Seasonal variation of gut Cyanophyta contents and liver GST expression of mud carp (*Cirrhina molitorella*) and Nile tilapia (*Oreochromis niloticus*) in the tropical Xiangang Reservoir (Huizhou, China)

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Liver glutathione S-transferase (GST) plays a major role in the detoxification of microcystins (MCs) via conjugation to glutathione (GSH). We evaluated the relationship between seasonal variation in fish gut contents and the expression of GST isoforms in mud carp (*Cirrhina molitorella*) and Nile tilapia (*Oreochromis niloticus*). We quantified the abundance and diversity of plankton in the water column and foregut of mud carp and Nile tilapia in the tropical Xiangang Reservoir between October 2007 and July 2008. The mRNA expression of 7 liver GST isoforms was determined by real-time RT-PCR. The gut contents of both species were dependent on the amount and type of phytoplankton and zooplankton in the water. The expression of liver GST genes in Nile tilapia and mud carp was positively and negatively correlated, respectively, with the abundance of toxic cyanobacteria in the fore-gut. The expression of liver GST mRNA was correlated to the abundance of toxic cyanobacteria in the gut contents of both species, suggesting that mRNA expression of GST isoforms could be used as a biomarker in Nile tilapia and mud carp to monitor cyanobacteria blooms in reservoirs.

glutathione S-transferase, seasonal variation, *Cirrhina molitorella*, *Oreochromis niloticus*, fish gut Cyanophyta content

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The occurrence of cyanobacterial water blooms in eutrophic lakes, reservoirs, and recreational waters is a common problem throughout the world. These blooms are often associated with toxigenic strains [1], which have been reported to induce poisoning, and even deaths of wild and domestic animals [2–5], as well as posing a risk to human health [6–8]. Cyclic hepatotoxic microcystins (MCs, 900–1000 Da) are one of the most common cyanotoxins [9,10].

MCs are taken up into the hepatocyte by multi-specific bile acid transporters and are detoxified in the liver by conjugation to glutathione via the action of glutathione-S-transferase (GST) then transported to the kidney and intest-

tine for excretion [11–14]. In mammals, 3 major families of glutathione transferase have been identified. These include 9 GSTs isoforms: alpha, mu, pi, theta, sigma, omega, zeta (GSTA, GSTM, GSTP, GSTT, GSTS, GSTO, GSTZ, respectively), the mitochondrial GST-kappa class (GSTK), and microsomal GSTs (mGST) [15]. In addition, a special GST, rho class (GSTR), was found in fish but has no homologue molecules in mammals [16]. In recent studies, the mRNA expression of GSTA, GSTR, GSTT, GSTM, GSTP, GSTK and MGS had been reported to play major roles in the detoxification of microcystins [17]. Some piscine GST isoenzymes have been identified as useful biomarkers for exposure to environmental pollutants. However, more information is needed to determine the relationship between

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MCs and teleost GSTs in the natural environment.

Nile tilapia (*Oreochromis niloticus*) feed selectively on cyanobacteria [18] and have the ability to digest and utilize cyanobacteria [19]. Mud carp (*Cirrhina molitorella*) feed on benthic organisms and detritus by scraping the surface of the sediment and filter-feeding on algae. In the natural environment, fish are frequently exposed to MCs directly or passively. Furthermore, during periods of rapid growth, some species may assimilate large quantities of MC-containing cyanobacteria [20,21]. However, the majority of information on the relationship between fish GST expression and MC tolerance is derived from laboratory studies. Hence, there is little data describing the relationship between Cyanophyta abundance in the fish gut and liver GST expression [22–29]. Our objective was to evaluate the mechanism for detoxification of MCs in the liver via conjugation to GSH by GST in fish in the wild. We analyzed seasonal changes in the abundance of cyanobacteria in the gut and transcription of several liver GSTs in Nile tilapia and mud carp in a tropical reservoir, Xiangang Reservoir (Huizhou, China),

1 Materials and methods

1.1 Study area and field sampling

The Xiangang Reservoir is located in Huizhou (Guangdong, China) and forms the mid and lower reaches of the Dongjiang River, a major tributary of the Pearl River. The total storage capacity of Xiangang Reservoir is $1.36 \times 10^8 \text{ m}^3$. The sampling site was located 7 km upstream of the dam (Figure 1). We collected samples between October 2007 and July 2008, and the cyanobacterial blooms occurred in April 2008.

The mud carp and Nile tilapia were immediately dissected and their livers were lysed and homogenized in 175 μL RNA Lysis Buffer (provided using the SV total RNA isolation system; Promega, USA). Their intestinal and viscera were sorted in 75% ethanol at room temperature (RT). The guts were separated and prepared in -20°C , with both ends being closed. We counted all mud carp and Nile tilapia and measured the total length (TL) of each individual. We collected samples of plankton using a 64- μm mesh plankton net. These were preserved in 5% formaldehyde at RT. Dominant phytoplankton and zooplankton were identified under a microscope. Water temperature and pH were recorded using an Orion Star series meter system (USA) and dissolved oxygen (DO) was measured using an Orion model 830A (Germany).

1.2 Water and gut contents analysis

The dominant phytoplankton and zooplankton in water were identified under a microscope [30]. The foregut (about 10 cm to esophagus) contents were transferred to an optical

Figure 1 Map of Xiangang Reservoir showing the sampling site.

plankton counter and saline was added prior to viewing under a microscope. We counted the number of phytoplankton and zooplankton per mL of foregut contents and water sample. The plankton was identified to the lowest possible taxon and grouped into the following categories: Cyanophyta, Chlorophyta, Bacillariophyta, Rotifera, Cladocera, and Copepoda. The species composition of gut and water samples is presented as a proportion of the total sample contents (%).

1.3 Amplification of the partial cDNA sequences of GSTs genes from the liver of mud carp

Total RNA was isolated from 30 mg sections of mud carp liver using the SV Total RNA Isolation System (Promega, USA). RNA extraction was performed following the manufacturer's protocols. Reverse transcription was performed with oligo (dT)₂₀ primer using a First Strand cDNA Synthesis Kit (Toyobo, Japan). Six pairs of degenerate primers (Table 1) were designed to clone partial GSTR, GSTT, GSTP, GSTM, GSTK, and mGST3 cDNA sequences with *Taq* polymerase (Takara, Japan). The PCR parameters were: 30 cycles at 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min, with an additional initial 3 min denaturation at 94°C and 5 min final extension at 72°C . PCR products of the expected length were purified on a 2.0% agarose gel and cloned into the pMD19-T Vector (Takara, Japan). The ligation mixtures were used to transform *E. coli* DH5 α using heat shock transformation. Positive transformants were selected and inserts were sequenced using an ABI Prism™

377 (Perkin Elmer, USA). Phylogenetic comparison of protein sequences was carried out using Mega 3.0 (<http://www.megasoftware.net>). A phylogenetic tree was constructed using the Neighbor-Joining (NJ) method.

1.4 Quantification of seasonal variation in liver GST mRNA expression

Gene-specific primers of mud carp GSTR, GSTM, GSTT, GSTP, GSTK, and mGST3 genes for real-time RT-PCR were designed using the Primer Designer Software 2.0, based on the cloned cDNA core sequences (Table 1). The primers of mud carp GSTA, beta-actin gene, Nile tilapia

GSTA, GSTR1, GSTR2, GSTM, GSTT, and beta-actin were designed using the sequences from GenBank EF100906, DQ365852, EU234530, EU107284, FJ436092, FJ231879, FJ177522, and AB037865, respectively (Table 1). Specific primers were synthesized by Sangon Company (Shanghai, China). Reverse transcription was performed with oligo (dT)₂₀ primers using a First Strand cDNA Synthesis Kit (Toyobo, Japan). The DyNamoTM Flash SYBR Green qPCR Kit (Finnzymes, Finland) was used for real-time PCR on a Chromo4 Real-Time Detection System (MJ Research, USA), following the manufacturer's instructions. PCRs were performed in a 20-μL volume containing 10 μL Master Mix (2×concentration), 0.5 μmol/L forward

Table 1 Primers for cloning mud carp GSTs partial cDNA sequences and real-time RT-PCR

Name of primer	Sequence of primer (GenBank ID.)
GSTR01F	5'-TGGAGGGTGATGATCGCGCTGGA(A/G)GA(A/G)AA(A/G)-3'
GSTR02R	5'-CGTGTCTGGACCCTCAGG(T/G)TT(T/C)TCCA(A/G)CCA-3'
GSTM01F	5'-GAATACACTGGTACTAAGTA(T/C)GA(A/G)GA(A/G)AA-3'
GSTM02R	5'-CAACAACATCATACATGATGAA(A/G)TC(T/C/A/G)AC(A/G)AA-3'
GSTT01F	5'-CGTCTCTGTTTACATTTTGC(T/C/A/G)AA(A/G)AA(A/G)AA-3'
GSTT02R	5'-CCACGCCTTGAGCTTGGGTCTGTT(T/C)TC(A/G)AA(T/C/A/G)AC-3'
GSTP01F	5'-TGCTCTTTTGACAGTTGCC(T/C/A/G)AA(A/G)TT-3'
GSTP02R	5'-CAGAAGGTCGAACAGGTTGTA(A/G)TC(T/C/A/G)GC(A/G)AA-3'
GSTK01F	5'-CGCACCGTGGAGCTCTT(T/C)TA(T/C)GA(T/C)GT-3'
GSTK02R	5'-GGAGGTACAGGGCCCATCCA(T/C)TT(T/C)TC-3'
mGST301F	5'-GTGATGT/CTA/G/C/TATGTATCTC/TGG-3'
mGST302R	5'-CCAGTGATAGTATCCCCA(T/C/A/G)GC(A/G)TA-3'
FQCM-GSTA01F	5'-AAGACCTTAAAGAACGGGC-3' (EF100906)
FQCM-GSTA02R	5'-GCTCAACTGGTTTCCAC-3'
FQCM-GSTR01F	5'-CTGAACAAGCGTCATCT-3' (FJ436068)
FQCM-GSTR02R	5'-CCATCTTCTCCAAGTATCC-3'
FQCM-GSTM01F	5'-GGTACTAAGTACGAGGAG-3' (FJ436073)
FQCM-GSTM02R	5'-CTCTCGTCTGCTCTTCTT-3'
FQCM-GSTT01F	5'-GGCTGAGAAGTTCCACAC-3' (FJ436070)
FQCM-GSTT02R	5'-CTCTACTGCATTGTCCAT-3'
FQCM-GSTP01F	5'-GTCTGTGAGGCTTCTCGT-3' (FJ436072)
FQCM-GSTP02R	5'-TCACCAACTAGGAATCCA-3'
FQCM-GSTK01F	5'-TGCTGTGTCGCTATAGAA-3' (FJ436074)
FQCM-GSTK02R	5'-TCGCATTCAATGAGCCTT-3'
FQCM-mGST301F	5'-TAGGTGGTGACGGAAGA-3' (FJ436075)
FQCM-mGST302R	5'-CCACGCGTAAGAGAACCT-3'
FQCM-ACT01F	5'-CGTGACATCAAGGAGAAG-3' (DQ365852)
FQCM-ACT02R	5'-GAGTTGAAGGTGGTCTCAT-3'
FQON-GSTA01F	5'-GCAATCCTGAATTACATCG-3' (EU234530)
FQON-GSTA02R	5'-ACACAGGAAGGTAGCGCTC-3'
FQON-GSTR101F	5'-CTTCACTCTCAGTTGTAAG-3' (EU107284)
FQON-GSTR02R	5'-TTGAATGTTGGAAGCTGTC-3'
FQON-GSTR201F	5'-GGTTGTCTCCAGAGCGTT-3' (FJ436092)
FQON-GSTR02R	5'-GCTGCTCTGTCATTATGC-3'
FQON-GSTM01F	5'-GGTACTAAGTATGAGGAG-3' (FJ231879)
FQON-GSTM02R	5'-AGTCTCTCCACACATGTT-3'
FQON-GSTT01F	5'-GATGCACGGCTCCAAGAT-3' (FJ177522)
FQON-GSTT02R	5'-CCACAATGGCGACCAGAT-3'
FQON-ACT01F	5'-AGAAGCTGTGCTACGTCG-3' (AB037865)
FQON-ACT02R	5'-ATGATGCTGTTGTAGGTG-3'

primer, 0.5 $\mu\text{mol/L}$ reverse primer, 0.3 μL ROX, cDNA equivalent to that generated from 1 μg total RNA, and ddH₂O to 20 μL . A melting curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. For every sample, quantitative PCR was performed in triplicates. Reactions were based on a three-step method: 95°C for 3 min; 40 cycles at 95°C for 20 s, 55°C for 20 s, and 72°C for 35 s. After confirming that target gene and control gene have the same amplification efficiency, the relative gene expression quantification was calculated by the $\Delta\Delta C_t$ method using Opticon Monitor 2.03 (MJ research, USA). The relative liver GST cDNA levels in mud carp and Nile tilapia were expressed as a ratio of GSTs/beta-actin mRNA (%).

1.5 Statistical analyses

We tested for differences among expressions of a single GST isoform different samplers from October 2007 to July 2008 using one-way analysis of variance (ANOVA) followed by a post hoc test of LSD and Duncan's multiple range test. Data were checked for normality and homogeneity of variances. Differences were considered significant if $P < 0.05$. Results are presented as the mean $\pm$ SE. All analyses were performed in SPSS13.0.

2 Results

2.1 Seasonal variation in water contents

Sampling and water quality parameters are illustrated in Table 2. The seasonal variation in species diversity and abundance of plankton in the water is illustrated in Figure 2. The dominant phytoplanktons were Cyanobacteria, Chlorophyta and Bacillariophyta. Toxic Cyanophyta was dominant in April, July, and October (65.0%, 83.8%, and 86.8%, respectively, of the total phytoplankton) whereas Chlorophyta were dominant in January (64.4%) (Figure 2). The zooplankton community consisted primarily of Rotifera, Cladocera, and Copepoda (Figure 2). Rotifera was dominant in April (8.6%) whereas Cladocera and Copepoda were dominant in October (2.4% and 2.2%, respectively) (Figure 2).

Table 2 Sampling and water quality parameters

Date	Mud carp		Nile tilapia		Temperature (°C)	DO (mg/L)	pH
	<i>n</i>	TL (cm)	<i>n</i>	TL (cm)			
Jan.	6	24.833 $\pm$ 0.691	6	21.92 $\pm$ 0.55	13.8	10.09	8.64
Apr.	5	32.75 $\pm$ 1.289	5	18.75 $\pm$ 0.75	25.9	8.77	8.66
Jul.	6	21.7833 $\pm$ 1.3951	4	15.63 $\pm$ 1.38	30.43	11.8	8.33
Oct.	4	23.375 $\pm$ 0.473	6	18.58 $\pm$ 0.93	25.4	8.77	7.54

Figure 2 Numerical composition (%) of plankton in water samples from Xiangang Reservoir from October 2007 to July 2008.

2.2 Seasonal variation in diets of mud carp and Nile tilapia

The time of collection, quantity and total length of mud carp and Nile tilapia are illustrated in Figure 3. We identified 7 food types in the foregut of the two fish species: toxic Cyanobacteria (primarily *Microcystis*, *Oscillatoria*, and *Anabaena*), other Cyanobacteria, Chlorophyta, Bacillariophyta, Rotifera, Cladocera, and Copepoda, and low numbers of zooplankton. The gut contents of the mud carp were dominated by toxic cyanobacteria in April and October (Figure 3(a)). Among the food items found in Nile tilapia, Chlorophyta were dominant in January (92.4%) and October

Figure 3 Seasonal changes in the proportion (%) of plankton in the gut of mud carp (a) and Nile tilapia (b) from Xiangang Reservoir.

(88.4%), whereas Chlorophyta and Bacillariophyta were equally dominant in July (Figure 3(b)). Toxic cyanobacteria contributed 91.7% during the cyanobacterial bloom season in April (Figure 3(b)).

2.3 Cloning and phylogenetic analysis of the GSTs partial cDNA sequences from mud carp liver

We used the mRNA sequences of mammalian GSTs to search the zebrafish and pufferfish genome and database for

homologous genes. Degenerate primers were then designed from conserved regions to clone partial GST cDNA sequences of mud carp by PCR (Table 1). The PCR products were subsequently cloned and sequenced (GenBank ID: FJ436068, FJ436073, FJ436070, FJ436072, FJ436074 and FJ436075). We constructed a phylogenetic tree with deduced amino acid sequences to investigate the relationship between mud carp GSTs and the different classes of GST enzymes from other vertebrates (Figure 4). The mud carp GST sequences had the highest similarity with those from

Figure 4 A phylogenetic tree showing the relationship between mud carp glutathione S-transferase (mud carp GST) with other classes of GSTs. The amino acid sequences were taken from GenBank: human (*Homo sapiens*): CAA46642 GSTA, NP_000844 GSTT, CAA30894 GSTP, AAH58881 GSTM, AAF65506 GSTK; mouse (*Mus musculus*): NP_598755 GSTT, NP_083831 GSTK; chicken (*Gallus gallus*): NP_990149 GSTA, NP_990696 GSTT; zebrafish (*Danio rerio*): XP_692427 GSTT, NP_001018349 GSTP, NP_001038525 GSTR, NP_997841 GSTM, NP_001002560 GSTK; common carp (*Cyprinus carpio*): ABD67512 GSTT, ABF57552 GSTP, ABD67511 GSTR, ABD67509 GSTM, ABD67508 GSTK, ABD67515 MGST3; and western clawed frog (*Xenopus tropicalis*): AAH87825 GSTT, NP_001080840 GSTM, NP_001091371 mGST3. The mud carp glutathione S-transferase (mud carp GST) is highlighted by black diamond. Scale Bar=0.2. The scale bar indicates the evolutionary distance between the groups.

common carp and zebrafish.

2.4 Relative liver GSTs mRNA expression in different seasons

The seasonal changes in the ratio of liver GSTs/beta-actin mRNA (%) of mud carp are illustrated in Figure 5. The expression of mud carp GST mRNA was lower in April, July, and October than in January. In January, GSTT mRNA expression was highest (79.4%), followed by GSTR (27.7%), GSTA (6.08%) and GSTP (5.10%). However, the mRNA expression of these GST genes decreased in April, with the exception of GSTK and GSTM. In July, the mRNA expression of GSTA, GSTT, and GSTP tended to increase. GSTR and GSTK mRNA expression was higher in October than in July (Figure 5).

The seasonal changes in mRNA expression in Nile tilapia are illustrated in Figure 6. The seasonal pattern of GST mRNA expression differed between Nile tilapia and mud

carp. Expression was higher in April and July than in January and October in Nile tilapia (Figure 6). GSTA and GSTR2 mRNA expression was higher in April than in July, January, and October. Similarly, GSTR1, GSTT, and GSTM mRNA expression was higher in July than in other months (Figure 6).

3 Discussion

In the present study, both mud carp and Nile tilapia consumed a large amount of algae in different seasons. Interestingly, the gut contents of mud carp and Nile tilapia were dominated by toxic Cyanobacteria in April, when cyanobacterial blooms were present. Our results were consistent with observations of bighead carp (*Aristichthys nobilis*) in Lake Donghu by Xie [31], who found that the proportion of cyanobacteria in food items increased when the proportion of cyanobacterial blooms increased in the water. Our results suggested that the seasonal changes in diet may be dependent on the abundance and availability of food items in the water.

Fish are persistently in direct contact with MCs when feeding on cyanobacteria. The liver GSTs of freshwater fish are responsible for the detoxification of MCs [11,32–34]. In our study, the mRNA expression of the liver GST in Nile tilapia was positively correlated with the abundance of toxic cyanobacteria in the fore-gut, particularly for GSTA and GSTR2 (Pearson correlation index = 0.99 and 0.97, respectively). Conversely, the expression of liver GST in mud carp was negatively correlated with the abundance of toxic cyanobacteria in the fore-gut, particularly for GSTA (Pearson correlation index –0.90). We hypothesize that differences in the pattern of GST expression between the two species are related to their specific resistance to MCs. Liang et al. [34] demonstrated that inducible expression of the liver GST gene was closely related to tolerance to MC-LR. The highly-resistant fish had inducible liver expression of either alpha- or rho class GST whereas the highly-sensitive fish had no inducible liver expression. Thus, we speculate that Nile tilapia is more resistant to MCs than mud carp [35]. Recently, researchers have successfully developed methods for the detection of MC(LR, RR)-GSH in fish organs. Combining this technique with molecular and metabolic evidence offers considerable promise in the future [36,37].

Expression of liver GST in mud carp was inhibited when high quantities of toxic cyanobacteria were present in the fore-gut. Lipopolysaccharide (LPS) may contribute to the inhibition of the GST expression. LPS reduces the capacity of MCs to conjugate with GSH [24]. It is also thought that LPS is involved in the *de novo* synthesis of GST [38]. LPS is simultaneously released with MCs from toxic cyanobacteria, and inhibits liver GST gene expression in phytoplanktivorous fish. This may be a result of co-evolution of the cyanobacteria and the fish feeding on them [33]. Regardless,

Figure 5 Relative mRNA expression of liver GSTs in mud carp from Xiangang Reservoir in different seasons. Columns represent the mean ratio of GSTs/beta-actin mRNA. Bars represent the SE. Different letters indicate a significant difference within enzyme types ($P < 0.05$).

Figure 6 Relative mRNA abundance of liver GSTs in Nile tilapia from Xiangang Reservoir in different seasons. Columns represent the mean ratio of GSTs/beta-actin mRNA. Bars represent the SE. Different letters indicate a significant difference within enzyme types ($P < 0.05$).

it is unclear whether the differences in the expression pattern of the two fish species results from the different responses of their GST genes to LPS exposure.

In conclusion, the gut contents of mud carp and Nile tilapia were largely dependent on the occurrence and abundance of phytoplankton and zooplankton in the water column. The expression of GSTs in mud carp was negatively correlated with the proportion of toxic cyanobacteria in the foregut. In contrast, the expression of GST isoforms in Nile tilapia was upregulated regardless of whether the proportion of toxic cyanobacteria was high or low. Our results suggest that liver GST mRNA abundance was correlated with the quantity of toxic Cyanobacteria in the gut contents of mud carp and Nile tilapia, depending on their resistance to MCs. Our results also suggested that the mRNA expression of GST isoforms could be used as biomarkers in Nile tilapia and mud carp.

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