Supporting Information

Adverse reproductive performance in zebrafish with increased bioconcentration of microcystin-LR in the presence of titanium dioxide nanoparticles

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Text S1: Characterization of n-TiO_2

The diameter sizes of n-TiO_2 was determined by transmission electron microscopy (TEM, JEOL2100F STEM/EDS, Tokyo, Japan), at an accelerating voltage of 100 kV. The crystal structure was confirmed by powder X-ray diffraction (XRD) using an X'Pert PRO XRD instrument (D8 Advance, Bruker, Germany). The average diameter and ζ potential in water (100 μg/L) were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern instruments, Worcestershire, UK). The surface area of n-TiO_2 was measured by the Brunauer-Emmett-Teller method using an ASAP 2020 physisorption analyzer (Micromeritics, Atlanta, US).

Text S2: Assessment of n-TiO_2 behavior in aquarium

The settling behavior of the n-TiO_2 particles were assessed in aquarium with the disturbance of fish for 48 hours, n-TiO_2 size distribution in three layers (based on the depth of exposure tank) were detected at 0, 12, 24, 36 and 48 h, respectively. All the water samples were sampled in the center of different water layers.

The size distribution and zeta potential of n-TiO_2 was examined by dynamic light scattering (Malvern instruments, Worcestershire, UK). In brief, 1.5 mL of water sample with n-TiO_2 particles (the water samples were analyzed immediately after sampling) was put in a clean cuvette, and the DLS was operated with a scattering angle of 90° from the incident laser beam. The zeta potential of the nanoparticles was measured at least seven times for each sample.

Text S3: MCLR analysis

The MCLR exposure solution was preceding filtrated through Whatman GF/C

filters (1.4 μm). Then the water was transferred to the new centrifuge tube and stored at -20 °C for further analysis. The liver, brain and gonad samples were freeze-dried for toxin extraction according to the previous studies[1], with minor modifications. The samples were extracted using methanol: butanol: water (20: 5: 75 v/v) and the extractions were passed through C18 cartridge. After being washed with 20 mL distilled water, they were eluted by using 20 mL of 20% methanol and finally 20 mL of 100% methanol. This last fraction was dried and dissolved in water for further analysis.

For the detection of MCLR, each well was added 50 μL of Microcystin-HRP enzyme conjugate solution. Then, 50 μL of the negative control, samples, and stander solution were added into the corresponding wells, respectively. The rabbit anti-Microcystin antibody solution was added into each well and the microplate should be attached with a parafilm for mixing the reagents on the platform of a shaking table for continuous mixing during incubation. After 30 min incubation (37 °C) in dark, the plate was washed five times with 1 cleaning solution. What followed was adding substrate solution (100 μL) to every well and shaking the plate vigorously to incubate (37 °C) for 30 min. The stop solution (100 μL) was added to every well in accordance with the order of adding the substrate solution. The plate was read at 450 nm using BioTek (BioTek Instruments, Inc, Beijing office. USA). All the experiments were repeated at least in triplicate.

Text S4: Histological observation

The ovaries and testes (n = 6, 2 from reach replicate tank) were fixed in 4 %

paraformaldehyde solution for 24 h. The samples were then dehydrated and paraffin-embedded, sectioned into 6 μm sections along the long axis of the gonad, and then the sections were stained with hematoxylin and eosin (H&E, Sigma–Aldrich, Shanghai, China). A total of nine tissue sections per sample were collected. Six samples from each sex and treatment group were randomly selected for histological and stereological analyses. The stage of oogenesis and spermatogenesis were identified and quantified using previously described methods[2]. Sections from all treatment groups were examined under a light microscope (Olympus MVX10), equipped with an Olympus Camedia C-5050 camera. Ovarian follicles were staged as: primary oocyte, cortical alveolar oocyte, early vitellogenic oocyte, late/mature oocyte. A total of three structural components of the testis were considered: spermatogonia, spermatocytes and spermatid. The percent of follicles at each stage of development was expressed as a percent of the total number of follicles, as well as the volume densities of each compartment in the testis estimated using Image Pro Plus 6.0 (Media Cybernetics Company, MD, USA), as previously described.

Text S5: Gene transcription analysis

Livers, brains and F1 larvae were homogenized in TRIzol reagent (TaKaRa, Dalian, China) and preserved at -80 °C for subsequent isolation of total RNA. Six fish with the same sex were sampled from each group and tissue of each fish was treated as a replicate. RNA concentrations and quality were validated by 260 nm absorbance and the 260/280 nm ratios, respectively. First-strand cDNA synthesis was carried out by using Takara reverse transcription kits (Dalian, China). QRT-PCR was done by

using SYBR Green Kits (Takara, Dalian, China) on an ABI Step One Plus system (Applied Biosystems, Foster City, CA). The sequences of primers of genes (such as *gapdh*, *erα*, *erβ*, *ar*, *gnrhr3*) were acquired from previous studies[3-5], the others used in this study were designed by ourselves with NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast). The thermal cycle was as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The different transcription levels were examined using the $2^{-\Delta\Delta Ct}$ method[6]. Our previous study showed the transcription level of glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was stable under MCLR exposure[7], and we chose it as an internal control.

References

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Figure S1. Histological examination of gonad development in zebrafish exposed to MCLR (0, 0.5, 4, 32 μg/L) alone, n-TiO_2 (100 μg/L) alone, and both of them for 21 days. The percentages of primary oocyte, cortical alveolar oocyte, early vitellogenic oocytes and late/mature oocyte in ovary of female zebrafish (A) and the percentages of spermatogonia, spermatocytes, and spermatid in testes of male zebrafish (B) were counted. Values represent the mean ± SE of three replicate samples for six individual fish from 3 replicate tanks.

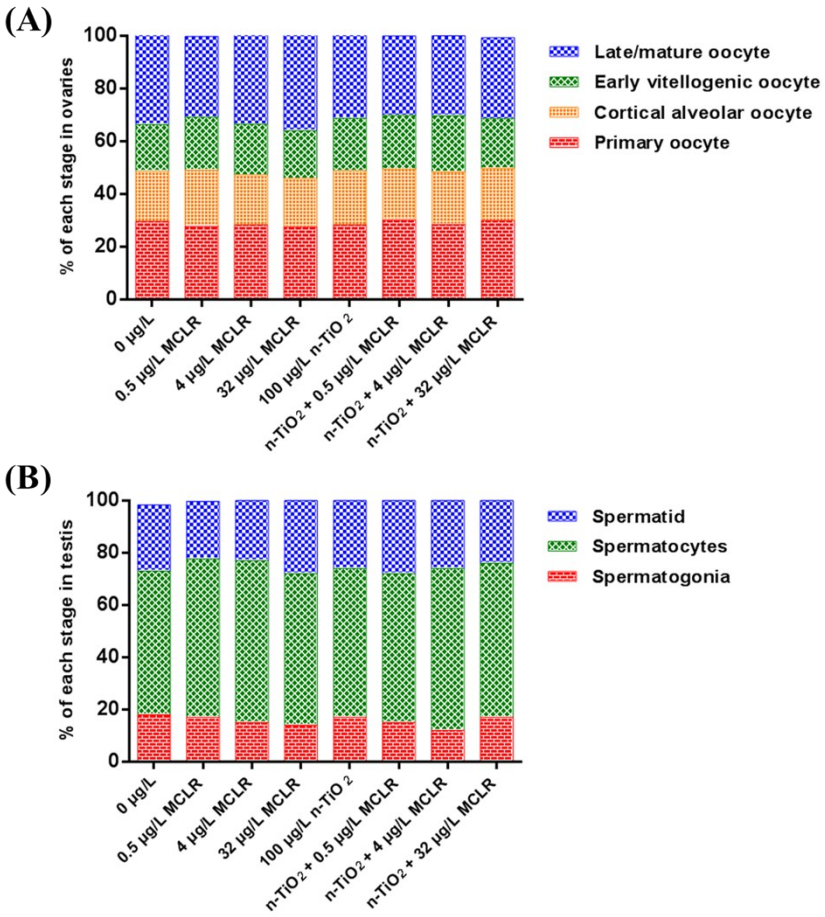

Figure S2. Cumulative number of eggs spawned per female zebrafish during 14-days pre-exposure period in different concentrations of MCLR (0, 0.5, 4, and 32 µg/L) with and without 100 µg/L n-TiO_2. Data are the mean values (mean ± SE) per female in each treatment group (n=3 replicate tanks). There was no significant difference among all groups.

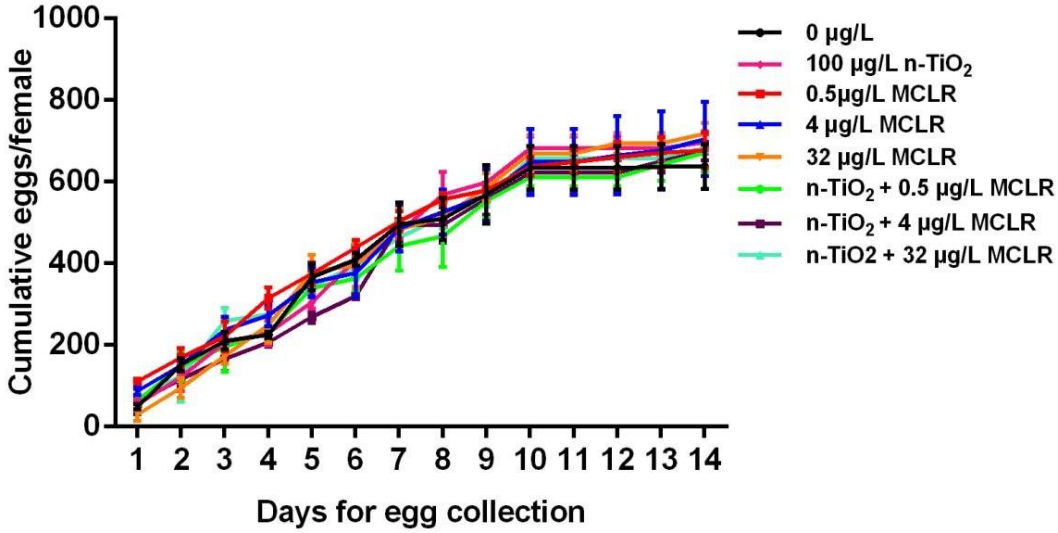

Figure S3. Based on the depth of exposure tanks, we divided the exposure solution into upper, middle and bottom layers. The average particle size and zeta potential of each layer were detected at various sampling point (0, 12, 24, 32 and 48 h). The average size of n-TiO_2 (A), (C) in the absence and presence of MCLR, respectively. The zeta potential of n-TiO_2 (B), (D) in the absence and presence of MCLR, respectively. Data are expressed as mean ± SE (n=3).

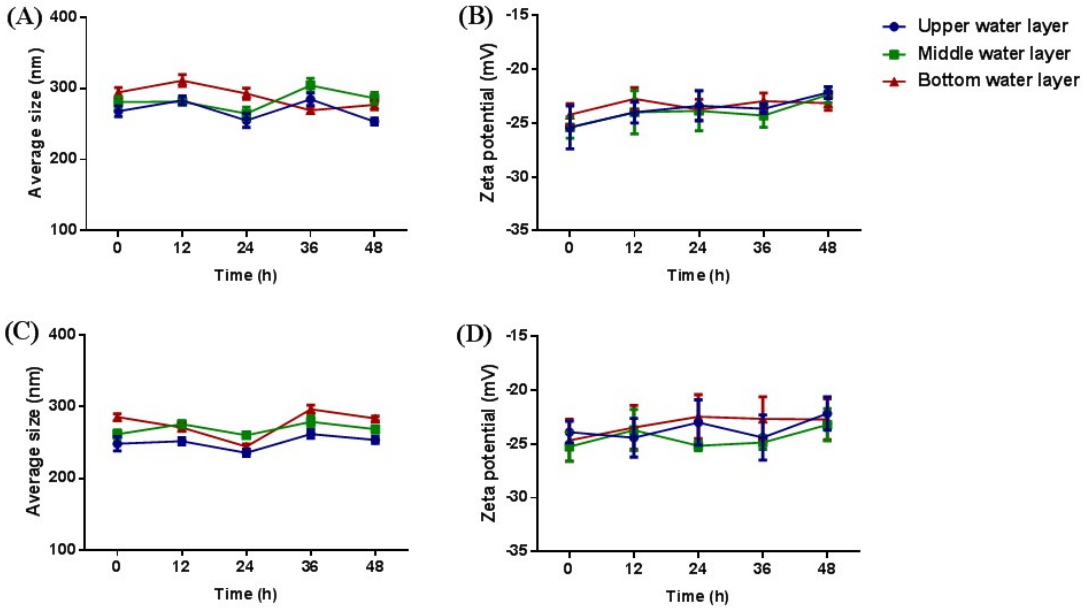

Table S1 Gene abbreviation

Gene name	Abbreviation
gonadotropin releasing hormone	*gnrh*
gonadotropin releasing hormone receptor	*gnrhr*
follicle stimulating hormone β	*fshβ*
luteinizing hormone β	*lhβ*
estrogen receptor	*er*
androgen receptor	*ar*
vitellogenin	*vtg*
20,22-desmolase	*cyp11*
cytochrome P450 17	*cyp17*
cytochrome P450 19	*cyp19a*
follicle stimulating hormone receptor	*fshr*
luteinizing hormone receptor	*lhr*
steroidogenic acute regulatory protein	*star*
3β-hydroxysteroid dehydrogenase	*3β-hsd*
17β-hydroxysteroid dehydrogenase	*17β-hsd*
glyceraldehyde 3-phosphate dehydrogenase	*gapdh*

Table S2. Sequences of primers of genes used in this study.

Gene name	Sequences of the primer (5'-3')	Accession number
gnrh2	Forward: actggtctcacggctggtat	NM_181439.4
	Reverse: aaatcacgaatgagggcatc	
gnrh3	Forward: cagcactggtcatacggttg	NM_182887.2
	Reverse: ccttcagcatccacctcatt	
gnrhr3	Forward: aacagacatgatcccgaagg	NM_001177450.1
	Reverse: aggttcccgaacacaaacag	
fshβ	Forward: acagcacacccagaaggtct	NM_205624.1
	Reverse: agctccccagtctgttgtgt	
lhβ	Forward: gagacggtatcggtggaaaa	NM_205622.2
	Reverse: aacagtcgggcaggttaatg	
erα	Forward: cactacggagccctcacttgcgga	NM_152959
	Reverse: gccctgaactgctccgacctc	
erβ	Forward: ttcacccctgacctcaagct	NM_174862
	Reverse: tccatgatgccttcaacacaa	
ar	Forward: tctgggttggaggtcctacaa	NM_001083123
	Reverse: ggtctggagcgaagtacagcat	
vtg1	Forward: gcactcacaaactggaaggc	NM_001044897.2
	Reverse: ggtccagtagccatcggtat	
vtg3	Forward: ggtggttcttggacttggtt	AF254638
	Reverse: cacaggagaggatgggattt	
star	Forward: aagggttggatcccaaaaac	NM_131663.1
	Reverse: tagctatgggtgggatgagg	
cyp11a	Forward: acagcctgctcagtgtcctt	NM_152953.2
	Reverse: agcaccgtcttcaggcttta	
cyp11b	Forward: tatacagagagcacctgggc	NM_152983.1
	Reverse: ctgcgtgtctctcgatgtgt	
cyp17	Forward: tggagctctttgcatgtttg	AY281362.1
	Reverse: agctcagcatctccacgttt	
cyp19a	Forward: ccgttcttatggcaggtgat	AF226620.1
	Reverse: ttgtgtggtcgatggtgtct	
3β-hsd	Reverse: agtgtcgcacatcgtctcag	AY279108.1
	Forward: cagtcggaccagcttttctc	
17β-hsd	Forward: catcatggacgtcaatctgc	AY306005.1
	Reverse: tctcacaagcgccctctatt	
fshr	Forward: gtctgtctgggcaacaaggt	NM_001001812.1
	Reverse: caccactattctcttcagctcgt	
lhr	Forward: tgctgtggagtgtgtttcgag	AY424302.1
	Reverse: gctctgggcgatttctattcttc	
gapdh	Forward: ctggtgacccgtgctgctt	NM_001115114
	Reverse: tttgccgccttctgcctta	

Table S3. The MCLR contents in the liver, brain and gonad of female and male fish exposed to MCLR (0, 0.5, 4, 32 μg/L) alone, n-TiO_2 (100 μg/L) alone, and both of them for 21 days. All data are expressed as mean ± SE (ng/g ww). The "*" indicates the statistical difference in comparison with the corresponding MCLR group without n-TiO_2 ($p < 0.05$).

Groups	Female			Male		
	Liver	Brain	Ovary	Liver	Brain	Testis
0.5 μg/L MCLR	0.31±0.05	0.22±0.03	0.16±0.02	0.32±0.03	0.20±0.01	0.22±0.04
0.5 μg/L MCLR+n-TiO_2	0.35±0.06	0.31±0.07	0.34±0.04	0.40±0.03	0.38±0.06	0.32±0.05
4 μg/L MCLR	0.58±0.10	0.45±0.03	0.39±0.03	0.78±0.09	0.61±0.10	0.56±0.10
4 μg/L MCLR+n-TiO_2	0.83±0.05*	0.71±0.06*	0.62±0.02*	1.05±0.11*	0.78±0.04*	0.70±0.11
32 μg/L MCLR	1.22±0.02	0.88±0.01	1.05±0.05	1.20±0.08	0.95±0.04	1.06±0.12
32 μg/L MCLR+n-TiO_2	1.53±0.11*	1.12±0.02*	1.32±0.08*	1.58±0.05*	1.29±0.05*	1.29±0.06*

Table S4. The transcription of genes in the liver, brain and gonad of female and male fish exposed to MCLR (0, 0.5, 4, 32 μg/L) alone, n-TiO_2 (100 μg/L) alone, and both of them for 21 days. All data are expressed as mean ± SE. The "*" indicates the statistical difference compared to the control ($p < 0.05$). And "#" indicates the statistical difference in comparison with the corresponding MCLR group without n-TiO_2 ($p < 0.05$).

Female

Tissues	Genes	0	n-TiO_2	0.5	n-TiO_2 +0.5	4	n-TiO_2 + 4	32	n-TiO_2 + 32
Brain	*gnrh2*	1.01±0.10	1.03 ± 0.11	1.02 ± 0.22	1.07 ± 0.20	1.20 ± 0.16	1.14 ± 0.13	1.18 ± 0.28	0.48 ± 0.12*#
	gnrh3	1.00 ± 0.03	1.11 ± 0.13	0.99 ± 0.12	0.92 ± 0.17	1.24 ± 0.15	0.94 ± 0.22	0.95 ± 0.15	0.55 ± 0.08*#
	gnrhr3	1.00 ± 0.03	0.86 ± 0.08	0.98 ± 0.08	0.98 ± 0.15	0.98 ± 0.05	0.95 ± 0.11	0.96 ± 0.16	1.27 ± 0.11
	fshβ	1.00 ± 0.02	1.01 ± 0.03	1.13 ± 0.11	1.13 ± 0.26	1.04 ± 0.23	1.25 ± 0.16	1.27 ± 0.25	1.56 ± 0.20*#
	lhβ	1.00 ± 0.04	0.94 ± 0.02	1.09 ± 0.16	0.91 ± 0.17	0.83 ± 0.11	1.14 ± 0.31	0.99 ± 0.06	1.09 ± 0.17
	erα	1.00 ± 0.01	0.99 ± 0.10	1.22 ± 0.18	1.12 ± 0.19	0.99 ± 0.20	1.19 ± 0.04	1.25 ± 0.18	2.45 ± 0.46*#
	erβ	1.00 ± 0.03	1.09 ± 0.07	1.24 ± 0.07	1.02 ± 0.09	1.23 ± 0.15	1.05 ± 0.09	1.35 ± 0.18	2.16 ± 0.63*#
	ar	1.00 ± 0.02	1.03 ± 0.04	1.08 ± 0.16	1.02 ± 0.06	1.25 ± 0.10	1.24 ± 0.09	1.26 ± 0.07	2.09 ± 0.40*#
Liver	*vtg1*	1.05 ± 0.22	0.92 ± 0.05	1.47 ± 0.72	1.39 ± 0.22	1.31 ± 0.31	1.20 ± 0.23	0.60 ± 0.19	1.07 ± 0.27
	vtg3	1.02 ± 0.11	0.98 ± 0.15	0.78 ± 0.14	0.76 ± 0.16	0.84 ± 0.11	0.54 ± 0.17*	0.6 ± 0.01*	0.31 ± 0.02*#
	erα	1.00 ± 0.02	0.95 ± 0.24	0.73 ± 0.11	1.06 ± 0.16	0.67 ± 0.17	0.71 ± 0.10	0.62 ± 0.05*	0.31 ± 0.07*#
	erβ	1.08 ± 0.10	1.07 ± 0.20	1.32 ± 0.41	1.49 ± 0.56	0.99 ± 0.34	0.54 ± 0.12	0.94 ± 0.24	0.53 ± 0.10
Ovary	*star*	1.02 ± 0.13	1.12 ± 0.05	0.87 ± 0.12	1.36 ± 0.42	1.05 ± 0.13	1.18 ± 0.10	1.99 ± 0.07*	2.65 ± 0.47*
	cyp11a	1.01 ± 0.09	1.02 ± 0.09	0.98 ± 0.14	1.58 ± 0.30	1.58 ± 0.16	1.40 ± 0.21	1.25 ± 0.30	1.45 ± 0.18
	cyp11b	1.00 ± 0.06	1.11 ± 0.07	1.28 ± 0.22	1.32 ± 0.16	1.22 ± 0.13	1.09 ± 0.19	1.21 ± 0.23	1.25 ± 0.16
	cyp17	1.00 ± 0.05	0.92 ± 0.12	1.04 ± 0.21	1.00 ± 0.21	0.95 ± 0.05	1.13 ± 0.20	1.07 ± 0.17	1.01 ± 0.05
	cyp19a	1.02 ± 0.13	0.97 ± 0.20	0.86 ± 0.10	1.50 ± 0.37	1.33 ± 0.13	1.74 ± 0.12*	1.54 ± 0.06*	2.77 ± 0.07*#
	3β-hsd	1.00 ± 0.01	1.14 ± 0.20	1.08 ± 0.05	1.17 ± 0.20	1.24 ± 0.20	1.60 ± 0.20*	1.42 ± 0.04*	2.77 ± 0.21*#
	17β-hsd	1.01 ± 0.06	1.01 ± 0.12	1.38 ± 0.06	0.94 ± 0.12	0.93 ± 0.23	1.06 ± 0.12	0.75 ± 0.13	0.98 ± 0.08
	lhr	1.00 ± 0.05	1.10 ± 0.18	1.07 ± 0.09	1.08 ± 0.12	1.12 ± 0.11	0.77 ± 0.05	0.49 ± 0.08*	0.39 ± 0.08*
	erα	1.00 ± 0.06	1.21 ± 0.17	0.74 ± 0.11	1.30 ± 0.11	1.18 ± 0.13	1.23 ± 0.05	0.82 ± 0.08	1.40 ± 0.33
	erβ	1.00 ± 0.02	1.06 ± 0.17	0.97 ± 0.28	0.94 ± 0.21	1.18 ± 0.23	0.70 ± 0.07	0.77 ± 0.14	0.86 ± 0.18

Male

Tissues	Genes	0	n-TiO_2	0.5	n-TiO_2 +0.5	4	n-TiO_2 + 4	32	n-TiO_2 + 32
Brain	*gnrh2*	1.00 ± 0.07	1.04 ± 0.12	1.00 ± 0.21	1.22 ± 0.15	1.17 ± 0.16	1.40 ± 0.60	0.99 ± 0.21	2.03 ± 0.39*#
	gnrh3	1.01 ± 0.10	0.96 ± 0.22	1.38 ± 0.44	1.13 ± 0.17	1.05 ± 0.06	0.94 ± 0.14	1.19 ± 0.16	1.19 ± 0.21
	gnrhr3	1.02 ± 0.10	1.01 ± 0.12	0.99 ± 0.09	0.92 ± 0.19	0.86 ± 0.09	0.92 ± 0.13	0.96 ± 0.15	0.97 ± 0.05
	fshβ	1.02 ± 0.11	1.07 ± 0.14	1.00 ± 0.12	0.96 ± 0.32	1.03 ± 0.35	0.86 ± 0.10	1.00 ± 0.03	0.58 ± 0.06
	lhβ	1.02 ± 0.11	1.12 ± 0.09	1.02 ± 0.04	1.06 ± 0.09	1.25 ± 0.39	1.17 ± 0.19	1.12 ± 0.24	1.11 ± 0.22
	erα	1.03 ± 0.14	1.11 ± 0.07	1.35 ± 0.37	1.17 ± 0.14	1.33 ± 0.28	1.10 ± 0.09	1.37 ± 0.21	1.30 ± 0.32
	erβ	1.00 ± 0.04	1.19 ± 0.07	1.08 ± 0.20	1.15 ± 0.15	1.24 ± 0.23	1.45 ± 0.11	1.70 ± 0.07	1.88 ± 0.36*#
	ar	1.00 ± 0.04	1.09 ± 0.13	0.92 ± 0.17	0.97 ± 0.10	0.97 ± 0.06	1.14 ± 0.17	0.98 ± 0.16	1.25 ± 0.19
Liver	*vtg1*	1.01 ± 0.08	1.10 ± 0.16	1.01 ± 0.11	1.20 ± 0.14	1.10 ± 0.15	1.01 ± 0.08	1.18 ± 0.16	0.96 ± 0.12
	vtg3	1.01 ± 0.06	1.03 ± 0.06	0.99 ± 0.10	1.08 ± 0.27	1.22 ± 0.15	1.15 ± 0.16	1.31 ± 0.26	1.07 ± 0.13
	erα	1.02 ± 0.11	1.09 ± 0.06	1.05 ± 0.07	1.14 ± 0.16	0.97 ± 0.22	1.34 ± 0.17	0.50 ± 0.09	1.14 ± 0.05
	erβ	1.00 ± 0.03	1.22 ± 0.10	0.97 ± 0.07	2.07 ± 0.41	0.92 ± 0.19	1.60 ± 0.09	0.98 ± 0.14	1.55 ± 0.37
Testis	*star*	1.01 ± 0.10	1.10 ± 0.08	1.10 ± 0.10	1.29 ± 0.16	0.95 ± 0.20	1.39 ± 0.20	1.06 ± 0.23	1.04 ± 0.25
	cyp11a	1.01 ± 0.09	1.09 ± 0.04	1.17 ± 0.14	1.19 ± 0.07	1.17 ± 0.11	1.10 ± 0.12	1.03 ± 0.09	1.17 ± 0.13
	cyp11b	1.00 ± 0.03	0.97 ± 0.04	0.95 ± 0.07	1.06 ± 0.15	1.06 ± 0.09	1.01 ± 0.16	1.26 ± 0.18	1.18 ± 0.03
	cyp17	1.00 ± 0.05	1.29 ± 0.22	1.19 ± 0.16	1.25 ± 0.14	1.17 ± 0.15	1.35 ± 0.21	1.20 ± 0.16	1.21 ± 0.16
	cyp19a	1.01 ± 0.08	1.00 ± 0.09	1.01 ± 0.16	1.13 ± 0.13	0.97 ± 0.02	1.06 ± 0.21	0.92 ± 0.07	1.45 ± 0.20*#
	3β-hsd	1.01 ± 0.08	1.07 ± 0.15	1.07 ± 0.09	1.24 ± 0.18	0.96 ± 0.24	1.31 ± 0.33	0.97 ± 0.24	1.52 ± 0.31
	17β-hsd	1.01 ± 0.06	1.04 ± 0.24	0.73 ± 0.13	1.06 ± 0.16	1.25 ± 0.20	1.14 ± 0.11	1.11 ± 0.29	1.72 ± 0.05*#
	fshr	1.00 ± 0.05	1.15 ± 0.13	1.18 ± 0.22	1.56 ± 0.22	1.11 ± 0.19	1.07 ± 0.08	1.15 ± 0.18	1.41 ± 0.22
	lhr	1.01 ± 0.07	1.05 ± 0.12	0.87 ± 0.13	1.12 ± 0.08	0.88 ± 0.02	1.30 ± 0.14	1.00 ± 0.16	1.38 ± 0.05*#
	erα	1.00 ± 0.02	1.16 ± 0.19	1.31 ± 0.18	1.11 ± 0.18	1.01 ± 0.12	1.69 ± 0.14*	1.13 ± 0.24	2.11 ± 0.16*#
	erβ	1.01 ± 0.08	1.08 ± 0.10	1.02 ± 0.13	0.95 ± 0.11	0.78 ± 0.09	0.98 ± 0.16	0.93 ± 0.13	1.25 ± 0.21