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Monitoring of 17α-methyltestosterone residues in tilapia's (*Oreochromis niloticus*) flesh and experimental water after its sex reversal

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Abstract

Tilapias are sexually reversed by hormonal treatment with 17α-methyltestosterone (MT) before introduction in culture unit to avoid over-breeding. This manipulation can be perceived as a real chemical hazard for consumers and environment. Therefore, this study was undertaken to evaluate the withdrawal of MT residues in tilapia's flesh treated with 65 mg of MT/kg of impregnated-feed for 28 successive days then enlarged for another three months post-treatment. At the 60th day post-treatment, the average of sex ratio in treated groups (97.78% males and 2.22% females) was significantly different ($P<0.001$) from the untreated one (48.57% males and 51.43% females). MT residues were analyzed using an ELISA method after liquid/solid extraction. The MT content in flesh was very low at the first day post-treatment (1.59 µg/kg), then continued to decrease significantly ($P<0.05$) and passed below the detection threshold (0.09 µg/kg) after 60 days post-treatment. The MT concentration in water's samples was below the detection threshold (0.16 µg/kg) and was insignificant from toxicological point of view. So from the data collected it can be suggested that MT treatment of tilapia carries no risk for human health and environment.

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Introduction

The Nile tilapia "*Oreochromis niloticus*" is the second most cultured fish species worldwide with an annual production exceeding 3.6 million tons in 2010 (FAO, 2012). Since the last decade, tilapia's breeding is in full rise in Algeria as an alternative compensation of the deficit of fishery products (Dergal *et al.*, 2013). Tilapias have many attributes that make them an ideal candidate for promoting aquaculture and development in Algeria. These include: fast growth, tolerance to a wide range of environmental conditions, resistance to stress and diseases, ability to reproduce in captivity, feeding on low trophic levels and good sensorial properties of flesh (Asad *et al.,* 2010; Junior *et al.,* 2012). Nevertheless, the precocious sexual maturity of tilapia can be the major drawback leading to the overpopulation of breeding ponds (Baroiller *et al.,* 2009). Consequently, the produced fish (mixed sex tilapia) are of unequal harvest size and reproductive females remain small because of mouth brooding (Baras *et al.,* 2001). For economical reasons, the manipulation of the sexual dimorphism in favor of the male proves to be an optimal solution (Baras and Melard, 1997). All-male populations are preferred because males grow faster and to a larger/more uniform size than equivalent populations of mixed sex fish or all-female stocks (Risto *et al.,* 2013). Sex reversal by oral administration of the synthetic steroid, 17α-methyltestosterone impregnated-feed proves to be the most simple, easiest, highly effective and reliable tool for the phenotypical masculinization of the newly-hatched tilapias resulting in a minimum of 95% male offspring (Marjani *et al.,* 2009; Celik *et al.,* 2011). The effectiveness of MT-treatment depends on the dose of hormone (Barry *et al.,* 2007), time of treatment initiation (7 to 12 days post-hatch) (Teichert-Coddington *et al.,* 2000) and treatment duration (usually 21 or 28 days) (Abucay and Mair, 1997). The use of the MT-hormone in the livestock production was often disputed by researchers because of their potential toxicity on the human health (carcinogenic properties and endocrine disrupter) and environmental hazards (Aman *et al.,* 2006; Hulak *et al.,* 2008; Zhang *et al.,* 2010; Barbosa *et al.,* 2013; Golan and Levavi-Sivan, 2014).

The group of anabolic steroids (including the MT) is prohibited by the European Union directive (2003/74/EC) (E.U, 2003) for animals fattening (De Brabander *et al.,* 2009; Risto *et al.,* 2013). Likewise, marketing of treated fish is restricted in India, Costa Rica and Ecuador (Megbowon and Mojekwu, 2012; Kefi *et al.,* 2013; Mlalila *et al.,* 2015). On the other hand, the US FDA authorizes the MT-hormone treatment on the basis of tangible scientific evidence (Mlalila *et al.,* 2015). In Algeria, there is no specific regulation for the use of anabolic steroids in livestock production. The risk of the MT can fit in the context of the national law n° 09-03 of February 25th, 2009 relating to the consumer protection and the repression of the frauds (*Official Journal* n° 15) (JORADP, 2009) as well as the executive decree n° 10-90 of March 10th, 2010 relating to the introduction of HACCP system into the establishments of foodstuffs (*Official Journal* n° 17) (JORADP, 2010). Therefore, our study principally focused on the optimization of an extraction method of MT residues from three different matrixes (water, fish's flesh and feed), which is compatible with an ELISA technique. Moreover, this work evaluates the withdrawal period of MT residues in tilapia's flesh after hormonal treatment in the laboratory conditions. Secondly, our study provides some replies on the environmental impact of the MT residues from released experimental water.

Material and methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the University of Liège (Belgium) (Authorization n° 1123/2011).

Experimental design

The experiment was conducted at the Research and Education Center in Aquaculture (CEFRA), University of Liege, Tihange, Belgium. Tilapia fry were obtained by artificial spawning from normal XX female and XY male. Eggs were hatched 10 days in 1.5 L Zug bottle supplied with water at 27°C ± 1°C.

Thereafter, alive fries (the survival rate was 70%) were removed from the bottle, weighed, counted, transferred and divided randomly into six glass aquariums of 50 L (± 230 fries per aquarium) which composed two independent recirculating systems. The first system contained tree aquariums for control (hormone free diet) and the three other aquariums constituted the second system fed MT-impregnated feed for 28 consecutive days. Each recirculating system was equipped with a vortex (mechanical and biological filters), supplied with continuously aerated running heated water (O_2 concentration > 6 ppm and T° = 27 ± 1 °C) and the lighting was provided for 12 hours. After the end of hormonal treatment (28 days), juveniles were shifted in two new independent recirculating systems (treated and control) composed of six glass aquariums of 250 L and managed to be under the same experimental conditions as the precedents. The photoperiod was changed for 14 hours of lighting and 10 hours of dark. Along the experiment (120 days), aquariums were cleaned and the food residues discarded daily. The temperature and the oxygenation of water were controlled and recorded daily. Levels of ammonia and nitrites in water were measured twice à week.

Medicated feed

The MT hormone-treated feed was prepared by dissolving 65 mg of 17α-methyltestosterone (*Acros Organics*, *Netherland*) in 600 ml of absolute ethanol (*Sigma, Germany*), and then incorporated into one kilogram of commercial powdered feed for tilapia fry (*Tilapia EX, Aqua Bio, Belgium*). The ethanol was air dried under ventilation hood for 24 hours, and the feed was stored in the refrigerator (4°C). During the experiment, four types of industrial dry feed (32% crude protein) were distributed depending on the granulometry of feed, which is suitable for the opening of the oral cavity. At the treatment period (28 days), optimal rations of medicated feed (for treated lots) and free diet powdered feed (for control lots) were adjusted using the following formula: $y = 5.818\ x^{-0.233}$ (data not published), where "y" is the weight of fish (g) and "x" is the biomass ratio (%). Feed was distributed manually over 8 hours per day.

Thereafter and from the cessation of treatment until the end of experiment, adjusted rations of free diet pellets at 5% of biomass of each aquarium were distributed using automatic belt feeders.

Zootechnical parameters

During the five months of the experiment, the mean body weight (recorded to the nearest gram), daily weight gain (DWG), specific growth rate (SGR) and food conversion rate (FCR) were monitored weekly. The survival rate of fish (%) was recorded on the termination of trial. The following formulas were used to calculate the above parameters (Chakraborty *et al.*, 2011).

DWG (g day^{-1}) = mean final weight (g) - mean initial weight (g) / days

SGR (% day^{-1}) = [(ln final weight - ln initial weight) / time (days)] × 100

FCR = total amount of dry feed consumed (g) / wet weight gain of fish (g)

Survival rate (%) = (final number of fish / initial number of fish) × 100

The sex ratio of juvenile tilapia was determined by microscopic analysis of gonadal squashes 60 days post treatment (Rougeot *et al.*, 2008). Briefly, one hundred fish per aquarium (treated and control) were randomly sampled and sacrificed using a lethal dose of anesthetic (*Benzocaine, Sigma*). After that, juvenile were sexed with the acetocarmine squash method of Guerrero and Shelton (1974).

Sampling

To control 17α-methyltestosterone residues from tilapia's flesh in both control and treated batches, fishes were sampled at 15 days intervals from the first day of treatment's cessation (29 days) until the third months post-treatment (90 days). Because of the smallest size of juveniles, ten individuals were randomly taken for the first and second sampling from each aquarium. Thereafter, only 5 fishes were randomly sampled from each aquarium. Fishes sampled from the same aquarium were anaesthetized (*Benzocaïne, Sigma-Aldrich*, ref. E1501) (200 mg l^{-1} of water).

Their flesh (skin and muscle in natural proportion) were collected, crushed and pooled. Each pooled sample was analyzed in triplicate (n = 3). In parallel, three pooled samples (10 tilapias by pool), taken from real marketable tilapias produced in a west of Algerian fish farm, were also analyzed.

To evaluate MT residues content in water, a dozen samples of water from the treated and controlled recirculating systems were taken before (7 days), during (21 and 27 days) and after the hormonal treatment (1 day, 2 and 5 weeks post treatment) from aquariums and from released water.

To evaluate the stability of methyltestosterone hormone during refrigerated storage (4°C) of feed, three samples of MT-impregnated feed were also collected. All the samples of flesh and water were preserved at -20°C until ELISA analysis.

Hormone analysis

MT residues from released water and flesh of tilapias were measured using a commercial available enzyme-linked immunosorbent assay (ELISA) kit (*METHYLTESTOSTERONE 2 hours*, Marloie, Belgium).

Optimization of buffer solutions

The sodium acetate buffer 50 mM was prepared by dissolving 4.1 g CH_3COONa in one liter of distilled water and the pH adjusted to 4.8 with acetic acid (20%). The phosphate buffer solutions (PBS-buffer) 67 mM was prepared by adding 2.9 g of sodium dihydrogen phosphate monohydrate ($Na_2PO4.H_2O$) to 8.19 g of disodium phosphate 2-hydrate ($Na_2HPO_4.2H_2O$) in one liter of distilled water. The PBS-buffer 20 mM was prepared by adding 0.87 g $Na_2PO4.H_2O$ to 2.44 g $Na_2HPO_4.2H_2O$ in one liter of distilled water. The pH of the two PBS-buffer solutions was adjusted to 7.2 with acetic acid (20%).

Extraction procedure

The described extraction procedure of ELISA Kit for urine was applied for water samples, except that the sample volume was doubled (1 ml of water instead of 0.5 ml for urine).

For flesh samples, the initial extraction procedure was slightly modified: 5 g of homogenate (tissue + 67 mM phosphate buffer pH 7.2) were used to extract the residue twice with 5 ml tert-butyl-methyl ether (TBM). The tubes were vigorously stirred (30mn), centrifuged (10 min. at 3000 *g*) and supernatants were transferred to another vial. The residue obtained after evaporation under a stream of nitrogen of the pooled ether phases, was dissolved in 1 ml methanol/distilled water (60/40) and diluted with 2 ml of 20 mM PBS buffer. The extract was cleaned up using Bakerbond SPE Octadecyl (C_{18}) 1 ml Solid Phase Extraction Columns (Baker, USA). The SPE columns were conditioned with 3 ml of methanol (100%) then equilibrated with 2 ml of 20 mM PBS buffer. The sample (3 ml) was charged onto the activated column and then the column was washed with 2 ml of methanol/distilled water (40 /60) before centrifugation. The sample extract was eluted slowly (15 drops/min) with 1 ml of methanol (100%) then evaporated until dryness at +60 °C under a mild stream of nitrogen. The residue was dissolved in 100 µl of ethanol and 900 µl of dilution buffer. Finally, 50 µl per well were charged in ELISA plate.

The same extraction method was applied for feed analysis. MT recovery from fish flesh and feed was measured using 3 control samples (tilapias and feed) spiked with 1, 3 and 5 µg/kg of MT.

Statistical analysis

The SAS software (Statistical Analysis System, 2000) was used for all statistical analyses. Time effect on each parameter (mean body weight, specific growth rate, daily weight gain and food conversion ratio) was assessed by general linear model (proc glm; SAS, 2000). The least square means (LSM) were calculated for each parameter according to time effect. Mean body weight was calculated for each week along.

The Chisquare ($\chi 2$) test was used in finding out the statistical significance of sex reversal rates ($P<0.05$) between treated and control batches.

Data of the MT residues concentrations were subjected to variance analyses (ANOVA one way), and differences between means were evaluated by the Tukey test (significance, $p<0.05$) using the statistical software SPSS 17.0 for Windows (SPSS Inc., Chicago, III, USA). The results of statistical analysis are shown as mean values ± standard deviation.

Results

Zootechnical parameters

The physicochemical properties of water used for experiment in two independent recirculating systems (control and MT treated) were within the acceptable range for fish growth.

The average values of temperature (27 ± 0.03°C), dissolved oxygen (6.77 ± 0.56 mg/L), nitrite (0.89 ± 0.75 mg/L), ammoniac (0.45 ± 0.30 mg/L) and pH (7.87 ± 0.09) released the good rearing management. During the experiment (4 months) and in spite of fish transfer between aquariums (from 50 L to 250 L), the survival rate remained 100% in both treated and controlled batches (Table 1).

Table 1. Growth parameters and survival rate of Nile tilapia (*Oreochromis niloticus*) in the experiment.

Parameters	Control group	Treated group
Initial weight (g)	0.0061 ± 0.002 [a]	0.0061 ± 0.002 [a]
Final weight (g)	39.69 ± 0.381[a]	41.58 ± 0.381[a]
DWG (g)	0.38 ± 0.01[a]	0.40 ± 0.004 [a]
SGR (% BW day^{-1})	8.36 ± 0.02 [a]	8.41 ± 0.01 [a]
FCR	0.41 ± 0.002 [a]	0.40 ± 0.04 [a]
Survival rate (%)	100 [a]	100 [a]

DWG: daily weight gain, SGR: specific growth rate. FCR: food conversion rate. Different letters on the same row indicate a statistically significant difference ($p<0.05$).

Growth performances are summarized in Table 1. Results of mean weight, daily weight gain, specific growth rate and food conversion rate were statistically similar ($P<0.05$) in both treated and untreated lots. Results of sex ratio rate are illustrated in Fig. 1.

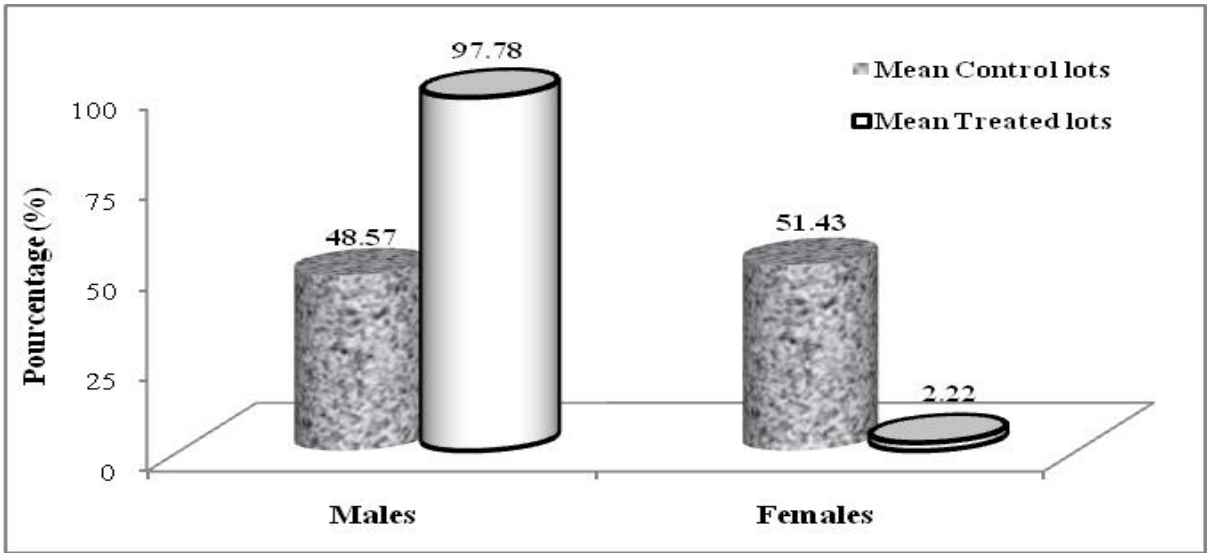

Fig. 1. The sex ratio rate (%) of juvenile tilapia (*Oreochromis niloticus*) following sex reversal treatment by 17α-methyltestosterone, mean (n = 3).

The mean number of male individuals in MT-treated groups was 97.78 ± 1.54% against 2.22 ± 1.54% of mean rate of female individuals. These values were significantly different (Chi2 test, P<0.001) from the untreated groups (48.57 ± 1.43% males and 51.43 ± 1.43% females). Fisher's Exact Test was also applied because of the lowest rate of females individuals (<5%) and confirm the highly significant difference between control and treated groups ($P< 2.51^{-20}$).

MT residues analysis

Following the obtained results from preliminary tests with the original ELISA method, the extraction procedure was optimized to improve the extraction recovery from the studied matrices (water, flesh and feed). Values of the recovery extraction were: 55 ± 9.54% from fish flesh, 73.85% from feed and higher than 85% from water. The performance parameters of the ELISA method were satisfactory in terms of reproducibility and linearity (R^2 = 0.998) as shown in Fig. 2. The respective limits of detection (LOD) in water and flesh were 0.16 ppb and 0.09 ppb and the respective limits of quantification (LOQ) were 0.25 ppb and 0.18 ppb.

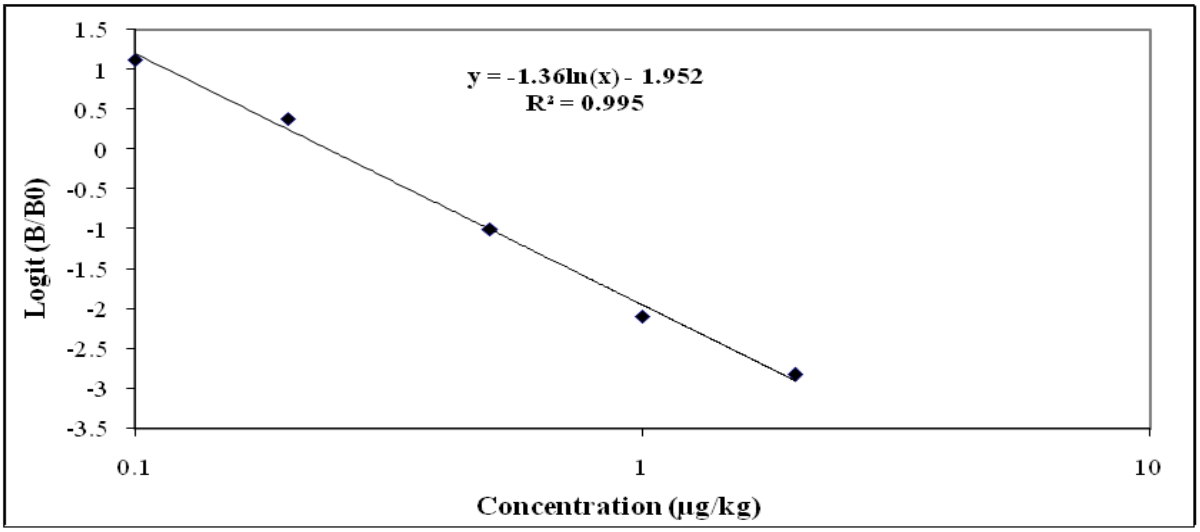

Fig. 2. Calibration curve of 17α-methyltestosterone ELISA test.

Figure 3 illustrates the results of the post treatment residual concentrations of 17α-methyltestosterone (µg/kg) in tilapia's flesh. Considering the 100% of cross-reaction between methyltestosterone and natural testosterone in the selected ELISA test, MT's values of treated tilapia are expressed after subtracting the corresponding physiological testosterone values from the control individuals. Results showed that the MT concentration in tilapia's flesh was very low (1.9 µg/kg) at the first sampling (one day post-treatment) then rapidly and significantly (p<0.05) decreased to 0.1 ppb then fell below the detection limit of the ELISA test (0.09 ppb) after two months post treatment in the triplicate treated groups. No MT residues were detected from the real samples of tilapia marketed and reared in Algeria.

The MT concentrations were below the LOD threshold (0.09 ppb) of the ELISA test. MT concentration value from MT-impregnated feed was 60 mg/kg, which justified the stability and good homogenization of the medicated feed used in this study. No MT residues were detected from the water samples by the ELISA test. The MT concentrations were below the LOD threshold (0.16 ppb).

Discussion

In aquaculture breeding and good management practices are paramount for fish growth. Water temperature and chemical quality in fishpond plays a vital role in the survival, growth performance and well being of tilapia (Khalil *et al.*, 2011).

Results of physicochemical parameters of water analysis were consistent with the standards range of ideal water for tilapia rearing provided by Hasheesh *et al.* (2011). In accordance with Asad *et al.* (2010), the survival rate of experimental fish in all aquaria remained 100%, which revealed that the MT treatment didn't affect the survival rate of fish.

The growth promoting efficiency of the MT anabolic steroid hormone in tilapia species is well documented.

Most of researchers agree that growth of treated fry of *Oreochromis niloticus* with 17α-methyltestosterone is superior than the untreated one (Marjani *et al.,* 2009; Asad *et al.,* 2011; Chakraborty *et al.,* 2011; El-Greisy and El-Gamal, 2012). The anabolic effect is attributed to the improvement of food conversion efficiency and the direct effect of MT on the gene expression in the muscle cells (Risto *et al.,* 2013). Contradictory to the previous studies, our work revealed that the anabolic effect was not evident within the experimental period.

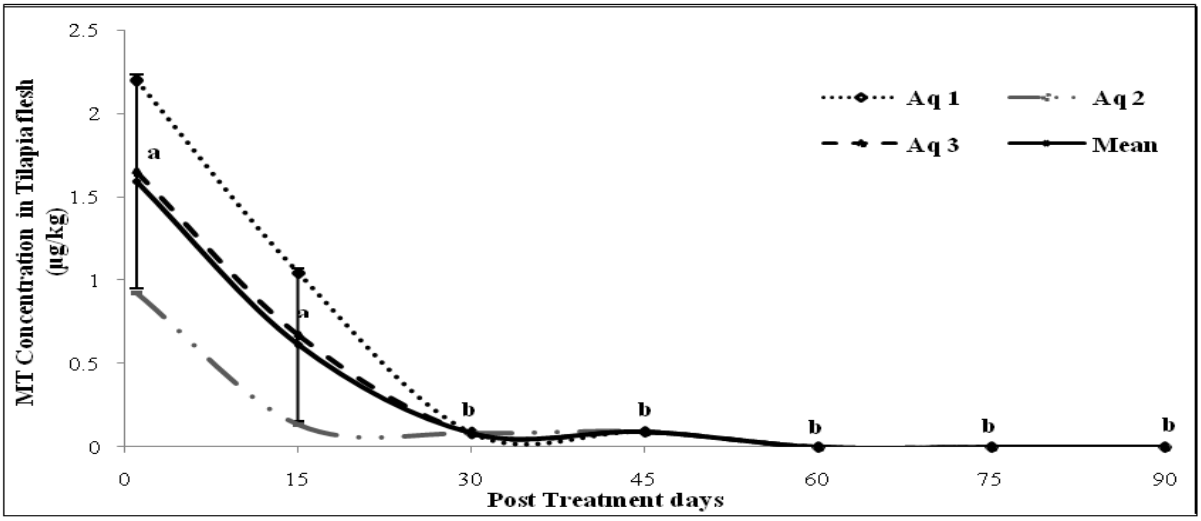

Fig. 3. Post treatment residual concentrations of 17α-methyltestosterone (µg/kg) in flesh of Nile tilapia (*Oreochromis niloticus*), Mean ± SD (n = 3). Different letters on the same curve indicate a statistically significant difference (Tukey test, $p<0.05$).

No significant difference was shown among growth parameters between control and treated individuals. Similar findings were reported by Phelps *et al.* (1992), Smith and Phelps (2001), Celik *et al.* (2011), Junior *et al.* (2012) and Kefi *et al.* (2013). This can be explained by the short period of experiment (only 4 month) and by the optimal meal distributed during the MT treatment compared to larger diets accessing the rate of 20% of biomass reported by Chakraborty *et al.* (2011). The role of MT in developing male sexual characters was highlighted in this study (~ 98%). The same results were obtained beforehand by Romerio *et al.* (2000) and Marjani *et al.* (2009), while testing 60 mg and 75 mg of MT. Kg^{-1} of impregnated-feed respectively. All male (100%) populations have been attained with MT-medicated feed at dose rate of 40 mg MT Kg^{-1} within a closed water system (Abucay and Mair, 1997), 50 mg MT Kg^{-1} (Bhandari *et al.,* 2006), and 70 mg MT Kg^{-1} of formulated feed with 40% crud protein (Asad *et al.,* 2010). An average of 95% male population was reported by Guerrero (1975), while treated tilapia fry with 30 mg MT Kg^{-1} of MT-medicated feed. A maximum of 93.7% male rate was described by Celik *et al.* (2011) while applying different doses (20-60 mg MT Kg^{-1} of medicated feed). The androgenic effect of 17α-methyltestosterone in tilapia fry is explained by the inhibition of the aromatase and the reducing estrogen levels during the sensitive period of sexual differentiation (from 10 to 38 days post-fertilization) (Mei and Gui, 2014; Genotte *et al.,* 2015; Rahma *et al.,* 2015).

Several methods have been developed and validated for the detection of anabolic steroid (17α-methyltestosterone) and their metabolites in various biological matrices. Costly and fastidious methods such as gas chromatography coupled to mass spectrometry (GC-MS) (Gomez *et al.,* 2013), liquid chromatography coupled to a diode array UV (Han *et al.,* 2012) or to mass spectrometry (Storey *et al.,* 2014) are generally solicited for the screening and confirmation of MT. Simple, fast, sensitive and cost-effective ideal assays for screening and quantification of MT are also available. Usually, immunoassays are recommended for screening of androgen residues and their metabolite (cross-reactivity) and are based on radioimmunoassay (RIA) applied with precaution against radioactive compounds (Contreras-Sanchez *et al.,* 2001). Enzyme-linked immunosorbent assay (ELISA) offer good repeatability, quick and potential of testing several samples at the same time (Lu *et al.,* 2006). In this work, we optimized the extraction procedure of the commercial ELISA kit (*METHYLTESTOSTERONE 2 hours*, Marloie, Belgium), which can serve as routine tool for laboratory or HACCP teams to screen MT residues from the three-studied matrix (feed, flesh and water). Lu *et al.* (2006) and Risto *et al.* (2013) or Kong *et al.* (2015) have developed an ELISA or indirect competitive enzyme-linked immunosorbent assay (icELISA) respectively, allowing the detection of the synthetic hormone accurately and at very low concentrations (ppt).

In this study, the kinetic of post treatment residual concentrations of MT in tilapia flesh was ascending from 2.19 µg kg^{-1} (in lot 1) to an undetectable concentration after 60 days post-treatment (in all lots). This finding is in line with previous evaluation studies of MT accumulation in whole fish since the end of seventies. Fagerlund and Dye (1979) and Johnstone *et al.* (1983) reported the rapid loss of MT in rainbow trout and tilapia respectively. In 1986, Goudie *et al.* due to radiolabelled methyltestosterone depletion in blue tilapia showed that after three weeks post treatment, the MT concentration equals 5 ng g^{-1}. Cravedi *et al.* (1993) reported that 68% of radiolabelled MT is rapidly extracted via bronchial elimination within 24 hours and the rest should been metabolized and extracted via bile or feces in trout. Green and Teichert-Coddington (2000) demonstrated by regression analysis of radioactivity depletion that concentrations of MT and metabolites in tilapia falls below 10 pg g^{-1} after 8-40 days of withdrawal. One year later, Vick and Hayton (2001) proved by pharmacokinetics of MT in rainbow trout that the oral bioavailability was 70% and the half-live was about 57 hours, which corrobrate the finding of the rapid depletion and efficiency of the MT-medicated feed. Dabrowski *et al.* (2004) showed the rapidly decrease of MT concentration from 3 µg g^{-1} to 0.5 µg g^{-1} within 8 weeks of withdraw in tilapia (confirmed results by our study). Chu *et al.* (2006), while developing an LC-MS method and controlled tilapia fillets (previously fed with MT-feed at 30mg MT kg^{-1} during 28 successive days) showed that level of MT was 0.13 ng g^{-1} after 14 days post treatment then fell below the LOD (0.04 ng g^{-1}).

Our study demonstrated that samples of released water from tilapia hatchery or from closed-loop aquaculture system were relatively harmless for environment. Certainly, water had undergone a mechanic and microbial filtration through the recirculating system. Most bacteria could biodegrade steroids as shown by Homklin *et al.* (2011, 2012) under electron acceptor conditions. In addition, the sensitivity of MT to photo-oxidation (Teichert-Coddington *et al.,* 2000) and the filtration through gravel or sand (Contreras-Sanchez *et al.,* 2001; Shore and Shemesh, 2003) played effective tools to minimize steroids concentration in water and sediment. On the other hand, incidental sex reversal of Tilapia nilotica (Abucay and Mair, 1997) and common carp (*Cyprinus carpio* L.) (Hulak *et al.,* 2008) were reported because of polluted water by MT.

In this study, the stability of a minimum of one month at 4°C of the MT-medicated feed (65 mg kg^{-1}) was in agreement with Barry *et al.* (2007) advocating several month for fish MT-impregnated feed at this storage temperature.

Conclusion

Our study has strengthened the scientific opinion about the harmlessness of using MT during sexual inversion of tilapia fry. There are no environmental or human risks while some good breeding and veterinary practices are applied in tilapia farms. MT-treatment (28 days) must be restricted only for the early fry stage (10 days post hatching).

The medicated dose must be limited at maximum of 65 mg kg^{-1} feed. To ensure safety of marketed tilapia fish, the enlargement period must be at least of 5 months. Tilapia farm workers must be aware about the dangers of hormone powder and must apply the instructions of use and storage. Finally, bacterial and physical systems of filtration must be established for released water from hatcheries. As global and interactive solution, implementing HACCP system in tilapia farms is desirable. The ELISA method optimized in this work can be applied for the screening of MT residues in feed, water and flesh matrix.

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References

Abucay JS, Mair GC. 1997. Hormonal sex reversal of tilapias: implications of hormone treatment application in closed water systems. Aquaculture Research **28,** 841-845. http://dx.doi.org/10.1046/j.1365-2109.1997.00878.x.

Aman CS, Pastor A, Cighetti G, De La Guardia M. 2006. Development of a multianalyte method for the determination of anabolic hormones in bovine urine by isotope-dilution GC-MS/MS. Analytical and Bioanalytical Chemistry. **386,** 1869-1879. http://dx.doi.org/10.1007/s00216-006-0739-0.

Asad F, Ahmed I, Saleem M, Iqbal T. 2010. Hormonal masculinization and growth performance in Nile tilapia (*Oreochromis niloticus*) by androgen administration at different dietary protein levels. International Journal of Agriculture and Biology **6(12),** 939-943.

Baras E, Melard C. 1997. Individual growth patterns of juvenile Nile tilapia, *Oreochromis niloticus* (L.): emergence and dynamics of sexual growth dimorphism. In: Fitzsimmons K, Ed. Tilapia Aquaculture. Northeast Regional Agricultural Engineering Service **106,** 169-177.

Baras E, Jacobs B, Mélard C. 2001. Effect of water temperature on survival, growth and phenotypic sex of mixed XX–XY/progenies of Nile tilapia *Oreochromis niloticus*. Aquaculture **192,** 187-199.

Barbosa IR, Lopes S, Oliveira R, Domingues I, Soares AMVM, Nogueira AJA. 2013. Determination of 17α-Methyltestosterone in freshwater samples of tilapia farming by High Performance Liquid Chromatography. American Journal of Analytical Chemistry **4,** 207-211. http://dx.doi.org/10.4236/ajac.2013.44026

Baroiller JF, D'Cotta H, Bezault E, Wessels S, Hoerstgen-Schwark G. 2009. Tilapia sex determination: where temperature and genetics meet. Comparative Biochemistry and Physiology Part A **153,** 30-38. http://dx.doi.org/10.1016/j.cbpa.2008.11.018.

Barry TP, Marwah A, Marwah P. 2007. Stability of 17α-methyltestosterone in fish feed. Aquaculture **271,** 523-529. http://dx.doi.org/10.1016/j.aquaculture.2007.05.001

Bhandari RK, Nakamura M, Kobayashi T, Nagahama Y. 2006. Suppression of steroidogenic enzyme expression during androgen-induced sex reversal in Nile tilapia (*Oreochromis niloticus*). General and Comparative Endocrinology **145,** 20-24. http://dx.doi.org/10.1016/j.ygcen.2005.06.014.

Celik I, Guner Y, Celik P. 2011. Effect of orally-administrated 17α-Methyltestosterone at different doses on sex reversal of Nile tilapia (*Oreochromis niloticus*, Linneaus 1758). Animal and Veterinary Advances **10(7),** 853-857.
http://dx.doi.org/10.3923/javaa.2011.853.857.

Chakraborty SB, Mazumdar D, Chatterji U, Banerjee S. 2011. Growth of mixed-sex and monosex Nile tilapia in different culture systems. Turkish Journal of Fisheries and Aquatic Sciences **11,** 133-140.
http://dx.doi.org/10.4194/TRJFAS.2011.0117.

Chu P-S, Lopez M, Serfling S, Gieseker CE, Reimschuessel R. 2006. Determination of 17α-Methyltestosterone in muscle tissues of tilapia, rainbow trout, and salmon using Liquid Chromatography-tandem Mass Spectrometry. Journal of Agricultural and Food Chemistry **54,** 3193-3198.
http://dx.doi.org/10.1021/jf052701r.

Contreras-Sanchez WM, Fitzpatrick MS, Schreck CB. 2001. Fate of methyltestosterone in the pond environment: detection of MT in pond soil from a CRSP site. In: Gupta A, McElwee K. Burke D, Burright J, Cummings X, Egna H, Editors. Eighteenth Annual Technical Report. Pond Dynamics/Aquaculture CRSP, Oregon State University, Corvallis, Oregon, 79- 82.

Cravedi JP, Delous G, Debrauwer L, Prome D. 1993. Biotransformation and branchial excretion of 17α-methyltestosterone in trout. Drug Metabolism *and* Disposition **2(21),** 377-385.

Dabrowski K, Rodriguez G, Lee K-J, Abiado MAG, Contreras-Sanchez WM, Marquez-Couturier G, Phelps RP. 2004. Studies on fate of methyltestosterone and its metabolites in tilapia and on the use of phytochemicals as an alternative method to produce a monosex population of tilapia. In: Harris R, Courter I, Egna H, Editors. Twenty-First Annual Technical Report. Aquaculture CRSP, Oregon State University, Corvallis, Oregon, 53- 60.

Dergal NB, Abi-Ayad S-ME-A, Degand G, Douny C, Brose F, Daube G, Rodrigues A, Scippo M-L. 2013. Microbial, biochemical and sensorial quality assessment of algerian farmed tilapia (*Oreochromis niloticus*) stored at 4 and 30°C. African Journal of Food Sciences **7(12),** 498-507.
http://dx.doi.org/10.5897/AJFS2013.1063

El-Greisy ZA, El-Gamal AE. 2012. Monosex production of tilapia, *Oreochromis niloticus* using different doses of 17α-methyltestosterone with respect to the degree of sex stability after one year of treatment. The Egyptian Journal of Aquatic Research **38,** 59-66.
http://dx.doi.org/10.1016/j.ejar.2012.08.005

EU. 2003. Directive 2003/74/EC of the European Parliament and of the Council amending Council Directive 96/22/EC concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of beta-agonists. Official Journal of the European Union L262, 19-21.

Fagerlund UHM, Dye HM. 1979. Depletion of radioactivity from yearling coho salmon *(oncorhynchus kisutch)* after extended ingestion of anabolically effective doses of 17α-methyltestosterone-1, 2-3 H. Aquaculture **18,** 303-315.

FAO. 2012. The state of world Fisheries and Aquaculture 2012. Rome. 209 p.

Gennotte V, Mafwila Kinkela P, Ulysse B, Akian Djetouan D, Bere Sompagnimdi F, Tomson T, Melard C, Rougeot C. 2015. Brief exposure of embryos to steroids or aromatase inhibitor induces sex reversal in Nile tilapia (*Oreochromis niloticus*). Journal of Experimental Zoology Part A: Ecological Genetics and Physiology **323,** 31-38.
http://dx.doi.org/10.1002/jez.1893

Golan M, Levavi-Sivan B. 2014. Artificial masculinization in tilapia involves androgen receptor activation. General and Comparative Endocrinology **207,** 50-55.
http://dx.doi.org/10.1016/j.ygcen.2014.04.026.

Gomez C, Pozo OJ, Marcos J, Segura J, Ventura R. 2013. Alternative long-term markers for the detection of methyltestosterone misuse. Steroids **78,** 44-52. http://dx.doi.org/10.1016/j.steroids.2012.10.008.

Goudie CA, Shelton WL, Parker NC. 1986. Tissue distribution and elimination of radiolabelled methyltestosterone fed to sexually undifferentiated Blue tilapia. Aquaculture **58,** 215-226.

Green BW, Teichert-Coddington DR. 2000. Human food safety and environmental assessment of the use of 17a-Methyltestosterone to produce male tilapia in the United States. Journal of the World Aquaculture Society **3(31),** 337-357.

Guerrero RDIII. 1975. Use of androgens for production of all male *Tilapia aurea* (Steindachner). Transactions of the American Fisheries Society **2,** 342-347.

Han Y, Ma Q, Lu J, Xue Y, Xue C. 2012. Optimization for subcritical fluid extraction of 17-methyltestosterone with 1,1,1,2-tetrafluoroethane for HPLC analysis. Food chemistry **135,** 2988-2993. http://dx.doi.org/10.1016/j.foodchem.2012.07.081.

Hasheesh WS, Marie M-AS, Abbas HH, Eshak MG, Zahran EA. 2011. An evaluation of the effect of 17α-Methyltestosterone hormone on some biochemical, molecular and histological changes in the liver of Nile tilapia; *Oreochromis niloticus*. Life Science Journal **8(3),** 343-358.

Homklin S, Ong SK, Limpiyakorn T. 2011. Biotransformation of 17α-methyltestosterone in sediment under different electron acceptor conditions. Chemosphere **82,** 1401-1407. http://dx.doi.org/10.1016/j.chemosphere.2010.11.068.

Homklin S, Ong SK, Limpiyakorn T. 2012. Degradation of 17α-methyltestosterone by *Rhodococcus sp*. and *Nocardioides sp*. isolated from a masculinizing pond of Nile tilapia fry. Journal of Hazardous Materials **221-222,** 35-44. http://dx.doi.org/10.1016/j.jhazmat.2012.03.072.

Hulak M, Paroulek M , Simek P, Kocour M, Gela D, Rodina M, Linhart O. 2008. Water polluted by 17α-methyltestosterone provides successful male sex inversion of common carp (*Cyprinus carpio* L.) from gynogenetic offspring. Journal of Applied Ichthyology **24,** 707-710. http://dx.doi.org/10.1111/j.1439-0426.2008.01107.x.

Johnstone R, Macintosh DJ, Wright RS. 1983. Elimination of orally administered 17α-Methyltestosterone by *Oreochromis mossambicus* (tilapia) and *Salmo gairdneri* (rainbow trout) juveniles. Aquaculture **35,** 249-257.

JORADP. 2009. Loi n° 09-03 du 25 février 2009 relative à la protection du consommateur et la répression des fraudes. Journal Officiel de la République Algérienne n°**15,** p.11.

JORADP. 2010. Décret exécutif n° 10-90 du 10 mars 2010 complétant le décret exécutif n° 04-82 du 18 mars 2004 fixant les conditions et modalités d'agrément sanitaire des établissements dont l'activité est liée aux animaux, produits animaux et d'origine animale ainsi que de leur transport. Journal Officiel de la République Algérienne n°**17,** p.18.

Junior RP, Vargas L, Valentim-Zabott M, Ribeiro RP, Da Silva AV, Otutumi LK. 2012. Morphometry of white muscle fibers and performance of Nile tilapia (*Oreochromis niloticus*) fingerlings treated with methyltestosterone or a homeopathic complex. Homeopathy **101,** 154-158. http://dx.doi.org/10.1016/j.homp.2012.05.005.

Kefi AS, Kang'Ombe J, Kassam D, Katongo C. 2013. Effect of 17 a-Methyl Testosterone on haematology and histology of liver and heart of *Oreochromis andersonii* (Castelnau, 1861). Journal of Marine Science: Research & Development **3(3),** 1-7. http://dx.doi.org/10.4172/2155-9910.1000130.

Khalil WKB, Hasheesh WS, Marie M-AS, Abbas HH, Zahran EA. 2011. Assessment of the impact of 17α-methyltestosterone hormone on growth, hormone concentration, molecular and histopathological changes in muscles and testis of Nile tilapia;*Oreochromis niloticus*. Life Science Journal **8(3),** 329-343.

Kong N, Song S, Peng J, Liu L, Kuang H, Xu C. 2015. Sensitive, fast, and specific immunoassays for methyltestosterone detection. Sensors **15,** 10059-10073.
http://dx.doi.org/10.3390/s150510059.

Lu H, Conneely GE, Crowe MA, Aherne M, Pravda M, Guilbault GG. 2006. Screening for testosterone, methyltestosterone, 19-nortestosterone residues and their metabolites in bovine urine with enzyme-linked immunosorbent assay (ELISA). Analytica Chimica Acta **570,** 116-123.
http://dx.doi.org/10.1016/j.aca.2006.03.108.

Marjani M, Jamili S, Mostafavi PG, Ramin M, Mashinchian A. 2009. Influence of 17-Alpha Methyl Testosterone on masculinization and growth in Tilapia (*Oreochromis mossambicus*). Journal of Fisheries and Aquatic Sciences **4(1),** 71-74.

Marwah P, Marwah A, Lardy H. 2005. Development and validation of a high performance liquid chromatography assay for 17-methyltestosterone in fish feed. Journal of Chromatography B **824,** 107-115.
http://dx.doi.org/10.1016/j.jchromb.2005.07.005.

Megbowon I, Mojekwu T. 2014. Tilapia sex reversal using methyl testosterone (MT) and its effect on fish, man and environment. Biotechnology **13,** 213-216.
http://dx.doi.org/10;3923/biotech.2014.213.216

Mei J, Gui JF. 2015. Genetic basis and biotechnological manipulation of sexual dimorphism and sex determination in fish. Science China Life Sciences **58,** 124-136.

Mlalila N, Mahika C, Kalombo L, Swai H, Hilonga A. 2015. Human food safety and environmental hazards associated with the use of methyltestosterone and other steroids in production of all-male tilapia. Environmental Science and Pollution Research. **22,** 4922-4931.
http://dx.doi.org/10.1007/s11356-015-4133-3.

Phelps RP, W Cole, Katz T. 1992. Effect of fluoxymesterone on sex ratio and growth of Nile tilapia, *Oreochromis niloticus* (L.). Aquaculture and Fisheries Management, **23(4),** 405-523.

Pozo OJ, Eenoo PV, Deventer K, Lootens L, Thuyne WV, Parr MK, Schanzer W, Sancho JV, Hernandez F, Meuleman P, Leroux-Roels G, Delbeke FT. 2009. Detection and Characterization of a new metabolite of 17-methyltestosterone. Drug Metabolism and Disposition **37(2),** 2153-2162.
http://dx.doi.org/10.1124/dmd.109.028373.

Montajami S. 2012. Assessment the impact of 17-methyl testosterone on the growth and survival rate of Golden barb fish, *Puntius gelius* (Hamilton, 1822). American-Eurasian Journal of Agricultural & Environmental Sciences **12(8),** 1052-1055. http://dx.doi.org/10.5829/idosi.aejaes.2012.12.08.65143.

Rahma A, Kamble MT, Ataguba GA, Chavan BR, Rusydi R, Siska M. 2015. Steroidogenic and thermal control of sex in tilapia (*O. niloticus*): A review. International Journal of Current Microbiology and Applied Sciences **4(1),** 214-229.

Risto U, Hajrulai-Musliu Z, Stojanovska-Dimzoska B, Dimitrieska-Stojkovic E, Todorovic A, Stojkovski V. 2013. Validation of screening method for determination of methyltestosterone in fish. Macedonian Veterinary Review **36(1),** 19-23.

Romerio M-PCS, Fenerich-Verani N, Santo De-Campos BE, Da-Silva AS. 2000. Masculinization of Nile tilapia, *Oreochromis niloticus*, using different diets and different doses of 17-alpha methyl testosterone. Revista Brasileira de Zootecnia **29(3),** 654-659.
http://dx.doi.org/10.1590/S151635982000000300003.

Rougeot C, Prignon C, Ngouana Kengne CV, Mélard C. 2008. Effect of high temperature during embryogenesis on the sex differentiation process in the Nile tilapia, *Oreochromis niloticus*. Aquaculture **276,** 205-208.
http://dx.doi.org/10.1016/j.aquaculture.2008.02.001.

Shore LS, Shemesh M. 2003. Naturally produced steroid hormones and their release into the environment. Pure and Applied Chemistry **75,** 1859-1871. http://dx.doi.org/10.1351/pac200375111859.

Smith ES, Phelps RP. 2001. Impact of feed storage conditions on growth and efficacy of sex reversal of Nile tilapia. North American Journal of Aquaculture **63,**242- 245.

Storey JM, Clark SB, Johnson AS, Andersen WC, Turnipseed SB, Lohne JJ, Burger RJ, Ayres PR, Carr JR, Madson MR. 2014. Analysis of sulfonamides, trimethoprim, fluoroquinolones, quinolones, triphenylmethane dyes and methyltestosterone in fish and shrimp using liquid chromatography-mass spectrometry. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences **972,** 38-47. http://dx.doi.org/10.1016/j.jchromb.2014.09.009.

Teichert-Coddington D, Manning B, Eya J, Brock D. 2000. Concentration of 17 alpha-methyltestosterone in hormone treated feed: effects of analytical technique, fabrication and storage temperature. Journal of the World Aquaculture Society **31(1),** 42-50.

Vick AM, Hayton WL. 2001. Methyltestosterone pharmacolinetics and oral bioavailability in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology **52,** 177-188. http://dx.doi.org/10.1016/S0166-445X(00)00146-6.

Zhang X, Zhang W, Xue L, Zhang B, Jin M, Fu W. 2010. Bioremediation of bacteria pollution using the marine sponge *Hymeniacidon perlevis* in the intensive mariculture water system of turbot *Scophthalmus maximus*. Biotechnology & Bioengineering **105(1),** 59-68. http://dx.doi.org/10.1002/bit.22522.