Residual levels of 17α-methyldihydrotestosterone in Nile tilapia (Oreochromis niloticus) fry following feeding supplementation

Nion Vinarukwong¹, Mintra Lukkana², Suthep Ruangwises³ and Janenuj Wongtavatchai¹*

Abstract: Intensive tilapia culture requires male-monosex population for its better yield and allowing more effective management of a single crop. Androgenic hormones are usually applied during the fish farming process to produce male-monosex population. This study investigated the residue of a synthetic androgenic steroid, 17α-methyldihydrotestosterone (MDHT) after a course of feeding supplementation in Nile tilapia (Oreochromis niloticus) fry at a dose of 80 mg/kg feed for 15 and 23 consecutive days. An analytical method using liquid chromatography tandem mass spectrometry was developed to determine the residual MDHT in tilapia fry at 1, 2, 3, 5, 7, 14 and 21 days after the last dose. The levels of MDHT on day 1 after hormone withdrawal were 3.198 ng/g in the 15-day treatment, and 3.224 ng/g in the 23-day treatment. MDHT was not detectable in fry after hormonal withdrawal for 5 days in both treatments (limit of quantitation, 0.95 ng/g), which suggests that negligible levels of MDHT will be present in Nile tilapia after 6–8 months hormonal withdrawal during the grown out period.

Subjects: Aquaculture; Food Chemistry; Food Analysis

Keywords: androgenic hormone; liquid chromatography tandem mass spectrometry; male-monosex tilapia; residue

ABOUT THE AUTHOR

Nion Vinarukwong, Mintra Lukkana, Suthep Ruangwises Dr. Janenuj Wongtavatchai, DVM, MS, PhD. Dr. Janenuj Wongtavatchai has worked for Chulalongkorn University, Thailand, since 1998. She has contributed 20 years of research and services to fish farmers through her work in the area of health management in aquaculture. She has developed a research pioneering the implementation of vaccination strategies in tilapia culture. In addition to her research, Dr. Janenuj Wongtavatchai has performed a scientific advisory role for regulatory bodies and FAO/WHO on the prudent use of veterinary drug in aquaculture. She has been awarded on the research utilization that has helped improve the production of commercial tilapia by the Thailand Research Fund and the Agricultural Research Development Agency of Thailand.

PUBLIC INTEREST STATEMENT

Intensive tilapia culture requires male-monosex population for its better yield and allowing effective management of a single crop. Male-monosex population is commonly acquired by feeding supplementation of androgenic hormones in tilapia fry. Despite the minimal use of androgenic hormones in farming process, hormonal residue in tilapia is necessary to be monitored to assure safety for consumption. We developed an analytical method to evaluate the residue of 17α-methyldihydrotestosterone (MDHT) after a course of feeding to early-stage fry at a dose of 80 mg/kg feed for 15 and 23 consecutive days. The levels of MDHT on day 1 after hormone withdrawal were 3.198 ng/g in the 15-day treatment, and 3.224 ng/g in the 23-day treatment. MDHT was not detectable in fry after hormonal withdrawal for 5 days in both treatments (limit of quantitation, 0.95 ng/g), which suggests that negligible levels of MDHT will be present in Nile tilapia after 6–8 months withdrawal during the growth stage.
1. Introduction

Male-monosex rearing of tilapia gives a higher growth rate, greater uniformity of size and better meat quality due to prevention of unwanted reproduction through undesirable sexual behavior and premature sexual maturation (Beardmore, Mair, & Lewis, 2003; Mlalila, Mahika, Kalombo, Swai, & Hilonga, 2015). There are various techniques that have been used to make male-monosex for tilapia (Dauda, Yakubu, & Oke, 2014), including manual sexing (Cnaani & Levavi-Sivan, 2009), hybridization (Mbiru et al., 2016), genetic manipulation (Pradeep et al., 2014), environmental manipulation (Wessels & Hörgsten-Schwark, 2007) and androgenic hormone feeding (Haffray et al., 2009).

Amongst different methods, synthetic androgenic hormone is widely used for production of male-monosex populations in aquaculture. This method offers reliable results, high success rate, easy handling and cost effectiveness for farming practice (Haffray et al., 2009). Different testosterone derivatives have been administered to fish at the early stage of fry, including 17α-methyltestosterone (MT) by feeding and bathing in Nile tilapia (Oreochromis niloticus) (Fitzpatrick, Schreck, & Gale, 2008; Mateen & Ahmed, 2015; Straus et al., 2013), feeding in Mozambique tilapia (Oreochromis mossambicus) (Marjani, Jamili, Mostafavi, Ramin, & Mashinchian, 2009) and bathing in Chinook salmon (Oncorhynhicus tsawytscha) (Baker, Solar, & Donaldson, 1988); 17α-ethynyltestosterone feeding in blue tilapia (Oreochromis aureus) (Guerrero, 1975); and 17α-methylprednisolone (MDHT) bathing (Fitzpatrick et al., 2008; Gale, Fitzpatrick, Lucero, Contreras-Sánchez, & Schreck, 1999) and feeding in Nile tilapia (Vinarukwong, Lukkana, & Wongtavatchai, 2018). MDHT is more potent than dihydrotestosterone and is highly androgenic and has a slight anabolic effect. This hormone was first used as a supplement in patients with abnormal levels of sex hormones (Wald, Meacham, Ross, & Niederberger, 2006). MDHT is also used by athletes and in racehorses because its anabolic effect can strengthen muscle (Hungerford et al., 2005). Androgenic hormones at a dose of 60–80 mg/kg feed given for 23 consecutive days are usually applied to produce male-monosex population in tilapia farming process (Department of Fisheries, Thailand, 2010); however, we have previously shown that MDHT at a decreased dosage, 80 mg/kg feed given for 15 consecutive days, successfully produces a male-monosex tilapia fry (Vinarukwong et al., 2018). The use of less androgenic hormones potentially results in less residual problems in the fish and aquatic environment.

Despite the production of male-monosex tilapia with androgenic hormones is practical and cost effective, the hormonal residue in fish is needed to be monitored for the minimum risk of health hazard in consumption. Determination of anabolic steroid hormones has been achieved by radioimmunoassay (RIA) (Khalil, Hasheesh, Marie, Abbas, & Zahran, 2011), enzyme-linked immunosorbent assay (Hungerford et al., 2005) and chromatographic assays. Liquid chromatography tandem mass spectrometry (LC–MS/MS) is an important analytical method for many hormones because of its high sensitivity and specificity (Bussy, Wassink, Scribner, & Li, 2017; Lohne, Andersen, Casey, Turnipseed, & Madson, 2013). LC–MS/MS has been used as a standard method for detection of hormonal residue in fish tissues and other matrices, such as testosterone in fish serum (Blasco, Carriquiriborde, Marino, Ronco, & Somoza, 2009), MT in tilapia, rainbow trout and salmon muscle (Chu, Lopez, Serfling, Gieseker, & Reimschuessel, 2006), carp muscle (Jiang, Lin, Fu, & Li, 2005) and fish feed (Marwah, Marwah, & Lardy, 2005). The purpose of this study is to develop an analytical method for the determination of MDHT residual amount in Nile tilapia fry using LC–MS/MS. The developed method is applied for comparative analysis of MDHT residual amount between a regular dosing period of 23 consecutive days and a minimum effective dosage of 15 consecutive days.

2. Material and methods

2.1. Fish dosing

MDHT (Sigma-Aldrich, Missouri, USA) was dissolved in 95.00% ethyl alcohol to prepare a solution for moistening commercial fish feed. The hormonal feeds were air dried and kept at 4°C in the dark and dry conditions. Eight thousand Nile tilapia fry were reared in 4 concrete tanks (1.00 m × 1.25 m × 0.80 m), allowing 2,000 fish/tank. Fish were fed 4 times a day at 13% body
weight (BW) per day. MDHT was given to the fry at 80 mg/kg feed for 15 or 23 consecutive days, 2 replicate tanks for each treatment. The water parameters were maintained as follows: temperature 29 ± 3°C, pH 7.0–8.0, dissolved oxygen 5.5–6.5 mg/L and ammonia (NH$_3$) ≤ 0.5 mg/L. Fish management was approved by an ethics committee of Chulalongkorn University Animal Care and Use Committee (CU-ACUC; Approval No. 11310046).

2.2. Sample collection
Five grams of fish samples was taken from each tank at 1, 2, 3, 5, 7, 14 and 21 days after hormone withdrawal. Fish were euthanized with an overdose of anesthetic agent, Aquanes® (Better Pharma, Bangkok, Thailand), and stored at −80°C until analysis. Samples from two replicate tanks were pooled together for an extraction.

2.3. Chemicals and reagents
Acetonitrile (LEDA, Spain) and methyl alcohol (Scharlau, Spain) were HPLC grade. Formic acid (Carlo Erba, Germany), tert-butylmethylether (TBME) (Merck, Germany), ammonium formate (Carlo Erba, Germany) were reagent grade. Standard MDHT (98.32% dry weight) and standard finasteride (99.50% dry weight) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used in preparing solutions was LC grade and purified with a Milli-Q water system (Millipore Corp., France). The mobile phase was prepared by dissolving 0.3153 g of ammonium formate in 1,000 mL of deionized water and adjusting to pH 3.5 with formic acid. Various mixtures of 5 mM ammonium formate and acetonitrile were used in the chromatographic system.

2.4. Analytical procedure
The method was modified from Chu et al. (2006). Samples were ground into fine powder with an equivalent amount of dry ice (w/w) using a tissue homogenizer (Pro Scientific, New Jersey, USA). An aliquot of 1 g homogenized tissue was transferred to a 5-mL centrifuge tube. Finasteride (50 µL at 500 ng/mL) as an internal standard and 950 µL of TBME were added to each sample. The mixture was vortex mixed for 30 s and centrifuged at 12,000 rpm at 10°C for 10 min. The clear supernatant was transferred to a microcentrifuge tube and evaporated to dryness in a speed vacuum concentrator at 50°C, 1.0 torr for 60 min. The residue was reconstituted with 500 µL of the acetonitrile, vortexed for 30 min, sonicated for 5 min and centrifuged at 14,000 rpm at 10°C for 10 min. The clear supernatant was transferred to an autosampler vial and 10 µL was applied into the LC–MS/MS system for each injection. Three injections of 10 µL were performed for each sample.

2.5. Liquid chromatography
The Shimadzu Prominence® HPLC system consisting of a binary gradient pump, a degasser, an autosampler, an API4000 mass spectrometer and LC solution® v. 1.22 SP1 software (Shimadzu Corp., Kyoto, Japan) was used in the study. The ammonium formate-acetonitrile gradient (5 mM ammonium formate pH 3.5: acetonitrile) was used as the mobile phase. The flow rate was 0.8 mL/min. A C$_8$ column (4.6 mm × 100 mm, 5 µm; Agilent Technologies, CA, USA) was held at 30°C and the autosampler temperature was maintained at 20°C. Detection was performed by MS at 305.3/269.3 m/z for MDHT and 373.5/355.4 m/z for finasteride. A typical injection sequence was performed in the following order: blank of calibration, calibration set, sample set and limit of quantitation (LOQ) sample set.

2.6. Method validation
All parameters were validated in accordance to the guidance for industry: Q2B validation of analytical procedures (US FDA, 1996). Accuracy and precision of the method were assessed with calibration curves using Nile tilapia samples fortified with MDHT at concentrations of 0.5, 1.0, 2.0, 3.0, 5.0, 7.5 and 10.0 ng/g and were calculated by least squares linear regression analysis of the peak area ratio (the ratio of the peak area of MDHT to the peak area of the internal standard in the sample) versus fortified concentrations of MDHT. The accuracy was assessed as percentage of recovery from the analysis of fortified MDHT standard in the Nile tilapia samples. The precision of the method expressed as % relative standard deviation (%RSD) was calculated using the equation RSD = 100 SD/μ, where SD and μ are the standard deviation and mean of concentrations of MDHT.
found in the Nile tilapia samples fortified with MDHT. Six regression lines, three for interday and three for intraday analyses, were constructed in this study. The LOQ for determination of MDHT was calculated using an equation \( \text{LOQ} = 10\sigma/S \), where \( \sigma \) is the standard deviation of the three y-intercepts, and \( S \) is the average of the three slopes of the regression lines.

### 2.7. Half-life calculation

The half-life \( (t_{1/2}) \) of MDHT in Nile tilapia fry was calculated using an equation as described by Jambhekar and Breen (2012), \( t_{1/2} = \ln(2)/k_e \) or \( t_{1/2} = 0.693/k_e \), where \( k_e \) is the elimination rate constant. The elimination rate constant is the slope of a semilogarithmic graph of the concentration–time data with a linear x-axis and a logarithmic y-axis.

### 3. Results and discussion

Chromatograms of MDHT standard, MDHT recovered from fortified samples and MDHT recovered from hormonal treated fry are shown in Figure 1. Table 1 summarizes the accuracy and precision for determination of MDHT in Nile tilapia samples fortified with three concentrations (1.0, 5.0 and 7.5 ng/g) of standard MDHT. The standard deviation of y-intercept (\( \sigma \)) for intraday analyses was 0.0225 and the average slope (\( s \)) was 0.2391. The calculated LOQ for intraday analyses was \((10 \times 0.0225)/0.2391 = 0.94 \text{ ng/g}\). Likewise, the LOQ for interday analyses was \((10 \times 0.0226)/0.2385 = 0.95 \). The average LOQ for determination of MDHT in this study was 
\[
\frac{0.94 + 0.95}{2} = 0.95 \text{ ng/g}.
\]

The residual amounts of MDHT were determined based on the calibration curve shown in Figure 2. For the 15-day treatment, MDHT residue levels decreased from 3.198 to 1.056 ng/g within 3 days after hormone withdrawal. For the 23-day treatment, these respective levels decreased from 3.224 to 1.046 ng/g. MDHT at a level above the LOQ (0.95 ng/g) was not found at 5 days or later after hormone withdrawal following both treatment courses (Table 2). The elimination rate constant of 15-day treatment was 0.554 and 23-day treatment was 0.563, the calculated half-lives of MDHT obtained from 15-day and 23-day treatments were 1.25 and 1.23 days, respectively.

LC–MS/MS technique was earlier employed successfully in an analysis of MT residue in Nile tilapia, rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar L.) (Chu et al., 2006). In this study, we developed LC–MS/MS procedure for determination of MDHT residue in Nile tilapia. The developed technique yielded 98.80% recoveries across the three concentrations of fortified MDHT indicating the method accuracy or the closeness of the mean obtained by this method to the true value of the analyte (US FDA, 2001). The precision values of our method (RSD = 2.15–12.92%) were in the criteria for the precision of an analytical method (RSD \( \leq \) 15%) (US FDA, 1996). Therefore, the method used in this study is appropriate for the quantitation and confirmation of MDHT residue in the Nile tilapia.

Feeding of Nile tilapia fry with MDHT at 80 mg/kg feed for 15 and 23 days resulted in similar levels of residual hormone that declined to less than the detectable limit (LOQ 0.95 ng/g) on day 5 after hormone withdrawal. The residual data suggest rapid clearance of the intake MDHT in Nile tilapia fry. The rapid clearance of MDHT was also reported in horse dosed with MDHT (1 mg/kg BW, per os); MDHT in urine was not detected at 2 days post-administration using GC–MS technique (LOQ 0.05 \( \mu \)g/mL) (Yamada et al., 2007).

The residue depletion data were used to extrapolate half-lives of a compound in different fish species: arsenobetaine in Atlantic salmon and Atlantic cod (Gadus morhua L.) (Amlund, Francesconi, Bethune, Lundebye, & Berntssen, 2006), nitrofuran in channel catfish (Ictalurus punctatus) (Chu, Lopez, Abrahom, El Said, & Plakas, 2008) and praziquantel in grass carp (Ctenopharyngodon idellus) (Xie, Zhao, Yang, & Hu, 2015). In this study, the estimated
half-lives of MDHT calculated from the depletion studies of 15-day treatments (1.23 days) and 23-day treatment (1.25 days) were comparable. The estimated half-life of MDHT in Nile tilapia obtained from the present study (mean 1.24 days) was much less duration compared to the rearing period of 6–8 months with MDHT-free diet. The 23-day treatment is usually employed in Nile tilapia nursery; however, a 100% masculinization of Nile tilapia fry was similarly achieved with the 15-day treatment (Vinarukwong et al., 2018). The minimum use of MDHT feeding is preferable and its residual level should be undetectable when the fish reach a marketable size.
4. Conclusion

Hormonal treatment is important for Nile tilapia production in several countries due to its economic effectiveness and pressure on the food supply. The present study showed that MDHT used for male phenotypic development was undetectable in fry at 5 days (LOQ 0.95 ng/g) after withdrawal of the hormonal diet in both 15-day and 23-day treatment courses. We report the current analysis in this context and as evidence that hormones can be used in this manner without hormonal residue in Nile tilapia meat at marketable size. The minimal use and long interval between hormonal administration and harvesting of fish allow time for hormonal elimination, which reduces the risk of a health hazard in consumption. Nevertheless, the chemical-free method for production of male-mono-sex Nile tilapia would be an ideal practice for aquaculture.

Table 1. Accuracy and precision for determination of MDHT in Nile tilapia samples

| MDHT added (ng/g) | Intraday (n = 3) | Interday (n = 3) |
|------------------|-----------------|-----------------|
|                  | Mean ± SD       | Mean ± SD       |
|                  | Found (ng/g)    | RSD (%)         | Recovery (%) | Found (ng/g)    | RSD (%)         | Recovery (%) |
| 1.00             | 0.994 ± 0.128   | 12.92           | 99.43        | 0.946 ± 0.111   | 11.73           | 94.58        |
| 5.00             | 4.956 ± 0.209   | 4.22            | 99.11        | 4.786 ± 0.103   | 2.15            | 95.71        |
| 7.50             | 8.037 ± 0.317   | 3.94            | 107.17       | 7.292 ± 0.353   | 4.84            | 97.22        |
| Slope            | 0.2391 ± 0.0053 |                 | 0.2385 ± 0.0107 |                 |
| y-intercept      | 0.1873 ± 0.0225 |                 | 0.1118 ± 0.0226 |                 |

RSD: Relative standard deviation; SD: standard deviation; limit of quantitation (LOQ) = 0.95 ng/g.

Figure 2. Linear regression analysis of the concentration of MDHT and peak area ratio (PAR).

Table 2. MDHT residue (ng/g) in Nile tilapia following oral administration at 80 mg/kg feed for 15 and 23 consecutive days

| Day after last dose | 15-day treatment | 23-day treatment |
|---------------------|------------------|------------------|
|                     | Mean ± SD        | RSD (%)          | Mean ± SD        | RSD (%)          |
| 1                   | 3.198 ± 0.051    | 1.60             | 3.224 ± 0.016    | 0.48             |
| 2                   | 2.065 ± 0.045    | 2.17             | 2.029 ± 0.027    | 1.31             |
| 3                   | 1.056 ± 0.029    | 2.75             | 1.046 ± 0.040    | 3.84             |
| 5                   | ND               | -                | ND               | -                |
| 7                   | ND               | -                | ND               | -                |
| 14                  | ND               | -                | ND               | -                |
| 21                  | ND               | -                | ND               | -                |

RSD: Relative standard deviation; ND: not detected; limit of quantitation (LOQ) = 0.95 ng/g.
Acknowledgments
This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program [Grant Number PHD/0126/2551], and CU Graduate School Thesis Grant, Chulalongkorn University, and Overseas Research Experience Scholarship for Graduate Student, Chulalongkorn University.

Funding
This work was supported by the Chulalongkorn University (TH) [CU Graduate School Thesis Grant] and Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program [Grant Number PHD/0126/2551].

Competing Interest
The authors declare no competing interests.

Author details
Nion Vinarkwong
E-mail: nion.vin@kmutt.ac.th

Mintra Lukkana
E-mail: lmimtra@gmail.com

Suthep Ruangwises
E-mail: suthep.r@chula.ac.th

Janenuj Wongtavatchai
E-mail: janenuj.wj@chula.ac.th

1 Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand.
2 National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives, Bangkok, 10900, Thailand.
3 Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand.

Citation information
Cite this article as: Residual levels of 17α-methyltestosterone in Nile tilapia (Oreochromis niloticus) fry following feeding supplementation, Nion Vinarkwong, Mintra Lukkana, Suthep Ruangwises & Janenuj Wongtavatchai, Cogent Food & Agriculture, 2018, 4: 1526436.

References
Amlund, H., Francesconi, K. A., Bethune, C., Lundebye, A. K., & Berntssen, M. H. (2006). Accumulation and elimination of dietary arsenobetaine in two species of fish, Atlantic salmon (Salmo salar L) and Atlantic cod (Gadus morhua L). Environmental Toxicology and Chemistry, 25(7), 1788–1794. doi:10.1897/05-107R1.1

Baker, I. J., Solar, I. L., & Donaldson, E. M. (1988). Masculinization of chinook salmon (Oncorhynchus tshawytscha) by immersion treatments using 17α-methyltestosterone around the time of hatching. Aquaculture, 72(3-4), 359–367. doi:10.1016/0044-8486(88)90224-4

Beardmore, J. A., Mair, G. C., & Lewis, R. I. (2001). Monosex male production in finfish as exemplified by tilapia: Applications, problems, and prospects. Aquaculture, 197(1–4), 283–301. doi:10.1016/S0044-8486(01)00590-7

Blasco, M., Carriquiriborde, P., Marino, D., Ronco, A. E., & Somoza, G. M. (2009). A quantitative HPLC-MS method for the simultaneous determination of testosterone, 11-ketotestosterone and 11β-hydroxyandrostenedione in fish serum. Journal of Chromatography B, 877(14–15), 1509–1515. doi:10.1016/j.jchromb.2009.03.028

Bussy, U., Wassink, L., Scriber, K. T., & Li, W. (2017). Determination of cortisol in lake sturgeon (Acipenser fulvescens) eggs by liquid chromatography tandem mass spectrometry. Journal of Chromatography B, 1040, 162–168. doi:10.1016/j.jchromb.2016.11.028

Chu, P. S., Lopez, M. I., Abraham, A., El Said, K. R., & Plakas, S. M. (2008). Residue depletion of nitrofurans drugs and their tissue-bound metabolites in channel catfish (Ictalurus punctatus) after oral dosing. Journal of Agricultural and Food Chemistry, 56(17), 8030–8034. doi:10.1021/jf801398p

Dauda, A. B., Yakubu, S. S., & Oke, A. O. (2014). Curbing the menace of prolific breeding in “aquatic chicken” (Tilapia): A way out to improve fish production in Nigeria. New York Science Journal, 7(6), 112–118.

Department of Fisheries, Thailand. (2010). Extension paper No. 2/2010. Department of Fisheries. Ministry of Agriculture and Cooperatives. Bangkok, Thailand. Retrieved April 14, 2018, from Fitzpatrick, M. S., Schreck, C. B., & Gole, W. L. (2008). Masculinization of tilapia through immersion in 17α-methyltestosterone or 17α-methylldihydrotestosterone. In: Deborah Burke, Brigitte Goetze, Danielle Clair and Hillary Egna. Pond Dynamics/Aquaculture Collaborative Research Support Program, Fourteenth Annual Technical Report. Corvallis, Oregon: Oregon Cooperative Fishery Research Unit, Oregon State University, pp. 93–98. Retrieved: pdacrsp.oregonstate.edu/pubs/technical/14tchpdf/14TR.pdf

Gale, W. L., Fitzpatrick, M. S., Lucero, M., Contreras-Sánchez, W. M., & Schreck, C. B. (1999). Masculinization of Nile tilapia (Oreochromis niloticus) by immersion in androgens. Aquaculture, 178(3–4), 349–357. doi:10.1016/S0044-8486(99)00136-2

Guerrero, R. D. (1973). Use of androgens for the production of all-male Tilapia aurea (Steindachner). Transactions of the American Fisheries Society, 104, 342–348. doi:10.1577/1548-8659(1975)104<342:UOAFTP>2.0.CO;2

Haffray, P., Petit, V., Guiguen, Y., Quillet, E., Rault, P., & Fostier, A. (2009). Successful production of monosex female brook trout Salvelinus fontinalis using gynogenetic sex reversed males by a combination of methyltestosterone immersion and oral treatments. Aquaculture, 290(1–2), 47–52. doi:10.1016/j.aquaculture.2009.01.029

Hungerford, N. L., Sorsait, B., Smart, C. G., McKinney, A. R., Ridley, D. D., Stenhouse, A. M., … McLeod, M. D. (2005). Analysis of anabolic steroids in the horse: Development of a generic ELISA for the screening of 17α-alkyl anabolic steroid metabolites. The Journal of Steroid Biochemistry and Molecular Biology, 96(3–4), 317–334. doi:10.1016/j.jsbmb.2005.03.007

Jambhekar, S. S., & Breen, P. J. (2012). Basic pharmacokinetics (2nd ed.). London: Pharmaceutical Press.

Jiang, J., Lin, H., Fu, X., & Li, M. (2005). Preliminary validation of high performance liquid chromatography method for detection of methyl-testosterone residue in carp...
muscle. *Journal of Ocean University of China*, 4(3), 248–251. doi:10.1007/s11802-005-0042-2

Kholl, W. K., Hasheesh, E. S., Marie, M. A. S., Abbas, H. H., & Zaran, E. A. (2011). Assessment 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.

Lohne, J. J., Andersen, W. C., Casey, C. R., Turnipseed, S. B., & Madson, M. R. (2013). Analysis of stilbene residues in aquacultured finfish using LC-MS/MS. *Journal of Agricultural and Food Chemistry*, 61(10), 2364–2370. doi:10.1021/jf3045878

Marjani, M., Jamili, S., Mostafavi, P. G., Ramin, M., & Mashinchian, A. (2005). Influence of 17α-methyl testosterone on masculinization and growth in tilapia (Oreochromis mossambicus). *Journal of Fisheries and Aquatic Science*, 4(1), 71–74. doi:10.3923/jfas.2009.71.74

Marwah, A., Marwah, P., & Lordy, H. (2005). Development and validation of a high performance liquid chromatography assay for 17α-methyltestosterone in fish feed. *Journal of Chromatography B*, 824(1–2), 107–115. doi:10.1016/j.jchromb.2005.07.005

Mateen, A., & Ahmed, I. (2015). Androgen sex reversal, subsequent growth and meat quality of Nile tilapia (Oreochromis niloticus). *Pakistan Journal of Agricultural Sciences*, 52(1), 199–202.

Mbiru, M., Limbu, S. M., Chenyambuga, S. W., Lamtane, H. A., Tamatamah, R., Madalla, N. A., & Mwandya, A. W. (2016). Comparative performance of mixed-sex and hormonal-sex-reversed Nile tilapia Oreochromis niloticus and hybrids (Oreochromis niloticus × Oreochromis urolepis hornorum) cultured in concrete tanks. *Aquaculture International*, 24(2), 557–566. doi:10.1007/s10499-015-9946-z

Milillo, N., Mahika, C., Kalombo, L., Swal, 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(7), 4922–4931. doi:10.1007/s11356-015-4133-3

Pradeep, P. J., Srijoya, T. C., Hassan, A., Chatterji, A. K., Withyachumarnkul, B., & Jeffs, A. (2014). Optimal conditions for cold-shock induction of triploidy in red tilapia. *Aquaculture International*, 22(3), 1163–1174. doi:10.1007/s10499-013-9736-4

Straus, D. L., Bowker, J. D., Bowman, M. P., Carty, D. C., Mitchell, A. J., Farmer, B. D., & Ledbetter, C. K. (2013). Safety of feed treated with 17α-methyltestosterone (17MT) to larval Nile tilapia. *North American Journal of Aquaculture*, 75(2), 212–219. doi:10.1080/15222055.2012.758211

US FDA. 1996. Guidance for industry Q2B, validation of analytical procedures: Methodology. Retrieved April 14, 2018, from http://www.fda.gov/downloads/drugs/guidances/ucm073384.pdf.

US FDA. 2001. FDA guidance for industry: Bioanalytical method validation guidance for industry. Retrieved April 14, 2018, from http://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf.

Vinarukwong, N., Lukkana, M., & Wongtavatchai, J. (2018). Decreasing duration of androgenic hormone feeding supplement for production of male monosex in tilapia (Oreochromis spp.) fry. *The Thai Journal of Veterinary Medicine*, 375–383. Retrieved from https://www.tci-thaijo.org/index.php/tjvm/article/view/146572.

Wald, M., Meacham, R. B., Ross, L. S., & Niederberger, C. S. (2006). Testosterone replacement therapy for older men. *Journal of Andrology*, 27(2), 126–132. doi:10.2164/jandrol.05036

Wessels, S., & Hörstgen-Schwark, G. (2007). Selection experiments to increase the proportion of males in Nile tilapia (Oreochromis niloticus) by means of temperature treatment. *Aquaculture*, 272, 580–587. doi:10.1016/j.aquaculture.2007.08.009

Xie, X., Zhao, Y., Yang, X., & Hu, K. (2015). Comparison of praziquantel pharmacokinetics and tissue distribution in fresh and brackish water cultured grass carp (Ctenopharyngodon idellus) after oral administration of single bolus. *BMC Veterinary Research*, 11(84), 1–6. doi:10.1186/s12917-015-0400-2

Yamada, M., Aramaki, S., Okayasu, T., Hosoe, T., Kurosawa, M., Kijima-Suda, I., Saito, K., & Nakazawa, H. (2007). Identification and quantification of metabolites common to 17α-methyltestosterone and mestanolone in horse urine. *Journal of Pharmaceutical and Biomedical Analysis*, 45(1), 125–133. doi:10.1016/j.jpba.2007.06.020
