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RESEARCH PAPER

Use of Bull Urine, Catfish Testis and Goat Testis to Replace Alcohol, Fishmeal and Steroid Hormone for the Production of Mono-Sex Tilapıa Fry

Anusha D. Perera[1,*], Ram C. Bhujel[1], Virak Visudtiphole[2]

[1]Asian Institute of Technology, Aquaculture and Aquatic Resources Management (AARM), Bangkok, Thailand.
[2]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 12120, Thailand.

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Corresponding Author
Tel.: +66900986147
E-mail: anu.perera22@gmail.com

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Abstract

One-week old swim-up fry of Nile tilapia (*Oreochromis niloticus*) were used for the trial. Six dietary feeds were assigned during the sex-reversal period – the standard sex-reversal diet without 17-α-methyltestosterone (MT) (C1), standard sex-reversal diet (C2), alcohol-replaced with bull urine included standard sex-reversal diet (BU), half MT-replaced with goat testis (GT), or catfish testis (CT1), all MT-replaced with catfish testis (CT2). Catfish or goat testis to fully replace the fishmeal and half of MT yielded acceptable ratio of the male population (96.6±1.0 and 94.8±0.9%, respectively), comparable to that produced from the standard diet (98.4±0.5%) (P>0.05). Bull urine can replace alcohol additionally required in the sex-reversal feed making process without compromising the fish sex efficiency. These results suggested the potential of the alternative natural products, bull urine, catfish, and goat testes, over the synthetic chemical conventionally used in the feed for sex-reversal of Tilapia.

Introduction

Tilapia and their hybrids (*Oreochromis* spp.) have become the most widely farmed aquatic species in the 21st century. They are commercially cultured in about 150 countries around the globe. They are a highly preferred species because they breed early and naturally without the need of hormone injection, survive well, grow fast in adverse conditions, and have no Y-bones in the muscle, which is easy to consume (Bhujel, 2014). *Oreochromis* spp. possess a district mouth-brooding characteristic. Their females incubate their own eggs and nurse their larvae in the mouth until the larvae can freely swim. However, early maturity and natural breeding become major problems for grow-out farmers when the males and females are stocked together in the same pond or tank. They start producing recruits when they are still small i.e. 50 g or so, causing overcrowding and stunting (Rana, 1988; Macintosh and Little, 1995). The stocked fish lose energy for reproduction instead for growth resulting in small-sized fish, regarded as weed fish often called poor-men's fish. The early maturity and uncontrolled breeding can be avoided by mono-sex culturing, using all-male fry (Mbiru et al., 2016). For tilapia, males grow and reach the marketable size faster than females benefitting the farmer due to the time saving and high market price for larger fish (Little et al., 2003; Bhujel, 2011; 2012; 2014; Mbiru et al., 2016).

All-male fry production technique has contributed to solve the problems and its adoption by public and private sectors has helped to boost the commercial farming of tilapia globally. Amongst the several techniques, hormonal sex-reversal is by far the most common procedure. In the common practice, a synthetic steroid hormone i.e. 17α methyltestosterone (MT) is dissolved in 95% ethanol and mixed with fishmeal-based high protein diet. The most common and effective dose of hormone is 60 mg/kg diet, which requires 240 mL of ethanol to ensure hormone to be evenly distributed in the feed (Macintosh and Little, 1995; Little et al., 2003; Bhujel, 2014). Nearly 5 kg fishmeal mixed with the MT hormone is required to produce 30,000 mono-sex fry which requires 1.2 liters of ethanol. Hence, 40 liters of ethanol and 10 g of MT are used for every million mono-sex fry produced. Efforts have been made to replace alcohol with acetone or other organic solvents and also animal urine, which also contains testosterone hormone. Efforts are also made to replace fishmeal by locally available ingredients such as silk-worm pupae, other insect meals and so on. Our previous study showed that Field cricket meal (FCM) can replace almost 80% of fishmeal (Perera and Bhujel, 2021). Ethanol was used as carrier of hormone and was evaporated after drying for 2-3 hours. The hormone was offered for 21 days to the fry immediately after the fry absorbed yolk-sac to ensure effective sex-reversal. Females were converted into males and males remained as males. Nevertheless, alcohol and the MT hormone are costly and have some limitations of availability and permission of usage in some countries due to religious beliefs (i.e. Islam) and points of view (Kefi et al., 2012). Some other issues are also the long-term exposure of staff and their health hazards (Mlalila et. al., 2014). Moreover, to the fish itself, the synthetic hormone causes histopathological changes on gill, liver, kidneys, and intestine (Suseno et al., 2020). High dose of MT often causes some side effects in the serum and muscles of the fish and also may cause low immunity (Sayed and Moneeb, 2015; Abo-Al-Ela et al., 2017). To mitigate the potential immunity compromise caused by MT, about 12 g Vitamin C has been recommended (Abo-Al-Ela et al., 2017; Perera and Bhujel, 2021). Despite the widespread use of MT in tilapia farming, concerns on the potential risks of the hormone and its residues in the target and non-target animals, human and the environment have been raised among the scientific communities (Mbiru et al., 2016). As the MT hormone is a steroid, some concerns exist regarding its potential effects and risks for the consumer, fish culturist and the environment (Megbowon and Mojekwu, 2014).

Many researches have tried to explore possibility of natural alternative products to replace or minimize the use of synthetic MT hormone e.g., Rohu testis (Khanal et al., 2014), common carp testis (Ranjan et al., 2015), bull testis (Phelps et al., 1996; Yustiati et al., 2018), hog testis (Meyer et al., 2008), ram testis (Haylor and Pascual, 1991), dried and frozen bull testis (Seazar et al., 2017) and so on. Some attempts have also been made to apply plant extracts as alternatives to MT to induce the sex reversal in Nile tilapia e.g., Mangosteen leaves (Khakong et al., 2011), papaya seed (Khalil et al., 2014), Khao Khreua i.e., *Butea superba* (Mengumphan et al., 2006; Badiola and Bhujel, 2012), Aloe Vera (Gabriel et al., 2017) and *Tribulus terrestris* seeds (Ghosal and Chakraborty, 2020). Among these works, some successes of sex conversion have been achieved, using animal products e.g., animal and carp fish testes in laboratory conditions.

However, they have not been as effective as the MT hormone. Consequently, no natural alternative product has yet been commercialized, nor adopted by the farmer, leaving more possibilities from other products to be explored. Any technology that uses locally available materials/ingredients and are cost effective would be adopted by farmers or practitioners. As bull urine and the testes of catfish and goats contains natural testosterones and are easily available in rural villages in many countries, they might be suitable to reduce the use of the steroid hormone, alcohol and the fishmeal required in the feed if don't affect the efficiency sex conversion. Therefore, potentials of bull urine, catfish testis and goat testis as the natural alternatives to replace animal protein ingredients and also steroid hormone partially or fully were examined in this study.

Materials and Methods

Experimental Fish and Rearing System

The experiment was conducted at the Aquaculture laboratory facility of the Asian Institute of Technology (AIT) (14.1° N and 100.6° E), Thailand, following the standard protocol of hormonal treatment of swim up fry. The treatment was carried out for 21 days, followed by 90-day nursing in the same tank to ensure that the fry were big enough for gonad squashing. In total, of the experiment was carried out for 112 (21+90 with 1 day in between) days during September 2020 – January 2021.

Free swimming fry (0.01±0.00 g) of Nile tilapia (*Oreochromis niloticus*) were obtained from the Department of Fisheries (DOF), Pathum Thani province in central Thailand. Swim-up fry were randomly distributed to 18 glass tanks (300 fish/tank, containing 100 L of dechlorinated freshwater. The experiment was set up according to the completely randomized design with six treatments and three replicates for each treatment. During the experiment, compressed air diffused was supplied to each glass tank through air-stones to maintain the dissolved oxygen (DO). The tanks were cleaned daily to remove uneaten food and feces. During the hormonal treatment, each group was offered with the assigned diet, five times per day for 21 days (i.e. 0.75, 1.5, 2.5 and 4.2 g per day for the periods of days 1-5, 6-10, 11-15 and 16-21 respectively). After that, all groups were further nursed for another 90 days, during

which all groups were fed with 30% protein commercial feed pellet for Tilapia (Charoen Pokphand, Thailand). At the end, growth performance of the fry in each tank was evaluated.

Experimental Diet

A total of six experimental diets were prepared (Table 1 and 2). 17-α methyl testosterone (HPLC grade, Sigma-Aldrich, Germany) was dissolved in 95% ethyl alcohol at a ratio of 60 mg/kg diet to prepare Diets C2, BU, GT, CT1. Diet C1 was basal diet consisting of fishmeal (FM), Field cricket meal (FCM), vitamins, mineral mixture and ethanol but without any hormone source. Fish meal (grade 1), mineral and vitamin mixtures were acquired from Weeramas Company Ltd., Thailand.

Diet C2 was the standard sex-reversal feed containing MT commonly used by tilapia hatcheries (Bhujel, 2014). Diet BU was prepared by replacing alcohol in C2 with bull urine (male) collected from a Bull farm in Pathum Thani province and stored at 4 ˚C. Diets GT and CT1 were formulated by replacing FM, FCM and half of the MT content in BU with goat testes or catfish testes, respectively. Diet CT2 was similar to CT1 but without MT. Catfish testes were collected as a byproduct from Testhong catfish farm in Pathum Thani. Goat testes were collected from a local meat market in Pathum Thani. The collected testes (goat testes and catfish testes) were chopped into a size of 1 mm^2 and air dried before used. The prepared diets were kept inside sealed plastic bags and stored at -20°C until they were used. After completion of the sex reversal period, fry were fed with the commercial pellet containing 30% crude protein 0.5-mm for 3 months until fish became around 10 g to be dissected for sex-testing using the gonad squash method (Guerrero and Shelton, 1974). Every 2 weeks, fish in each aquarium were group-weighted to adjust feed according to their biomass.

Average water temperature during this experiment was maintained at 27-28°C with 12:12 light: dark photoperiod cycle using fluorescent tubes as a light source. Average dissolved oxygen, total ammoniacal nitrogen concentration and pH were in the ranges of 6.54-6.73 mg/L, 0.23-0.32 mg/L and 7.72-7.74, respectively. were. There were no significant differences in water quality parameters among the treatments and these ranges are suitable to promote good growth of Nile tilapia.

Sex Determination

After the nursing period, the fish were dissected and isolated male (testes) and female (ovary) gonads. Three-hundred fish from each treatment were

Table 1. Composition of the experimental diets for sex reversal (21 days)

Ingredients	No MT + 80% FCM (C1)	100% MT + 80% FCM (C2)	100% MT + 80% FCM + bull urine (BU)	50%MT + Goat testes (GT)	50% MT + catfish testes (CT1)	NO MT+ catfish testes (CT2)
FM (g)	19.34	19.34	19.34	0.00	0.00	0.00
FCM (g)	77.36	77.36	77.36	0.00	0.00	0.00
Catfish testis (CT) (g))	0.00	0.00	0.00	0.00	96.70	96.70
Dried goat testis (GT) (g)	0.00	0.00	0.00	96.70	0.00	0.00
Vitamin C (g/100g)	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix (g)[1]	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix (g)[2]	0.30	0.30	0.30	0.30	0.30	0.30
Total (g)	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Solvents used to dissolve steroid hormone						
Alcohol (mL)	12	12	0	18	18	24
Bull urine (mL)	0	0	12	0	0	0
MT standard solution	0	12	12	6	6	0

[1] Mineral premix (g/kg of diet): calcium biphosphate, 20 g; sodium chloride, 2.6 g; potassium chloride, 5 g; magnesium sulphate , 2 g; ferrous sulphate, 0.9 g; zinc sulphate, 0.06 g; cupric sulphate, 0.02 g; manganese sulphate, 0.03 g; sodium selenite, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004 g.

[2] Vitamin premix (IU or mg/kg of diet): vitamin A, 500,000 IU; vitamin D3, 100,000 IU; vitamin E, 10,000 IU; vitamin K,800 mg; vitamin B1,250 mg; vitamin B2, 1200 mg; vitamin B6, 750 mg; vitamin B12, 5 mg; vitamin B5, 3000 mg; vitamin B3, 2150 mg; biotin, 25 mg; folic acid, 300 mg; inositol, 25,000 mg; Selenium, 30 mg; Iron, 20,000 mg; Zinc, 32,000 mg; Copper, 2000 mg.

Table 2. Proximate compositions (%) of feeds on dry matter basis (Mean±SE)

	C1	C2	BU	GT	CT1	CT2
Dry matter (%)	91.8±0.1^{d}	91.6±0.1^{d}	82.3±0.0^{a}	89.2±0.1^{c}	89.2±0.1^{c}	87.2±0.0^{b}
Moisture (%)	8.2±0.1^{a}	8.4±0.1^{a}	17.7±0.0^{d}	10.8±0.0^{b}	10.8±0.1^{b}	12.8±0.0^{c}
Crude protein (%)	56.1±0.4^{a}	56.6±0.6^{a}	55.8±0.2^{a}	67.6±0.4^{b}	69.9±0.4^{c}	69.9±0.2^{c}
Crude lipid (%)	16.3±1.7^{b}	17.8±0.6^{b}	18.9±1.1^{b}	12.2±0.2^{a}	10.7±0.0^{a}	10.7±0.1^{a}
Crude fiber (%)	4.9±0.0^{c}	4.9±0.1^{c}	4.5±0.1^{b}	0.2±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}
NFE [1]	14.0±1.6^{b}	12.1±0.1^{ab}	10.6±1.3^{ab}	10.7±0.2^{ab}	10.3±0.4^{ab}	9.3±0.3^{a}
Crude Ash (%)	8.7±0.01^{a}	8.6±0.02^{a}	10.1±0.02^{e}	9.2±0.0^{c}	9.0±0.03^{b}	9.9±0.03^{d}
GE [2] (kJ / kg)	5284±89^{a}	5379±25^{a}	5379±59^{a}	5414±5^{a}	5382±5^{a}	5348±7^{a}

anesthetized using 60 mg/L of MS222 (Ethyl 3-aminobenzoate methanesulfonate). Three randomly selected gonad samples were placed on a slide and stained with indigo-carmine (Guerrero and Shelton, 1974; Wassermann and Afonso, 2002). Then, the stained tissue was observed under light microscope (Huma Scope Advanced ^{LED} 40X power with 0.5X CCD adapter) to evaluate the quality of sex-reversal fry (Bhujel et al., 1998)

MT Hormone Analysis

Chemicals and Regents

Standard MT powder and internal standard testosterone were purchased from Sigma-Aldrich (Germany). Acetonitrile, methyl alcohol, MTBE (Methyl tert-butyl ether) and ethanol were all HPLC grade (Sigma-Aldrich, Germany).

Sample Extraction

Water and fish samples were taken from each tank on the 7th and 21st days of the sex-reversal period and stored at -80°C until analysis. Two hundred and fifty mL of water sample were collected from each tank and filtered through 0.2 µm syringe membrane filter (Whatman). Extraction of the hormone from whole fish was modified from Chu et al. (2006) and Vinarkwong et al. (2018). The whole fish was grounded in liquid nitrogen. One gram of the ground tissue was mixed with 0.4 µg of testosterone and 950 µL of MTBE. The mixture was vortex 30 sec and centrifuged at 16,000x g force (RCF) at 10°C for 10 min. The clear supernatant was collected and evaporated to dryness, using a N_2 gas blower. The residue was reconstituted with 500 µL of acetonitrile. The sample was vortex for 30 min, sonicated for 5 min and centrifuged at 19,000x g force (RCF) at 10°C for 10 min. The clear supernatant was then collected.

Analytical Procedure

The method was modified from Vinarukwong et al. (2018) and Chu et al. (2006). High performance liquid chromatography (HPLC, CERI, Tokyo) with L-column ODS (C18, 5µm, 12nm) 4.6 mm inner diameter (I.D.) × 150 mm length (L) was held at 40°C. Detection of analysis was at UV 210 nm and the flow rate was 0.8 mL/min. A mixture of acetonitrile and Milli Q water (60/40) was used as mobile phase and methanol was used as a blank sample. Methanol was used to dissolve the MT hormone standard. The standard solutions of MT were prepared at the following concentrations: 0.05, 0.1, 0.5, 1.0, 10, 50 µg/L. Ten µL of samples were injected into the column using an auto-sampler. Each sample was run in triplicate.

Histological Analysis

Three fish from each treatment were sampled and dissected at the 112th day. Their testes, liver and intestine were isolated and immediately fixed in Bouin's solution for 48 hours and then transferred to 70% alcohol solution. Afterwards, the samples were dehydrated in a series of alcohol solutions, placed in xylene and then embedded in paraffin. The tissue blocks were sectioned with a thickness of 0.5µm and stained with Hematoxylin and Eosin (H&E). Tissue sections were observed under a CX 31 Olympus (Japan) microscope.

Data Analysis

MS Excel was used for the data compilation and statistical package for social sciences (SPSS ver. 22.0 Inc.) was used for the statistical analysis. All results were considered significant either at the 5% or 1% significance levels. One-way analysis of variance (ANOVA) was performed to evaluate the effects of factors, and linear and non-linear regression were used to determine the cause-and-effect relationship; and Turkey's HSD post-hoc test was used for the group comparison.

Results

Sex-reversal

The average sex ratio of fry in C1 fed without any source of steroid hormones was approximately 58: 42 (male: female) which was close to the natural ratio of 50:50 (male: female) (Table 3). In contrast, all other treatments produced 88.1 to 98.4% male, significantly higher than the C1 group ($P<0.05$). However, percent of males in the CT2 group, whose diet contained catfish testes but MT, were significantly lower than those of the groups fed with MT-containing diets.

During the first 21 days of sex reversal feeding and 90 days nursing trials, all experimental diets were well ingested by the fish. The initial average weight of fry was 0.01±0.00 g and the final weight ranged from 0.14-0.17

Table 3. Percentage of males determined by using the gonad squash method

	C1	C2	BU	GT	CT1	CT2
Sample 1	60.0	98.4	100.0	95.4	98.5	89.6
Sample 2	60.7	99.2	96.9	93.9	96.0	86.6
Sample 3	54.6	97.5	98.4	95.2	95.3	88.0
Average	58.4 ± 1.9^{a}	98.4 ± 0.5^{c}	98.4 ± 0.9^{c}	94.8 ± 0.4^{c}	96.6 ± 1.0^{c}	88.1 ± 0.9^{b}

and 2.12-3.78 g during sex-reversal and nursing period, respectively (Table 4). All the groups fed with any source of steroid hormones during the sex-reversal period had significantly higher weight gain and SGR in both phases than the C1 group, in which no source of steroid hormones was included.

Survival rate of the fry ranged from 65.6-78.4 and 79.1-86% during the sex-reversal and nursing period, respectively. Feed conversion ratio (FCR) of the fry cultures during sex-reversal ranged from 1.21-1.69 among the different groups. The BU group fed with the bull urine-containing diet showed the lowest FCR during this period, compared with the other groups. Compared with that during sex reversal, FCR during the nursing period was in the higher range between 1.65-1.93 and the values were significantly ($P<0.05$) different among the treatments.

Biweekly growth curve (Figure 1) clearly showed that the fry fed with the Control diet without MT grew significantly slower than all the other groups during nursing period. Addition of MT, bull urine, Catfish testis, and goat testis have benefit on growth performance in addition to the sex-reversal.

Table 4. Growth performance during sex-reversal phase and subsequent nursing of the fry fed with different sex-reversal diets

Sex-reversal (21 days)	C1	C2	BU	GT	CT1	CT2
IW (mg)	0.01±0.00 ^{a}	0.01±0.00 ^{a}	0.01±0.00^{a}	0.01±0.00 ^{a}	0.01±0.00 ^{a}	0.01±0.00 ^{a}
FW (g)	0.14 ±0.00 ^{a}	0.16±0.00 ^{ab}	0.16±0.00^{c}	0.16 ±0.00^{ab}	0.17±0.00^{b}	0.17±0.00^{bc}
WG (g/fish)	0.13±0.00 ^{a}	0.14±0.00 ^{b}	0.14±0.00^{b}	0.15±0.00^{b}	0.15±0.00 ^{b}	0.15±0.00 ^{b}
Survival (%)	72.0±1.0 ^{b}	70.3±0.7 ^{ab}	78.4±0.7 ^{c}	65.6±2.4 ^{a}	68.7±1.3 ^{ab}	69.9±0.9 ^{ab}
FCR	1.69±0.00 ^{d}	1.53±0.00 ^{cd}	1.21±0.00 ^{a}	1.55±0.10^{cd}	1.45±0.00^{bc}	1.37±0.00^{b}
FCE	0.59±0.01 ^{a}	0.65±0.01 ^{ab}	0.83±0.01 ^{d}	0.65±0.02^{ab}	0.69±0.02^{bc}	0.73±0.01 ^{c}
PER	0.97±0.01^{bc}	1.06±0.02 ^{c}	1.22±0.01 ^{d}	0.85±0.03^{a}	0.88±0.02 ^{ab}	0.91±0.01^{ab}
SGR (%)	11.3±0.1 ^{a}	11.7±0.0 ^{b}	11.8±0.0^{bc}	12.0±0.1^{cd}	12.0±0.0^{cd}	12.1±0.1^{d}
Nursing phase (90 days)						
IW (g)	0.17±0.00 ^{a}	0.19 ±0.00^{bc}	0.19±0.00 ^{b}	0.19±0.00^{bcd}	0.20±0.00 ^{cd}	0.20±0.00 ^{d}
FW(g)	2.12±0.00 ^{a}	2.90±0.00 ^{b}	3.07±0.10^{bc}	3.32±0.00 ^{cd}	3.78±0.00 ^{de}	3.56±0.10 ^{de}
WG (g/fish)	1.96 ±0.00 ^{a}	2.71±0.00 ^{b}	2.88±0.10^{bc}	3.12±0.00 ^{cd}	3.58±0.00 ^{e}	3.36±0.10 ^{de}
Survival (%)	79.1±0.6 ^{a}	82.9±1.2 ^{ab}	82.0±1.8 ^{ab}	85.8±1.6 ^{ab}	84.9±1.0 ^{ab}	86.0±2.0 ^{b}
FCR	1.93±0.10 ^{c}	1.65±0.00 ^{a}	1.70±0.00 ^{ab}	1.87±0.00^{bc}	1.66±0.00 ^{a}	1.76±0.10^{abc}
FCE	0.39±0.02 ^{a}	0.35±0.01 ^{c}	0.34±0.00^{bc}	0.31±0.01 ^{ab}	0.35±0.01 ^{c}	0.33±0.00^{abc}
PER	0.83±0.03 ^{ab}	0.91±0.00 ^{b}	0.89±0.00 ^{ab}	0.81±0.00 ^{a}	0.87±0.00 ^{ab}	0.90±0.00 ^{b}
SGR (%)	2.01±0.00 ^{a}	2.7±0.01 ^{b}	2.22±0.00^{bc}	2.26±0.00 ^{c}	2.34±0.00 ^{d}	2.29±0.00 ^{cd}

Note: All values are mean ± SE, calculated from three replicates. Values for each experimental group in the same row followed by different superscripts are significantly ($P<0.05$) different. SE=standard error. IW= Initial weight; FW= Final weight; WG=weight gain; FCR= Feed conversion ratio; FCE= Feed conversion efficiency; PER= Protein efficiency ratio; SGR= Specific growth rate

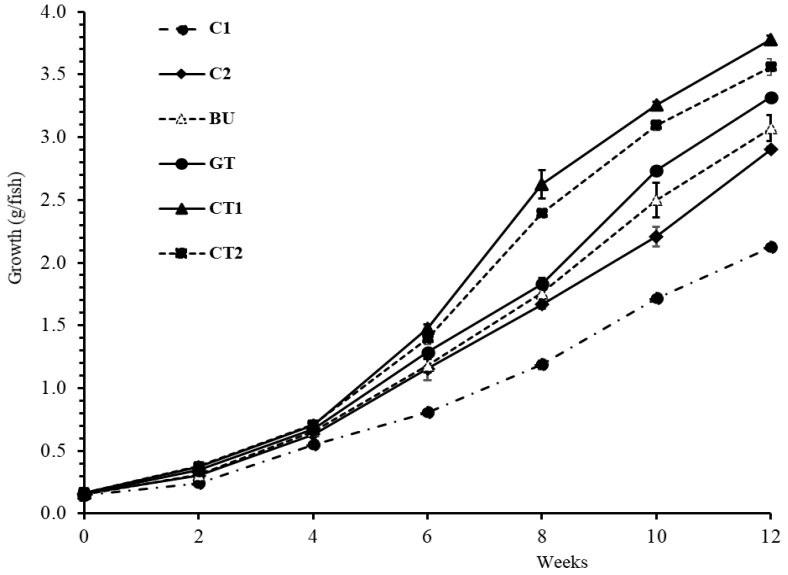

Figure 1. Growth curve of the tilapia fry/fingerling during the nursing period

Histological analysis

Liver of the fry offered with MT-containing diets (C2, BU, GT and CT2) showed signs of congestion, necrosis, hemorrhage, hemolysis, and congested blood vessels (Figure 2) while that of the fry fed with no-MT diets (C1 and CT1) was in perfectly healthy condition. Clear hemolysis and hemorrhage were evident in liver of the 100%-MT diet group (C2). On the other hand, testicular tissue of the 3-month-old males was all similar and in normal condition (Figure. 3).

Analysis of MT Residuals in Water and Tissue Samples

Representative examples of chromatograms of an MT standard, MT extracted from the rearing water sample and from a whole fish sample are shown in Figure 4. Testosterone (T) was used as an internal standard to calculate the efficiency of the extraction.

The residual amounts of 17-α MT and added T in water and fry were calculated, using linear regression equations (For MT: y=8010x+50.518, R^2=0.99; for natural testosterone T: y= 7863.2x+3766.8, R^2=0.99),

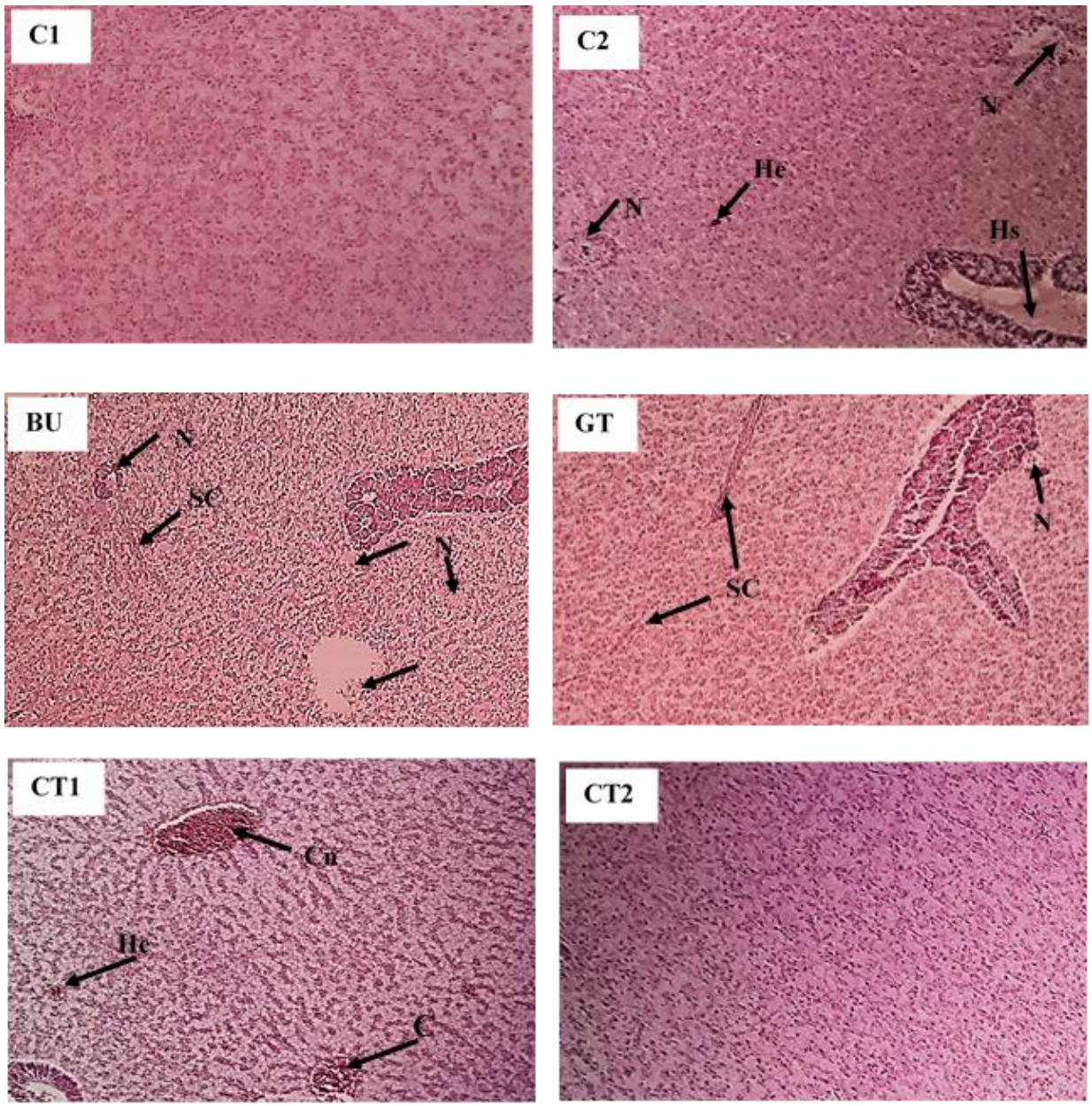

Figure 2. Histological changes of dissected liver from *O. niloticus* after the nursing period. Light micrographs of a histological samples with Hematoxylin and Eosin (40×). (C1) Liver tissue of C1 showing normal histological structure of hepatocytes, a quite homogeneous cytoplasm and sinusoids. (C2) Liver tissue of *O. niloticus* subjected to 60 mg MT/kg diet shows hemolysis (Hs), necrosis (N), and hemorrhage (He) (BU) Liver tissue of *O. niloticus* exposed to 60 mg MT.kg diet with bull urine shows necrosis (N), and sinusoidal congestion (SC) (GT) Liver tisue of *O. niloticus* exposed to 30mg MT/kg diet with goat testis shows necrosis (N) and sinusoidal congestion (SC). (CT1) Liver tissue of *O. niloticus* exposed to 30mg MT/kg diet with catfish testis shows congested blood vessel (Cn), and hemorrhage (He). (CT2) Liver tissue of *O. niloticus* subjected to 0% MT diet with catfish testis showing normal histological structure.

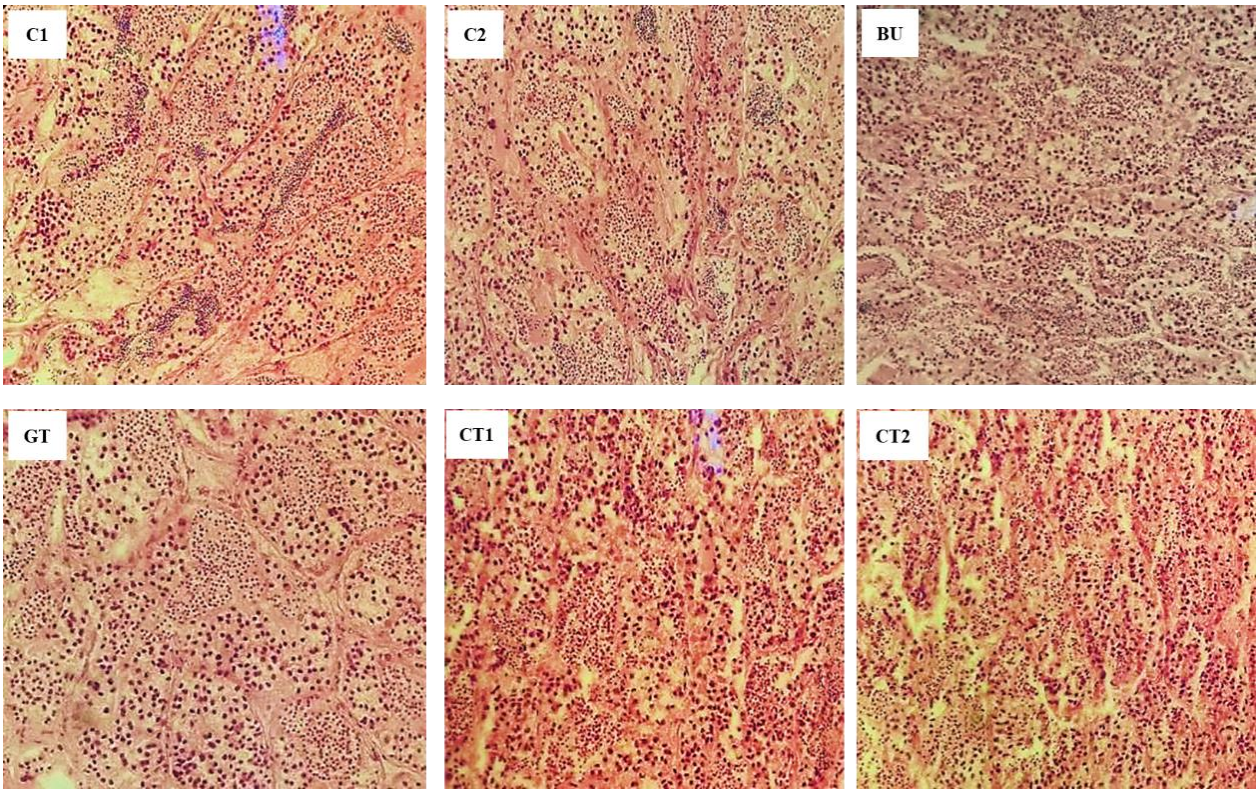

Figure 3. Testicular histology of 3-month-old male tilapia (n=6). Light micrographs of histological samples with Hematoxylin and Eosin (40×). **(C1)** Control C1, No MT hormone, **(C2)** Standard diet 60 mg MT/kg diet, **(BU)** standard MT diet 60 mg MT per diet+ bull urine, **(GT)** goat testis + 30 mg MT /kg diet, **(CT1)** Catfish testis +30 mg MT/kg diet, **(CT2)** Catfish testis + No MT.

derived from the plots of standard solutions MT residue amounts in water and fry at the 7th and 21st days were showed in Table 5. During sex reversal, MT was detected only in the water and fish samples derived from the groups fed with MT-added diets (C2, BU, GT and CT1). The levels of MT in the water and tissue were in accordance with the amounts of MT included in the diets. Interestingly, the levels of MT residual in the rearing water of all groups at Day 21 were lower than those at Day 7. This trend was more evident in the fish tissue, in which the levels of all groups decreased to the non-detectable level at Day 21. Residues of natural testosterone (NT) present in the animal testes were detected neither in the water nor in the fish.

Discussion

A synthetic steroid hormone known as MT hormone is widely used for the commercial production of mono-sex tilapia seed in Asia, because it ensures production of all-male or nearly all-male (99-100%) fry. Earlier research used fishmeal as the sole protein source in conjunction with MT hormone to produce sex-reversal fry (Macintosh and Little, 1995; Little et al., 2003; Bhujel, 2014). However, due to problems with fishmeal, the current research is using a combination of 20% fishmeal and 80% fermented cornmeal (FCM) as the main protein sources (Perera & Bhujel, 2021), along with different sources of testosterone to avoid adverse effects. The practice has been adopted around the world due to its efficiency in producing predictable results. Any alternatives would not be accepted by the commercial producers unless the new method assures the result of over 95% male populations when tested on the mass-scale production. Many attempts have failed to meet this high requirement. Therefore, the present study was an attempt using a different approach i.e., refining the current method instead of exploring the complete replacement. The present study clearly indicated that partial (50%) replacement of the MT as well as other problematic ingredients i.e., alcohol and fishmeal could be the practical solution. In the previous study (Perera and Bhujel, 2021), field cricket meal was found to be suitable to replace up to 80% of fishmeal. The present study was to continue to find solutions for other ingredients such as alcohol and MT hormone in addition to the use of fishmeal. Bull urine has been found to be as effective as ethanol to replace half of its volume. The first half of the ethanol is necessary for MT hormone to be dissolved as it is steroid and requires an organic solvent. A preliminary trial was conducted and has been found successful when ethanol was fully replaced by acetone (Bhujel, personal communication). As acetone is not an alcohol, it is acceptable by Muslims communities, and it is a byproduct produced after burning the fat in the body of human.

Regarding the alternative to synthetic steroid MT hormone, so many efforts have been made to find solutions especially herbal or plant base, but no method is considerably effective (Gabriel et. al., 2015). Some

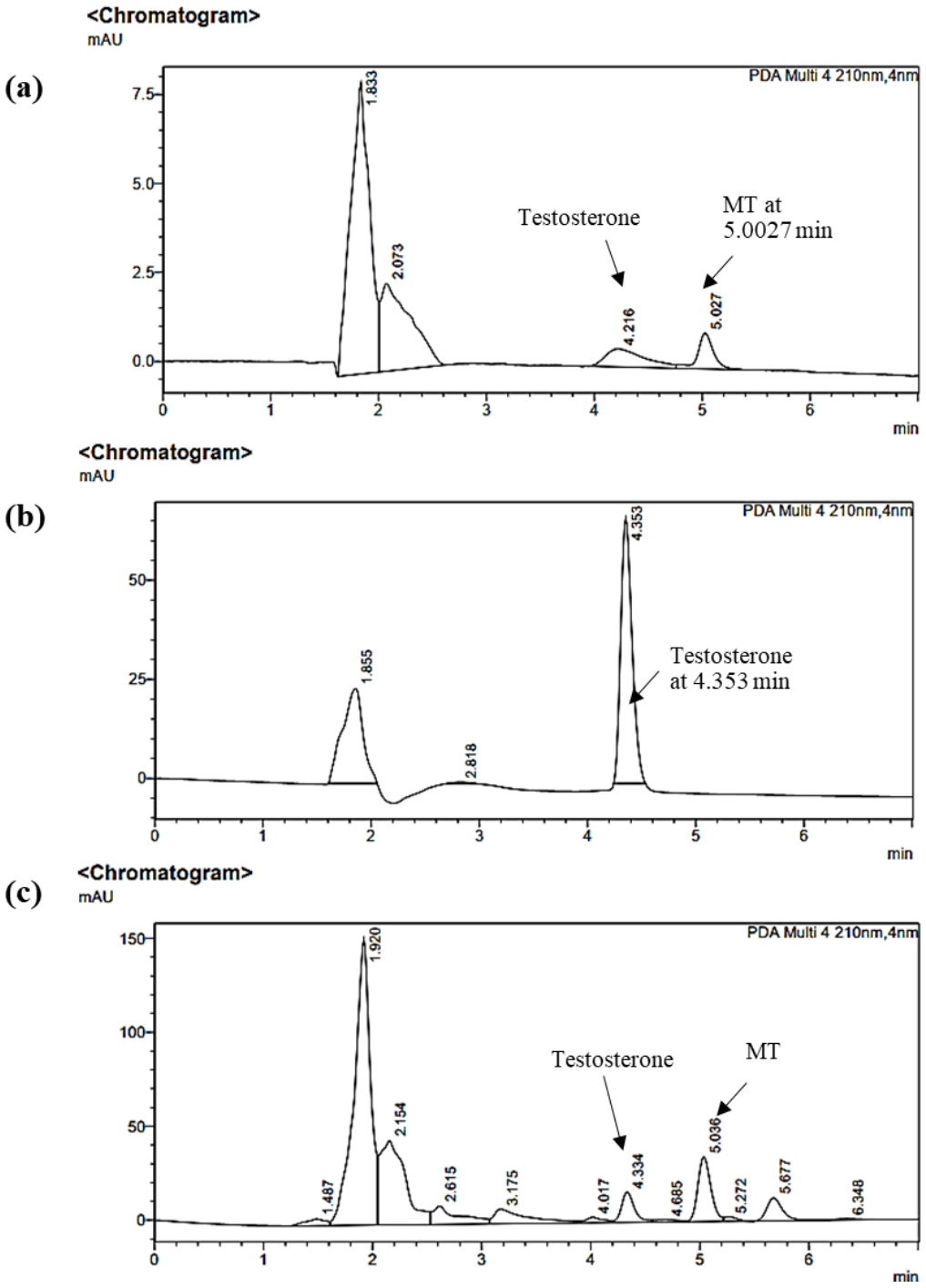

Figure 4. HPLC chromatograms of (a) standard solution of MT (1 mg/L) containing Testosterone (T) as an internal standard (IS) (b) T recovered spike from the IS-only solution (60 mg/L) (c) MT recovered spike of the extract from the fry of tilapia (MT at 5.0036 min; T at 4.334 min).

Table 5 Determination of MT residue in water and fry on Day 7 and Day 21 during the experiment (Mean±SE).

Treatment	MT Residual (μg/L) in rearing water				MT Residual (μg/g) in Nile tilapia			
	Day 7	RSD%	Day 21	RSD%	Day 7	RSD%	Day 21	RSD%
C1	ND	-	ND	-	ND	-	ND	-
C2	468.1^{c}±10.3	3.81	315.8^{b}±16.2	8.90	2.35^{b}±0.14	8.06	ND	-
BU	406.1^{b}±10.9	4.64	260.3^{b}±4.7	3.12	2.31^{b}±0.11	6.53	ND	-
GT	246.3^{a}±11.6	8.17	184.5^{a}±6.1	5.73	1.23^{a}±0.03	3.59	ND	-
CT1	284.8^{a}±7.4	4.53	180.5^{a}±19.0	18.20	1.33^{a}±0.06	5.97	ND	-
CT2	ND	-	ND	-	ND	-	ND	-

Note: ND = non-detectable. RSD = Relative standard deviation

concerns about the MT use still remaining. MT has been reported to have an inverse relationship between antibody production or immune response with the increased dose of MT (Abo-Al-Ela et al., 2017; Abo-Al-Ela, 2018; Suseno et al., 2020). MT has also been found to have effects on histopathological changes on gill, liver, kidneys, and intestine (Kefi et al., 2012; Suseno et al., 2020). At the same time, there are some claims that it has side effects such as oxidative stress, deterioration on the environment and human health as MT affects the phagocytosis process (Sayed & Khalil, 2016). Therefore, many attempts to explore natural alternative solutions have been made continuously such as use of animal testis and plant origin products.

Regarding the efforts on animal-based products, uses of ram and hog testes has been found to produce up to 85 and 83% male population, respectively (Hylor and Pascual, 1991). Another report showed that using goat testis resulted in 60% males with 66.7% survival rate of fish (Ladu and Madara, 1994). In contrast, Bull testis was tested by Phelps et al. (1996) and reported that it was an ineffective source of testosterone. The amount of Testesterone from the testis remaining in the feed depends on the feed preparation techniques. Along the line on the possibility of testis application, more recent study was on the use of freeze-dried bull testes, which was offered to the fry for 30 days. Nevertheless, its efficiency was only 73% male population males (Seazar et al., 2017). None of these were as effective as compared to that of MT. Even if the testis of hog or bull works, some communities do not accept those testes for examples, hog testes for Muslims and bull testes for Hindus. In the present study, goat and fish testes were selected due to their more universal acceptance.

In the present research, goat testis in combination with 50% less MT of the commonly used dose produced considerably higher percentage of males i.e. 94.8% with similar survival during sex-reversal but higher survival during the nursing period, compared with C1 and CT2. The amount of MT residue in the fish body was also the lowest on the 7th day. Reduction in the application dose by half decreased the residual hormone by almost half in the fish body and also culturing water. Hence, the goat testis in combination with 50% MT in the sex-reversal diet is recommended for farmers as it would give additional benefits of cost reduction.

More importantly, other attempts using testes of various fish species are even more relevant, practical and likely more acceptable. For example, results obtained from the testis of rohu (*Labeo rohita*) were 91.4% male (Khanal et al., 2014). However, the result was s lower than the acceptable level of the commercial famers. Similarly, the testis of common carp (*Cyprinus carpio*) was tried (Ranjan et al., 2015), which gave higher percentage of males i.e. 95.8±7.2% by 100% replacement of MT with the carp testis for 30-35 days. It is reasonably high and can be acceptable in many countries. However, Common carp supply is not widely available enough in many countries to serve the demand, especially in Africa, where it is not a common species. Therefore, testes of African catfish, a common species in Africa, were instead tried in this study. The results showed that using solely catfish testes without MT produced males by 88.1% in the fry population. However, reduction of the MT dose by half from the standard sex-reversal diet formula combined with catfish testes can further increase the percentage of males to 96.6%, comparable to that of the standard diet ($P>0.05$) and meeting the acceptable level of farmers. Reduction of the MT dose in the diet additionally helped to decrease the MT residue in the fish and the culturing water. Reduction of MT by half from the standard diet combined with inclusion of catfish testes are hereby recommended for the sex-reversal diet in Tilapia. This diet formula could be applicable in countries where catfishes are produced or consumed in large quantities, if the testes collection system or unit in slaughtering house could be put in place similar to the collection of pituitary glands, which is used for induced breeding of many other species. As a suggestion for further studies, testing the use of these additives to obtain mono-sex progeny in rainbow trout culture should be considered instead of using of gametes of sex-reversed brood stocks referring Inanan and Yılmaz (2018); Inanan et al. (2016).

Present results showed that a higher amount of MT residue in the water on the 7th day compared to the 21st day. This could be accounted for the higher amount of uneaten feed remaining in the water as earlier-staged fry are offered a higher feeding rate based on their body weight. An earlier report indicated that the MT residue amount in sex-reversal ponds was about 617.4 µg/L (Barbosa et al., 2013), but the current range was about 50% lower than this due to the daily maintenance of water quality. Earlier, MT residue in fish sample was reported to be around 6 µg/kg at the age of 3-4 months (Suseno et al., 2020). Nevertheless, the amount of the present results was higher than this amount on the 7th day, but not detectable on the 21st day. Pandian and Kirankumar (2003) reported that exogenous steroid amount of 5 µg/kg were too risky for human, whereas total testosterone and estradiol amount was 3 µg/kg (Jamieson, 2009). In this scenario, those results suggested that MT residues in fish and water should be maintained at the lowest possible during the early stage, so that it would not pose any risk when they get older and marketable size. Therefore, it suggests that there might be a room to reduce feed thereby the hormone during earlier stages of hormone feeding. However, more research is needed to prove it.

Gonad histology did not show any significant changes among the treatment, but histological analysis of the liver tissue results showed deteriorating effects of MT on the liver tissue. Liver is a target organ for drug metabolism so is easily prone to be damaged by the drug and hormone administrated to the animal. This study showed reduction of MT dose can minimize the damage.

Testosterone was more abundant in African elephant urine (Ganswindt et al., 2002). A minimum

detection level was set at 2 ng/ml for T in urine of male veal calves (Scippo et al., 1993). Ethanol can be naturally produced from glucose by indigenous microorganisms already present in the urine (Saady et al., 1993). An advantage of urine might be that it may serve as solvent to dissolve steroid hormone since it contains substances such as urea, uric acid, creatine and so on. It might help to reduce the use of ethanol while preparing MT feed for sex-reversal as well as improve the immunity due to the balance proximate composition of bull urine (Mohanty et al., 2014). The present study used bull urine to replace ethanol and resulted in significantly higher percent of males 98.4%, s similar to that fed with the standard MT diet. Components of steroid compounds in urine and additionally, ethanol or other compounds can be produced to dissolve steroid MT hormone. For the commercial production, it would create a greater impact and would be acceptable to mono-sex tilapia farmers. Additionally, testosterone already present in the urine helps to induce the male sex ratio. Moreover, bull urine might be easily acceptable in some communities, especially South Asia where cow urine is considered as holy water and has been more used for other applications (Mohanty et al., 2014).

The current experiment used 80% FCM and 20% Fishmeal as the main protein sources for diet C1, C2 and BU to get higher percentage of growth and survival rate (Perera & Bhujel, 2021). The amino acids profile and proximate composition of FCM show that it has adequate nutrients for fish growth, which are close to the values of earlier reports (Wang et al., 2005; Taufek et al., 2016a; 2017; Khan, 2018, Perera and Bhujel, 2021). Fish fed with the testes have significantly higher specific growth rate than those fed with FCM. This could be attributed to the higher protein contents in catfish and goat testes. Catfish and goat testes contain 67-70% protein while FCM had 55-56% protein as showed by the results from the proximate analysis of the feed ingredients in this study. Use of catfish testis or other fish testes, which are normally wasted along with viscera, has additional benefits that they could completely replace fishmeal in the diet of tilapia for sex-reversal. The only question is how they can be collected from slaughterhouses.

There may be an opportunity for some people to collect the testes of all fish species as a new business or as a source of additional income. Further research is needed to examine whether a combination of fish or goat testes with bull urine or other animal urine could help reduce alcohol use. Additionally, there is a need to test acetone to replace the remaining 50% of the required alcohol. Catfish testes were collected after squeezing the sperm, but collecting fresh testes without squeezing the sperm may yield better results in terms of sex reversal. Similarly, testing various combinations of probiotics or enzymes with fish or animal testes may be helpful as well. Therefore, more research should be conducted in that direction.

Conclusions

MT hormone caused some histopathological changes in liver and was detectable in the water and fish body. However, the concentrations did not exceed the limits of synthetic steroid and the MT hormone was not detectable after the 21st day in the fish body. The present study suggests that locally available resources, such as bull urine, catfish testis, and goat testis, can partially or fully replace alcohol, synthetic hormones, and fishmeal. When the sex-reversal diet is composed of 80% field cricket meal and 20% fishmeal, bull urine can reduce at least 50% volume of ethanol without compromising the sex-reversal efficiency (98.4% male). Use of African catfish testis can fully replace fishmeal and reduce the use of synthetic MT hormone by 50%. Almost 97% male population could be achieved, which can be acceptable to the commercial sector. Similarly, if goat testis was used to replace all fishmeal and 50% MT hormone, efficiency of sex-reversal was still reasonably high i.e. nearly 95%, which might be still acceptable for the farmers in some countries.

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