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Transcriptome response of pubertal male *Clarias macrocephalus* to dietary 17α-methyltestosterone

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Abstract

We studied the mechanism of the effects of 17α-methyltestosterone (MT) on previously reported enhanced growth and maturation of the Asian catfish *Clarias macrocephalus*. A total of 18 pubertal male (3 fish·tank^{-1}) catfish (ABW=125.97 g) were stocked into six experimental circular tanks and fed with a basal diet (control) or a basal diet containing 60 mg MT kg^{-1} diet for 90 days. At the termination of the experiment, livers were excised, and total RNA was extracted, evaluated, and reverse transcribed to cDNA. Six libraries of cDNA (3 for the control and 3 for the MT-treated group) were subjected to bioinformatics. Differentially expressed genes (DEGs) were identified using similarity scores of the *de novo* assembled transcript with those in the seven public databases. There were 141 up- and 72 down-regulated DEGs, mostly belonging to uncharacterized/not yet identified proteins. Thus, DEGs in the top enriched KEGG pathways were used to characterize the effects of MT. Upregulated DEGs included tyrosine hydroxylase (TH), Cytoplasmic Polyadenylation Element Binding (CPEB), Fas-associated death domain (FADD), Major Histocompatibility Complex class 1 (MHC-1), other immune-related genes, among others. Down-regulated DEGs included Cytochrome P450 family one subfamily A member 1 (CYP1A1), Alternative oxidase (AOX), UDP-glycosyltransferase (UGT), Acyl-CoA Synthase Bubblegum Family Member 1 (ACSBG), Stearoyl-CoA Desaturase (SCD), among others.

In conclusion, MT-treated male *Clarias macrocephalus* exhibited up-regulation of pathways protecting cellular conditions like cell proliferation, survival, development and homeostasis processes, for development and homeostasis processes. MT also affected changes in bile acid production and the inhibition of the production/conversion of testosterone. MT resulted in the down-regulation of a serotonergic system that possibly affected gonadal development, inhibition of the retinoid enzyme that would otherwise diminish the full effects of MT or its metabolite, and inhibition of the formation of estrogen, leading to down-regulation of several estrogen-related KEGG

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pathways. The results provide valuable information about the key genes for use as biomarkers of maturation and reproduction for the Asian catfish and contribute to our understanding of the molecular mechanisms and regulative pathways behind these two processes in fish.

Introduction

17α-methyltestosterone (MT) is a synthetic androgen combining both androgenic and estrogenic effects. It is usually used as a therapeutic medicine to treat breast cancer, delayed puberty and hypogonadism in males, and postpartum breast pain. MT was also widely used for clinical therapy in animal livestock farms. A few years ago, MT was widely used in aquaculture for inducing fish into males for its pronounced effect on masculinization in China (Ong et al., 2012; Xu et al., 2018). Previously, we have reported an evaluation of MT as a growth and reproductive promoter (Bautista et al., 2022a) and quantified doses for maximal responses. To elicit maximal specific growth rate (SGR), gonadosomatic index (GSI), fertilization rate (FR), and hatching rate (HR), dosages of 58.3, 75.0, 90.6, and 78.2 $mg \cdot kg^{-1}$ MT were estimated using a quadratic model in our report.

Other researchers have reported unfavorable effects of MT in other species, while a few, including our report, find it not resulting in any negative alterations in the physiology of the fish. Higher MT concentrations or prolonged treatment could result in paradoxical feminization in which there is gonadal intersexuality in which, instead of inducing the female to become a functional male, the other way around happens. Other adverse effects include testis degeneration (Sayed et al., 2018), decreased GSI (Ahmad et al., 2002), production of vacuolated seminiferous lobules mainly due to testis degeneration (Shen et al., 2015), production of sterile males in grouper and milkfish (Tan-Fermin et al., 1994).

Very little is known about the effects of MT at the molecular level in fish. In the cyprinid fish, *Pseudomonas parva*, MT at 200 ngL^{-1} exhibited more obvious disruption effects on males than females, mainly reflected in the immune system (Wang et al., 2020). These authors found three common pathways, cell adhesion, cytokine-cytokine receptor interaction, and neuroactive ligand-receptor interaction, in both sexes after MT exposure. Gao et al. (2015) studied the effects of MT on the gonad transcriptome response of rare minnow *Gobiocypris rarus*. In males, 268 DEGs with 108 upregulated transcripts and 160 downregulated transcripts were detected upon MT exposure. Four DEGs, namely, ndufa4, slc 1a3a, caskin-2, and rpt3, were found in *G. rarus* of both sexes. These authors observed that MT seemed to affect genes involved in pathways related to physiological processes in the gonads of *G. rarus* which included the electron transfer of Complex IV, endothelial cell activation, axon growth and guidance, and proteasome assembly and glutamate transport metabolic. In male Mandarin fish *Siniperca chuatsi*, MT also activated the expression of male-related genes, such as dmrt1, hsd17b3, hsd11b2, cyp11a1, cyp11b, amh, amhr2, sox9 and gsdf (Zhu et al., 2022). Some of these genes were activated during the spermatogenesis stage, suggesting that the up-regulation of these male-related genes may be the result of sex differentiation rather than the cause. Most studies on the transcriptomic response of fish upon MT exposure are related to sex reversal, and only a few dealt with its effects on gonadal development.

In light of the above, the present study aimed to determine some mechanisms of the transcriptomic response of the pre-pubertal male Asian catfish *Clarias macrocephalus* to dietary MT.

Materials and Methods

Experimental fish and set up

A total of 18 immature male (3 $fish \cdot tank^{-1}$) Asian catfish (*Clarias macrocephalus*) (ABW=125.97 g) were randomly distributed and stocked into six experimental circular tanks (70 cm diameter x 25 cm height) containing 50 L freshwater (salinity of 0 parts per thousand, ‰). Experimental fish were acclimatized to a basal (i.e., no MT) diet for three days before the experiment.

Induced spawning and artificial fertilization

As described previously (Bautista et al., 2022a), females were injected intramuscularly with gonadotropin and dopamine inhibitor (0.5 ml·kg^{-1}; Ovaprim™, Syndel Lab. Ltd., USA) into the dorsal muscle. Stripping was done the following morning, and the dry method of artificial fertilization was carried out. Male Asian catfish were sacrificed, testis excised, cut into small pieces, squeezed onto the stripped eggs in a small bowl, gently stirred with chicken feathers for about 5 min, and the fertilized eggs incubated.

Feeding trials

Two experimental diets were used: a commercial diet with no MT and a commercial diet with 60 mg 100 g^{-1}. The diets were prepared as described previously (Bautista et al., 2022b). Briefly, the commercial feed was pulverized, steamed, and air-dried. MT was dissolved in ethyl alcohol, mixed thoroughly with the steamed pulverized feed, passed through a meat grinder, air-dried, and cut into desired sizes. The control diet was prepared in the same manner except for adding distilled water instead of the hormone solution. The prepared feeds were stored in the freezer until use.

Feeding was *ad libitum,* wherein the amount of feed was measured every first day of the week and was used as the basis for the feeding rate for the whole week. Feeding was monitored and adjusted to stop feeding when no feeding activity was detected or add more feeds when feeds were consumed quickly. Diets were given thrice daily (at 0800h, 1400h, and 2000h). Water temperature, pH, and salinity were measured three times a week, while nitrite and ammonia were monitored once a week. Sampling was done on the first day of stocking and every 15 days by bulk-weighing the fish until the end of the experiment.

RNA Extraction

Total RNA was extracted, and assessed its quality, quantity, and integrity as described previously (Bautista et al., 2022a,b). The absorbance ratio at OD260/280 (Genova Nano Spectrophotometer; Jenway, UK) was determined, and those near 2.0 were selected for subsequent analysis. RNA integrity was evaluated by detecting distinct gel red-stained 28s and 18s ribosomal RNA (rRNA) bands using Bio-Rad Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (Biorad, CA, USA). The RNA samples were then desiccated in a solution of RNAstable® (Biomatrica, San Diego, CA) for 24-h before sending the samples to Novogene Bioinformatics in Beijing, China, for RNA sequencing and bioinformatic analyses.

cDNA Library Construction and Sequencing

cDNA libraries were constructed in which mRNA was enriched using oligo(dT) beads. After fractionation, the mRNA sequences were cut into shorter fragments of approximately 150 bp. These cleaved RNA fragments synthesized the first strand using random hexamers and reverse transcriptase. The second strand was generated by adding a custom synthesis buffer (Illumina) with dNTPs, RNase H, and *Escherichia coli* polymerase I via nick translation. cDNA was purified using AMPure XP beads (Illumina, San Diego, USA) and subjected to end repair, A-tailing, ligation of adapter sequences, and polymerase chain reaction (PCR) enrichment of the purified products to yield the final cDNA library. The cDNA libraries were generated from three replicates of each catfish strain for sequencing on Hiseq Illumina 4000 platform, generating ~150 bp paired-end reads.

Bioinformatics Analysis

a) *Data filtering and De Novo Transcriptome Assembly*

After removing reads with adapter contamination and uncertain and low-quality nucleotides, six libraries were constructed. Clean reads were *de novo* assembled first to generate a reference sequence using Trinity software (Grabherr et al., 2011). The Corset software was used to perform hierarchical clustering to remove redundancy. The most extended transcripts in each cluster were selected to represent a particular unigene.

b) Gene Functional Annotation

Functional annotation of the contigs or genes was done using seven databases. Identities of the genes were determined based on similarity scores with the available sequences in the databases, namely, Nr (NCBI non-redundant protein sequences), Nt (NCBI nucleotide sequences), Pfam (Protein family), Swiss-Prot, KOG/COG (Cluster of Orthologous Groups of Proteins, COG; and eukaryotic Orthologous Groups, KOG), GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genome).

c) Gene Expression Analysis

Bowtie software was initially used to map out reads to the assembled transcriptome. RSEM software was used to analyze the mapping results of Bowtie and quantify gene expression levels by counting the reads that belong to the exon of a particular gene in each sample. The same software also converted the reads into fragments per kilobase of transcript sequence per million mapped reads (FPKM) values.

d) Analysis of Differentially Expressed Genes (DEGs)

The read count values from the gene expression analysis were used as input data for identifying DEGs using DESeq software. This method is based on a negative binomial distribution wherein the *p*-values were normalized first using the *q*-value. A q-value <0.05 and |log2 (fold-change)| >1 was set as a threshold to classify significant differential expressions. GO enrichment of DEGs was conducted using GOseq R packages based on the Wallenius non-central hypergeometric distribution. KOBAS software was used to examine the statistical enrichment of DEGs in KEGG pathways.

e) KEGG Pathway Enrichment Analysis

In this study, the pathways affected by MT treatment in male *C. macrocephalus* were identified by enrichment analysis using the KEGG database. The significantly enriched metabolic or signal transduction pathways mediated by the DEGs were identified.

Results

Transcriptome sequencing and sequence assembly

To identify the genes involved in the Asian catfish response to dietary MT, we created six *c*DNA libraries from *m*RNAs extracted from the liver of catfish fed the control diet and MT diet. For each group, high-throughput RNA sequencing resulted in 52.50 - 60.63 million paired-end reads (**Table 1**). After filtering for low-quality reads and adapter sequences, 51.48-59.41 million clean reads (97.21-98.80% from raw data) were generated. From the clean data of the six libraries, about 80.53 to 82.16% of clean sequences were successfully mapped to the *de novo* assembled transcript (**Table 5**). The assembly was performed using the combined clean reads from the six libraries. *De novo* assembly using the Trinity software produced 76,274 unigenes (including contigs and singletons ranging from 301 to 18,360 bp; **Tables 2 and 3**), with an average length of 1,195 bp (N50=2126 bp; **Table 3**). The 76,274 unigenes were searched against 7 public databases (**Table 4**). Of these unigenes, 29,764 (39.02%) showed significant BLASTx matches in the NCBI_NR database, and 21,832 (24.62%) showed significant matches in the Swiss-Prot database. There were 46,958 unigenes (61.56%) annotated in at least one database, which means that 29,316 unigenes (38.44%) were without BLASTx hits. Large numbers of the unigenes were revealed to be short fragments (300-500 bp; 38.13%), and some of these might be non-coding RNA sequences or new genes.

Table 1 Basic statistics of RNA-seq reads obtained from Illumina HiSeq4000.

Sample	Raw Reads	Clean Reads	Clean Bases	Error(%)	Q20(%)	Q30(%)	GC (%)
C1	57439776	55572250	16.7G	0.03	97.36	92.47	48.30
C2	58154699	57285060	17.2G	0.03	97.06	91.86	47.93
C3	60634990	59413731	17.8G	0.03	96.92	91.57	47.52
T60_1	59639811	58926695	17.7G	0.03	97.10	92.01	47.49
T60_2	52502414	51478044	15.4G	0.03	97.06	91.87	47.29
T60_3	57077393	55484168	16.6G	0.03	96.96	91.71	47.77

Table 2 Overview of the Number of Transcripts and Unigenes in Different Length Intervals.

Transcript length interval	300-500bp	500-1kbp	1k-2kbp	>2kbp	Total
No. transcripts	43,617	34,655	26,954	36,724	141,950
No. Unigenes	29,085	22,803	11,775	12,611	76,274

Table 3 Overview of the length distribution of transcripts and unigenes.

Type	Min Length	Mean Length	Median Length	Max Length	N50	N90	Total Nucleotides
Transcripts	301	1,549	839	18,360	2,804	587	219,837,002
Unigenes	301	1,195	619	18,360	2,126	457	91,158,362

Table 4 Statistics of successfully annotated genes in *Clarias macrocephalus.*

	Number of Unigenes	Percentage (%)
Annotated in NCBI_NR	29764	39.02
Annotated in NT	40072	52.53
Annotated in KO	15714	20.6
Annotated in Swiss-Prot	21832	28.62
Annotated in PFAM	22723	29.79
Annotated in GO	22717	29.78
Annotated in KOG	9662	12.66
Annotated in all Databases	6072	7.96
Annotated in at least one Database	46958	61.56
Total Unigenes	76274	100.00

Similarity analysis of C. macrocephalus liver transcriptome

Using the BLASTx tool, the organism distribution showed that the unigenes' similarity matched sequences from a range of species. Most of the unigenes had the highest homology to genes from five fish species with 36.6% hits to the iridescent shark catfish *Pangasionodon hypopthalmus*, followed by the channel catfish *Ictalurus punctatus* (19.9%), yellow catfish *Tachysurus fulvidraco* (6.4%), the cyprinid Kanglang white minnow *Anabarilius graham* (2.8%), rainbow trout *Oncorhynchus mykiss* (2.6%) and others (31.9%)(**Figure 1**). These results showed the relatively close phylogenetic relationship of *C. macrocephalus* and these teleosts. It also showed that similarity between these species may have been due to the availability of the near complete genome which consequently supported a higher gene count. Also, these results could be partially explained by the lack of genomic information for *the Clarias* spp. Annotation for *Clarias batrachus* was only 0.43% (data not shown) which was included in the annotation for "other" (31.9%).

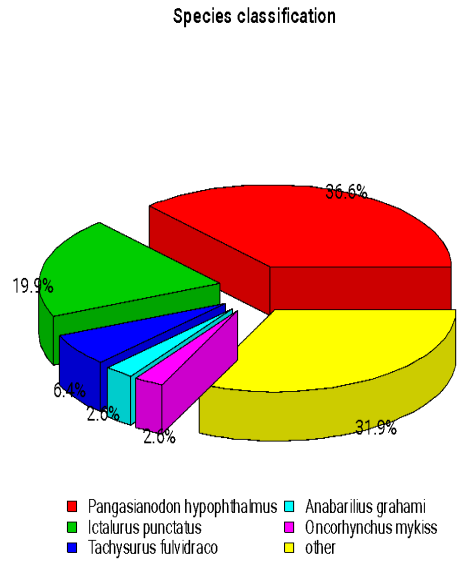

Figure 1 Species distribution results of homology search of RNA-seq sequences against NR database.

Table 5 Summary of the RNA-seq reads and mapping of the six libraries with the de novo assembled transcripts of *Clarias macrocephalus*.

Library	Total reads	Total mapped
C1	111,144,500	91,308,918 (82.15%)
C2	114,570,120	92,375,948 (80.63%)
C3	118,827,462	97,122,040 (81.73%)
T60_1	117,853,390	95,549,346 (81.07%)
T60_2	102,956,088	82,913,182 (80.53%)
T60_3	110,968,336	89,766,834 (80.89%)

Differentially expressed genes (DEGs)

Transcripts were considered significant DEGs when FDR $\leq$ 0.001 and log2 ratio $\geq$ 1. Most unigenes (>90%) were not differentially expressed between MT-treated and control groups. In contrast, 141 transcript-derived unigenes were up-regulated, and 72 were down-regulated (**Figure 2**). In the list of the top 20 up-regulated and top 20 down-regulated DEGs (data not shown), a considerable proportion still needs to be characterized/identified. Only 9 out of 20 were identified, and those unknown proteins fall within the 39.46% unmapped unigenes (**Table 5**). Thus, genes identified in the top 20 enriched KEGG pathways were used to characterize the possible molecular effects of MT on male catfish.

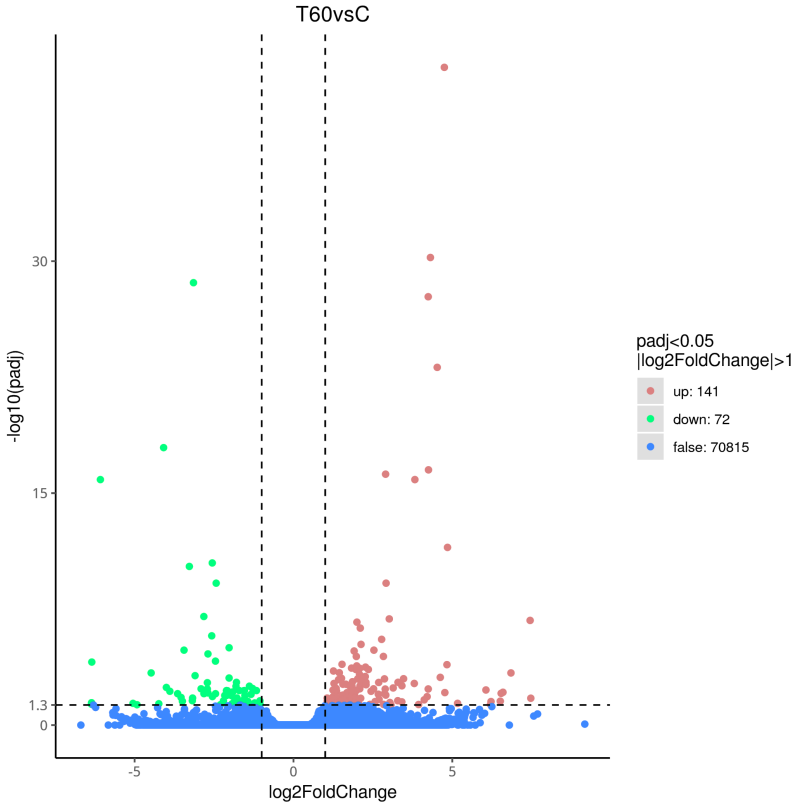

Figure 2 Volcano plot of DEGs between MT60 and control group. Red: up-regulated; Green: down-regulated; Blue: no significant differential expression.

Functional annotation of DEGs

GO items were not significantly enriched after MT exposure. However, top 20 enriched downregulated GO items included oxidoreductase activity, cell wall, thylakoid, embryo development, cell motility, and locomotion, among others (data not shown). In contrast, enriched upregulated GO items include cell-cell signaling, cell wall organization or biogenesis, transmembrane transport, symbiont process, signal transduction, plasma membrane, and endoplasmic reticulum, among others.

After MT exposure of the male catfish, up-regulated enriched KEGG pathways, included were NF-kappa B signaling pathway, viral myocarditis, phagosome, isoquinoline alkaloid biosynthesis, progesterone-mediated oocyte maturation, PPAR signaling pathway, primary bile acid biosynthesis, calcium signaling pathway, adipocytokine signaling pathway, among others (**Table 6**). Eleven KEGG pathways were significantly enriched and were down-regulated, namely, tryptophan metabolism, retinol metabolism, progesterone-mediated oocyte maturation, PPAR signaling pathway, drug metabolism-cytochrome P450, metabolism of xenobiotics by cytochrome P450, breast cancer, estrogen signaling pathway, steroid hormone biosynthesis, chemical carcinogenesis and metabolic pathways (**Table 7**).

Table 6 Top 20 enriched KEGG pathways, name and count of up-regulated DEGs and classes A and B categories of each pathway.

ID	KEGG_A_class	KEGG_B_class	Description	padj	Gene count	Gene name
ko00790	Metabolism	Metabolism of cofactors and vitamins	Folate biosynthesis	0.32932424	2	GGH, TH
ko04320	Organismal Systems	Development and regeneration	Dorso-ventral axis formation	0.32932424	2	CPEB, ORB CPEB, ORB
ko04710	Organismal Systems	Environmental adaptation	Circadian rhythm	0.32932424	2	NR1D1 PRKAB
ko05030	Human Diseases	Substance dependence	Cocaine addiction	0.32932424	2	TH GRIN1
ko05330	Human Diseases	Immune disease	Allograft rejection	0.32932424	2	MHC1 IGH
ko05320	Human Diseases	Immune disease	Autoimmune thyroid disease	0.32932424	2	MHC1 IGH
ko04060	Environmental Information Processing	Signaling molecules and interaction	Cytokine-cytokine receptor interaction	0.32932424	3	CCL19, ELC IL10RB, CD210B IL13RA2, CD213A2
ko02024	Cellular Processes	Cellular community - prokaryotes	Quorum sensing	0.32932424	1	ACSL, fadD
ko04061	-	-	Viral protein interaction with cytokine and cytokine receptor	0.32932424	2	CCL19, ELC IL10RB, CD210B
ko04080	Environmental Information Processing	Signaling molecules and interaction	Neuroactive ligand-receptor interaction	0.32932424	3	ADRA1B GLRA1 GRIN1
ko05031	Human Diseases	Substance dependence	Amphetamine addiction	0.32932424	2	TH GRIN1
ko04920	Organismal Systems	Endocrine system	Adipocytokine signaling pathway	0.32932424	2	PRKAB ACSL, fadD
ko04020	Environmental Information Processing	Signal transduction	Calcium signaling pathway	0.32932424	3	ADRA1B IGH GRIN1
ko00120	Metabolism	Lipid metabolism	Primary bile acid biosynthesis	0.32932424	1	CYP7A1
ko03320	Organismal Systems	Endocrine system	PPAR signaling pathway	0.32932424	2	CYP7A1 ACSL, fadD
ko04914	Organismal Systems	Endocrine system	Progesterone-mediated oocyte maturation	0.32932424	2	CPEB, ORB CPEB, ORB
ko00950	Metabolism	Biosynthesis of other secondary metabolites	Isoquinoline alkaloid biosynthesis	0.32932424	1	TH
ko04145	Cellular Processes	Transport and catabolism	Phagosome	0.32932424	3	MHC1 IGH CALR
ko05416			Viral myocarditis	0.32932424	2	MHC1 IGH
ko04064	Environmental Information Processing	Signal transduction	NF-kappa B signaling pathway	0.32932424	2	CCL19, ELC IG

Table 7 Top 20 enriched KEGG pathways, name, count of down-regulated DEGs and classes A and B categories of each pathway.

ID	KEGG_class_A	KEGG_class_B	KEGG_Desc.	padj	Gene Count	Gene name
ko00250	Metabolism	Amino acid metabolism	Alanine, aspartate and glutamate metabolism	0.10994877	4	CYP1A1 E1.13.11.11, TDO2 E1.13.11.11, TDO2 AOX
ko00254	Metabolism	Biosynthesis of other secondary metabolites	Aflatoxin biosynthesis	0.15726003	2	CYP1A1 UGT AOX
ko00471	Metabolism	Amino acid metabolism	D-Glutamine and D-glutamate metabolism	0.15726003	2	CYP8B1 ACSBG SCD, desC
ko00440	Metabolism	Metabolism of other amino acids	Phosphonate and phosphinate metabolism	0.24025876	2	PGR, NR3C3 PGR, NR3C3 PIK3R1_2_3
ko00830	Metabolism	Metabolism of cofactors and vitamins	Retinol metabolism	0.24025876	3	CYP1A1 UGT
ko05410	Human disease	Cardiovascular disease	Hypertrophic cardiomyopathy	0.24025876	4	PGR, NR3C3 PGR, NR3C3 PIK3R1_2_3
ko00620	Metabolism	Carbohydrate metabolism	Pyruvate metabolism	0.2691585	3	PGR, NR3C3 PGR, NR3C3 PIK3R1_2_3
ko00260	Metabolism	Amino acid metabolism	Glycine, serine and threonine metabolism	0.32065085	3	CYP1A1 UGT
ko00061	Metabolism	Lipid metabolism	Fatty acid biosynthesis	0.33246632	2	UGT AOX
ko04727	Organismal Systems	Nervous system	GABAergic synapse	0.33246632	3	CYP1A1 UGT
ko05206	Human disease	Cancer:overview	MicroRNAs in cancer	0.33246632	6	ACSBG SCD, desC
ko00220	Metabolism	Amino acid metabolism	Arginine biosynthesis	0.33246632	2	AOX
ko00100	Metabolism	Lipid metabolism	Steroid biosynthesis	0.33246632	2	PIK3R1_2_3 AOX
ko04964	Organismal Systems	Excretory system	Proximal tubule bicarbonate reclamation	0.33246632	2	PGR, NR3C3 PGR, NR3C3
ko00232	Metabolism	Biosynthesis of other secondary metabolites	Caffeine metabolism	0.39001493	1	CYP8B1
ko00640	Metabolism	Carbohydrate metabolism	Propanoate metabolism	0.39001493	2	BCL6 PIK3R1_2_3
ko00860	Metabolism	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism	0.39001493	2	PIK3R1_2_3 SCD, desC
ko04146	Cellular Processes	Transport and catabolism	Peroxisome	0.39001493	3	UGT
ko03018	Genetic Info Processing	Folding, sorting and degradation	RNA degradation	0.39001493	3	ACSBG
ko00680	Metabolism	Energy metabolism	Methane metabolism	0.39001493	2	AOX

Discussion

Previous studies of the effects of MT in fish have focused mainly on its interference in gonadal development and sex hormone synthesis (Amano et al., 1994; Lai et al., 2018; Passini et al., 2018) and the genes that play roles in these processes (Lyu et al., 2019). The general observation in these studies was that MT could disrupt sex hormone synthesis and the development of germ cells in fish of both sexes (Gao et al., 2015). In the rare minnow, *Gobiocypris rarus*, reduced spermatocyte numbers after 57.73 ngL^{-1} MT exposure for seven days were observed, suggesting that MT could suppress spermatogenesis (Liu et al., 2014; Gao et al., 2015). This is in agreement with the findings in *Astyanax bimaculatus* (Rivero-Wendt et al., 2013). We could not ascertain this observation in the present study with male *C. macrocephalus* since we did not attempt to count the spermatocyte number but only tested the ability of the testis from the MT-exposed male to fertilize the eggs from the non-MT-treated females.

Since most DEGs are not yet characterized proteins, we had to look at the genes identified in enriched KEGG pathways (**Tables 6** and **7**). The gene count in the list of enriched pathways indicated the important up- and down-regulated pathways. The top-upregulated gene by count is tyrosine hydroxylase (TH). TH catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), the initial and rate-limiting step in the biosynthetic pathway of catecholamines including dopamine, noradrenaline, and adrenaline. These catecholamines are essential in various physiological and behavioral functions in the nervous and endocrine systems (Kobayashi, 2001).

Specifically, it has the potential to recover spermatogenic loss and improve spermatogenesis by recovery of the endocrine axis and testicular homeostasis in rats, leading to better semen quality. This could be why the present study showed that MT-treated male catfish exhibited up-regulated TH, which might be behind its significant performance in producing heavier and longer testis, presumably leading to higher FR and HR.

Another up-regulated gene is the Cytoplasmic Polyadenylation Element Binding (CPEB), which controls translation in development, health, and disease, specifically in gametogenesis, cancer etiology, synaptic plasticity, learning, and memory (Ivshina et al., 2014). In *Drosophila*, the CPEB proteins Orb and Orb2 play critical roles in oogenesis and in neuronal function, as do related proteins in *Caenorhabditis* el*egans* and *Aplysia*, and perhaps also in the present study. Orb2 is the protein that affects spermatogenesis, is expressed in the adult germline, and has essential functions in programming the progression of spermatogenesis from meiosis through differentiation. It is required for meiosis, and the orderly differentiation of the spermatids after meiosis is complete. Since both CPEB and its protein Orb are overexpressed in the male catfish in the present study, it probably led to the observed higher reproductive performance of MT-treated catfish over that of the control catfish (Bautista et al., 2022b).

Apoptosis is initiated by binding a ligand such as the Fas ligand (FasL) to its receptor. It induces trimerization of the Fas receptors, which recruits the Fas-associated death domain (FADD). In the present study, these genes were overexpressed (i.e., three counts). The Fas/FADD complex then binds to an initiator caspase through interactions between the death effector domain of FADD and these caspase molecules. Caspase-8 or -10 then activates the executioner caspases-3 and -7, resulting in cellular disassembly (i.e., apoptosis)(Shetty et al., 2001). Apoptosis in spermatogenesis also happens, and one of the possible mechanisms for this event is that testosterone acts on androgen receptor-positive somatic cells, causing the apoptosis of spermatogonia. Apoptosis could be beneficial by getting rid of bad spermatogonia and keeping the good ones.

The Major Histocompatibility Complex class 1 (MHC-1) gene was overexpressed in the present study. Its function is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T cells. The consequences are deleterious to the pathogens. In humans, an influence of MHC-linked genes on male gamete quality and function has been demonstrated by Van der Ven et al. (2000). Human leukocyte Antigen G (HLA-G) is a non-classical MHC-1 antigen and is assumed to play significant roles in human spermatogenesis and early embryonic development (Yao et al., 2014).

Immune-related genes such as Immunoglobulin Heavy Locus (IGH), Chemokine Ligand (CCL19), and Epstein-Barr virus-induced molecule-1 ligand chemokine (ELC) were all overexpressed in the present study. In the present study, MT treatment in male catfish might have enhanced these two immune genes, which could provide protection against pathogens. IGH loci consist of repeated and highly homologous sets of genes of different types that rearrange in developing B cells to produce an individual's highly variable repertoire of expressed antibodies designed to bind to a vast array of pathogens (Watson and Breden, 2012). In contrast, the CCL19 gene plays a role in inflammatory and immunological responses and normal lymphocyte recirculation and homing. It may play an important role in the trafficking of T-cells in the thymus and T-cell and B-cell migration to secondary lymphoid organs; it binds to the chemokine receptor CCR7. The majority of chemokines attract lymphocytes, activated cells, or memory cells. ELC, another CC chemokine highly expressed in lymphoid tissues, may function as a lymphocyte chemoattractant, perhaps promoting cell compartmentalization within these tissues (Ngo et al., 1998).

The Achaete-scute Homolog 1 (ACSL1) gene increases its expression under high-fat feeding conditions (Mashek et al., 2006). As an anabolic agent, MT promotes the metabolism of fatty acids toward storage. Perhaps this condition predisposes the catfish to overexpress the ACSL1 gene in their livers in the present study. Expression of the ACSL1

gene is subject to tissue-specific regulation. The other forms of ACSL contribute to the activation of fatty acids (FAs) that have different metabolic fates in each tissue. Overexpressing ACSL1 in rat primary hepatocytes increased oleate incorporation into phospholipid and diacylglycerol while decreasing incorporation into cholesterol esters; it also increased the incorporation of *de novo* synthesized FA into glycerolipids.

Under-expressed genes in the present study included Cytochrome P450 family 1 subfamily A member 1 (CYP1A1) gene. Lee et al. (2007) evaluated the effects of *Panax ginseng* extracts on DNA damage in rats; expression of cytochrome P450 (CYP) 1A1 and reproductive toxicity was evaluated in the testis of rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxinthe (TCDD). DNA damage and the expression of CYP1A1 mRNA were significantly increased in rat testes; TCDD induces pathological and genotoxic damage in rat testes, while *P*. *ginseng* extract treatment exhibits a therapeutic capacity to reduce these effects via reducing CYP1A1 mRNA. The therapeutic effect of *P. ginseng* could be similar to the down-regulation of the CYP1A1 gene in MT-treated catfish in the present study.

The alternative oxidase (AOX) gene was down-regulated in MT-treated male catfish in the present study. In tunicates, AOX conferred a substantial disadvantage associated with decreased production of mature sperm. With the AOX gene being under-expressed, sperm differentiation could have proceeded, usually with spermatozoids released to the seminal vesicle. High AOX expression was detected in the outermost cell layer of the testis sheath, which the authors hypothesize to have disrupted a signal required for sperm maturation in tunicates (Saari et al., 2017). The downregulation of AOX could then be considered beneficial for the male catfish in the present study allowing the whole process of sperm maturation usually proceeds.

The UDP-glycosyltransferase (UGT) gene had a count of 5 across the list of top enriched down-regulated KEGG pathways in the present study. The roles of UGT in *Drosophila* include xenobiotic metabolism, nicotine resistance, olfaction, cold tolerance, sclerotization, pigmentation, and immunity (Ahn & Marygold, 2021). These UGTs are highly expressed in organs of detoxification (e.g., liver, kidney, intestine) and can be induced by pathways that sense demand for detoxification and modulation of endobiotic signaling molecules (Meech et al., 2017). The under-expressed UGT gene in the present study may underscore the assumption that the liver cells did not recognize MT as toxic.

The Acyl-CoA Synthase Bubblegum Family Member 1 (ACSBG) gene activates fatty acids with C16 to C24 (Moriya-Sato et al., 2000). It has been suggested to play a significant role in brain development and reproduction (Tang et al., 2001). ACSBG2 plays an important role in spermatogenesis, and testicular development is associated with male infertility (Fraisl et al., 2006) and exhibits a more exclusive expression pattern being highly expressed in the testis (Pei et al., 2006). In contrast, ACSBG1 is found to be mainly restricted to the brain, adrenal glands, gonads, and spleen in mice and humans (Moriya-Sato et al., 2000). In the present study, the down-regulation of these enzymes in male catfish liver suggests normal fertility.

Stearoyl-CoA Desaturase (SCD) is an enzyme that participates in the conversion of saturated fatty acids into unsaturated fatty acids in mammalian adipocytes (Svendsen et al., 2020). Fatty acid (FA) metabolism disorders in infertile men are potentially associated with deregulations in the expression of rate-limiting genes such as desaturases. Monounsaturated fatty acids (MUFA) (palmitoleic acid; C16:1 and oleic acid; C18:1) can be obtained via endogenous synthesis from their saturated FA (SFA) substrate precursors. This process requires a desaturation reaction catalyzed by SCD (Svendsen et al., 2020), and SCD expression affects sperm formation and the acrosome reaction. It provides an energy source for spermatogenesis (Saether et al., 2003). Over-expression of SCD in the testicular tissue of men may result in elevated MUFA levels in addition to an increase in androgen hormone levels and a decrease in estrogen hormone levels. These events can affect sperm motility and viability and lead to male infertility. Whether low or high levels of SCD expression can impair spermatogenesis has not been addressed (Bagheri et al., 2021). Previously, we have shown that male catfish exhibited acceptable sperm viability

by producing heavy and long testis, resulting in good fertilization and hatching rates of fertilized eggs under the condition of under-expressed SCD, suggesting that low-level SCD expression could not have impaired spermatogenesis (Bautista et al., 2022b).

Another down-regulated gene in the present study was phosphatidylinositol 3-kinase p85 alpha regulatory subunit 1 gene (PIK3R1,2,3). Phosphoinositide 3-kinase (PI3K) plays a critical role in tumorigenesis, and the PI3K p85 regulatory subunit exerts both positive and negative effects on signaling. Expression of Pik3r1, the gene encoding p85, is decreased in human prostate, lung, ovarian, bladder, and liver cancers, consistent with the possibility that p85 has tumor suppressor properties. In mice with a liver-specific deletion of the Pik3r1 gene, they exhibited enhanced insulin and growth factor signaling and progressive changes in hepatic pathology, leading to the development of aggressive hepatocellular carcinomas with pulmonary metastases. p85 regulatory subunit of PI3K functions as an inhibitor of growth factor signaling and a novel tumor suppressor in the liver and most likely in other cancers as well (Taniguchi et al., 2010). Down-regulating this gene, such as in male catfish in the present study, renders the growth factor signaling free from inhibition but also free from suppressor of novel tumor and other cancers in the liver.

Up-regulated KEGG pathways

Enriched up-regulated KEGG pathways show that the immune system, specifically the endocrine system, apparently dominated the pathways, and so did signal transduction of the immune system.

The NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) transcription factor family is a regulator of many cellular signaling pathways, providing a mechanism for the cells in response to a wide variety of stimuli linking to inflammation (Shih et al., 2015). It exerts effects on almost all cell types in the body, playing an essential function in inflammation, immune responses, cell cycle, and cell survival (Sen and Baltimore, 1986; Li and Verma, 2002; Kaltschmidt et al., 2005; Ledoux and Perkins, 2014). This pathway is implicated in cell proliferation and survival, demonstrated by constitutively active NF-κB signaling in many tissues (Ben-Neriah and Karin, 2011). The NF-κB is inactive in the cytoplasm. One of the triggers of this signaling pathway is the serine phosphorylation by serine/threonine protein kinase, the gene of which was also up-regulated in the present study. The activated NF-κB will translocate from the cytoplasm to the nucleus and will do its function of cell proliferation and survival.

Autophagy is a process in eukaryotes by which cytoplasmic cargo is sequestered inside double-membrane vesicles (called phagosomes) by phagocytosis and delivered to the lysosome for degradation (Yang & Klionsky, 2010); phagosomes were up-regulated in the present study. Autophagy has essential roles in survival, development, and homeostasis processes and is induced by stress and development. It can often exert cytoprotective functions to support the re-establishment of cell status, and its inhibition may induce cell death. Concomitant with the stimulated autophagy is stimulated phagocytosis, which is mainly receptor-dependent and is regulated by the calcium ion (Nunes & Demaurex, 2010). It follows naturally that calcium signaling was also a top pathway up-regulated in the present study.

Isoquinoline alkaloids are derived from phenylalanine and tyrosine and are formed from the precursor 3,4-dihydroxytyramine (dopamine) (Khan & Kumar, 2015). These alkaloids are derived from plants and are inhibitors of the AKR1C3 enzyme. AKR1C3 (also known as 17β-hydroxysteroid dehydrogenase type 5) is an enzyme responsible for the pre-receptor regulation of steroid hormone action (Skarydova et al., 2014). As an up-regulated pathway in the present study, the biosynthesis of isoquinoline alkaloids in the present study may have inhibited the AKR1C3 enzyme and, thus, prevented its action in the conversion of MT to testosterone and further to 17β-estradiol by aromatase.

Peroxisome Proliferator-Activated Receptor (PPAR) is a nuclear receptor that has been demonstrated to regulate lipid homeostasis and tumorigenesis, among others. There are three isotypes of PPARs named PPARα, PPARβ/δ, and PPARγ (Monrose et al, 2021). Within the testis, all PPARs are expressed in both somatic and germ cells (Froment et al.,

2006). PPARβ/δ are expressed in Leydig, Sertoli, and germ cells. In the testis, PPARγ protein is detected at a high level in Sertoli cells, and a weak expression is noticed in spermatocytes. Pparγ mRNA was highly expressed in late spermatids, while Pparβ mRNA was highly expressed in round spermatids. The upregulation of the PPAR signaling pathway in the catfish liver in the present study indicates the primary role of MT in the testicular physiology of this fish. However, it was also listed as a downregulated KEGG pathway, an indication of fine-tuning its regulation of the process.

Bile acids (BAs) are synthesized from cholesterol in liver hepatocytes and are secreted into the small intestine to emulsify and promote the absorption of dietary lipids. Bile acids play key homeostatic roles in glucose metabolism, cholesterol, and lipid metabolism, xenobiotic detoxification of toxins, and lifespan extension (Tiwari & Maiti, 2009; Goldberg et al., 2010). Ohshima et al. (1996) demonstrated that methyltestosterone caused a decrease in basal bile flow and bile acid secretion and increased basal cholesterol secretion and the biliary cholesterol-to-phospholipid ratio. This indicates that the up-regulated primary bile synthetic pathway in the catfish liver in the present study referred more to the increased proportion of cholesterol in the bile acid than to the secretion of bile per se.

Down-regulated KEGG pathways

Tryptophan metabolism was the top significantly enriched KEGG pathway in the present study due to dietary MT; specifically, it was down-regulated. Tryptophan oxygenase (tryptophan pyrrolase), which catalyzes the conversion of *L*-tryptophan to formylkynurenine, is regulated by adrenocorticosteroids, estrogens, and androgens (Rose, 1972). Elevated expression pattern of tryptophan hydroxylase (form 2) mRNA correlated well with the increased levels of 5-hydroxytryptophan (5-HTP) and serotonin (5-HT) in the brain of MT-treated male African catfish *Clarias gariepinus* compared to control males (Raghuveer et al., 2011). These authors hypothesize that the serotonergic system is involved in brain sex differentiation in teleosts. Although sex reversal was too late to happen in immature adult male catfish, the tryptophan metabolic pathway being down-regulated in MT-treated *Clarias macrocephalus* male in the present study support the hypothesis of Raghuveer et al. (2011) regarding sex reversal in fish. In adult male catfish, we hypothesize that MT's effect on the brain's serotonergic system could have effects on gonadal development.

In a study by Wu et al. (2020), transcriptome analysis identified some pathways putatively involved in spermatogenesis after MT treatment of orange-spotted grouper; the retinol metabolism pathway was identified as one of the three pathways. Vitamin A and provitamin A (β-carotene) are metabolized to specific and active retinoid derivatives (i.e., enzymes) that function in either vision or growth and development (Duester, 2001). These enzymes fall into three categories, and one of the categories is short-chain dehydrogenase/reductase (SDR) enzyme families, and several SDRs have androgens rather than retinoids as predominant substrates suggesting a function in androgen metabolism as well as retinoid metabolism. This could be an indication that androgens which include MT or its metabolite, could be acted upon by retinoid derivatives/enzymes, and this process probably underwent down-regulation as a result of MT treatment, as observed in the present study.

In the present study, MT treatment resulted in the down-regulation of two KEGG pathways: drug metabolism-CYP and metabolism of xenobiotics by CYP. The P450 aromatase enzyme, a product of the CYP19 gene, is responsible for converting C19 steroids (androstenedione, testosterone, and 16-α-hydroxyandrostenedione) into estrone, estradiol-17β, and estriol, respectively (Simpson et al., 1994). MT inhibits aromatase activity dose-relatedly and is a competitive inhibitor (Mor et al., 2001). Cytochrome P450-mediated side chain cleavage enzyme (*cyp11a1*) and cytochrome P450 aromatase (*cyp19a1a*)(Liu et al., 2014; Zheng et al., 2016) are considered steroidogenesis genes, considered as one of the crucial genes in mediating the disrupting effects of MT. This explains the results in the present study of MT treatment resulting in the down-regulation

of the other top significantly-enriched KEGG pathways, namely, breast cancer, estrogen signaling pathway, steroid hormone biosynthesis, and chemical carcinogenesis. Aromatase, encoded by c*yp19a1a*, is responsible for a critical step in converting androgens to estrogen (Lau et al., 2016). In the report of Wang et al. (2020), upon MT exposure, females had the same trend of *cyp19a1a* transcriptional profile and E2 (i.e.,17β-estradiol) level, indicating the crucial effect of aromatase in converting T to E2 in *P. parva*. The inconsistent trends of *cyp19a1a* transcriptional profile and E2 level in male fish in their study were attributed to the weak role of E2 in male fish. In teleost fish, *cyp11b* and *cyp17a1* are critical genes in the androgen synthetic pathway, while 11β-HSD2 (11β-hydroxysteroid dehydrogenase 2) is the key gene for 11-KT (11-ketotestosterone) synthesis. Thus they had an essential influence on testicular development and spermatogenesis (Tokarz et al., 2013). In *P. parva*, the testicular mRNA expression level of these three genes was down-regulated almost in all MT treatment groups, which agrees with the results in the present study on *Clarias macrocephalus* males.

In conclusion, MT-treated male *Clarias macrocephalus* exhibited up-regulation of some pathways that protect the cellular conditions such as cell proliferation, survival, development, and homeostasis processes (via NF-κB transcription factor family, phagosomes and isoquinoline alkaloids) for development and homeostasis processes. MT also caused changes in the characteristics of bile acids produced, inhibiting the production/conversion of testosterone that otherwise would have resulted in fat mass in catfish. In contrast, MT resulted in the down-regulation of the serotonergic system that could affect gonadal development, inhibition of the retinoid enzyme that would otherwise diminish the full effects of MT or its metabolite, and inhibition of the formation of estrogen by aromatase leading to down-regulation of several estrogen-related KEGG pathways. The results of the present study provide valuable information about the key genes for use as biomarkers of maturation and reproduction for the Asian catfish and contribute to our understanding of the molecular mechanisms and regulative pathways regulating these two processes in fish.

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