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Sterility and its implication in tilapia aquaculture: a review

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Tilapia, popularly known as "the aquatic chicken", are fast growing, hardy fish tolerating a wide range of environmental conditions and hence assumes great importance in world fisheries. Though native to Africa, Israel and Jordan, they have been introduced to several tropical and temperate climatic conditions in the world. Tilapia, belonging Cichlid family, has about 100 species and subspecies, of which *Oreochromis niloticus* (Nile tilapia), *O. mossambicus* (Mozambique tilapia), *O. aureus* (blue tilapia) and hybrids between them are the most important with focus on major aquaculture efforts. These species account for 99.5% of global tilapia production. China is the world's largest tilapia producer and the USA, the world's largest tilapia consumer.

Why Tilapia?

The global tilapia aquaculture production was 3, 20, 000 metric tons in 2010 (Fitzsimmons, 2010) making it an important fish for aquaculture after carp and salmon. The demand for Tilapia can be attributed to their ease of culture, high protein content, rapid growth rate, large size, omnivorous diet, means of reproduction and production ability, resistant to disease, palatability, tolerance to high stocking density and a variety of environmental conditions. Their growing demand together with variety of production systems and the high price for the species has encouraged many aquaculturist to enter to tilapia aquaculture worldwide.

Why sterility?

Under aquaculture conditions, tilapia reaches sexual maturity early and starts reproducing with multiple annual spawning before they reach marketable size. Growth and reproduction in fish are closely related and the prolific breeding of tilapia together with high survival of the

larvae would result in stunted growth of fish thus reducing yield and economic value. Controlling reproduction has been a major challenge in tilapia culture for many years. Tilapia is also notorious as invasive species in many countries. Tilapia is also reported as problematic invasive species in Australian and American waters. In India, tilapia was introduced to fill the unoccupied niche but soon it has spread to all over the country due to its prolific breeding.

Culture of monosex progeny, preferably males which grow faster and to a larger size than females, is a possible solution to regulate uncontrolled reproduction in tilapia culture. In addition to the faster growth rates, males are also more uniform in size and more energy efficient as females are smaller in size and also tend to waste energy during breeding. Various techniques like i) manual sexing, ii) hybridization, iii) sex reversal and iv) genetic manipulation are currently in use to produce mono-male tilapia culture. All these techniques have advantages and disadvantages and yet to be widely used in aquaculture.

Rendering the fish sterile by manipulation of reproduction would help to increase growth by reducing energy consumption for reproduction and thus benefit aquaculture (Rottmann et al., 1991). Sterility in fish can also prevent backcrosses of hybrids with their parents and ensure reproductive containment in transgenic escapees. Sterility can be achieved either by ploidy manipulation to produce sterile triploids or the use of transgenics by gene “knock-out” or “gene knock-down”

Triploidy induced sterility

Triploidy, the presence of three sets of chromosomes instead of two in an organism, has attracted considerable attention as a tool to induce sexual sterility in tilapia. Triploidy is induced by the application of hydrostatic pressure, temperature or chemical (colchicine or cytochalasin B) shock at the specific time and duration after the egg is fertilized mainly to disrupt the normal extrusion of a polar body from the egg (polar body I – PBI or plar body II – PBII) during meiosis. Induction of PBI triploidy is more challenging compared with PBII triploidy due to the fragile nature of embryos at this earlier developmental stage. The presence of the odd set of chromosomes presumably hinders the normal pairing of homologous chromosomes and thus disrupts the development of the gametes rendering the animals functionally sterile (Benfey, 1999). According to the EU regulations, polyploids are not considered genetically modified

organisms (GMOs). The success rate of sterility varies with species, sex, method of treatment to induce triploidy and also on the egg quality. Sterility by triploidy is more successful in females compared to males. Triploid females are characterized by ovaries with few oogonia and primary oocytes, which fail to mature while in triploid males, though the size of the testes does not differ significantly from diploids, they too are functionally sterile with few spermatids. The disadvantages are the occasional productions of mature oocytes by triploid females (Schafhauser-Smith and Benfey, 2001) and also that some embryos can still be diploid after treatment or can have mosaic tissue with both diploid and triploid cells. Triploidy is not feasible for some species resulting in negative impact performance traits. An alternative to the inconsistent induction of triploidy by physical treatments is the crossing of tetraploid fish with diploid fish. Tetraploid fish however, generally have poor early survival, growth, and male fertilizing ability.

Triploidy in tilapia is quite difficult and resulted in higher proportions of females. The "all-female" triploid technique in tilapia is less satisfactory as males grows faster and larger and has higher market demand. Growth, survival and gonadal development of triploid tilapia were poorer than those of diploids.

Transgenic methods for reproductive containment

Transgenic sterilization is attained mainly by the targeted disruption or decrease of hormone genes in the sexual reproduction pathway and the sterility thus induced can be rescued by exogenous hormone administration. Transgenic containment can also be accomplished by gonad-specific transgene excision. Gene function can be altered theoretically by targeting the gene coding for a crucial reproductive hormone (gene knockout) or its specific gene message (gene knockdown) (Maclean et al., 2003). **Gene knockout** is the recognition and replacement of gene sequence by defective copy via homologous recombination. The technique involves the use of a construct which renders the fish infertile in wild by a reversible reproductive control by disrupting gametogenesis or embryogenesis. This approach, though well established in mouse but in fish, embryonic stem cell lines with chimerical competence are few and hence studies on knockout in fish is difficult. Pluripotent embryonic stem cell-like cell lines have been reported in several teleosts including zebrafish (Fan et al., 2004), medaka (Hong and Schartl, 2007), sea perch (Chen et al., 2007), gilthead sea bream (Bejar et al., 2002), red sea bream (Chen et al., 2003), Japanese flounder (Chen et al., 2004), turbot (Chen et al., 2005), and Asian sea bass

(Parameswaran et al., 2007). Though the *in vivo* germ line competence of these cell lines are not yet confirmed, regulating the generation, maintenance of stem cells and inducing them to differentiate in the correct orientation has great potential for novel reproductive containment techniques. In **gene knock down**, the targeted gene is not destroyed or disrupted as in a knockout experiment, but its expression is severely diminished in gene "knockdown" strategy. Commonly possible methods are RNA interference (RNAi), ribozyme and antisense.

Inactivation targets to induce sterility

In fish, the hormonal cascade controlling gonadal development and gametogenesis involves the synthesis of gonadotropin-releasing hormone (GnRH) from the hypothalamus which stimulates the release of gonadotropin (GtH), namely follicle stimulating hormone (FSH) and luteinizing hormone (LH) from pituitary gland, which in turn regulates gonadal differentiation and maturation by the production of sex steroids. GnRH is a multifunctional peptide and usually two or three forms (GnRH1, GnRH2 and GnRH3) are present in the brain of vertebrates including fish. Targeted removal of GnRH subunits regulating gonadotropin release would result in sterile broodstock, the fertility of which could be recovered by treatments of sterile with exogenous GnRH forms and this forms the basis of transgenic sterilization. In tilapia, both GnRH1 (seabream GnRH) and GnRH3 (salmon GnRH) regulates gonadotropin release forming targets for knock down to induce sterility. However, the success rate has been reported to be higher with 'sea bream type'. Compounds like gamma-aminobutyric acid (GABA), GABA receptor agonists, or GABA receptor antagonists have been tried to disrupt the establishment of the gonadotropin-releasing hormone system during early development to inhibit sexual maturity.

In addition to GnRH, targeting the GtHs also induces sterility. FSH and LH in teleosts are heterodimeric glycoproteins, each consisting of two subunits, the α-and β-subunits. While the α-subunits are identical in FSH and LH, the difference in β-subunits confers the specificity of the hormones with FSH controlling gonadal recrudescence and growth whereas LH predominantly regulating gonadal development and maturation. The LH β-subunit is the preferred target for gene inactivation to produce sterility, where the fertility could be recovered by hormonal administration. However, 100 per cent success with sterilization methods has not been achieved yet. Sterility in some cases might also adversely affect performance of transgenic fish.

In recent years, molecular factors regulating reproduction like kisspeptin and its receptor (*GPR54*) (Parhar et al., 2004) which positively regulates GnRH, and gonadotropin-inhibiting hormone - GnIH (Tsutsui et al., 2007) which negatively regulates GnRH have been identified. GtH release can be inhibited by dopamine (Trudeau, 1997) and stimulated by gamma-aminobutyric acid (Popesku et al., 2008) or by neuropeptide Y (Li et al., 2012). Studies to explore their function to regulate reproduction mechanism and methods to suppress these signalling molecules can lead to novel methods to induce sterility in fish.

Common possible transgenic inactivation approaches to knock down the expression of targeted gene for sterility are RNA interference (RNAi), ribozyme and antisense.

Gene knock down via RNAi:

This was first reported by Fire et al. (1998) in *Caenorhabditis elegans*, where introduction of specific double stranded RNA could inhibit the activity of genes containing homologous sequences. This interference is post transcriptional and appears to be highly effective in *C. elegans* and Drosophila (Misquitta and Paterson, 1999). The ability of dsRNA to inhibit specific gene functions in zebrafish has been reported (Zhao et al., 2001), however, attempts to use RNAi to ablate the reporter gene expression in tilapia expressing lacZ was not effective.

Gene knock down via antisense:

The technique involves inserting the DNA sequence of the target gene into a transgenic construct in reverse orientation so that a complementary mRNA transcript is transcribed which binds to the target mRNA transcript, resulting in a knockdown in the level of target gene expression. The quality and the amount of the antisense determine the efficiency of knockdown of the specific gene expression. Disadvantage of the technique includes failure to induce 100% inhibition of the target gene expression at RNA or protein level. Gene knockdown via antisense RNA is most effective only against relatively rare messenger RNA species (Branch, 1998). In fish, the first report on the use of antisense approach for reproductive sterility was in rainbow trout (*Oncorhynchus mykiss*) to antagonize the *in vivo* production of GnRH3 (Uzbekova et al., 2000) which resulted in asynchronous maturation among the antisense treated fish.

Targeted knockdown of GnRH1, GnRH3 and the LH β-subunit mRNA via antisense sequences in Nile tilapia (*Oreochromis niloticus*) was tested (Maclean et al., 2002) by developing antisense constructs using strong, constitutively-expressing promoters (β-actin, histone 3), and one using the promoter sequences from the target gene. The antisense constructs were as follows: (a) tilapia β-actin promoter/tilapia LH β-subunit gene antisense, (b) carp β-actin promoter/tilapia GnRH3 gene antisense, (c) tilapia β-actin promoter/tilapia GnRH1 gene antisense, (d) tilapia histone 3 promoter/tilapia GnRH1 gene antisense and (e) tilapia GnRH1 promoter/tilapia GnRH1 gene antisense. Preliminary results indicated a reduction in fertility in female fish expressing GnRH3 antisense sequence (construct b) when crossed with wild-type males compared to non-transgenic control females. Reduction of sterility was more in transgenic males with complete sterility in some cases (Wong and Van Eenennaam, 2008).

Gene knock-down via ribozyme

Conditional loss of gene function can be mediated at the mRNA level by ribozymes. Ribozymes are *trans*-acting antisense RNA molecules with catalytic activity capable of selectively binding (solely to complementary RNA target in ribozyme) and facilitating the cutting of specific messages at target sites (Takagi et al., 2001) thus reducing the mRNA expression. Hammerhead ribozymes, are the most widely used type of ribozymes, which cleave their targets at NHH sites where N can be any base and H can be any base except G. Ribozymes directed down-regulation or "knockdown" in the expression of targeted genes has been reported in zebrafish (Xie et al., 1997) and rainbow trout (Boonanuntanasarn et al., 2005). Disadvantage in using ribozymes is the determination of the best target site on the messenger to knockdown. In tilapia, the only target sequence reported to be sensitive to naturally occurring hammerhead ribozyme activities is GUC (guanine, uridine, cytosine triplet). Ribozymes against the five GUC codons in the mRNA of GnRH1 of tilapia has been tested (Maclean et al., 2002).

If sterility is considered in the strict sense, only triploidy and transgenesis are the available approaches. However, triploidy in tilapia is "leaky" process also resulting in females. Transgenic sterilization is difficult and many of them are still under research. Ascertaining the efficiency of the use, antisense, ribozyme or RNAi approach for sterility is not possible as many of the studies are still in the preliminary stage and with few peer-reviewed literatures available on the same. More works are needed to determine the possibility of using it for aquaculture. As an

alternative, production of monosex population can be considered to prevent the prolific breeding. Monosex population induced by hormones, hybridization and temperature can be considered.

Hormones induced sterility

Hormonal sex reversal is widely used for producing monomale population of tilapia. Male steroid hormones or their analogues are generally incorporated either with larval feeds and administered to larvae with undifferentiated gonadal tissue for sufficient time to enable sex reversal and produces individuals that grow and function reproductively as males. The procedure must be initiated before the primal gonadal tissue starts differentiating into ovarian tissue. It can also be done through immersion of fertilized eggs or sac fry. Using this technique farms can produce populations of greater than 90% male fish. However, due to the possible impact on health and as potential environmental contaminant, the use of hormones for sex reversal has been under criticism. Studies on the impact of 17-alpha methyltestosterone (MT) incorporated in feed for sex reversal in tilapia, revealed considerable leakage of MT into pond water and sediments (Contreras-Sanchez et al., 2001). Hormone (or hormone metabolites) leaching from uneaten food can induce sex reversal in exposed untargeted organisms resulting in biased sex ratios.

Mono-sex tilapia through hybridization: Hybrid vigour was the main reason for early hybridization works with tilapia. However, unexpected all or nearly all-male progeny was obtained from interspecific crosses between certain species of tilapia. Monosex hybrids result from unique differences in the genetic basis of sex determination in a number of closely related tilapia species. Crosses yielding 100% male offspring are:

Male parent		Female parent
Oreochromis aurea /O. hornorum	x	*Oreochromis niloticus*
Oreochromis aurea /O. hornorum	x	*Oreochromis mossambicus*

Disadvantage of the technique is the difficulties in maintaining pure lines of two species for a long period of time.

Temperature induced monosex population: Several studies have reported that elevated temperature favours masculinity in *O. niloticus* and other species. Though the mechanism is not clear, a possible explanation for the influence of temperature on the sex differentiation is the

"brain sexualization" hypothesis suggested by many researchers. The high temperatures down regulate the activities of brain aromatase (*cyp19b*), the terminal enzyme in the estrogen biosynthetic pathway that catalyzes the formation of estrogen from androgen (Chang et al., 2005). Another hypothesis regarding the temperature influence on the early sexual differentiation is its direct effect on the future gonadal cells either somatic and/or germinal germ cells (Rougeot et al., 2008). Further investigations are necessary to explain the heat shock induced abnormal sex ration in tilapia.

YY male or Genetically male tilapia (GMT) technology

The YY male or GMT technology involves a genetic breeding programme combining the hormone feminization (resulting in genetic XY female production) followed by mating with normal males and progeny testing, to generate novel 'YY' male genotypes (Genetically male tilapia). Males from this batch when mated with female tilapias produce only male offsprings. The "YY male technology" provides a robust and reliable solution to the serious problem of early sexual maturation, unwanted reproduction and overpopulation in tilapia culture. GMT have advantage over sex reversed tilapia in providing higher yield, survival, better food conversion ratio and also of GMT are higher than sex reversed tilapia and also have the uniform size, having more uniform harvest size distribution. Disadvantage of the techniques includes the excess time consumed and involving labour intensive protocols.

Trojan Y Chromosome – The Trojan Y chromosome strategy makes use of a genetically engineered female fish with multiple Y chromosomes. A female fish with two Y chromosomes (Trojan Y) in the target population mating with males of the target population would result in the production of all male progeny, half of which are super males (males with two Y chromosomes, making them sterile) (Gutierrez and Teem, 2006). Models indicate that for fish species that mature and reproduce once a year, the timeframe for extinction is about 70 years if the Trojan Y fish is stocked at 1.66% of the total population annually (Teem et al. 2013).

Daughterless Gene – Daughterless gene technology is a form of sex ratio distortion, where a transgene disrupts a key step in sexual development (i.e., expression of aromatase enzyme) to produce all-male offspring (Thresher and Bax 2003; Thresher 2008). The transgene is inheritable

to future generations (Thresher and Bax 2003) and progressively skews the population sex ratio resulting in decline of reproductive output of the population (Burt 2003).

Conclusion

Obtaining 100% sterility is a great challenge in aquaculture due to the strong selection pressure for the reversion to fertility (Brunner et al., 2007). Triploidy in tilapia is difficult and not preferred due to the possible all-female progeny. Transgenic approaches are still in the experimental stage and not available for commercial application. However, gene knock down approaches may represent the most reliable method to guarantee the reproductive containment. Ablating the genes responsible in reproduction such as GnRH or LH and the possibility to restore fertility by exogenous hormone administration is an appealing option for inducing sterility. The Trojan Y chromosome and the daughterless gene strategy reduce the number of females in a target population, ultimately leading to local extinction of the population. While many of these approaches are feasible, the assurance of sterility by these complex methods in aquaculture is difficult. More research to provide science-based estimates of the reproductive containment of these techniques are required. Monosex male population production by temperature and hormone induced methods are alternatives. Hormone induced sex reversal is the most popular method, though 100% male progeny cannot be assured. However, considering the economics and ease of operation and also percentage of success, hormone induced sex reversal appears more feasible.

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