Effects of toxic *Microcystis aeruginosa* on the expression of Hox genes in *Daphnia similoides sinensis*

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**Abstract**
Lake eutrophication and cyanobacterial blooms have become worldwide environmental issues. Under cyanobacterial blooms (especially *Microcystis*), *Daphnia* spp. can transfer beneficial information to their offspring in order to improve adaptability. *Hox* genes are important regulatory factors of transcription in metazoans, and are involved in the growth and development of organisms. However, the mechanisms of *Microcystis* on the expression of *Hox* genes in *Daphnia* are unclear. In this study, the effects of *Microcystis aeruginosa* on *Hox* gene expression in the mothers and offspring (F1) of two *Daphnia similoides sinensis* clones were investigated using a mixed diet of *M. aeruginosa* and *Scenedesmus obliquus*. Compared with the 100%S food treatment, the survival rates at the end of the experiment of clone 1-F1 in the food treatments containing *M. aeruginosa* were significantly lower, but it was significantly higher for clone 2-F1 in the 20%M + 80%S food treatment. Moreover, the survival rates at the end of the experiment of clone 1-F1 in the food treatments containing *M. aeruginosa* were significantly higher than those of their mother. Based on previous transcriptome data, 14 *Hox genes* of *D. similoides sinensis* were identified, including *Abd-B*, *CDX-1*, *Dll*, *HOX-1*, *HOX-2*, *HOXA1*, *HOXA2*, *HOXB3*, *HOXB3-2*, *HOXB7*, *HOXC4*, *HOXC7*, *HOXC8*, and *HOXD10*. The expressions of *Abd-B*, *HOX-2*, *HOXA1*, *HOXC7*, and *HOXD10* of clone 2-mothers were 2.9–22.5 times as high as in the 100%S food treatment, whereas the expressions of *CDX-1*, *HOX-1*, *HOXB3*, and *HOXD10* of clone 1-mothers were 4.8–13.1 times at same food level. The expression of *HOXA2*, *HOXC7*, *HOXC8*, and *HOXD10* of clone 1-F1 in the 40%M + 60%S food treatment was 8.2–21.1 times as high as in the 100%S food treatment. However, compared with the 100%S food treatment, the expressions of *CDX-1* in the mothers and *F1* of clone 2 and *HOXB7* in the mothers of clone 1 in the food treatments containing *M. aeruginosa* were significantly lower (*p < .05*). Our results suggest that the offspring (F1) produced by *D. similoides sinensis* mother pre-exposed to toxic *M. aeruginosa* had stronger adaptability to *M. aeruginosa* than their mothers. Moreover, *Hox gene* expressions of *D. similoides sinensis* had obvious differences between clones under stress of toxic *M. aeruginosa*. 
1 | INTRODUCTION

Hox genes are important regulatory factors of transcription in metazoan animals and comprise a large family of highly conserved DNA transcription factors (Affolter et al., 1990). In vertebrates, the Hox gene family is often displayed in multiple cluster form, and participates in the regulation of embryonic development and morphological diversity (Krumlauf, 1994; McGinnis & Krumlauf, 1992). In metazoans, the target sites of the Hox gene homology domain are connected with specific DNA sequences (Affolter et al., 1990), which can regulate cell fates (Batas, 1993) and affect cell recognition via genetic address (Lawrence, 1992; Lawrence & Morata, 1983). Hox genes were first identified in Drosophila melanogaster (McGinnis et al., 1984; Scott & Weiner, 1984), and Papillon and Telford (2007) studied the expression and evolution models of Hox3 and ftz genes in Daphnia pulex.

Animal mothers can transfer environmental information to their offspring so that their offspring can produce adaptive responses to environmental heterogeneity in terms of phenotype, physiology, behavior, and reproduction (Agrawal et al., 1999; Frost et al., 2010; Mousseau & Fox, 1998). In birds, lizards, insects, and crustaceans, maternal effects play an important role in their population adaptation to the environment (Badyaev et al., 2002; Mousseau & Dingle, 1991; Schwarzenberger & Elert, 2013; Uller, 2004). Boersma et al. (2000) observed that large-sized Daphnia magna could produce larger offspring as well as produce larger ephippia in order to improve their hatching rates. D. magna can improve net reproduction efficiency and fitness of their offspring after short-term exposure to the pesticide fenvalerate (Pieters & Liess, 2006). Furthermore, Badyaev (2008) found that the adaptability of a passerine bird to the environment obtained through maternal effects could be preserved for a long time before genetic evolution took place.

In recent decades, cyanobacterial blooms by species such as M. aeruginosa have become more frequent and severe in lakes due to eutrophication, leading to suppressed population dynamics of various Daphnia species (Deng et al., 2008; Hansson et al., 2007; Liess & Hillebrand, 2004; Przytul ska et al., 2015). Cyanobacteria often release toxins such as microcystin (MC) which inhibits protein phosphorylation, affects physiological metabolism, and changes chromosomal structure, resulting in genotoxicity (Lankoff et al., 2004; Peng et al., 2018; Zegura et al., 2003). Microcystin (MC) can be accumulated in consumers through the food chain and can even affect human health (Christoffersen, 1996; Gilroy et al., 2000; Jorgensen, 1999; Reynolds, 1994). Usually, M. aeruginosa has an inhibitory effect on the life-history traits of Daphnia species (Gustafsson & Hansson, 2004; Jiang et al., 2013; Li et al., 2014; Lyu, Meng, et al., 2016; Yang et al., 2011). However, some studies have indicated that single-cell or small-colony Microcystis spp. can be fed by Daphnia spp. to favor their growth and reproduction (Chen & Xie, 2003; Hanazato, 1991; Li et al., 2014). Other studies have even shown that the offspring of Daphnia species can obtain more adaptability to toxic M. aeruginosa via maternal effect (Lyu, Guan, et al., 2016; Lyu et al., 2017). In Daphnia carinata, the offspring of the mothers pre-exposed to M. aeruginosa had quicker defensive responses than did their mothers previously unexposed to M. aeruginosa (Jiang et al., 2013). Gustafsson et al. (2005) found that the offspring of D. magna pre-exposed to M. aeruginosa had shorter time to maturation and a greater number of offspring. Schwarzenberger et al. (2009) observed that the offspring produced by the mothers pre-exposed to M. aeruginosa up-regulated the expression of target genes in D. magna, and suggested that the maternal effect was a short-term adjustment strategy to the environment.

In summary, M. aeruginosa could affect life-history traits and expression levels of some genes in Daphnia, but it was unknown how toxic M. aeruginosa affected the expression levels of Hox genes in Daphnia species and whether these genes of their offspring from the mother pre-exposed to M. aeruginosa had the adaptability to toxic M. aeruginosa. 14 Hox genes have been identified in D. similoides sinensis based on previous transcriptome data (Zhang et al., 2016), including Abd-B, CDX-1, Dll, HOX-1, HOX-2, HOXA1, HOXA2, HOXB3, HOXB3-2, HOXB7, HOXC4, HOXC7, HOXC8, and HOXD10. In this paper, our goal is to compare the influences of M. aeruginosa on Hox genes of mothers and F1 in two D. similoides sinensis clones, and to examine the adaptability of F1 from pre-exposed mothers to toxic M. aeruginosa and the differences between two clones.

2 | MATERIALS AND METHODS

2.1 | Collection, identification, and culture of D. similoides sinensis

Lake sediment from the 0- to 1-cm layer was collected from Lake Junshan in Jiangxi province (28°9′41″–28°46′13″N, 116°1′15″–116°33′38″E) in August 2015 using an 8.4-cm-diameter columnar gravity corer (Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences). The sediment was washed using 200 mesh (0.074 mm) in the laboratory, and the residue was examined using a microscope (Olympus, Japan) in order to identify the ephippia of D. similoides sinensis according to the methods of Benzie (2005) and Gu et al. (2013). Ephippia containing resting eggs of D. similoides sinensis were incubated at 25 ± 1 °C in...
aerated tap water in an intelligent light incubator (Saifu, Ningbo, China). *S. obliquus*, a nontoxic microalgae species, was used as a food source.

### 2.2 Culture of *M. aeruginosa* and *S. obliquus*

*Microcystis aeruginosa* was obtained from Lake Junshan in August 2015. A single colony of *M. aeruginosa* was chosen in the laboratory, and then cultured in BG-11 medium in an intelligent light shaker incubator (QZB-98B, China) at (28 ± 1) °C with illumination of a 12:12 h light/dark cycle. *M. aeruginosa* which were single or two cells in morphology were collected at the exponential phase of population growth and stored at 4°C.

*Scenedesmus obliquus* was obtained from the Freshwater Algae Culture Collection (Institute of Hydrobiology, Chinese Academy of Sciences), and cultured in BG-11 medium in an intelligent light incubator (Saifu, Ningbo, China) at 25°C, with a 12:12 h light/dark cycle, then collected at the exponential phase of population growth and stored at 4°C.

### 2.3 *D. similoides sinensis* mother experiment

Two *D. similoides sinensis* ephippia containing resting eggs were randomly selected, and then hatched in a 50-ml beaker in an intelligent light incubator at 25°C with a 12:12 light/dark cycle, respectively. The individual hatched from each ephippium containing resting eggs represented one clone, and each clone was respectively cultured through parthenogenesis. Two clones from different resting eggs were employed in the experiment. Third generation youngs (<12 h old) produced by each clone were used as experimental animals in the mother experiment. Three food treatments were designed based on biomass content: 100% *S. obliquus* (100% S), serving as a control, 20% *M. aeruginosa* + 80% *S. obliquus* (20% M + 80% S), and 40% *M. aeruginosa* + 60% *S. obliquus* (40% M + 60% S). The total biomass of each food treatment was 40 mg/L wet weight. There were three replicates in each food treatment, yielding a total of 18 experimental groups (2 clones x 3 food treatments x3 replicates). At the beginning of the experiment, 20 young females (<12 h old) at third generation were randomly placed in each 250-ml beaker. The culture medium was 200 ml aerated tap water (over 48 h). Therefore, 180 youngs were employed for each clone in the mother experiment. The experiments were carried out in an intelligent light shaker incubator (QZB-98B, China) at (25 ± 1) °C and 12:12 light/dark cycle. All neonates produced by the mothers in each 250-ml beaker were promptly removed during the experiment. The survival rates of the mothers were calculated daily and lasted at the end of the 14-day experiment. The culture medium was replaced every two days before *D. similoides sinensis* mothers became pregnant, from which point on it was replaced daily. The cultural density (20 young females) of *D. similoides sinensis* and temperature (25°C) in this experiment are according to our previous experimental designs (Peng et al., 2018; Xu et al., 2018).

On the fourteenth day, 12-h-old neonates produced by the mother in the 20% M + 80% S food treatment were removed and placed in new 250-ml beakers for an offspring (F1) experiment. At the end of the mother experiment, all *D. similoides sinensis* mothers in each food treatment were pooled into an EP tube and stored in liquid nitrogen for later measurement of *Hox* genes.

### 2.4 *D. similoides sinensis* F1 experiment

In the mother experiment, owing to fewer offspring produced in the 40% M + 80% S food treatment, the offspring (<12 h old, F1) produced by the mothers of two *D. similoides sinensis* clones in only the 20% M + 80% S food treatment on the fourteenth day were collected and regarded as experimental animals in the F1 experiment, and 180 individuals (F1) in each clone were employed. The F1 experimental designs were the same as described in the mother experiment. After 14 days, all F1 females in each food treatment were pooled into an EP tube and stored in liquid nitrogen for later measurement of *Hox* genes.

### 2.5 RNA isolation and cDNA synthesis

Total RNA of all mothers and offspring (F1) of *D. similoides sinensis* in the experiments was extracted using the MiniBEST universal RNA kit (TaKaRa, Dalian, China). DNase I in the kit was used to avoid genomic DNA contamination. A spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific, USA) was used to check the concentration and purity of RNA. Total RNA samples were stored at -80°C. Single-stranded cDNA templates were synthesized using the PrimeScript™ RT kit (TaKaRa, Dalian, China), and cDNA template samples were stored at -20°C.

Quantitative real-time PCR of *D. similoides sinensis* *Hox genes* was performed in a LightCycler® 96 PCR device (Roche, Switzerland), using a 2×SYBR® Premix Ex Taq kit (Tli RNase H Plus; TaKaRa, Dalian China). The 10 μL RT PCR reaction contained 5 μL of 2×SYBR®Premix Ex Taq (Tli RNaseH Plus), 1.0 μL of the DNA template (1 ng/μL), 0.2 μL of each upstream and downstream primer (10 μM), and 3.6 μL of ddH2O. The amplification conditions consisted of an initial step for one cycle of 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s. Fluorescence was measured using a melting curve from 55°C to 95°C in order to detect single gene-specific peaks and primer-dimer peaks. The qRT-PCR primers (Table 1) were designed using Beacon Designer 7.9 (PREMIER Biosoft International, CA, USA), and the results were analyzed using LightCycler® 96 SW 1.1 software. *D. similoides sinensis* *Hox gene* expression was quantified using the Q-Gene method in Visual Basic software based on Microsoft Excel. *DsimGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *DsimACT* (actin) were selected as reference genes (Muller et al., 2002; Simon, 2003). Three biological replicates were used for each sample.
Compile PI/Mw in ExPASy software (https://web.expasy.org/compute_pi/). Alignment, similarity, and homology analyses were performed using genes (Table 2). Amino acid sequences of molecular weight and isoelectric point were predicted using the joining in MEGA6 software, and a heatmap was constructed using uploaded in Dryad Digital Repository (https://doi.org/10.5061/dryad.6hdr7sr2n).

### 2.6 Gene identification and sequence analyses

The homologous genes were searched and compared in NCBI (https://www.ncbi.nlm.nih.gov/). Reading frames and functional domains based on the complete sequence information of these homologous genes were predicted using the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) from the NCBI database. Sequence alignment, similarity, and homology analyses were performed using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalX. Molecular weight and isoelectric point were predicted using the Compile pl/Mw in ExPASy software (https://web.expasy.org/compute_pi/) (Table 2). Amino acid sequences of *D. similoides sinensis* *Hox* genes were predicted using Primer Premier 5. The phylogenetic tree of *D. similoides sinensis* Hox genes was constructed using neighbor-joining in MEGA6 software, and a heatmap was constructed using Hemi software (Druga et al., 2016; Tamura et al., 2013; Xu et al., 2018). The sequences of *D. similoides sinensis* Hox genes had been uploaded in Dryad Digital Repository (https://doi.org/10.5061/dryad.6hdr7sr2n).

### 2.7 Statistical analyses

All statistical analyses were performed using SPSS 20.0. Two-way ANOVA was employed to analyze the influences of food treatment, mother-F1 generation, and their combinations on the survival rates at the end of the experiment and each Hox gene expression of each *D. similoides sinensis* clone. For each clone, multiple comparisons (Tukey’s HSD) were also used to test the differences of the survival rates at the end of the experiment and each Hox gene expression of both mothers and F1 among different food treatments, respectively.

### 3 RESULTS

#### 3.1 Survival rates of two *D. similoides sinensis* clones under different food treatments

The survival rates of the mothers and F1 in clone 1 showed a gradual dropping trend with the increasing of *M. aeruginosa* concentration. However, it was an opposite pattern in clone 2 (Figure 1).

For clone 1, both food treatment and mother-F1 generation affected significantly the survival rates at the end of the experiment (Food treatment: *F* = 118.429, *p* = .000; Mother-F1 generation: *F* = 75.571, *p* = .000), but their combinations had no significant effect (*F* = 1.857, *p* = .198). Multiple comparisons (Tukey’s HSD) showed that, compared with those in the 100%S food treatment, the survival rates at the end of the experiment of both mothers and F1 in the 40%M + 60%S food treatment were significantly lower (mothers: *p* < .001; F1: *p* < .0001), and it was also significantly lower (*p* < .001) in the 20%M + 80%S food treatment for F1. However, the survival rates at the end of the experiment of F1 in the food treatments containing *M. aeruginosa* were significantly higher than those of the mothers (20% M + 80%S: *p* = .0346; 40%M + 60%S: *p* = .0019).

For clone 2, food treatment affected significantly the survival rates at the end of the experiment (*F* = 7.600, *p* = .007), but both mother-F1 generation and their combinations of food treatment and mother-F1 generation had no significant effects (mother-F1 generation: *F* = 0.400, *p* = .539; their combinations: *F* = 0.400, *p* = .679). Multiple comparisons (Tukey’s HSD) showed that the survival rates at the end of the experiment of F1 in the 20%M + 80%S food treatment were significantly higher than those in the 100%S food treatment (*p* = .0128).

### TABLE 1 The qRT-PCR primer sequences of *D. similoides sinensis* in the experiment

| Name     | Sequence                        | Name     | Sequence                        |
|----------|---------------------------------|----------|---------------------------------|
| HOX-1-F  | CACGGTAAATTCGCAATC              | HOX-1-R  | GTAGTCGGGTTTGTATTTG             |
| CDX-1-F  | TTTCCATTAGCTGCTACA              | CDX-1-R  | TTTCTCCACGGCTTCTTCA             |
| HOXA2-F  | AATATGGAGGTTTCTCTACT            | HOXA2-R  | TGAAGATGTCGGTGGTTG              |
| HOX7-F   | CATCATGACGATCATCACAA            | HOX7-R   | CGGATGGCTTGGTATGTT              |
| HOX8-F   | GCAACAACAGCAACAATCA             | HOX8-R   | CAAACAGTACGCTATGTC              |
| Abd-B-F  | GCGGATGAAACAGAAGAAG             | Abd-B-R  | GATGATGTCGAGTTGATG              |
| HOXB3-F  | GGCACGGATGCTCATTCA              | HOXB3-R  | AAGAGTATGGTATGTTG               |
| HOX-2-F  | AGATGACAGTACGATGCTTAC           | HOX-2-R  | CGTTTGGTATGATGAG                |
| DII-F    | ATCGCTTAAATTACCGTGTTG           | DII-R    | CAGGGGATGATGGATCAGT             |
| HOXC4-F  | TTCTCACAAATCCAGCTATCT           | HOXC4-R  | TCTCTCGGGTCCATTCC               |
| HOXA1-F  | CGACCGGAAATCAACAG               | HOXA1-R  | ACTGAAATGGTGGTATGUG             |
| HOXD10-F | CTGATGACGCACTACGGAA             | HOXD10-R | GTAGATGTCGATGAG                 |
| HOXB3-2-F| CATCATGACGATCATGCTC             | HOXB3-2-R| GAAGAGTATGGAGCGGTATG             |
| HOXC8-F  | CCTCGGCTCCTGTTGTATC             | HOXC8-R  | GTCCCGTGGTGTGGT                 |
| GAPDH-F  | TCGTCCTCAAATGCTTCTT             | GAPDH-R  | CGCGGATCACAGCCTT               |
| ACT-F    | CATCCACCATGAGATTTAAG            | ACT-R    | CTGTCGATCCTTCTTG                |

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For clone 2, food treatment affected significantly the survival rates at the end of the experiment (*F* = 7.600, *p* = .007), but both mother-F1 generation and their combinations of food treatment and mother-F1 generation had no significant effects (mother-F1 generation: *F* = 0.400, *p* = .539; their combinations: *F* = 0.400, *p* = .679). Multiple comparisons (Tukey’s HSD) showed that the survival rates at the end of the experiment of F1 in the 20%M + 80%S food treatment were significantly higher than those in the 100%S food treatment (*p* = .0128).
| Gene Name | ORF (aa) | Length (bp) | Complete ORF | PI | MW (kDa) | Best Blastx Match |
|-----------|----------|-------------|--------------|----|----------|-------------------|
| HOX-1     | 89       | 730         | N            | 10.45 | 97.7     | Homeobox protein Hox-C4 JAN74469.1 Daphnia magna 8.00E-31 100 -1 |
| CDX-1     | 220      | 1537        | N            | 9.95  | 25.12    | Homeobox protein CDX-1 JAN85504.1 Daphnia magna 1.00E-113 98 1 |
| HOXA2     | 852      | 2961        | Y            | 6.49  | 92.36    | Homeobox protein Hox-A2 JAN79144.1 Daphnia magna 0 89 1 |
| HOXC7     | 142      | 654         | Y            | 9.84  | 15.7     | Putative Homeobox protein CHOX-7 KOB75552.1 Operophtera brumata 3.00E-48 69 -2 |
| HOXB7     | 373      | 1124        | N            | 9.22  | 41.04    | Homeobox protein Hox-B7a KDR08069.1 Zootermopsis nevadensis 8.00E-21 80 3 |
| Abd-B     | 441      | 1777        | N            | 9.30  | 45.37    | Putative Homeotic abdominal-B protein KZS21965.1 Daphnia magna 8.00E-134 99 2 |
| HOXB3     | 709      | 2131        | Y            | 6.1   | 77.31    | Homeobox BarH 2-like protein KZS03900.1 Daphnia magna 2.00E-38 85 2 |
| HOX-2     | 222      | 821         | N            | 7.94  | 24.05    | Putative Homeobox protein Hox-C4, partial JAN47684.1 Daphnia magna 1.00E-39 98 3 |
| DII       | 347      | 1250        | Y            | 8.83  | 38.44    | Homeobox protein Hox-B1, putative XP_002431233.1 Pediculus humanus corporis 5.00E-30 78 3 |
| HOXC4     | 171      | 518         | N            | 8.55  | 19.7     | Homeobox protein Hox-C4 JAN74469.1 Daphnia magna 1.00E-70 82 3 |
| HOXA1     | 579      | 3231        | Y            | 8.88  | 58.99    | Homeobox protein Hox-A1 JAN46366.1 Daphnia magna 1.00E-92 99 2 |
| HOXD10    | 366      | 1533        | N            | 11.76 | 57.21    | Homeobox protein CDX-1 JAN85504.1 Daphnia magna 3.00E-53 94 3 |
| HOXB3-2   | 127      | 1024        | N            | 10.22 | 36.85    | Putative homeotic HOX3 protein KZS03900.1 Daphnia pulex 8.00E-33 71 2 |
| HOXC8     | 387      | 1825        | N            | 10.81 | 68.22    | Predicted: homeobox protein MSX-2-like XP_019879395.1 Aethina tumida 1.00E-41 78 -2 |
3.2 Identification and characterization of *D. similoides sinensis* Hox genes

Based on previously published transcriptome data (Zhang et al., 2016), 14 Hox genes of *D. similoides sinensis* were identified, including Abd-B, CDX-1, Dll, HOX-1, HOX-2, HOXA1, HOXA2, HOXB3, HOXB3-2, HOXB7, HOXC4, HOXC7, HOXC8 and HOXD10, among which Dll, HOXA1, HOXA2, HOXB3, and HOXC7 had complete ORF. The Hox gene sequences with the complete ORF-binding domain covered the entire homeodomain region, and the remainder covered all or part of homeodomain. The 14 sequences consisted of full-length 89–852 amino acid sequences, with molecular weight (MW) ranging from 17.7 to 97.7 kDa and isoelectric points (pI) ranging from 6.1 to 11.76 (Table 2).
TABLE 3  Two-way ANOVA results on the effects of food treatment, mother-F1 generation, and their combinations on the relative expression of 14 *D. similoides sinensis* Hox genes

| Clone | Genes | Factors                        | df | F     | p    |
|-------|-------|--------------------------------|----|-------|------|
| Clone 1 | Abd-B | Generation                     | 1  | 0.124 | .731 |
|        |       | Food treatments                 | 2  | 1.685 | .226 |
|        |       | Generation × Food treatments    | 2  | 1.464 | .270 |
|        | CDX-1 | Generation                     | 1  | 44.527| .000 |
|        |       | Food treatments                 | 2  | 6.193 | .014 |
|        |       | Generation × Food treatments    | 2  | 6.153 | .014 |
|        | Dll   | Generation                     | 1  | 7.844 | .016 |
|        |       | Food treatments                 | 2  | 0.890 | .436 |
|        |       | Generation × Food treatments    | 2  | 0.042 | .959 |
|        | HOX-1 | Generation                     | 1  | 91.504| .000 |
|        |       | Food treatments                 | 2  | 11.522| .002 |
|        |       | Generation × Food treatments    | 2  | 12.295| .001 |
|        | HOX-2 | Generation                     | 1  | 2.982 | .110 |
|        |       | Food treatments                 | 2  | 2.701 | .108 |
|        |       | Generation × Food treatments    | 2  | 2.694 | .108 |
|        | HOXA1 | Generation                     | 1  | 5.555 | .036 |
|        |       | Food treatments                 | 2  | 2.730 | .105 |
|        |       | Generation × Food treatments    | 2  | 3.319 | .071 |
|        | HOXA2 | Generation                     | 1  | 18.463| .001 |
|        |       | Food treatments                 | 2  | 23.563| .000 |
|        |       | Generation × Food treatments    | 2  | 19.562| .000 |
|        | HOXB3 | Generation                     | 1  | 30.085| .000 |
|        |       | Food treatments                 | 2  | 17.714| .000 |
|        |       | Generation × Food treatments    | 2  | 18.987| .000 |
|        | HOXB3-2 | Generation                     | 1  | 12.077| .005 |
|        |       | Food treatments                 | 2  | 4.308 | .039 |
|        |       | Generation × Food treatments    | 2  | 4.694 | .031 |
|        | HOXB7 | Generation                     | 1  | 11.046| .006 |
|        |       | Food treatments                 | 2  | 8.550 | .005 |
|        |       | Generation × Food treatments    | 2  | 8.696 | .005 |
|        | HOXC4 | Generation                     | 1  | 16.380| .002 |
|        |       | Food treatments                 | 2  | 0.374 | .696 |
|        |       | Generation × Food treatments    | 2  | 0.421 | .666 |
|        | HOXC7 | Generation                     | 1  | 27.088| .000 |
|        |       | Food treatments                 | 2  | 7.643 | .007 |
|        |       | Generation × Food treatments    | 2  | 0.888 | .437 |
|        | HOXC8 | Generation                     | 1  | 6.456 | .026 |
|        |       | Food treatments                 | 2  | 4.080 | .044 |
|        |       | Generation × Food treatments    | 2  | 5.102 | .025 |
|        | HOXD10 | Generation                     | 1  | 165.122| .000 |
|        |       | Food treatments                 | 2  | 157.520| .000 |
|        |       | Generation × Food treatments    | 2  | 97.613| .000 |

(Continues)
3.3 | Phylogenetic tree analysis of D. similoides sinensis Hox genes

A neighbor-joining tree of Hox genes was constructed based on the amino acid sequences from D. similoides sinensis, D. pulex, D. magna, Pelodiscus sinensis, Zootermopsis nevadensis, Operophtera brumata, Latimeria menadoensis (Koh et al., 2003), Litopenaeus vannamei (Sun et al., 2015), Drosophila melanogaster (http://flybase.org/), and Homo sapiens (https://www.genenames.org/). HOX-1 and HOX-2 are not included in the phylogenetic tree because of their short amino acid sequences. Abd-B, CDX-1, DII, HOXA1, HOXA2, HOXB3, HOXB3-2, HOXB7, HOXC4, HOXC7, HOXC8, and

| Clone 2 | Genes | Factors | df | F  | p   |
|---------|-------|---------|----|----|-----|
| Abd-B   |        | Generation | 1  | 5.956 | .031 |
|         |        | Food treatments | 2  | 4.077 | .045 |
|         |        | Generation × Food treatments | 2  | 2.508 | .123 |
|         |        | Generation | 1  | 15.341 | .002 |
|         |        | Food treatments | 2  | 20.799 | .000 |
|         |        | Generation × Food treatments | 2  | 1.222 | .329 |
|         |        | Food treatments | 2  | 0.713 | .510 |
|         |        | Generation × Food treatments | 2  | 1.337 | .299 |
|         |        | HOX-1 | Generation | 1  | 4.296 | .060 |
|         |        | Food treatments | 2  | 1.932 | .187 |
|         |        | Generation × Food treatments | 2  | 3.533 | .062 |
|         |        | HOX-2 | Generation | 1  | 5.035 | .044 |
|         |        | Food treatments | 2  | 1.201 | .335 |
|         |        | Generation × Food treatments | 2  | 0.478 | .631 |
|         |        | HOXA1 | Generation | 1  | 8.089 | .015 |
|         |        | Food treatments | 2  | 4.885 | .028 |
|         |        | Generation × Food treatments | 2  | 3.994 | .047 |
|         |        | HOXA2 | Generation | 1  | 4.765 | .050 |
|         |        | Food treatments | 2  | 1.042 | .383 |
|         |        | Generation × Food treatments | 2  | 2.221 | .151 |
|         |        | HOXB3 | Generation | 1  | 14.150 | .003 |
|         |        | Food treatments | 2  | 8.705 | .005 |
|         |        | Generation × Food treatments | 2  | 7.903 | .006 |
|         |        | HOXB3-2 | Generation | 1  | 7.123 | .020 |
|         |        | Food treatments | 2  | 5.724 | .018 |
|         |        | Generation × Food treatments | 2  | 6.038 | .015 |
|         |        | HOXB7 | Generation | 1  | 0.099 | .758 |
|         |        | Food treatments | 2  | 1.750 | .215 |
|         |        | Generation × Food treatments | 2  | 0.578 | .576 |
|         |        | HOXC4 | Generation | 1  | 2.687 | .127 |
|         |        | Food treatments | 2  | 0.411 | .672 |
|         |        | Generation × Food treatments | 2  | 0.485 | .627 |
|         |        | HOXC7 | Generation | 1  | 11.363 | .006 |
|         |        | Food treatments | 2  | 2.848 | .097 |
|         |        | Generation × Food treatments | 2  | 3.521 | .063 |
|         |        | HOXC8 | Generation | 1  | 15.266 | .002 |
|         |        | Food treatments | 2  | 16.214 | .000 |
|         |        | Generation × Food treatments | 2  | 19.089 | .000 |
|         |        | HOXD10 | Generation | 1  | 6.262 | .028 |
|         |        | Food treatments | 2  | 6.530 | .012 |
|         |        | Generation × Food treatments | 2  | 5.062 | .025 |

Note: Bold values indicates p < .05 is significant; p < .01 is very significant.
HOXD10 were respectively clustered into different clades with orthologs in other species (Figure 2).

### 3.4 Hox gene expression in the mothers and F1 of two D. similoides sinensis clones under different food treatments

For clone 1, food treatment and mother-F1 generation affected significantly the relative expression of CDX-1, HOX-1, HOXA2, HOXB3, HOXB3-2, HOXB7, HOXC8, HOXD10 genes as well as their combinations (Table 3). Moreover, both food treatment and mother-F1 generation affected significantly the relative expression of HOXC7 gene (Table 3). In clone 1-mothers, compared to that in the 100%S food treatment, 11 Hox genes (CDX-1, Dll, HOX-1, HOX-2, HOXA1, HOXB3, HOXB3-2, HOXC4, HOXC7, HOXC8, and HOXD10) were up-regulated in the food treatments containing *M. aeruginosa* (20%M + 80%S and 40%M + 60%S), whereas the HOXA2 was only up-regulated in the 40%M+60%S food treatment (Figure 3). The expressions of CDX-1, HOX-1, HOXB3, and HOXD10 of clone 1-mothers in the 40%M+60%S food treatment were 4.8–13.1 times as high as in the 100%S food treatment. Multiple comparisons (Tukey’s HSD) showed that the expressions of CDX-1, HOX-1, HOXB3, and HOXD10 in the 40%M + 60%S food treatment were significantly higher than those in the 100%S food treatment (p < .05), whereas the expression of only HOX-1 in the 20%M + 80%S food treatment was significantly higher than in the 100%S food treatment. Moreover, the expressions of both HOXB3 and HOXD10 in the 40%M + 60%S food treatment were significantly higher than those in the 20%M+80%S food treatment (p < .05). However, HOXB7 was significantly lower in the food treatments containing *M. aeruginosa* than in the 100% S food treatment (p < .05). In clone 1-F1, the expressions of only CDX-1 and HOXA2 were up-regulated in the 20%M + 80%S food treatment, whereas the other Hox genes were down-regulated. Compared to the 100%S food treatment, the expressions of nine Hox genes (AbdB, CDX-1, Dll, HOXA2, HOXB7, HOXC4, HOXC7, HOXC8, and HOXD10) were up-regulated in the 40%M+60%S food treatment (Figure 3). The expression of HOXA2, HOXC7, HOXC8, and HOXD10 of clone 1-F1 in the 40%M+60%S food treatment was 8.2–21.1 times as high as in the 100%S food treatment. Multiple comparisons (Tukey’s
HSD) showed that the expressions of HOXA2, HOXC7, HOXC8, and HOXD10 in the 40%M+60%S food treatment were significantly higher than those in the 100% S food treatment (p < .05). In addition, the expressions of HOXA2, HOXB7, HOXC7, HOXC8, and HOXD10 in the 40%M+60%S food treatment were significantly higher than those in the 20%M+80%S food treatment (p < .05).

For clone 2, food treatment and mother-F1 generation affected significantly the relative expressions of HOXA1, HOXB3, HOXB3-2, HOXC8, and HOXD10 genes as well as their combinations (Table 3). Moreover, both food treatment and mother-F1 generation affected significantly the relative expressions of Abd-B and CDX-1 genes (Table 3). In clone 2-mothers, the expressions of 10 Hox genes (Abd-B, DII, HOX-2, HOXA1, HOXA2, HOXB3, HOXB3-2, HOXB7, HOXC7, and HOXD10) in the food treatments containing M. aeruginosa were up-regulated compared to that in the 100%S food treatment (Figure 3). The expressions of Abd-B, HOX-2, HOXA1, HOXC7, and HOXD10 of clone 2-mothers in the 40%M+60%S food treatment were significantly higher than those in the 100%S food treatment (p < .05), as were Abd-B, HOX-1, HOX-2, HOXA1, HOXA2, HOXC7, HOXC8, and HOXD10 in the 40%M+60%S food treatment. However, the expression of CDX1 in the food treatments containing M. aeruginosa (20%M + 80%S and 40%M + 60%S) was significantly lower than that in the 100%S food treatment. In clone 2-F1, the expressions of 7 Hox genes (HOX-1, HOX-2, HOXB3, HOXB3-2, HOXB7, HOXC8, and HOXD10) in the 20%M + 80%S food treatment were up-regulated compared to that in the 100%S food treatment. The expressions of 10 Hox genes (Abd-B, HOX-1, HOX-2, HOXA1, HOXB3, HOXB3-2, HOXC4, HOXC7, HOXC8, and HOXD10) in the 40%M + 60%S food treatment were up-regulated compared to those in the 100%S food treatment (Figure 3). Multiple comparisons (Tukey’s HSD) showed that the expression of CDX1 in the food treatments containing M. aeruginosa (20%M + 80%S and 40%M + 60%S) was significantly lower than that in the 100%S food treatment (p < .05), whereas it was only significantly lower in the 20%M + 80%S food treatment for HOXB3.

A phylogenetic tree constructed based on amino acid sequences from vertebrates and invertebrates showed that Hox genes had evolved into different functions after multiple genomic duplication or genomic doubling events. Abd-B, CDX-1, DII, HOXA1, HOXA2, HOXB3, HOXB3-2, HOXB7, HOXC4, HOXC7, HOXC8, and HOXD10 of D. similoides sinensis were clustered into different clades with orthologs from other species. There was an orthologous relationship between HOXB3 from D. similoides sinensis and HsHOXB3 from H. sapiens (Sun et al., 2015), and HOXB3-2 had an orthologous correlation with LmHOXB3 from L. menadensis (Koh et al., 2003). HOXC4 from both D. similoides sinensis and D. magna were clustered into a separate clade with Dfd from L. vannamei (Sun et al., 2015), suggesting that these three species were orthologs. Orthologous relationships between HOXA1 from both D. similoides sinensis and D. magna and Lab from D. pulex were also observed. Moreover, Abd-B from D. similoides sinensis were clustered into a clade with 10 Hox genes from D. melanogaster.

4 | DISCUSSION

4.1 | Identification and phylogenies of D. similoides sinensis Hox genes

In this study, 14 Hox genes of D. similoides sinensis were identified based on previous transcriptomic data (Zhang et al., 2016; Table 2). In the shrimp L. vannamei, there were 13 Hox gene protein sequences at the transcriptomic level (Sun et al., 2015). However, 39 Hox gene sequences in Ichthyophis bananicus were found based on genomic data (Wu et al., 2015). Therefore, the 14 Hox genes in D. similoides sinensis in this study might be underestimated based on the data of the transcriptome rather than the genome.

4.2 | Effects of food treatment and clone on the survival rate and the Hox gene expressions of D. similoides sinensis

Usually, the survivals of Daphnia are restrained in the presence of M. aeruginosa. Survival rate and life span of D. galeata dropped obviously with the increase in M. aeruginosa concentration (Han et al., 2012). Rohrlack et al. (2001) found that the median survival time of different Daphnia species was closely related to their microcystin ingestion rate. In this study, compared with the 100% S food treatment, the survival rates at the end of the experiment of clone 1-mothers and clone 1-F1 in the 20%M + 80%S and 40%M + 60%S food treatments were significantly lower, whereas it was significantly higher for clone 2-F1 in the 20%M + 80%S food treatment. Peng et al. (2018) observed also that the mother exposed to toxic M. aeruginosa enhanced the fitness of D. similoides sinensis offspring to Microcystis and had the differences among clones. Similarly, different genotypes of D. galeata showed different tolerance to M. aeruginosa (Druga et al., 2016). However, D. similoides sinensis offspring to D. similoides sinensis had the differences between species or clones. Moreover, it had potential limitations using only the survival rate to evaluate the adaptability of D. similoides sinensis offspring to M. aeruginosa in this study, and more the life-history parameters should be employed to study the mechanism.

Microcystis can affect related gene expression of Daphnia spp. (Druga et al., 2016; Lyu et al., 2015; Schwarzenberger et al., 2009; Schwarzenberger & Elert, 2013; Xu et al., 2018). Schwarzenberger et al. (2009) observed that the presence of dietary microcystins led to the up-regulation of two genes (glyceraldehyde-3-phosphate dehydrogenase and ubiquitin-conjugating enzyme) which involved in the basic metabolism of D. magna. Some gene expression of Daphnia species to toxic M. aeruginosa showed the differences between
clones (Druga et al., 2016; Xu et al., 2018). In this study, in the 40%M + 60%S food treatment, the survival rates at the end of the experiment of clone 1-mothers were significantly lower than those of clone 2-mothers (p < .05), and the expression of Abd-B in clone 2-mothers was higher than in clone 1-mothers. In insects, Abd-B is able to regulate the development of the posterior nodules (Hou et al., 2004), affecting the ecdisis and survival. Moreover, in this study, Clone 2-mother and Clone 2-F1 had similar survival rates under 20%M+80%S food treatment, whereas their Hox gene expression patterns are different under the same condition. Therefore, the expression patterns of Hox genes may be related to the tolerance of D. similoides sinensis offspring to M. aeruginosa and have the differences between clones.

Daphnia spp. have an inductive defense mechanism against M. aeruginosa, which can transfer environmental information and tolerance to M. aeruginosa to their offspring, and reduce the toxic effects of M. aeruginosa (Gustafsson et al., 2005; Jiang et al., 2013; Schwarzenberger & Elert, 2013). Compared with the mothers un-exposed to M. aeruginosa, the offspring from mothers exposed to M. aeruginosa have a shorter time to maturation and produce more offspring, and so had greater fitness for an adverse environment (Gustafsson et al., 2005). Schwarzenberger and Elert (2013) observed that cyanobacterial protease inhibitors could lead to an increase in protease gene expression of D. magna offspring. Arginine kinase transcript level of D. magna offspring whose mothers had been previously exposed to M. aeruginosa were significantly higher than those of mothers fed with pure S. obliquus (Lyu et al., 2015). The Hox genes, as a family encoding transcriptional regulator, could regulate the growth and development of crustaceans as well as body formation (Hou et al., 2004). Dll is an important gene regulating the growth of arthropods (Hou et al., 2004), and could similarly regulate appendage development in insects (Hughes & Kaufman, 2002). Vachon et al. (1992) found also that the abdomen appendages in insects might not be developed if Dll was inhibited by other Hox genes. In this study, compared to those in the 100%S food treatment, the expression of Dll of clone 1-mothers and clone 1-F1 in the 40%M+60%S food treatment was up-regulated, suggesting that the increasing expression level of Dll may protect the development of Daphnia appendages. This result may be consistent with which the survival rates at the end of the experiment of clone 1-F1 was higher than that of their mothers in the 40%M + 60%S food treatment. Moreover, compared to the 100% S food treatment, the gene expression of Abd-B and HOXB7 of clone 1-F1 were up-regulated in the 40%M + 60%S food treatment, but down-regulated in clone 1-mothers, suggesting that these offspring (F1) may have greater tolerance than their mothers under higher M. aeruginosa concentration.

5 CONCLUSIONS

In this study, 14 Hox genes of D. similoides sinensis were identified based on previous transcriptome data, including Abd-B, CDX-1, Dll, HOX-1, HOX-2, HOXA1, HOXA2, HOXB3, HOXB3-2, HOXB7, HOXC4, HOXC7, HOXC8, and HOXD10. In clone 1-mothers and clone 1-F1, the survival rates at the end of the experiment of D. similoides sinensis in the food treatments containing M. aeruginosa were significantly lower than those in the 100%S food treatment (p < .05). Moreover, the survival rates at the end of the experiment of clone 1-F1 in the food treatments containing M. aeruginosa were higher than those of the mothers. However, there were no significant differences in the survival rates at the end of the experiment of D. similoides sinensis clone 2-mothers between the 100%S food treatment and food treatments containing M. aeruginosa (p > .05). Compared to the 100%S food treatment, the expression of Abd-B in clone-2 mothers was significantly higher in the 40%M + 60%S food treatment, whereas they were down-regulated in clone 1-mothers. Therefore, it is likely that the down-regulation of Abd-B in clone 1-mothers might be responsible for a significant decrease in the survival rates at the end of the experiment under higher M. aeruginosa concentrations.

The expressions of Abd-B, HOX-2, HOX7, and HOXD10 in clone 2-mothers in the 40%M + 60%S food treatment were significantly up-regulated compared to that in the 100%S food treatment, whereas the expressions of CDX-1, HOX-1, HOXB3, and HOXD10 were significantly up-regulated in clone 1-mothers. Moreover, the expressions of HOX2, HOX7, HOX8, and HOXD10 of clone 1-F1 in the 40%M + 60%S food treatment were significantly higher than those in the 100%S food treatment. However, compared with the 100%S food treatment, the expressions of CDX-1 in clone 2-mothers and clone 2-F1 and HOXB7 in clone 1-mothers in the food treatments containing M. aeruginosa were significantly lower. Our results suggest that the offspring (F1) produced by D. similoides sinensis mothers pre-exposed to toxic M. aeruginosa had stronger adaptability to M. aeruginosa than their mothers. Moreover, Hox gene expressions of D. similoides sinensis had obvious differences between clones under the stress of toxic M. aeruginosa. Although our experimental results are satisfactory and rational, it has the potential limitations to reveal the adaptability of D. similoides sinensis offspring to M. aeruginosa in the study when we only compared F1 from the mothers in the 20%M + 80%S food treatment with the 100%S food treatment. Therefore, further studies need to be promoted in the future.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

Xiaoxue Xu: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Investigation (lead); Methodology (equal); Software (equal); Visualization (equal); Writing – original draft (lead);
Writing – review & editing (equal). Yaqin Cao: Investigation (supporting); Methodology (supporting). Huiying Qi: Investigation (supporting); Methodology (supporting). Daogui Deng: Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Funding acquisition (lead); Methodology (equal); Project administration (equal); Supervision (equal); Validation (supporting); Writing – original draft (supporting); Writing – review & editing (equal). Yan-nan Zhang: Conceptualization (equal); Formal analysis (supporting); Methodology (equal); Software (equal); Supervision (equal); Validation (supporting). Jianxun Wu: Investigation (supporting); Methodology (supporting). Shuxiu Peng: Investigation (supporting); Methodology (supporting); Visualization (supporting). Zhongze Zhou: Conceptualization (supporting); Project administration (equal); Supervision (equal).

DATA AVAILABILITY STATEMENT
Fourteen Hox gene sequences are identified in D. similoides sinensis through the previous transcriptome data (Zhang et al., 2016. https://doi.org/10.1038/srep34241). Reading frames and functional domains were predicted using the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) from the NCBI database. The sequence data of D. similoides sinensis in this study have been deposited in Dryad Digital Repository (https://doi.org/10.5061/dryad.6hdr7sr2n).

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