Effect of 17α-methyltestosterone (MT) on osmoregulatory responses and apoptosis in genetically improved farmed tilapia (GIFT), *Oreochromis niloticus* (L.)

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Androgenic compounds can affect osmoregulatory response and apoptosis in fish. In the present study, we exposed genetically improved farmed tilapia (GIFT) *Oreochromis niloticus* (L.) to 17α-methyltestosterone (MT, 0.5 and 5 mg/L) for 7, 14 and 21 days for understanding the phenomenon. The activities of Na^+/K^+ ATPase (NKA) and Ca^{2+}/Mg^{2+} ATPase (CMA) were measured in the gill, kidney and intestine to evaluate the change in osmoregulation of GIFT, and genotoxicity was also detected. Results showed that organic NKA were significantly decreased in 5 mg/L MT exposure groups. The intestine NKA was significantly increased (0.5 mg/L MT). MT exposures increased the CMA of kidney and intestine (0.5 mg/L), together with gill CMA (5 mg/L MT). The results of genotoxicity assay showed gill *atp*1*a*1*a* and *nkcc*2 transcripts significantly increased, while intestine *atp*1*a*1*a* and *fxyd*7 transcripts revealed significant increases for MT exposure groups. Caspases proteins demonstrated significant increases at 7th and 21st day, and their transcripts were enhanced in 0.5 mg/L MT exposure groups. The results have evidently demonstrated that chronic exposure of MT could result in organic osmoregulatory response and hepatic apoptosis in GIFT *O. niloticus*.

Keywords: Aquatic pollution, Fish farming, Masculinisation, Nile tilapia

The artificial androgenic compound, 17α-methyltestosterone (MT) is often used to induce masculinization of both secondary sexual characters and gonads in aquatic field studies[1]. For example, male tilapia and Yellow catfish has grown faster than females[2], and MT-immersion for sexually immature hatched larvae has been used to produce mono sex male population. The normal dose of MT used in fish farming was 60 mg/L. The residual MT can be detected in waste water obtained from the Beijing area of China[3]. Nile tilapia, *Oreochromis niloticus* (L.), (Fam. Cichlidae) is adapted to freshwater and seawater[4]. It is sensitive to the oxidative stress of pollutants and can be treated as a kind of ideal model fish for toxicity experiment[5]. Genetically improved farmed tilapia (GIFT), a tropical species, is suitable for culture in warm waters, and is sensitive to aquatic environmental factors[6]. Aquatic organisms are known to have well-developed osmoregulatory apparatus to regulate fluid and ion transport when faced with changes in water osmolality and to maintain a constant body osmolality. Ion-osmoregulation is vital for the maintenance of tissue and cellular functions. The gills of fish are recognized as highly efficient ion-osmoregulatory apparatus evolved to adapt to large changes in external osmolality[7].

Fish genotoxicity, the more sensitive indicator to pollution[8], might be a useful biomarker in eco-toxicological studies. Oxidative stress is known to generate reactive oxygen species (ROS), mainly in the mitochondria, and Na^+/K^+ ATPase (NKA) and Ca^{2+}/Mg^{2+} ATPase (CMA) are associated with ion transporting[9]. Osmoregulatory responses in their main target organs (gill, kidney and intestine) are sensitive to heavy metals[10]. Redox imbalance has been shown to induce apoptosis in male mouse somatic and spermatogonial stem cells11. Zhou *et al*.[12] reported that bisphenol A (BPA) inhibits germ cell nest breakdown by interfering with oxidative stress and apoptosis pathways.

There is limited scientific data concerning the toxicity to understand the possible cytotoxic effects

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clearly, including oxidative stress, osmoregulatory response and apoptosis. The present study hypothesized that organic osmoregulatory response and hepatic apoptosis could be impaired by MT exposure via detecting tissue NKA and CMA, hepatic caspases protein and their transcriptional assay.

Materials and Methods
Experimental design
Fertilized eggs of GIFT, *Oreochromis niloticus* were obtained from Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences, Yixing. One-month old *O. niloticus* juveniles were used in the experiment and were acclimatized in the aquarium facility with dechlorinated tap water at 28±1°C, with 14:10 h light/dark cycle. The experimental fish were offered feed twice a day (purchased from Jiangsu Zhe Ya Food. Co. Ltd., China). The experimental conditions were as follows: pH, 7.1±0.5 units; dissolved oxygen (tested by YSI 556MPS, USA), 7.16±0.16 mg/L; total phosphate, 2.16±0.17 mg/L; total nitrogen and ammonia nitrogen (by Nessler's reagent spectrophotometry), 0.52±0.15 and 0.44±0.06 mg/L, respectively; total water hardness (ICP-OES, Optima 7000, PerkinElmer, USA), 194.3±13.0 mg/L CaCO$_3$; COD$_{Mn}$ 12.45 ± 0.65 mg/L. The GIFT tilapia (*n*=90, 69.47±6.13 g, 9.25±1.02 cm) were assigned to three groups in triplicate (*n*=10 per aquarium per time point per concentration). Each group fish were exposed to 0 (control), 0.5 and 5 mg/L MT groups (Sigma-Aldrich, USA), respectively for 7, 14 and 21 d. There were no statistically significant differences in body weight or length in the exposure experiment. During the experiment, no fish mortality was observed.

Fish sampling
At the end of each experimental period, fishes were sacrificed by transaction of spinal cord according to the decision of Ethic Committee of Nanjing Agricultural University. Samples for both gene expression (*n*=3, gill, kidney, intestine and liver) and biochemical analysis (*n*=3, gill, kidney, intestine and liver) were collected. Particularly those tissue samples (gill, kidney, intestine and liver) for gene expression studies were homogenized using Trizol reagent (Invitrogen, USA), frozen in liquid nitrogen and stored at −80°C immediately until the ATPase analyses. Four tissues (gill, kidney, intestine and liver) were homogenized in ice-cold buffer containing 20 mM Tris–HCl, 0.25 M sucrose, and 1 mM EDTA

(pH 7.7) with a ratio of 1/10 at 10,090×g for 2-3 min. Homogenates for four tissues were centrifuged at 13,000×g (4°C) for 20 min. The supernatants for four tissues were collected for determination of total protein levels, ATPase activity and Caspases protein measurement. The experimental methodology was approved by the Institutional Animal Care and Use Committee of the Ministry of Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences. The tests were undertaken in accordance with the National Legislation for Fish Welfare established by the Ministry of Science and Technology of the People's Republic of China (approval ID: 2011AA1004020012).

Determination of osmoregulatory response
The supernatant was analyzed for NKA (cas no. A070-2-2) and CMA (cas no. A070-3-2) activities using the commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The detection protocol followed the manufacturers' instruction. The NKA and CMA activities were quantified spectrophotometrically with a PowerWave XS2 (BioTek instruments Inc, Vermont, USA). Total protein levels were determined according[13] and bovine serum albumin was used as a standard.

Determination of caspases
Hepatic caspase-3, 8 and 9 activities were measured using caspases-3 (cas no. H076), 8 (cas no. H081) and 9 (cas no. H082) Activity Assay Kits (Nanjing Kaiji Biotechnology, China), respectively as previously described[14]. Briefly, liver samples were homogenized in lysate buffer and the homogenate were centrifuged at 15,000×g for 20 min at 4°C. Then, the supernatants were collected. Caspase-3, 8 and 9 activities was measured by using substrate peptides Ac-DEVD-pNA[15].

RNA extraction, reverse transcription (RT) and qRT-PCR
Total RNAs were extracted from all fish gill, kidney, intestine and hepatocytes of GIFT juveniles from MT exposure, the control groups. Total RNA quality check, DNAase treatment, reverse transcription for RNA, cDNAs normalization, qRT-PCR and reference gene selection was performed following Zheng *et al*.[5]. *β-actin* was the most stable reference gene under exposure of MT in our study[5]. The qRT-PCR primers for *β-actin*, *atp*1*a*1*a*, *nkcc*2, *fxyd*7, *caspase* 3, *caspase* 8 and *caspase* 9 are mentioned in Table 1. Caspase gene expression was measured in hepatocytes, while *nkaα1a*, *nkcc1* and

Table 1 — The primers of tilapia used for qRT-PCR in the study

Gene	Primers	Tm (°C)	Accession No.	Product (bp)
β-actin	F: GGTGGGTATGGGTCAGAAAGA R: GCTGTCGTGAAGGAGTAG	59.1	NM_001100.4	124
*atp*1*a*1*a*	F: GCTCCAGAGAGGATTTTGGAC R: CTCCAAGACCTCCCAACTCA	58.7	XM_005266431.4	121
*nkcc*2	F: CAAAGGCTACGGCAAGAACAAT R: AACATCACCCCACAGCAGAGAA	61.9	XM_021467734.1	258
*fxyd*7	F: CTTGCTGTCATTATGTTTGTCT R: GGTACTTCTGTCTTTGGGATTT	56.0	NM_001201424.1	122
caspase 3	F:GGAGTGGACGATACAGACGC R:CGCTGATTCATGCCTGTACTC	60.0	XM_017029506.1	194
caspase 8	F:CAAGCCGATGCCTCAAAC R:ACTTTCCGCTGCTATTTCC	56.8	NM_001206941.2	159
caspase 9	F: CTTCAGCGGAACAGGGTTA R: GAAGGCACTCCAGAAATAAGG	57.0	NM_001007404.2	202

Table 2 — Tissue NKA and CMA activities of *O. niloticus* tissues under MT exposure[*]

Day	Treatment	Gill activity (U/gprot)		Kidney activity (U/gprot)		Intestine activity (U/gprot)	
		NKA	CMA	NKA	CMA	NKA	CMA
7 d	Con	25.66±6.32^a	7.95±1.24^b	30.25±4.92^a	5.12±1.10^b	20.14±3.22^b	4.62±1.10^b
	0.5 mg/L MT	26.00±5.21^a	13.31±1.65^a↑	26.45±2.65^b↓	10.46±0.95^a↑	28.14±2.34^a↑	9.52±1.08^a↑
	5 mg/L MT	19.41±6.04^b↓	11.26±1.23^a↑	19.78±4.19^c↓	6.47±1.00^b	19.54±3.21^b↓	5.61±1.28^b
14 d	Con	25.45±4.25^a	7.02±1.47^b	24.18±3.91^a	6.24±0.58^b	20.47±3.64^b	6.49±0.96^b
	0.5 mg/L MT	19.61±2.32^b↓	4.95±1.24^c↓	25.87±3.47^a	10.47±1.09^a↑	25.31±2.14^a↑	16.47±1.98^a↑
	5 mg/L MT	19.97±1.84^b↓	17.39±1.53^a↑	14.62±2.73^b↓	5.21±0.96^b	16.32±1.05^c↓	4.67±1.83^b
21 d	Con	25.88±3.21^b	12.61±2.31^b	26.96±2.43^a	9.24±1.51^b	29.68±2.65^b	6.75±2.11^b
	0.5 mg/L MT	29.02±3.55^a↑	8.52±2.63^c↓	24.65±2.11^a	14.87±5.32^a↑	33.24±5.32^a↑	9.95±2.47^a↑
	5 mg/L MT	20.12±3.18^c↓	15.41±1.96^a↑	17.11±2.10^b↓	7.32±1.52^b	17.10±1.74^c↓	6.48±1.55^b

[* "↑" stands for up-regulation, and "↓" stands for down-regulation. The different lower-case letters indicate statistically significant differences (P <0.05]

fxyd7 were completed in three tissues (gill, kidney and intestine). The changes of expression levels of these genes after MT exposure were calculated following the reported research[16].

Statistical analysis

Data were tested for normality of distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test) prior to analysis. The data were dealt with one-way ANOVA analysis followed by the LSD test (Ahmad et al., 2006) with SPSS Statistics 25.0 (SPSS Inc., Chicago, IL USA), with P <0.05 indicating an significant difference (different lower-case letter).

Results

NKA and CMA activities

All the experimental data are shown as the mean ± standard deviation of the mean (SD). The tissue NKA (Na$^+$/K$^+$ATPase) and CMA (Ca^{2+}/Mg^{2+} ATPase) activities (gill, kidney and intestine) are depcited in Table 2. Gill NKA activities in 0.5 mg/L MT (17α-methyltestosterone) exposure groups were significantly decreased (P <0.05, 14 d) and induced (P <0.05, 21 d), respectively when compared with the controls. The values of kidney NKA activities were lowest in the 0.5 mg/L MT exposure groups at 7 d when compared with the controls (P <0.05), while intestine NKA activities were significantly induced for 0.5 mg/L MT exposure groups all through the exposure time when compared with the controls (P <0.05). NKA activities were significantly decreased in 5 mg/L MT exposure groups at all exposure time points when compared with the controls (P <0.05).

Kidney and intestine CMA activities were significantly increased for 0.5 mg/L MT exposure groups when compared with the controls (P <0.05). Gill CMA activities were significantly increased (P <0.05, 7 d) and decreased (P <0.05, 14 d and 21 d) for 0.5 mg/L MT exposure groups, respectively when compared with the controls. Gill CMA activities were significantly induced for 5 mg/L MT exposure groups when compared with the controls (P <0.05).

Caspases activities

The activities of hepatic caspase 3 were revealed in Fig. 1A. Caspase 3 activities showed significant increase for 0.5 mg/L MT exposure at 7 d and 14 d when compared with the controls (P <0.05), while those only revealed significant increase for 5 mg/L
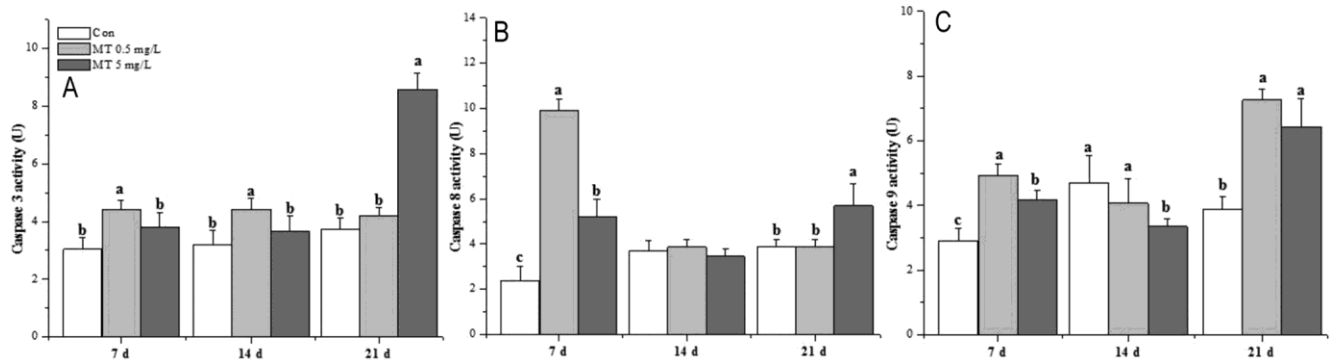
Fig. 1 — (A-C) Caspase 3, 8 and 9 activities of *Oreocromis niloticus* tissues under MT exposure. [The different lower-case letters indicate statistically significant differences ($P <0.05$)]

MT exposure at 21 d when compared with the controls ($P <0.05$). Caspase 8 activities showed significant increase for 0.5 mg/L MT exposure at 7 d (increased by 417%, $P <0.05$), while those revealed significant increase for 5 mg/L MT exposure 7 d (increased by 218%, $P <0.05$) and 21 d (Fig. 1B, $P <0.05$) when compared with the controls. Caspase 9 activities showed significant increase for MT exposure (0.5, 5 mg/L) both at 7 d and 21 d (Fig. 1C, $P <0.05$), while those demonstrated significant decrease for 5 mg/L MT exposure at 14 d (decreased by 140%, $P <0.05$) when compared with the controls.

Osmoregulatory response and apoptosis

The gene expression profiles of osmoregulatory response signaling pathway (*atp*1*a*1*a*, *nkcc*2 and *fxyd*7) were revealed in Fig. 2. Gill *atp*1*a*1*a* and *nkcc*2 transcripts showed significant increase ($P <0.05$) when compared with the controls, while intestine *atp*1*a*1*a* and *fxyd*7 transcripts revealed significant increase for MT exposure at all the exposure time, except for 0.5 mg/L MT exposure at 7 d ($P <0.05$) when compared with the controls. However, Kidney and intestine *nkcc*2 transcripts demonstrated as significant decrease for both 0.5 and 5 mg/L MT exposure groups at 21 d ($P <0.05$) when compared with the controls. Kidney *atp*1*a*1*a* transcripts only showed significant increase for 5 mg/L MT exposure groups at 7 d ($P <0.05$) when compared with the controls.

The hepatic gene expression profiles of apoptosis signaling pathway (*caspase* 3, *caspase* 8 and *caspase* 9) were revealed in Fig. 3. Both, *caspase* 3 and *caspase* 8, transcripts of hepatocyte showed significant increase and decrease in 0.5 and 5 mg/L MT exposure groups at 7 d ($P <0.05$), while *caspase* 9 revealed significant increase only for 5 mg/L MT exposure ($P <0.05$) when compared with the controls. *caspase* 3, *caspase*

8 and *caspase* 9 transcripts were revealed significant increase only in 0.5 mg/L MT exposure groups at 14 d ($P <0.05$), and those showed significant increase in a dose-dependent manner at 21 d ($P <0.05$) when compared with the controls.

Discussion

We know MT has the potential to induce oxidative stress, our previous study showed that hepatic antioxidant enzymatic activities were increased in tilapia under MT exposure[17]. The present study firstly focused on related organic osmoregulatory response and hepatic apoptosis in tilapia under MT (17α-methyl-testosterone) exposure. The exposure period and the concentration were chosen according to the previous studies[17,18]. Our results have demonstrated that MT stimulates adaptive response, eg., increased expression of some antioxidant enzyme genes; oxidation production and the depletion of antioxidant enzymes. However, antioxidative responses were different between higher MT (5 mg/L) and lower MT (0.5 mg/L) treated groups. The current data together with our previous data[17,18] suggest that higher concentration of the testing toxicants or even long-term exposure could alert impairment of tissues[18].

Usually, the biomarker genes tested to verify the potent signaling pathway involved in the cascade of osmoregulatory response. These genes for this study contains: *atp1a1a*[9,19], *nkcc*2[19,20] and *fxyd*[21,22]. When fish gills face acute transfer, phosphocreatine will act as an energy source to meet the osmoregulatory demand through the activity of NKA activity. Hence, *atp*1*a*1*a* (NKA), *nkcc*2 (NKA transporter) and *fxyd*7 (NKA transport regulator) were detected in three osmoregulatory response related tissues in the present study. NKA activity showed the induced impairment of osmoregulation in lowest copper exposure groups,
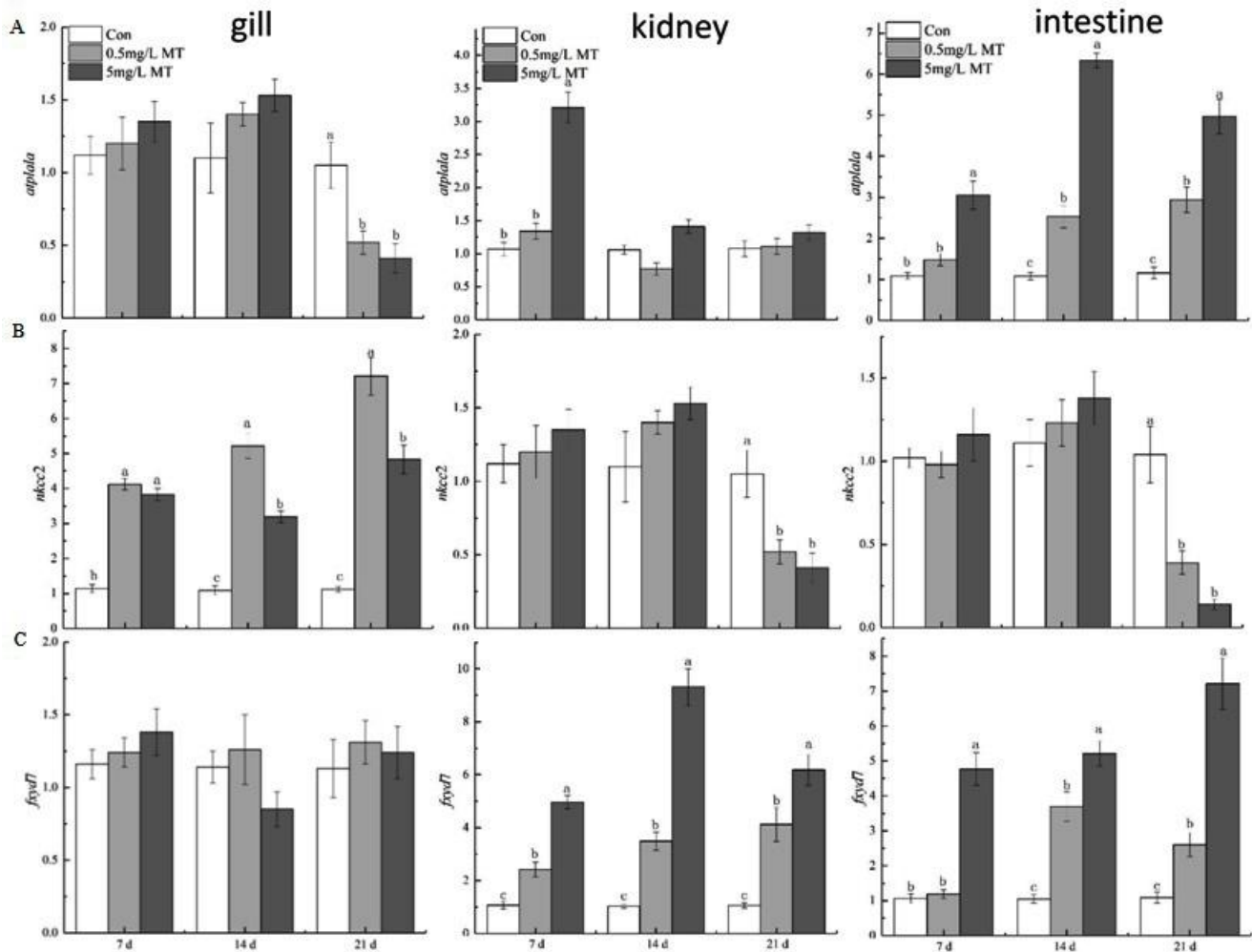
Fig. 2 — Transcriptional osmoregulatory response related gene expression profiles in different *O. niloticus* tissues under MT exposure. (A) *atp*1*a*1*a*; (B) *nkcc*2; and (C) *fxyd*7
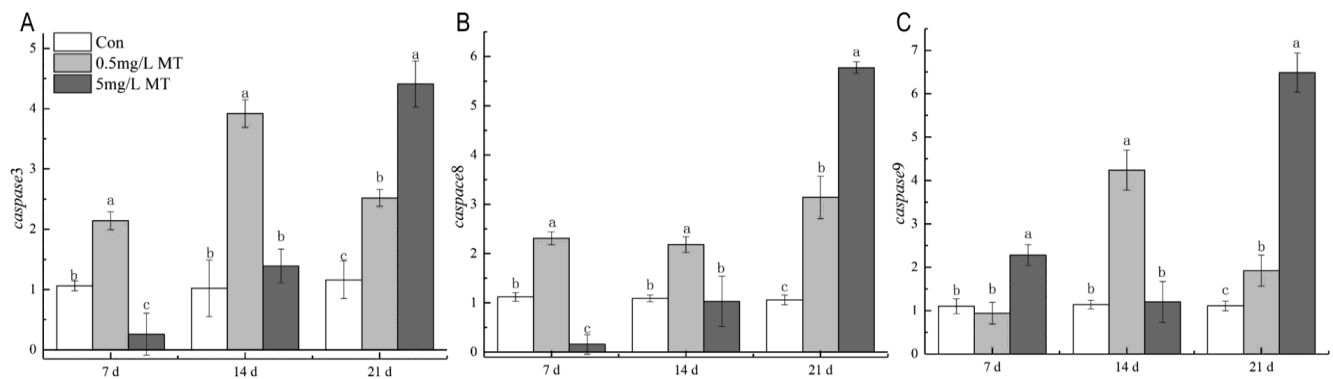

Fig. 3 Hepatic apoptosis related gene expression profiles in *O. niloticus* tissues under MT exposure. (A) *caspase* 3; (B) *caspase* 8; and (C) *caspase* 9.

but not to higher exposure levels[23]. The decline in NKA was presumably mediated by the combined action of deficient mitochondrial electron transport chain activity[24]. Renal NKA was found increased in

tilapia under cadmium exposure[25]. Branchial NKA could be used as a sensitive biomarker to assess the health status of tilapia population[23], and tilapia gill NKA and Ca^{2+} ATPase could act as sensitive

biomarkers in metal contaminated waters[26]. The key transporters associated with the NaCl transport process were thought to be Na$^+$/K$^+$/2Cl-cotransporter 1 (NKCC2, *nkcc*2)[27]. By interacting with NKA, the phospholemman (FXYD) domain-containing ion transport regulator (*fxyd*) was involved in teleost osmoregulation[21]. FXYD7 (*fxyd*7) was mainly expressed in brain, and the recent study found that FXYD7 could decrease K$^+$ affinity, thus it contributed to stimulation of the enzyme at elevated extracellular K$^+$ concentrations[22]. As of now, the knowledge and function of FXYD7 in tilapia is limited.

The results showed NKA and CMA activities (gill, kidney and intestine) decrease and increase, respectively; kidney and intestine CMA activities were only increased in 0.5 mg/L MT exposure groups in the present study. Gill NKA and CMA activities were found decreased in 0.5 mg/L MT exposure groups, in alignment with the studies on copper exposure[28] and lead exposure[29]. The results also showed gill, intestine *atp*1*a*1*a* and gill *nkcc*2 transcripts, kidney and intestine *fxyd*7 displayed significant increase in the present study as reported earlier[9,19,20,22]. Gene transcriptional levels of ion pumps (such as Na$^+$/K$^+$ ATPase α3-subunit) were revealed as significant increase in tilapia under various stress[19,20]. Two Na$^+$/K$^+$ ATPase α1 isoforms (α-1a and α-1b) are mainly expressed in gill, and α-1a (Na$^+$/K$^+$ ATPase α1 polypeptide 1a, *atp*1*a*1*a*) is mainly expressed in freshwater tilapia[9]. The present study demonstrated NKA and CMA activities could act as sensitive biomarkers of organic osmoregulatory response[30,31] for different concentration of MT exposure in tilapia.

Xenobiotic compounds produced hepatotoxicity in tilapia for recent years[32,33], which resulted in apoptosis by detecting the activities and transcripts of the caspase family[34,35]. Apoptosis was revealed upon tilapia branchial mitochondrion-rich cells in the form of osmoregulatory response under salt water[36]. In the gill cells of euryhaline fish (tilapia), abrupt changes in environmental change modify the sphingomyelin turnover and control the production of free ceramide[14]. In addition, the latter one serves as an important stress signaling molecule resulted in apoptosis[37]. A significant activation of caspase-3, -8, -9 activities was evident in toxicant exposure groups[32,34,35]. Caspase-3 could be used as oxidative stress related predictor of pollution in *Oreochromis niloticus*[34], which was upregulated in the tilapia serum upon exposure to hyperosmotic solutions[35]. When taking tilapia to the perfluorooctane sulfonate (PFOS)

and perfluorooctanoic acid (PFOA) exposure, a significant activation of caspase-3, -8, -9 activities was evident in both PFOS and PFOA exposure groups[32], which suggested pollutants to induce apoptosis with the involvement of caspases in tilapia hepatocytes[32,33]. However, caspase 3 in serum was upregulated when fish had been taken to seawater compared with fresh water groups. It demonstrated that prolonged apoptosis occurred in tilapia exposed to elevated salinity *in vitro*[38]. It means that caspase proteins were induced usually when fish faced to osmoregulatory stress. Hepatic caspase activities (-3, -8 & -9) and their transcripts were enhanced in tilapia under MT exposure in the present study, which demonstrated hepatic apoptosis[18,39,40] were impaired in GIFT under MT exposure. However, the the current data cannot be considered to be a irrefutable evidence. There could be other pathways which may also be affected by MT exposure. MT decreased strippable male ratio, enhanced hatching rates with subsequent oxytocin treatment (5 IU/kg i.v.)[41], changed the expression of the arginine vasotocin receptors[42] and aquaporins (water associated membrane protein channels)[43]. These proteins (oxytocin[44], arginine vasotocin[45] and aquaporin[46,47]) may also be relative to osmoregulatory stress.

Conclusion

The above study of the impact of androgenic compound 17α-methyltestosterone (MT) on the osmoregulatory response and apoptosis in genetically improved farmed tilapia (GIFT), *Oreochromis niloticus* has demonstrated significantly decrease and increase in the organic (5 mg/L MT) and intestine (0.5 mg/L MT) NKA (Na$^+$/K$^+$ ATPase) by 17α-methyltestosterone (MT) exposure. Also, it did increase the CMA (Ca^{2+}/Mg^{2+} ATPase) of kidney and intestine (0.5 mg/L), together with gill CMA (5 mg/L MT) as well. Gill *atp*1*a*1*a* and *nkcc*2, intestine *atp*1*a*1*a* and *fxyd*7 transcripts significantly have also shown increase. Caspases proteins demonstrated significant increase at 7th and 21st day, and their transcripts were found elelvated in 0.5 mg/L MT exposure groups. MT could result in organic osmoregulatory response and hepatic apoptosis in GIFT, while the irrefutable evidence need to be taken.

Conflict of interest

Authors declare no competing interests.

References

1 Lagadic L, Coutellec MA & Caquet T, Endocrine disruption in aquatic pulmonate molluscs: few evidences, many challenges. *Ecotoxicology*, 16 (2007) 45.

2 Chen CH, Li BJ, Gu XH, Lin HR & Xia JH, Marker-assisted selection of YY supermales from a genetically improved farmed tilapia-derived strain. *Zool Res*, 40 (2019) 108.

3 Chang H, Wan Y, Wu S, Fan Z & Hu J, Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. *Water Res*, 45 (2011) 732.

4 Sardella BA, Kültz D, Cech JJ Jr & Brauner CJ, Salinity-dependent changes in Na^+/K^+-ATPase content of mitochondria-rich cells contribute to differences in thermal tolerance of Mozambique tilapia. *J Comp Physiol B*, 178 (2008) 249.

5 Zheng Y, Wu W, Hu G, Zhao Z, Meng S, Fan L, Song C, Qiu L & Chen J, Hepatic transcriptome analysis of juvenile GIFT tilapia (*Oreochromis niloticus*), fed diets supplemented with different concentrations of resveratrol. *Ecotoxicol Environ Safety*, 147 (2017) 447.

6 Zheng Y, Hu G, Wu W, Zhao Z, Meng S, Fan L, Song C, Qiu L & Chen J, Transcriptome analysis of juvenile genetically improved farmed tilapia (*Oreochromis niloticus*) livers by dietary resveratrol supplementation. *Comp Biochem Physiol C*, 223 (2019) 1.

7 Ng HM, Ho JCH, Nong W, Hui JHL, Lai KP & Wong CKC, Genome-wide analysis of MicroRNA-messenger RNA interactome in *ex-vivo* gill filaments, *Anguilla japonica*. *BMC Genomics* 21 (2020) 208.

8 Chakravarthy S, Sadagopan S, Nair A & Sukumaran SK, Zebrafish as an *in vivo* high-throughput model for genotoxicity. *Zebrafish*, 11 (2014) 154.

9 Dave PH & Kwong RWM, Cadmium exposure reduces the density of a specific ionocyte subtype in developing zebrafish. *Chemosphere*, 244 (2020) 125535.

10 Baysoy E, Atli G & Canli M, The effects of salinity and salinity+metal (chromium and lead) exposure on ATPase activity in the gill and intestine of tilapia *Oreochromis niloticus*. *Arch Environ Contam Toxicol*, 64 (2013) 291.

11 Das J, Kang MH, Kim E, Kwon DN, Choi YJ & Kim JH, Hexavalent chromium induces apoptosis in male somatic and spermatogonial stem cells via redox imbalance. *Sci Rep*, 5 (2015) 13921.

12 Zhou C, Wang W, Peretz J & Flaws JA, Bisphenol A exposure inhibits germ cell nest breakdown by reducing apoptosis in cultured neonatal mouse ovaries. *Reprod Toxicol*, 57 (2015) 87

13 Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem*, 193 (1951) 265.

14 Genestier L, Prigent AF, Paillot R, Quemeneur L, Durand I, Banchereau J, Revillard JP & Bonnefoy-Bérard N, Caspase-dependent ceramide production in Fas- and HLA class I-mediated peripheral T cell apoptosis. *J Biol Chem*, 273 (1998) 5060.

15 Peterson QP, Goode DR, West DC, Botham RC & Hergenrother PJ, Preparation of the caspase-3/7 substrate Ac-DEVD-pNA by solution-phase peptide synthesis. *Nat Protoc*, 5 (2010) 294.

16 Livak KJ & Schmittgen TD, Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25 (2001) 402.

17 Zheng Y, Qu J, Qiu L, Fan L, Meng S, Song C, Bing X & Chen J. Effect of 17α-methyltestosterone (MT) on oxidation stress in the liver of juvenile GIFT tilapia, *Oreochromis niloticus*. *SpringerPlus* 5 (2016) 338.

18 Zheng Y, Chen J, Liu Y, Gao J, Yang Y, Zhang Y, Bing X, Gao Z, Liang H & Wang Z, Molecular mechanism of endocrine system impairment by 17α-methyltestosterone in gynogenic Pengze crucian carp offspring. *Ecotoxicol Environ Safety*, 128 (2016) 143.

19 Moorman BP, Lerner DT, Grau EG & Seale AP, The effects of acute salinity challenges on osmoregulation in Mozambique tilapia reared in a tidally changing salinity. *J Exp Biol*, 218 (2015) 731.

20 Seale AP, Stagg JJ, Yamaguchi Y, Breves JP, Soma S, Watanabe S, Kaneko T, Cnaani A, Harpaz S, Lerner DT & Grau EG, Effects of salinity and prolactin on gene transcript levels of ion transporters, ion pumps and prolactin receptors in Mozambique tilapia intestine. *Gen Comp Endocrinol*, 206 (2014) 146.

21 Hu P, Li S, Zhong Y, Mu X, Gui L & Zhang J, Identification of *fxyd* genes from the spotted scat (*Scatophagus argus*): molecular cloning, tissue-specific expression, and response to acute hyposaline stress. *Comp Biochem Physiol B*, 174 (2014) 15.

22 Li B, Hertz L & Peng L, Cell-specific mRNA alterations in Na^+, K^+-ATPase α and β isoforms and FXYD in mice treated chronically with carbamazepine, an anti-bipolar drug. *Neurochem Res*, 38 (2013) 834.

23 Wu SM, Tsai JW, Tzeng WN, Chen WY & Shih WY, Analyzing the effectiveness of using branchial NKA activity as a biomarker for assessing waterborne copper toxicity in tilapia (*Oreochromis mossambicus*): A damage-based modeling approach. *Aquat Toxicol*, 163 (2015) 51.

24 Maiti AK, Saha NC & Paul G, Effect of lead on oxidative stress, Na^+/K^+ATPase activity and mitochondrial electron transport chain activity of the brain of *Clarias batrachus* L. Bull *Environ Contam Toxicol*, 84 (2010) 672.

25 Garcia-Santos S, Monteiro S, Malakpour-Kolbadinezhad S, Fontaínhas-Fernandes A & Wilson J, Effects of Cd injection on osmoregulation and stress indicators in freshwater Nile tilapia. *Comp Biochem Physiol C*, 167 (2015) 81.

26 Eroglu A & Canli M, Effects of Cd, Zn and Cd + Zn combination on ATPase activitiy in the gill and muscle of tilapia (*Oreochromis niloticus*). *Bull Environ Contam Toxicol*, 91 (2013) 420.

27 Hwang P & Lee T, New insights into fish ion regulation and mitochondrion-rich cells. *Comp Biochem Physiol A*, 148 (2007) 479.

28 Kulac B, Atli G & Canli M, Response of ATPases in the osmoregulatory tissues of freshwater fish *Oreochromis niloticus* exposed to copper in increased salinity. *Fish Physiol Biochem*, 39 (2013) 391.

29 Kaya H & Akbulut M, Effects of Waterborne Lead Exposure in Mozambique Tilapia: Oxidative Stress, Osmoregulatory Responses, and Tissue Accumulation. *J Aquat Anim Health*, 27 (2015) 77.

30 Damsteegt EL, Wylie MJ & Setiawan AN, Does silvering or 11-ketotestosterone affect osmoregulatory ability in the New Zealand short-finned eel (*Anguilla australis*)? *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*, 204 (2018) 1017-1028.

31 Kaya H, Aydın F, Gürkan M, Yılmaz S, Ates M, Demir V & Arslan Z, A comparative toxicity study between small and large size zinc oxide nanoparticles in tilapia (*Oreochromis niloticus*): Organ pathologies, osmoregulatory responses and immunological parameters. *Chemosphere* ,144 (2016) 571.

32 Liu C, Yu K, Shi X, Wang J, Lam PK, Wu RS & Zhou B, Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*). *Aquat Toxicol*, 82 (2007) 135.

33 Piner P & Üner N, Oxidative stress and apoptosis was induced by bio-insecticide spinosad in the liver of *Oreochromis niloticus*. *Environ Toxicol Pharmacol*, 36 (2013) 956.

34 Franco JL, Trevisan R, Posser T, Trivella DB, Hoppe R, Martins Rosa J, Fernandes Dinslaken D, Decker H, Inês Tasca C, Bainy Leal R, Freire Marques MR, Dias Bainy AC & Luiz Dafre A, Biochemical alterations in caged Nile tilapia *Oreochromis niloticus*. *Ecotoxicol Environ Saf*, 73 (2010) 864.

35 Kumar VB, Jiang IF, Yang HH & Weng CF, Effects of serum on phagocytic activity and proteomic analysis of tilapia (*Oreochromis mossambicus*) serum after acute osmotic stress. *Fish Shellfish Immunol*, 26 (2009) 760.

36 Inokuchi M & Kaneko T, Recruitment and degeneration of mitochondrion-rich cells in the gills of Mozambique tilapia *Oreochromis mossambicus* during adaptation to a hyperosmotic environment. *Comp Biochem Physiol A*, 162 (2012) 245.

37 Mizushima N, Koike R, Kohsaka H, Kushi Y, Handa S, Yagita H & Miyasaka N, Ceramide induces apoptosis via CPP32 activation. *FEBS Lett*, 395 (1996) 267.

38 Kammerer BD & Kültz D, Prolonged apoptosis in mitochondria-rich cells of tilapia (Oreochromis mossambicus) exposed to elevated salinity. *J Comp Physiol B*, 179 (2009) 535.

39 Zheng Y, Yuan J, Gu Z, Yang G, Li T & Chen J, Transcriptome alterations in female Daphnia (*Daphnia magna*) exposed to 17β-estradiol. *Environ Pollut*, 261 (2020) 114208.

40 Zheng Y, Yuan J, Meng S, Chen J & Gu Z, Testicular transcriptome alterations in zebrafish (*Danio rerio*) exposure to 17β-estradiol. *Chemosphere*, 218 (2019) 14.

41 Viveiros AT, Jatzkowski A & Komen J. Effects of oxytocin on semen release response in African catfish (*Clarias gariepinus*). *Theriogenology*, 59 (2003) 1905.

42 DeAngelis R, Dodd L, Snyder A & Rhodes JS, Dynamic regulation of brain aromatase and isotocin receptor gene expression depends on parenting status. *Horm Behav*, 103 (2018) 62.

43 Xiong Z, Li B, Wang L, Zeng X, Li B, Sha X & Liu H, AQP8 and AQP9 expression in patients with polycystic ovary syndrome and its association with *in vitro* fertilization-embryo transfer outcomes. *Exp Ther Med*, 18 (2019) 755.

44 Martos-Sitcha JA, Wunderink YS, Gozdowska M, Kulczykowska E, Mancera JM & Martínez-Rodríguez G, Vasotocinergic and isotocinergic systems in the gilthead sea bream (*Sparus aurata*): an osmoregulatory story. *Comp Biochem Physiol A Mol Integr Physiol*, 166 (2013) 571.

45 Martos-Sitcha JA, Cádiz L, Gozdowska M, Kulczykowska E, Martínez-Rodríguez G & Mancera JM, Arginine Vasotocin and Cortisol Co-regulate Vasotocinergic, Isotocinergic, Stress, and Thyroid Pathways in the Gilthead Sea Bream (*Sparus aurata*). *Front Physiol*, 10 (2019) 261.

46 Ellis LV, Bollinger RJ, Weber HM, Madsen SS, Tipsmark CK, Differential Expression and Localization of Branchial AQP1 and AQP3 in Japanese Medaka (*Oryzias latipes*). *Cells*, 8 (2019) 422.

47 Kokou F, Con P, Barki A, Nitzan T, Slosman T, Mizrahi I & Cnaani A, Short- and long-term low-salinity acclimation effects on the branchial and intestinal gene expression in the European seabass (*Dicentrarchus labrax*). *Comp Biochem Physiol A Mol Integr Physiol*, 231 (2019) 11.