International journal of agricultural sciences and veterinary medicine

ISSN: 2320-3730

Vol-3 Issue-01 Feb 2014

The 17-alpha-methyl testosterone hormone's impact on commercially important tilapia (Oreochromis niloticus) and how its breeding habitat has changed over time

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Abstract:

The fate of hormone 17 α Methyl testosterone in the flesh of merchantable fish (greater than or equal to 250 g) and in the tilapia culture medium of the Senegal River Valley (Oreochromis niloticus) is studied in this publication. Fish fry of this species are fed for 28 days with a food supplemented with 17 α-methyl testosterone in the following proportions (30, 60 and 90 mg MT/ kg feed). The fish are then given a non-hormonal diet for another five months. The evaluation of hormone residues on fish flesh by ELISA was done in the lab of Prof. Grant VANDENBERG in Quebec, Canada by ELISA that of farmed water at LACOMEV of Inter States of Sciences and Veterinary Medicine School of Dakar by HPLC. The results of these analyzes indicate that low levels of MT in the flesh of market-sized fish 60 days after cessation of the supply of hormone-free feed to fish, and levels of MT concentrations in samples below detection limit (1.3 µg I mL).

Keywords: Oreochromis niloticus, 17α, methyltestosterone, parts, enzyme-linked immunosorbent assay, high-performance liquid chromatography.

INTRODUCTION

Modern tilapia fish farming requires, among other things, the use of high-quality fingerlings and high- performance, producer-accessible feeds (Aqua Fish, 2014). Although these inputs are used by most farmers in sub-Saharan Africa, they are of low quality (Anonymous, 2010, UEMOA, 2013). Conversely, mixed-sex fingerlings are reduced in quality and production due to the use of feed that does not function well. marine life. In fact, mixing male and female individuals in the same farm leads to significant differences in size of fish produced on tilapia fish farms. The presence of females leads to uncontrolled reproduction, over-recruitment of juveniles, competition for food and space, and stunted natural growth of the fish stock. That development is impeded by the need to procreate. As a result, the stock cannot reach market-size. In this instance, the biomass of recruits may even surpass that of adults, making up as much as 70% of the total biomass of fish caught. Studies have shown that male tilapias grow about twice as fast as females (Brawand et al, 2014). Therefore, the use of the 17α methyl testosterone hormone (17 α MET) in modern tilapia farms, enables the transformation of females into males. This practice is common in modern fish farming of this species worldwide (Bolivar et al, 2004). It provides both a large number of male fingerlings (up to 90% of the fingerlings treated with this hormone), rapid growth, and fish of the same size (Smith and Phelps, 1997; Hussain et al., 2005). The use of hormones in animal production is banned in many countries, especially in many European countries and in

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ISSN: 2320-3730

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the United States. Potential hazards to public health, the environment and biodiversity are often cited as justification. As stated by the FAO in 2011.Research for non-hormonal sex control based on genetic and / or environmental approaches is carried out in research laboratories for mass production of male fingerlings. Recently, those studies have led to the development of YY male-to-male progeny production technology that is untreated by hormones (Marjanovic et al, 2016). Raising tilapia fingerling's conditioning temperature makes it possible to invert the sex (females into males). This fact has led scientists of the AQUATROP group from the Intrepid unit of CIRAD (Agricultural Research Center for International Development) to initiate research on markers related to sex and thermo-sensitivity. This study has enabled the identification and isolation of molecular markers for early phenotypic sexing in tilapia. In a near future, the results of this study will enable the marketing of kits for the homogenous production of YY- tilapia fingerlings for genetic sex control and / or the selection of thermosensitive progeny breeders for hormone-free sex control by temperature. While expecting these kits to be available on the market, the masculinizing hormone, 17 α MET, continues to be widely used in tilapia farms around the world, for example in Africa by two of the largest fish-farming countries namely Egypt and Nigeria . This scientific article examines the impact of this hormone on market-size fish and on the fish farming environment.

MATERIAL AND METHODS

Equipment

Tilapia *O.niloticus* belongs to the family of cichlids. This species has been thoroughly studied to define its breeding pattern (Beveridge et al., 2000, Bolivar et al., 2004 Costa-Pierce et al., 1997, Costa-Pierce et al., 2000, Falk et al., 1996, Fishelson et al., 1983, Fitzsimmons et al., 2000, Fitzsimmons, 1997 Lazard, and J. Legendre , M., 1996, Pullin and Lowe-McConnell, 1982, Pullin et al., 1988, Watanabe et al., 2002)

That species is grown in more than 100 countries with a production reaching nearly 4.3 million tons over a world production of farmed fish estimated at 59.8 million tons (FAO, 2006), making it the second major species in global aquaculture, after carp.

We harvested spawners used in this study from the Senegal River delta to obtain market-sized tilapia (Fig. 1). We carried out the breeding at the Hann Bel-Air ISRA / IRD fish farm in Dakar, as part of the implementation of the Adaptation, Transfer and Dissemination of Tilapia male fingerlings Mass Production Technologies project (*Oreochromis niloticus*).This project was funded by the National Agricultural Agrofood Research Fund (FNRAA) of WAAPP2 between 2016 and 2017. Industrial food branded "RANNAN FISH" packaged in 20 kg bags was used to feed the fish. That meal is composed of fishmeal, fish oil, wheat flour, rice bran, soy flour, corn flour. It contains carotenoids, vitamins 45 B1, B2, C and E, Calcium, Magnesium, Biotin and other trace elements. The protein and lipid contents of the food are listed in Table 1.

The daily intake (D.I.) is calculated based on the following formula (FAO, 2002), D.I = (A.W x F.R. / 100) x Number of individuals

A.W: average weight (g); F.R: feed rate.

The feed rate and frequency at the beginning of the experiment are the same for the four batches, but this rate varies according to the average weight of the larvae. It accounts for 14% of biomass for the larvae, 11% for 2 gfingerlings, 10% for 5 g fingerlings and 6% for individuals of about 40 g (spawner). For the latter group, thefeed is in pellet form with a daily intake of 29 g. For the average weight of a freshly hatched larva, is 10 mg (this Fig. is confirmed by FAO, 2002). The food ration is given twice a day over a period of 28 days and the remainder

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ISSN: 2320-3730

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is recovered by siphoning after measuring the temperature.

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Methods

Residues of the hormone 17 α MET on market-sized fish and the breeding environment were determined using ELISA kits, which are more accessible while allowing the analysis of several samples at a time.

Hormonal treatment of fingerlings

Three diets have been formulated. Their difference lies in the amount of methyl testosterone mixed with ethanol (0.5 l):

*diet 1: food without hormone;

*diet 2: 30 mg of MT after dilution + 0.5 l of ethanol;

*diet 3: 60 mg of MT after dilution + 0.5 l of ethanol (the reference formula);

*diet 4: 90 mg of MT after dilution + 0.5 l of ethanol.

Thus, these different doses 30, 60 and 90 mg of 17 α-methyl testosterone are derived from the 10 ml taken from each of the initial solutions, obtained after a dilution of respectively 0.5; 1 and 1.5 g of MT in 166 ml of ethanol for each dose. The solution obtained is then diluted in 490 ml of 95% ethanol and mixed directly in 1 kg of food.Ethanol is then evaporated by drying while avoiding exposure to the sun as androgens break down when exposed to sunlight or high temperatures. These operations are performed with gloves and a mask to prevent direct contact with the hormone.

Searching for 17 MET residues in the flesh of market-size fish

In order to monitor the residues of 17 α MET in tilapia flesh, a random sampling (post-treatment) of a minimum of 5 fish (n = 5) per aquarium was carried out every 15 days: D1 (end of treatment) to D150. The samples (selected fish) are sacrificed with a part of their flesh being crushed and stored in the freezer at -40°C. These tissue fragments are removed from the freezer for homogenization in an acetonitrile solution (1: 1, weight: volume) and then crushed with a BASIC ULTRA-TURAX® T10 (Cravedi et al, 1989, Chu et al. al, 2006) in order to extract the MET if present in the flesh of the fish. The resulting homogenate is placed in a centrifuge for 15 minutes at a 4 ° C temperature (Morales and Moyano, 2010). After centrifugation, the supernatant is gently recovered to be placed in Eppendorf tubes and stored in the freezer at -20 ° C, waiting for analysis through the ELISA method at the Grant Vandenberg Laboratory of the Faculty of Science Agriculture and Food in Laval University of Quebec-Canada.

The ELISA (Enzyme Linked Immuno Sorbent Assay) test is based on the competition between a free molecule present in the homogenate and a complex of the same molecule bound to an enzyme for binding to a specific limited number of the molecule to be assayed. The substrate of the enzyme is added to the analyzed sample, which degrades to release a colored metabolite (in yellow) after an incubation phase. The absorbance of the sample is determined with a spectrophotometer using a plate reader (VARIOSKAN) at a given wavelength (450 nm). Hormone analysis was performed using the My BioSource ® ELISA kit (MBS7224868).

Residue research of 17 α MET in farmed water

Forty-eight (48) samples of tilapia farming water were collected from the eight (8) treated and control aquariums, then put into 200 ml PET bottles. Samples were collected every week from D7 (during treatment) to D50. The water samples were well frozen until the end of the breeding.

At the time of sample processing, a standard stock solution of 1 mg / ml in methanol is prepared. Intermediate standard solutions with a concentration level between 5 and 30 µg / ml are derived from the standard solution and stored in a freezer. The stock standard solution is stored

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in the freezer for a maximum of three weeks. The supplemented solutions are prepared with the same concentration level as the intermediate standard solutions, from the stock standard solution.

Detection of the MET residues in farming water is then carried out by defrosting. The cartridges containing the water samples are then placed in a centrifuge (at 4000 rounds per minute (rpm) with a flow rate of 2 ml/min).We collect 2 ml from the supernatant in each sample. Then comes the washing stage with 2 ml of ultrapure water (UPW) and the cartridge is then dried before elution with 2 ml of acetonitrile. The eluates are collected in vials for reading in the HPLC chain.

Data entry and processing

Data collected on fish flesh and water used for fish farming were captured on EXCEL (Microsoft Excel® 2013). We created new variables, allowing for descriptive statistical analysis (calculation of mean, standard deviation, confidence intervals and their limits, the minimum and the maximum). Chi-Square test and variance analyses (ANOVA) are then performed using the Rx64 3.1.3 software to determine the ratio of treated batches to control's.

Results

MET Residue Analyzes in the flesh of market-size Fish

We identified a negative correlation between the 450 nm DO, the concentration of the hormone α MET 17 in theflesh of market-size fish and the

Discussions

Evaluation of residues of 17α methyl testosterone in the flesh of market–sizefish

The residue content of the hormone MET 17α in tilapia flesh after treatment declines rapidly from a maximum concentration level of 89.53 ng / liter (Batch 1); 92.59 ng / liter (Batch 2) and 98.97 ng / liter (Batch3) to undetectable concentration levels 5 months after stopping the hormonal treatment. The samples of tilapia flesh

breeding environment. Thus, variations in 17α-MET concentration (x) explain nearly 97% of variations in DO concentration (y). This leads to the following linear relationship: y = -0.1755 x
+ 1.7227 (Fig. 2).

The results expressed in FIG. 3 illustrate the concentration values detected by the ELISA test in treated fish after deduction of the physiological MET concentrations values in the corresponding controls. From the eighty-eight (88) samples that were analyzed, it appears from this graph (Fig. 3) that the average concentrations of MET are very high (89.53ng / ml; 92.59ng / ml; 97ng / ml respectively in batches 1, 2 and 3) from the first sampling (one day after halting the treatment). This concentration remains statistically unchanged ($P < 0.05$) for the first fifteen
(15) days. Then, it decreases significantly ($P < 0.05$) and reaches 0.28 ng / ml; 1.14 ng / ml and 2.24 ng / ml respectively in batches 1, 2 and 3 one month after stopping the hormonal treatment. It remains statistically ($P < 0.05$) unchanged until the end of the experiment (5 months post treatment). Two months after the end of the treatment, the MET is no longer detectable by the ELISA test (<LOD).

Analysis of water samples

In the forty-eight (48) water samples analyzed by HPLC at the LACOMEV laboratory of the EISMV in Dakar, 17α-methyl testosterone was not detected at all. The samples were all negative (Fig. 4).

were surely negative after a breeding period of 6 months. These results are consistent with those obtained by Dergal et al. (2015) and Rizkalla et al. (2004) who reported that monosex tilapia *Oreochromis niloticus* samples did not differ from control (untreated) samples after 5 months of breeding and concentrations were below the detectable level (0.09 µg / kg and 3 mg / kg). After thirty years of study, it became obvious that MET clearance is very fast in different species of fish. These findings are consistent

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with results obtained by Fagerlund and Dye (1979) and Johnstone et al. (1983) who noted the disappearance of more than 95% of MET traces, in the viscera of *Oncorhynchus kisutch* salmon, tilapia *Oreochromis mossambicus*, Salmo gairdneri trout fed with food containing MET, 50 hours after the feeding. Goudie et al. (1986) reported that juvenile tilapia *Oreochromis aureus* treated with radioactive MET for 30 days displayed sign of rapid depletion of radioactivity in the muscle. Likewise , Gulla et al. (2007) detected no hormone residues in the flesh of trout fed with 30 mg of 17 MET α per kg of fish feed for 35 days. Thus, Cravedi et al. (1993) confirmed that 17α MET hormone is rapidly transformed and excreted through feces and gills in *Oncorhynchus mykiss* trout based on radioactivity experiments to assess the pharmacokinetics of the hormone and its metabolites. Recently, Dabrowski et al. (2004) demonstrated a rapid fall in MET concentration from 3 µg / kg to 0.5 µg / kg after a waiting period of 8 weeks. This latest result was confirmed in the current study, with hormone concentration falling below the threshold of the ELISA test after waiting for two months. Chu et al. (2006) demonstrated that the concentration of MET in the tilapia fillets *Oreochromis sp*., *Oncorhynchus mykiss* trout and Salmo salar Atlantic salmon, fed with medicated feed at 30 mg / kg for 28 days, was 0.13. mg / kg after a waiting period of 14 days and passed below the threshold of quantification (0.04 ug / kg per LC / MS) 3 weeks after stopping the hormonal treatment. Guerrero (2008) demonstrated that the concentrations of the hormone MET 17α fell to a normal level, 5 days after the interruption of MET supply. The assessment of the depletion regression of radioactivity revealed that the hormone residues present no side effects for public health. Concentrations of 17α MET and its metabolites intilapia fall below the threshold of 100 mg / kg after 6 to 50 days (Teichert et al, 2001). The same authors in 2001, analyzing tilapia fillets, estimated that there is very small MET 17α concentration in a portion of edible tissue (57 to 143 g of fillet) and is expressed in ppt (parts per trillion), i.e 1.2 to 3.4 ng of the hormone after a waiting period of 21 days. In the same vein, Vick and Hayton (2001) demonstrated through a pharmacokinetic study of MET in rainbow trout (*Oncorhynchus mykiss*) that its oral bioavailability would be 70% and that its half-life would be 57 hours. The results of this study buttress the fact of the hormone depletes rapidly. They also highlight the effectiveness of the food containing 17α MET. Molecular biology analyses also revealed that methyl testosterone could induce DNA fragmentation and molecular genetic variability (using PCR) in tilapia testicular tissues (*Oreochromis niloticus*). These genetic modifications were more important during the first fourmonths of the study in treated tilapia. In addition, histological examinations revealed no structural changes, or any evidence of hormone accumulation in sections of treated and untreated fish muscle throughout the experiment (8 months of study) (Wagdy et al, 2011).

Regarding residues of hormone 17α MET in farming water, increasing attention is being paid to the impact of compound pharmaceutical actives, including hormones released in the environment via discharges of wastewater (Heberer, 2002). Although it has been established that the use of the hormone does not result in the accumulation of residues in the tissues of treated fish, their release into the environment and consumer reaction are still a matter of concern. As it is very common to overfeed fingerlings, the residual 17α MET hormone in

foods containing that hormone from the masculinization process can accumulate in the ponds and be released into the receiving water body when water from the pond is discharged or when the ponds are cleaned.

Evaluation of 17α methyl testosterone residues in farming water

In this study, water samples released from the eight aquariums were relatively harmless to the environment. Forty-eight (48) water samples taken during and after the hormonal treatment were analyzed by HPLC and 17α, MET hormone was not detected at all with values below the limit of determination (1,3µg / ml).

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These data can be explained by the fact that fingerlings consumed almost all the food distributed per day. In addition, the water was recycled through mechanical filters. These results are consistent with those of Dergal (2015) with the ELISA test revealing no trace of hormone residues in farming water. In six (6) out of twenty-six tilapia water samples (26) collected by Barbosa et al. (2013) and analyzed by HPLC, the 17α-methyl testosterone was at a level above the limit of quantification i.e. 59.9> 2000 µg / liter. Phelps et al.(1999) and Contreras-Sanchez et al. (2001) demonstrated that, after a conventional treatment, tilapia juveniles treated with the MET 17α at a 60 mg / kg dose for 28 days, displayed an average concentration level of 4.46 µg / g in water, 9.7 µg / g in soil and 4.6 µg / g in sediment 20 days after treatment. These values are minimal and the majority of fungi and bacteria can metabolize this steroid. Green and Teichert-Coddington (2000) demonstrated that 17α MET hormone in water israpidly diluted due to photo oxidation and bacterial degradation. White et al. (2006) reported that hormone levels decrease following a combination of photolysis, microbial degradation, plant uptake, soil uptake or sequestration. Recently, Contreras-Sanchez (2010) reported that hormone 17α MET in sediments can be quickly cleaned from the system using UV-type sterilizers, sunlight or activated carbon. Concentrations were below 5 ng
/ ml in water after 48 hours. In the same year (2010), in the course of a laboratory experiment, the same author demonstrated that bacteria *Bacillus cereus* and *Pseudomonas fluorescens* degrade 99% of the hormone in aquarium water after 20 days of treatment. In addition, it only takes 16 days for Pseudomonas aeruginosa to degrade 97% of the hormone. Homklin et al. (2011, 2012) who demonstrated that the majority of bacteria could biodegrade steroids have confirmed this.

On the other hand, Fitzpatrick et al. (2000) reported that MET persisted for more than 8 weeks in the environment. Previous findings (Abucay et al., Abucay and Mair, 1997) revealed that the persistence of 17α MET in the environment could induce an accidental sexual inversion. This could affect other tilapia fingerlings which were not treated in neighboring fish farms. Hulack et al. (2008) on a common carp farm (*Cyprinus carpio* L.) reported the same finding. In addition, hormone 17α MET can be converted and metabolized to estrogen andinduce feminization of fingerling (Rinchard et al., 1999). But it should be noted that in water steroid hormones are rapidly absorbed in sediments, or are reduced to inorganic compounds via mineralization. Hormone concentrations were significantly reduced, from 166 to 7 ng per liter for 17α MET hormone following filtration through a gravel and sand filter (Shore and Semesh, 2003).

Conclusion

This study on the evolution of 17α MET on tilapia farming environment and on market-size fish (slightly more than 200 g) reveals a lack of residues on fingerlings with different levels of hormone concentration per kilogram of feed (30, 60, 90 mg)

No trace of the hormone was detected in the water samples used for fish farming in the 08 aquariums used forthe study. Residues of the hormone MET 17α in tilapia flesh after treatment declines rapidly from a maximum concentration level of 89.53 ng / liter (batch 1); 92.59 ng / liter (batch 2) and 98.97 ng / liter (batch 3) up to undetectable concentration levels 5 months after halting the hormonal treatment. These results of the analyses are encouraging but we should carry out further analyses such as HPLC associated with mass spectrometry. We should also replace the ELISA test by the so-called ion chromatography method.

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ISSN: 2320-3730

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