African Journal of Biotechnology Vol. 12(36), pp. 5496-5502, 4 September, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4119
ISSN 1684-5315 ©2013 Academic Journals

Review

Male tilapia production techniques: A mini-review

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Accepted 4 June, 2012

Tilapia culture has been growing over the past decades as an excellent source of high-quality protein. Some of the Tilapia´s advantages are the ability to breed and produce new generations rapidly, tolerate shallow and turbid waters, resist a high level of disease and be flexible for culture under many different farming systems. These characteristics are the main reasons for its commercial success. However, one of them contributes to the major drawback of pond culture: the high level of uncontrolled reproduction that may occur in grow-out ponds. Uncontrolled reproduction yields to stunted growth and unmarketable fish due to offspring competing with the initial stock for food, besides other problems like less dissolved oxygen, greater release of ammonia and feces, heterogeneous sizes and overpopulation stress. Monosex production has been preferred in order to deal with these issues. Males are preferred because they grow almost twice as fast as the females. This paper reviews monosex male production techniques and their results, comprising environment manipulation, hybridization, sex reversal and genetic manipulation. The choice of a particular technique would depend on the legislation of each country. This review's should help to select the appropriate technique depending on the market target and the commercial technology available.

Key words: Monosex production, hybridization, sex reversal, environmental and genetic sex determination.

INTRODUCTION

Mainly taking the form of fish farming, aquaculture has skyrocketed in the past three decades. It is growing at 9% annually and is projected to contribute 41% (53.6 million tonnes) of the world's fish production by 2020 (Krishen et al., 2009). Today, low-income food-deficit countries, mostly in Asia, account for nearly 85% of the world's aquaculture production. Scientists began their research by focusing on Nile Tilapia because of its ability to breed and produce new generations rapidly, its tolerance for shallow and turbid waters, its high level of disease resistance and its flexibility for culture under many different farming systems (Yosef, 2009; Soto-Zarazúa et al., 2010a). The major drawback of pond culture is the high level of uncontrolled reproduction that may occur in grow-out ponds. Monosex culture is one the basic methods of controlling Tilapia populations that have been carried out in some countries for aquaculture purposes. This technique includes manual separation of sexes, environmental manipulation, hybridization, hormone augmentation (sex reversal) and genetic manipulation methods such as androgenesis, gynogenesis, polyploidy and transgenesis. None of these methods is consistently 100% effective, and thus a combination of methods is suggested.

Males are preferred because they grow almost twice as fast as females, which may be caused by a sex-specific physiological growth capacity, female mouth-brooding or the more aggressive feeding behavior of males. Expected survival for all-male culture is 90% or greater. A disadvantage of male monosex culture is that fingerlings have to be grown until it is possible to distinguish the female and male juveniles (at least up to 50 g) and then the female juveniles are discarded. The percentage of females mistakenly included in a population of mostly male Tilapia affects the maximum attainable size of the original

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Figure 1. Tilapia sex differentiation (photo by Rob. L. Elliott).

stock in grow-out phase. The density for male monosex culture varies from 10,000 to 50,000/ha or more, at proper feeding rates. Densities of around 10,000/ha allow the fish to grow rapidly without the need for supplemental aeration. About six months are required to produce 500 g fish from 50 g fingerlings, with a growth rate of 2.5 g/day (Fortes, 2005). A sex (male or female) is heterogametic if it has two different sex chromosomes (XY) and is homogametic if it has a matching pair of sex chromosomes (XX or YY). Without external influence Tilapia sex will be determined genotypically as female if it has two X chromosomes and will be male if it has XY chromosomes. Fish have certain plasticity during sex differentiation since several functional sex phenotypes can be generated by diverse mechanisms (Baroiller and D'Cotta, 2001). Thus, we can obtain phenotypically XX sex-reversed males and YY supermales.

Male Tilapia production has an economic importance to its producers and sellers. The increase in employment in the sector outpacing world population growth and employment in traditional agriculture is a crucial source of income and livelihood for hundreds of millions of people around the world (Soto-Zarazúa et al., 2011). It could play an important role to provide food security for the general population as an excellent source of high-quality protein (FAO, 2010; Soto-Zarazúa et al., 2010b). The aim of this review was to help choose a particular technique for the male Tilapia production based on a comparison between the techniques currently available, including the traditional techniques such as manual sorting and hybridization, to high-tech techniques which modify the genetic structure of the fish to produce male Tilapia.

MANUAL SORTING

Some species of the genus Tilapia can be easily sorted into males and females. Either the colors are sufficiently differentiated to serve as reliable sex indicators or the structure of the anal papilla is used; the opening of the oviduct is distinguishable in the female and is not present in the male as shown in Figure 1. Turning the fish over, it is possible to look at the secondary sex organs (shaped like a small cone) located behind the anus. Male tilapia (left on Figure 1) has a simple papilla, while the female (right on Figure 1) have a slightly wider organ with a wide opening to allow eggs to eject during mating. To make it easier to distinguish the ovarian opening, it is possible to use a dye such as gentian violet. Using a cue tip that is dipped in a violet dye and smeared lightly over the papilla from front to back, the dye outlines the openings while improving visibility.

With experience it is possible to notice the sex of even small immature fish with speed even though human error is always present. A second check is made when the fish have grown somewhat larger and distinctive sex-coloration is more discernible. Since this technique fails if there is a single female present in the raising pond, care must be taken to ensure that there are no females left over from a previous stocking. The sexing of small Tilapias, although feasible, is tedious and not entirely reliable (Hickling, 1963). Additionally, it is stressful for the fish. While it is an easy technique, it is extremely laborious and human accuracy varies from 80 to 90%, which leads to the presence of females in the pond. Therefore, this method is rarely used (Penmann and McAndrew, 2000). Moreover, this technique may be useful in small populations, but in commercial practice their use increases the cost of skilled labour and increases the risk of human error, leading to uncontrolled reproduction.

ENVIRONMENTAL MANIPULATION FOR SEX DETERMINATION

Tilapia is a thermo-sensitive species and its male to female ratio increases with temperature and/or ovarian differentiation is induced by low temperatures. Fish show particularities in their temperature sex determination patterns since monosex populations are generally not produced at extreme temperatures, suggesting the existence of strong temperature genotype interactions. Temperature treatments must be applied at a critical sensitive period, relatively similar to the hormone sensitive period. Molecular mechanisms of thermo-sensitivity could be addressed in Tilapia species (example *Oreochromis niloticus*), where aromatase gene expression is down-regulated by masculinising temperature treatments. Furthermore, in Tilapia, the gene expression of 11β-hydroxylase (a key enzyme involved in the synthesis of 11-oxygenated androgens) does not appear to be affected by temperature treatments (Baroiller and D´Cotta, 2001).

Table 1. Hybridization to produce all-male progeny.

Crosses (female x male)	Result
O. niloticus × *O. variabilis* *O. nigra* × *O. urolepis hornorum* *O. vulcani* × *O. urolepis hornorum* *O. vulcani* ×*O. aureus*	98 to 100% (Fishelson, 1962; Pruginin, 1967; Pruginin et al., 1975; Hsiao, 1980; Hulata et al., 1983, 1993)
O. niloticus × *O. urolepis hornorum*	All-male progeny (Wohlfarth et al., 1990)
O. niloticus × *O. aureus*	All-male progeny (Wohlfarth, 1994)

Meanwhile, a strong effect of temperature on sex differentiation has been demonstrated in various Tilapia species and in a hybrid (Baroiller et al., 1995a, b; Baroiller and Clota, 1998; Desprez and Mélard, 1998; Wang and Tsai, 2000). Baroiller et al. (1995a) demonstrated that Tilapias were sensitive to temperature during the critical period of sex differentiation. It was possible to masculinise XX progenies (100% females) with elevated temperatures above 32°C, giving functional male phenotypes. High temperatures could efficiently masculinise some progenies if started around 10 days post fertilization and if applied for at least 10 days, with longer periods being just as effective (Baroiller et al., 1995a, b; Tessema et al., 2006; Wessels and Hörstgen-Schwark, 2008). However, if a treatment was applied for a 10-day period but begun at 7 days post fertilization, it had no effect on sex ratios (Baroiller et al., 1995a, b). This window for temperature sensitivity coincides with the gonad sensitivity towards other external factors, notably hormones. Like temperature, hormonal treatments or the use of aromatase inhibitors during sex differentiation can override the genetic sex determination, inversing sex and producing functional phenotypes (Nakamura, 1975; Baroiller et al., 1999; Guiguen et al., 1999).

The temperature sensitivity of Nile Tilapia during sex differentiation is not seen in all progenies and can be heritable in Nile Tilapia (Wessels and Hörstgen-Schwark, 2008). Together, these studies showed that sex in the Nile Tilapia is governed by the interactions of three components, a complex genetic sex determination system with a major determinant locus and some minor genetic factors, as well as the influence of temperature. Unfortunately most of these studies showing sensitivity to temperature are hindered because the sex determination mechanisms of most of these species have not been able to be well characterized and physiological, genetic or ecological studies cannot be carried out to better understand the component of this environmental sensitivity (Baroiller et al., 2009). Although studies are underway, this technique is not reliable due to multiple variables that need to be taken into consideration and there are other treatments that are simpler and achieve better results.

HYBRIDIZATION

Hybridization takes advantage of qualitative variances to improve genetics in Tilapia by crossing two closely related but distinct subspecies of fish. If the sex determination system is different, the hybridization between a female homogametic and a male homo-gametic produces only male offspring (Wohlfarth and Hulata, 1991; Trombka and Avtalion, 1993). Hybrids from Oreochromis with two opposing "sex chromosomes" models (XX/XY in *O. niloticus* and *O. mossambicus* and WZ/ZZ in *O. aureus* and *O. urolepis hornorum*) exemplify the complexity of sex determination model in their pure species. Wohlfarth and Hulata (1991) suggests that the genetic mechanism of sex determination in Tilapias is analogous to that in platy fish, depending on variation in both sex chromosome and autosomally carried factors. Empirical evidence for this is not available for Tilapias due to the absence of sex-linked markers. In such a complex system, the chances of producing “super males”, which generate all-male broods with any female, in either intraspecific combination, appear small. A super male Tilapia (YY) could be attained by feminizing genetic males (XY) with estrogens and then breeding them with normal males (XY), which leads to three different possibi-lities: females (XX), males (XY) and super males (YY). This strategy has various limitations to consider. To create an YY bank, several generations have to be analyzed and the technique is not 100% effective, which implies the possibility that other factors directly influence sexual determination (Green et al., 1997).

There are more than 100 tilapia species but the most prominent for aquaculture are the Nile tilapia (*O. niloticus*), the Mozambique tilapia (*O. mossambicus*), and the blue tilapia (*O. aureus*) *O. niloticus* or one of its subspecies are commonly preferred in tropical freshwater while *O. aurea* has increased cold tolerance so it is grown in subtropical freshwater. Table 1 shows some of the crosses that lead to all-male progeny and their best results reportted. Among the major constraints in producing hybrids are: maintaining the purity of brood stocks, limited fecundity of parent fish which restricts fry production and diffi-

culty in producing sufficient number of hybrid fry due to spawning incompatibilities between the parent species. In as much as not all crosses produce 100% males, the hybrids may still be subjected to manual separation of sexes or hormone augmentation. Some advantages of hybridization are that it saves time, space and feeds, but it is not a perfect solution (Fortes, 2005).

SEX REVERSAL OR HORMONE AUGMENTATION

This method can be performed by oral administration of feed incorporated with androgen and eggs or fry immersion in different concentrations of the male hormone. The principle behind this method lies on the fact that at the stage when the Tilapia larvae are said to be sexually undifferentiated (right after hatching up to about 2 weeks or up to the swim-up stage), the extent of the androgen (male hormone) and the estrogen (female hormone) present in a fish is equal. Thus, augmenting one of the hormones that is originally present in the fish will direct the fish to either male or female depending upon the hormone introduced. Accordingly, if the Tilapia larvae are fed with feeds that are incorporated with male hormone as example 17α-methyltestosterone, the fish will develop into phenotypic male physically and function as male but possess the female genotype (XX). In the same way, if a female hormone is mixed with the feed that is taken by the fish, then the fish will be directed to phenotypic female physically and functions as female, but possesses the male genotype (XY). This is commonly referred to as "sex reversal".

Different steroids have been used over the years to induce sex reversal even if 17α-methyltestosterone is the most common (Pandian and Varadaraj, 1990) for *Oreochromis mossambicus*; 17α-ethynyltestosterone (Shelton et al., 1981) with *O. aureus*; 17α-methylan-drostendiol (Varadaraj and Pandian (1987) with *O. mossambicus*; mibolerone (Torrans et al., 1988) with *O. aureus*; norethisterone acetate (Pandian and Varadaraj, 1990) with *O. mossambicus*; fluoxymesterone with *O. niloticus* (Phelps et al., 1992); trenbolone acetate with *O. aureus* (Galvez et al., 1996). Production of male tilapia through the use of androgens is very effective. Sex reversed "male" reached similar average weights as genetically male tilapia (Mair et al., 1995) and it does not require that a portion of the production be discarded as in manual selection, or that two separate stocks of fish be maintained as in hybridization (Phelps and Popma, 2000). The presence of hormone residue in adult fish has not yet been studied, thus its effect on consumers is not yet known and so his use is restricted. Hormones may also be difficult to obtain in some countries and hatchery facilities and skilled labour is required (Fortes, 2005).

Sex reversal by oral administration of feed incorporated with methyl testosterone is probably the most effective and practical method for the production of all male Tilapia, However, the technique has some limitations such as the uniform age of fish that should be used at the first feeding stage to ensure high reversal rate and less control of reversal efficiency especially when done in the natural environment where natural feed is present. Moreover, widespread use of large quantities of sex reversal hormones in hatcheries may pose a health risk to workers (Mair, 1997; López et al., 2007). This technique has achieved successful results up to 100% and feed with the male hormone is commercially available or can be prepared. One of its disadvantages though is the possibility of contaminating the water through wastewater due to non-consumed feed.

Another variant of the oral administration is the use of live bait that has been raised in an artificial environment enriched with male steroids. This technique has been used with Nile Tilapia fry and has obtained levels of masculinisation up to 99% (Contreras-Sánchez et al., 2004). Sex reversal by the immersion technique is achieved by immersing the eggs in different concen-trations of 17-αmethyl testosterone exposed for different times. The mechanism of action of the immersion technique is that the hormone is absorbed through passive diffusion across the lipid membrane of the eggs. During the embryonic development, gonadal differen-tiation can be affected by the administration of steroid sex hormone (Jobling, 1995) in the holding water. Strussman and Nakamura (2003) pointed out that the mechanism of action of exogenous steroids during sex differentiation is not sufficiently clear. Cagauan et al. (2004) evaluated sex reversal of Nile Tilapia *O. niloticus* by immersing the eggs in different concentrations of 17-αmethyl testosterone. Highest percent male of 91% was attained at 800 µg L-1hormone concentration at 96-h immersion time comparable with the 88 to 89% in 400 to 600 µg L-1hormone concentration at the same immersion time. Sex reversal by egg immersion may lessen the duration of treatment and lower the cost of hormone used relative to the traditional technique of sex reversal by oral admini-stration. However, this technique presents conflicting results possibly due to the rapid early development that limits the window of opportunity (Contreras-Sánchez, 2001) and these results are lower than those obtained with the immersion of fry (Gale et al., 1996; Fitzpatrick et al., 1999; López et al., 2007) and with the use of feed hormone (Manosrioi et al., 2004; Jiménez and Arredondo, 2000; Torres and Marquez, 2006).

Similarly to egg immersion, Gale et al. (1996) demonstrated that fry immersion for just three hours in 17α-methyldihydrotestosterone on two days resulted in masculinisation of Nile Tilapia with a success rate of 93%. It is therefore important to consider the number, time and duration of the immersion. Fitzpatrick et al. (1999) reported 90% of males obtained using treatments with 0.5 mg/L methyl testosterone in two immersions, with duration of 2 h each one, between day 10 and 13 after fertilization. López et al. (2007) also obtained 92.6% of males

using 1.8 mg methyl testosterone/L, with an immersion of 4 h, between 10 and 14 days after fertilization. This alternative technique of administering the sex reversal hormone may be of great help in hatcheries employing artificial incubation because of the greater control of sex reversal and lower risk to health of workers. On the other hand, these results are worse than using hormone on feed and this could yield to an uncontrolled reproduction with all the problems earlier mentioned.

GENETIC SEX DETERMINATION

The study of the influence of gene expression in performance traits like growth rate, feed conversion efficiency, body conformation, disease resistance and sex determination is an opportunity to meet the demands of fish production while ensuring profitability (Liu, 2007). Genetics is a field of study in constant development. Functional genomics is an emerging discipline that studies the effects of gene expression, genomic controls and transcriptional profiles and it is being applied to cultured fish species. The challenge is to determine exactly what each gene does in terms of the development and physiological functioning of the organism (Murphy, 2002).

A specific trait is the result of many genes working together and some genes involved in sex determination have already been found. Cyp19 is a gene that encodes P450 aromatase, the key enzyme catalyzing the conversion of androgens into estrogens (Tong and Chung, 2003). Estrogens play a crucial role in ovarian differentiation of non-mammalian vertebrates, including fish. There are results that suggest that Foxl2 affects the ovarian differentiation of the Nile tilapia by regulating aromatase expression and possibly the entire steroidogenic pathway. Foxl2 and Cyp19a1 are co-localized spatially and temporally in the female making them the earliest known markers for ovarian sex differentiation. Foxl2 can be considered as the pro-ovary, but anti-testis gene because the disruption of Foxl2 could stimulate the XX tilapia to reverse its sex from female to male partially or completely (Wang et al., 2008). Ijiri et al. (2007) studied the gonadal expression of 17 genes thought to be associated with gonadal sex differentiation in vertebrates, confirmed the role of FoxI2 and Cyp19a1a in ovarian differentiation and concluded that DMRT1 have a crucial role in testicular differentiation.

Kobayashi et al. (2008) also examined the expression profiles of tDMRT1 and Sox9a during gonadal sex differentiation and hormone-induced sex reversal. This study indicated that tDMRT1 is expressed in the germ-cell-surrounding cells and medullary-cell-mass cells, which differentiate into the efferent duct during testicular differentiation, irrespective of the genetic sex and suggest that Sox9a is not involved in the differentiation of the intratesticular efferent duct. They concluded that DMRT1 is a superior molecular marker for somatic testicular differentiation, and that DMRT1 and Sox9a play different roles in the testicular differentiation of tilapia. In addition, Wang et al. (2010) clarified the role of DMRT1 in their study by showing that it suppresses the female pathway by repressing aromatase gene transcription and estrogen production in the gonads of tilapia and possibly other vertebrates. Although, presently, it is not technically feasible to produce all male populations of tilapia by suppressing or activating genes, the knowledge needed to do this is being developed. Kuramochi et al. (2011) induced male-specific nest-building behavior in 70% of females treated with 11-ketotestosterone (11-KT) or methyl testosterone. This treatment increased the number of gonadotropin-releasing hormone type III (GnRH3) neurons, which presents sexual dimorphism in females to a level similar to that in males. These results indicate androgen-dependent regulation of GnRH3 neurons and nest-building behavior, suggesting that GnRH3 is importantly involved in sex reversal of male-specific reproductive behavior. However, the endocrine mechanism underlying sex reversal of reproductive behaviours remains unsolved.

CONCLUSIONS

In Tilapia culture, the desired goal efficiency rate for each technique to produce male Tilapia is between 98 and 100%. The technique employed in the majority of the developing countries is sex reversal because of its easy employment and high rate of success. Among the different techniques used for sex reversal (oral administration, egg immersion and fry immersion) the one that shows the best results is the fry immersion, with a higher success rate and without the risk to employees due to contact with the hormone during the preparation of the feed. Some other advantages of these techniques are: it takes less period of time, less water quality and quantity is needed, less hormone consumption and no influences from fish feeding behavior. Due to various environmental issues related to hormone use such as the possible effects of treatment residues on water quality and biodiversity, the countries with legislation against the use of hormones tend to use nonhazardous, consumer and environment-friendly methods to obtain all-male populations through genetic control. These methods, even if approved, are out of the common aqua farmer's technical capabilities.

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