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The extent to which immunity, apoptosis and detoxification gene expression interact with 17 alpha-methyltestosterone

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ABSTRACT

Innate immunity is the first line of defence against invasion by foreign pathogens. One widely used synthetic androgen for the production of all-male fish, particularly commercially valuable Nile tilapia, *Oreochromis niloticus*, is 17 alpha-methyltestosterone (MT). The present study investigates the effect of MT on innate immunity, cellular apoptosis and detoxification and the mortality rate, during and after the feeding of fry with 0-, 40-and 60-mg MT/kg. Expression analysis was completed on interleukin 1 beta (*il1β*), interleukin 8 (*il8*), tumour necrosis factor alpha (*tnfα*), *CXC2*- and *CC*-chemokines, interferon (*ifn*), myxovirus resistance (*mx*), toll-like receptor 7 (*tlr7*), immunoglobulin M heavy chain (*IgM* heavy chain), vitellogenin (*vtg*), cellular apoptosis susceptibility (*cas*) and glutathione *S*-transferase α1 (*gstα1*). Expression analysis revealed that MT had a significant impact on these genes, and this impact varied from induction to repression during and after the treatment. Linear regression analysis showed a significant association between the majority of the tested gene transcript levels and mortality rates on the 7th and 21st days of hormonal treatment and 2 weeks following hormonal cessation. The results are thoroughly discussed in this article. This is the first report concerning the hazardous effect of MT on a series of genes involved in immunity, apoptosis and detoxification in the Nile tilapia fry.

1. Introduction

Worldwide, the Nile tilapia (*Oreochromis niloticus*) is a commercially important fish. It has excellent culture characteristics as it can efficiently utilize low protein feed, reproduce easily and tolerate a wide range of environmental conditions, stresses and infections [1]. Although many tilapia species are cultured, *Oreochromis niloticus* (Linnaeus, 1758) is the most preferred in more than 50 countries [2]. Currently, aquaculture is a promising global agricultural industry [3]. Nile tilapia aquaculture production around the world resulted in 2,537,492 tons in 2010 and 3,425,121 tons in 2013. Aquaculture production of Nile tilapia in Egypt represented about 81%, 88% and 86% of tilapia yields in 2010, 2012 and 2013, respectively [4].

There is a need for monosex culturing, especially of all-male fish. All-male monosex culture is characterized by greater uniformity in growth, size and body weight, better flesh quality, more rapid growth and the lack of spawning [5–8]. Androgens, such as 17 alpha-methyltestosterone (MT) [9–12], carp testis (natural androgen), are widely used in this respect [13] as well as trenbolone [14] or nonsteroidal aromatase inhibitors, such as fadrozole [15,16], which efficiently produce a higher percentage of male fish. MT has been used for the production of monosex stock in several species, such as yellow catfish [17], spotted scat [18], medaka [19], Atlantic cod [20], and guppy (*Poecilia reticulata*) [21]. In Nile tilapia, masculinization processes begin when larvae are just finished the yolk sac absorption, at which time, they are fed a diet containing MT at doses of 40, 50, 60 or 70 mg MT/kg until 28 days of age

Abbreviations: MT, 17 alpha-methyltestosterone; 11-kT, 11-ketotestosterone; EDCs, endocrine disrupting chemicals; E2, 17 β-estradiol; EE2, 17 α-ethinyl estradiol; il1β, interleukin 1 beta; il8, interleukin 8; tnfα, tumour necrosis factor alpha; ifn, interferon; mx, myxovirus resistance; tlr7, toll-like receptor 7; vtg, vitellogenin; cas, cellular apoptosis susceptibility; gstα1, glutathione S-transferase α1; dph, days post-hatching.

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[9,10,22–24].

There is a great deal of concern about the use of androgens in aquaculture with regard to both the environment and the immunity of the fish. First, several androgens and oestrogens derived from animal wastes are widely polluting the environment, particularly drained agroecosystems [25]. Both 17 β-trenbolone and MT have been detected in the environment [25], producing environmental negative effects [26]. Second, regarding health concerns and immunity, Chen, et al. [27] demonstrated a severe endocrine disruption resulting from the use of MT. MT has a genotoxic effect on human lymphocytes; in a dose dependent manner, it increases the frequency of sister chromatid exchanges and decreases cell cycle kinetics [28]. 11-ketotestosterone (11-kT) inhibits innate immune responses in common carp [29] and three-spined sticklebacks [30], and reduces Immunoglobulin M (IgM) production in various teleost fish species [31–33].

Moreover, antibody producing cells were significantly decreased in Chinook salmon leukocytes cultures containing MT, testosterone or mibolerone [34,35]. High doses of testosterone causes immunosuppression and a compromised T-cell immune response [36,37]. Testosterone microencapsulation implants in gilthead seabream showed a precocious pro-inflammatory tendency, but later, mixed pro-/anti-inflammatory activation [38].

Cytokines are immune modulators and include interleukin1 beta, (first isolated fish interleukin, Il1β), interleukin 8 (Il8), tumour necrosis factor alpha (Tnfα), CXC2- and CC-chemokines, interferon (Ifn) and myxovirus resistance (Mx) [39–43]; they are involved in chemotaxis, complement activation and pathogen killing and opsonization in the phagocytosis process [44,45]. Previously, short exposure to endocrine disrupting chemicals (EDCs) such as chemical pollutants or 17 β-estradiol (E2) induced antioxidant activity and innate immune gene expression, including *il1β*, *il8*, *tnfα*, *CC*-chemokine, *ifnγ* and *mx*, in a concentration-dependent manner in newly hatched zebrafish [43,46]. The other immunity member, toll-like receptors (TLRs), which are present on phagocytic and epithelial cells, are responsible for pathogen recognition [47–49]. Tlr7 can recognize viruses [50], bacteria [51] and parasites [52].

The immunoglobulins of fish contain heavy and light polypeptide chains [53]. IgM is required to combat bacterial and viral infections [54]. In the pre-larval stages of tilapia, IgM is derived from the maternal fish [55]. In Nile tilapia, circulating IgM is significantly affected by an increase or decrease in water temperature and salinity [56]. In humans, it has been found that females have higher plasma levels of IgM than males [57].

The precursor of yolk proteins, vitellogenin (Vtg) is not only considered a nutrient source that is used by the developing embryos, but it also has an important role in innate immunity [58,59]. The expression of *vtg* is induced following exposure to 17 α-ethinyl estradiol (EE2) in the sand goby [60] and cadmium in rainbow trout [61].

Many genes are involved in apoptosis, including cellular apoptosis susceptibility (*CAS*), which has a function in cell proliferation and apoptosis [62] and in protection against apoptotic cell death [63].

Glutathione *S*-transferase (GST) plays a vital role in detoxification and antioxidant defence [64,65]. A synthetic anabolic steroid, stanozolol, increases glutathione reductase activities [66]. Kurtz, et al. [30] found that high levels of circulating 11-kT causes even more oxidative stress. Furthermore, Gst activity has value as a biomarker of environmental pollution [67].

Synthetic steroids caused high mortality in many species [Reviewed by 68]. MT has shown a significant negative effect on the survival rate of fathead minnows [69], and caused a mortality rates, 54.43% and 71.25% for doses of 30 and 50 mg/kg of feed, respectively, in Sabaki tilapia [70].

Based on this information, a better understanding of immune related, cellular apoptosis and detoxification gene expression responses to orally administrated MT in Nile tilapia (*Oreochromis niloticus*) fry is necessary to clarify the relationship between androgens and immunity. The goal of this study is to evaluate the relative change in mRNA levels of *il1β*, *il8*, *tnfα*, *CXC2*- and *CC*-chemokines, *ifn*, *mx*, *tlr7*, *IgM* heavy chain, *vtg*, *cas*, and *gstα1* as well as the fish mortality rate induced by the hormone during and after the use of MT with doses of 0-, 40- and 60-mg/Kg of diet for 21 days.

2. Material and methods

2.1. Fish and culture facilities

This work was reviewed and approved by the Animal Care and Welfare Committee of Kafrelsheikh University, Egypt. All experiments were carried out in accordance with the general guidelines and recommendations of EU Directive 2010/63/EU for animal experiments and the Canadian Council on Animal Care [71].

Nile tilapia (*Oreochromis niloticus*) larvae, with an average weight of 0.01 g, were collected from the commercial fish hatchery of Dr. Salah Ibrahim, Kafrelsheikh, Egypt. About 2520 two-day-old larvae were stocked in nine glass tanks (280 fry in each tank). Each glass tank (80 × 45 × 40 cm) was filled with about 130 L of dechlorinated tap water. The aquaria were continuously aerated, provided with heaters having thermostats to maintain the water temperature at 28 ± 1 °C, and half of the water was exchanged daily to ensure high water quality. The photoperiod was a 14 h light:10 h dark cycle. During the experimental period, the water quality parameters were set as follows: dissolved oxygen 6.7 ± 1.4 mg/L and pH 7 ± 0.4. These parameters were stabilized during the experiments.

2.2. Experimental setup

The larvae were left five days for acclimatization and yolk sac absorption. The fry were fed a powdered mixed ration (40% crude protein) contain a commercial meal (Joe FiD Co., Egypt; 30.0% crude protein, 6.1% crude lipid, 4.5% pure crude fibre, total energy ≥ 4080 kcaL/kg) plus fish meal (Triple Nine, Denmark). The feeding protocol was 2–3 times per day at a daily rate of 30% of body weight.

Three different concentrations (0 mg, 40 mg and 60 mg) of 17 alpha-methyltestosterone (MT) (Argent laboratories Inc., Philippines) were dissolved in 50 ml of absolute ethanol, sprayed and mixed well with each kilogram of ration to create a concentration of 0- (control), 40- and 60-mg MT/kg feed. The feed mixtures were left to completely dry at room temperature, packed in air-tight black containers, and stored in the refrigerator until use. At seven days post-hatching (dph) in which the acclimatization period is finished, every three aquaria were assigned to one group and fed with one of three concentrations of MT. Group 1 received the ration containing the solvent only and served as a control group, while group 2 received 40 mg of MT/kg feed and group 3 received 60 mg of MT/kg feed for 21 days (28 dph). Then, all groups of fry were fed with the same diet, without addition of hormone or ethanol, until 42 dph (the end of the experiment). The assessment of three groups was performed after one week of hormonal treatment (14 dph), at the end of hormonal treatment (28 dph) and 14 days following the cessation of hormonal treatment (42 dph). The daily mortality was also recorded.

2.3. RNA extraction and reverse transcription (RT)

The fry had been fasting for 18 h before each sample was taken.

The total RNA was isolated from the entire fry homogenate ($n = 3$ from each treated group) using TRIzol reagent (easy-RED™, iNtRON Biotechnology, Korea), according to the manufacturer's protocol. RNA integrity and quality were verified through visual inspection of rRNA bands (18S and 28S) in ethidium bromide-stained 1% agarose. The inspection confirmed that RNA was intact. The SensiFAST™ cDNA synthesis kit (Bioline, United Kingdom) was used for reverse transcription (RT) of the isolated RNA following the manufacturer's instructions. The synthesized cDNA was kept at − 40 °C.

2.4. Quantitative real-time PCR (qRT-PCR)

Gene specific primers of *CXC2*-chemokine, interferon (*ifn*), myxovirus resistance (*mx*), toll-like receptor 7 (*tlr7*) and vitellogenin (*vtg*) were designed by primer 3 plus [72]. All sequences of gene specific primers, along with the remaining tested genes, including interleukin 1 beta (*il1β*), interleukin 8 (*il8*), tumour necrosis factor alpha (*tnfα*), *CC*-chemokine, immunoglobulin M heavy chain (*IgM* heavy chain), cellular apoptosis susceptibility (*cas*), glutathione S-transferase α1 (*gstα1*) and β-actin (as a reference gene) are shown in Table 1.

Next, quantitative real-time PCR (qRT-PCR) was performed using the SensiFast™ SYBR Lo-Rox kit (Bioline, United Kingdom) and the Stratagene MX3000P real-time PCR system. The reaction mix consisted of 10 μl of SensiFast™ SYBR Lo-Rox, 0.5 μM of each primer and 2 μl of cDNA. The qRT-PCR cycling parameters were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, annealing for 1 min at 60 °C for all genes, excluding *tnfα* and *cas*, which was 57 °C. The fluorescent signal was measured at the annealing/extension step. Dissociation curve analyses were performed beginning at 65 °C and ending at 95 °C, with incremental increases of 0.5 °C every 5 s to validate the specificity of the PCR amplicons. For all of tested genes, the dissociation curve analysis demonstrated that only one peak existed at the corresponding melting temperature, indicating that the PCR was specifically amplified (data not shown). The reactions were performed duplicately on triplicate samples. The amount of target gene transcript was normalized to β-actin transcript. A threshold cycle (Ct) -based relative quantification of all genes was analysed by the formula $2^{-\Delta\Delta Ct}$ [80]; where $\Delta\Delta C_t = (C_t \text{ (target)} - C_t \text{ (β-actin)})$ treated $- (C_t \text{ (target)} - C_t \text{ (β-actin)})$ control.

2.5. Statistical analysis

An unpaired Student's *t*-test with Welch's correction (without the assumption of equal SDs) was used to determine the statistically significant differences between the treated and control groups. Simple linear regression analysis was performed to identify the relationship between normalized gene expressions and mortality rate. The relative expression data were transformed using log-base-two to ensure that the requirements of homogeneity of variance and normalized statistical distributions were met. GraphPad Prism software version 6 (GraphPrism Software, La Jolla, California, USA) was used for the statistical analysis, and the results were expressed as mean ± SEM. Differences were considered to be statistically significant at *P* values less than 0.05. The significant difference over the solvent controls was also estimated to be $P < 0.04$, $P < 0.006$ and $P < 0.0006$.

3. Results

The modulation of 17 alpha-methyltestosterone (MT) in three different concentrations (0-, 40- and 60-mg/kg of diet) on Nile tilapia were estimated through the analysis of the relative expression of genes involved in innate immune response, apoptosis and detoxification. In all of the genes that were studied, excluding interleukin 8 (*il8*), a dose-dependent induction in mRNA transcript levels at the 7th day of hormonal treatment (14 days post-hatching, dph) was followed by a fluctuating decrease at the 21st day of treatment (28 dph) and after 14 days of hormonal cessation (42 dph).

To characterise the expression patterns of pro-inflammatory mediators, such as cytokines (including interleukin 1 beta (*il1β*), *il8*, tumour necrosis factor alpha (*tnfα*), *CXC2*- and *CC*-chemokines, interferon (*ifn*) and myxovirus resistance (*mx*)), the mRNA levels of these genes in Nile tilapia fry were determined at the 7th and 21st day of treatment and after 14 days of hormonal cessation. On the 7th day of hormonal treatment, fry that were fed a 40- or 60-mg/kg diet, the levels of the tested cytokines increased significantly, excluding the up-regulation of *CXC2*-chemokine (at 40 mg/kg) was insignificant (Fig. 1 and Fig. 2A–C). To illustrate, *il1β*, *il8*, *tnfα*, *CXC2*- and *CC*-chemokines, *ifn* and *mx*, showed significant increases of 3.3-, 1.7-, 1.97-, 1.3-, 2.1-, 2.5- and 1.23-fold, respectively, at a dose of 40 mg/kg ($P < 0.04$) and 4.3-, 1.8-, 3.1-, 3.3-, 4.3-, 4.4- and 3.4-fold, respectively, at a dose of 60 mg/kg ($P < 0.04$). These increases were followed by declines to approximately the normal or subnormal levels (down-regulation) on the 21st day of treatment, but *il8* showed a marked increase, and the only point of significance ($P < 0.04$) that identified was at 60 mg/kg in *il1β*, *il8* and *ifn*, which showed 1.7-, 2.2- and 0.5-fold changes, respectively, and in *CXC2*-chemokine (0.68-fold change) at 40 mg/kg. However, after 14 days of hormonal cessation, all studied cytokines down-regulated and this ranging from 0.74- to 0.25-fold in both treated groups, excluding *il8* and *CC*-chemokine at a dose of 40 mg/kg in which they were close to the solvent control level (Figs. 1–2A–C).

In addition, the transcription levels of toll-like receptor 7 (*tlr7*), immunoglobulin M heavy chain (*IgM* heavy chain) and vitellogenin

Table 1
Sequences of primer pairs used in the quantitative real-time PCR reactions.

Target gene	Forward primer (5\–3\)	Reverse primer (5\–3\)	Accession No.	Reference
il1β	TGCACTGTCACTGACAGCCAA	ATGTTCAGGTGCACTATGCGG	DQ061114.1	[73]
il8	GCACTGCCGCTGCATTAAG	GCAGTGGGAGTTGGGAAGAA	NM_001279704.1	[74]
tnfα	GGTTAGTTGAGAAGAAATCACCTGCA	GTCGTCGCTATTCCCGCAGATCA	NM_001279533.1	[75]
CXC2-chemokine	CTATCCATGGAGCCTCAGGT	CTTCTTGAGCGTGGCAATAA	XM_003452201	This article
CC-chemokine	ACAGAGCCGATCTTGGGTTACTTG	TGAAGGAGAGGCGGTGGATGTTAT	FF279635.1	[76,77]
ifn	AGCACAACGTAGCTTTCCCT	TAAACAGGGCAAACAGGTCA	XM_003460533.2	This article
mx	GGATCCTGATGGAGAGAGGA	GCATTTGACCACCATGTAGC	XM_003460517.2	This article
tlr7	TCAGCAGGGTGAGAGCATAC	ACATATCCCAGCCGTAGAGG	XM_005477981.1	This article
IgM heavy chain	AGGAGACAGGACTGGAATGCACAA	GGAGGCAGTATAGGTATCATCCTC	KJ676389.1	[78]
vtg	AGACCCTCAGTTGCTGGAGT	CGGTGTCGAGAGCTGAGTAG	FJ709597	This article
cas	CAGTCTGTGAAAGGCCACACTATAAG	TCATTGGCTTGTGTTATTCCATGCTTCTG	AF547173	[63]
gstα1	TAATGGGAGAGGGAAGATGG	CTCTGCGATGTAATTCAGGA	EU234530	[64]
β-actin	CAGCAAGCAGGAGTACGATGAG	TGTGTGGTGTGTGGTTGTTTTG	XM_003455949.2	[79]

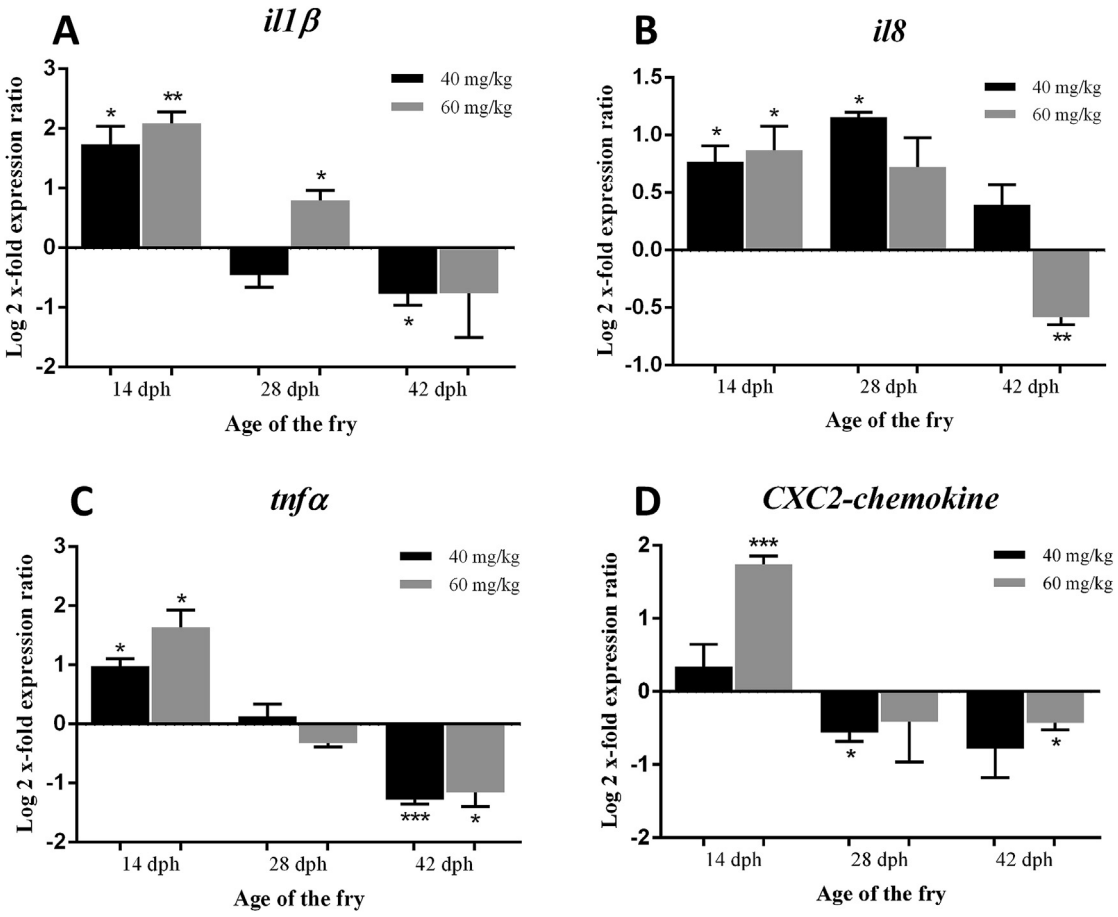

Fig. 1. The relative gene expression levels of interleukin 1 beta (*il1β*) (A), interleukin 8 (*il8*) (B), tumour necrosis factor alpha (*tnfα*) (C) and *CXC2*-chemokine (D) in Nile tilapia fry fed 40 and 60 mg MT/kg compared to control (0 mg MT/kg, represented by *x*-axis) at day 7 (14 dph) and day 21 (28 dph) of hormonal treatment and 14 days after treatment cessation (42 dph). The β-actin was used as a reference gene to normalize the data and the data shown as mean ± SEM (*, **, ***, on the data bars indicate a significant difference over solvent controls, $P < 0.04$, $P < 0.006$ and $P < 0.0006$, respectively).

(*vtg*) were determined (Fig. 2D; and Fig. 3A and B). In both treated groups, the mRNA levels were significantly increased at the 7th day of treatment. Their fold change resulting from doses of 40- and 60-mg/kg as follows: 1.55 and 2.89, respectively, for *tlr7*; 1.5 and 4.17, respectively, for *IgM* heavy chain; 4.2 and 7.2, respectively, for *vtg*. Next, at the 21st day of treatment, the transcription levels decreased but remained higher than those of the solvent control ($P < 0.04$). However, this excludes the mRNA level of *IgM* heavy chain at a dose of 40 mg/kg. Its induction at the 7th day of treatment was accompanied by down-regulation at the 21st day of treatment ($P > 0.05$). The mRNA levels of *tlr7*, *IgM* heavy chain and *vtg* were markedly down-regulated in both doses ($P < 0.04$) after 14 days of hormonal cessation. This excludes *IgM* heavy chain (60 mg/kg dose, 1.18-fold, $P > 0.05$).

The expression level of cellular apoptosis susceptibility (*cas*), which is involved in apoptosis regulation was strongly induced at the 7th day of treatment (40 mg/kg, 1.6-fold, $P < 0.04$; 60 mg/kg 2.5-fold, $P < 0.04$) and then showed a sharp, significant reduction (40 mg/kg, 0.11-fold, $P < 0.0006$; 60 mg/kg, 0.49-fold, $P < 0.006$) at the 21st day of treatment. The low expression level was maintained, but with 0.78-fold ($P > 0.05$) and 0.3-fold ($P < 0.0006$) levels after 14 days of treatment cessation compared to the solvent control for the lowest and highest doses, respectively (Fig. 3C). A similar behaviour of expression was noticed in the detoxification gene that was studied, glutathione *S*-transferase α1 (*gstα1*) which was 40 mg/kg, 1.9-fold, $P < 0.04$ and 60 mg/kg, 2.3-fold, $P < 0.04$ at the 7th day of treatment. By the 21st day of treatment, the transcript levels were suppressed (40 mg/kg, 0.52- fold, $P < 0.006$; 60 mg/kg, 0.74-fold, $P < 0.04$). In addition, after treatment had been stopped for two weeks, the expression remained lower than that of the solvent control (40 mg/kg, 0.7- fold, $P > 0.05$; 60 mg/kg, 0.84-fold, $P > 0.05$; Fig. 3D).

During the experiment, the fry didn't show any signs of disease or infection. Mortality rates were 5, 9 and 10.4% at the 7th day of treatment; 9.2, 23.5 and 26% at the 21st day of treatment; 20, 36.3 and 47.14% after 14 days of hormonal cessation at the doses, 0-, 40- and 60-mg/kg of diet, respectively. A significant correlation ($P < 0.05$) was observed between mortality rates and the expression of the majority of tested genes when subjected to linear regression analysis. The coefficient of determination (R^2) for all of the genes in the case of 40 or 60 mg/kg treatments are provided in Table 2. In the case of 40 mg/kg, *il1β*, *tnfα*, *ifn*, *mx* and *vtg* showed a significant, highly positive correlation ($P < 0.001$). A moderate significant correlation was detected in the *CXC2*-chemokine, *tlr7* ($P < 0.007$), *IgM* heavy chain ($P < 0.005$) and *gstα1*. Low or no correlation was found between mortality and the *il8*, *CC*-chemokine and *cas*. In case of 60 mg/kg, the tested genes showed a significant, highly positive correlation ($p < 0.005$). This excludes *CC*-chemokine, *IgM* heavy chain and *gstα1*, all of which demonstrated a significant moderate correlation ($P < 0.03$). This indicates that mortality is negatively affected by most of the gene expressions that were studied in this article.

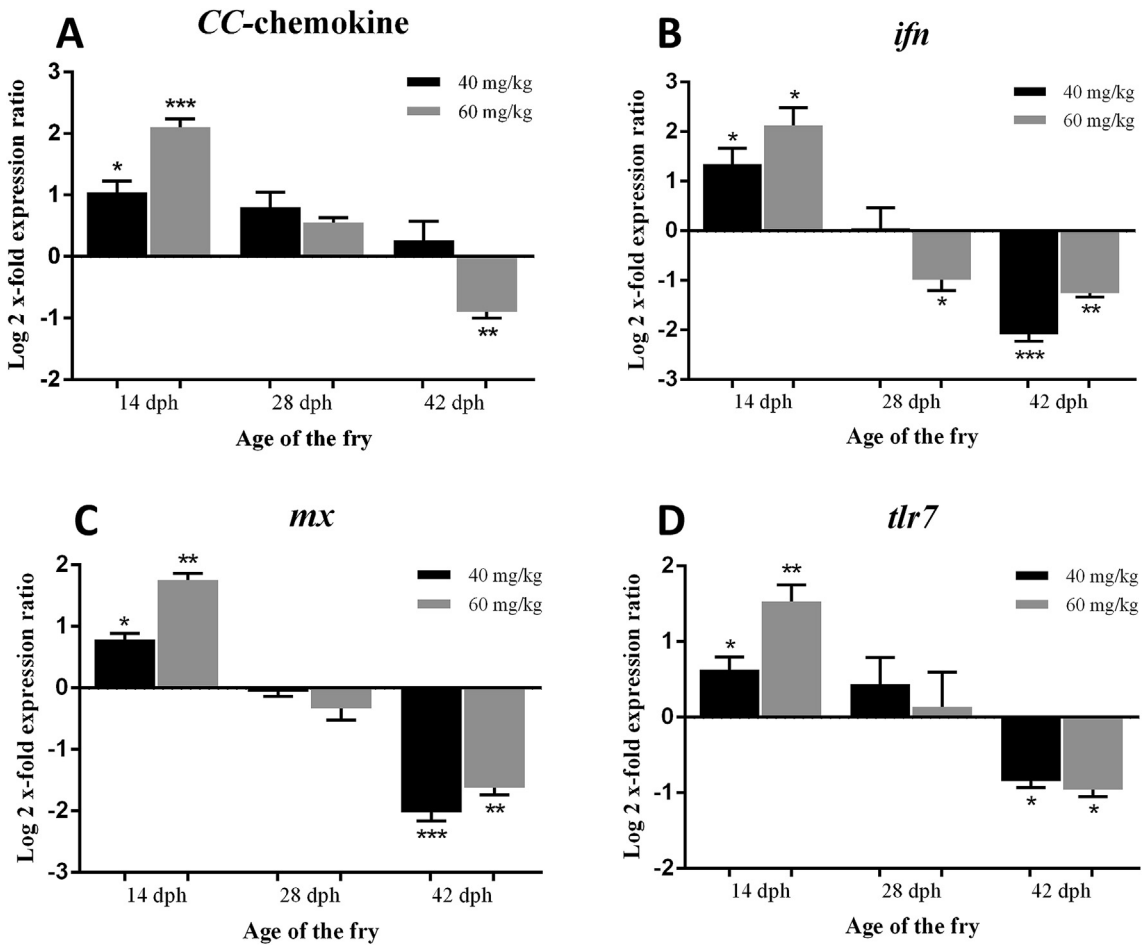

Fig. 2. The relative gene expression levels of *CC*-chemokine (A), interferon (*ifn*) (B), myxovirus resistance (*mx*) (C) and toll-like receptor 7 (*tlr7*) (D) in Nile tilapia fry fed 40 and 60 mg MT/kg compared to control (0 mg MT/kg, represented by *x*-axis) at day 7 (14 dph) and day 21 (28 dph) of hormonal treatment and 14 days after treatment cessation (42 dph). The β-actin was used as a reference gene to normalize the data and the data shown as mean ± SEM (*, **, ***, on the data bars indicate a significant difference over solvent controls, $P < 0.04$, $P < 0.006$ and $P < 0.0006$, respectively).

4. Discussion

Aquaculture is a promising and an important industrial sector. It provides a consistent supply of aquatic species for human consumption. Nile tilapia, *Oreochromis niloticus*, is one of the most important cultured freshwater fish in various countries [77]. Producing a monosex culture that is all-male is vital for aquaculture farming because of their advantages such as the male fish have over female fish. 17 alpha-methyltestosterone (MT) is widely used in the production of all-male Nile tilapia fry [8,10,81,82].

It is well-known that the immune system plays an essential role in the protection against pathogens and external environmental conditions in fish. Fish mainly depend on innate immunity. However, the administration of hormones, especially androgens, in fish has been implicated as a negative regulator of both innate and adaptive immunity. Since infections in aquaculture can cause great losses in fish stock, an immunity deficiency caused by the administration of hormones is likely to endanger commercial fish farming. Studies have reported that testosterone, an important androgen, had a suppressive effect on immunity, particularly on viral and host antigens [83–85]. Sullivan, et al. [86] showed that androgens caused a reduction in the total number of T-cells, lapositive lymphocytes, suppressor/cytotoxic T-cells, helper T-cells and B-cells. The immunosuppressive effect of testosterone is caused by increasing the activity of suppressor T-cells, without directly affecting mature T-cells [87]. However, androgen-deprivation, improved lymphoid haematopoiesis, and, thus, on augmented the immune response [88] by increasing the levels of T-cells and making them proliferate more vigorously in response to antigens [89].

Cytokines are a large category of proteins that are crucial to many immunological processes. In particular, there is a cross-talk between cytokines. To explain, Hong and Secombes [90] found that the administration of the recombinant trout interleukin 1 beta (Il1β) induced gene expression of *il1β*, interleukin-8 (*il8*) and myxovirus *resistance* (*mx*); Il8 induced the transcripts of immunoglobin M (*IgM*) [91] and *Il1β* [92]. In addition, recombinant interferon (Ifn) increased the expression of *il1β*, *il8*, tumour necrosis factor alpha (*tnfα*), *CC*-chemokines and *ifn* [93]. Likewise, a reciprocal induction of expression was found between *il8* and *tnfα* [94]. Meanwhile, the recruitment and activation of phagocytes were mediated by Tnfα which induced of the endothelial cell expression of *CXC*- and *CC*-chemokines [95]. This type of harmonious interaction was seen between the tested cytokines and almost of the remaining tested genes in our study which all of the tested cytokines showed an early up-regulation and a late down-regulation.

Interestingly, in our study, after 7 days of exposure to MT, all of the studied gene mRNA levels were elevated in the Nile tilapia fry. This observation may give an explanation to the elevated levels of both IgA in orchiectomised rats [96] and of IL1β and TNFα in

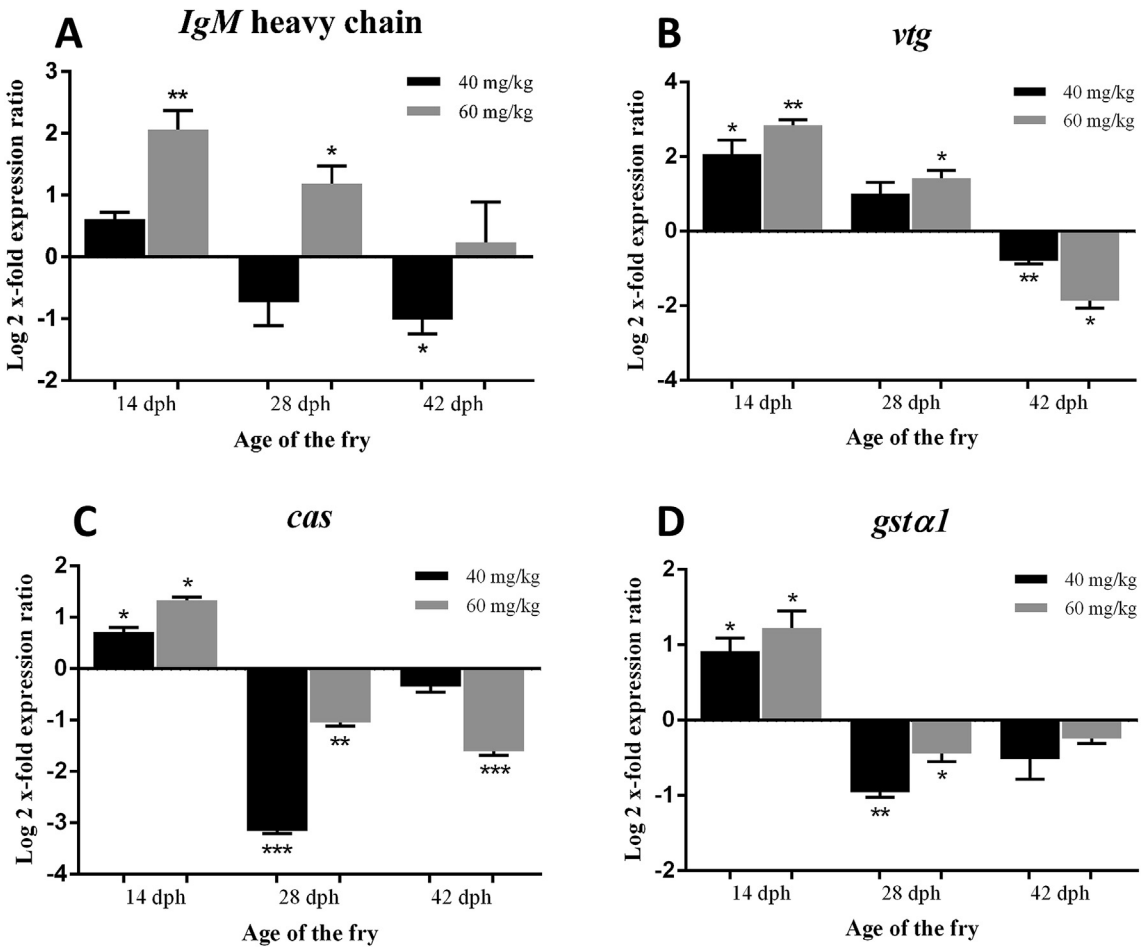

Fig. 3. The relative gene expression levels of immunoglobulin M heavy chain (*IgM* heavy chain) (A), vitellogenin (*vtg*) (B), cellular apoptosis susceptibility (*cas*) (C) and glutathione S-transferase α1 (*gstα1*) (D) in Nile tilapia fry fed 40 and 60 mg MT/kg compared to control (0 mg MT/kg, represented by *x*-axis) at day 7 (14 dph) and day 21 (28 dph) of hormonal treatment and 14 days after treatment cessation (42 dph). The β-actin was used as a reference gene to normalize the data and the data shown as mean ± SEM (*, **, ***, on the data bars indicate a significant difference over solvent controls, $P < 0.04$, $P < 0.006$ and $P < 0.0006$, respectively).

Table 2
R square values of linear regression analysis between cumulative mortality and normalized target gene expression level (fold change).

Treatment	Gene name					
	il1β	*il8*	*tnfα*	*CXC2*-chemokine	*CC*-chemokine	*ifn*
40 mg MT/kg of diet	0.7979	0.1580	0.9244	0.5221	0.4283	0.8814
60 mg MT/kg of diet	0.7715	0.7546	0.8431	0.5624	0.9749	0.7013
Treatment	Gene name					
	mx	*tlr7*	*IgM* heavy chain	*vtg*	*cas*	*gstα1*
40 mg MT/kg of diet	0.9173	0.6595	0.6995	0.8637	0.08932	0.5155
60 mg MT/kg of diet	0.9315	0.8318	0.5758	0.9632	0.8221	0.5228

ovariectomised mice [97] following the administration of testosterone and oestrogen, respectively. Krasnov, et al. [98] observed that there was a sharp decrease in lice infection during sexual maturation of Atlantic salmon. Also, increased levels of *ifn* and *mx* mRNA were observed in Atlantic salmon during the smolting process [99]. This could have resulted from a naturally short elevation in androgen and oestrogen levels.

In our study, the delayed effect of MT on the cytokines in the Nile tilapia was similar to the direct negative effect that testosterone had on the macrophage by reducing the expression of *IL1β*, *TNFα* [100] and IL8 production [101]. Furthermore, dihydrotestosterone inhibited the mRNA expression of *TNF* [102]. Another study found that juvenile Atlantic cod that were chronically exposed to water discharged from the offshore oil industry (produced water) had significantly up-regulated mRNA expressions of *il1β*, *il8* and *IgM* light chains [103]. However, these observations could be due to disparate body responses to different chemicals.

Our results found that MT significantly enhanced the expression of *ifn*, *mx* and the toll-like receptor 7 (*tlr7*) on the 7th day of treatment, although the levels rapidly declined to the normal on the 21st day and reached subnormal after 14 days post-treatment. In fact, according to Christeff, et al. [104], IFN treatment reduced the serum androgen concentrations in humans. Thus, most of the early immune defences, particularly, *ifn*, *mx* and *tlr7*, which are the main

players in antiviral immunity, were dramatically affected by MT.

Low concentrations of 17 α-ethinyl estradiol (EE2) were seen to induce *il1β*, *il8* and *tnfα* mRNA levels in non-activated macrophages, whilst EE2 suppressed *il1β* in activated macrophages [105]. Diazinon, an insecticide, could accelerate mRNA synthesis of *Infγ*, although the translation might be impaired [106].

A Long-term high dose of oral testosterone reduced IgM levels in rainbow trout [31]. Although, in our study, the fry received 60 mg/kg of MT in their diet, showed higher *IgM* heavy chain mRNA levels than the control throughout the study. On the contrary, the fry received 40 mg/kg of MT in their diet showed down-regulation of *IgM* heavy chain on the 21st day of treatment and 14 days post-treatment and this findings agree with the decrease in serum IgM levels seen in men who received oral synthetic testosterone for 6 months [107].

Vitellogenin (Vtg) is an immune related protein and a biomarker that is used to indicate environmental toxicity. Exposure of pengze crucian carp to MT for 4 weeks and juvenile fathead minnows to MT for 3 weeks showed significant increases in *vtg* transcripts compared with the controls [108,109]. In our study, fry that were fed either 40 mg or 60 mg of MT expressed the same effect at day 7 and day 21 of treatment but downregulated after 14 day of treatment cessation.

Previously, Mandarin fish's Tnfα protein induced programmed cell death in HeLa cells [110]. The attenuation of cellular apoptosis susceptibility (CAS) protein reduced the cell's sensitivity to apoptosis by TNF [111]. Meanwhile, the toll-like receptors (TLRs) can induce programmed cell death [44]. Some mammalian viruses can escape from the immune responses by targeting IFN or apoptosis [112]. Besides the detoxification function of glutathione *S*-transferase α1 (Gstα1) is also included in innate immune responses [113]. Both the *cas* and *gstα1* genes showed evidence of up-regulation at 1 week post-treatment of MT. These earlier responses were comparable to the early anti-apoptotic and antioxidant protective effects seen in rats given synthetic testosterone [114] and also, the oxidative response in the liver of largemouth bass following the EE2 feeding [115]. Another potential explanation for this elevation is the anabolic effect of MT, which causes the *cas* to be profusely expressed by the proliferating cells [63]. It is worthwhile to note that, in our study, the increase in both *cas* and *gstα1* genes fell sharply to markedly low levels at day 21 of treatment and after 14 days after hormonal cessation. These results agree with earlier reports in which oral MT (40 and 200 mg/kg of body weight/day) given for 28 days clearly increased the apoptotic germ cells in rat [116].

Our study looked at how a change that affected the immunity of Nile tilapia fry clearly affected its survival. We found a strong correlation between the majority of gene transcript levels and mortality rate. These results agree with the findings of Pandian, et al. [117] which showed that hormonal induction of sex reversal caused low survival rates of converted male and female fish. Juvenile rainbow trout that were given 17 β-estradiol (E2) in feed showed a reduction in survival rate after being challenged with *Yersinia ruckeri* bacteria compared with control populations [118]. Similarly, Junior, et al. [119] showed that the survival rates for Nile tilapia were 54.1% for the control population and 50.3% for the population fed MT for 28 days. On the other hand, increasing the dose of MT, increased the mortality in convict cichlid [120]. In addition, it was shown that fish fed an E2-treated diet had higher survival rate than fish fed an MT-treated diet [121]. At the same time, the mortality rate of septic males (70%) was higher than the mortality rate of septic females (26%) [122], which might be due to elevated systemic anti-inflammatory mediators in females [123]. Mice that were deficient in IgM showed a 70% mortality rate with a reduced TNFα and neutrophil recruitment and an increased peritoneal bacterial load after caecal ligation and puncture [124]. These was along with our regression analysis results, which reveal a significant high and moderate correlation for *TNFα* ($R^2 = 0.92$, 0.84 for 40 and 60 mg/kg, respectively) and *IgM* ($R^2 = 0.7$, 0.58 for 40 and 60 mg/kg, respectively), respectively.

Gene expression studies are known as powerful methods in toxicology and environmental biomonitoring [125,126]. Our results showed that the Nile tilapia fry that were fed 60 mg MT/kg had a lower immunity and higher mortality than the fish fed 40 mg MT/kg. To the best of our knowledge, this paper represents the first study about how MT affects gene expression from the immune, apoptosis and antioxidant views. Our findings established how these systems could be affected by synthetic androgens. There was a striking difference between the early- and late-gene responses toward MT. The analysis of these genes could be taken as an indicator of environmental toxicity. We should be aware that these frequently used endocrine disrupting chemicals (EDCs) not only affect the immune system of fish, but also markedly influence the antioxidant system and even the survival rates.

5. Conclusion

In conclusion, the immune system is considered to be the army of the body protecting the body against a variety of pathogens that cause disease. Various studies showed that hormones and chemicals can alter the capacity of fish to respond to infection. Regardless, further investigations are needed to evaluate the immunological response after a pathogenic challenge. Our results showed that MT disrupted the immunity and detoxification systems and was a negative regulator of innate immunity in Nile tilapia. The effect of MT was reflected in the gene expression of several genes that were relevant to the immune, apoptosis and antioxidant systems. MT treatment was also associated with a higher mortality rate. Further studies should be done to link the change of these gene transcript levels with other immunological parameters and pathogen resistance.

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