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Changes in plasma thyroid hormones and cortisol levels in crucian carp (*Carassius auratus*) exposed to the extracted microcystins

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ABSTRACT

The endocrine response of crucian carp injected intraperitoneally with extracted microcystins (MC) was investigated in this study. Fish were injected intraperitoneally either with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 600 μg microcystins per kg body weight. The plasma levels of triiodothyronine (T_3), thyroxine (T_4), free triiodothyronine (FT_3), free thyroxine (FT_4), and cortisol were determined at 0, 1, 3, 12, 24, and 48 h post-administration of MC-containing extract. Treated fish displayed abnormal behaviors, such as a startle response and disoriented swimming, as well as changes in ventilation rates. Plasma cortisol concentrations of fish in both dose groups significantly increased after administration of extracted MC and remained high throughout the experiment, which suggested that MC elicited a stress response in treated fish. The profiles of cortisol changes in treated fish appeared to be dose dependent, indicating that fish in the high dose group experienced greater MC-induced disturbance. Mortality occurred after 12 h in the high dose group. Plasma levels of T_4, T_3, FT_4, and FT_3 did not vary significantly between the control fish. In contrast to this, fish exposed to MC-containing extract showed significant declines in T_3, FT_4, and FT_3 levels in a dose-dependent manner throughout the experiment. Plasma T_4 levels, however, did not vary significantly in the low dose group, whereas they decreased significantly at 48 h post injection in the high dose group. This study demonstrates that administration of microcystins-containing extract causes a stress response and reduces the plasma levels of thyroid hormones in crucian carp. These results illustrate that microcystins exerted potent effects on the endocrine system of crucian carp, through activating their hypothalamus-pituitary- interrenal axis and disturbing thyroid function.

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1. Introduction

Microcystins (MCs) are a family of potent hepatotoxins produced by several genera of freshwater cyanobacteria, including *Microcystis*, *Nodularia*, and *Anabena*. More than 75 structural variants of MCs are currently known (Svrcek and Smith, 2004), which differ principally in the two L-amino acids at positions 2 and 4 (Sivonen and Jones, 1999). The most common form is microcystin-LR (MC-LR) (Dawson, 1998). Exposure to toxic cyanobacteria or administration of MCs can cause fish death (Lindholm et al., 1999), hepatotoxic effects (Solter et al., 1998; Li et al., 2005; Gupta and Guha, 2006), kidney damage (Kotak et al., 1996; Fischer and Dietrich, 2000), haematological and biochemical changes (Vajcova et al., 1998; Malbrouck et al., 2003; Zhang et al., 2007), growth inhibition (Bury et al., 1995; Zhao et al., 2006), oxidative stress (Ángeles et al., 2005), and reproductive injury (Ding et al., 2006).

The impact of xenobiotic compounds on endocrine effects has attracted growing interest in recent years. However, few researchers have studied the effects of MCs on endocrine responses in teleosts. Waterborne toxicants generally are known to cause endocrine disturbances in fish (Zhou et al., 2000; van der Oost et al., 2003; Thangavel et al., 2005), and hormone regulation may be impaired due to exposure to environmental pollutants (Folmar, 1993; Ernst et al., 2007; Bury et al. (1996) reported that short-term exposure (96 h) to lysed toxic *Microcystis aeruginosa* cells led to elevations in plasma cortisol and glucose levels of brown trout (*Salmo trutta*) and Ernst et al. (2007) showed that toxic cyanobacteria could elicit a classic stress response in the form of abnormal behavior and increased ventilation rates, thereby disturbing the normal physiological processes of fish.

In fish, thyroid hormones (THs) such as triiodothyronine (T_3) and thyroxine (T_4) are involved in a variety of physiological

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processes (Griffin, 2000; Power et al., 2001; Liu and Chan, 2002; Eales, 2006). Numerous studies have demonstrated that environmental contaminants affect plasma TH levels and cause thyroid dysfunction in fish (Waring and Brown, 1997; Zhou et al., 2000; Thangavel et al., 2005). Furthermore, an elevated level of cortisol, which is a common endocrine response during stress induced by waterborne pollutants, has been shown to interfere with thyroid function in fish (Walpita et al., 2007). Previous investigations demonstrate that toxins produced by some strains of phytoplankton, such as domoic acid, can alert the thyroid function of rodents by affecting neuroendocrine regulation of THs (Arufe et al., 1995; Alfonso et al., 2000). However, measurement of TH levels during MC exposure in aquatic animals has never been reported.

To date, it is not clear whether MCs can disrupt the endocrine system in fish. But, decline in TH levels and increase in cortisol levels caused by toxins usually reduce the physiological functions in animals. We hypothesized that microcystins would probably disrupt circulating thyroid hormones and cortisol levels, which in turn influence the physiological functions in fish proved by previous studies (Bury et al., 1995; Zhao et al., 2006; Ernst et al., 2007). Therefore the aim of this study was to elucidate the possible mechanism behind reductions of physiological performance in fish exposed to toxic cyanobacteria by evaluating the effects of extracted MCs on plasma TH and cortisol levels in crucian carp (*Carassius auratus*).

2. Materials and methods

2.1. Toxin

Cyanobacteria (mainly composed of *Microcystis* spp.) were collected from surface blooms of Lake Dianchi, Yunnan in China. Crude MCs in the cyanobacteria were extracted three times with 75% (V/V) methanol and suspended in distilled water for use in the experiment. To determine the MC content in cyanobacteria, the extract was filtered through a glass microfiber filter (Whatman GF/C, Maidstone, UK) and the filtrate was diluted 1:5 with distilled water. Next, the diluted filtrate was applied to a 5 g reversed phase octadecylsilance (ODS) column, which was previously activated with 50 ml of methanol (100%) followed by 50 ml distilled water. The cartridges then were washed with 50 ml distilled water. MCs were eluted from the cartridges with 100 ml 100% methanol and then evaporated to dryness. The residue was dissolved in 10 ml distilled water and used for the final detection. Quantitative analysis of MCs was performed using a reverse-phase high-performance liquid chromatography. Concentration of MCs was determined by comparing the peak areas of the test samples with those of MC-LR and MC-RR standards (Wako Pure Chemical Industries, Japan). The total MC content was 1.41 mg g^{-1} dry weight (DW), with MC-RR and -LR values of 0.84 and 0.50 mg g^{-1} DW, respectively. The extracted microcystins (mainly MC-RR and -LR) were resuspended in 0.75% NaCl solution to 75 μg ml^{-1} and 300 μg ml^{-1}, respectively.

2.2. Fish and toxin administration

Healthy crucian carp weighing 265 ± 22.6 g were collected from a local fish hatchery in Wuhan City, China and were transported to the laboratory at College of Fisheries in Huazhong Agricultural University. Fish were acclimated for 15 days before the start of the experiment. During the acclimation and experimental period, water temperature (25 ± 1 °C), dissolved oxygen (6.8 ± 0.7 mg L^{-1}), and ammonia concentration (range trace to 0.5 mg L^{-1}) were recorded daily. Fish were fed with pellet food at a rate of 2.0% of the body weight per day. Feeding was terminated 2 days before initiation of the experiment, and no food was supplied to fish during the experimental period.

In the present study, fish were exposed to sublethal and lethal concentrations of extracted MCs. Fish in the sublethal group ($n = 120$) and the lethal group ($n = 120$) were injected intraperitoneally with *Microcystis* extracts corresponding to 150 and 600 μg microcystins per kg body weight (BW). The injection volume used in each treated fish was 2 ml kg^{-1}. Fish ($n = 120$) injected intraperitoneally with 0.5 ml of 0.75% sodium chloride served as the control. Fish in each group were distributed equally into twelve 200 L tanks (10 fish per tank).

2.3. Ventilation rate and behavior observations

The ventilation rate of control and MC-treated fish was determined at the initial time point, which served as the basal rate, and at five different time points during the experiment by counting the opercular ventilatory movement of six individuals per tank for 1 min each. Fish behavior in the control and treatment groups was observed daily throughout the experiment. Dead fish were registered to calculate mortality and removed from the tank.

2.4. Blood sampling

At each sampling point, twenty fish (from two tanks) from each treatment and the control were anaesthetized in 150 mg L^{-1} buffered solution of tricaine methane sulfonate (MS222, Sigma) and sampled at 0, 1, 3, 12, 24, and 48 h post injection of MC solution. Blood samples (1.0 mL) were taken by caudal puncture using heparinized syringes and then centrifuged at 3000*g* for 15 min at 4 °C. The separated plasma was stored at −75 °C until the hormone assay could be performed.

2.5. Hormone assay

Plasma thyroxine (T_4) and triiodothyronine (T_3) concentrations were determined in duplicate by radioimmunoassay (RIA) using the methods of Crane et al. (2004). Plasma cortisol concentration was determined by RIA using the methods of Baker and Rance (1981). Plasma free T_4 (FT_4) and free T_3 (FT_3) concentrations were determined in duplicate according to Ebbesson et al. (1998) and Plohman et al. (2002).

2.6. Statistical analysis

The data collected from the two treatment groups and the control group are presented as mean ± SD for each group. The data from control fish and fish exposed to extracted MCs were tested for statistical differences using a one-way ANOVA followed by Duncan's multiple comparison tests using the STATISTICA software package (Version 6.0, Statsoft, Inc.). Statistical differences were determined at the $p < 0.05$ or $p < 0.01$ levels for all analyses and indicated with * and **, respectively.

3. Results

3.1. Mortality and behavior

No mortality occurred in either the control or the sublethal group during the experimental period. However, in the lethal group, 5.0%, 8.3%, and 18.3% fish mortalities were observed at 12, 24, and 48 h post injection, respectively.

Crucian carp in the sublethal and lethal dose groups responded to exposure to MCs with irregular swimming behavior. Some of the

treated fish displayed an increased startle response and hectic swimming over the duration of the experiment. Furthermore, treated fish appeared to become increasingly disoriented after 12 h post injection, especially in the lethal dose group. Furthermore, much more individuals with abnormal behavior were present in the lethal dose group than in the sublethal dose group. Treated fish in the sublethal dose group seldom displayed an imbalanced swimming behavior.

In the lethal dose group, ventilation rates were significantly elevated from 1 to 3 h post injection and then significantly decreased compared to the initial time point and the control fish (Fig. 1). Ultimately, opercular ventilatory movements of fish in the lethal dose group gradually reduced in frequency and magnitude. In the sublethal dose group, significantly elevated ventilation rates occurred only at 12 h (Fig. 1). In contrast, control fish exhibited normal behavior and constant ventilation rates during the experimental period.

3.2. Plasma cortisol

After administration of MCs, plasma cortisol levels were significantly higher in the treatment groups than in the control group throughout the 48 h period of the experiment (Fig. 2). For the first 24 h post injection, plasma cortisol levels were significantly higher in the lethal dose group than in sublethal dose group; by 48 h post injection, the plasma cortisol level in the lethal dose group decreased but was still significantly higher than that of the control. However, the plasma cortisol concentration in the sublethal dose group remained high throughout the experiment, exceeding the level of the lethal dose group at the end of the experiment.

3.3. Plasma T_4 and T_3

No significant changes in plasma T_4 levels of fish were observed in the sublethal dose and the control groups over the course of the 48 h experiment (Table 1), and there was no significant difference

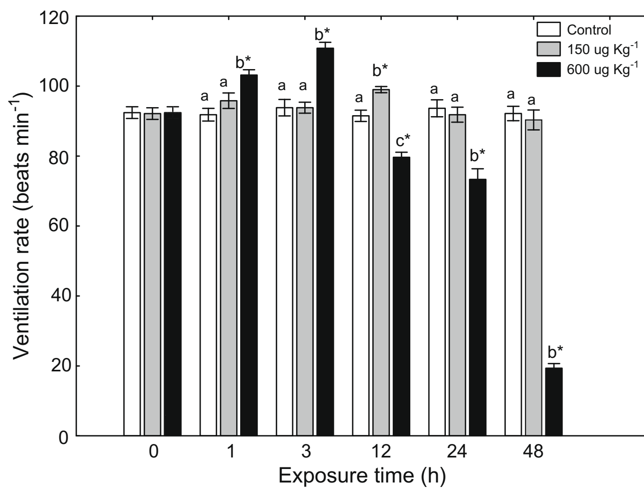

Fig. 1. Ventilation rates of crucian carp, *Carassius auratus*, injected intraperitoneally with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 600 μg MC kg^{-1} BW. Values are expressed as mean ± SD. There was no significant change in ventilation rate in control group during the experimental period. Values indicated with different letters at the same time point are significantly different at $p < 0.01$. * indicates significant differences at $p < 0.05$ from $t = 0$ h.

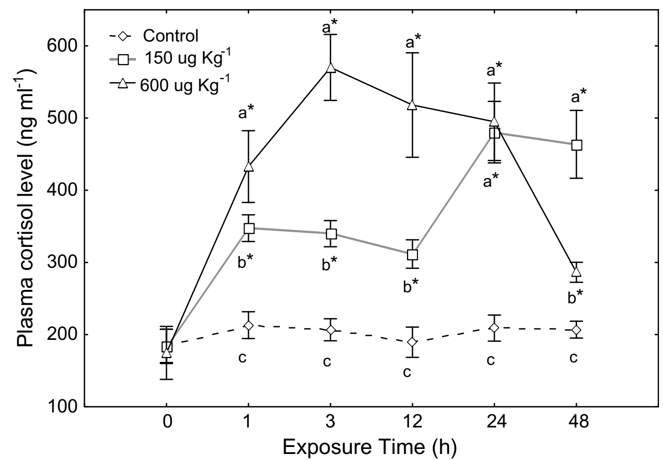

Fig. 2. Plasma cortisol levels of crucian carp, *Carassius auratus*, injected intraperitoneally with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 600 μg MC kg^{-1} BW. Values are expressed as mean ± SD. There was no significant variation in control values over the 48 h period. Values indicated with different letters at the same time point are significantly different at $p < 0.01$. * indicates significant differences at $p < 0.05$ from $t = 0$ h.

Table 1
Plasma thyroxine (T_4), triiodothyronine (T_3), free thyroxine (FT_4), and free triiodothyronine (FT_3) levels of crucian carp, *Carassius auratus*, injected intraperitoneally with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 600 μg MC kg^{-1} BW

Experimental groups	Exposure time					
	0 h (n = 10)	1 h (n = 10)	3 h (n = 10)	12 h (n = 10)	24 h (n = 10)	48 h (n = 8)
T_4 (ng ml^{-1})						
Control	11.08 ± 4.72	9.12 ± 3.83	9.37 ± 1.57	9.25 ± 4.21	10.55 ± 3.14	11.31 ± 3.53
150 μg kg^{-1}	10.78 ± 3.46	8.65 ± 4.48	9.25 ± 2.81	10.94 ± 2.26	9.94 ± 1.41	8.22 ± 1.44
600 μg kg^{-1}	10.42 ± 3.02	10.04 ± 2.50	8.35 ± 2.15	6.21 ± 1.69	7.30 ± 2.21	2.15 ± 1.08**
T_3 (ng ml^{-1})						
Control	1.84 ± 0.26	2.01 ± 0.14	1.93 ± 0.10	1.91 ± 0.08	1.86 ± 0.17	1.87 ± 0.19
150 μg kg^{-1}	1.90 ± 0.35	1.81 ± 0.18	1.77 ± 0.36	1.58 ± 0.25*	1.23 ± 0.29**	0.87 ± 0.12**
600 μg kg^{-1}	1.88 ± 0.18	1.42 ± 0.51	1.03 ± 0.37**	1.03 ± 0.19**	0.96 ± 0.24**	0.71 ± 0.10**
FT_4 (pg ml^{-1})						
Control	8.52 ± 0.66	8.76 ± 0.44	8.07 ± 0.65	7.90 ± 0.54	8.39 ± 1.18	7.80 ± 0.69
150 μg kg^{-1}	8.23 ± 0.72	7.98 ± 0.61	5.01 ± 0.54**	5.86 ± 0.18**	4.69 ± 0.89**	2.12 ± 0.69**
600 μg kg^{-1}	8.91 ± 0.83	1.63 ± 0.44**	1.09 ± 0.33**	1.38 ± 0.36**	1.67 ± 0.57**	1.30 ± 0.27**
FT_3 (pg ml^{-1})						
Control	2.13 ± 0.20	2.43 ± 0.15	2.29 ± 0.14	2.52 ± 0.09	2.37 ± 0.19	2.25 ± 0.27
150 μg kg^{-1}	2.26 ± 0.32	1.95 ± 0.19**	1.21 ± 0.28**	1.29 ± 0.21**	1.03 ± 0.21**	0.68 ± 0.12**
600 μg kg^{-1}	2.34 ± 0.43	1.92 ± 0.29**	0.96 ± 0.14**	0.90 ± 0.21**	0.43 ± 0.09**	0.48 ± 0.07**

Values are given as mean ± SD. Significant differences between control fish and treated fish are indicated (*$p < 0.05$ and **$p < 0.01$) for each time point.

in plasma T_4 levels between the sublethal dose group and the control group. In the lethal dose group, plasma T_4 levels tended to decrease over the course of experiment, but the decrease was not statistically significant compared to the control and the initial level. However, the plasma T_4 level at 48 h post administration of MCs was significantly lower in the lethal dose group than in the control and the initial level.

Plasma T_3 levels of fish in both dose groups significantly decreased after MC administration (Table 1). Plasma T_3 levels at each sampling point were significantly lower in the lethal dose group than in the sublethal dose group. Plasma T_3 levels were significantly lower in the treatment groups than in the control group as of 3 h and 12 h post injection in the lethal and sublethal dose groups, respectively.

3.4. Plasma free T_4 and free T_3

Plasma FT_4 and FT_3 levels of fish decreased significantly in both dose groups during the experiment (Table 1), but no significant changes occurred in the control group. The plasma FT_4 level in the lethal dose group declined after MC administration, reached a minimum at 1 h post injection, and remained at a low level thereafter. In the sublethal dose group, the plasma FT_4 level significantly declined at 3 h post injection and reached a minimum at the end of the experiment. At each sampling point, the plasma FT_4 level was significantly lower in the lethal dose group than in the sublethal dose group. The temporal trends of plasma FT_3 level in both dose groups were similar to those of plasma FT_4. Acute reduction in the plasma FT_3 level in both dose groups occurred at 1 h post injection and reached a minimum at the end of the experiment.

4. Discussion

In the present study, crucian carp exposed to sublethal and lethal doses of MCs exhibited an acute increase in plasma cortisol levels. Cortisol, known as a stress hormone, plays a major regulatory role in metabolism and is mediated by the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997; Mommsen et al., 1999). An elevated plasma cortisol level is a primary indicator of a stress response in fish (Barton, 2002). The results of this study suggest that crucian carp exposed to either sublethal or lethal doses of MCs suffer physiological stress. This finding is in agreement with results of previous studies of brown trout and the European whitefish, *Coregonus lavaretus* (Bury et al., 1996; Ernst et al., 2006, 2007). On the other hand, Bury et al. (1996) reported that plasma cortisol levels of the brown trout (*Salmo trutta*) returned to the control level after 24 h exposure to toxic cyanobacteria cells, whereas in the present study, plasma cortisol concentrations of the treated fish were consistently higher than those of the control fish throughout the experiment. In general, plasma cortisol concentrations reflect the magnitude of the stress response in fish, and once adaptation to the stressors has occurred plasma cortisol concentrations return to the pre-stressed levels despite the ongoing presence of the stressors (Donaldson, 1981).

Other signs of fish stress induced by toxic cyanobacteria include an elevated ventilation rate and irregular swimming behavior Barreto and Volpato (2004); Ernst et al (2007), and MC-treated fish in the current study exhibited such signs. The alterations in behaviors in the MC-treated fish appeared to be dose dependent. Increased ventilation rate is an indicator for enhanced stress, irrespective of the type of stressor present (Barreto and Volpato, 2004). However, compared to the control and the sublethal dose groups, ventilation rate in the lethal dose group decreased significantly as of 12 h post injection paralleling with fish death. This may suggest that the reduced ventilation is more likely the result of exhaustion of adaptation energy during the severe stress responses induced by extracted microcystins. Therefore, it can be inferred that fish suffered more severe stress in the lethal dose group than in the sublethal dose group.

Stress responses are considered to be adaptive tools that enable a fish to cope with disturbances and maintain a homeostatic state. If the stressor is overly severe or long lasting to the point that the fish is not capable of regaining homeostasis, then the responses themselves may become maladaptive and threaten the fish's health (Barton, 2002). In this study, high mortality occurred in the lethal dose group, whereas fish survived in the sublethal dose group. It concluded that the failure of the MC-exposed fish to adapt to the severe MC-induced stress eventually resulted in fish death. This result hence demonstrated that the severity of stress was higher in the lethal dose group.

Mommsen et al. (1999) reported that the toxicant-exposed fish in their study exhibited atrophied pituitary corticotropes. Thus, it appears that prolonged hyperactivity of the cortisol-producing cells associated with long-term pollutant exposure will lead to an exhaustion of the pituitary-interrenal axis in fish (Hontela, 1997; Mommsen et al., 1999). In this study, cortisol levels in the lethal dose group dropped at the end of the experiment, and fish in this group had higher mortality and lower ventilation rates compared to the sublethal dose group. However, the decline of cortisol levels did not allow fish in the lethal dose group to overcome the physiological disturbances induced by MC exposure, which likely is indicative of interrenal exhaustion from severe stress caused by exposure to crude MC extract.

In the present study, intraperitoneal injection of MCs into fish decreased plasma concentrations of T_4 and T_3, and the declines in concentration appeared to be dose dependent over the course of the experiment. This finding indicates that MCs probably disrupted the synthesis or secretion of the circulating TH and the conversion of T_4 to T_3 in treated fish. Previous studies have demonstrated that environmental contaminants can result in thyroid dysfunction of fish (Zhou et al., 2000; Coimbra et al., 2005). A lethal dose of toxicants or lethal stress would severely disrupt thyroidal function and thereby cause decline of the peripheral circulating T_4 level (Thangavel et al., 2005; Walpita et al., 2007). In the current study, a significant decrease in T_4 levels in the lethal dose group occurred at 48 h post injection. T_4 is the main form of TH secreted from the thyroid gland. Results hence suggested that a lethal dose of MCs disturbed secretion of thyroxine, possibly causing hypothyroidism after a certain time after exposure to toxin.

T_3, the active form of THs, appears to be produced largely by peripheral enzymatic monodeiodination of T_4 mainly in the liver and other tissues (Van der Geyten et al., 1998). In general, a decline in plasma T_3 levels is mostly due to a drop in thyroidal T_4 production and secretion and/or changes in peripheral TH metabolism. At most sampling points in this study, significant decreases in plasma T_3 levels paralleled the relatively stable plasma T_4 levels in MC-treated fish. This finding suggests that a slight variation in plasma T_4 levels in the sublethal dose group and a reduction in thyroidal T_4 secretion in the lethal dose group could play only a minor role in the regulation of circulating T_3 levels. The decreased T_3 levels in the treated fish thus appear consistent with reduced hepatic T_4 monodeiodination. Previous studies have shown that hepatic damage reduces hepatic T_4 deiodination in animals (Liu and Wang, 1991), which in turn causes a decline in peripheral T_3 production. MCs are primarily hepatotoxins, and acute exposure to MCs via intraperitoneal injection causes severe hepatic lesions in fish (Carbis et al., 1996; Solter et al., 1998; Fischer and Dietrich, 2000; Li et al., 2005). Stress could provoke an acute drop in circulating T_3 levels by decreasing hepatic outer ring deiodination activity (Walpita et al., 2007). Given the decline in plasma T_3 concentrations associated with the occurrence of physiological stress observed

in this study and considering previous studies demonstrating hepatic lesions caused by MC exposure, it can be inferred that the basic hepatic regulation of the thyroid in crucian carp was probably disrupted by exposure to MCs.

Free TH levels are preferred clinically as indices of thyroidal status (Refetoff, 1979; Eales and Shostak, 1985). In this study, both free T_4 and free T_3 in the treatment groups decreased significantly in a dose-dependent manner, indicating that the amount of THs available for target tissues of fish in the two dose groups was less than that in the control group. An insufficiency in THs would lead to abnormal growth, development, and metabolism in fish exposed to environmental contaminants (Park et al., 2006). A reduction in fitness and growth suppression has been reported in *C. lavaretus* and *Cyprinus carpio*, respectively, exposed to MCs (Li et al., 2004; Malbrouck and Kestemont, 2006; Ernst et al., 2007). Considering the present results, low plasma levels of THs resulting from exposure to MCs might have contributed partly to the physiological dysfunction observed in crucian carp.

5. Conclusion

This study demonstrated that administration of cyanobacterial extract containing microcystins to crucian carp resulted in a stress response manifested as an elevated cortisol level and reduced plasma concentrations of thyroid hormones. Injection with sublethal and lethal dose of microcystins led to abnormal behavior and changes in ventilation rates. Moreover, the severity of adverse effects occurred in a dose-dependent manner. These results illustrate that MCs have potent effects on the endocrine system of fish; they activate the HPI axis and disturb thyroid function in crucian carp. The observed drop in TH levels that accompanies the stress responses may be implicated in the observed growth suppression and reduction in fitness previously reported in MC-exposed carp.

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