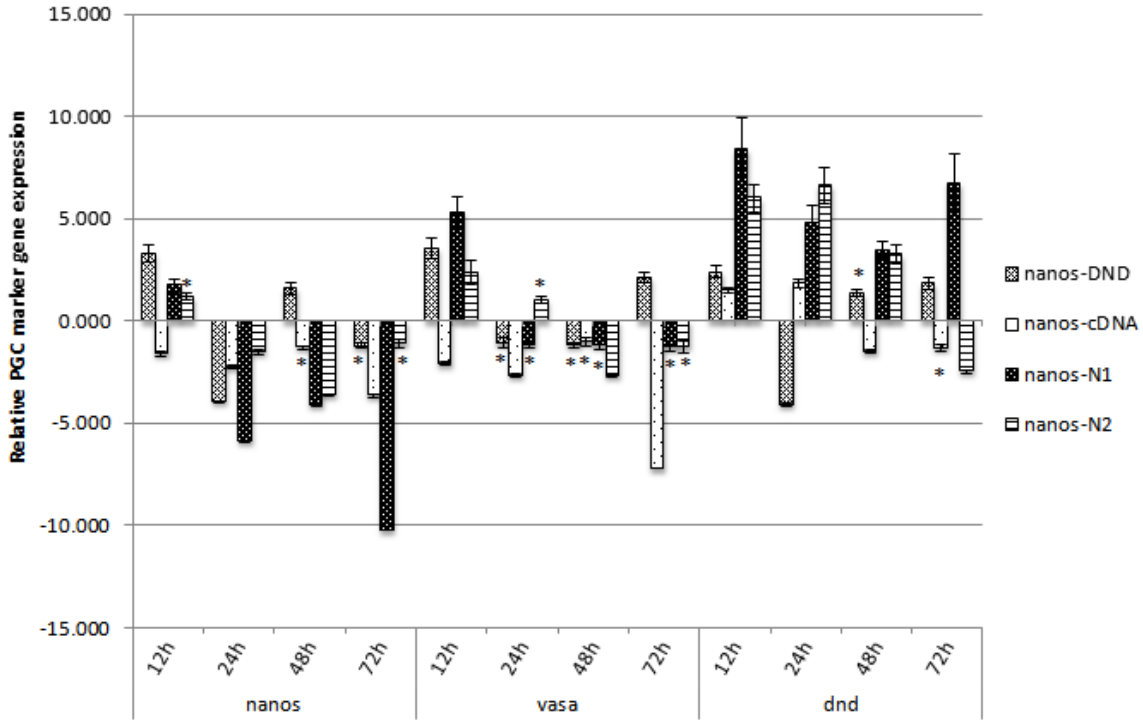

Fig.9. Effect of same promoter channel catfish *nanos* (nanos) on different shRNA structures (channel catfish *dead end* (*dnd*), *nanos* cDNA (cDNA), 5'sh *nanos* (N1), and 3'sh *nanos* (N2) expression. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

Racemase

Racemase driven constructs tended to have stronger expression than *nanos* (Fig. 10). Oddly, RM-*dnd* was effective at down-regulating the off target *nanos*. This construct and the RM-cDNA had the strongest down-regulation of *nanos*, usually 4-21X. Most of the constructs down regulated *vasa*, but not as strongly as *nanos* and again up-regulated *dnd*. RM-cDNA most strongly, upwards to 10X, up-regulated *dnd*.

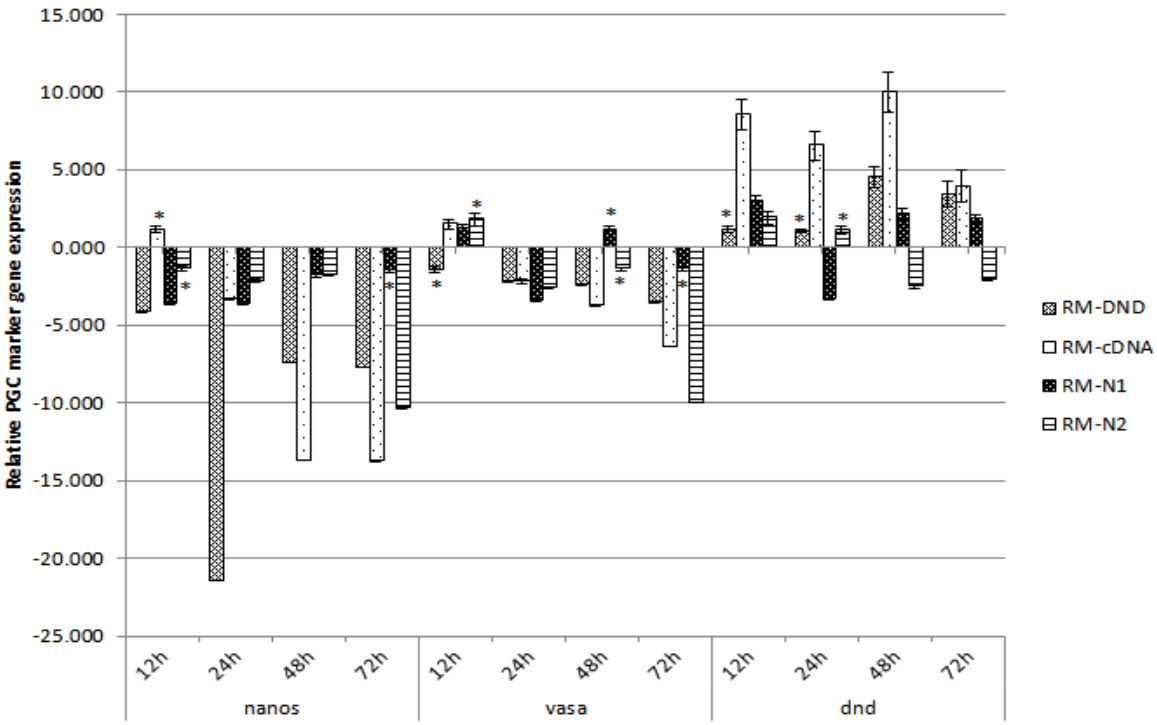

Fig.10. Effect of same promoter zebrafish racemase (RM) on different shRNA structures (channel catfish *dead end* (*dnd*), *nanos* cDNA (cDNA), 5'sh *nanos* (N1), and 3'sh *nanos* (N2)) expression. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio*, using knockdown constructs with shRNAI. Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

M

M driven constructs exhibited a similar pattern of regulation as the previous constructs (Fig. 11). M-DND and M-sh3' down-regulated *nanos* ($P<0.05$) at all-time points and downwards to 18X. Again, the sh5' construct had observed down-regulation of greater magnitude that the sh3' construct. All constructs also down-regulated the off target *vasa*,

but not as strongly as *nanos*. Effects on *dnd* were small and sporadic.

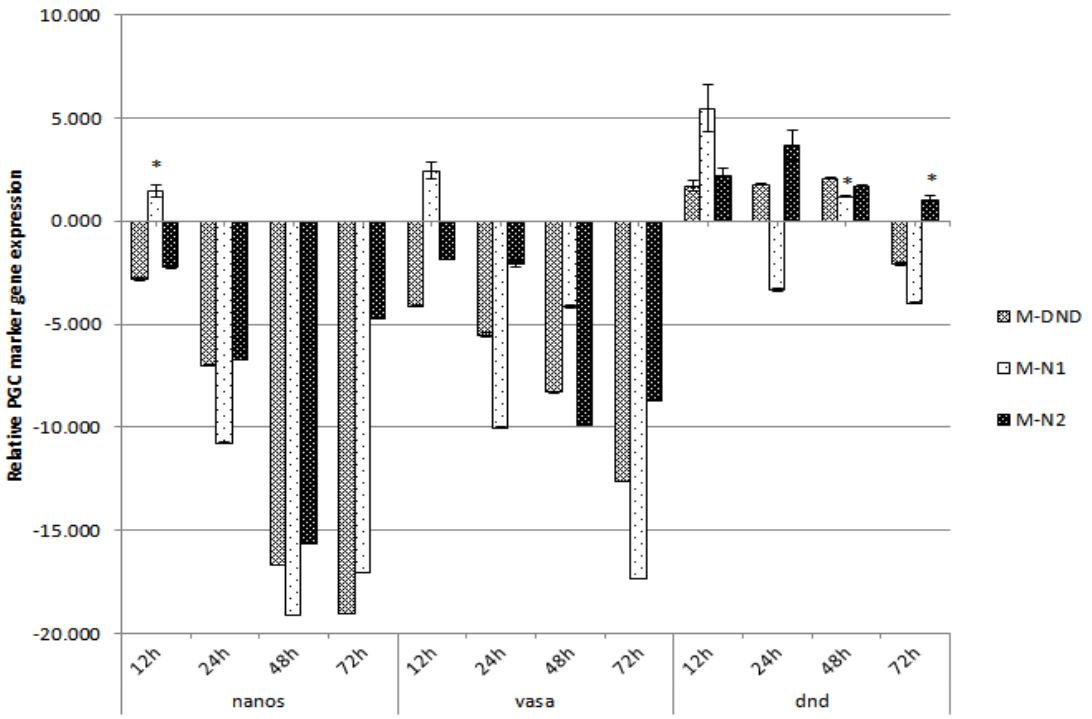

Fig.11. Effect of same promoter yeast CTR3 (M) on different shRNA structures (channel catfish *dead end* (dnd), 5'sh *nanos* (N1), and 3'sh *nanos* (N2)) expression. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio,* using knockdown constructs with shRNAI. Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

MCTR

Only two MCTR constructs were evaluated but they gave similar results as other constructs in the study (Fig. 12). MCTR-DND and MCTR-cDNA both down-regulated *nanos* as much as 20X, and both down regulated the off target, *vasa*, to a lesser extent. Both had variable effects on *dnd*, but at 12 hpf the cDNA construct up-regulated *dnd* 25X ($P<0.05$).

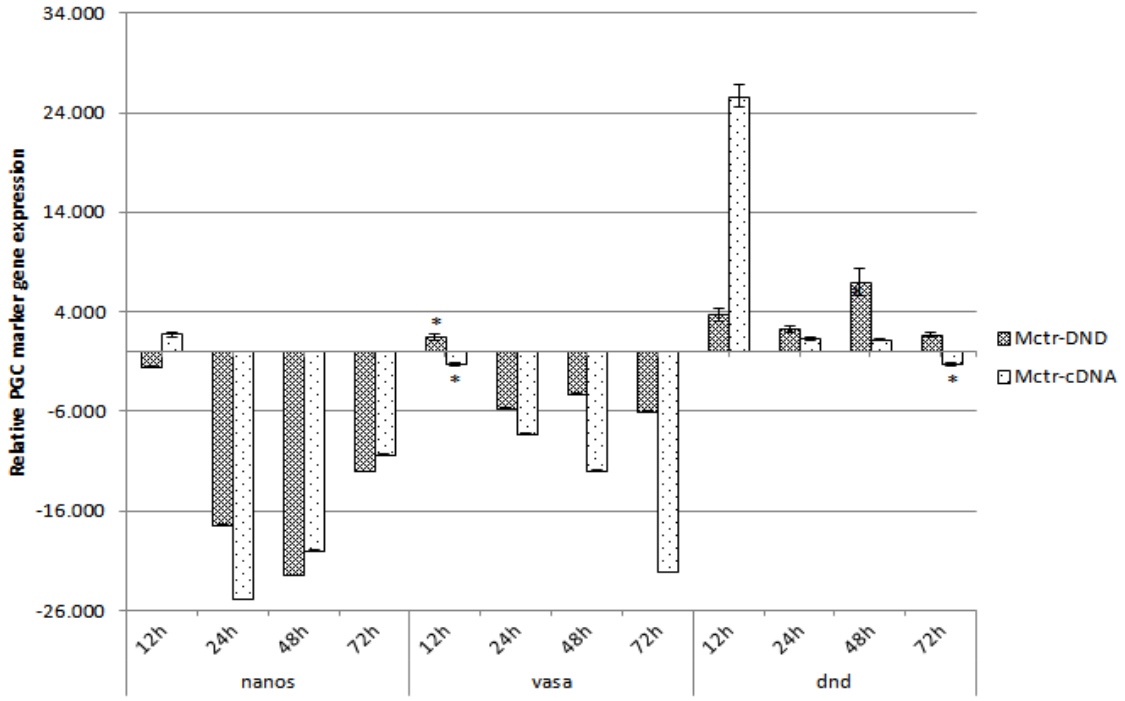

Fig.12. Effect of same promoter yeast CTR3 with reduced sensitive (MCTR) on different shRNA structures (channel catfish *dead end* (dnd) and *nanos* cDNA (cDNA)) expression. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 12, 24, 48 and 72 hours after fertilization during embryonic development of common carp, *Cyprinus carpio,* using knockdown constructs with shRNAI. Relative PGC genes expressions were expressed as fold change over control samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.5 Sex maturity

In the control group, males showed 100% sexual maturity at 19 months of age and females showed 25% maturity (Table 2). Low number of fish prevented meaningful analysis in many cases. However, M-DND and Nanos-N2 treated males had less ($P< 0.05$) sexual maturity than control males, and MCTR-DND males had less ($P=0.07$) sexual maturity than control males. In the case of females, no differences ($P>0.05$) in sexual maturity were observed between control and females treated with knock down constructs. If sexes are pooled, ADSS-N2 treated fish also had less ($P<0.07$) sexual maturity than controls.

Table 2. Sexual maturity of putative 19-month sterile transgenic common carp (*Cyprinus carpio*) electroporated with different knockdown constructs. Im: immature or sterile; m: sexually maturecontaining gametes; m%: maturity percentage. Percentage mature is significantly different from the non-electroporated controls (*P<0.05, **P<0.01,***P<0.001;Fisher's Exact Test and Fisher's Multi-treatment Exact Test).

Construct	Male			Female			Pooled by Sex	
	Im	M	M%	Im	M	M%	Im	M
ADSS N2	2	0	0	4	0	0	6	0**
M N1	1	0	0	1	0	0	2	0
RM DND	1	0	0	2	0	0	3	0
RM cDNA	1	0	0	1	0	0	2	0
RM N1	5	3	37.5	2	1	33.3	7	4
RM N2	2	0	0	2	0	0	4	0
M DND	3	0	0.0*	3	1	25	6	1
MCTR DND	4	1	20.0**	9	0	0	13	1*
MCTR cDNA	1	0	0	1	0	0	2	0
Nanos DND	1	0	0	na	na	na	1	0
Nanos cDNA	1	0	0	1	0	0	2	0
Nanos N1	2	0	0	na	na	na	2	0
Nanos N2	3	0	0.0*	1	0	0	4	0
Control	0	3	100	6	2	25	6	5

If knock down constructs are pooled among promoters, reduction in sexual maturity rate for electroporated embryos was more obvious for males and pooled sex, but not females (Table 3). Differences in male maturity existed among groups (P=0.01), and all four knock down types, N1, N2, nanos cDNA and DND had less sexual maturity (P<0.05) than control males. Differences in maturity for pooled sexes existed among groups (P=0.01), and three knock down types, N2 (P<0.05), nanos cDNA (P<0.07) and DND (P<0.05) had less sexual maturity (P<0.05) than pooled control sexes.

Table 3 Sexual maturity of putative 19-month sterile transgenic common carp (*Cyprinus carpio*) electroporated with different knockdown constructs pooled by knockdown element. Im: immature or sterile; m: sexually mature containing gametes; m%: maturity percentage. Percentage mature is significantly different from the non-electroporated controls (*P<0.05, **P<0.01,***P<0.001; Fisher's Exact Test and Fisher's Multi-treatment Exact Test). N1and N2: short hairpin structures targeting 5' and 3' of based on channel catfish *nanos* gene; cDNA: channel catfish *nanos* cDNA; *Dnd:* short hairpin structure of based on channel catfish *dead end* gene.

Gene	Male			Female			Pooled		
	Im	M	M%	Im	M	M%	Im	M	M%
N1	8	3	27.2*	3	1	25.0	11	4	26.7
N2	7	0	0.0*	7	0	0.0	14	0	0.0*
cDNA	3	0	0.0*	3	0	0.0	6	0	0.0**
Dnd	9	1	10.0*	14	1	6.7	23	2	8.3*
Control	0	3	100.0	6	2	25.0	6	5	45.4

4. Discussion

4.1 Normal pattern of PGC marker genes expression

A significant quantity of *vasa*, *nanos* and *dnd* mRNA was present in the unfertilized common carp eggs. After fertilization and within 6 hours, there was a significant, but not dramatic up-regulation of *nanos* and *vasa* (approximately 1.5X, Fig. 3). Both *nanos* and *vasa* genes were progressively down regulated from 6 hpf to 72 hpf (2X at 12h, 3-5X at 24 h and 5-7X at 48 h). At 72 hpf, both of *nanos* and *vasa* were expressed at their lowest level with *nanos* down-regulated about 7X and *vasa* down regulated approximately 11X compared to the 0 h control. It appears that *vasa* and *nanos* genes shut down early in embryogenesis, and their corresponding mRNA, which could have been as much as 40% of maternal origin depending upon rates of degradation, and during the remainder of embryogenesis after 6 hpf these mRNAs are degraded in a linear fashion. However, maternally derived *nanos* mRNA in zebrafish degrades rapidly before gastrulation but having strong signal using Northern blot (Koprunner, et al., 2001), so perhaps very little rather than 40% of the *vasa* and *nanos* mRNA that was detected at 6hpf (the time of gastrulation in the current study) was of maternal origin. This raises the question of what is the purpose of the relatively high levels of *nanos* and *vasa* in the common carp egg and presumably the zebrafish, if it is going to be replaced or supplemented by embryonically derived *nanos* and *vasa* mRNA around the time of gastrulation? If it is being supplemented, this could mean that the maternal levels, although easily detectable, are

not adequate for development and PGC migration. For some reason, up-regulation of *nanos* and *vasa* at gastrulation appears important.

Results of Xu et al (2005) examining the *vasa* homolog from gibel carp (*Carassius auratus gibelio*) were also similar to ours. *Vasa* expression was high in unfertilized eggs and continued throughout cleavage to the early gastrula stage, then dramatically declined to barely detectable level during neurula, muscle, heart beating, prehatch and hatch stage as measured by RT-PCR.

There are two types of maternal *nanos* mRNA in zebrafish, *nos1* and *nos2*. *Nos1* has been involved to PGC migration and survival during embryogenesis by knockdown experiment; while *Nos2* is undetectable in the PGC during the first 2 days post-fertilization (Koprunner et al.,2001). 3'UTR of *nos1* has been constructed into a construct fused with green fluorescent protein (GFP) to visualize the PGCs migration because of its 3' UCR specifically stabilization in the PGCs. Kawakami et al., (2011) had developed 3'UTR of common carp *nanos* gene fused with GFP to visualize PGCs migration behavior in goldfish host embryos. They had also demonstrated that common carp (cc) cells labeled with GFP-ccnanos-3' UTR mRNA colocalized with cells expressing *vasa* mRNA, thus concluding the identity of the GFP-labeled cells as PGCs. Our result may further indicate that *nanos* gene had the same expression pattern as that of *vasa* during early embryo development (Fig.3).

Dnd gene was also down-regulated during early embryo development (Fig. 4). *Dnd* gene was down-regulated more dramatically at each time point compared with that of *nanos* and *vasa*. Even by 6hpf significant (P=0.08) down-regulation or degradation of *dnd* mRNA has begun (-1.36X). This accelerated between 6 hpf and 12 hpf (-71X). It appeared to be more important to clear the embryo of *dnd* mRNA than *vasa* and *nanos* as at 24, 48 and 72 hpf down-regulation of *dnd* was -144, -355 and -580X, respectively. *Dnd* was still detectable after this strong down-regulation.

These observations on embryonic expression and levels of *nanos*, *vasa* and *dnd* mRNA might have implications for different knock down strategies to prevent migration of

PGCs and their potential success. If the maternal mRNA is of sufficient quantity and fully functional, knockdown of embryonic expression of these genes may not impede PGC migration. However, if knockdown of the early embryonic expression of *nanos*, *vasa* or *dnd* is important for stopping PGC migration or if the knock down constructs could accelerate degradation of existing mRNA, an impact on PGC migration or fertility could be made. To better understand the pattern of expression observed in this study and its implications, expression of *nanos*, *vasa* and *dnd* should be examined from 0-6 hpf.

4.2 Effect of knock down constructs on PGC marker genes expression

Overall, M, and Mctr promoters drove the strongest expression of the knock down constructs as measured by the degree of up- or down expression followed by RM promoter, and then Nanos promoter. Thus these two yeast promoters worked quite well in a common carp genomic background. Not surprisingly from a phylogenic standpoint, the zebrafish promoters were also effective in common carp embryos.

Nanos, M, MCTR and Racemase promoter driving *Dead end* shRNAi were almost always ineffective in knocking down the target, *dnd*, and had the opposite effect, up-regulation of *dnd*. There is no obvious reason why these genes would up-regulate *dnd*. Activation of interferon pathway, one of the antiviral responses, could be used to explain that most of the gene constructs up-regulated *dnd* gene in this study (Karpala et al. 2005). dsRNAs activate gene expression by targeting noncoding regulatory regions in gene promoters, which can increase gene transcription (Li et al., 2006). Therefore, it could be speculated that *dnd* short-hairpin structure may produce RNA sequences that are complementary to parts of *dnd* promoter, thus increasing dnd gene transcription during early embryo PGC knockdown process. Perhaps, the expression of *dead end* shRNAi is actually slowing degradation rather than elevating expression through some kind of blockage, binding or competition with the mechanisms, pathways or enzymes metabolizing the *dnd* mRNA.

Overexpression of *nanos* cDNA also appeared to up- regulate *dnd*. The MCTR driven construct had strong up-regulation (25X) on *dnd* at 12 h, but little or no effect during the

rest of development. The RM driven construct had the most consistent up-regulation of *dnd*. Again, perhaps the increase in *nanos* mRNA slowed the degradation of the existing *dnd* mRNA rather than upregulating *dnd*.

Knock down of *nanos* and *vasa* was prevalent with the dnd shRNAi constructs (Fig. 5) with the knock down of *nanos* being stronger. *Dnd* shRNAi construct driven by promoters M, Mctr and RM knocked down *nanos* upwards to 7-22X at all-time points with MCTR appearing to be the most consistent. This unexpected result could be explained by these constructs silencing *nanos* and *vasa* via off-targeting effects (Karpala et al.,2005) . Introduction of foreign dsRNA into an organism could trigger a number of antiviral immune responses, which contribute to induction, activation and regulation of antiviral mechanism through the interaction between dsRNA associated with viral replication and the host. Such an antiviral mechanism induces knockdown of any genes from which the processes of RNAi are to be derived in addition to the non-specific gene silencing effects (Karpala et al., 2005; Thresher et al., 2009).

Nanos, mctr and rm promoter worked in concert with nanos cDNA to knockdown the PGC markers *nanos* and *vasa*. Consistent with the results for the *dnd* shRNAi constructs, MTCR drove the strongest knock down of *nanos* and *vasa.* This yeast copper control promoter was one of the most consistent and stronger promoters in this study.

sh5' *nanos* gene knocked down *nanos*, *vasa* at 24, 48 and 72 h. This combination also knocked down the off-target *dnd* at 24 and 72 h, although it up-regulated *dnd* at 12 and 48 h.

Nanos promoter with sh5' *nanos* gene also knocked down *nanos* at 24 h, 48 h and 72 h, but not as strikingly as M. This *nanos* construct did not affect *vasa* mRNA levels, but was the only construct that up-regulated *dnd* gene at all timepoints. The RM promoter construct knocked down *nanos* at 12 h, 24 h and 48 h, but not dramatically.

Again, sh3' *nanos* driven by M was the most consistent, knocking down *nanos* at all timepoints, and it also knocked down the off-target *vasa* at all timepoints. The ADSS driven construct was also quite effective for knocking down *nanos* from 12 hpf to 48 hpf.

4.3 Sexual Maturity

Several constructs exhibited promise for knocking down expression of *nanos* and *vasa* or overexpressing *dnd* or altering the degradation dynamics of these mRNAs. Sexual development was measured to see if these alterations at the mRNA level affected sexual maturation as measured by ovarian development or sperm production.

At 19 months of age, the majority of the male common carp should be expressing sperm, and a fraction of the females should be sexually mature and exhibit ovarian growth sufficient to appear gravid. Replication was limited for this aspect of the study as a cold front rapidly lowered the temperature when the majority of the fingerlings in this study were about 2.5 cm in length resulting in heavy mortality.

Although replication was low, the data analysis indicated that some of these constructs reduced fertility and sexual maturation at 19 months of age. Putative sterilization was more effective in males than females. M-DND, Nanos-N2 and MTR-DND treated males had less sexual maturity than control males. Based on the patterns of gene expression in the embryos, it is not clear if these are target or off target effects. In the case of females, although there appeared to be trends in reduction of sexual maturity, no differences in rate of sexual maturity were observed between control and females treated with knock down constructs. If sexes are pooled, ADSS-N2 treated fish also had less sexual maturity than controls.

If knock down constructs are pooled among promoters, reduction in sexual maturity rate for electroporated embryos was obvious for males and pooled sex, but not females. All four knock down types, N1, N2, *nanos* cDNA and DND had less sexual maturity than control males.

5. Conclusions

The knock down constructs evaluate reduced or in some cases increased mRNA of PGC related proteins, but did not eliminate mRNA of primordial germ cell (PGC) proteins because they were not 100% effective or because of the presence of residual mRNA.

This alteration of mRNA levels for *nanos*, *vasa* and/or *dnd* sterilized a large percentage of common carp males at 19 months of age, but the effects on female fertility were inconclusive. These constructs were electroporated into one-cell embryos, and with the known dynamics of DNA introduction into fish embryos (Dunham 2011), the expression and biological effects on these embryos could be realized without gene integration. Thus, fish need to be evaluated at older ages elucidate the sexual effects on females, F1 transgenic individuals need to be produced and evaluated to confirm the results from genomic expression rather than cytoplasmic expression and lastly, the repressibility of the constructs needs to be evaluated to allow production of fertile transgenic brood stock to complete a system of repressible transgenic sterilization.

Acknowledgement

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III Reversible Transgenic Sterilization in Channel Catfish

Abstract

Knockdown approaches utilizing overexpression and shRNAi approaches were investigated to attempt reversible transgenic sterilization in channel catfish, *Ictalurus punctatus*. Two primordial germ cell marker genes, *nanos* and *dead end* were the target knockdown genes and an off-target gene, *vasa*, their expressions were evaluated in temporal manner using real-time PCR. Sodium chloride, cadmium chloride, copper sulfate and doxycycline were evaluated as repressor compounds. Constructs were designed driven by promoters from different species and were electroporated into embryos. Some of the constructs had knocked down PGC marker genes expression and some of the constructs had repressed the PGC marker genes in the presence of repressor chemicals. For some systems and gene constructs, rate of sexual maturity appeared higher in three-year-old brood fish treated with repressors as embryos than for corresponding controls indicating potential for some of these reversible transgenic sterilization systems to be functional. Evaluation of F1 transgenic channel catfish with these constructs will be needed to confirm the results observed in this initial generation of fish and experiments. Perfection of this system would allow environmentally safe application of transgenic catfish, hybrids and domesticated catfish would be applicable to other aquatic organisms.

Key words: Transgenic sterilization, RNAi, Channel Catfish embryos, PGC gene expression

1. Introduction

Channel catfish (*Ictalurus punctatus*), is the primary aquaculture species produced in the United States, consecutively ranked from 2006 to 2010 as the sixth most consumed seafood in the US (NFI, 2010). American producers are facing difficulty keeping pace with competition from Southeast Asia. A $3.4 billion deficit in fish and other seafood products was encountered in 2010, accounting for almost a tenth of US total trade deficit of $37.3 billion, and almost half of trade deficit in fish products with Southeast Asian countries.

Exploiting fish genetics can greatly contribute to production efficiency and enhancing production. Genetic enhancement of farmed fish has advanced to the point that it is now impacting aquaculture worldwide, however, potential maximum improvement in overall performance is not close to being achieved (Dunham et al. 2001). Transgenic technology holds to improve the production and profit to aquacultured fish.

Channel catfish growth has been improved via growth hormone gene transfer (Dunham et al. 1998), bacterial disease resistance improved with cecropin gene transfer (Dunham et al. 2002). Transgenic fish have been developed that have improved growth, color, disease resistance, tolerance of heavy metals, survival in cold and body composition, and that can produce pharmaceutical proteins (Dunham and Liu 2006; Dunham 2008).

Public fears that transgenic fish would pose environmental and ecological risk if escaped and unconfined, allowing transgenic genotype to expand the geographic range and become an exotic species essentially. The fitness of transgenic fish will determine whether or not they impact the environment. Key factors determining fitness of transgenic fish include reproductive performance, foraging ability, swimming ability and

predator avoidance and these traits have been the same or weaker compared to controls (Dunham et al. 1995; Dunham, 2003).Regardless of fitness and potential environmental risk, guaranteed containment will likely be necessary for transgenic fish to be commercialized. Wong and Van Eenennaam (2008) reviewed that containment methods and applications, including physical containment, physicochemical containment and genetic containment.

Genetic containment options include triploidy and transgenic sterilization. Transgenic sterilization is a better option to reduce the risk than triploidy because triploidy can decrease performance and minimizes, but does not eliminate risk because fertile brood stock are necessary (Dunham, 2003; Dunham 2004). Additionally, triploidy is not feasible on a commercial scale for channel catfish and many other fishes.

Transgenic sterilization is currently the most promising option to confine various biotechnological forms of fish by achieving reproductive confinement via genetic disruption of gondal development, formation of gametes or blockage of embryonic development (Thresher et al. 2004; Templeton 2005; Chaimongkol 2009; Thresher et al. 2009). Repressible transgenic sterilization is essential as reproductive capacity needs restoration to both produce fish as well as perpetuate future generations. One such system is the sterile feral technology based on a modified tet-off system, in which components of the constructs are fused so that a specific promoter is coupled to a repressible element that in turn drives expression of a blocker gene, antisense RNA, dsRNA, sense RNA or ribozyme to an early key developmental gene (Thresher et al. 2001; Dunham 2004, 2010; Thresher et al. 2009). In the absence of a repressor, the construct functions, the blocker gene turned on, the target gene disrupted and gonads do not develop or embryos die. If the blocker gene does not transcribe, the gonad develops or the embryos live. Sterile feral system has been demonstrated with the repression of the knockout function in zebrafish, oysters, channel catfish and common carp (Thresher et al. 2009).

Doxcycline (dox) was administrated into water during incubation of embryo development in the tet-off system. Embryonic development is rescued in the presence of dox while the embryo experiences abnormal development or dies out without the dox. The beauty of the

tet-off system is that it is manageable, inducible, and repressible in a temporal and spatial way (Wong and Eenennaam, 2008; Thresher et al. 2009; Gama Sosa et al. 2010). The mechanism of the tet-off system works as follows: the primary part of the system is tet repressor, which is constructed by a tet transcriptional activator (tTA) , fusing with tetR DNA binding domain with the activation domain of the herpes simplex virus VP16 transcriptional activation protein. tTA is expressed under the control of a tissue specific promoter, such as bone morphogenetic protein 2 (zBMP2) or other embryogenesis or gametogenesis promoter in a temporal and spatial way. The second part has transgene sequences, including tet response element (TRE), a cytomegalovirus (CMV) minimal promoter (PminCMV) with the tetO sequences, followed by a transgene (blocker gene). The system is turned on without doxcycline. In this scenario, tTA binds to TRE, activating CMV promoter, which in turn results in transcription of the blocker gene. By adding the dox, tTA is prevented binding to the TRE, and transcription of the trangene is repressed.

Continuous dox treatment could lead to environmental issues and could be expensive on a commercial scale. An additional drawback of the tet-off system is that it includes short stretches of bacterial and viral sequences, which regulators and the public fear. Alternative repressor systems may be necessary to alleviate these concerns. Repressors such as salt and copper sulfate were considered in the current study, because salt and copper sulfate are inexpensive and widely used to treat fungus in catfish hatcheries (Su et al 2012). Cadmium chloride based systems may have potential as transferrin is strongly down-regulated by cadmium chloride in fish (Carginale et al. 2002). If these compounds are not found at high levels in the natural environment occupied by the species, the promoters are turned off in their presence and high concentrations do not kill the developing embryos, these compounds could be used as repressors to restore fertility of the transgenic knockout embryos.

Primordial germ cell gene markers include *nanos, vasa*, and *dead end* and others. Their unique characteristics such as origins, migration route, and specification, gene expression profiles along with results of knockout studies (Yoon et al, 1997; Koprunner et al 2001; Weidinger et al, 2003; Slanchev, et al 2005; Saito et al 2006; Richardson and Lehmann,

2010) suggest they would be suitable targets for knockout and a source of early embryonic promoters.

The overall goal was to develop repressible systems that can transgenically sterilize channel catfish. The objectives of this study were to determine the normal pattern of gene expression of nanos, vasa and dead end in channel catfish embryos, evaluate shRNAi constructs and a cDNA construct designed for knockdown of nanos and dead end (theoretically,disrupting migration of the PGCs and preventing germ cell development), examine off target effects, repress the effects of the sterilization constructs on expression of *nanos* and *dead end*, and determine sexual maturity in 3-year-old channel catfish exposed to transgenic sterilization constructs during embryonic development.

2. Materials and Methods

2.1 Construct and Experimental Design

A 7 X 4 X 2 factorial design below had been utilized in the study.

Tet-off vasa		*dead end* ds RNA		
Tet-off nanos		*nanos* ds RNA 3’		repressor molecule
CTR3				
CTR3 (reduced)	X	*nanos* ds RNA 5’	X	
ADSS2				
RM		*nanos* cDNA		no repressor molecule
Transferrin				

Seven sets of constructs driven by 7 different promoters were designed. These promoters included, Zebrafish ADSS2 (ADSS) and racemase (RM) (both salt responsive), yeast CTR3 (M) (copper sensitive) and Mctr (reduced, copper less sensitive), Salmon *transferrin* (*T*) (cadmium sensitive), channel catfish *nanos* (*nanos*) and *vasa* (*vasa*).

The 7 promoters were linked to 4 knockout constructs (28 constructs), 3 that target channel catfish *nanos* and one that targets catfish *dead end*. Two knockouts were double stranded short hair-pin structure RNAis, sh-nanos targeting the 5’ *nanos* sequence (N1) and sh-nanos targeting the 3’ *nanos* sequence (N2); a full length *nanos* cDNA (cDNA)

and a double stranded short hair-pin structure RNAi targeting *dead end* (*dnd*). Three knock out systems target *nanos* and one targets *dead end* (*dnd*).

Table 4 Promoter system, knockout gene, construct name, repressible treatment and control treatment for 28 transgenic sterilization constructs electroporated into channel catfish, *Ictalurus punctatus*, embryos. Embryos were incubated in the regular Holtfreter solution (with 3.5ppt saline) for all treated and control groups except for the ADSS and RM groups for which Holtfreter's was altered to contain 4ppt salinity and 0.3ppt salinity for treated and controlled groups, respectively.). These constructs were driven by promoters included, Zebrafish ADSS2(ADSS) and racemase (RM) (both salt responsive), yeast CTR3 (M) (copper sensitive) and Mctr (copper less sensitive), Salmon transferrin (T) (cadmium sensitive), channel catfish nanos (nanos) and vasa (vasa). The main genes fused in the constructs included, double stranded short hair-pin structure RNAis, sh-nanos targeting the 5' *nanos* sequence (N1) and sh-nanos targeting the 3' *nanos* sequence (N2), a full length *nanos* cDNA (cDNA) and a double stranded short hair-pin structure RNAi targeting channel catfish *dead end* (*dnd*).

System(promoter)	Knockout Gene	Construct name	Treatment	Control
Adss	shRNAi *dnd*	adss-dnd	4 ppt salinity in Holtfreter solution, 10 ppm doxycycline (dox)	0.3 ppt salinity in Holtfreter solution, 10ppm dox
	Nanos cDNA	adss-cDNA		
	shRNAi 5'	adss-N1		
	shRNAi 3'	adss-N2		
RM	*dnd*	RM-dnd		
	cDNA	RM-cDNA		
	N1	RM-N1		
	N2	RM-N2		
M	*dnd*	M-dnd	3.5 ppt salinity in Holtfreter solution, 0.1 ppm $CuSO_4$, 10 ppm dox	3.5 ppt salinity in Holtfreter solution, 10 ppm dox
	cDNA	M-cDNA		
	N1	M-N1		
	N2	M-N2		
MCTR	*dnd*	MCTR-dnd		
	cDNA	MCTR-cDNA		
	N1	MCTR-N1		
	N2	MCTR-N2		
T	*dnd*	T-dnd	3.5 ppt salinity in Holtfreter, 0.1 ppm $CdCl_2$, 10 ppm dox	3.5 ppt salinity in Holtfreter solution, 10 ppm dox
	cDNA	T-cDNA		
	N1	T-N1		
	N2	T-N2		
Nanos	*dnd*	nanos-dnd	3.5 ppt salinity in Holtfreter solution, 100 ppm dox	3.5 ppt salinity in Holtfreter solution, 10 ppm formalin treatment for 15-30 min before change water
	cDNA	nanos-cDNA		
	N1	nanos-N1		
	N2	nanos-N2		
Vasa	*dnd*	vasa-dnd		
	cDNA	vasa-cDNA		
	N1	vasa-N1		
	N2	vasa-N2		
Control	No gene	Control	10 ppm dox	

A key to each of the 7 systems are the repressor molecules. Zebrafish ADSS2 and racemase are strongly down-regulated by sodium chloride (Hoshijima and Hirose, 2007), yeast CTR3 is strongly down-regulated by copper (Labbe et al. 1997) and transferrin is strongly down-regulated by cadmium chloride in fish (Carginale et al. 2002). Channel catfish *nanos* and *vasa* promoters were tethered to tet-off system and their expression drove tTA which binds with TRE to express the knock out constructs. Theoretically, this system is deactivated by doxycycline.

Electroporated embryos will be divided into 2 groups for each of the 28 sterilization constructs. One group will be untreated and the other group will be incubated in the appropriate repressor compound. The following amount of compounds were administered into the Holtfreter's solution: 100 ppm dox based on for the tet-off systems (Thresher et al. 2009), 4 ppt NaCl for the salt systems (Su et al. 2012), 0.1 ppm copper sulfate and 0.1 ppm cadmium chloride based on toxicity trials we conducted in 2008.

Waste $CdCl_2$ was collected using appropriate procedures and disposed of by the Auburn University Environmental Safety Unit. Even though waste $CdCl_2$ must be disposed of, a large number of potential repressed, fertile brood stocks can be produced in a limited amount of static water without creating an environmental hazard.

2.3 Plasmid DNA preparations

Plasmids were transformed with One Shot® Top10 chemically competent *E.coli* cell, following the procedures recommended by the protocol. About 100 to 200 µl transformation mix was used to plate on the LB agar (10.0 g Tryptone, 5.0 g yeast extract, 5.0 g NaCl, 15.0 g agar in 1.0 L solution) plates. A single colony was picked up from each plate to conduct a mini-prep plasmid DNA extraction (Qiagen mini-prep). Plasmids were linearized with *SfiI* (20,000 units/ml, BioLabs) following the product's protocol with little modification. For each plasmid digestion, a mixed 50 ul reaction solution contains 1 ug of plasmid DNA, NEBuffer 4(10×), BSA (100×) and the volume adjusted using water to 50 µl.

DNA agarose gel electrophoresis was used to analyze the plasmid DNA (using uncut plasmid, *SfiI* cut plasmid). Once the size of plasmid was confirmed, a maxi-prep plasmid DNA extraction was performed using Qiagen maxi-prep kit. Plasmid DNA was linearized following the same enzyme digestion protocol. After plasmid linearization, phenol-chloroform-ethanol method was used to inactivate *SfiI* enzyme and purify the DNA. The quantity of DNA was measured using a UV-spectrophotometer.

Each plasmid was prepared separately in two tubes for the purpose of twice electroporation. One was diluted in the 2.0 ml (0.9%) saline with the concentration 50 µg/ml. The other for the second electroporation was prepared in 9.0 ml TE buffer (5 mM Tris-HCl, 0.5 M EDTA, pH=8.0) with a concentration 50 µg/ml.

2.4 Brood stock preparation

Sexually mature male and female channel catfish were harvested from Fisheries Genetics Unit, Auburn University. Six AU-20 females were artificially spawned (Kristanto et al. 2009, Su et al. 2012b). Sperm was collected from all 3 AR and 3 Th males and diluted with 9 ppt saline (1000 Ml VisIV container 0.9% sodium chloride, injection, USP). Sperm was refrigerated till use. Water temperature was 25-27 °C and oxygen concentration was around 5-8 ppm. Females were induced with LHRHa at 30 µg/kg BW for the priming dose and 12 h later at 150 µg/kg for the resolving dose, and eggs collected (Kristanto et al. 2009, Su et al. 2012b).

2.5 Electroporation

Electroporation was performed with the Baekon 2000 macromolecule transfer system. Parameters were 6 kV, 27 pulses, 0.8 sec burst, 4 cycles, 160 µsec (Powers et al., 1992). One-1.5 ml of each sperm (TH and AR) was dropped in the tube containing 2.0 ml DNA construct and was gently shaken. Sperm and DNA constructs were mixed at least 5

minutes before pouring into petri dish (electroporation cuvette). Approximately 8-10 ml of water was added to reduce the salt concentration.

Noncontact mode of electroporation with the electrode 1 to 2 mm above the buffer was applied to electroporate sperm with plasmid. Eggs were fertilized within 1-2 minutes after electroporation of the sperm. Sixty minutes after fertilization, 100-200 embryos were loaded as a thin layer into the petri dishes, 2-4 ml of DNA solution was added. The embryos were allowed to incubate in the plasmid DNA for 10 minutes. Seventy minutes post fertilization, the embryos were electroporated using the exact same procedure as for the sperm. There were 3 replicates for each embryo transfection.Same amount of sperm within 2 ml saline (0.9%) was electroporated using the same setting for the first time. Egg embryos were fertilized using electroporated sperm. After 60min, control non-transgenic embryos were electroporated with TE buffer for the second electroporation. Embryos were then moved into 8.0L tubs with 5.0L Holtfreter's solution, and incubated statically. The tubs were aerated and the embryos gently agitated with compressed air delivered through airstones. Dead embryos were removed every 24 hours. Holtfreter's solution was changed daily.

2.6 Embryo treatent

Embryo treatment began within 4-5 hours after completion of fertilization process. Treated transgenic Adss and RM groups were incubated in 4 ppt salinity in Holtfreter's solution, and another 3 control transgenic embryo replicates were incubated in 0.3 ppt salinity in Holtfreter solution. M and MCTR group were treated with 0.1 ppm copper sulfate in 3.5 ppt salinity of Holtferter solution (NaCl 3.5 g, $NaHCO_3$ 0.2 g, KCl 0.05 g, $MgSO_4$ 333 µl (300 g in 500 ml), $CaCl_2$ 333 µl (150 g in 500 ml), pH: 7~7.5 in 1.0 L decholorined water). T group was treated with 0.1 ppm $CdCl_2$ for only 3 days before hatch. 10.0 ppm doxycycline was used to treat the following treated and non-treated transgenic sterile embryos, which included ADSS, RM, M, MCTR, and T groups. Nanos and vasa groups were treated with 100 ppm doxycycline for the treated transgenic embryos while non-treated transgenic embryos received 10.0 ppm formalin for 30 min daily. Non-transgenic channel catfish was treated with 10 ppm doxycycline.

2.7 Embryo collection, RNA extraction and Real-time PCR

Embryos were collected at three different time points, 24 h, 48 h and 120 h post fertilization for treated transgenic, non-treated transgenic and non-transgenic control for all the 59 groups, respectively. Approximately, 20 embryos from each sample were collected from each tub for each treatment, placed into 1.5 ml sterile micro centrifuge tube and submerged into liquid nitrogen for each time point. Samples were kept at -80 °C after collection until RNA extraction.

Samples were ground into powder in the presence of liquid nitrogen and around 0.3 ml powder from each sample was used to extract RNA using TRIzol® Reagent (Ambio, cat #: 15596-018). The quality and quantity of RNA was measured using DNA agarose gel and UV- spectrophotometer. All extracted samples had an A260/280 ratio greater than 1.8, and were diluted to 500 ng/ul.

RNA was reverse transcribed into cDNAs by iScript Synthesis Kit (Bio-Rad, USA, cat #: 170-8891). qRT-PCR was performed on a C1000 Thermal Cycler (Bio-Rad, USA) by using SsoFast EvaGreen supermix kit (Bio-Rad, USA, cat: #172-5201) following the manufacturer's instructions with modification. Ribosome 18s mRNA was used as an internal control. Primers used for relative *nanos*, *vasa* and *dnd* expression were listed in table 5.

Table 5. Primers used for qRT-PCR to test knockdown effect on the PGC marker genes relative expression in the transgenic channel catfish (*Ictalurus punctatus*) embryos. *Dnd*: short for *dead end* gene. *Nanos, vasa* and 18s ribosome mRNA from channel catfish could be traced down with the NCBI accession IDs.

Gene	GID	Forward Sequence	Reverse Sequence
Nanos	CK419278.1	TACGCCCGAGATTTCTCATC	ACCTTGAGGTGATGGAGGTG
Vasa	FD175080.1	CGTTATTGGTCGTGGAAAGG	GGCACTGAACATGAGGGTTT
DND	unpublished	AGCTGTGTCTCAGCGATCTG	GCACTGACGTCCTTCTCCTT
18S	GQ465834.1	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG

2.8 Sexual maturity

Fry were grown in aquarium until they reached 10cm, marked and stocked in earthen ponds in 2009. When channel catfish were 3-year olds, sex and state of sexual maturity

was determined based upon both primary and secondary sexual characteristics (Kelly 2004).

2.9 Statistical analysis

Crossing-point (Ct) values were exported into Microsoft Excel sheet from Bio-Rad CRX Manager (Version 1.6.541.1028, 2008). The relative expression ratio of target gene was analyzed for significance test using a randomization test in the REST (Pfaffl, et al., 2002) software on the assumption that PCR had 100% efficiencies, and randomization was performed at 1000 times to capture significance at the level of P<5%. The results were graphed with corresponding standard errors. Two different ways of relative expression analysis were performed. Transgenic treated and non-treated data were normalized to non-transgenic data of the same time point as the first data analysis, respectively. Data from transgenic treated were compared to data of transgenic non-treated in a pair-wise fashion.

Fisher's Exact Test was used to test the sexual maturity of 3-year-old potentially transgenic channel catfish. Probablity levels evaluated were P=0.05 and P=0.10.

3. Results

3.1 Expression of *nanos, vasa* and *dead end* in normal channel catfish embryos

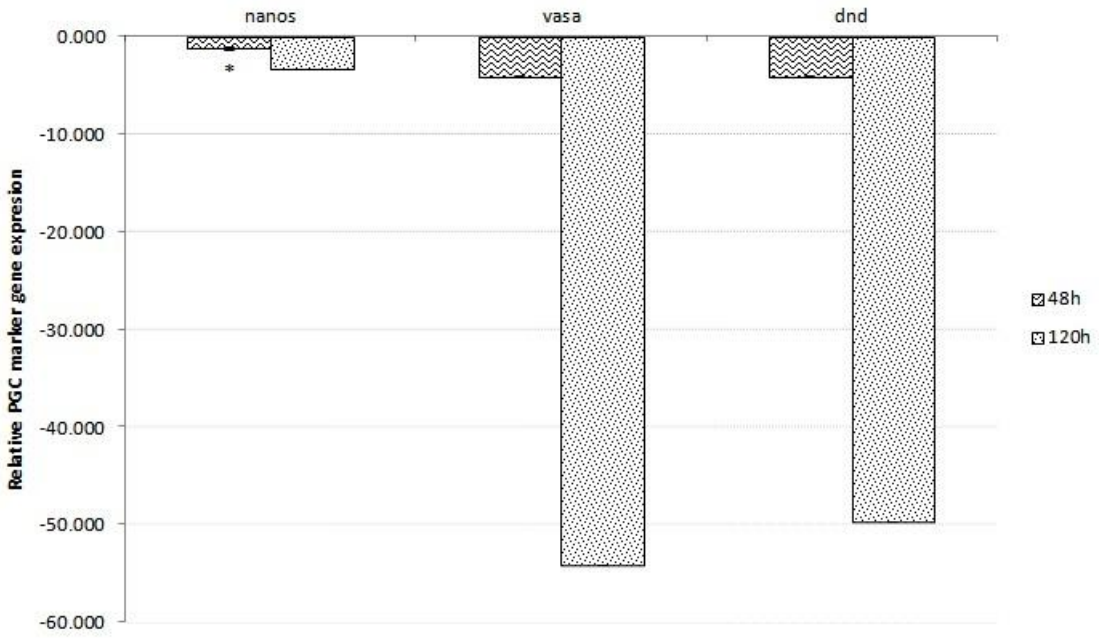

Fig.13. Analysis of relative expression of *nanos, vasa* and *dead end* (*dnd*) gene during embryonic development of channel catfish (*Ictalurus punctatus*) using real-time PCR in electroporated non-transgenic eggs. The samples were analyzed at 24 h, 48 h and 120 h post fertilization (hpf). Relative *nanos*, *vasa* and *dnd* genes expression at 48 hpf and 120 hpf were expressed as fold change over 24 hpf sample as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE; all the fold changes were significant at the level of $P< 0.05$ except that of *nanos* at 48hpf ($P>0.05$) using Pairwise Fixed Reallocation Randomization Test.

Initial levels of *nanos, vasa and dead end* at 24 hpf were very low compared to the control gene 18s (Fig.13; table 6). At 48 and 120 hpf *nanos* was declining relatively slowly, -1 and -3X, respectively. Levels of *vasa and dead end* were about 4X lower at 48 hpf than at 24 hpf, and were dramatically lower, -50X and -54X at 120 hpf compared to 24 hpf (Fig.13; table 6).

Table 6. Average Ct values (N=3) for PGC marker genes expression across three time points in the electroporated non-transgenic Channel Catfish (*Ictalurus punctatus*) embryos.

Time	18s	*nanos*	*vasa*	*Dnd*
24 hpf	11.39	28.22	27.49	30.59
48 hpf	11.13	28.32	29.29	32.41
120 hpf	10.56	29.17	32.42	35.40

3.2 Comparison of same construct between transgenic treated vs non-transgenic control and transgenic control group vs non-transgenic control

3.2.1 ADSS system

ADSS-DND knock-downed the target, *dnd*, 3X to 5X at 24 hpf and 48 hpf in treated and control electroporated embryos relative to non-transgenic electroporated controls. The treated group also had reduced expression at 120 hpf, but not the control. Adss-DND treated and control groups had significant knock-down for *nanos* and *vasa* at 48 hpf and 120 hpf 1.7X to 7.5 X (Fig. 14), but little or no knockdown occurred at 24 hpf.

Adss-dnd treated and control had the same level of expression of *dnd* at 24 and 48 hpf, and treated continued to be down-regulated at 120 hpf, but not the control (Fig. 14). In the case of the off- target, *nanos*, expression level differences between control and treated groups was not significant or minimal. For the off target *vasa*, the treated group was down-regulated compared to the corresponding electroporated control at 24 hpf, but as expected, was up- regulated at both 48 hpf and 120 hpf compared to the control.

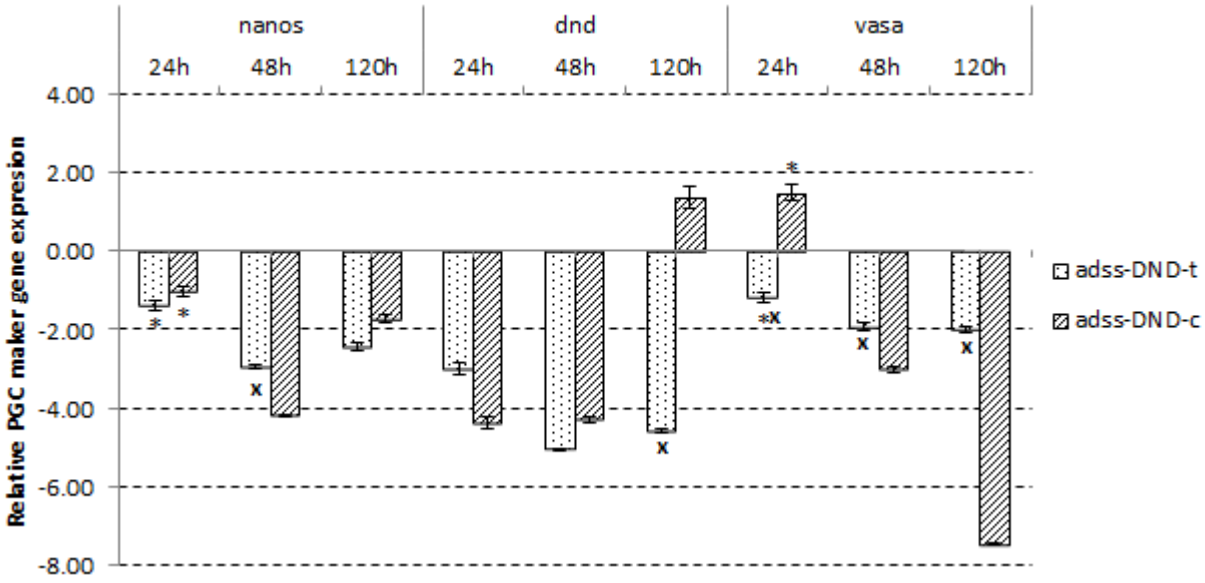

Fig.14. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated adss-dnd transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization, respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Adss-cDNA treated and control strongly up-regulated *nanos* gene expression at all three time points as expected (Fig. 15). Adss-cDNA treated up-regulated *nanos* from 167X to 747X at the three time points, reaching highest value at 48hpf. Adss-cDNA control progressively up-regulated *nanos* expression from 32X to almost 300X. Treated and control constructs knocked-down *dnd* from around 2X to 10X all the time. Treated and control had little affect on *vasa* gene at 24 and 48 h, but -8.3X and -3.0X at 120 hpf, respectively.

When treated with salt, ADSS-cDNA electroporated embryos had higher expression levels of *nanos* than those that were not treated with salt at 24 and 48 hpf (Fig. 15), which is opposite of what was expected. For the off-target gene, *dnd*, treated embryos had lower expression than untreated at all time points as expected, and for *vasa*, had lower expression than untreated controls at 2 of 3 time points, namely 48 hpf and 120 hpf.

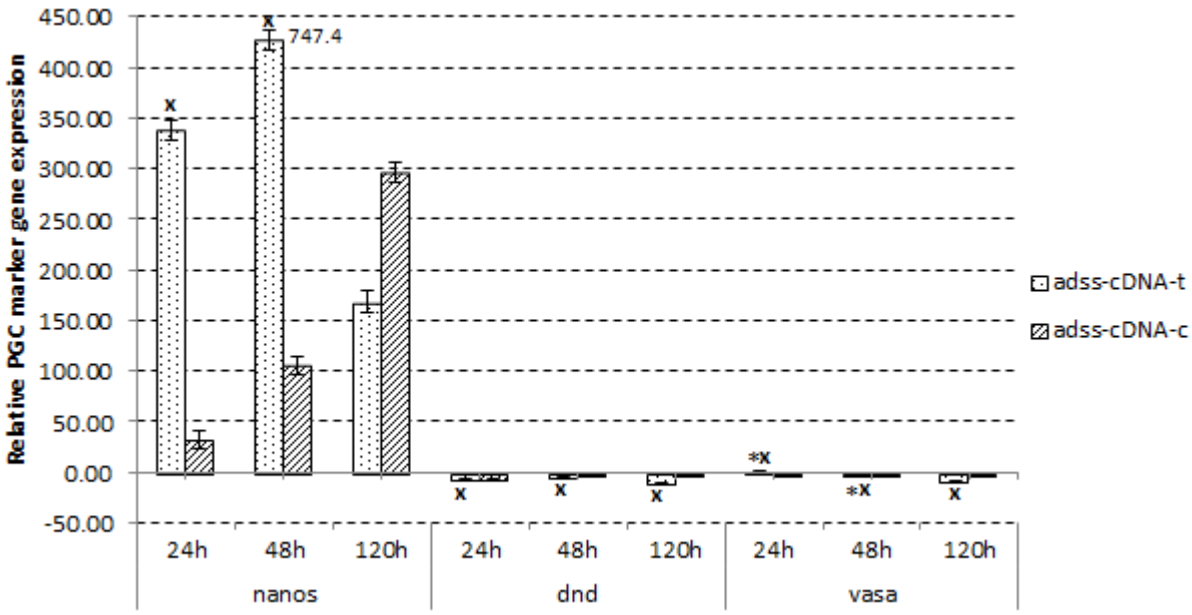

Fig.15. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated adss-cDNA transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). Relative expression of *nanos* (48 hpf) in adss-cDNA treated: 747.40±10.00. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Adss-N1 was relatively ineffective at knocking down *nanos* at 24 and 48 hpf (Fig. 16), but had significant knockdown at 120 hpf. Only ADSS-N1 control had almost 2X knockdown effect on *nanos* at 120 hpf. Both of them had around 1.5X knockdown effect on *vasa* at 120 hpf. Treated and control groups showed similar knockdown patterns on *dnd* decreasing expression from 7X to 3.5X for salt treated and 4.5X to 1.6X for the control. Differences between the salt treated and control group were minimal and inconsistent.

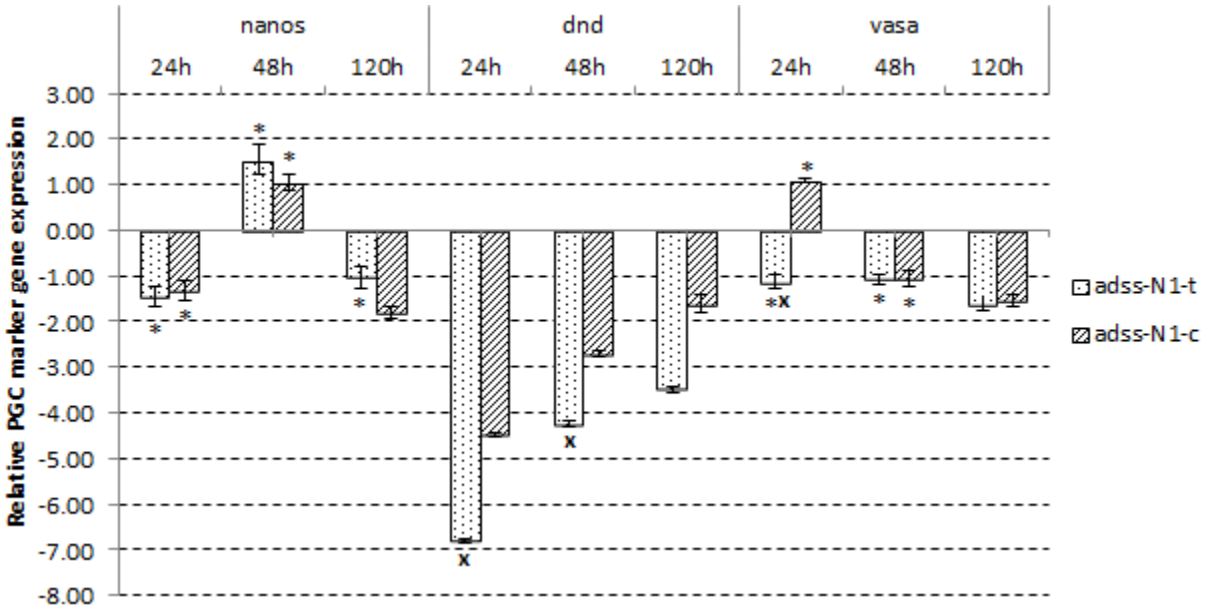

Fig.16. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated adss-N1 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Adss-N2 control had a 2X knockdown of *nanos* and *vasa* at 24 hpf (Fig. 17). Adss-N2 control had variable effects on *nanos* and *vasa* at all time points, knockdown for *nanos* 2.3X (24 hpf), for *dnd* 4X (24 hpf) and 3X (48 hpf). However, it up-regulated *nanos* (6.5X, 120hpf) and *dnd* (3X, 120 hpf). Salt treated embryos knocked down *nanos* and *dnd* all the time, around 2X knockdown effect on *nanos* while knowdown *dnd* from 8.4X to 1.6X. Salt treatment tended to increase the knock down rather than the expected decrease in knock down of the PGC markers.

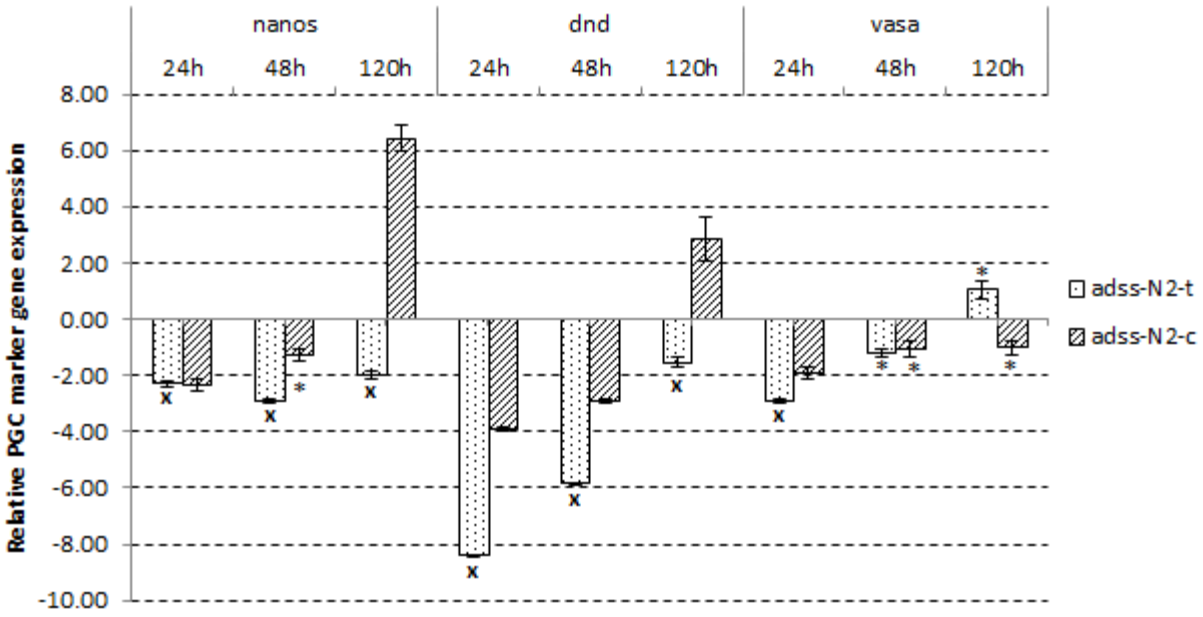

Fig.17. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated adss-N2 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos, dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

3.2.2 RM system

RM-DND (Fig. 18) treated and control electroporated groups knocked down *dnd* all the time from 3X to 5X except for the treated at120 hpf, and knocked down *vasa* only at 120 hpf, 3.3X and 2X, respectively. RM-DND treated knocked down *nanos* for around 2X (24 hpf) and 2.5X (48 hpf), but up-regulated *nanos* to 164X at 120 hpf. RM-DND control continuously down-regulated *nanos* for 1.1X (24 hpf), 2.7X (48 hpf) and 3.3X (120 hpf). The salt treatment did not block the down-regulation of the untreated electroporated control except at 120hpf, salt treated RM-dnd up-regulated *nanos* to 548X and *dnd* to 2.3X compared to the untreated electroporated control (Fig.18).

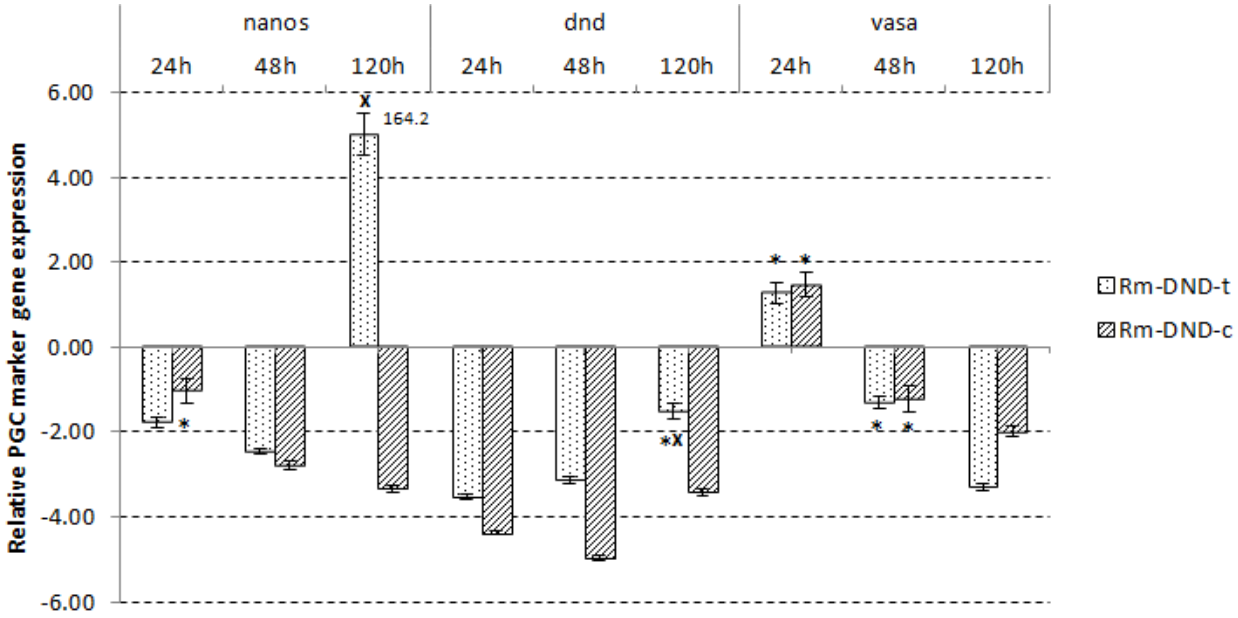

Fig.18. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated RM-DND transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). Relative expression of *nanos* (120 hpf) in RM-DND treated: 164.20±1.00. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

RM-cDNA salt treated and control groups up-regulated *nanos* from 30X (120 hpf) upwards to 510X (48 hpf). For the control group, the effect was continuously increasing, from 33X (24 hpf) to 384X (120 hpf). Both of treatments down-regulated *dnd* at the time from 1.9X to 6X. They had little effect on knockdown *vasa* for 4.8X and 8.8X at 120 hpf, respectively. When treated with salt, RM-cDNA considerably up-regulated *nanos* 8.49X (24 hpf) compared to the untreated electroporated control, and this trend continued at 48 hpf, which was opposite of expectations. At all other time points for the 3 PGC genes, the effects of the salt treatment were minimal and inconsistent (Fig. 19).

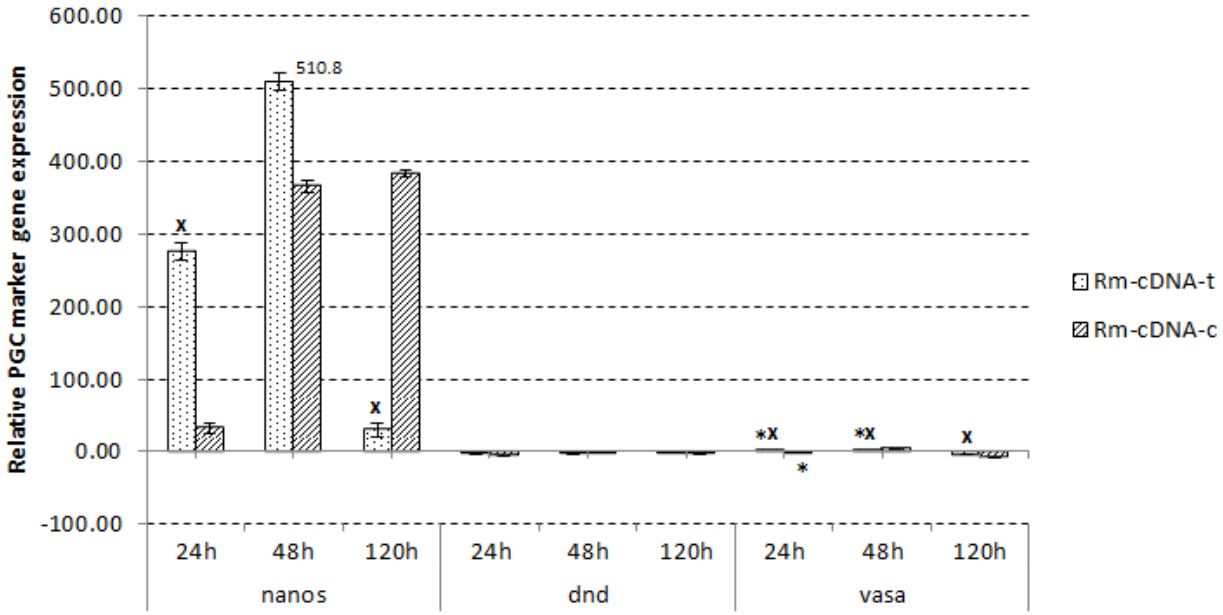

Fig.19. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated RM-cDNA transgenic channel catfish (*Ictalurus punctatus*) embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). Relative expression of *nanos* (48 hpf) in Rm-cDNA treated: 510.83±10.00. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

RM-N1(Fig.20) treated and control knocked down *dnd* at the time points from 2X to 6X. They had little effect on *vasa* gene with a knockdown effect 2X for treated at 120 hpf. These treatments had the opposite of effect on *nanos* than expected, both treatments down-regulated *nanos* 2.2X (48 hpf) but up-regulated *nanos* 4X for treated and 2.5X for control (120 hpf). Differences existed between the salt treated and untreated electroporated eggs, but they were biologically small (Fig. 20).

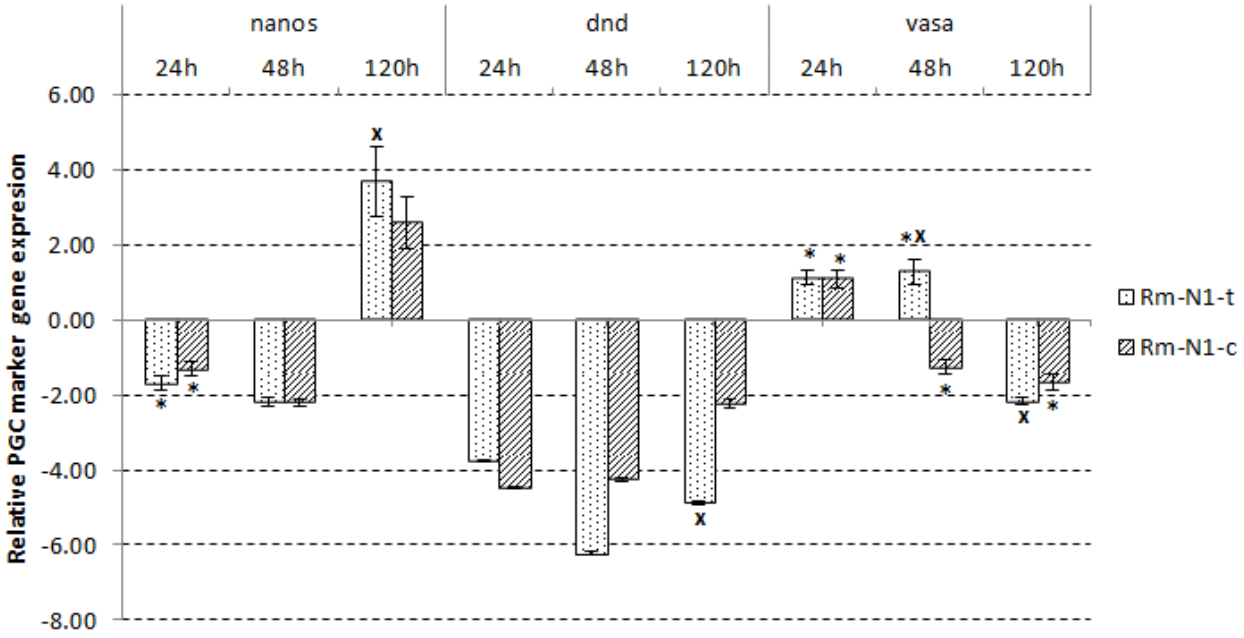

Fig.20. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated Rm-N1 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

RM-N2 (Fig. 21) showed much more promise than RM-N1. At 24 hpf and 48 hpf, control group down-regulated the target, *nanos*, around 2.2X, and *vasa* from 2X to 3X. RM-N2 treated and control knocked down the off-target, *dnd*, all the time from 2X to 5.5X. The salt treated group up-regulate *nanos* at 24 and 48 hpf as desired, as well as all time points for *vasa* in comparison between salt treated and control group (Fig. 21). Differences between salt treated and control expression were minimal for *dnd.*

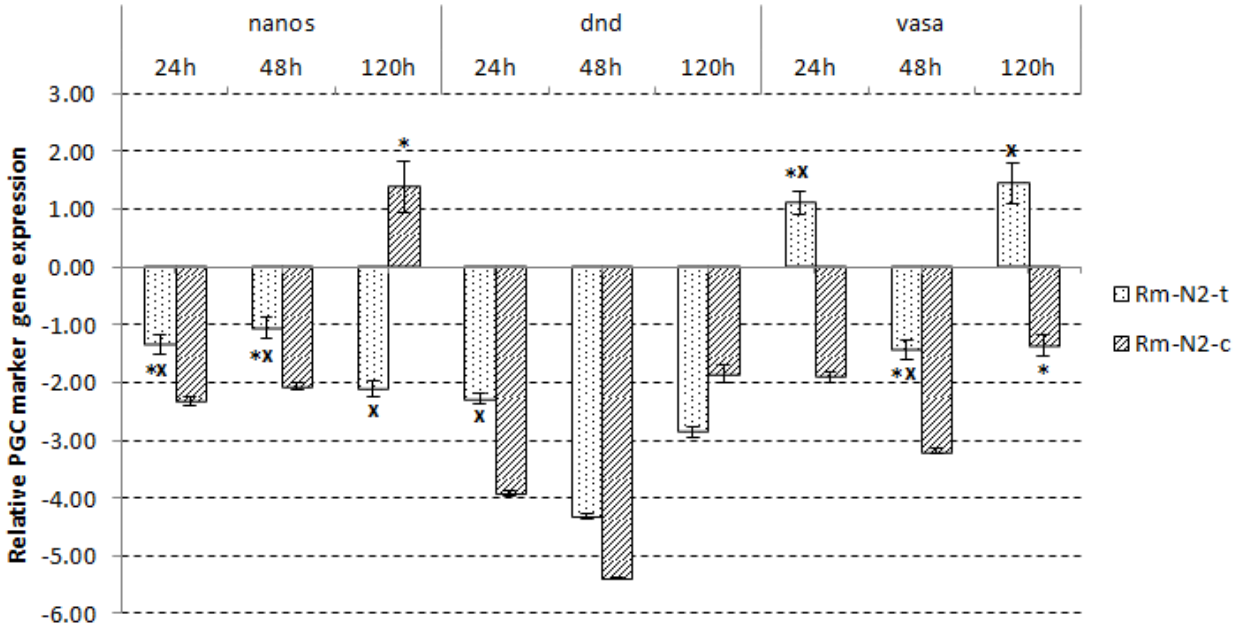

Fig.21. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated Rm-N2 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

3.2.3 M system

M-DND (Fig. 22) control down-regulated *dnd* 3X to 7X for the 3 time points and only 2.2X at 48 hpf for *nanos.* Treated group down-regulated *dnd* continuously from 6X to 14X, and for the off-target, *nanos*, 2X to 6X. Treated and control down-regulated *vasa* only at 120 hpf for 10X (treated) and 5X (control). Copper application did not turn off the M promoter, and had the opposite effect as, generally, the treated embryos had greater down-regulation than the non-treated controls (Fig. 22).

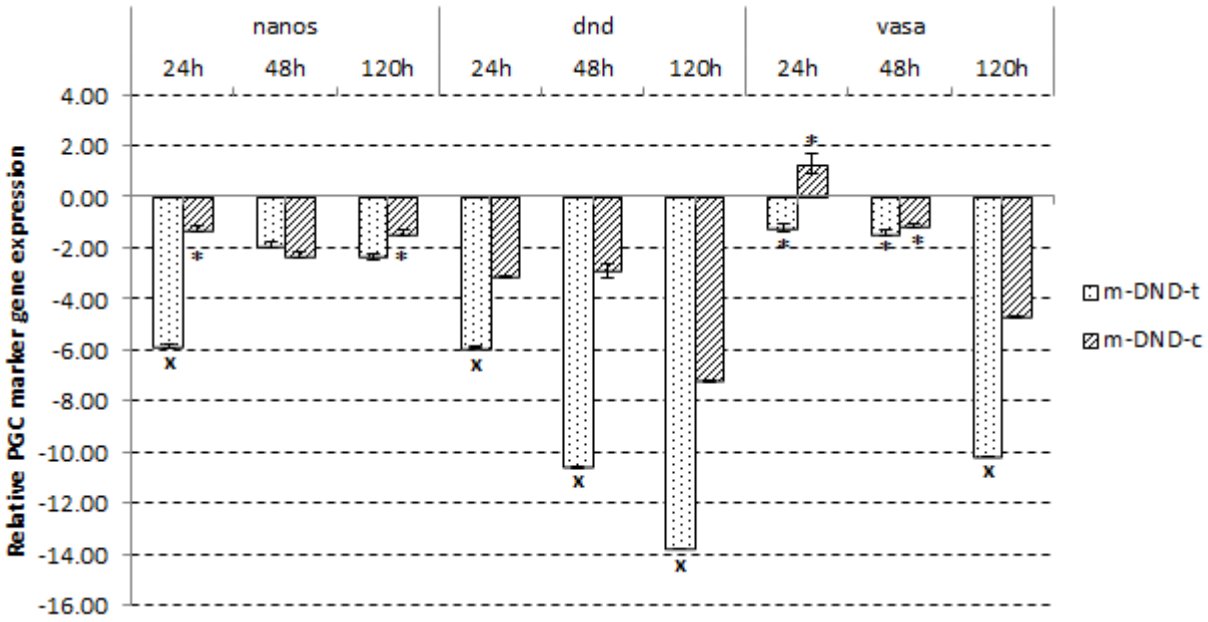

Fig.22. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated M-DND transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

M-cDNA (Fig. 23) treated and control groups up-regulated *nanos* expression from 80X to 700X. Both groups had off-target effects, and progressively down-regulated *dnd* from 3X to 9X for copper treated and from 6X to12X for control. Effects on *vasa* were small and inconsistent. Differences in expression between the copper treated and control embryos were either insignificant or biologically small (Fig. 23).

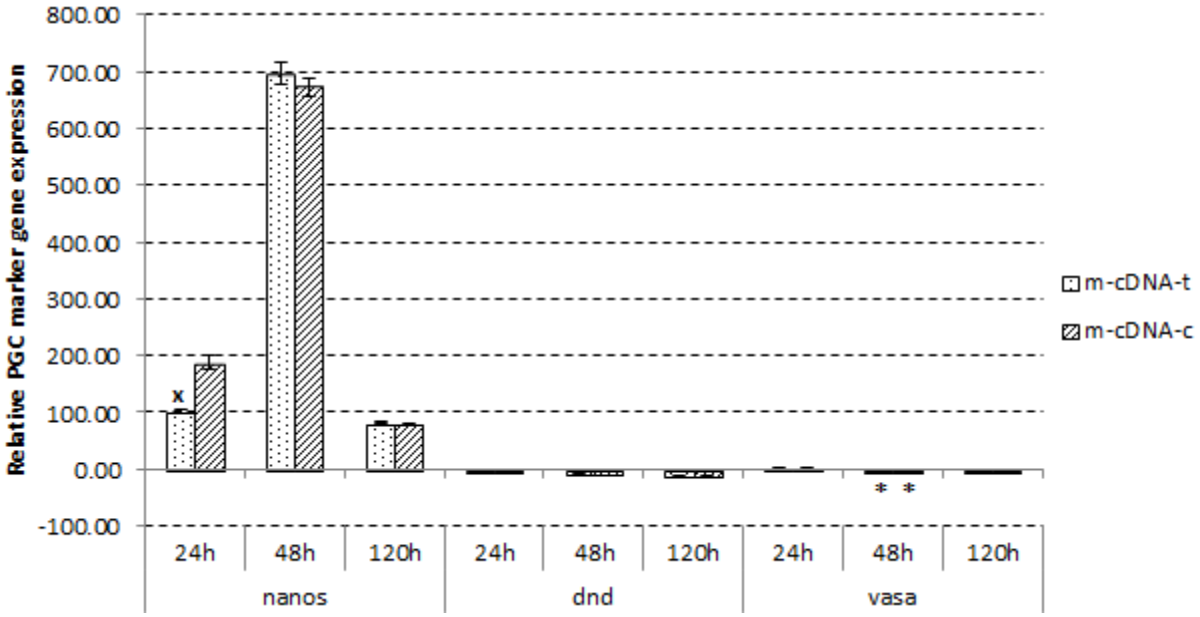

Fig.23. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated m-cDNA transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Copper treated and control M-N1 knocked down *nanos* for 2.3X (treated) and 3.5X (control) at 48hpf but up-regulated *nanos* for 20X (treated) and 37X (control) at120 hpf (Fig. 24). M-N1 had insignificantly down-regulated *vasa* all the time from 1.1 to 1.4 ($P>0.05$). Treated and control groups were knocked down for *dnd* from 3.7X to 9.5X for the treated and from 4X to 7X for the control at 24 and 48 hpf. They both were up-regulated for *dnd* for 3X expression at 120 hpf. Copper had minimal effects on the expression of this construct (Fig.24).

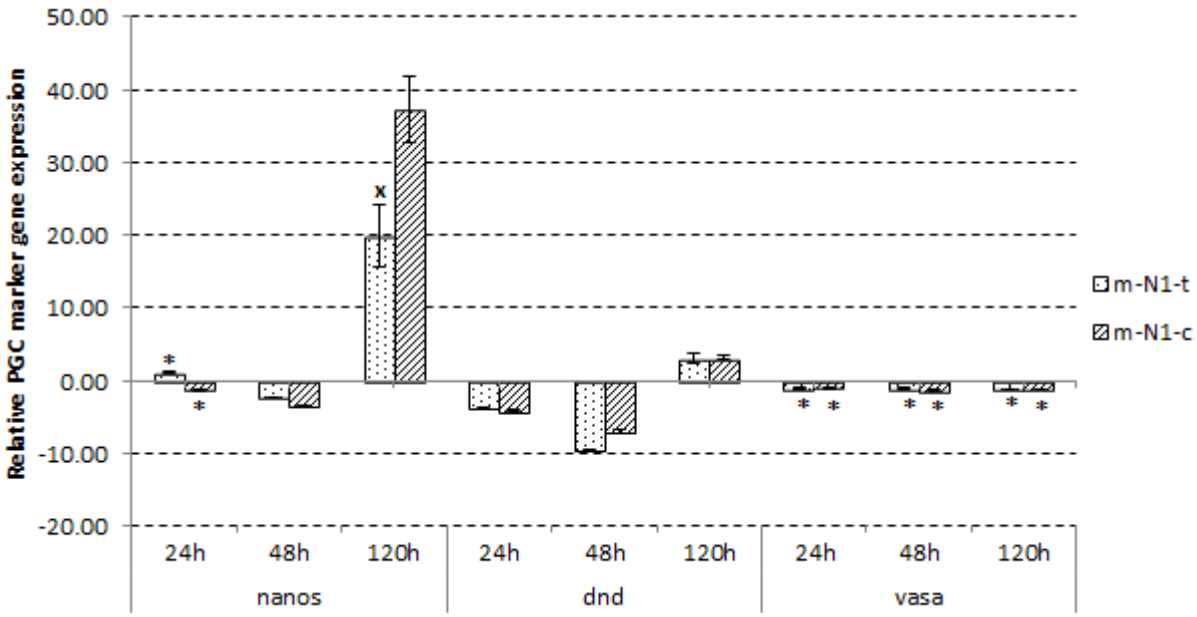

Fig.24. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated m-N1 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Copper treated and control M-N2 embryos were knocked down at 24 and 48 hpf for the target, *nanos* (Fig. 25). Both treatments knocked down for *dnd*, from 3X (24 hpf) to 4.2X (48 hpf) for the treated and from 3.5X (24 hpf) to 6.5X (48 hpf) for the control. They were both up-regulated for *dnd* 3X (120 hpf) and *vasa*, close to 2X (24hpf). The copper treatment appeared to have promise in this case, as several times it up-regulated (observed mean or signicantly different mean) the expression compared to the non-treated electroporated control.

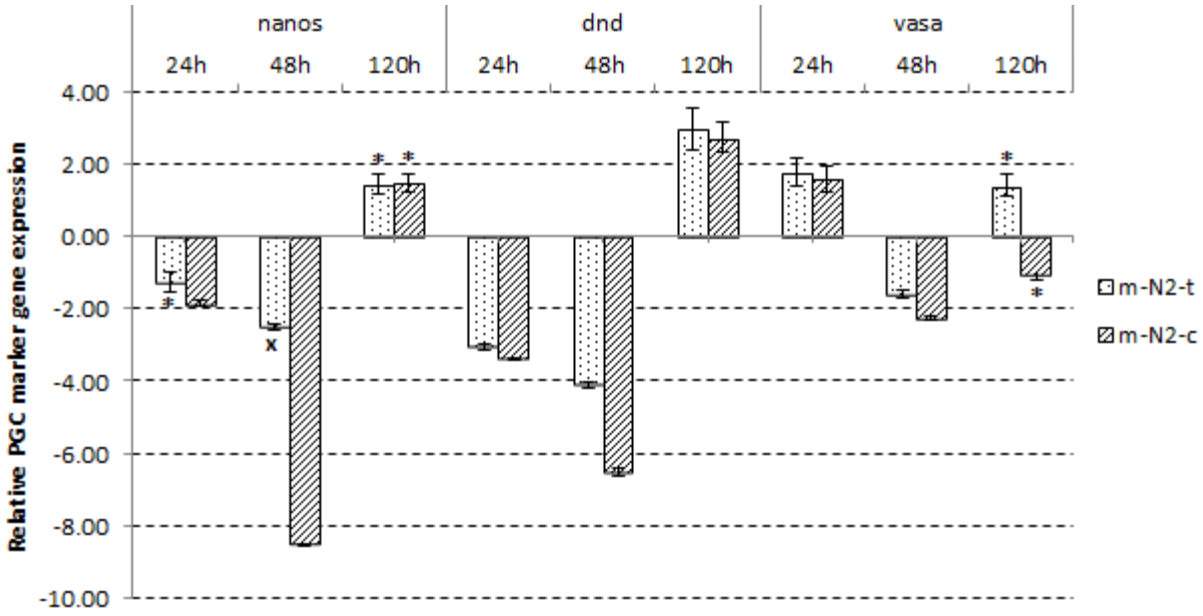

Fig.25. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated m-N2 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

3.2.4 Mctr system

Electroporated control MCTR-DND (Fig. 26) progressively knocked down the target, *dnd*, from 4X to 15X and the off-target, *nanos* all the time from 1.8X to 4.3X. Treated group increasingly knocked down *dnd* over time from 1X to 20X all the time. Both treatments had little effect on *vasa* expression ($p>0.05$). The constructs responded to the copper and up-regulated *nanos* relative to the control at 24 h and 120 h, and *dnd* at 24 h but not at the other time points.

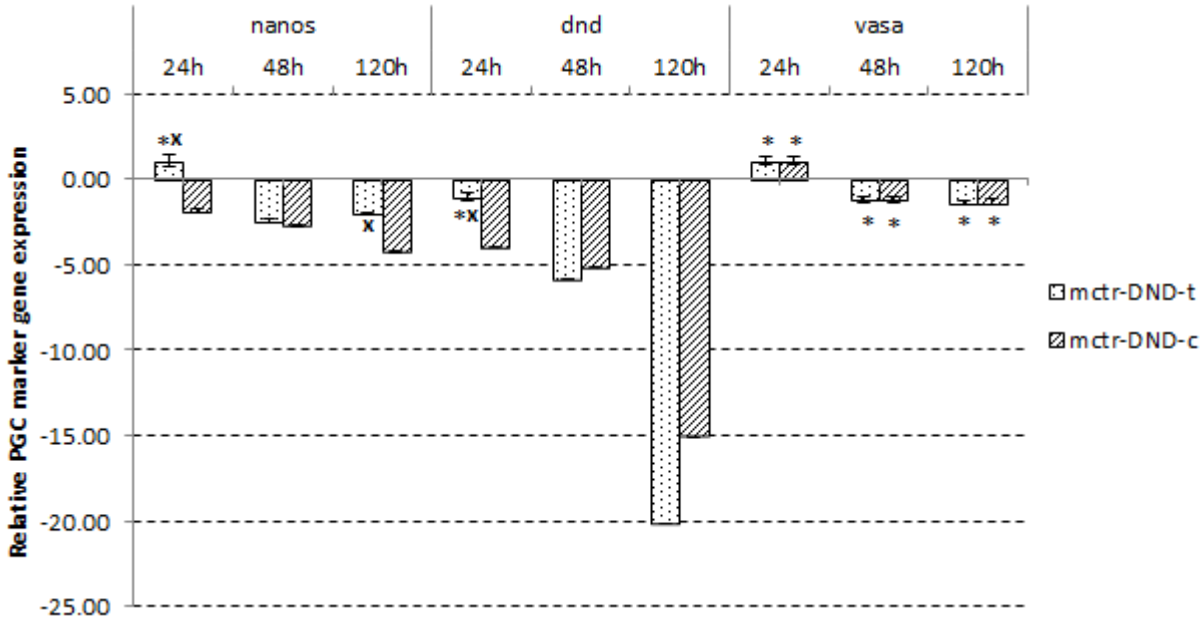

Fig.26. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated mctr-DND transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Mctr-cDNA worked well (Fig. 27) and up-regulated *nanos* expression in the treated and control at all 3 time points. For the copper treated embryos, the expression was decreasingly from 147X to 36X; while in the untreated transgenic group, the expression was increasingly from 117X to 366X as expected. They both knocked down off-target, *dnd*, decreasingly from 6.7X (48 hpf) to 3.6X (120 hpf) (treated) and from 10.7X (48 hpf) to 3.3X (120 hpf) (control). Both treatments had a sporadic effect on *vasa*, but down-regulated 1.3X and 1.6X each (48 hpf, $p<0.05$). Treated up-regulated *vasa* for 2X (24 hpf). Significant differences were observed in the expression of copper treated and un-treated embryos for the target, *nanos*, at 48 hpf and 120 hpf.

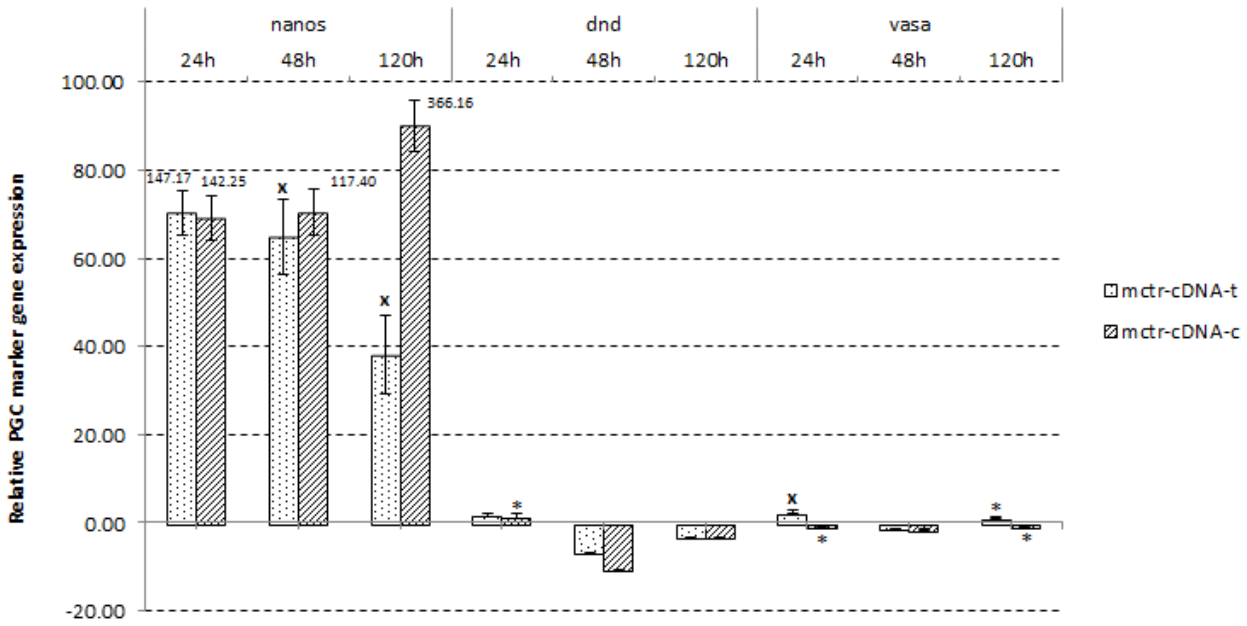

Fig.27. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated mctr-cDNA transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). Relative expression of *nanos* (24hpf) in mctr-cDNA control: 142.25±26.17; relative expression of *nanos* (24 hpf) in mctr-cDNA treated: 147.17± 21.77; relative expression of *nanos* in mctr-cDNA control 117.40±15.34 (48 hpf), and 366.16±22.82 (120 hpf). **X** on the standard error bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Mctr-N1 (Fig. 28) treated and control groups were knocked down for *nanos*, 3.3X and 5.8X, respectively, at 120 hpf. Both the treated embryos were progressively knocked down for *dnd* from 2.5X (48 hpf) to 9.3X (120 hpf) for the treated and from 7X (48 hpf) to 11X (120 hpf) for the transgenic control. Significance was found in the expression of *dnd* due to treatment at 48 hpf. Both treatments knocked down for *vasa* 4X and 2.3X at 48 hpf ($P<0.05$), respectively.

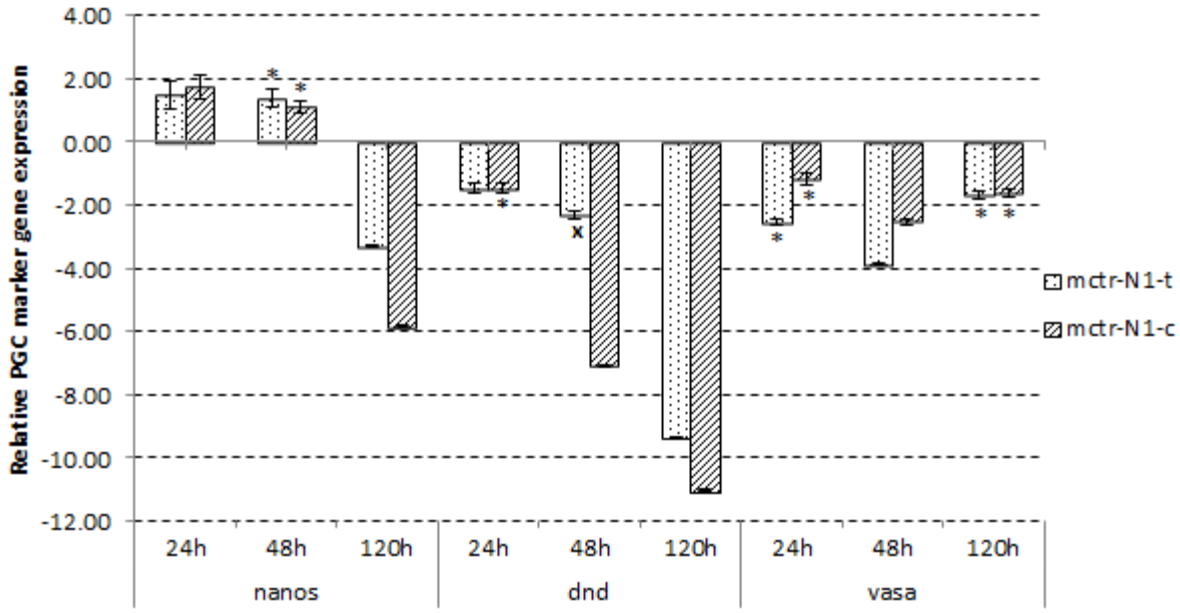

Fig.28. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated mctr-N1 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos, dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Control Mctr-N2 embryos were knocked down for *nanos* 1.8X (48 hpf), 8.4X (120 hpf) and 1.7X for *vasa* (48hpf) (Fig. 29). This construct knocked -down *dnd* decreasingly from 7.6X (48 hpf) to 4.6X (120 hpf) for treated and from 8.6X (48 hpf) to 4.7X (120 hpf) for the control. The copper treated groups were knocked down for *nanos* (2.3X, 120hpf) and *vasa* (2.4X, 48hpf). At 120 hpf, this construct responded to copper as expected in regards to *nanos* expression.

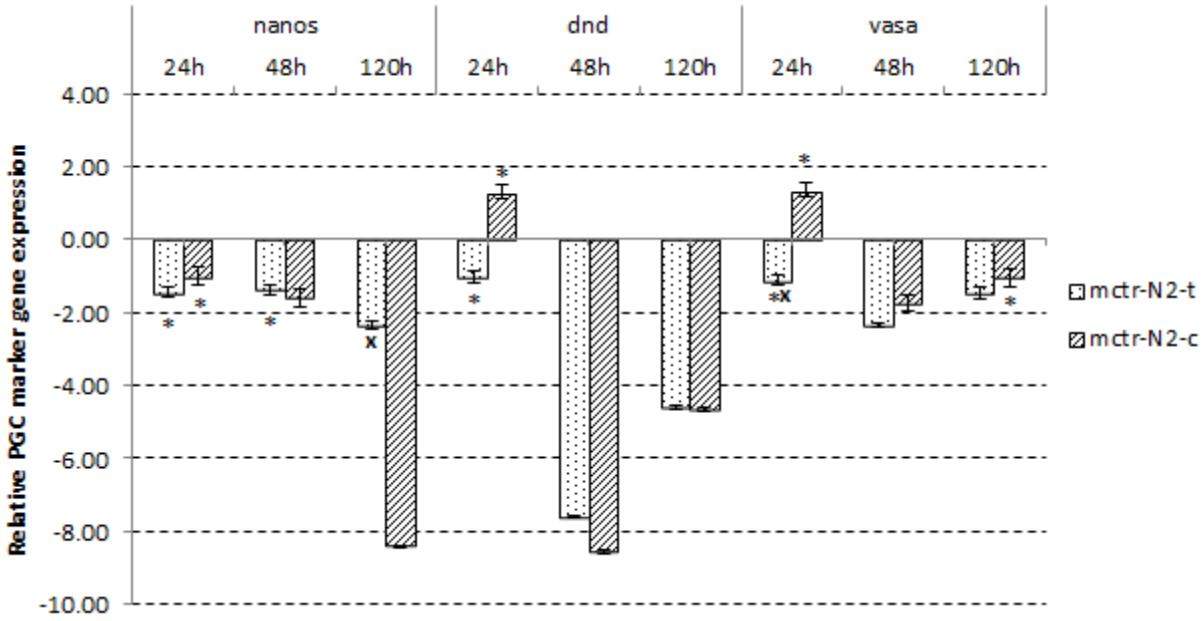

Fig.29. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated mctr-N2 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

3.2.5 T system

Treated and control of T-DND (Fig. 30) knocked down *dnd* at all time points, roughly 3X to 6X. In general, both groups were knocking down the off-targets *vasa and nanos.* Cadmium did not repress this construct (Fig. 30), but significant differences were found between the treatments for *nanos* at 48 hpf and 120 hpf, and for *vasa* at 48 hpf due to Cadmium.

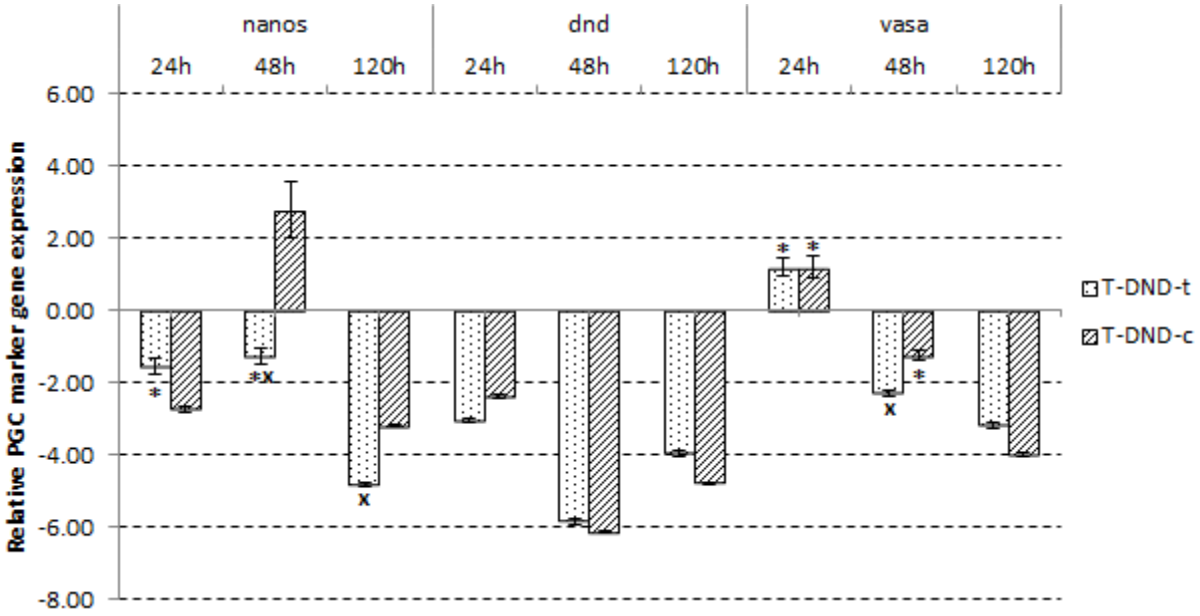

Fig.30. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated T-DND transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Like other cDNA fused constructs, T-cDNA (Fig. 31) up-regulated *nanos* all the time, but dramtically from 18X up to 2900X for the treated, and 275X to 1741X for the control treatments. Treated groups were knocked down for *vasa* expression from 1.5X (24 hpf) to 5X (120 hpf), and 2.3X (48 hpf) and 1.7X (120 hpf) for the control. Cadmium successfully repressed expression of T-cDNA for the target at 24 hpf, but was not very effective at other time points and with the off- targerts (Fig. 31).

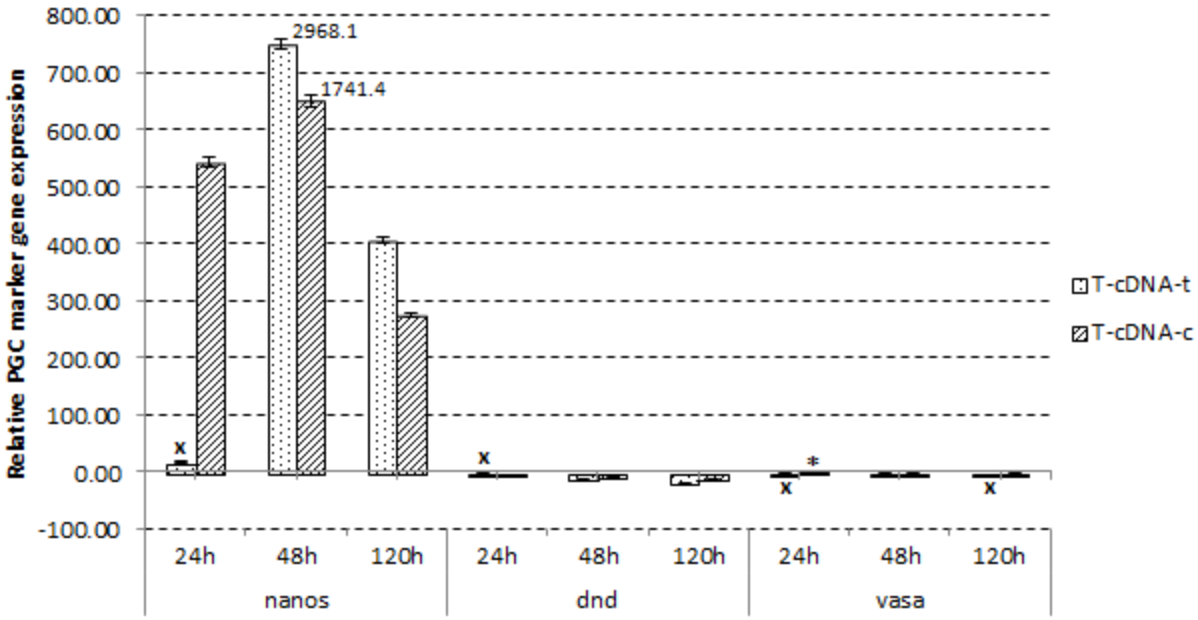

Fig.31. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated T-cDNA transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). Relative expression of *nanos* (48 hpf) in T-cDNA treated: 2968.07± 10.001; Relative expression of *nanos* (48 hpf) in T-cDNA control: 1741.45± 10.001. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Again, N-1 constructs did not function as expected (Fig. 32).Both treatments knocked the target, *nanos*, for 1.8X (treated) and 3.6X (control) at 24 hpf. They knocked down *vasa* for 1.6X (treated) and 2.2X (control) at 48 hpf. Treated embryos were dramatically up-regulated for *nanos* expression from 1X (48 hpf) to 250X (120 hpf). T-N1 (Fig. 32) down-regulated *dnd* expression 3X for the treated embryos at both 24 hpf and 48 hpf, but the control was down regulated 3.6X to 4.6X for all time points. Cadmium was able to repress the construct when the embryos were 120 hpf (Fig. 32).

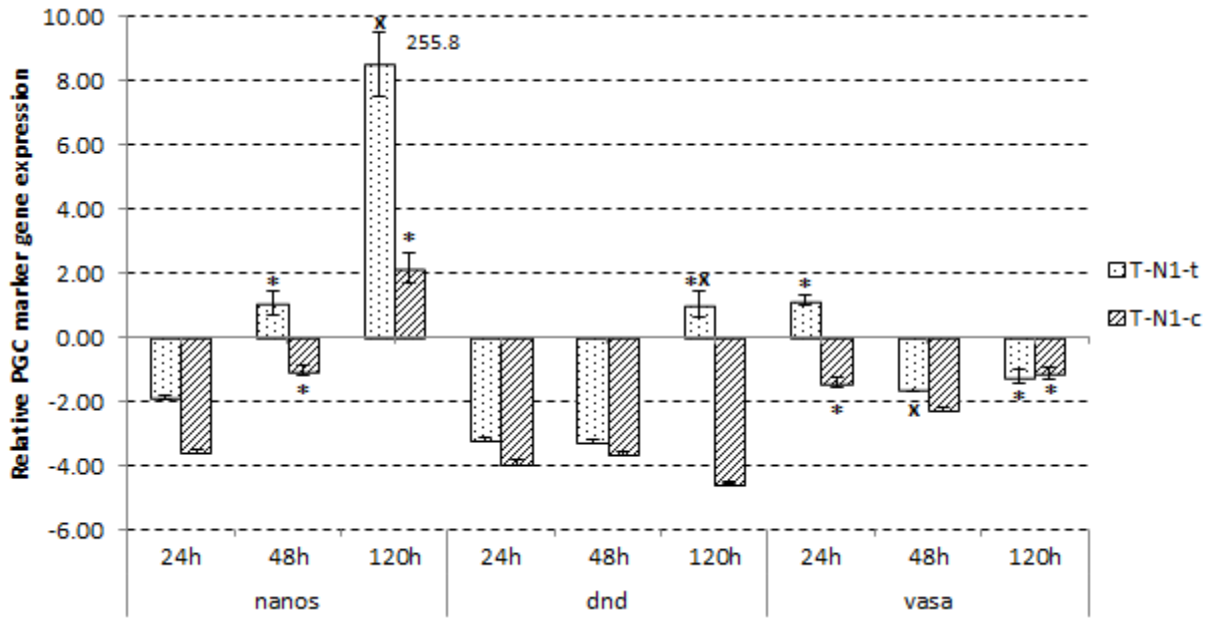

Fig.32. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated T-N1 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos, dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). Relative expression of *nanos* (120 hpf) in T-N1 treated: 255.83± 10.001. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

T-N2 was also relatively ineffective (Fig. 33). Again, cadmium was able to repress the construct for the target when the embryos were 120 hpf .

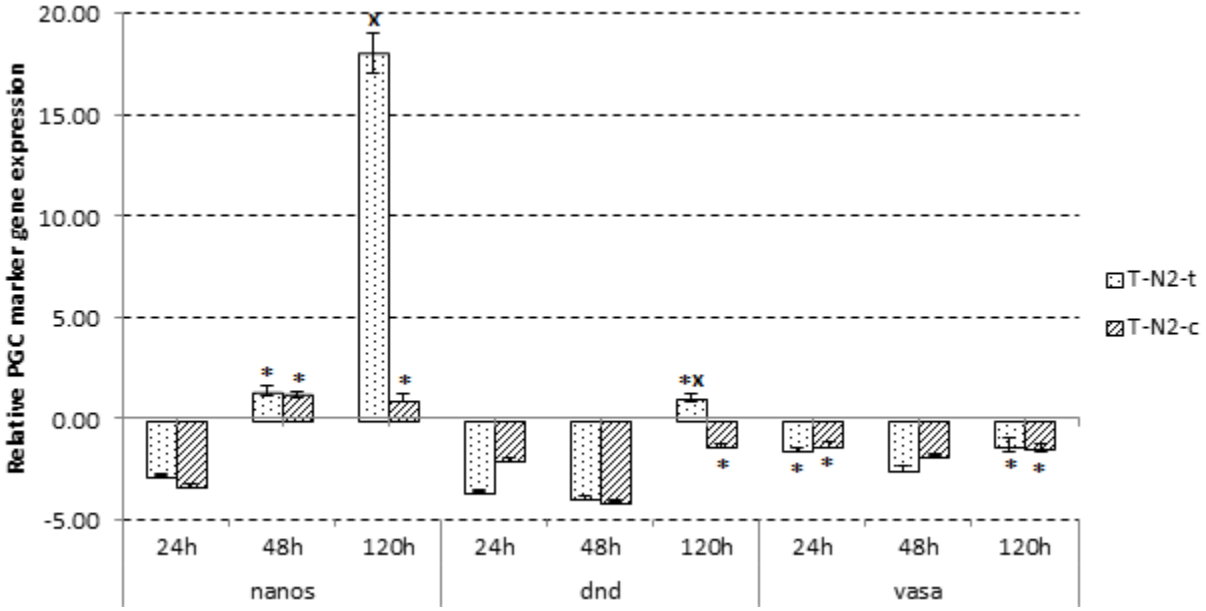

Fig.33. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated T-N2 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos, dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

3.2.6 Nanos system

Nanos-dnd (Fig. 34) knocked down *dnd* from 4.6X to 3.4X for the treated group and from 5.5X to 1.8X for the control at 48 hpf and 120 hpf. Off target effects were observed. The treated embryos were up-regulated *nanos* for 1.6X (24 hpf) and 1.8X (120 hpf), while the untreated control was up-regulated for *nanos* 2.4X (24 hpf) and 12X (120 hpf), both of them slight down-regulated *nanos* at48 hpf ($p>0.05$). Treated embryos were up-regulated for *vasa* 1.7X at24hpf, but downregulated 10X at 48hpf. The control treatment had a similar effect on *vasa*, up-regulated at 24 hpf 2.5X but down-regulated 5.7X at 48hpf. Doxycycline did not repress this construct in the expected manner (Fig. 34).

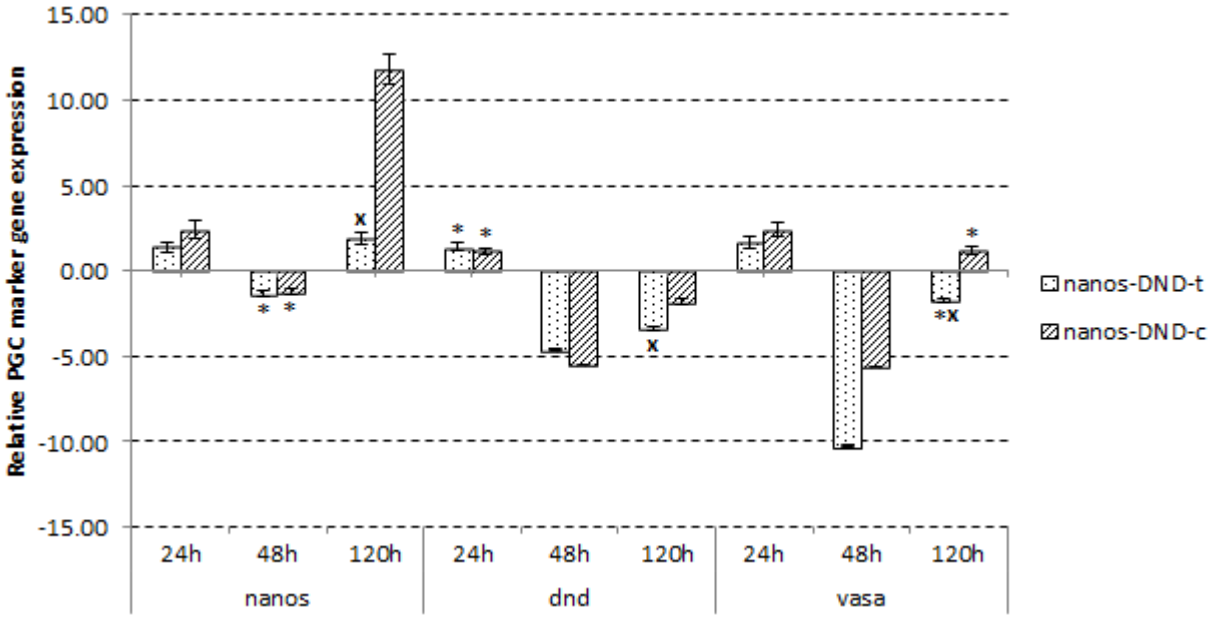

Fig.34. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated nanos-DND transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X**

on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Nanos-cDNA (Fig. 35) dramatically up-regulated *nanos* expression at all time points,128X to 1394X, for the treated embryos, and 61X to 580X for the control embryos. Both were knocked down from 2X to 5X for *dnd* at 48 hpf to 120 hpf . Both up-regulated about 3.5X for *vasa* at 24 hpf. Treatment with doxycycline enhanced expression, which was the opposite of the expected effect.

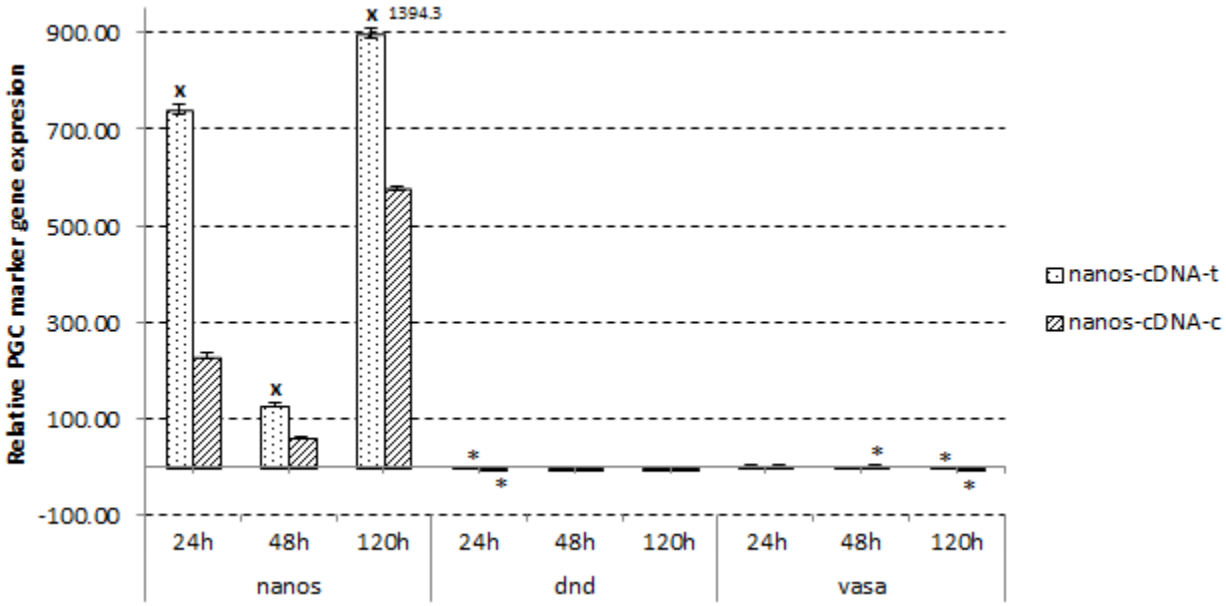

Fig.35. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated nanos-cDNA transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). Relative expression of *nanos* (120 hpf) in nanos-cDNA treated: 1394.30± 10.001. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Nanos-N1(Fig. 36) was inconsistent in its effects, and had off target effects. Doxycycline also had inconsistent effects in regards to repressing this knock down construct.

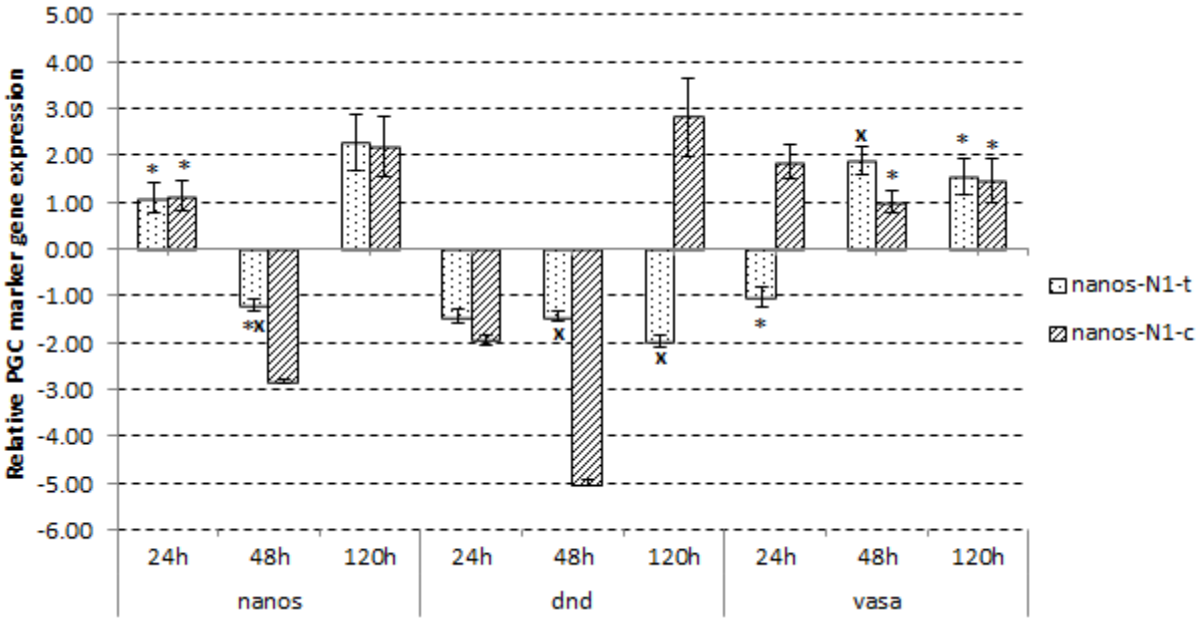

Fig.36. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated nanos-N1 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Oddly, Nanos-N2 regulated *nanos* 3X down (48 hpf) but 14X up (120 hpf) for the control (Fig. 37). This construct had the off-target effect of down-regulating *dnd* for 1.5X to 3.2X at 24 hpf and 48 hpf. Again, doxycycline was inconsistent and minimal in its repression of the construct.

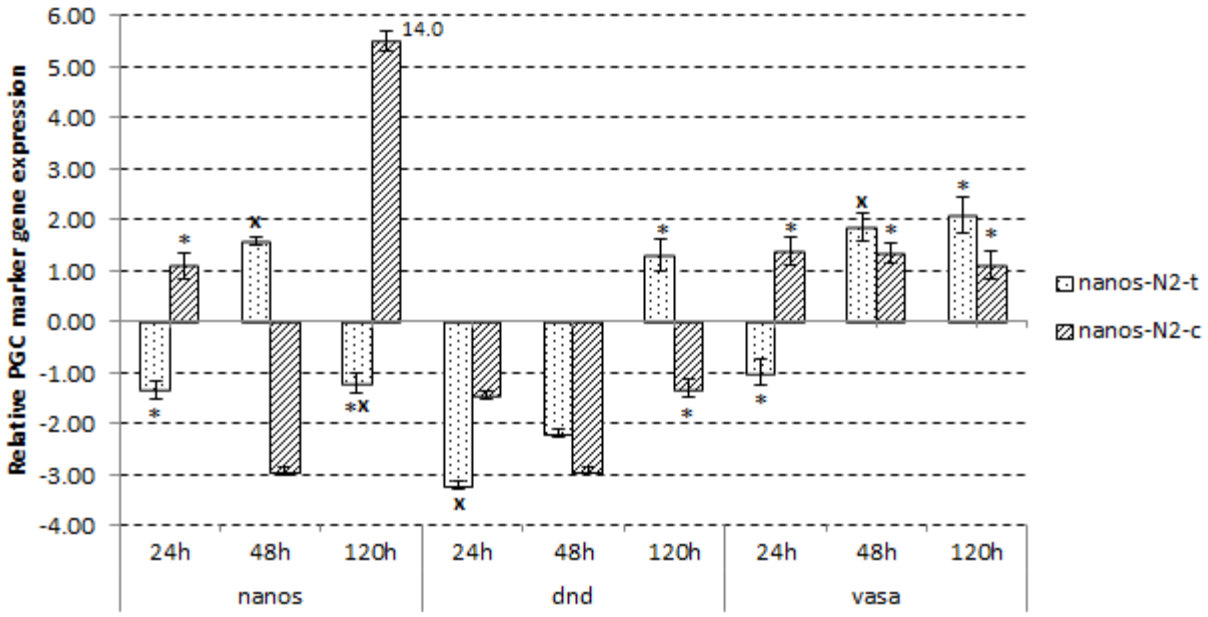

Fig.37. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated nanos-N2 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). Relative expression of *nanos* (120 hpf) in nanos-N2 treated: 14.04± 2.74. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

3.2.7 Vasa system

Vasa-dnd (Fig. 38) knocked down *dnd* progressively from 1.6X to 8.8X for the treated embryos and 3.3X to 7X for the control embryos at 24 hpf and 48 hpf. After that, treated embryos were decreasingly down-regulated for *dnd* to 3.3X at 120 hpf, while control embryos were up-regulated for *dnd* to 2.2X at 120 hpf. The construct had no effect on *vasa* expression. Both treatments down-regulated *nanos* 340X for treated and 75X for control at 120 hpf. Doxycycline was ineffective as a repressor of the construct.

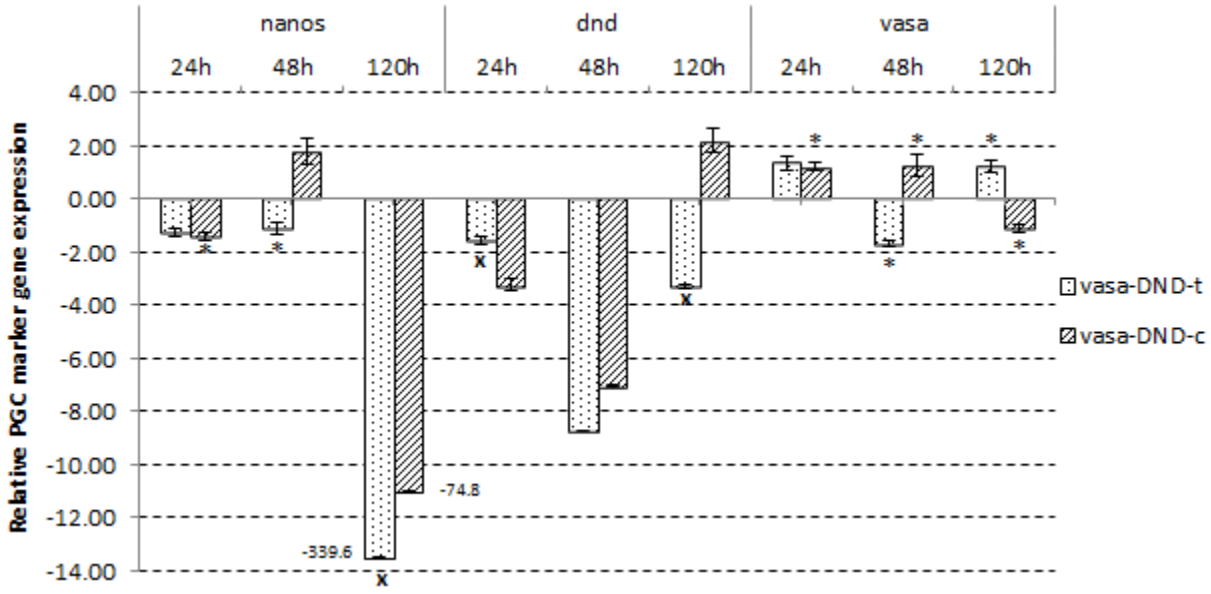

Fig.38. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated vasa-DND transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). Relative expression of *nanos* (120 hpf) in vasa-DND treated: 339.62± 0.101; Relative expression of *nanos* (120 hpf) in vasa-DND control: 74.77± 0.101. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Vasa-cDNA (Fig. 39) dramatically up-regulated *nanos* almost all the time (except control at 120 hpf), from 14X to 480X in the treated embryos, and from 11X to 122X in the control. Both treatments had the off-target effect of progressively knocking down *dnd* expression from 3.3X to 4.5X for the treated and from 3X to 13.3X for the control at 24 hpf to 48 hpf, but not at 120 hpf. There was no effect on *vasa*, and doxycycline tended to enhance rather than repress expression (Fig. 39).

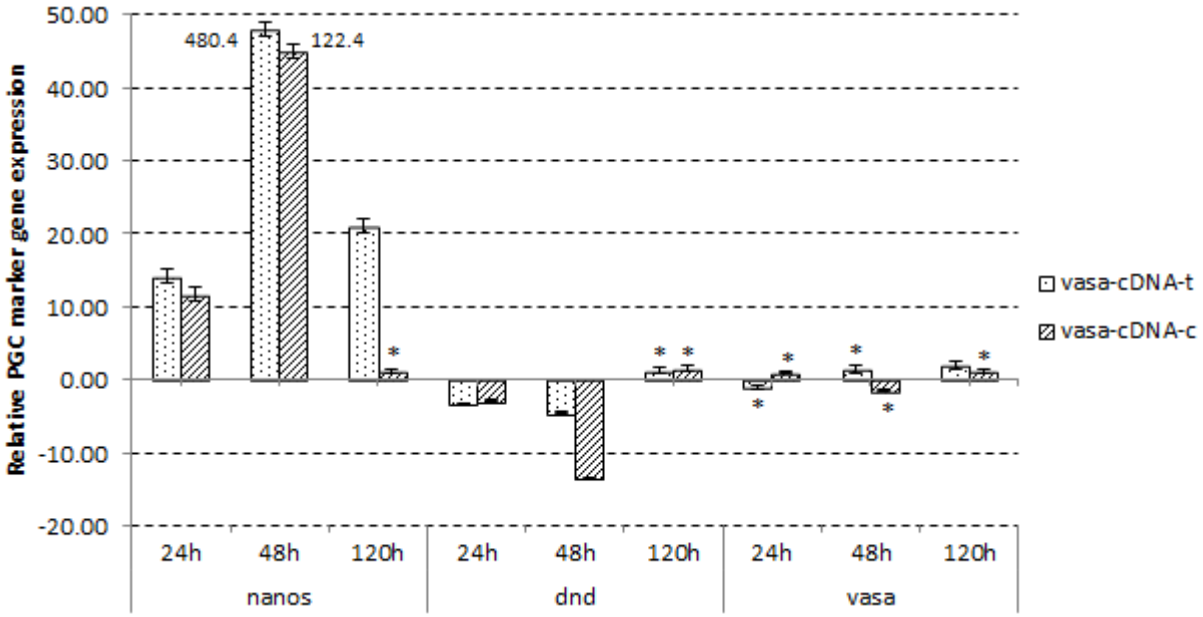

Fig.39. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated vasa-cDNA transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test(PFRR). Relative expression of *nanos* (48 hpf) in vasa-cDNA treated: 480.36± 10.001; relative expression of *nanos* (48 hpf) in vasa-cDNA control: 122.41± 10.001. **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Vasa-N1 (Fig. 40) knocked down *nanos* and *dnd* all the time in the treated group. For *nanos*, the knock- down ranged from 1.9X to 2.5X, and for *dnd* from 3.8X to 6.5X. *Vasa* was only knocked down 3.2X at 120hpf.

Vasa-N1 (Fig. 40) control knocked down *nanos* only dramatically of 6.3X at 120hpf, and that of *dnd* range from 2.6X to 11X with the greatest value 11X at 48hpf, and had knockdown effect on *vasa* of 3.8X (48 hpf) and 2.3X (120 hpf), with up-regulated effect on *vasa* at 24hpf. Although significant difference in doxycycline treatment effect was observed red, it was relatively ineffective as a repressor for this construct.

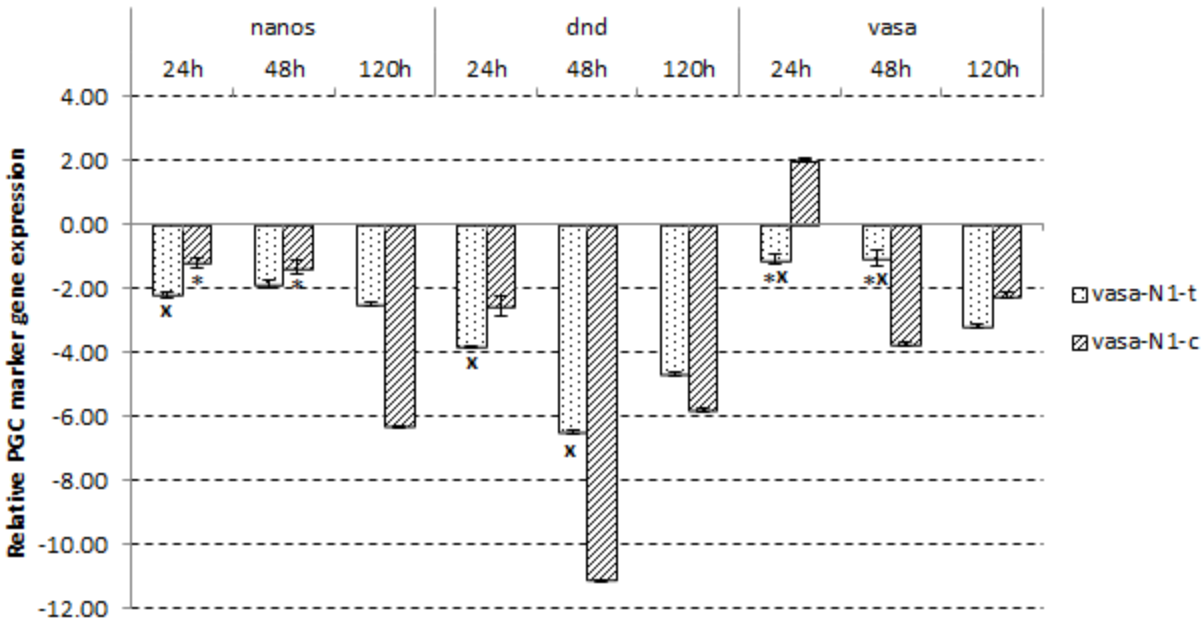

Fig.40. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated vasa-N1 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos, dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

Treated and control of Vasa-N2 (Fig. 41) increasingly knocked down *nanos* at all time points, ranging from 1.9X to 4.2X. Both treatments knocked down *dnd* all the time. Knock down for treated embryos ranged from 3.3X to 11X, and for control embryos from 3X to 13X with the greatest value at 48hpf for both. Both treatments knocked down *vasa* 3.5X (control) and 4.7X (treated) at 48 hpf. No significant repression of the construct was obtained from the exposure to doxycycline.

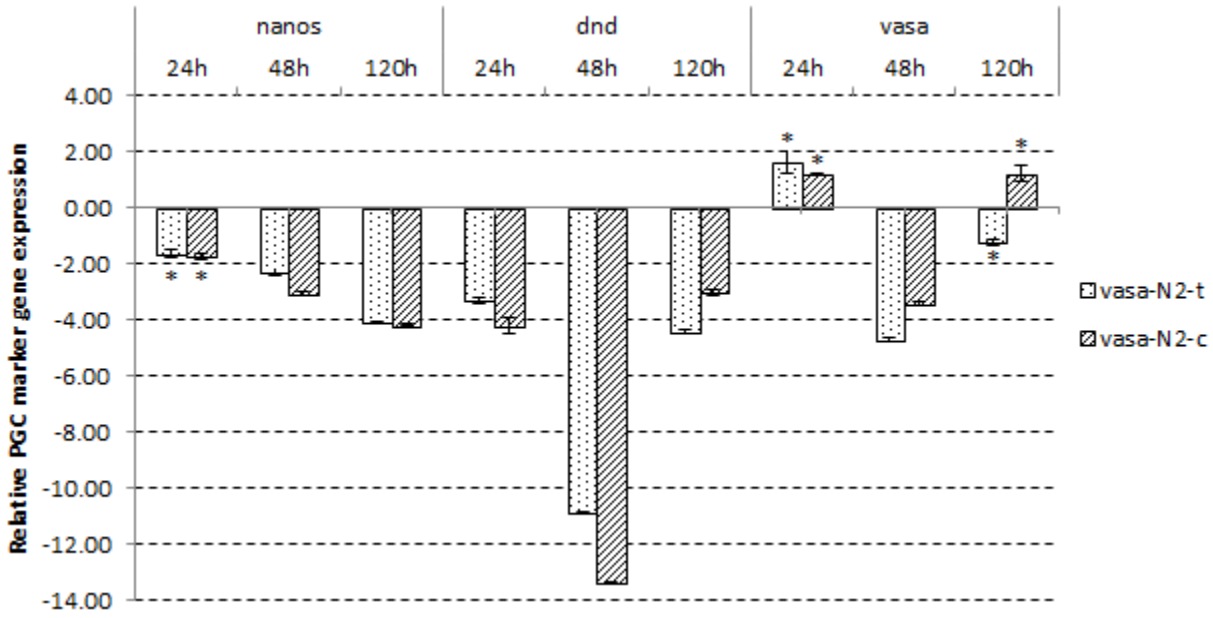

Fig.41. Relative primordial germ cell (PGC) marker genes, *nanos*, *dead end* (*dnd*) and *vasa* expression in the treated and non-treated vasa-N2 transgenic channel catfish *(Ictalurus punctatus)* embryos. The samples were analyzed at 24, 48 and 120 h post fertilization (hpf), respectively. Relative *nanos*, *dnd* and *vasa* genes expression were expressed as fold change over non-transgenic control samples of the same time point. Relative fold changes were expressed as mean ±SE; all the values were significant at the level of $p < 0.05$ unless where marked with asterisk using Pairwise Fixed Reallocation Randomization Test (PFRR). **X** on the SE bar indicated the relative expression between treated and control was significantly different from each other using PFRR.

3.3 Comparison of expression of each knockdown by promoters designed for each repressor

3.3.1 DND

Adss and RM promoters (Fig. 42) were exposed to salt. Neither functioned as designed, except RM-dnd at 120 hpf.

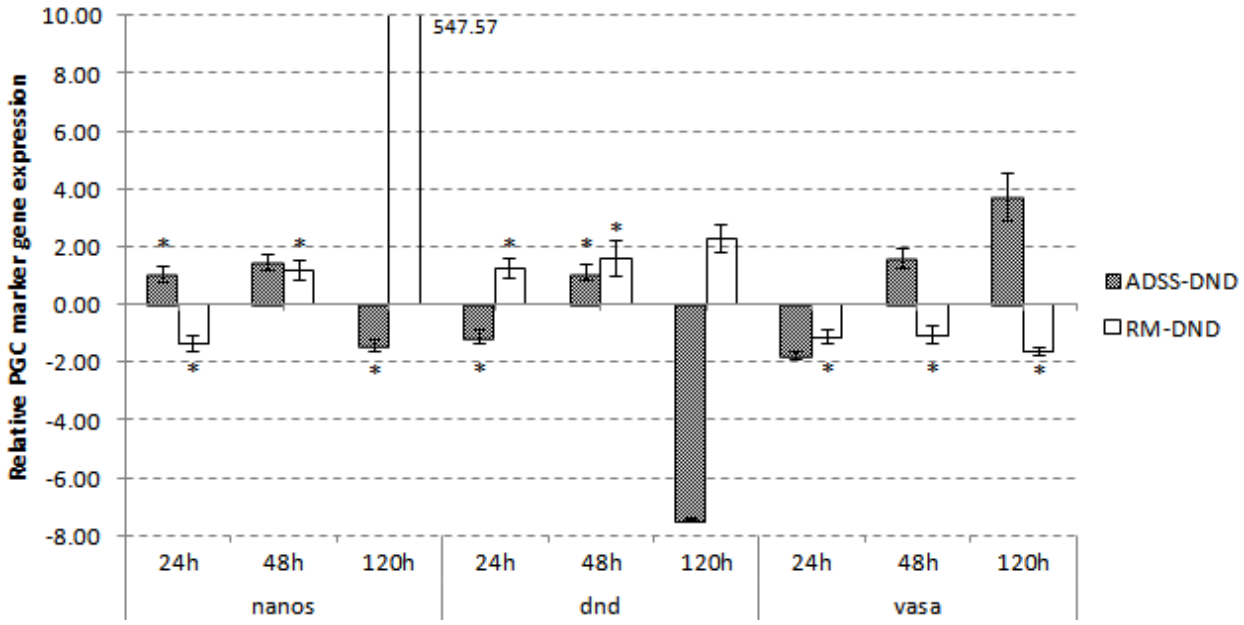

Fig.42. Effect of different promoter zebrafish ADSS2 (adss) and racemase (rm) on channel catfish *dead end* (*dnd*) shRNA structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test. Relative expression of *nanos* (120 hpf) in RM-DND: 547.57± 10.001.

In general, M promoter had down-regulated PGC marker genes most of time, while Mctr up-regulated PGC marker genes (Fig.43). Copper sulfate increased the knock down significantly at all-time points for M-dnd rather than moderating knock down as hypothesized.

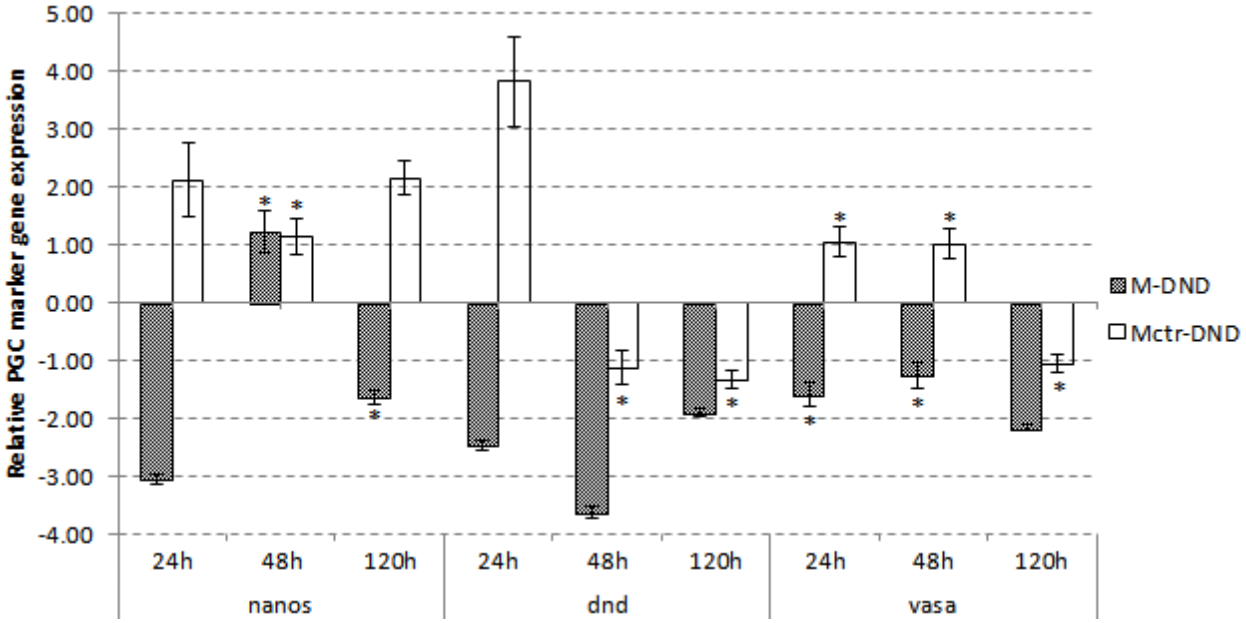

Fig.43. Effect of different promoter yeast (copper specific transcriptional repression) CTR3 promoter (M) and CTR3 promoter but less sensitive (Mctr) on channel catfish *dead end* (*dnd*) shRNA structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus,* using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

Nanos-dnd and vasa-dnd did not function as designed except at 120 hpf down regulation was enhanced by the addition of doxycycline (Fig.44) for both the target *dnd* and the off-target *nanos*. The transferrin-dnd did not strongly respond to the repressor, cadmium chloride (Fig.45).

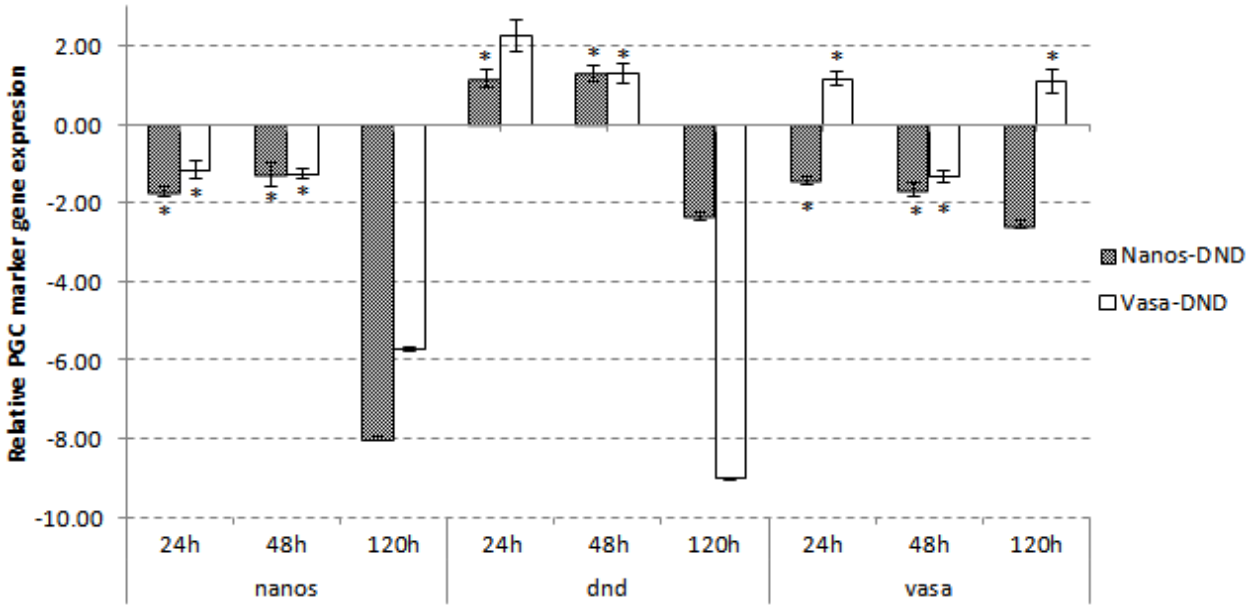

Fig.44. Effect of different promoter channel catfish nanos and channel catfish vasa on channel catfish *dead end* (*dnd*) shRNA structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

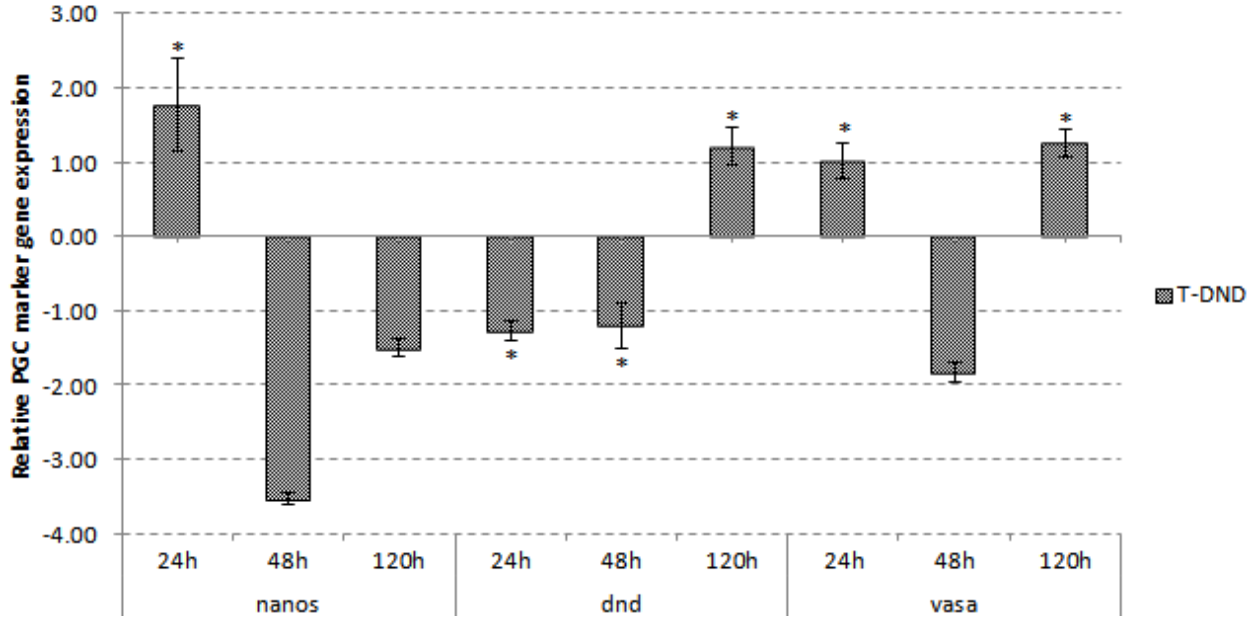

Fig.45. Effect of promoter salmon transferrin on channel catfish *dead end* (*dnd*) shRNA structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over

transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.3.2 cDNA

Adss-cDNA and RM-cDNA up-regulated *nanos* early in development (24 and 48 hpf), and expression was enhanced rather than repressed by salt, but RM-cDNA was repressed by salt at 120 hpf (Fig.46). In general, The M system was not repressed by copper sulfate; however the Mctr system did respond to the copper at 48 and 120 hpf (Fig. 47).

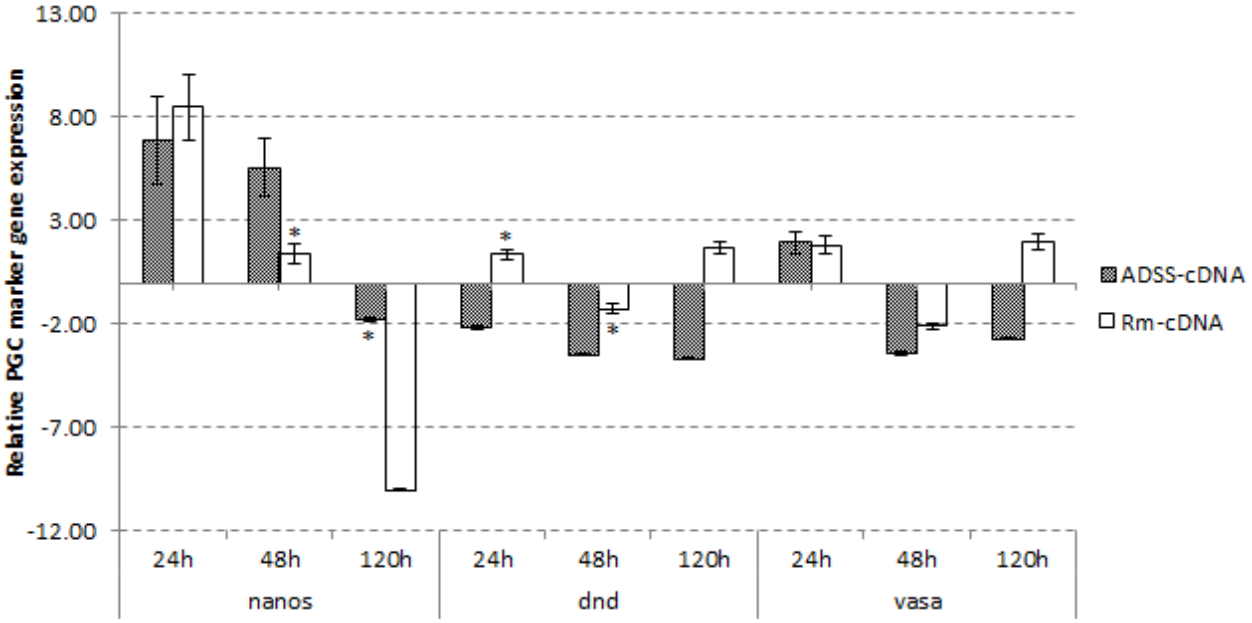

Fig.46. Effect of different promoter zebrafish ADSS2 (adss) and racemase (rm) on channel catfish *nanos cDNA* overexpression structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos*, *vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

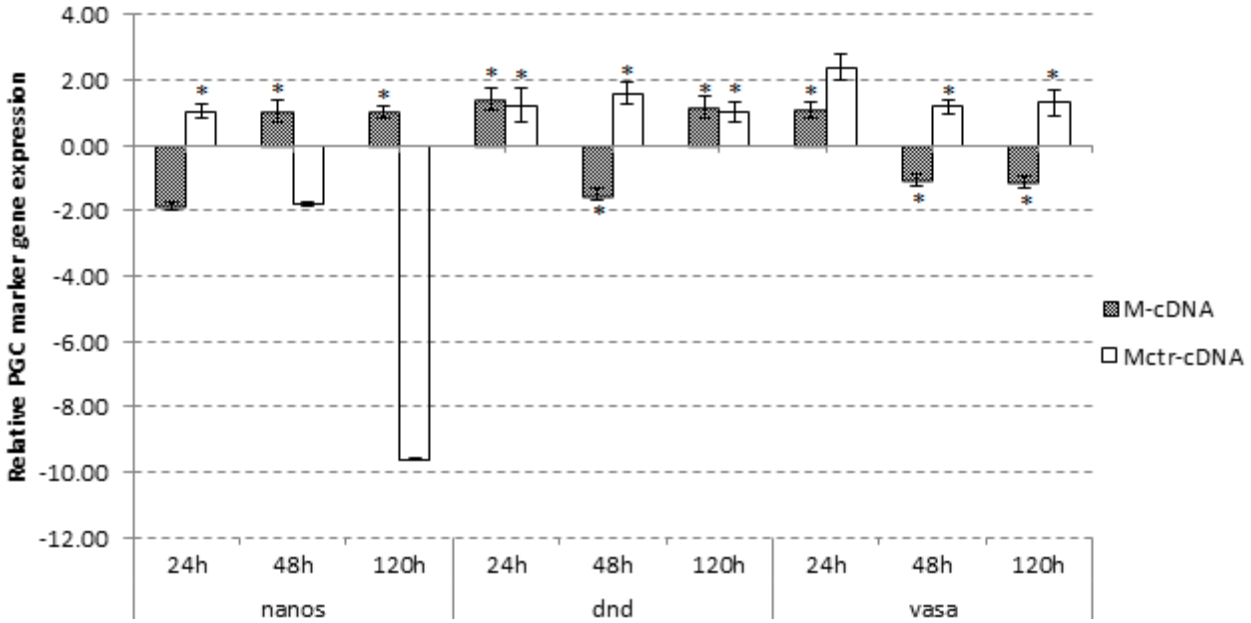

Fig.47. Effect of different promoter yeast (copper specific transcriptional repression) CTR3 promoter (M) and CTR3 promoter but less sensitive (Mctr) on channel catfish *nanos cDNA* overexpression in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos*, *vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

Doxycyline enhanced expression of Nanos-cDNA and Vasa-cDNA systems for *nanos* rather than repressing expression (Fig.48).

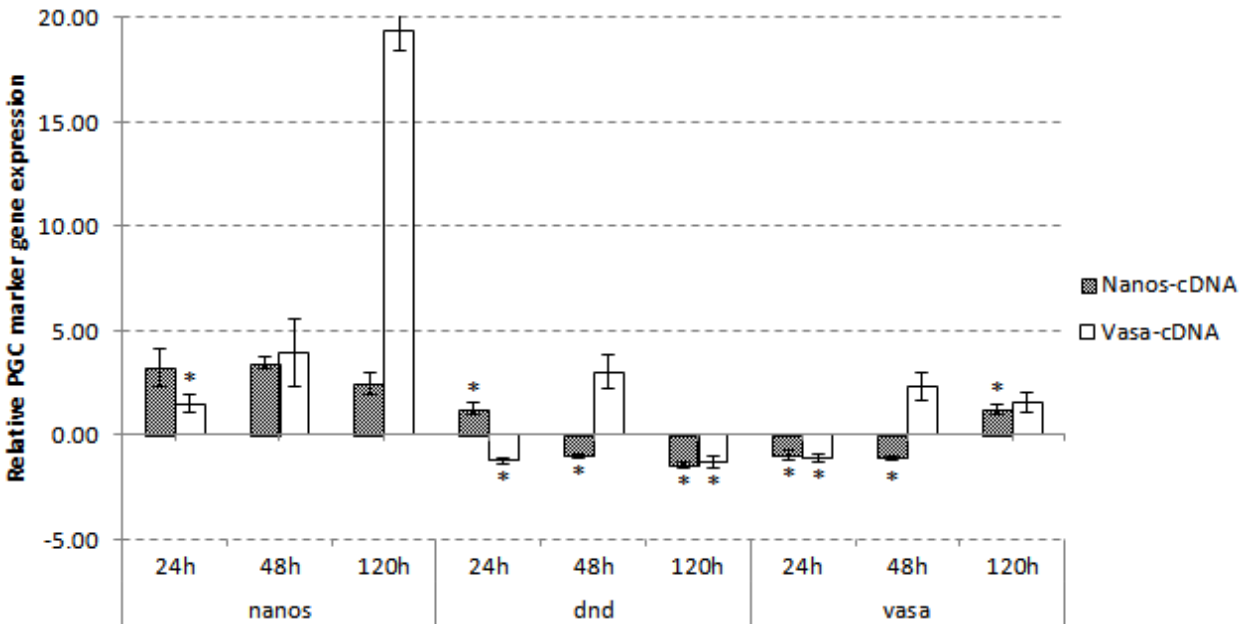

Fig.48. Effect of different promoter channel catfish nanos and channel catfish vasa on channel catfish *nanos cDNA* overexpression structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

Transferrin-cDNA showed promise for functioning as designed as cadmium chloride strongly repressed expression at 24hpf, but had little effect later in development (Fig.49).

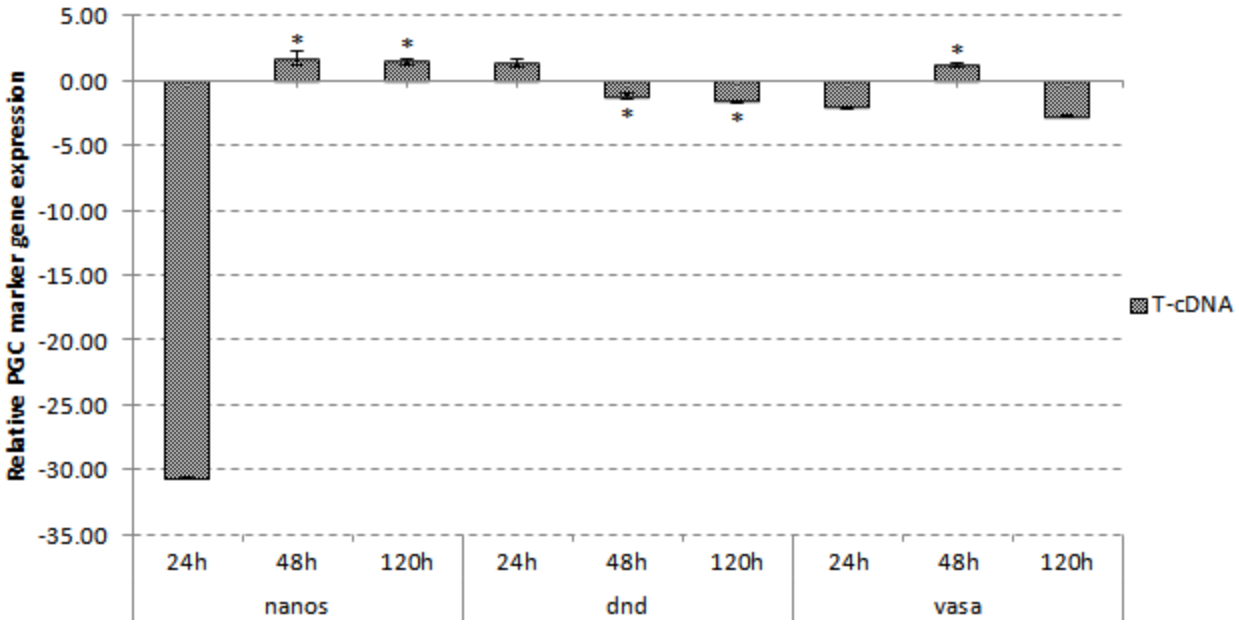

Fig.49. Effect of promoter salmon transferrin on channel catfish *nanos cDNA* overexpression structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus,* using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.3.3 N1 and N2

Expression of the knock down and its repression was inconsistent for all systems for N1 (Fig.16, 20, 24,28,32,36 and 40). However, T-N1showed some promise as N1 was strongly (118X) repressed at 120 hpf (Fig. 50). RM-N2 systems exhibited promise as salt had a small repressing effect on target expression at 24 and 48 hpf (Fig.51). ADSS-N2 was repressed by salt at 48 hpf and 120 hpf (Fig.51).

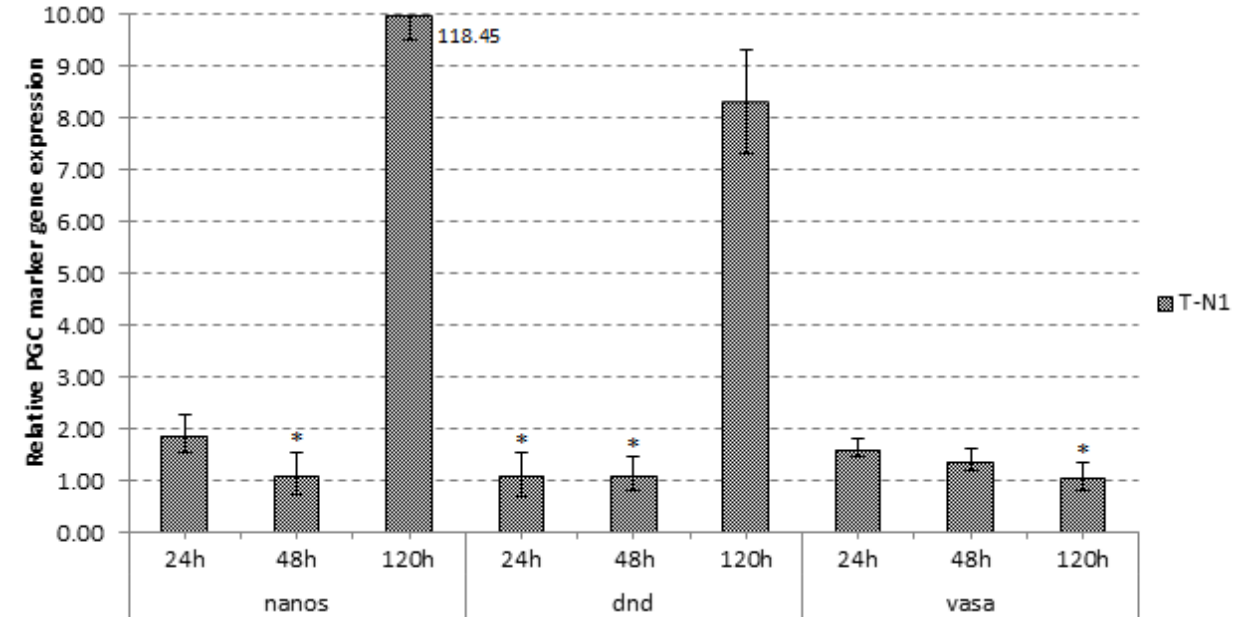

Fig.50. Effect of promoter salmon transferrin on channel catfish *nanos*1 ds-sh RNA (N1) (targeting 5' *nanos*) structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus,* using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test. Relative expression of *nanos* (120 hpf) in T-N1: 118.45± 10.001.

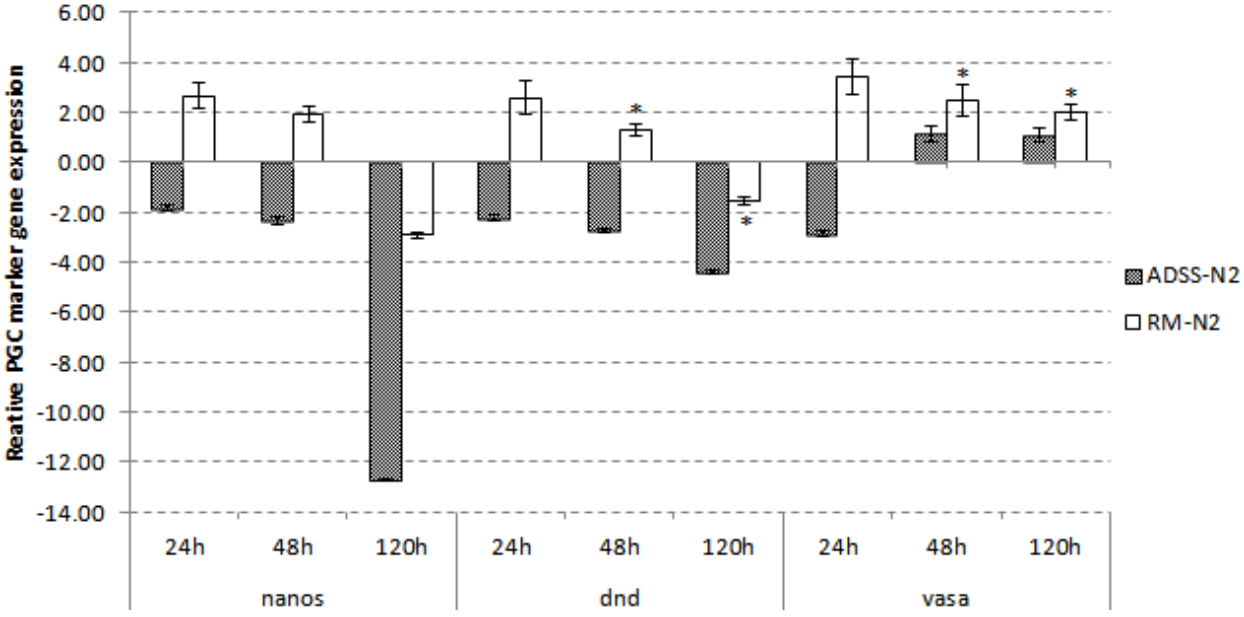

Fig.51. Effect of different promoter zebrafish ADSS2 (adss) and racemase (rm) on channel catfish *nanos*1 ds-sh RNA (N2) (targeting 3' *nanos*) structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish,

Ictalurus Punctatus, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

Copper sulfate was inconsistent in repressing M-N2 and Mctr-N2 as it either had no effect or at one time point for each construct it repressed knock down as expected (Fig.52). Doxycycline did not repress N2 except it strongly repressed nanos-N2 at 120 hpf (Fig.53). Cadmium chloride did not repress T-N2, and had the opposite effect, but strongly repressed down regulation at 120 hpf (Fig.54).

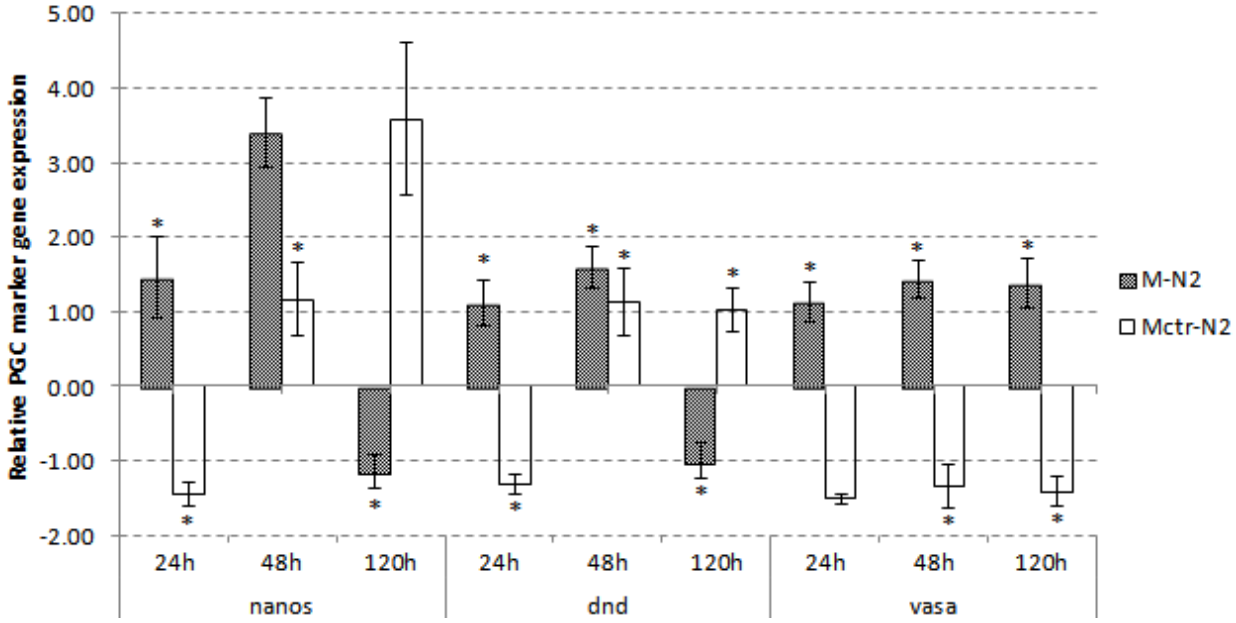

Fig.52. Effect of different promoter yeast (copper specific transcriptional repression) CTR3 promoter (M) and CTR3 promoter but less sensitive (Mctr) on channel catfish *nanos*1 ds-sh RNA (N2) (targeting 3' *nanos*) in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

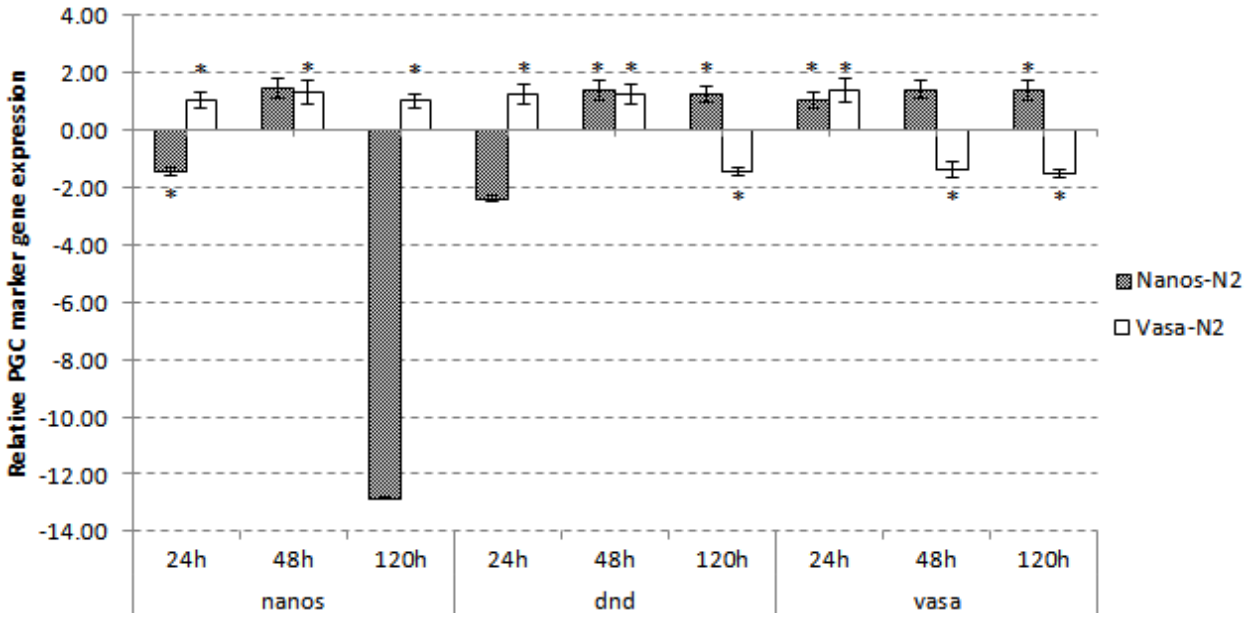

Fig.53. Effect of different promoter channel catfish nanos and channel catfish vasa on channel catfish *nanos*1 ds-sh RNA (N2) (targeting 3' *nanos*) structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

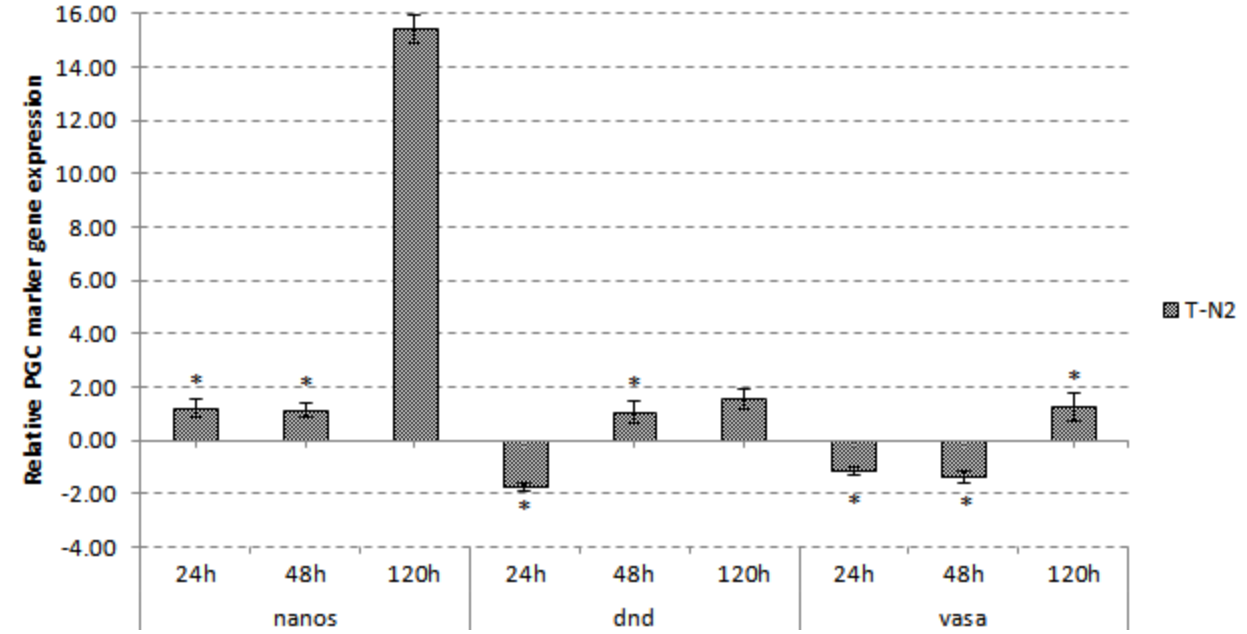

Fig.54. Effect of promoter salmon transferrin on channel catfish *nanos*1 ds-sh RNA (N2) (targeting 3' *nanos*) structure in knock down PGC marker genes expression in transgenic channel catfish *(Ictalurus punctatus)* embryos. Expression of PGC genes (*nanos, vasa* and *dead end)* were measured at 24, 48 and 120 h post fertilization (hpf) during embryonic development of channel catfish, *Ictalurus Punctatus*, using knockdown constructs with shRNAi. Relative PGC genes expressions were expressed as fold change of transgenic treated over transgenic non-treated samples taken at the same time point as normalized to change

in the expression of 18s control. Relative fold changes were expressed as mean ±SE. All the values were significant at the level of $p < 0.05$ unless marked with asterisk using Pairwise Fixed Reallocation Randomisation Test.

3.4 Sexual maturity of 3-year-old channel catfish

The percentage of gravid males and females at 3 years of age for fish treated with or without repressor compounds is found in Table 7. Some trends emerged, such as 80% of ADSS-cDNA females treated with salt being gravid, and 56% of the knockout electroporated controls being gravid, but this observed difference was not significant ($P>0.05$). Mctr-dnd and Mctr-N2 demonstrated expected trends, but the means of the treated and control fish were not different ($P>0.05$). If Mctr knockouts and sexes are pooled, percentage of fish gravid, 58%, was higher ($P<0.10$) for repressed fish than fish that did not receive the repressor compounds, 26%. When treatments and sexes were pooled by the knockout type, N1, percentage of fish gravid, 37%, was lower ($P<0.05$) for repressed fish than fish that did not receive the repressor compounds, 66%. When treatment and sex were pooled by the knockdown type N2, gravid female from treatment showed higher percentage of gravid ($P>0.05$).

4. Discussion

Normal expression

From 24 to 120 hpf levels of *nanos* mRNA dropped naturally. *Vasa* and *dead end* mRNA levels also dropped at a slightly faster rate from 24 to 48 hours, and then were dramatically reduced about 50X between 48 and 120 hpf. mRNA of these PGC markers is either being down-regulated or mRNA that is either maternally derived mRNA or being generated earlier in embryogenesis is being degraded. Since, several of the constructs in this study further reduce mRNA levels of *nanos, dead end* and *vasa*, some transcription of these genes must be occurring throughout embryogenesis, and degradation of mRNA for these genes cannot be the only explanation for the decreasing levels of mRNA. Maternally derived *nanos* mRNA in zebrafish degrades rapidly before gastrulation (Koprunner, et al., 2001), supporting the conclusion that the decreases in the

Table 7. Percentage of gravid, 3-year-old male and female channel catfish, *Ictalurus punctatus*, electroporated with knockout constructs for *nanos* and *dead end* and then receiving (t) or not receiving repressor compounds. These constructs were driven by promoters included, Zebrafish ADSS2(ADSS) and racemase (RM) (both salt responsive), yeast CTR3 (M) (copper sensitive) and Mctr (copper less sensitive), Salmon transferrin (T) (cadmium sensitive), channel catfish nanos (nanos) and vasa (vasa). The main genes fused in the constructs included, double stranded short hair-pin structure RNAis, sh-nanos targeting the 5' *nanos* sequence (N1) and sh-nanos targeting the 3' *nanos* sequence (N2), a full length *nanos* cDNA (cDNA) and a double stranded short hair-pin structure RNAi targeting channel catfish *dead end* (*dnd*). * Means significantly different (P=0.05, Fisher's Exact Test). ** Means significantly different (P=0.10, Fisher's Exact Test). NG: not gravid individual. G: gravid.

Construct	Male			Female			Pooled sexes		
	G	NG	%	G	NG	%	G	NG	%
ADSS-cDNA (t)	3	1	75	4	1	80	7	2	78
ADSS-cDNA	5	2	71	5	4	56	10	6	63
MCTR-dnd (t)	2	0	100	-	-	-	2	0	100
MCTR-dnd	3	5	38	0	2	0	3	7	30
MCTR-N2 (t)	3	3	50	2	2	50	5	5	50
MCTR-N2	2	4	33	0	3	0	2	7	22
N2 pooled (t)	10	10	50	5	9	36	15	19	44
N2 pooled	10	8	56	1	9	10	11	17	39
N1 pooled	16	8	67	11	6	65	27	14	66
N1 pooled (t)	3	4	43	4	8	33	7	12	37*
MCTR pooled (t)	5	3	63	2	2	50	7	5	58
MCTR pooled	5	9	36	0	5	0	5	14	26**

nanos, *vasa* and *dead end* mRNA levels in channel catfish are due to natural and transgenic down regulation and not just degradation.

The decrease in mRNA for these 3 proteins in channel catfish embryos was similar to that of common carp embryos (Su, 2012) for *nanos* and *dead end*, except that the down regulation of *vasa* was like that of *dead end* and accelerated almost identically during the last half of embryonic development. Regulation of the PGC markers was not exactly the same in common carp and channel catfish and it is possible that the role of *vasa* is not exactly the same in these two species.

These observations on embryonic expression and levels of *nanos*, *vasa* and *dnd* mRNA might have implications for different knock down strategies to prevent migration of PGCs and their potential success. Effectiveness of the knockdown may be tempered by the naturally decreasing levels of *nanos*, *dead end* and *vasa*. In light of these findings, a

more detailed study of the temporal expression of these 3 proteins might be beneficial for identifying the transgenic sterilization constructs with the best probability of success.

Knock down

Several of the knockdown constructs were able to reduce the expression of *dead end*. Adss-dnd and vasa-dnd were able to down regulate *dead end* early in embryonic development, but oddly, not at 120 hpf. It is not known, when are the most critical time points to potentially disrupt the migration and kill the PGCs. RM-dnd, Mctr-dnd, M-dnd and T-dnd were able to down regulate *dead end* at all 3 time points. Nanos-dnd was not able to down-regulate *dnd* at 24 hpf, but functioned as expected during the rest of development. Levels of knock down were similar for all constructs with MCTR giving slightly more consistent and stronger down regulation. As expected, the zebrafish, salmon, channel catfish and yeast promoters were able to drive transcription in channel catfish embryos.

The Adss promoter was able to overexpress *nanos cDNA* increasingly over time reaching a 300X up regulation by 120 hpf, and a similar results were observed for RM-cDNA, vasa cDNA and nanos cDNA in varying fashion at different time points. Mctr *up-regulated nanos at all the times a little more consistant than did M-cDNA* , M-cDNA up regulated *nanos* at all 3 time points with a peak of 700X at 48 hpf. The most dramatic up regulation of the cDNA constructs was driven by the T promoter, which elevated *nanos* levels 250-1741X.

N1 was the 5' target for the nanos shRNAi. Adss, RM, nanos, and T N1 constructs were relatively ineffective in changing *nanos* expression. Mctr-N1 had inconstistant effect knockdown nanos, only knockdown at 120 hpf whereas m-N1 had little effect early in development, but then strongly up-regulated *nanos* at 120 hpf. Vasa N1 had little effect early in development, but then down regulated *nanos* as expected at 120 hpf. Overall, this series of constructs designed to cleave a 5' mRNA target of *nanos* were ineffective.

N2 was the 3' target for the nanos shRNAi. RM-N2 had significant but small (-2X) down regulation of *nanos* early in development, but up-regulated *nanos* at 120hpf, and a similar

pattern was observed for the other salt sensitive promoter Adss. Mctr-N2 progressively down regulated *nanos* over time as did M-N2, but more strongly until 120 hfp at which time there was no effect on expression. T-N2 did not affect *nanos* expression. Opposite of the expected results, nanos-N2 up-regulated rather than down-regulated *nanos*. However, vasa-N2 down regulated *nanos* increasingly at all time points.

Several shRNAi constructs knocked down *dead end* as expected, and *nanos* could be overexpressed. The approach using a 5' shRNAi target for *nanos* was not effective, and the approach using the 3' shRNAi target appears more promising to decrease expression of *nanos*.

Repression

Adss and RM are salt sensitive promoters, which down regulated when exposed to salt in zebrafish embryos (Hoshijima and Hirose, 2007). Zebrafish Adss was not very responsive when exposed to salt in channel catfish embryos. Opposite of expectations, the constructs Adss-cDNA and RM-cDNA strongly up-regulated rather than decreasing expression. RM-dnd responded to salt and appeared to be repressed and significantly (P=0.05) at 120 hpf. RM-N2 also responded to salt at 24 and 48 hpf, and was repressed at these time points.

The constructs or DNA sequences ligated and downstream of the salt sensitive promoters appear to affect their response to salt. Other factors that could affect their sensitivity are the insulators and border elements in the constructs.

Mctr and M are yeast promoters that should be down regulated by copper sulfate (Labbe et al. 1997). The Mctr constructs did not repress when exposed to copper sulfate except for the N2 and N1 constructs at 120 hpf. Our expectation would be that repression this late in development might be too late to restore fertility to the embryos. However, the trend for both of these treatments was for the treated fish to have a higher observed rate of gravidness at 3 years of age compared to untreated controls, suggesting that repression at 120hpf might be adequate to obtaint the desired biological effect. M down regulated even more strongly when exposed to copper sulfate instead of being repressed in the case

of the *dnd* shRNAi. Similar to Mctr-N2, one time point for M-N2 was repressed as expected. Overall, the Mctr and M constructs were not usually repressed by copper.

Nanos and vasa drove the tet-off system (Thresher et al. 2009) which is regulated and repressed by doxycycline. The vasa and nanos shRNAi constructs did not regularly repress as designed. Opposite of expectations and similar to the situation with the salt sensitive promoters, the application of the repressor, in this case doxycycline enhanced rather than repressed the production of *nanos* mRNA for both nanos-cDNA and vasa-cDNA. A similar tet-off approach utilizing early developmental promoters from zebrafish that were designed to kill embryos by disrupting body development with cDNA and shRNAi knock downs successfully disrupted development and killed channel catfish, common carp and zebrafish embryos (Thresher et al. 2009). However, expression patterns and mRNA data of these constructs are not available to compare to our results, making it more difficult to predict if the biological effects that are desired are possible with the unexpected expression patterns.

T is a cadmium responsive promoter from salmon (Carginale et al. 2002). Similar to the other promoter systems, T did not repress when exposed to cadmium chloride, and again actually responded to cadmium chloride by enhancing expression of *nanos* cDNA.

The results indicate that we can greatly depress the expression of *nanos*, *dead end* and *vasa*. This should result in the disruption of migration and the death of PGCs followed by sterilization of the fish. It is difficult to predict which constructs would work best as there were many off- target knock downs that could result in sterilization from constructs that did not function as designed. However, a few constructs such as RM-dnd and RM-N2 both knocked down and repressed which theoretically, could result in a repressible transgenic sterilization system.

The age of the embryos and thus the changing transcriptome affected the expression of the knock downs as the degree of efficacy and direction of regulation changed over time for many constructs. These could likely be resolved as a myriad of epistatic effects. In

several cases, knockdown occurred at 24 and 48 hpf followed by up-regulation at 120 hpf.

The basis of these initial experiments is that foreign DNA initially replicates strongly within embryos, expresses in the cytoplasm and degrades later on in development (Houdebine and Chourrout, 1991). Copy numbers of plasmid DNA autonomously replicates exceeding numbers of embryonic cells number in the embryos development (Dunham, et al 1992). Thus, genetic and biological effects of the constructs can be observed in P1 embryos even without integration, especially for genes active in development.

Sexual Maturity

Sterilization was assessed by evaluating the secondary sexual characteristics of the fish. If fish treated with repressor compounds have a higher rate of sexual maturity than those that are not treated, this would be evidence of the potential for a workable and repressible transgenic sterilization system.

Trends were observed in this regards as percentage of sexually mature and repressor treated Adss-cDNA, Mtctr-dnd and Mctr-N2 was numerically, but not statistically higher than controls. When Mtcr treatments were pooled the difference between treated and control groups was statistically different. The opposite expectation, higher rates of sexual maturity for untreated controls was observed for N1 groups pooled. This could be a result of the repressing compound, doxcycline, having the unexpected opposite effect of enhancing expression making sterilization of the treated fish even more likely than in the control.

5. Conclusions

Sexual maturity and sterilization of the fish electroporated with these knockout constructs for *nanos* and *dead end* need to be spawned and the gonads examined to confirm fertility or sterilization. F1 transgenic embryos need to be evaluated for two reasons. Any P1 transgenics generated will be mosaic (Dunham 2011) complicating biological effects and

these constructs may behave differently in the nucleus compared to the cytoplasm for which the results in the experiment were obtained. Additionally, copy number will be much lower in F1 transgenics compared to the transient cytoplasmic transgenics in this experiment, which could affect the results. Pleiotropic effects are possible (Muir and Howard, 2002; Sundström et al.2007; Guan et al. 2008) and should be evaluated in the future. These initial results indicate potential for a repressible transgenic sterilization system targeting gametes, but whether or not this system will be 100% effective cannot be conclusively demonstrated until the reproductive traits of repressed and untreated F1 transgenic channel catfish for these knockdowns are evaluated.

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