Transcriptomic analysis dissects the mechanistic insight into the Daphnia clonal variation in tolerance to toxic Microcystis

Kai Lyu,1 Lei Gu,1 Hui Wang,1 Xuexia Zhu,1 Lu Zhang,1 Yunfei Sun,1 Yuan Huang,1 Zhou Yang 1,2*
1Jiangsu Key Laboratory for Biodiversity and Biotechnology, School of Biological Sciences, Nanjing Normal University, Nanjing, China
2Department of Ecology, College of Life Science and Technology, Jinan University, Guangzhou, China

Abstract
Cyanobacteria have become more prevalent than other phytoplankton in freshwater assemblages during summer. In such conditions, cyanobacterial traits may reduce zooplankton fitness and the energy flow efficiency from primary producers to aquatic herbivores. Cladocerans, as the dominant zooplankton grazers in freshwater ecosystems, exhibit clonal variation in their tolerance to cyanobacteria with an increasing gradient in eutrophication history. Hitherto, research on the full modes of action (MoAs) of Daphnia clonal differences in tolerance to toxic Microcystis still remains in its infancy. We conducted fitness and transcriptome analyses on two Daphnia clones, clone TH09 and TH14. A significant decline in body growth rate was detected in the sensitive clone TH09 compared with the tolerant clone TH14 at the presence of toxic Microcystis. Furthermore, transcriptional analysis indicated that major MoAs such as glutathione metabolism, protein processing in endoplasmic reticulum, amino sugar/nucleotide sugar metabolism, and arachidonic acid metabolism were linked to the tolerance fitness in Daphnia similoides. These results provided mechanistic insights into the pathways of genetic and biological processes involved in cyanobacteria tolerance in the Daphnia clonal variation, and propose that the genetic architecture of this fitness-related trait would be helpful to clarify how zooplankton clones adapt to harmful algal blooms.

Phytoplankton composition in assemblages has shifted to increased cyanobacterial dominance, and therefore disrupt food-web processes during summer because of global warming and remarkable increases in nutrient fluxes (Kosten et al. 2012). As the most common bloom-forming cyanobacterium, Microcystis spp. can perform the biosynthesis of the hepatotoxin, microcystin (MC) (Carmichael 1997; Codd 2000). MC inhibits protein phosphatases (Toivola et al. 1994; Runnegar et al. 1995) and damages DNA through the promotion of oxidative stress (Zegura et al. 2003). In addition, Microcystis is known for containing protease inhibitors that act against the key digestive serine proteases in the alimentary canal of arthropod herbivores (Agrawal et al. 2001). Most herbivorous zooplanktons, such as Daphnia, are negatively affected by toxic cyanobacteria, as cyanobacteria could contribute to enhanced mortality, abnormal development, and lower reproduction (Lürling and van der Grinten 2003; Dao et al. 2010).

Of note, the uniform understanding of this negative relation between cyanobacterial and zooplankton abundance has been questioned in some lab- and field-scale investigations (Gustafsson et al. 2005; Samelle and Wilson 2005; Jiang et al. 2016; Lyu et al. 2016b), revealing that Daphnia had the ability to gain tolerance to dietary toxic Microcystis. The reproductive success of Daphnia is characterized by cyclical parthenogenesis, which is involved in alternation between sexual reproduction and parthenogenesis. Therefore, it is reasonable to assume that Daphnia populations consist of coexisting clones. Different Daphnia clones, that is, genotypes, exhibit differences in their individual fitness after exposure to cyanobacteria (reviewed by Ger et al. 2014). The initial evidence for clonal variation in tolerance to cyanobacteria was reported by Gilbert (1990), where an Anabaena strain inhibited the population growth rate in one Daphnia clone without affecting another. Another previous work studied individuals raised from diapaused eggs deposited in Lake Constance sediments and found that their tolerance to cyanobacteria was changed by the historical gradient of eutrophication (Hairston et al. 1999). Subsequent studies then indicated the improving

*Correspondence: yangzhou@njnu.edu.cn

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Additional Supporting Information may be found in the online version of this article.
tolerance of *Daphnia* to toxic cyanobacteria was considerably dependent on clonal variation (Sarnelle and Wilson 2005).

Although we recognize the importance of gene function and annotation, we have marginal knowledge on their changes in response to ecological or environmental fluctuations, and subsequently their influence on phenotypic variation as well as macroscopic population dynamics (Miner et al. 2012). Therefore, it is essential to evaluate the transcriptional responses to changes in environment together with tractable and well-characterized ecologies (Colbourne et al. 2011). Under the scenario of toxic *Microcystis*, the discovery of the full modes of action (MoAs) of *Daphnia* clonal differences in tolerance are currently in its infancy. The previous studies of molecular cyanobacterial responses have mainly focused on the identification of candidate genes (Schwarzenberger et al. 2010, 2012, 2014; Lyu et al. 2015). For example, Schwarzenberger et al. (2012) investigated that the inter-clonal variation of *Daphnia* tolerance to protease inhibitors in cyanobacteria strongly depended on the residual activity of digestive proteases. They subsequently revealed that ATP-binding cassette (a gene family for substrate transporta-
tion) RNA expression was linked with the microcystin tolerance in four *Daphnia* clones by feeding dietary supplement-
mentation with pure microcystin (Schwarzenberger et al. 2014). However, it remains unclear whether other potential novel MoAs exit in *Daphnia* tolerance to live cyanobacteria cells.

Since *Daphnia*, a global grazer of cyanobacteria, inhabits a central position in pelagic food webs (Ger et al. 2014), it is essential to directly reveal the fundamental MoAs of the potential of *Daphnia* to resist live cyanobacteria cells and therefore to possibly suppress cyanobacterial blooms (Sarnelle 2007). The present study aimed to demonstrate the important genes and the biological pathways related to *Microcystis*-tolerance in the *Daphnia* clonal variation. To compare the expression and observe the parallel body growth rates, which have been proposed as an appropriate proxy for *Daphnia* population fitness (Lampert and Trubetskova 1996), RNA-seq was executed separately to sample experimental animals from two *Daphnia* clones which were both fed with toxic *Microcystis*. Of note, the exposure to live *Microcystis aeruginosa* cells, rather than the waterborne cyanobacteria extract or microcystin, was chose in our study, because estimating the effects of dietary live *Microcystis* is helpful to obtain a more authentic knowledge of aquatic environmental influence (Sarnelle and Wilson 2005; Sarnelle et al. 2010; Asselman et al. 2012).

**Materials and methods**

**Organisms’ origin and maintenance**

The two clones of *Daphnia similoides* in the present experiment were sampled at Lake Taihu (120° 09′ E, 31° 10′ N; East China) and were named clone TH09 and clone TH14, respectively. This *Daphnia* species was widely distributed in fresh water in low latitudes (Makino et al. 2017; Rizo et al. 2017; Xu et al. 2018). The lake (i.e., Lake Taihu) experiences annual heavy cyanobacterial blooms, during which *Microcystis* constitutes over 50% of the summer phytoplankton biomass (Liu et al. 2011; Deng et al. 2014). Genetic distinctness was examined in the two clones by using a PCR method (Schwenk 1993) prior to the optimized modification. The two clonal cultures were established from one parthenogenetic mother respectively and were fed by high-quality food *Scenedesmus obliquus* (FACHB-416; 1.5 mg C L\(^{-1}\) per day). Only offspring from the third brood were used in culture and exposure. These clones grew at 25°C, under a 14 : 10 h light/dark cycle, and in M4 medium (Elendt and Bias 1990) in the laboratory acclimated for more than 5 months in order to reduce possible disturb caused by maternal effects and environmental variance before the clone exposure. Meanwhile, the *Daphnia* medium was mildly aerated with clean oxygen for 1 d prior to use and renewed completely two times per week.

The high-quality alga *S. obliquus* and the toxic *M. aeruginosa* strain PCC7806 were purchased from the Fresh-water Algae Culture Collection of the Institute of Hydrobiol-
ogy at the Chinese Academy of Sciences. Cyanobacterium *M. aeruginosa* grew in the form of single cell (5–6 μm diameter spheres) or paired cells, so that it prevented mechanical interference from large-sized colonies that was probably to impact *Daphnia* ingestion. Furthermore, we executed a pilot experiment under proposal by the previous study (Gan et al. 2010), wherein we evidenced that the *M. aeruginosa* strain biosynthes-
size two kinds of MCs, namely, MC-LR as well as MC-RR. Each cell produced 3.6 pg of each MC variant. *S. obliquus* and *M. aer-
uginosa* were stored in 400 mL of BG-11 medium (Stanier et al. 1971). The algae were grown semi-continuously at 25°C under fluorescent light (40 μmol photons m\(^{-2}\) s\(^{-1}\)) and in a 14 : 10 light/dark cycle. The strains were centrifuged in the exponential stage and fed to the zooplankton. The dietary algal densities were determined with a blood cell counting chamber under an inverted Nikon microscope at a magnification of ×400 (Lyu et al. 2015).

**Experimental design**

We conducted a body growth experiment to investigate the *D. similoides* tolerance performance, according to the previous study (Lampert and Trubetskova 1996). Three replicates were conducted and these animals were released within 12 h. Subsequently, 20 neonates from each beaker were transferred to 1 L of M4 medium. These neonates were exposed to the following food treatments for 7 d at a total food concentration of 1.5 mg C L\(^{-1}\): (1) 100% *S. obliquus*; (2) a mixture of 50% *M. aeruginosa* + 50% *S. obliquus*. We monitored the medium parameter (Mean value: e.g., pH 7.8, dissolved oxygen = 7.0 mg L\(^{-1}\), temperature 25°C and water hardness [as CaCO\(_3\)] of 276 mg L\(^{-1}\) every 4 h and renewed exposure medium daily, in order to keep parameters steady. Three biological replicates were analyzed for each food treatment, which was administered to
each clone in a steady photoperiod of 14 : 10 h (light/dark) cycle and at 25°C. The body growth rates (BGRs) of the *D. similoides* clones were determined from the body dry weight of a subsample of the animals at the beginning and end of the experiment (Day 7), calculated based on the study (Schwarzenberger et al. 2014). Number of molting was examined by counting shed carapaces. Living *D. similoides* individuals were snap-frozen after the BGR measurements. In the present study, we compared the transcriptomic change between the *D. similoides* clone TH09 and TH14 fed with *M. aeruginosa*. Subsequently, the living individuals of the two clones exposed to MC-producing *M. aeruginosa* were subjected to RNA extraction and RNA-seq analyses.

**RNA sampling and cDNA library construction**

*D. similoides* RNA was isolated by using TRizol Reagent (Takara, Japan) in accordance with the manufacturer’s instructions. The total RNA quality and quantity of the two groups of samples were evaluated by electrophoresis on 1.2% agarose gels, and the absorbance at 260/280 nm was determined using a spectrophotometer (Nanodrop, Thermos Scientific). Only samples with OD260/OD280 = 1.8–2.0, OD260/OD230 ≥ 2.0 and RIN ≥ 8.0 were used for sequencing. Further details of RNA sampling and cDNA generation are provided in the Supporting Information.

**Illumina sequencing, assembly, and annotation**

The cDNA library was sequenced on the Illumina HiSeq 2500 sequencing platform that generated approximately 100 bp paired end (PE) raw reads by LC Sciences (Houston, U.S.A.). The raw sequence data were uploaded to the NCBI Sequence Read Archive (SRA). Further details of sequence analysis are provided in the Supporting Information.

**Analysis of differentially expressed genes**

To analyze the differentially expressed genes in *D. similoides* clones in the presence of toxic *M. aeruginosa*, the number of reads for each of the contigs was converted to reads per kilo base per million (RPKM) by using RSEM 1.2.3 (Wang et al. 2017). Differentially expressed genes (DEGs) were further annotated by Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, as demonstrated in the Supporting Information.

**Validation of the RNA-seq profiles by real-time quantitative PCR (qPCR)**

To validate the RNA-Seq results, 10 genes (see Table S4 in Supporting Information) were randomly selected to confirm the expression profiles of *D. similoides* clones in response to *M. aeruginosa* by qPCR. The *D. similoides* clones were treated as described above. qPCR was performed with an SYBR Green Master kit (Takara, Japan) according to the manufacturer’s protocol. The primers for qPCR are listed in Supporting Information Table S4. qPCR was carried out in a total volume of 20 μL (10 μL of 2 × SYBR Premix Ex Taq, 1 μL cDNA mix, 0.5 μL of each primer [10 μM], and 8 μL of sterile distilled H2O). The PCR program was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. qPCR method with β-actin (see Lyu et al. 2016a for the primer sequences) as an internal control was used to explore mRNA expression levels.

**Data analysis**

Two-way (*Daphnia* clone difference and food treatment) ANOVA followed by Holm-Sidak test was employed to detect significant differences in BGR changes and number of molting. Data of BGR and number of molting satisfied assumptions of normality and equal variances before the ANOVA test. A significance level of *p* < 0.05 was applied. All statistical analyses were performed using SigmaPlot v11.0 (Systat Software).

**Results**

**Changes in BGR and number of molting**

Significant BGR (*p* = 0.019) and number of molting (*p* < 0.001) differences between clones TH09 and TH14 exposed to *M. aeruginosa* were detected (Fig. 1; Supporting Information Table S1), which indicated that clone TH14 was more tolerant to toxic *M. aeruginosa* than clone TH09. In contrast, the BGR of clone TH09 (0.63 ± 0.075) was significantly higher than that of clone TH14 (0.42 ± 0.013) (Fig. 1A; *p* < 0.001) under good food conditions. For the clone TH09, the BGR in toxic *M. aeruginosa* treatment was significantly lower than that in *Scenedesmus* (Fig. 1A; Supporting Information Table S1; *p* < 0.001). At the presence of toxic *Microcystis*, number of molting in the sensitive clone TH09 was significantly lower than that in the tolerant clone TH14 (Fig. 1B; *p* < 0.001).

**Transcriptome sequencing and assembly**

To screen for the genes related to *Microcystis* tolerance, we isolated mRNA from clones TH09 and TH14 after 7 d exposure to the mixture containing 50% MC-producing *M. aeruginosa* and 50% *S. obliquus*. Two transcriptome libraries were constructed by using total RNA and subjected to Illumina deep sequencing. After quality control and clipping adapter, an amount of 86,978,248 clean reads remained, comprising 46,438,962 in clone TH09 and 40,539,286 in clone TH14 (Supporting Information Tables S2). These reads were transferred in the de novo assembly by the ABySS program. The assembly created 40,653 contigs with an average length of 3265 bp (N50 = 1364 bp). After splicing and redundancy filtration, the contigs were intergraded into 17,738 unigenes, each of which had an average length of 1617 bp (N50 = 2658 bp). The unigenes length distribution was shown in Supporting Information.
Information Fig. S1. These assembly sequences represent transcription and can be used for further analysis. After assembly, all the unigenes were analyzed. A cut-off E-value of $10^{-5}$ was used for BLAST analysis in the NCBI nucleotide sequences (NT), NCBI non-redundant protein sequences (NR), and Swiss-Prot databases (Supporting Information Table S3). The results showed that 12,598 (71.02%) unigenes attained significant BLAST hits in the NR database and 9552 (53.79%) hits in the Swiss-Prot database. Furthermore, 9446 unique protein hits were identified after searching the NR and Swiss-Prot databases. Only 4871 unigenes were not identified in the three databases.

Differential expression analysis

To identify whether the DEGs involved in *D. similoides* tolerant to toxic *M. aeruginosa*, pairwise comparisons for differential expression analysis were conducted between clones TH09 and TH14 fed by *M. aeruginosa*. In this pairwise comparison, 1864 genes were differentially expressed, comprising 689 upregulated genes and 1175 downregulated genes (Fig. 2).

GO and KEGG enrichment analysis of DEGs

In the present study, 1864 DEGs were assigned GO terms. The significant GO terms divided into the three fields of biological process, cellular component, and molecular function were shown in Fig. 3. Among the biological processes, 169 (25%) of the DEGs were distributed to apoptotic process (GO: 0006915), and 107 DEGs were distributed to immune response (GO: 0006955). In the field of molecular function, approximate 20% of the DEGs were belonged to protein homodimerization activity (GO: 0042803) function, with another 19% involved in calcium ion binding (GO: 0005509). In cellular component, the high-enriched GO terms were plasma membranes (GO: 0005886, 23.8%) and integral to membrane (GO: 0016021, 23.7%).

The analysis of pathway enrichment was helped to deeply recognize biological functions of the DEGs. In the present study, five significant ($p < 0.05$; Table 1) pathways were identified through the comparison of clones TH09 and TH14, including glutathione metabolism, protein processing in endoplasmic reticulum, amino sugar/nucleotide sugar metabolism and arachidonic acid metabolism.

Validation of RNA-seq by qPCR

To validate the DEGs identified by RNA-seq, the expression of 10 target genes randomly selected from those with differing expression patterns and from genes of interest based on functional enrichment and pathway results, was confirmed by qPCR (Table 1). The melting-curve analyses revealed a single product for all tested genes. As shown in Fig. 4, all qPCR results were in good agreement with the Illuminia RNA-seq analysis. For example, the expression of genes (glutathione S-transferase D2, gamma-glutamyltransferase 1, serine/threonine-protein kinase PDIK1L, chitinase 10, chitin synthase 2, hematopoietic prostaglandin D synthase, and group XIIB secretory phospholipase A2) showed an immediate significant decline in clone TH09 compared with TH14 in the presence of toxic *Microcystis*.

Discussion

According to the hypothesis of the arms race, the degree of outbreak and spread of cyanobacterial blooms relies extensively on the dynamic interplay between the capacity of the toxic algae to fend off the herbivore consumers and the resistance ability of the herbivore consumers to tolerate the toxicity. On the one hand, the toxicity and morphology are considered as key traits in the reduction of grazing losses of cyanobacteria by zooplankton (Yang et al. 2006; Jang et al. 2007; Pineda-Mendoza et al. 2014), although cyanotoxins have other various functions, including iron chelator (Utkilen and Gjølme 1995), photosynthesis or other light related processes (Young et al. 2005), and intercellular intraspecies communication (Schatz et al. 2007; Zilliges et al. 2008). On the other hand, zooplankton often exist mutually with harmful algal blooms.
(HABs), suggesting that they have multiple abilities to behaviorally reduce the ingestion of hazardous doses and to be physiologically tolerant to the ingested toxin. In line with the previous studies (Hietala et al. 1997; Chislock et al. 2013; Jiang et al. 2013, 2015), the present population-level results clearly showed clonal variation in Daphnia fitness in response to toxic Microcystis. Of note, the clones were cultured individually under identical conditions. Dissolved oxygen, pH, and temperature in the medium were steadily maintained as exposure medium were renewed daily. Therefore, it is conceivable that significant BGR differences between the two clones would be arise from genetic factors, rather than their inducible tolerance. The present clones were originated from individual D. similoides adults isolated from the cyanobacterial-polluted lake, which were rational considered as DNA-level distinct, despite DNA traits in each clone were not monitored by genomic analyses.

Live cyanobacterial cells have been observed to decline zooplankton fitness through the following three basic routines: (1) large colony formation and filamentous morphologies can block filtering appendages and thereby inhibit grazing (DeMott et al. 2001); (2) nutritional deficiency in the cyanobacterial cells (Von Elert and Wolffrom 2001); and (3) cyanobacteria produce toxic secondary metabolites (e.g., MC and digestive serine proteases) (Rohrlack et al. 2001; Yang et al. 2012). The frequently observed mechanical clog on filtering apparatus (DeMott et al. 2001) in Daphnia did not appear in the present study as Microcystis we used was mostly consisted of single and paired cells. Hence, the clonal variation in BGR against toxic Microcystis results from toxic metabolites and nutritional deficiency. We then attempted to acquire valuable global-scale transcriptomic evidence by comparing the clones TH09 and TH14 at the presence of dietary Microcystis, in order to support the clonal variation of Daphnia in response to cyanobacterial effects. Given the advantages in GO annotation and KEGG pathway classification, we identified and validated certain sensitivity-related genes, and the most important pathways involved clonal variation in response to toxic Microcystis were further discussed below. Due to the limited scale of the present study, we encourage future research incorporating more treatments, temporal endpoints across zooplankton taxa, and improved gene annotation to enrich the dynamic processes of MoAs of zooplankton tolerance to toxic cyanobacteria.

**Glutathione metabolism**

Intracellular MCs suppress protein phosphatases (An and Carmichael 1994), which, along with generation of oxidative stress, is treated as the major MoAs of their toxicity (Rohrlack et al. 2004; Lyu et al. 2014). A possible mechanism for buffering the toxic effects of MCs is linked with glutathione S-transferases (GSTs), which catalyzes the biotransformation of MCs with glutathione; this process is widely proposed as the first step in detoxification found in many aquatic livings, including plants, invertebrates, and fish (Pflugmacher et al. 1998; Ortiz-Rodriguez and Wiegand 2010; Lyu et al. 2016a). Our study further revealed that glutathione metabolism was significantly ($p = 0.0049$) enhanced in the tolerant clone TH14 as GST D2, aminopeptidase N, thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) and gamma-glutamyltransferase 1 (GGT1) expression were significantly ($p < 0.001$) increased compared to the sensitive clone TH09...
Fig. 5; Table 1). Based on the finding that antioxidation as well as biotransformation are the two main processes of GST in MCs detoxification (Plugmacher et al. 1998), it is conceivable that the enhancement in GST D2 gene expression in the tolerant clone compared with the sensitive was observed when the animals are fed with the toxic *Microcystis*. Of note, another important element in the detoxification process is glutathione (GSH). Given the GSH depletion caused by conjugation with MC, the cells must continue with GSH re-synthesis to meet the detoxification requirements (Ding et al. 2000). In our study, aminopeptidase N and GGT1, which are involved in GSH synthesis (Lee et al. 2004), were up-regulated in the tolerant clone TH09; thus, GSH synthesis was enhanced. This suggested that these effects inhibited the MC toxicity in the cell.

**Protein processing in endoplasmic reticulum**

In the pairwise comparison of clone TH09/TH14, the expression levels of serine/threonine-protein kinase-PDII (STPK-PDII) and tyrosine-protein kinase (TPK) were significantly (*p < 0.001*) reduced, whereas that of heat shock protein 70 Bbb (HSP70B) was significantly increased (*p < 0.001*). In protein processing in endoplasmic reticulum, the STPK-PDII serves as a sensor to detect the unfolded protein response (Fig. 5). Furthermore, TPK can phosphorylate cytosol or nucleus proteins at tyrosine residues during the process (Radha et al. 1996). These enzymes play an essential role in the homeostatic maintenance of stress response and protein folding, which are crucial when the number of unfolded proteins increases (Walter and Ron 2011). As reported previously (Asselman et al. 2012; Lyu et al. 2016c), exposure to toxic...
Microcystis inhibited STPK-PDK1L and TPK expression in the sensitive clone TH09 and interfered with normal protein folding and repair. In addition, since HSP70B serves as a molecular chaperone, minimizes protein aggregation, and repairs and protects cellular proteins from stress damage (Mayer and Bukau 2005), it was to be expected that increased HSP70B expression was induced by an abundance of unfolded proteins. Accordingly, the protein processing in endoplasmic reticulum pathway was identified as statistically significant (Table 1), indicating that the repair of dysfunctional protein folding was enhanced in the tolerant clone.

Amino sugar and nucleotide sugar metabolism

While the inhibited chitinase 10, beta-N-acetylglucosaminidase and chitin synthase 2 in clone TH09 compared to clone TH14, the increased amino-sugar and nucleotide-sugar metabolism pathway was found in the tolerant clone TH14 (Table 1). Microcrustaceans growth and immunity require molting in some species (Duneau and Ebert 2012). The present study found the number of molting in the sensitive clone TH09 was significantly lower than that in the tolerant clone TH14, at the presence of toxic Microcystis (Fig. 1B), suggesting that Microcystis disordered molting in sensitive clone worse than the tolerant clone. The prerequisite events during molting are the aged cuticle separation from the underlying epidermis and an increased levels in several enzymes and hormones, collectively named as molting fluid (Stevenson 1972). During molting, chitinase digests the chitin in the old cuticle and chitin synthase catalyzes the generation of new cuticles (Fig. 5). The opposite functions of chitin synthase 2 and chitinase 10 suggest that their activities must be highly coordinated in integument during the molting-cycle (Bade and Stinson 1978). In addition, beta-N-acetylglucosaminidase acts synergistically with endochitinases,

Table 1. Key genes of significant enrichment of KEGG function pathways in comparison of clone TH09 and TH14 at the presence of Microcystis dietary.

| Function pathway                        | p value   | Gene name                                                                 | Contig ID   | log2FC by transcriptomics |
|----------------------------------------|-----------|----------------------------------------------------------------------------|-------------|---------------------------|
| Glutathione metabolism                 | 0.0049    | Glutathione S-transferase D2                                               | c9881_g1    | −2.707                    |
|                                        |           | Aminopeptidase N                                                           | c12223_g1   | −2.363                    |
|                                        |           | Thyrotrpin-releasing hormone-degrading ectoenzyme                          | c12287_g1   | −3.554                    |
|                                        |           | Gamma-glutamyltransferase 1                                               | c12098_g1   | −1.736                    |
| Protein processing in endoplasmic      | 0.0093    | Serine/threonine-protein kinase PDK1L                                     | c4789_g1    | −2.451                    |
| reticulum                              |           | Tyrosine-protein kinase                                                   | c12519_g1   | −1.796                    |
|                                        |           | Heat shock protein 70 Bbb                                                  | c8343_g1    | 1.242                     |
| Amino sugar and nucleotide sugar       | 0.0133    | Chitinase 10                                                               | c14639_g1   | −2.802                    |
| metabolism                             |           | Beta-N-acetylglucosaminidase                                               | c7415_g1    | −1.365                    |
|                                        |           | Chitin synthase 2                                                          | c15208_g1   | −2.312                    |
| Arachidonic acid metabolism            | 0.0243    | Hematopoietic prostaglandin D synthase                                     | c9865_g2    | −2.802                    |
|                                        |           | Group XIB secretory phospholipase A2                                      | c12403_g3   | −1.365                    |
|                                        |           | Gamma-glutamyltransferase 5                                                | c12098_g1   | −2.312                    |

Fig. 4. Validation of RNA-seq data by using qPCR. The mRNA levels of selected target genes in clone TH14 were compared to clone TH09 which were assigned a relative value of 1 (represented by the dashed line). The results were analyzed according to ANOVA by t-test (p < 0.05).
which cleave crystalline chitin into chitooligosaccharides. They may also alleviate the inhibition of chitinases through the accumulation of chitotriose and chitotetraose in the molting fluid (Fukamizo and Kramer 1985). In our study, these enzymes were up-regulated in the tolerant clone TH14, which directly explaining that the tolerant clone had greater superiority in individual fitness than the sensitive clone (Fig. 1).

Arachidonic acid metabolism (AAM)

Living cyanobacteria contain lipopolysaccharide (LPS) in their outer cell layers. Previous studies have revealed that bacterial LPS, a potentially inflammatory toxin (Mayer et al. 2011), increased the release of arachidonic acid and its metabolites (Rosenberger et al. 2004). Here, several AAM-responsive genes (i.e., hematopoietic prostaglandin D synthase (H-PDS), group XIIB secretory phospholipase A2 (GXSPA2), and gamma-glutamyltransferase S) were significantly ($p < 0.001$) induced in the tolerant clone TH14 compared with the sensitive clone (Fig. 5; Table 1). H-PGDS requires GSH for the reaction; it is inactive with other thiol compounds. As the inhibition of GSH production in the sensitive clone was witnessed in the present study, we cannot exclude the possibility that the down-regulated H-PDS gene expression resulted from feedback regulation by adequate H-PDS protein. That is, the increase in H-PGDS expression in the tolerant clone TH14 resulted in enhanced prostaglandin formation (Dawson 1998), and potentially mediated inflammation triggered by microcystin. In addition, GXSPA2, as an atypical member of the secreted phospholipase A2 family, catalyze the hydrolysis of glycerophospholipids at the $S_n$2 position to release arachidonic acid and other free fatty acids (Schaloske and Dennis 2006). Based on these reports, we speculated that the enhancement of AAM in the tolerant clone TH14 contributed to the development of resistance against cyanobacterial LPS inflammation.

Conclusions

Through the application of a transcriptome-wide top-down strategy, we had access to recognize the genes and complicated pathways involved in cyanobacteria tolerance in *Daphnia* populations. As expected, not only did we discover evidence compatible with previous results (Asselman et al. 2012, 2017; Schwarzenberger et al. 2014), but we also identify novel candidate pathways that accounted extensively for the tolerance of *Daphnia* to cyanobacteria. There was a total of 1864 DGEs that highlighted the pathways of glutathione metabolism, protein processing in endoplasmic reticulum,
amino sugar/nucleotide sugar metabolism, and arachidonic acid metabolism (Fig. 5). These significantly changed pathways suggested the strong tolerability of clonal variation was involved in cyanotoxin detoxification, the activation of protein fold repair, inflammation mediation, and molting/growth maintenance (Fig. 5). Our results emphasized the important roles of novel candidate pathways, which have often been missed in previous zooplankton-cyanobacteria studies.

Data accessibility statement
Sequence data is uploaded to the Sequence Read Archive (SRA) with accession PRJNA446001.

Author Contributions
KL conceptualized and designed the experiment together with ZY. Experiments as well as data analyses were conducted by KL, LG, HW, XZ, LZ, YS, and HY. The first draft of the manuscript was written by KL and commented by ZY. All authors have read the final version of the article.

References
Agrawal, M. K., D. Bagchi, and S. N. Bagchi. 2001. Acute inhibition of protease and suppression of growth in zooplankter, Moina macrocopa, by Microcystis blooms collected in Central India. Hydrobiologia 464: 37–44. doi:10.1023/A:1013946514556
An, J., and W. W. Carmichael. 1997. The cyanotoxins. Adv. Bot. Res. 27: 211–257. doi:10.1016/S0065-2296(08)60282-7
Chislock, M. F., O. Sarnelle, B. K. Olsen, E. Doster, and A. E. Wilson. 2013. Large effects of consumer offense on ecosystem structure and function. Ecology 94: 2375–2380. doi:10.1890/13-0320.1
Codd, G. A. 2000. Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. Ecol. Eng. 16: 51–60. doi:10.1016/S0925-8574(00)00089-6
Colbourne, J. K., and others. 2011. The ecoresponsive genome of Daphnia pulex. Science 331: 555–561. doi:10.1126/science.1197761
Dao, T. S., L. C. Do-Hong, and C. Wiegand. 2010. Chronic effects of cyanobacterial toxins on Daphnia magna and their offspring. Toxicon 55: 1244–1254. doi:10.1016/j.toxicon.2010.01.014
Dawson, R. M. 1998. The toxicology of microcystins. Toxicon 36: 953–962. doi:10.1016/S0041-0101(97)00102-5
DeMott, W. R., R. D. Gulati, and E. Van Donk. 2001. Daphnia food limitation in three hypereutrophic Dutch lakes: Evidence for exclusion of large-bodied species by interfering filaments of cyanobacteria. Limnol. Oceanogr. 46: 2054–2060. doi:10.4319/lo.2001.46.8.2054
Deng, J., B. Qin, H. W. Paerl, Y. Zhang, J. Ma, and Y. Chen. 2014. Earlier and warmer springs increase cyanobacterial (Microcystis spp.) blooms in subtropical Lake Taihu, China. Freshw. Biol. 59: 1076–1085. doi:10.1111/fwb.12330
Ding, W. X., H. M. Shen, and C. N. Ong. 2000. Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. Environ. Health Perspect. 108: 605–609. doi:10.1289/ehp.00108605
Duneau, D., and D. Ebert. 2012. The role of moulting in parasite defence. Proc. R. Soc. B Biol. Sci. 279: 3049–3054. doi:10.1098/rspb.2012.0407
Elendt, B.-P., and W.-R. Bias. 1990. Trace nutrient deficiency in Daphnia magna cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of D. magna. Water Res. 24: 1157–1167. doi:10.1016/0043-1354(90)90180-E
Fukamizo, T., and K. J. Kramer. 1985. Mechanism of chitin oligosaccharide hydrolysis by the binary enzyme chitinase system in insect moulting fluid. Insect Biochem. 15: 1–7. doi:10.1016/0020-1790(85)90037-X
Gan, N., Q. Huang, L. Zheng, and L. Song. 2010. Quantitative assessment of toxic and nontoxic Microcystis colonies in natural environments using fluorescence in situ hybridization and flow cytometry. Sci. China Life Sci. 53: 973–980. doi:10.1007/s11427-010-4038-9
Ger, K. A., L. A. Hansson, and M. Lürling. 2014. Understanding cyanobacteria-zooplankton interactions in a more eutrophic world. Freshw. Biol. 59: 1783–1789. doi:10.1111/fwb.12393
Gilbert, J. J. 1990. Differential effects of Anabaena affinis on cladocerans and rotifers: Mechanisms and implications. Ecology 71: 1727–1740. doi:10.2307/1937581
Gustafsson, S., K. Rengefors, and L.-A. Hansson. 2005. Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. Ecology 86: 2561–2567. doi:10.1890/1359-1746(2005)86[2561:ICFTOT]2.0.CO;2

Hairston, N. G., and others. 1999. Lake ecosystems: Rapid evolution revealed by dormant eggs. Nature 401: 446–446. doi:10.1038/46731

Hietala, J., C. Lauren Matta, and M. Walls. 1997. Sensitivity of Daphnia to toxic cyanobacteria: Effects of genotype and temperature. Freshw. Biol. 37: 299–306. doi:10.1207/s15327415fb0701_10

Jiang, X., H. Gao, L. Zhang, H. Liang, and X. Zhu. 2016. Rapid evolution of tolerance to toxic Microcystis in two cladoceran grazers. Sci. Rep. 6: 25319. doi:10.1038/srep25319

Kosten, S., and others. 2012. Warmer climates boost cyanobacterial dominance in shallow lakes. Glob. Chang. Biol. 18: 118–126. doi:10.1111/j.1365-2486.2011.02488.x

Lampert, W., and I. Trubetskova. 1996. Juvenile growth rate as a measure of fitness in Daphnia. Funct. Ecol. 10: 631–635. doi:10.1046/j.1365-2435.1996.001006.x

Lee, D.-H., R. Blomhoff, and D. R. Jacobs. 2004. Review is serum gamma glutamyltransferase a marker of oxidative stress? Free Radic. Res. 38: 535–539. doi:10.1080/10715760410001694026

Liu, X., X. Lu, and Y. Chen. 2011. The effects of temperature and nutrient ratios on Microcystis blooms in Lake Taihu, China: An 11-year investigation. Harmful Algae 10: 337–343. doi:10.1016/j.hal.2010.12.002

Lürling, M., and E. van der Grinten. 2003. Life-history characteristics of Daphnia exposed to dissolved microcystin-LR and to the cyanobacterium Microcystis aeruginosa with and without microcystins. Environ. Toxicol. Chem. 22: 1281–1287. doi:10.1002/etc.5620220614

Lyu, K., X. Zhu, R. Chen, Y. Chen, and Z. Yang. 2014. Molecular cloning of manganese superoxide dismutase gene in the cladoceran Daphnia magna: Effects of microcystin, nitrite, and cadmium on gene expression profiles. Aquat. Toxicol. 148: 55–64. doi:10.1016/j.aquatox.2013.12.031

Lyu, K., L. Zhang, X. Zhu, G. Cui, A. E. Wilson, and Z. Yang. 2015. Arginine kinase in the cladoceran Daphnia magna: cDNA sequencing and expression is associated with resistance to toxic Microcystis. Aquat. Toxicol. 160: 13–21. doi:10.1016/j.aquatox.2014.12.023

Makino, W., N. Maruoka, M. Nakagawa, and N. Takamura. 2017. DNA barcoding of freshwater zooplankton in Lake Kasumigaura, Japan. Ecol. Res. 32: 481–493. doi:10.1007/s11284-017-1458-z

Mayer, A. M., J. A. Clifford, M. Aldulescu, J. A. Frenkel, M. A. Holland, M. L. Hall, K. B. Glaser, and J. Berry. 2011. Cyanobacterial Microcystis aeruginosa lipopolysaccharide elicits release of superoxide anion, thromboxane B2, cytokines, chemokines, and matrix metalloproteinase-9 by rat microglia. Toxicol. Sci. 121: 63–72. doi:10.1093/toxsci/kfr045

Miner, B. E., L. De Meester, M. E. Pfrender, W. Lampert, and N. G. Hairston. 2012. Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proc. R. Soc. B Biol. Sci. 279: 1873–1882. doi:10.1098/rspb.2011.2404

Ortiz-Rodriguez, R., and C. Wiegand. 2010. Age related acute effects of microcystin-LR on Daphnia magna biotransformation and oxidative stress. Toxicon 56: 1342–1349. doi:10.1016/j.toxicon.2010.07.020

Pflugmacher, S., C. Wiegand, A. Oberemm, K. A. Beattie, E. Krause, G. A. Codd, and C. E. Steinberg. 1998, 1425. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: The first step of detoxication. Biochim. Biophys. Acta 56: 527–533. doi:10.1016/j.toxicon.2010.07.020

Pineda-Mendoza, R. M., G. Zuniga, and F. Martinez-Jeronimo. 2014. Inochemicals released by Daphnia magna fed on Microcystis aeruginosa affect mcyA gene expression. Toxicon 80: 78–86. doi:10.1016/j.toxicon.2014.01.008

Radha, V., S. Nambirajan, and G. Swarup. 1996. Association of lyn tyrosine kinase with the nuclear matrix and cell-cycle-dependent changes in matrix-associated tyrosine kinase activity. FEBS J. 236: 352–359. doi:10.1111/j.1365-2000.1996.0352.x
Rizo, E. Z. C., Y. Gu, R. D. S. Papa, H. J. Dumont, and B.-P. Han. 2017. Identifying functional groups and ecological roles of tropical and subtropical freshwater Cladocera in Asia. Hydrobiologia 799: 83–99. doi:10.1007/s10750-017-3199-y

Rohrlack, T., E. Dittmann, T. Borner, and K. Christoffersen. 2001. Effects of cell-bound microcystins on survival and feeding of Daphnia spp. Appl. Environ. Microbiol. 67: 3523–3529. doi:10.1128/AEM.67.8.3523-3529.2001

Rohrlack, T., K. Christoffersen, M. Kaebernick, and B. A. Neilan. 2004. Cyanobacterial protease inhibitor microvillin J causes a lethal molting disruption in Daphnia pulex. Appl. Environ. Microbiol. 70: 5047–5050. doi:10.1128/AEM.70.8.5047-5050.2004

Rosenberg, T. A., N. E. Villacreses, J. T. Hovda, F. Bosetti, G. Weerasinghe, R. N. Wine, G. J. Harry, and S. I. Rapoport. 2004. Rat brain arachidonic acid metabolism is increased by a 6-day intracerebral ventricular infusion of bacterial lipopolysaccharide. J. Neurochem. 88: 1168–1178. doi:10.1111/j.1471-4159.2002.02613.x

Runnegar, M., N. Berndt, and N. Kaplowitz. 1995. Microcystin uptake and inhibition of protein phosphatases: Effects of chemoprotectants and self-inhibition in relation to known hepatic transporters. Toxicol. Appl. Pharmacol. 134: 264–272. doi:10.1006.taap.1995.1192

Sarnelle, O. 2007. Initial conditions mediate the interaction between Daphnia and bloom-forming cyanobacteria. Limnol. Oceanogr. 52: 2120–2127. doi:10.4319/lo.2007.52.5.2120

Sarnelle, O., and A. E. Wilson. 2005. Local adaptation of Daphnia pulicaria to toxic cyanobacteria. Limnol. Oceanogr. 50: 1565–1570. doi:10.4319/lo.2005.50.5.1565

Sarnelle, O., S. Gustafsson, and L. A. Hansson. 2010. Effects of cyanobacteria on fitness components of the herbivore Daphnia. J. Plankton Res. 32: 471–477. doi:10.1093/plankt/fbp151

Schaloske, R. H., and E. A. Dennis. 2006. The phospholipase A2 superfamily and its group number system. Biochim. Biophys. Acta 1761: 1246–1259. doi:10.1016/j.bbalip.2006.07.011

Schatz, D., Y. Keren, A. Vardi, A. Sukenik, S. Carmeli, T. Börner, E. Dittmann, and A. Kaplan. 2007. Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins. Environ. Microbiol. 9: 965–970. doi:10.1111/j.1462-2920.2006.01218.x

Schwarzenberger, A., A. Zitt, P. Kroth, S. Mueller, and E. Von Elert. 2010. Gene expression and activity of digestive proteases in Daphnia: Effects of cyanobacterial protease inhibitors. BMC Physiol. 10: 6. doi:10.1186/1472-6793-10-6

Schwarzenberger, A., C. J. Kuster, and E. Elert. 2012. Molecular mechanisms of tolerance to cyanobacterial protease inhibitors revealed by clonal differences in Daphnia magna. Mol. Ecol. 21: 4898–4911. doi:10.1111/j.1365-294X.2012.05753.x

Schwarzenberger, A., T. Sadler, S. Motameny, K. Ben-Khalifa, P. Frommolt, J. Altmüller, K. Konrad, and E. von Elert. 2014. Deciphering the genetic basis of microcystin tolerance. BMC Genomics 15: 776. doi:10.1186/1471-2164-15-776

Schwenk, K. 1993. Interspecific hybridization in Daphnia: Distinction and origin of hybrid matriline. Mol. Biol. Evol. 10: 1289–1302. doi:10.1093/oxfordjournals.molbev.a040076

Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol. Rev. 35: 171–205.

Stevenson, J. R. 1972. Changing activities of the crustacean epidermis during the molting cycle. Am. Zool. 12: 373–379. doi:10.1093/icb/12.2.373

Toivola, D. M., J. E. Eriksson, and D. L. Brautigan. 1994. Identification of protein phosphatase 2A as the primary target for microcystin-LR in rat liver homogenates. FEBS Lett. 344: 175–180. doi:10.1016/0014-5793(94)00382-3

Utikilen, H., and N. Gjølme. 1995. Iron-stimulated toxin production in Microcystis aeruginosa. Appl. Environ. Microbiol. 61: 797–800.

Von Elert, E., and T. Wolf. 2001. Supplementation of cyanobacterial food with polyunsaturated fatty acids does not improve growth of Daphnia. Limnol. Oceanogr. 46: 1552–1558. doi:10.4319/lo.2001.46.6.1552

Walter, P., and D. Ron. 2011. The unfolded protein response: From stress pathway to homeostatic regulation. Science 334: 1081–1086. doi:10.1126/science.1209038

Wang, Y., Y. Xiu, K. Bi, J. Ou, W. Gu, W. Wang, and Q. Meng. 2017. Integrated analysis of mRNA-seq in the haemocytes of Spiroplasma eriocheiris in response to Spiroplasma eriocheiris infection. Fish Shellfish Immunol. 68: 289–298. doi:10.1016/j.fsi.2017.07.036

Xu, X., Y.-N. Zhang, S. Peng, J. Wu, D. Deng, and Z. Zhou. 2018. Effects of Microcystis aeruginosa on the expression of nuclear receptor genes in Daphnia similoides sinensis. Ecotoxicol. Environ. Saf. 150: 344–352. doi:10.1016/j.ecoenv.2017.12.033

Yang, Z., Z. Feng, X. Shi, and H. Cao. 2006. Morphological response of Microcystis aeruginosa to grazing by different sorts of zooplankton. Hydrobiologia 563: 225–230. doi:10.1007/s10750-005-0008-9

Yang, Z., K. Lu, Y. Chen, and D. J. Montagnes. 2012. The interactive effects of ammonia and microcystin on life-history traits of the cladoceran Daphnia magna: Synergistic or antagonistic? PLoS One 7: e32285. doi:10.1371/journal.pone.0032285

Young, F. M., C. Thomson, J. S. Metcalf, J. M. Lucoq, and G. A. Codde. 2005. Immunogold localisation of microcystins in cryosectioned cells of Microcystis. J. Struct. Biol. 151: 208–214. doi:10.1016/j.jsb.2005.05.007

Zegura, B., B. Sedmak, and M. Filipi. 2003. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon 41: 41–48. doi:10.1016/S0041-0101(02)00207-6
Zilliges, Y., J.-C. Kehr, S. Mikkat, C. Bouchier, N. Tandeau de Marsac, T. Börner, and E. Dittmann. 2008. An extracellular glycoprotein is implicated in cell-cell contacts in the toxic cyanobacterium Microcystis aeruginosa PCC 7806. J. Bacteriol. 190: 2871–2879. doi:10.1128/JB.01867-07

Acknowledgments

We acknowledge the anonymous reviewers for their very constructive comments. This study was supported by the National Natural Science Foundation of China (31730105), “333 High Level Talent Project” in Jiangsu Province (BRA2017452), Natural Science Youth Foundation of Jiangsu Province (BK20170572), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Conflict of Interest
None declared.

Submitted 12 April 2018
Revised 15 July 2018
Accepted 06 August 2018

Associate editor: Susanne Menden-Deuer