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Microcystin-LR affects the hypothalamic-pituitary-inter-renal (HPI) axis in early life stages (embryos and larvae) of zebrafish☆

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ABSTRACT

Frequencies and durations of blooms of cyanobacteria are increasing. Some cyanobacteria can produce cyanotoxins including microcystins (MCs). MCs are the most common toxic products of hazardous algal blooms (HABs), with the greatest potential for exposure and to cause toxicity. Recently, MCs have been shown to disrupt endocrine functions. In this study, for the first time, effects of MC-LR on the hypothalamic-pituitary-inter-renal (HPI) axis during early embryonic development (embryos/larvae) of zebrafish (*Danio rerio*), were investigated. Embryos/larvae of zebrafish were exposed to 1, 10, 100, or 300 μg MC-LR/L during the period of 4–168 h post-fertilization (hpf). Exposure to 300 μg MC-LR/L resulted in significantly greater concentrations of whole-body cortisol than those in controls. Expressions of genes along the HPI axis and mineralocorticoid receptor (MR-) and glucocorticoid receptor (GR-) centered gene networks were evaluated by use of quantitative real-time PCR. Expression of mRNA for crh was significantly down-regulated by exposure to 300 μg MC-LR/L, while expressions of crhbp, crhr1, and crhr2 were significantly up-regulated, relative to controls. MC-LR caused significantly lesser levels of mRNA for steroidogenic genes including hmgra, star, and cyp17, but expression of mRNA for hsd20b was significantly greater than that of controls. Treatment with MC-LR also altered profiles of transcription of MR- and GR-centered gene networks, which might result in multiple responses. Taken together, these results demonstrated that MC-LR affected the corticosteroid-endocrine system of larvae of zebrafish. This study provided valuable insights into molecular mechanisms behind potential toxicity and endocrine disruption of MCs.

1. Introduction

In recent years, due to activities of humans, including changes in climate and releases of contaminants, frequencies, durations, areas and intensities of blooms of cyanobacteria have been increasing (Buratti et al., 2017). Cyanobacteria can produce extracellular products that are bioactive compounds, called cyanotoxins, which can be harmful to humans, animals and plants. Microcystins (MCs) are the most commonly observed cyanotoxins. MCs are cyclic heptapeptides with 5 non-protein amino acids and 2 variable protein amino acids, sharing the general structure of cyclo-(-D-Ala^1-L-X^2-D-isoMeAsp^3-L-Z^4-Adda^5-D-isoGlu^6-Mdha^7). Methylations, hydroxylations, epimerizations and amino acid replacements lead to structural diversity of MCs, such that more than 100 various congeners have been detected in lakes or cell cultures (Miller et al., 2017). Among these reported congeners, microcystin-LR (MC-LR) is regarded as one of the most commonly occurring, widely distributed and abundant, with the greatest potential for exposure and to cause toxic effects (Chen and Xie, 2016). The total MC concentrations in natural water ranged from a few μg/L to several hundreds of μg/L (Heiskary et al., 2014; Miller et al., 2017; Oehrle et al., 2017;

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Turner et al., 2018).

Since MCs are released from cells of cyanobacteria into surface waters, the most likely initial contacts with organisms is via direct exposure in aquatic environments, especially eutrophic lakes (Pavagadhia and Balasubramanian, 2013). Aquatic animals, including fishes and amphibians, live all or part of their lives in water, thus, exposure to MCs can affect their fitness and survival. In addition to long-term, direct contact with MCs over the body surface, fish are also exposed to toxins when they pass into the body across gill membranes during respiration. Also, some phytoplanktivors, such as silver carp (*Hypophthalmichthys molitrix*) and omnivorous species like Nile tilapia (*Oreochromis niloticus*) can ingest cyanobacteria. A route of indirect exposure of aquatic species to MCs, is through aquatic food webs, by consumption of MC-contaminated aquatic organisms.

Traditionally, because liver is a primary target organ and MCs accumulate there, the primary mechanisms of adverse effects have been various forms of hepatotoxicity. MCs can also damage other organs of fish, including kidney, brain, testis, and ovary, and cause harmful outcomes in fertility, fecundity and development of early life stages (Malbrouck and Kestemont, 2006; Pavagadhia and Balasubramanian, 2013; Chen et al., 2016). Recently, both MC-LR and MC-RR have been reported to affect synthesis of cholesterol in zebrafish (*Danio rerio*), the precursor of all 5 classes of steroid hormones (glucocorticoids, mineralocorticoids, androgens, estrogens, and progestagens), which might result in perturbation of synthesis of steroid hormones and disruption of endocrine systems (Pavagadhi et al., 2013; Chen et al., 2016). It has been found that MCs disrupted endocrine functions of fishes and amphibians (Bury et al., 1996; Jia et al., 2014, 2018; Zhao et al., 2015b; Hou et al., 2017). MC-LR impairs reproduction of zebrafish, Nile tilapia (*Oreochromis niloticus*), and frog (*Rana nigromaculata*) by modulating the hypothalamic-pituitary-gonad (HPG) axis (Zhao et al., 2015b; Chen et al., 2016; Hou et al., 2016, 2017; Liu et al., 2016a, 2018; Su et al., 2016; Jia et al., 2018). Exposure to MC-LR or MC-RR can also disrupt functions of the thyroid axis. Specifically, MCs can affect thyroid follicle epithelial cells, decrease concentrations of thyroid hormones, thyroxin (T4) and triiodothyronine (T3), alter transcription of genes and activities of iodothyronine deiodinases and disrupt signal transduction along the hypothalamic-pituitary-thyroid (HPT) axis (Li et al., 2008; Yan et al., 2012; Liu et al., 2015a,b,c, 2016; Xie et al., 2015; Cheng et al., 2017). Previous studies have found that concentrations of cortisol in blood plasma were significantly greater in brown trout (*Salmo trutta*) exposed to lysed cells of *Microcystis aeruginosa* PCC 7820 (containing 24–42 μg MC-LR/L), and crucian carp (*Carassius auratus*) exposed to extracts of *Microcystis* spp. that contained 150 or 600 μg/kg body mass of MCs by intraperitoneal injection (Bury et al., 1996; Li et al., 2008). These results suggested that MCs might exert potent effects on adrenal endocrine system. However, the potential mechanisms by which MCs affect cortisol are largely unknown.

The adrenal endocrine system is primarily regulated by the hypothalamic-pituitary-adrenal (HPA) axis, which is implicated in maintaining homeostasis of corticosteroids (glucocorticoids and mineralocorticoids) by modulating their synthesis/production, secretion/release, transport, and metabolism (Nesan and Vijayan, 2013). Mineralocorticoids have been implicated in regulation of hydro-mineral balance at a systemic and cellular level; whereas glucocorticoids regulate various cellular and physiological functions, being involved in metabolism, cell growth, development, behavior and the cardiovascular system (Bury and Sturm, 2007). Activation of the corticosteroid stress axis is a highly conserved sequence of coordinated signaling events and behavioral and physiological responses among a variety of organs throughout vertebrate evolution, which can lead to secretion of stress hormones in response to intrinsic or extrinsic stimuli (Nesan and Vijayan, 2013). In response to physical, chemical, or biological stimulus, a series of molecular and biochemical events along the HPA axis includes hypothalamic secretion of corticotropin-releasing hormone (CRH), which acts on corticotropes (corticotrophs) in the anterior pituitary and results in synthesis and secretion of corticotropin (adrenocorticotropic hormone, ACTH), a product from post-translational cleavage of the precursor protein pro-opiomelanocortin (POMC) (Alderman and Bernier, 2009; Alsop and Vijayan, 2009). CRH stimulates secretion of ACTH via specific G-protein coupled receptors (CRHR1 and CRHR2) and is further modulated by a shared binding protein (CRHBP, Alderman and Bernier, 2009). Subsequently, ACTH binds to melanocortin type 2 receptor (MC2R, also called the ACTH receptor) on the inter-renal steroidogenic cells of head kidney to activate the steroidogenic signaling pathway, and synthesize and secret corticosteroids in fish. In turn, cortisol, the main circulating corticosteroid in teleosts, binds to corticosteroid receptors, mineralocorticoid (MR) and glucocorticoid (GR) receptors (Nesan and Vijayan, 2013). Both MR and GR belong to the superfamily of nuclear receptors, which act primarily as ligand-dependent transcription factors in multi-cellular animals, to affect expression of their downstream genes in target tissues and thus playing key roles in many physiological processes (Zhao et al., 2015a; Baker and Katsu, 2017). Homologues of mammalian neuroendocrine pathways, the brain-sympathetic-adrenomedullary and HPA axes, the brain-sympathetic-chromaffin cell axis and hypothalamic-pituitary-inter-renal (HPI) axis, respectively, have been found in fish (Steenbergen et al., 2011). HPA (mammals) and HPI (fish) axes share extensive homologies in terms of the general functional organization and physiology of responses to stressors via similarities in anatomy, connectivity, and molecular constituents. Like mammals, fish respond to various stressors through increased concentrations of cortisol, which, as in humans, is the main corticosteroid. In fish, cortisol is produced and released by steroidogenic inter-renal cells of head kidney, which are the adrenocortical homologue of mammals (Steenbergen et al., 2011). Furthermore, during early ontogenies of fishes, head kidney and progenitors of the immune system become closely arranged (Steenbergen et al., 2011). In a variety of species, including humans, rodents, and fishes, cross-talk among the nervous, immune and endocrine systems have been reported (Viltart and Vanbesien-Mailliot, 2007; Steenbergen et al., 2011). Several studies have shown that MCs accumulated in brain (including the hypothalamus), head kidney (pronephros) and exerted neurotoxic and immunotoxic effects on fishes *in vivo* and *in vitro* (Wright et al., 2004; Lei et al., 2008a,b; Wei et al., 2008, 2009; Wang et al., 2010; Rymuszka et al., 2007, 2010; Rymuszka and Adaszek, 2012; Qiao et al., 2013a; Faltermann et al., 2016). Therefore, it can be postulated that MCs impair corticosteroid-endocrine systems of fishes through the neuro-endocrine system and HPI axis.

In this study, for the first time, effects of MC-LR on the HPI axis during early life stages (embryos/larvae) of zebrafish were investigated. Embryos/larvae of zebrafish have been suggested as an appropriate model species in which to study stress and to assess effects and mechanisms of action of endocrine-active environmental pollutants. In the present study, concentrations of whole-body cortisol were measured in larvae of zebrafish exposed to environmentally relevant concentrations of MC-LR. In addition, expressions of genes along the HPI axis and MR- and GR-centered gene networks were studied. Results of the current study provide a better understanding of the molecular mechanisms behind potential toxicity of MCs and also help in management decisions on MCs in aquatic environments.

2. Materials and methods

2.1. Chemicals and reagents

Purified MC-LR with purity ≥95% was obtained from Taiwan Algal Science Inc. (China). Iodine [^{125}I] Cortisol Radioimmunoassay (RIA) Kits were purchased from Beijing North Institute of Biological Technology (Beijing, China). Bicinchoninic acid (BCA) protein kits were bought from Nanjing Jiancheng Bioengineering, Inc. (Nanjing, Jiangsu, China). TRIzol reagent was purchased from Invitrogen (USA). PrimeScript RT reagent kits and SYBR® Premix Ex Taq™ GC (Perfect Real Time) kits were obtained from TakaRa (Japan). All of the other chemicals and reagents used in the present study were of analytical or higher grades.

2.2. Exposure of zebrafish to MC-LR

Adult (5-month old), AB strain, zebrafish were maintained as described previously (Chen et al., 2017b) with minor modifications. Briefly, zebrafish were cultured in glass tanks with a closed flow-through system with dechlorinated and aerated tap water at 28 ± 1 °C with a light:dark cycle of 12:12 h. Fish were fed freshly hatched brine shrimp twice daily. To ensure consistency in developmental stages, all fertilized eggs used were obtained by artificial fertilization and examined by use of stereo microscopy to select embryos which had normally developed to the blastula stage (4 h post-fertilization, 4 hpf) for subsequent experiments. Embryos were randomly distributed into glass beakers containing 100 mL of MC-LR solution (0, 1, 10, 100 or 300 μg MC-LR/L). There were four replicates for each treatment, and each beaker included 100 embryos. Exposure was during the period from 4 to 168 hpf. The range of exposure concentrations was selected, based on results of previous studies (Li et al., 2011; Yan et al., 2012; Xie et al., 2015). Half of the water in each beaker was replaced every 48 h by fresh water of appropriate concentration of MC-LR. The period of exposure was chosen based on prior knowledge that: (1) expressions of genes tested in the present study in HPI axis became detectable before 96 hpf in zebrafish; (2) zebrafish HPI axis became active at 97 hpf (Fig. 1) (Alsop and Vijayan, 2009). During the exposure period, heart rate (72 hpf), mortality (168 hpf), and malformations (168 hpf) were recorded. Mortality was identified by coagulation of embryos or disappeared heartbeat, and malformation included spinal curvature and pericardial edema. The heart rate (beats/minute) was measured under a stereo microscope (Leica M205FA) and recorded by a stopwatch. Finally, larvae of zebrafish were anesthetized in ice-cold water and immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Detection of concentrations of MC-LR

Quantitative analysis of MC-LR in water was performed by competitive Enzyme-Linked ImmunoSorbent Assay (ELISA) according to the methods described in previous studies (Chen et al., 2017b). Before and during the exposure, concentrations of MC-LR were monitored after renewal of water. A sample of 200 μL of water was collected from each beaker and stored at −20 °C until analysis. Concentrations of MC-LR were determined by use of a commercially available microcystin plate kit (Beacon Analytical Systems, Inc., Saco) with a SpectraMax M5 Microplate Reader (Molecular Devices, CA, USA). The minimum detection limit (MDL) for MCs is 0.1 μg/L.

2.4. Quantification of cortisol

Extraction of cortisol was conducted by use of previously described methods with minor modifications (Alsop and Vijayan, 2008). Briefly, 30 larvae from each beaker were pooled as one replicate (n = 3) and were thawed, homogenized in phosphate-buffered saline (PBS, pH 7.4, 4 °C) for 30 s with a rotor-stator homogenizer. Diethyl ether was added to the homogenate, and the supernatant was collected and evaporated to reduce the volume of solvent. Cortisol was reconstituted in PBS and was measured by use of an Iodine [^{125}I] Cortisol Radioimmunoassay (RIA) Kit (Beijing, China) with a GC-911 γ-counter (Zhongjia, China). Content of cortisol was normalized to the protein content of the corresponding sample, which was measured with a bicinchoninic acid (BCA) protein kit (Nanjing, China), with bovine serum albumin (BSA) as the standard.

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

Thirty larvae from each beaker were pooled as one replicate (n = 3). qRT-PCR was performed by use of previously described methods with minor modifications (Chen et al., 2014, 2017b). Briefly, isolation of total RNA was conducted by use of TRIzol reagent (Invitrogen, USA). Concentrations of total RNA were determined by measuring reading value of the absorbance at 260 nm (A260) by use of a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc, MA, USA) and purity of RNA was assessed by the A260/280 ratios with values between 1.8 and 2.1. Integrity of RNA was estimated by visual inspection of bands of two ribosomal RNAs (rRNAs), 28S and 18S, on a 1% agarose gel with GelRed staining. First strand, complementary DNA (cDNA) was synthesized by reverse transcription of total RNA with oligo-dT primers and random 6 mers by use of a PrimeScript RT reagent Kit (TakaRa, Japan) on a T100™ thermal cycler (Bio-Rad, Pleasanton, CA, USA).

Sequences of primers used for mRNA expression analysis were designed with Primer Premier 5.0 (Premier, Canada) (Table 1). qRT-PCR was performed by use of SYBR® Premix Ex Taq™ GC (Perfect Real Time) kit (TakaRa) on a Bio-Rad CFX96 Real-Time System (Bio-Rad). The thermal cycle was set as follows: 3 min initial denaturation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 15 s, and primer annealing and extension at 72 °C for 20 s. To confirm that single PCR products were formed, melt curves of

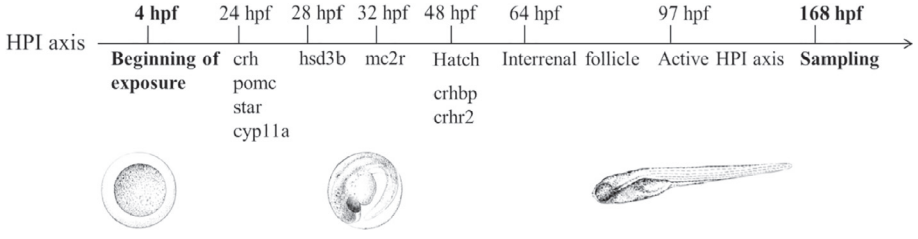

Fig. 1. Overview of timing of initial detection for components of the hypothalamic-pituitary-inter-renal (HPI) axis during development of zebrafish (Alsop and Vijayan, 2009) and experimental design.

Table 1
Primer sequences of target genes along the hypothalamic-pituitary-inter-renal (HPI) axis in zebrafish.

Gene	Primer Sequence (5′-3′)		GenBank number	Amplicon size (bp)	References
	Forward	Reverse			
crh (corticotropin-releasing hormone)	TTCCACCGCCGTATGAATGT	CGAGCCGGATGAAGTACTCC	NM_001007379.1	126	–
crhr1 (corticotropin releasing hormone receptor 1)	GCGGGTCAGTTTCCACAGTA	CCCTGGGTTTTGCAGAGGAT	XM_017354749	136	–
crhr2 (corticotropin releasing hormone receptor 2)	TGACGAGCCACCTGTTTCTC	CACAGGTCAGGAGTTAGGCG	NM_001113644	143	–
ui (urotensin 1)	AACATGATCGAAATGGCGCG	AGTGTGAATGTCGGATGCGT	NM_001030180	179	–
avt (arginine vasotocin)	TCGTCTGCCTGCTACATCCA	TCCGGCTGGGATCTCTTG	NM_178293	56	Fuzzen et al., 2010
trh (thyrotropin-releasing hormone)	GCCCAGCTTTACCCCAATGT	ATTCAGCGCTACCTACTTTTGGA	NM_001012365	65	Fuzzen et al., 2010
crhbp (corticotropin-releasing hormone binding protein)	GGATAACGAGATCAGCCCGG	ACCCTCTACGGCCACCATAT	NM_001003459	124	–
pomc (pro-opiomelanocortin)	GCCCCTGAACAGATAGAGCC	CTCGTTATTTGCCAGCTCGC	AY158003.1	192	–
pc1 (prohormone convertase 1)	TGGCTGCTGGGATATTTGC	GAAGGTCCCGCCATGTCA	NM_001137662	63	Fuzzen et al., 2010
pc2 (prohormone convertase 2)	TTAGTAGCAACGACCCATACCCTTA	CGGGTACCATGACTGTTAAACCA	NM_001142266	67	Fuzzen et al., 2010
hmgra (hydroxy-methylglutaryl-CoA reductase a)	AGAACAGGAGACGCAATGGG	TTGGCTGGAATGGTGGTCTC	BC155135	197	–
hmgrb (hydroxy-methylglutaryl-CoA reductase b)	CCAACTGCTCCCTGATCAAT	TTTAAGAATCGCGAGGCACT	NM_001014292	203	Liu et al., 2011
star (steroidogenic acute regulatory protein)	ACCTCATCCCACCCATAGCT	GGCAAAGTGGAGGTGACAGA	NM_131663.1	151	–
cyp17 (cytochrome P450, family 17)	GACCCAGGACGCTTTCTGAA	TTTGCAAAATCCACGCCAGG	AY281362.1	148	–
hsd11b2 (hydroxysteroid 11-beta dehydrogenase 2)	GGAGAGGGAGCCAAGCATTT	AAGTTTGGCCTTGGTGTCGA	NM_212720	117	–
hsd20b2 (hydroxysteroid 20-beta dehydrogenase 2)	AAGAGCTTGCCAAACGAGGA	TGCCTGAATAACGTGCGTCT	XM_689815	119	–
cyp21a2 (cytochrome P450, family 21, subfamily A, polypeptide 2)	AATGGTCTGACTTTGCTGGGA	CAGCCTTTCCACTGTAGTCTCG	XM_021466882.1	222	–
cyp11al (cytochrome P450, family 11, subfamily A, polypeptide 1)	CACCTCACCCATGCTGTACCTA	GGTCAGCCTGGTTAAAGATGC	NM_152953.2	107	–
cyp11b (cytochrome P450, family 11, subfamily B)	TGGAAGCGAGCTGTCACGTT	TCGCTCCACAGCCGAAATG	NM_001080204.1	98	–
mc2r (melanocortin 2 receptor)	CCTCTTGGTGATTGTGGCTGTG	AAAGGGCCGCGTAAGTTCAG	NM_180971.1	168	–
hsd3b (hydroxy-delta-5-steroid dehydrogenase, 3 beta)	AGGCACGCAGGAGCACTACT	CCAATCGTCTTTCAGCTGGTAA	AY279108.1	60	–
mr (mineralocorticoid receptor)	ATTGGGCCTAGTGCAAAATG	TCTCTGTTTGGCTCGGTCTT	EF567113	249	Liu et al., 2011
ube2i (ubiquitin-conjugating enzyme E2I)	TGGAAAGAGGGAAGATGTGG	CGAATGAAGTGAAGGGGTGT	NM_131351	155	Ma et al., 2015
hsd11b3 (11beta-hydroxysteroid dehydrogenase-type 3)	TGGTGAAGTATGCCATCGAA	GCAAAGCTTTTTGAGCCATC	AY578180	162	Ma et al., 2015
hpse (heparanase)	CGGCAGTCTGAACAGATGAA	AACACGGGACAAATCCACAT	NM_001045005	153	Ma et al., 2015
egfr (epidermal growth factor receptor)	AACGCAAATAATGGCAGGAC	TCTCCAGAACCACAGTGCAG	AY332223	191	Ma et al., 2015
adrb2a (adrenoceptor beta 2, surface a)	CGGCCAGTATCGAGACACTC	AGCACAATGAAGCGAGCTCT	NM_001102652	112	–
adrb2b (adrenoceptor beta 2, surface b)	TTCTTTTGCGAGTTTTGGACG	GTAGGAACGAGATAAGGGCGG	NM_001089471.1	193	–
gr (glucocorticoid receptor)	CTACGTTGAACAGGCTGGGT	AGGTCTGGAGCGAAACACAG	EF567112.1	199	–
pepck (phosphoenolpyruvate carboxykinase)	TGCCTGGATGAAATTTGACA	GGCATGAGGGTTGGTTTTTA	NM_213192.1	106	Seiliez et al., 2013
tat (tyrosine aminotransferase)	GCCTGAATCCAGTGATGCCGTC	GATTGCTCAGTGACCAGCCGTT	NM_001077554.1	118	Zhang et al., 2015
hsp90aa1 (heat shock protein 90, alpha (cytosolic), class A member 1)	GGATCTGGTGATCCTGCTGT	TCCAGAACGGGCATATCTTC	NM_131328	180	Ma et al., 2015
rela (v-rel avian reticuloendotheliosis viral oncogene homologue A)	TATAAGCCACACCCACACGA	GAATGGGTTGTTTTGCGTCT	AY163839	174	Ma et al., 2015
dap3 (death associated protein 3)	TCGACCGTTCATGTAAACCA	CTGGATGCTGAGACACCTGA	NM_001098737	174	Ma et al., 2015
tgfb1 (transforming growth factor, beta 1)	GTCCGAGATGAAGCGCAGTA	GGAGACAAAGCGAGTTCCCA	AY178450	184	–
gapdh (glyceraldehyde-3-phosphate dehydrogenase)	GATACACGGAGCACCAGGTT	GCCATCAGGTCACATACACG	NM_001115114	163	Liu et al., 2013

amplification products were analyzed at the end of each PCR reaction. qRT-PCR reactions were carried out in triplicate for each selected gene. The housekeeping gene gapdh was stable and unaffected by exposure to MC-LR. Therefore, it was used as the endogenous assay control for normalization of gene expression data. Relative expressions of the genes were calculated by use of the $2^{-\Delta\Delta CT}$ method.

2.6. Statistical analyses

Statistical analyses were performed by use of the SPSS package19.0 (SPSS, Chicago, IL, USA). All values were expressed as the mean ± standard error (SE). Data were examined to determine if they met the assumption of normality, by use of the Kolmogorov-Smirnov test. The assumption of homogeneity was checked by use of Levene's test. If necessary, data were log-transformed to approximate normality. Nonparametric analyses were carried out if data could not meet the normality even after transformation. Differences between control and MC-LR treatment groups were tested by One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Two levels of statistical significances were defined as those with $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results

3.1. Quantification of MC-LR in exposure solutions

During exposures, actual concentrations of MC-LR in 1, 10, 100 and 300 μg/L treatment groups were 0.88 ± 0.08, 8.24 ± 0.75, 81.75 ± 10.05 and 250.84 ± 15.34 μg/L, respectively. MC-LR was not detected in the control group. Thus, all exposure concentration data are presented as nominal concentrations.

3.2. Developmental toxicity

Exposure to MC-LR did not affect rates of mortality or

malformation in larvae of zebrafish exposed to 1, 10, 100 or 300 μg MC-LR/L (Fig. 2). Significant lesser heart rates were observed in larvae exposed to 300 μg MC-LR/L.

3.3. Whole-body cortisol levels

Exposure to 300 μg MC-LR/L caused significantly greater concentrations of whole-body cortisol (1.66-fold) relative to those of controls (Fig. 3), but no significant alteration was detected in larvae of zebrafish exposed to lesser doses (1, 10, or 100 μg MC-LR/L), relative to that of controls.

3.4. Expressions of genes related to hypothalamic and pituitary hormones

Expression of mRNA for crh was significantly down-regulated in larvae of zebrafish exposed to 10, 100, or 300 μg MC-LR/L, relative to that of the control (Fig. 4A). Relative to controls, treatment with 300 μg MC-LR/L resulted in significantly up-regulated expressions of mRNA of crhbp, crhr1, and crhr2, but down-regulated expression of mRNA of avt. Relative to the controls, significant down-regulation of expressions of mRNA of pomc was observed upon exposure to 10, 100 or 300 μg MC-LR/L. Expressions of mRNA of trh, ui, pc1, or pc2 were not altered by exposure to MC-LR.

3.5. Expressions of genes related to steroidogenic pathways

Treatment with 100 or 300 μg MC-LR/L resulted in significantly down-regulation of expressions of mRNA of mc2r, hmgra and star relative to those in controls (Fig. 4B). Expression of mRNA for cyp17 was significantly down-regulated when exposed to 300 μg MC-LR/L, while expression of mRNA for hsd20b was significantly up-regulated. Relative to expressions in controls, no statistically significant alterations of expression of mRNA for hmgrb, cyp11a, hsd3b, cyp21a2, cyp11b, or hsd11b were observed in larvae exposed to MC-LR.

3.6. Expressions of genes in GR and MR pathways

Expression of gr was significantly less in larvae of zebrafish exposed to 100 or 300 μg MC-LR/L relative to that of the control (Fig. 5A). MC-LR caused significant down-regulation of expressions of dap3 in larvae of zebrafish exposed to 10, 100 or 300 μg MC-LR/L. When exposed to 100 or 300 μg MC-LR/L, expressions of mRNA for rela and tat were significantly greater than those of the controls. Expression of mRNA for pepck was significantly up-regulated by exposure to 300 μg MC-LR/L, while expression of mRNA for hsp90aa1 was significantly down-regulated. Expressions of hpse or tgfb1 were not changed by exposure to MC-LR.

When exposed to 300 μg MC-LR/L, expression of mRNA for mr was significantly greater than that of the controls (Figs. 5B and 6). Treatment with 100 or 300 μg MC-LR/L resulted in significant down-regulations of ube2i relative to that of controls. Expression of mRNA for hsd11b3 was down-regulated in larvae of zebrafish

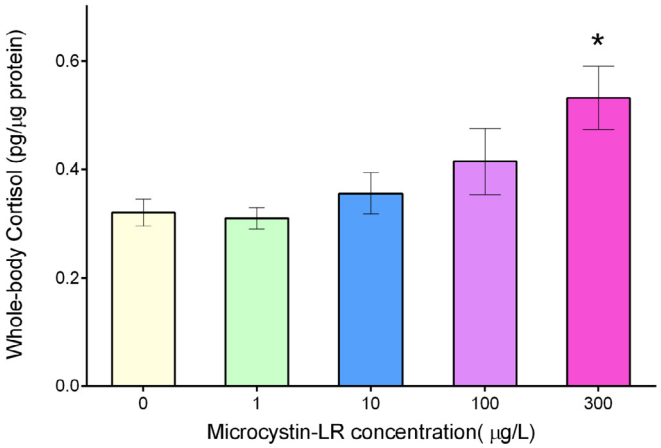

Fig. 3. Whole-body concentrations of cortisol in larvae of zebrafish exposed to 1, 10, 100, or 300 μg MC-LR/L (microcystin-LR). Values are presented as the mean ± standard error (SE). * indicates $p < 0.05$ versus control.

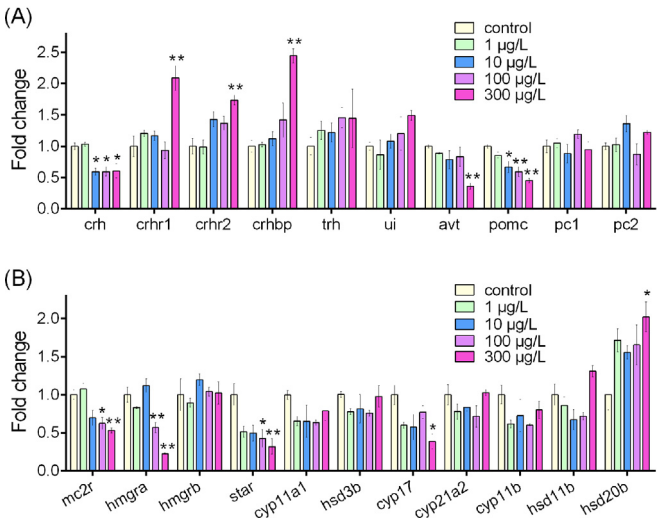

Fig. 4. Expressions of genes related to hypothalamic and pituitary hormones (A) and steroidogenic pathways (B) in larvae of zebrafish exposed to 1, 10, 100, or 300 μg MC-LR/L (microcystin-LR). Quantitative real-time PCR was used to quantify numbers of copies of mRNA for target genes. Gapdh was used as internal control. Values are presented as the mean ± standard error (SE). * indicates $p < 0.05$ versus control, and ** indicates $p < 0.01$ versus control.

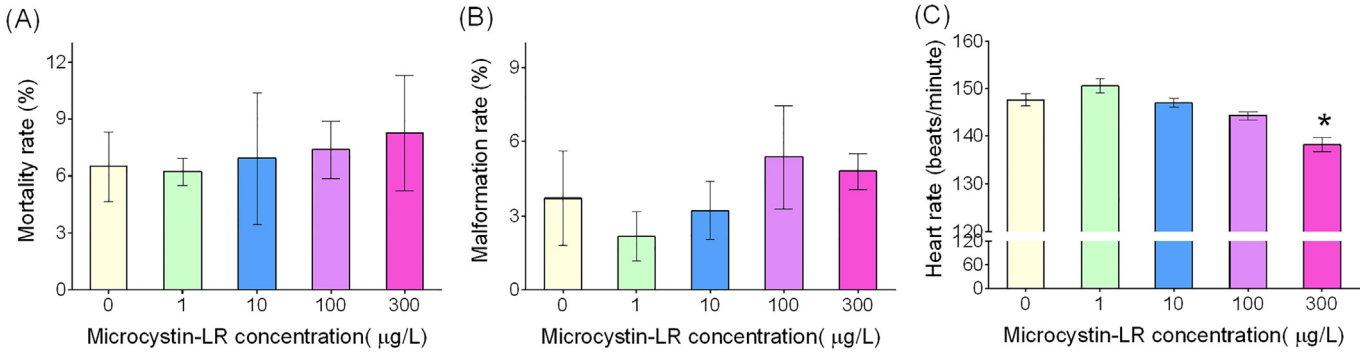

Fig. 2. Mortality rate (A), malformation rate (B), and heart rate (C) of larvae of zebrafish exposed to 1, 10, 100, or 300 μg MC-LR/L (microcystin-LR). Values are presented as the mean ± standard error (SE). * indicates $p < 0.05$ versus control.

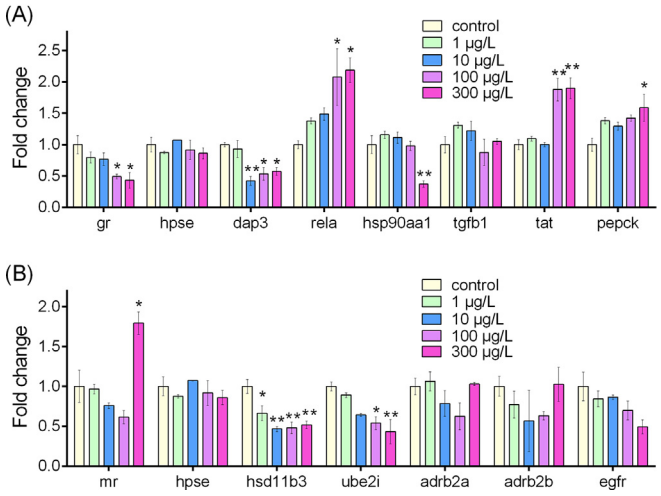

Fig. 5. Expressions of genes in glucocorticoid (GR) and mineralocorticoid receptor (MR) pathways in larvae of zebrafish exposed to 1, 10, 100 or 300 μg MC-LR/L (microcystin-LR). Quantitative real-time PCR was used to determine numbers of copies of mRNA for target genes. Gapdh was used as internal control. Values are presented as the mean ± standard error (SE). * indicates $p < 0.05$ versus control, and ** indicates $p < 0.01$ versus control.

exposed to 1, 10, 100 or 300 μg MC-LR/L. There were no differences in expressions of mRNA for hpse, egfr, adrb2a, or adrb2b in larvae exposed to MC-LR, relative to expressions of mRNA for those genes in controls.

4. Discussion

In order to maintain internal homeostasis in a complex and continually changing environment, vertebrates have evolved a conserved adaptation known as the stress response (Fuzzen et al., 2010). In fishes, the stress response is modulated by an endocrine coping mechanism, the hypothalamic-pituitary-inter-renal (HPI) axis, which regulates concentrations of cortisol circulating in blood. In the present study, effects of exposure to MC-LR during early embryonic development on the HPI axis in larvae of zebrafish (*Danio rerio*) were investigated. The overall results showed that MC-LR significantly affected both concentrations of cortisol and transcription of genes along the HPI axes during early life stages (embryos/larvae) of zebrafish, indicating that general stress was induced, and altered HPI axis function might affect metabolism, osmoregulation and development.

Compared to the hypothalamic-pituitary-gonad (HPG) or -thyroid (HPT) axis, few studies have focused on impacts of MCs on the HPI axis. The HPI axis and its end product, cortisol, have important functions in multiple physiological processes, not only in regulation of metabolism, osmoregulation and hydro-mineral balance, but also affecting behavior, immunity, growth, development, and reproduction (Fuzzen et al., 2010). In the present study, no lethality or malformations were observed in embryos/larvae of zebrafish exposed to 1, 10, 100, or 300 μg MC-LR/L, which indicated that exposures were indeed less than the threshold for overt toxic effects on general health of exposed fish. Results of previous studies have shown that MC-LR at concentrations more than 500 μg/L caused lethality and malformations (Li et al., 2011). However, a decrease of heart rate was observed at lesser concentrations, 200 and 300 μg

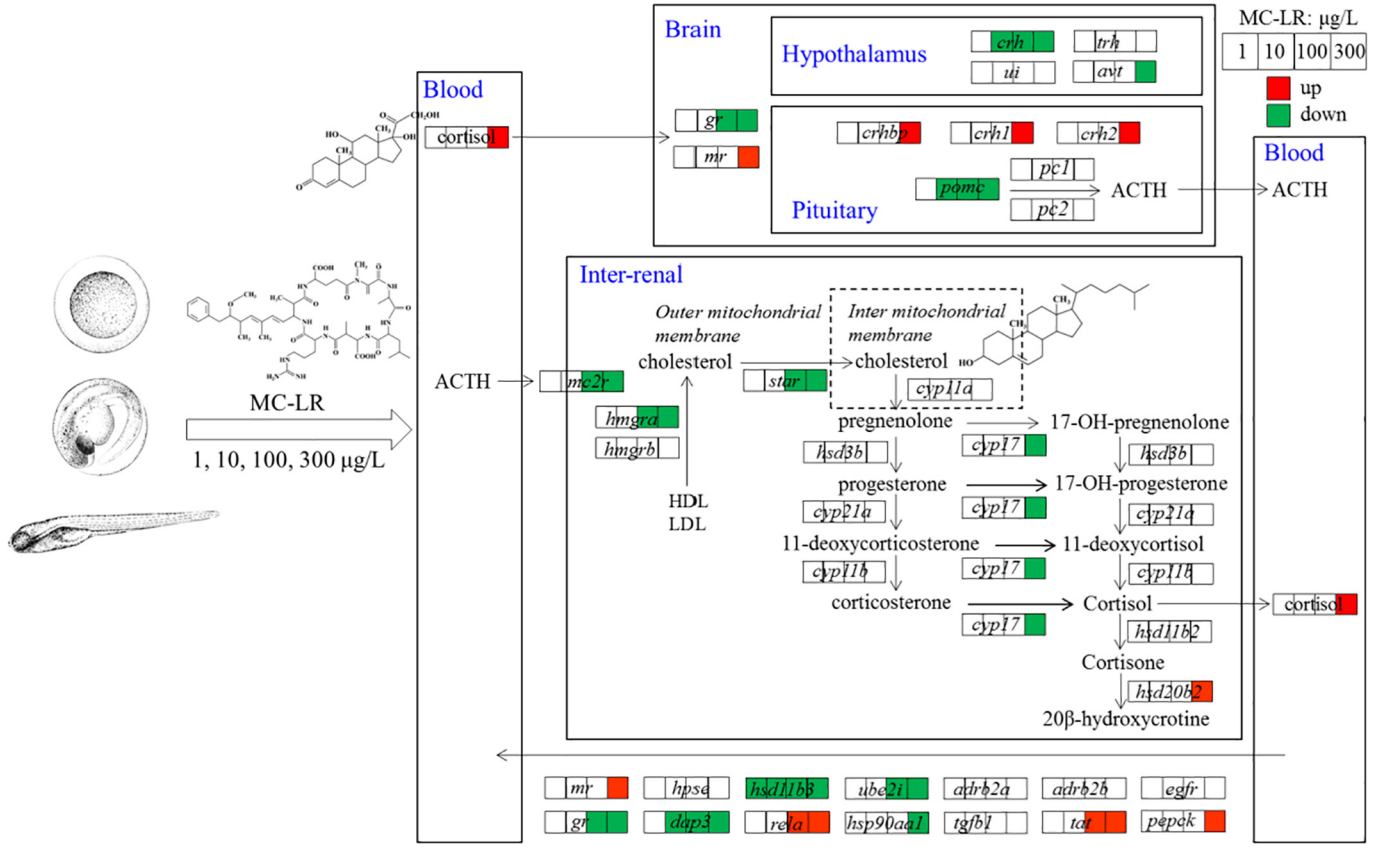

Fig. 6. Effects of microcystin-LR (MC-LR) on the hypothalamic-pituitary-inter-renal (HPI) axis in larvae of zebrafish.

MC-LR/L in this study and a previous study (Li et al., 2011). Depressed heart rate and embryonic heart failure were also reported in Japanese medaka (*Oryzias latipes*) and *Sinocyclocheilus graham* (Saraf et al., 2018; Zi et al., 2018). It has been found that embryo exposure to elevated cortisol leads to lesser heart rate and cardiac performance dysfunction in zebrafish (Nesan and Vijayan, 2012). As expected, in this study, concentrations of whole-body cortisol were significantly greater in larvae of zebrafish exposed to MC-LR until 168 h post-fertilization (hpf). Thus, these results suggest that MCs can cause adverse outcome such as bradycardia and heart failure through disturbing the cortisol signaling pathway. This finding is consistent with results of previous studies of brown trout exposed to lysed cells of *Microcystis aeruginosa* PCC 7820 (containing 24–42 μg MC-LR/L), and crucian carp exposed to extracts of *Microcystis* spp. that contained 150 or 600 μg/kg body mass of MCs by intraperitoneal injection (Bury et al., 1996; Li et al., 2008).

As the primary neurohormone, corticotropin-releasing hormone (CRH) produced in the hypothalamus stimulates pituitary secretion of corticotropin (adrenocorticotropic hormone, ACTH). ACTH then binds to melanocortin type 2 receptor (MC2R) on the steroidogenic inter-renal cells of head kidney, which is analogous to the mammalian cortex of the adrenal gland, and stimulates synthesis and release of cortisol in fish. This pathway, referred to as the HPI axis, is activated following exposure to diverse stimuli and is homologous to and virtually identical to the hypothalamus-pituitary-adrenal (HPA) axis of tetrapods (Alderman and Bernier, 2009; Bury and Sturm, 2007). Besides CRH, additional peptides are also capable of inducing the secretion of ACTH by the pituitary. These include: urocortin I (UI), thyrotropin-releasing hormone (TRH), angiotensin II (ANG II), arginine vasotocin (AVT), which is homologue of arginine vasopressin (AVP) in mammals, and isotocin (IST), which is homologous to mammalian oxytocin (Steenbergen et al., 2011). In the current study, results of which are presented here, abundances of transcripts of crh, avt, pomc, mc2r, hmgra, star, and cyp17 were significantly less in fish exposed to MC-LR, relative to unexposed controls, while greater expressions of mRNAs for crhr1, crhr2, crhbp, and hsd20b were observed. Thus, it is suggested that exposure to MCs causes the observed effects via negative feedback, which could inhibit production of CRH by the hypothalamus, release of ACTH by the pituitary, or steroidogenesis in the head kidney. Negative feedback is one possible mechanism that allows living organisms to maintain relatively stable physiological status when facing stress (Liu et al., 2013). In previous studies, MCs have also been reported to alter expression of mRNA for crh (Yan et al., 2012; Xie et al., 2015; Liu et al., 2015b, 2016b). Expression of mRNA for crh was significantly up-regulated in larvae of zebrafish exposed to 500 μg MC-LR/L at 96 hpf (Yan et al., 2012), while it was significantly down-regulated in larvae of zebrafish exposed to 300, 1000 or 3000 μg MC-RR/L at 96 hpf (Xie et al., 2015). Exposure to 1, 5 or 25 μg MC-LR/L resulted in significantly greater expression of mRNA for crh in 1-month-old, juvenile zebrafish for 7, 14, 21 or 28 days (Liu et al., 2015b), but caused significant lesser mRNA expression of crh in 3-month old, adult zebrafish for 14, 21 or 28 days (Liu et al., 2016b). These differences in responses might be due to variants and concentrations of MC, different temporal patterns of expression, different developmental stages, and/or different durations of exposure. Therefore, to understand mechanisms, time-course transcriptional experiments might be needed.

Unlike mammals, cortisol acts as both corticosteroids (glucocorticoids and mineralocorticoids) in teleosts (Bury and Sturm, 2007). The majority of physiological processes regulated by corticosteroids are mediated via corticosteroid receptors: glucocorticoid (GR) and mineralocorticoid receptors (MR) (Bury and Sturm, 2007). These receptors act as ligand-dependent transcription factors and are responsible for translation of the corticosteroid (cortisol) signal to the genome. In this study, exposure to MC-LR caused up-regulation of expression of mr but down-regulation of associated genes ube2i and hsd11b3, while hpse, egfr, adrb2a, or adrb2b showed no significant changes. However, gr and its downstream genes hsp90aa1 and dap3 were down-regulated while tat, pepck, and rela were up-regulated. Therefore, further investigations might be required to explore the relationship between gr, mr and their associated downstream genes in embryos/larvae of zebrafish exposed to MCs in future.

Effects of chemicals on cross-talk among the HPI, HPG, and HPT axis have been characterized in both larvae and adults of zebrafish (Liu et al., 2011, 2013). In zebrafish, chemical-induced adverse impacts on one endocrine axis can have indirect effects on other endocrine axes. Therefore, results of this study suggested that MCs also disrupted HPG or HPT axes. It has been reported that exposure to MCs can result in changes of amounts of hormones, including 17β-estradiol, testosterone and thyroid hormone (Yan et al., 2012; Qiao et al., 2013b; Liu et al., 2015a,b, 2016b; Xie et al., 2015; Zhao et al., 2015b; Chen et al., 2016; Chen et al., 2017a; Hou et al., 2016; Liu et al., 2016a; Su et al., 2016; Cheng et al., 2017). Exposure to MCs can also inhibit growth and development of oocytes, changes in mating and spawning behavior, and inhibit reproduction of fishes (Chen et al., 2016). Apart from regulating the HPI axis, it has also been demonstrated that CRH is a potent stimulator of pituitary thyrotropin (TSH) secretion in non-mammalian vertebrates (De Groef et al., 2006; Liu et al., 2011, 2013). In fishes, CRH is a key linker between HPI and HPT axis and is a more pronounced factor than thyrotropin-releasing hormone (TRH) for promoting TSH secretion and regulating HPT axis. Alteration of CRH could cause deregulation of TSH in zebrafish and Chinese rare minnow exposed to MCs (Yan et al., 2012; Liu et al., 2015a,b, 2016b; Xie et al., 2015; Cheng et al., 2017). Also, in vertebrates, brain, adrenal/inter-renal tissue and/or gonads (testis or ovary), partly, share certain parts of steroidogenesis-related pathway composed of the same genes, such as star, hmgra, hmgrb, hsd3b, and cyp17 (Liu et al., 2013). Therefore, it is difficult to determine if effects of MCs on steroidogenesis-related gene expression occur in brain, inter-renal, gonads, or all three. Due to their smaller size, when zebrafish embryos/larvae are used as research subjects, total RNA had to be isolated from the whole body for subsequent cDNA synthesis and qRT-PCR. Because this would represent a number of organs and tissues, it is not possible to determine which might have been the target of effects of MCs. Nevertheless, results of this study indicated that MCs could modulate steroidogenesis and endocrine function in larvae of zebrafish. Further studies might use *in situ* hybridization to investigate effects of MCs on steroidogenesis in different tissues including brain, inter-renal tissue and/or gonads of zebrafish larvae (Park et al., 2008; Alderman and Bernier, 2009). Although study of interactions among the HPG, HPI, and HPT axes was beyond the scope of this study, the implications of interactions among endocrine axes are important and the molecular mechanisms warrant further investigations.

It's widely accepted that the main acute toxic mechanisms of MCs are inhibition of protein phosphatases 1 and 2A (PP1 and PP2A) and induction of oxidative stress by covalent binding of Mdha[7] of MCs to thiol of PP1, PP2A, and GSH (Chen and Xie, 2016). Recently, increasing studies reported that environmentally relevant concentrations of MCs (from a few μg/L to several hundreds of μg/L) can exert endocrine-disrupting effects on fish and amphibians, which suggest that MCs may be endocrine disrupting chemicals (EDCs) (Yan et al., 2012; Jia et al., 2014, 2018; Liu et al., 2015a,b, 2016b; Xie et al., 2015; Zhao et al., 2015b; Hou et al., 2017). EDCs are a group of potentially hazardous substances that include natural and synthetic chemicals and have the ability to mimic endogenous hormones and interfere with their biosynthesis,

metabolism, or action at environmentally relevant concentrations (le Maire et al., 2010). MC-LR, a cyclic heptapeptide, is a structural analog to cholesterol and cortisol (Fig. 6). Therefore, it's possible that the endocrine-disrupting effects are due to their ability to simulate cholesterol or cortisol and modify their synthesis, metabolism, and transport. MC-LR may also act via nuclear receptors GR and MR, and thus affecting the HPI axis. This finding addresses ecological consequences of environmentally relevant concentrations of MCs on fish and other animals.

The affected genes at level of regulation and adverse effects at level of phenotype is a classical and controversial question in toxicological studies. Currently, questions such as “What do these molecular alterations really mean in terms of adverse effects?” are still not sufficiently answered. In this study, several genes along the HPI axis and MR- and GR-centered gene networks (crh, pomc, dap3, and hsd11b3) were affected at much lower concentrations than others, especially hsd11b3 has been modulated already at 1 μg/L. On the other hand, a lot of other genes (crhr1, crhr2, crhbp, avt, hsd20b, hsp90aa1, pepck, and mr) have been affected only at the greatest tested concentration, 300 μg/L. Therefore, the effective concentrations for modulation of different genes vary. Nevertheless, adverse outcomes (decrease of heart rate and elevated cortisol level) were only observed in larvae of zebrafish exposed to 300 μg MC-LR/L. More studies are needed to clarify the correlation of expression of genes and apical adverse effects.

5. Conclusions

In summary, this is the first study demonstrating that exposure to MC-LR resulted in disruption of corticosteroid-endocrine system in early life stages (embryos/larvae) of zebrafish. MCs could alter transcription of genes associated with hypothalamus-pituitary-inter-renal (HPI) axis, including neurocrine pathways, steroidogenic pathways and receptor signaling pathways. These results suggest that MCs have endocrine-disrupting effects. This study raises concerns about the potential ecological risks of increasingly occurred cyanobacterial blooms and frequently detected MCs in natural water sources worldwide. The effects of MCs on the endocrine system and cross-talk of endocrine axis (HPG, HPI, and HPT) warrant further investigations.

Conflicts of interest

None.

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