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Dietary exposure of the water flea *Daphnia galeata* to microcystin-LR

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ABSTRACT

Harmful substances like the cyanotoxin microcystin-leucine-arginine (MC-LR) are commonly found in eutrophic freshwater environments, posing risks to aquatic organisms. The water flea, *Daphnia*, is a well-established model organism for environmental toxicology research. Nevertheless, there is currently insufficient research on the genes that respond to MC-LR in *Daphnia galeata*. This study aimed to gain insights into the notable genes that react significantly to MC-LR. In this study, we generated an extensive RNA-Seq sequences isolated from the *D. galeata* HK strain, Han River in Korea. This strain was nourished with a diet of the green microalga *Chlorella vulgaris* and treated with pure MC-LR at a concentration of 36 ug/L. The transcriptome profile in response to the MC-LR treatment was obtained and 336 differentially expressed genes were subjected to Gene Ontology (GO) and euKaryotic Orthologous Groups of proteins analyses. GO enrichment analysis showed that chemical stimulus, amino sugar metabolic and catabolic process, oxidative stress, and detoxification were highly enriched, in reverse, proteolysis and fucosylation were underpresented. Detoxification process related genes such as peroxidase-like, chorion, and thyroid peroxidase-like were enriched for eliminating or neutralizing MC_LR from an organism's body. Furthermore, functional protein classification revealed an upregulation of lipid and inorganic ion transport processes, while amino acid and carbohydrate transport processes were found to be downregulated. These findings offer insights into how organisms respond to ecotoxic stimuli, providing valuable information for understanding adaptation or defense pathways.

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Introduction

Daphnia are considered an indicator species of water toxicity (Wan et al. 2021), and their survival and reproductive success can reflect the health of aquatic ecosystems (Dao et al. 2010; Cao et al. 2014; Henning-Lucass et al. 2016). Studying the survival of *Daphnia* exposed to environmental toxins is of great importance in the field of ecotoxicology (Cui et al. 2018; Zimmermann et al. 2020; Wan et al. 2021). Researchers gain insights into the overall state of aquatic environments by understanding how environmental toxins affect the development, reproduction, growth, and life cycles of *Daphnia* (Lurling 2003; Herrera et al. 2015; Schwarzenberger and Martin-Creuzburg 2021). Excessive nutrient input to freshwater has increased the frequency of cyanobacterial blooms, which produce biological toxins referred to cyanotoxins that affect the feeding, growth, and life history parameters of the grazer *Daphnia* (Ma et al. 2014; Huisman et al. 2018; Feist and Lance 2021).

Previous studies have shown that when an organism is exposed to microcystin-leucine-arginine (MC-LR), a common cyanotoxin, its cells can attempt to detoxify the toxin by conjugating it with glutathione (GSH). This process involves the binding of MC-LR to the thiol (sulfhydryl) group of GSH, forming a covalent bond (Plugmacher et al. 1998). The resulting glutathione conjugate is generally less toxic than the original MC-LR molecule, which helps protect the organism from the harmful effects of the toxin. The glutathione conjugate of MC-LR can then be further processed and ultimately eliminated from the organism's body through various biochemical pathways, including transport and excretion mechanisms. This detoxification process is an essential defense mechanism that organisms employ to counteract the toxic effects of MC-LR and other xenobiotics (foreign substances). However, the efficiency of this detoxification process can vary among species, and some may be more susceptible to the harmful effects of MC-LR than others. Understanding the detoxification pathways of MC-LR and its interactions with cellular components, like glutathione, is of great interest in environmental and toxicological research, particularly in

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the context of aquatic ecosystems where cyanobacterial blooms and microcystin contamination can occur.

The water flea *Daphnia* is a well-established model organism for environmental toxicology. Nevertheless, there is currently insufficient research on the genes that respond to MC-LR in *Daphnia galeata*, a widespread *Daphnia* species found in Korea. Therefore, to gain insights into these genes, we generated an extensive RNA-Seq dataset and performed a comparative transcriptome analysis. The RNA-Seq method provides high-resolution data on gene expression and allows the identification of novel transcripts. This ecotoxicology experiment provides opportunities for developing predictive models and identifying biomarkers of stress response for freshwater assessments in Korea using *Daphnia galeata*.

Materials and methods

Field sample collection and laboratory culture

Zooplankton samples from Han River in Korea were collected in a layer 0–1 m deep using a plankton net with an opening diameter of 30 cm and mesh size of 50 µm. The sampling site was located at 37°34′50″ N 127° 10′42″ E (Figure 1). On 7 June 2023, the field environmental conditions included a temperature of 24°C, pH of 7.27, dissolved oxygen (DO) of 5.2 mg/L, electrical conductivity (EC) of 372 µs/cm, and total dissolved solids (TDS) of 186 ppm. *Daphnia* (*Daphnia*) *galeata* Sars, 1863 (Kim 1988; Tanaka 1992; Zuykova et al. 2010; Kirdiashcheva and Kotov 2013; Błędzki and Rybak 2016) is a dominant species in the Han River of Korea (NIER 2006, 2017). The *D. galeata* collected from Han River in Korea was named *D. galeata* HK clone for our laboratory culture. This HK clone was classified according to *Daphnia* (*Daphnia*) *galeata* Sars, 1863. We used diagnostic characters – such as the presence of an ocellus, a pointed head helmet, the rostrum shape, particular features of ephippial females, and the presence of postabdominal claw without fine external combs in which the spine size of combs 1, 2, and 3 are equal (Figure 2). The classification was subsequently confirmed using molecular phylogenetic analysis (Figure 3). Female individuals were isolated with the aid of a stereomicroscope SZ40 (Olympus, Japan) for parthenogenetic reproduction. In the laboratory, all clones were cultured at 21 ± 1°C and a light intensity ranging from 540 to 1000 lux with a 16:8 light:dark photoperiod. For the clonal culture, five to ten clones per 0.5 L of moderately hard water were maintained (NIER 2017). The culture medium was renewed twice a week and prepared a day before use, with DO concentrations ranging from 5.5 to 8.0 mg/L. *Daphnia* HK clones were fed every three days with a mixture of the pure green alga *Chlorella vulgaris* and YCT (Yeast, Alfalfa, and Tetramin) (EvergreenTOP, Korea).

Figure 1. Sampling location of *Daphnia galeata* HK in Korea. The small orange box in the left panel corresponds to the large orange box (right panel). The white dot in the right panel indicates the sampling site (37°34′50"N, 127°10′42"E).

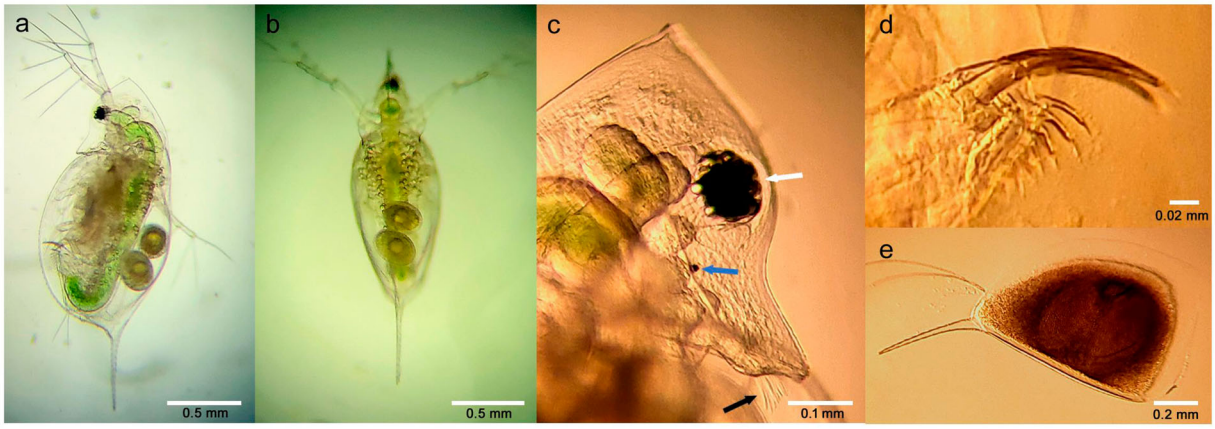

Figure 2. Morphological characteristics of female *Daphnia galeata* HK under a microscope. (a) A lateral view showing the broad oval carapace and bluntly pointed helmet. (b) A dorsal view. (c) A close-up view of the head showing the reduced antennules and only sensory setae protruding from under the pronounced rostrum. The white arrow indicates the large compound eye, the blue arrow indicates the ocellus, and the black arrow indicates sensory setae. (d) The postabdominal claw is shown with its fine, evenly sized combs. (e) A close-up view of an ephippium showing two ephippial eggs inside.

The growth observations of Daphnia *HK strain*

To record growth stages, we measured the body length, height, and apical spine length on days 3, 6, 9, 12, 15, 18 and 21. Also, we monitored the reproduction success of *Daphnia* by counting the number of observed eggs. For these experiments, five *Daphnia* possessing 8 or 10 eggs,

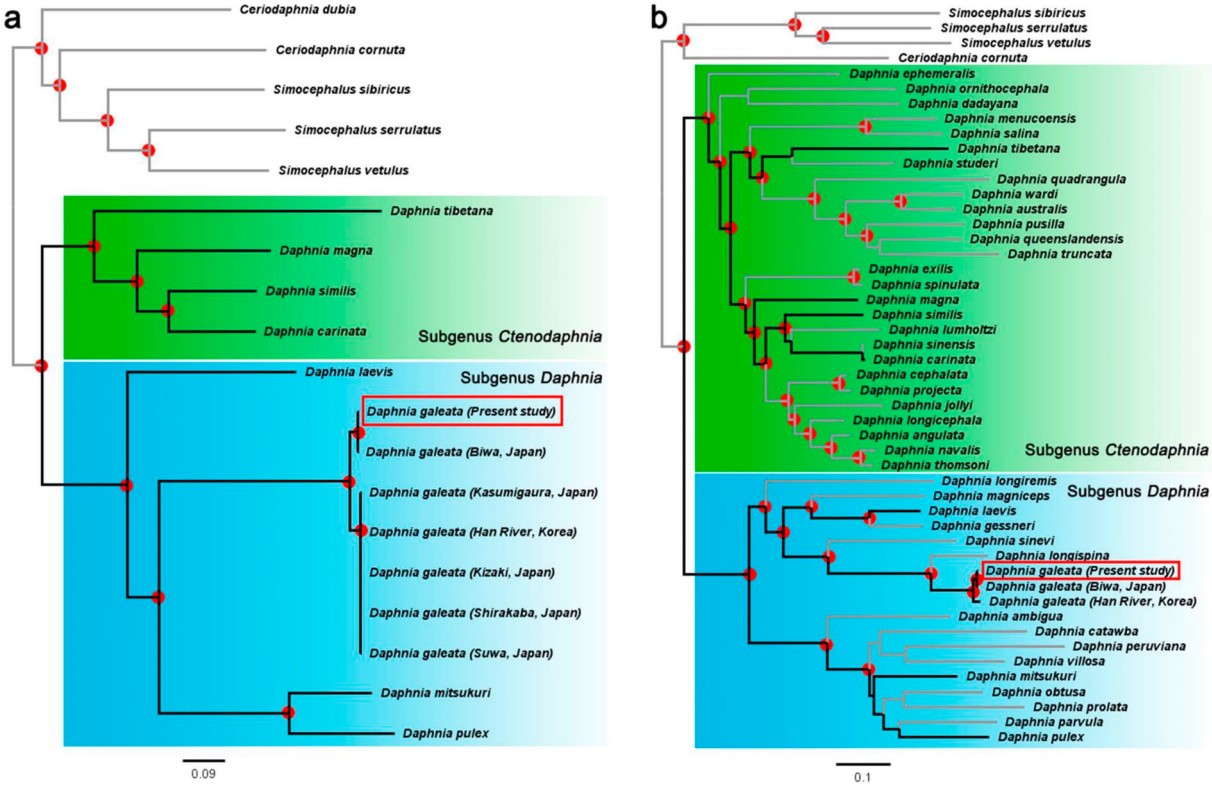

Figure 3. Maximum-likelihood phylograms based on *Daphnia* mitochondrial gene sequences. (a) A phylogenetic tree inferred based on an alignment of the nucleotide sequences of 13 mitochondrial genes. (b) A phylogenetic tree inferred based on an alignment of the partial nucleotide sequences of the mitochondrial 12S and 16S rRNA genes and the COX1 gene. In (b), the individuals used in the phylogeny of (a) are indicated by black bold lines. Nodes with the bootstrap support values greater than 85% are indicated with a red dot. The *D. galeata* HK clone used in this study is indicated with a red box. Species from the genera *Simocephalus* and *Ceriodaphnia* were used as the outgroups.

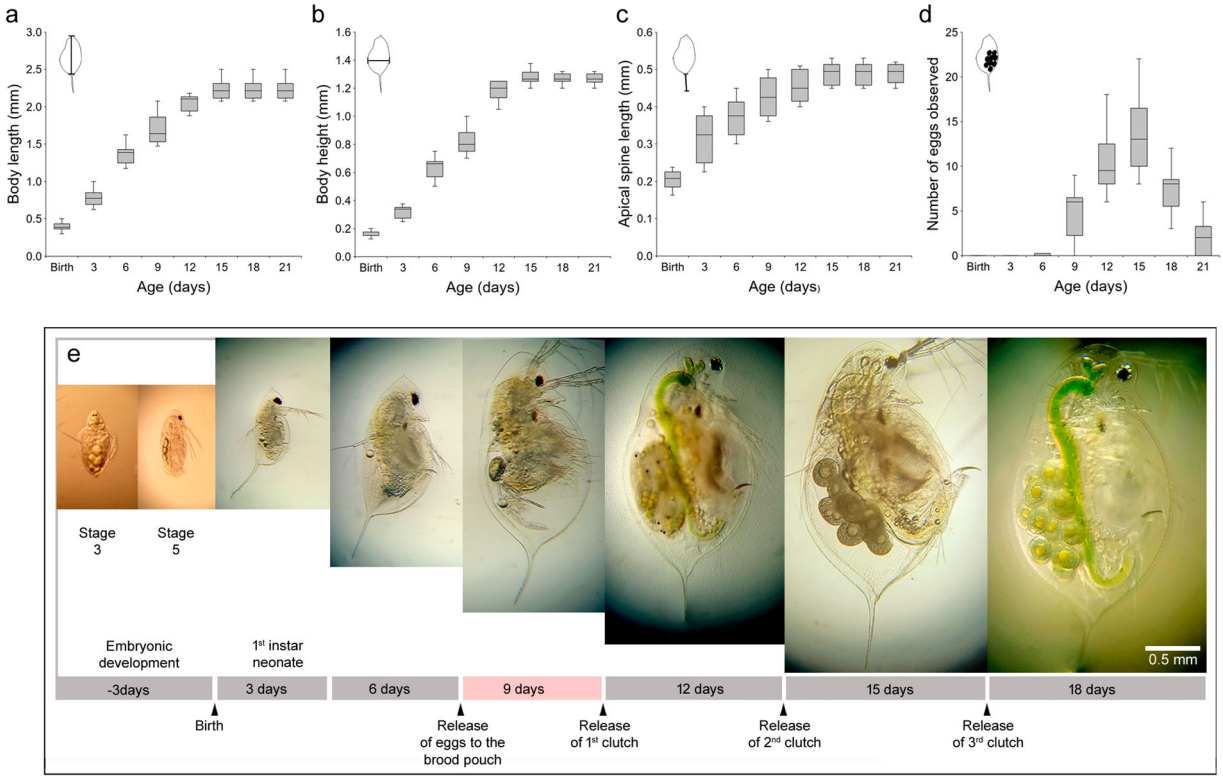

Figure 4. Boxplots of the growth and reproduction characteristics of *Daphnia galeata* HK and photomicrographs showing growth stages. (a) Body length measured from the point of the helmet to the base of the apical spin. (b) Body height measured from the dorsal to the ventral side of the body at its widest point. (c) Apical spine length measured from the base to the tip. (d) The number of eggs produced by *D. galeata* HK. (e) Embryonic development stages 3 and 5 and 3-, 6-, 9-, 12-, and 18-day-old adult daphnids. Below the photos, there is a timeline annotating the life stages based on studies of *D. magna* (Campos et al. 2018). To measure the sizes, we used 10 Daphnia. The relevant morphometric measurement ranges are illustrated in the upper left of each boxplot.

isolated using a microscope, were cultured in 500 ml of the culture medium. The next day, neonates were collected to perform a semi-static test. Ten neonates cultured in 500 ml of culture medium were fed every 3 days, and the medium was renewed every 3 days to measure morphological sizes and count the number of eggs (Figure 4). After 9 days, the first instar and subsequent neonates were separated from their mothers and cultured separately.

Short term (24 h) exposure to MC-LR

For this experiment, we used 9-day-old mature *D. galeata* HK (Figure 2). The mRNAs of *Daphnia* HK clones exposed to MC-LR were sequenced to obtain transcript expression. For the MC-LR exposure experiment, a concentration of 36 µg/L MC-LR (MC-LR) was used, and a positive control (EtOH; 36 µl/L EtOH) and a negative control (Ctrl; no treatment) were included. After treatment, cultures were incubated for 24 h in 50 ml of the test medium with 15 *Daphnia* HK clones that had reached maturity (8–9 days of age). The MC-LR (> 95% purity, ALX-350-012) was purchased from Enzo Biochem (USA) and dissolved in an organic solvent, absolute ethanol (99.9%) at a ratio of 1:1 (w/v). The dissolved MC-LR was stored at −20°C for 6 months.

Transcriptome sequencing, data filtering and de novo assembly

After the toxicant exposure tests, surviving daphnids in each condition were retrieved and immediately preserved in 1.8 ml of RNAlater™ Stabilization Solution (Thermo Fisher Scientific, USA). Short-read mRNA sequencing was performed on multiple individuals of *D. galeata*: 15 individuals each from the Et-OH treatment and Ctrl groups, and 14 from the MC-LR treatment group. For each experiment group, a paired-end library was generated using the TruSeq Stranded mRNA library kit (Illumina, USA) with an insert size of approximately 155 bp (Jung et al. 2020; Kim et al. 2022; Jeon et al. 2023). The mRNA sequencing was conducted on the Novaseq6000 platform (Illumina, USA), generating 101 bp reads from both ends. Sequencing data filtering was performed using fastp (ver. 0.23.4) (Chen et al. 2018). Adapter sequences (–detect_adapter_for_pe) and bases with a

mean quality lower than Q20 (–cut_mean_quality 20, –cut_window_size 4) were removed. In addition, reads shorter than 75 bp (–length_required 75) and/or that contained an undetermined base 'N' (–n_base_limit 0) were also filtered out. *De novo* transcriptome assembly was carried out using SPAdes (ver. 3.15.5) (Bushmanova et al. 2019) in RNA-seq mode (–rna) with two auto-determined k-mer parameters (-k 33,49). Quality of the transcriptome assembly was assessed using BUSCO (ver. 5.3.0) (Manni et al. 2021) with the Arthropoda lineage database (arthropoda_odb10, as of September 2020) containing a total 1,013 BUSCO marker genes.

Molecular phylogenetic analysis

Phylogenetic trees of the genus *Daphnia* were constructed based on the nucleotide sequences of the mitochondrial genes. For this analysis, GetOrganelle (ver. 1.7.7.0) (Jin et al. 2020) was used to assemble the mitochondrial genome of *D. galeata* HK using their transcriptome sequencing data with a previous mitochondrial genome of *D. galeata* available on GenBank (LC177071.1; from Biwa Lake, Japan) as a reference. In addition, both publicly available mitochondrial genomes and partial sequences of the mitochondrial 12S, 16S rRNA and cytochrome c oxidase subunit 1 (COX1) genes from other daphnids were retrieved from the database. The information of the sequences used are summarized in Table S1. Multiple sequence alignments were initially generated using MAFFT (ver. 7.520) (Katoh and Standley 2013) with the L-INS-i algorithm, and then the alignments were concatenated into 13 mitochondrial genes or three partial genes, using a custom-written Perl script conCat.pl (Eyun 2017). The concatenated sequence alignments used for phylogenetic inferences are available in Supplemental File 1. The maximum-likelihood (ML) analyses were performed using RAxML-NG (ver. 1.2.0) (Kozlov et al. 2019), applying the substitution model GTR + F + I + G4 for both alignment datasets. The best tree for each ML analysis was selected among the initial 15 random and 15 parsimony trees. Bootstrap support values were estimated with the option '–bs-trees autoMRE' and presented as the transfer bootstrap expectation values (Lemoine et al. 2018). Tree visualization was performed using FigTree (ver. 1.4.4; available at http://tree.bio.ed.ac.uk/software/figtree).

Differentially expressed gene analysis and functional annotation

Open reading frames (ORFs) of assembled transcriptome contigs were predicted to generate unigene sequences with a minimum protein length of 100 amino acids using TransDecoder (ver. 5.7.1) (https://github.com/TransDecoder/TransDecoder). The ORF redundancies with less than 95% sequence similarity were eliminated (-c 0.95) using cd-hit-est (ver. 4.8.1) (Li and Godzik 2006). Contig and unigene completeness were assessed and compared with BUSCO (ver. 5.3.0) based on the single-copy orthologs represented in the arthropoda_odb10 database. The filtered reads of each sample were mapped to the unigene set using Bowtie2 (ver. 2.5.1) (Langmead and Salzberg 2012) with '–very-sensitive' for read count calculations. The mapping results were normalized using the Cufflinks package (ver. 2.1.1) (Trapnell et al. 2010). Fragments per kilobase of transcript per million mapped reads (FPKM) values were used to estimate gene expression levels. The criteria for identifying significantly differentially expressed genes (DEGs) was set at a p-value < 0.05 and $|\log_2$-(fold-change)$|$ between samples ≥ 1. Gene annotation and functional DEG enrichment analyses were performed on the KOBAS website (http://bioinfo.org/kobas) with a Benjamini–Hochberg adjusted p-value < 0.05 as the criteria for significance based on the genome of *Daphnia pulex* (Bu et al. 2021). Gene ontology enrichment and graphical visualization of the results and gene characteristics were performed with ShinyGO (ver. 0.77) with an FDR cutoff of 0.05 (http://bioinformatics.sdstate.edu/go). The number of euKaryotic Orthologous Groups of proteins (KOGs) in each regulated group was counted and assigned to their specific categories using eggNOG-mapper (ver. 2.1.11) (Cantalapiedra et al. 2021). The genes in the significantly affected KOG categories were identified and annotated by BLAST against the NCBI nr database.

Results

Morphological identification keys of a female Daphnia galeata *HK*

A typical planktonic species, *D. galeata* is widely distributed and very frequently reported from Korea, commonly in lakes, reservoirs, fishponds, and rivers, but not from temporary waters (Kim 1988; Błędzki and Rybak 2016). The most recent morphological description of *D. galeata* and discussion of its taxonomic status was released in 2013 (Kirdiashcheva and Kotov 2013). To identify *D. galeata* daphnids to species, we used the pointed shape of the head helmet (Figure 2a), the medial crest located high on the head (Figure 2b), the presence of a single ocellus (Figure 2c, blue arrow), the large compound eye (Figure 2c, white arrow), pointed rostrum shape (Figure 2c), the presence of postabdominal claws without fine external combs (Figure 2d), and the particular features of ephippium

(Figure 2e). Distinctively, in this taxon, the first antennae of females are located noticeably nearer the end of the rostrum (Figure 2c, black arrow) and the bases of the first antennae are uniquely positioned along the sides of the medial crest when compared to those of its closest relatives, such as *D. cucullata* and *D. hyalina* (Kirdiashcheva and Kotov 2013). In this study, 9-day-old *D. galeata* HK clones showed one to four eggs under the carapace. On average, a body length with an apical spine was 2.143 mm and a body heigh was 0.9 mm at this age (Figure 2). The identification of *D. galeata* HK was consistent with all diagnostic characters of this taxon. Despite this morphological concordance, we applied molecular phylogenetics to corroborate the species classification (Figure 3).

Phylogenetic analysis

To perform genetic classification of the *Daphnia galeata* HK clone, two phylogenetic trees of *Daphnia* were constructed using an ML method. The first tree used a dataset consisting of 13 concatenated mitochondrial (mt) genes and included 19 species, with a total of 11,201 positions in the final dataset (Figure 3a) (Plessis et al. 2023). The second ML analysis used a dataset consisting of three concatenated partial mt genes (12S, 16S rRNAs, and COX1), comparing 49 species using a total of 3,864 positions in the final dataset (Figure 3b). The nucleotide sequences were aligned with MAFFT (Katoh and Standley 2013), and when aligned sequences contained gaps, the sequences corresponding to the gaps were included from all other sequences. Sequences from the genera *Simocephalus* and *Ceriodaphnia* made up the outgroups in both trees. The genus *Daphnia* is divided into two subgenera: *Ctenodaphnia* and *Daphnia*. Using molecular methods, Kotov and Taylor (2011) showed that this splits dates back to the Mesozoic. Both of our trees showed a genetical separation between subgenus *Ctenodaphnia* – including *Daphnia* (*C.*) *magna* Straus, 1820 (green boxes in Figure 3) – and subgenus *Daphnia* – including *Daphnia* (*D.*) *pulex* Leydig, 1860 and *Daphnia* (*D.*) *galeata* G.O. Sars, 1863 (blue boxes in Figure 3) – which is consistent with the split seen in a previous study (Błędzki and Rybak 2016). Also, the tree based on three partial mt genes showed high compatibility with the tree based on the full lengths of 13 mt gene sequences. Therefore, mitochondrial 12S rRNA, 16S rRNA, and COX1 appear to be sufficient for species identification within the genus *Daphnia*. In summary, the species identification of *D. galeata* HK was verified by comparative phenotypic (Figure 2) and genetic (Figure 3) analyses, and the two results confirmed each other.

The life cycles of Daphnia galeata *HK in the culture conditions*

To investigate the developmental phenomena in *D. galeata* HK, the body length, body height, and the size of an apical spine were measured and the number of eggs under the carapace were counted at three-day intervals for 21 days (Figure 4). In this experiment, from 3 to 21 days, the median total body length (not including the apical spine) grew from 0.597 to 2.744 mm, body heigh from 0.168 to 1.280 mm, and apical spine size from 0.205 to 0.503 mm. At day 21, total body lengths with the apical spine were 2.600 to 2.950 mm and body heights were 1.250 to 1.320 mm (Figure 4a–c). Adult female *Daphnia* with mature ovaries release eggs 13 min after molting (Hiruta and Tochinai 2014; Campos et al. 2018). Eight out of ten mature females formed subitaneous eggs by day 9, with 3–9 eggs observed (Figure 4d). The genus *Daphnia* molts four to six times before reaching maturity, but continues to molt and grow in regular intervals throughout their life (Sumiya et al. 2016; Zuykova et al. 2017). During the subculture, embryos were forced out of the carapace to observe embryo developmental traits from the egg stage to stage 5. *Daphnia galeata* HK clones showed two red eyes at stage 3, two brown eyes at stage 4, and one black eye at stage 5 (Figure 4e). This experiment showed the escape of a juvenile stage at day 9 under our laboratory culture conditions (Figure 2), revealing the maturation time of the Korean strain *D. galeata* HK.

Gene expression profiling

Comparative transcriptome studies provide high-resolution data on transcript levels and allow the identification of novel transcripts expressed under experimental conditions in the laboratory or in response to environmental changes in nature (Orsini et al. 2016; Toyota et al. 2016; Cui et al. 2018). We performed RNA sequencing (RNA-Seq) to obtain comprehensive data on gene expression changes in response to cyanotoxin MC-LR exposure. Paired-end Illumina RNA sequencing generated a total of 116.4 million reads under the MC-LR treatment, and negative (Ctrl) and positive (EtOH) controls produced 151.8 and 116.0 million reads, respectively (Table S2). Low-quality reads and adapter sequences were filtered, leaving 11.3–14.4 Gbp per treatment containing 112–142.7 million high-quality reads (Table S2). The filtered reads were assembled *de novo* into full-length

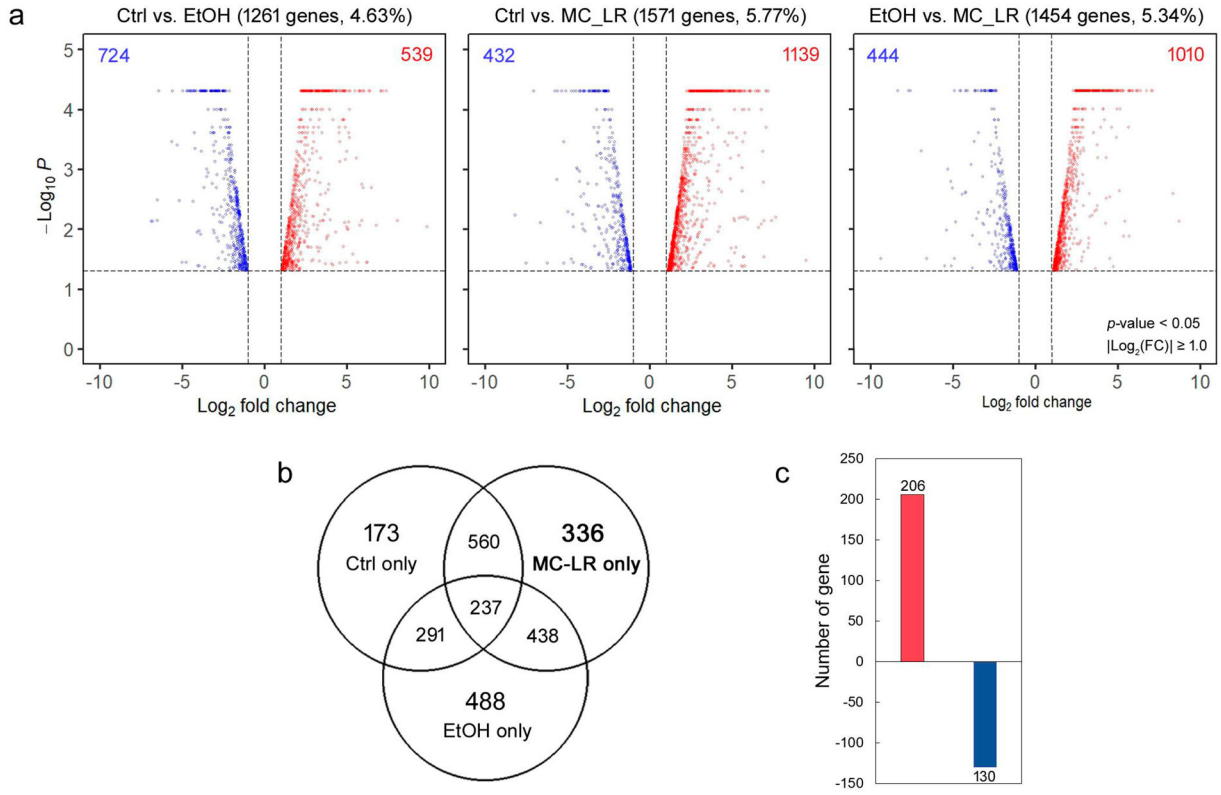

Figure 5. Comparison of differentially expressed genes in *Daphnia galeata* HK following treatment with microcystin-LR (MC-LR) or ethanol (EtOH) or in an untreated control (Ctrl). (a) Volcano plots of the three pairwise comparisons. The number and percentage of genes is indicated above each plot. Red and blue circles represent highly upregulated or downregulated genes in female *D. galeata* HK, respectively. (b) A Venn diagram showing the number of shared or unique differentially expressed genes for each treatment. (c) Unique genes expressed after exposure to MC-LR.

transcripts, and predicted ORFs were extracted, resulting in a total of 29,163 unigenes (Table S2). Under cyanotoxin MC-LR exposure, we identified significant up- or down-regulated transcripts based on two-fold or greater changes with a p-value ≥ 0.05. A total of 1,263 (Ctrl vs. EtOH), 1,571 (Ctrl vs. MC-LR), and 1,454 (EtOH vs. MC-LR) genes were differentially expressed across the three comparisons (Figure 5a). In the daphnids treated with MC-LR, 336 unique DEGs were identified (Figure 5b). The number of up-regulated genes was more than 1.5 times that of the down-regulated genes (Figure 5c).

Functional enrichment and frequency analysis

To determine the function of specific unigenes expressed in response to MC-LR, Gene Ontology (GO) enrichment and euKaryotic Orthologous Groups of proteins (KOGs) analysis were performed. GO-enrichment analysis is a powerful bioinformatics approach to understand the biological significance of a set of genes (Mi et al. 2013) and KOG analysis is a useful for functional and evolutionary studies to classify the database of clusters of orthologous groups of proteins encoded in Eukaryotes (Tatusov et al. 2000).

Firstly, in our GO-enrichment analysis, 248 (73.8%) unigenes were annotated to the *D. pulex* genome and divided into 166 up-regulated and 82 down-regulated unigenes (Table S3 and S4). Only 176 unigenes were assigned to GO terms, including 73 to biological processes, 43 to cellular components, and 60 to molecular functions. In biological processes (GO:0008150), metabolic processes (parent GO:008152) and responses to stimulus (parent GO:0050896) were highly enriched (Figure 6). Amino sugar metabolic process (GO:0006040) genes were over-represented, including amino sugar biosynthetic process (GO:0046349), N-acetylglucosamine metabolic process (GO:0006044), and amino sugar catabolic process (GO:0046348) genes. Also, responses to stimulus were over-represented, including response to oxidative stress (GO:0006979) and cellular oxidant detoxification (GO:0098869) genes. Proteolysis (GO:0006508) and fucosylation (GO:0036065) genes were under-represented. In general, proteolysis is the hydrolysis of proteins into smaller polypeptides and/or amino acids

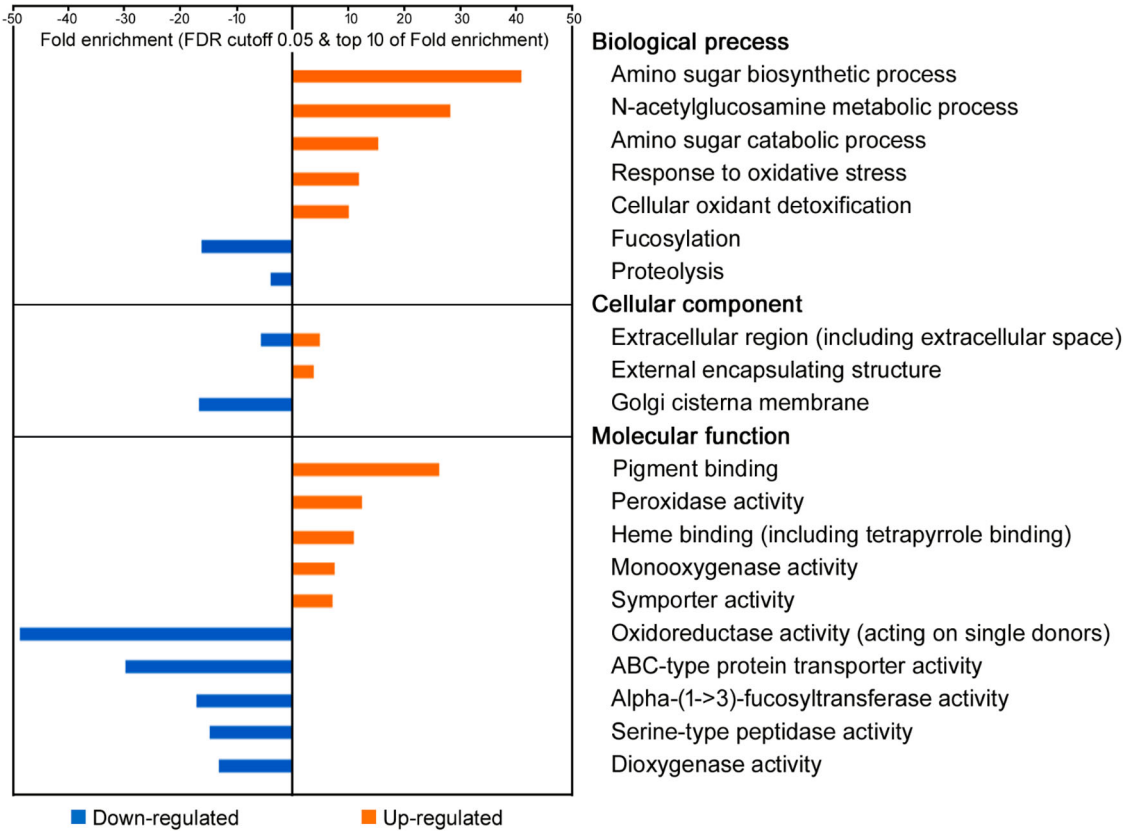

Figure 6. Gene ontology enrichment analysis with 336 differentially expressed genes specific to MC-LR-treated *Daphnia galeata* HK daphnids. Over- and under-represented genes in each GO-term are shown as orange and blue bars, respectively. The individual GO terms are categorized as belonging to cellular components, biological processes, and molecular functions.

by cleavage of their peptide bonds, and fucosylation is a subclass of glycosylation (GO:0070085). In Cellular Components (GO:0005575), extracellular region (GO:0005576) genes, including extracellular space and matrix (GO:0031012) genes were both over- and under-represented, but none of the assigned unigenes overlapped with each other (Figure 6). Golgi cisterna membrane genes, which are polarized between receiving cargo from the endoplasmic reticulum and sending cargo forward to post-Golgi organelles, were down-represented (Ito et al. 2014). In molecular functions (GO:0003674), the GO terms of DEGs coincided those assigned under the biological process and cellular component categories. For instance, peroxidase activity (GO:00004601) and monooxygenase activity (GO:0004497) are associated with the response to oxidative stress and oxidant detoxification, categories in biological processes, and both were over-represented in this study (Figure 6). In addition, α-(1->3)-fucosyltransferase activity (GO:0046920) is related to fucosylation (a biological process) and was down-represented. Transporter activity (GO:0005215), the parent GO term of symporter activity (GO:0015293) and ABC-type protein transporter activity (GO:0008320), were enriched under molecular functions, but not under biological processes. Among the subclasses of oxidoreductase activity pathway (GO:0016491), monooxygenase (GO:0004497), oxidoreductase (GO:0016684), oxidoreductase (GO:0016702), and peroxidase (GO:0004601) activity genes were over-represented. However, dioxygenase (GO:0051213) and oxidoreductase (GO:0016702) activity genes were under-represented.

Secondly, KOGs analysis showed that 211 unigenes were assigned to KOG database and divided into 135 up-regulated and 76 down-regulated genes (Figure 7). The M and Q class only involved in the upregulation. In the M class, C–C motif chemokine ligand 2 is one of several cytokine genes, which are a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes in human (Zhang et al. 2010b). The hexosyl transferases [chs1] is responsible for chitin synthesis in cuticle of fungi, nematodes, and arthropods (Zhang et al. 2010a). The Q class includes the secondary membrane or lipid transporters. The sulfonylurea receptor [Sur], the removal or sequestration of unwanted toxic compounds, forms

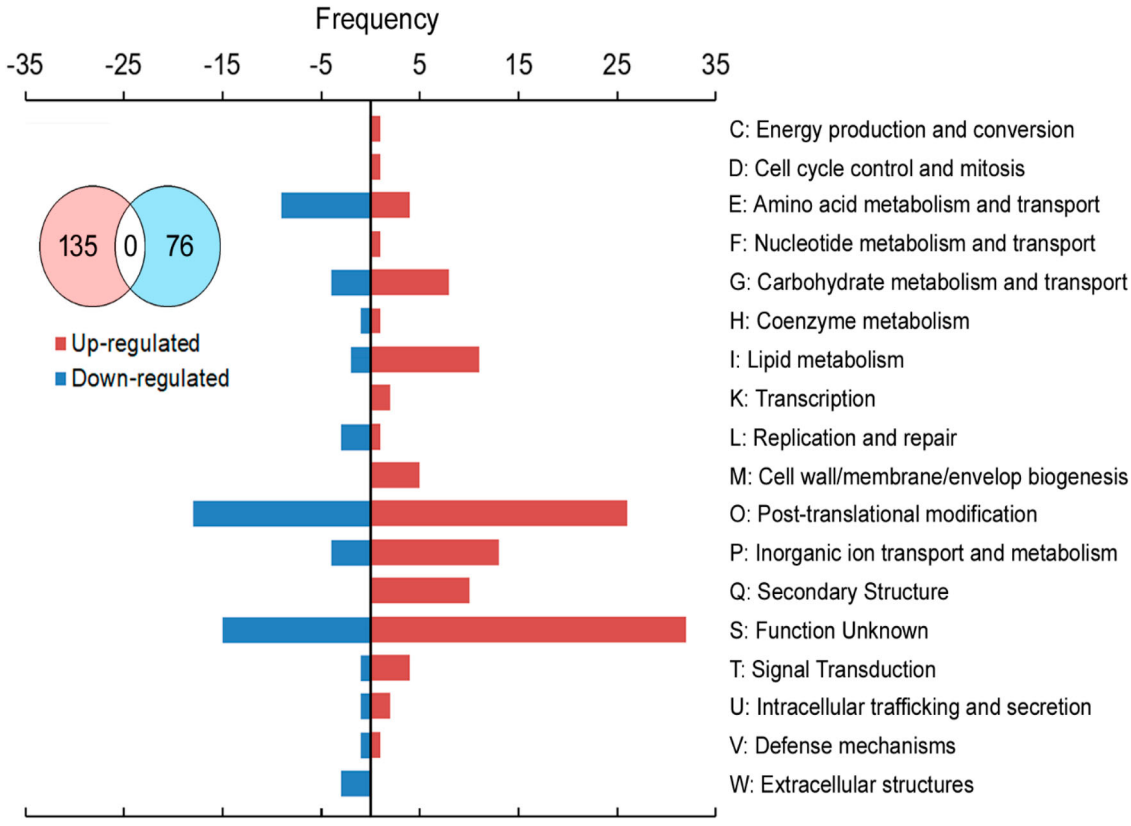

Figure 7. KOG category with differentially expressed genes specific to MC-LR-treated *Daphnia galeata* HK daphnids. Up- and Down-represented genes in each KOG category are shown as pink and sky bars, respectively. The individual KOG are categorized as belonging to C to W.

a large tetrameric complex with an inward-complex functions in regulations of the activity of the channel by sensing cellular ATP levels (Wilkens 2015). The lipid transporter, ATP binding cassette, class A3 [ABCA3] plays a critical role in the regulation of pulmonary surfactant homeostasis (Beers and Mulugeta 2017). In reverse, the W class only involved in the upregulation, C1q and tumor necrosis factor related protein 3 [C1QTNF3] in human involved in several processes, including cellular triglyceride homeostasis, regulation of cytokine production, and negative regulation of gluconeogenesis (Wölfing et al. 2008). None of the assigned unigenes overlapped between up- and down-regulated genes (Table S5). Excluding unknown function as the S class, the high frequency was post-translational modification as the O class. Interestingly, some of unigenes involved in the same pathway, but having unique genes each (marked in dark grey on Table S5). For instance, in the O class, triacylglycerol [PNLIP], the peptidase S1 family [Tpsg1], and serine-type endopeptidase activity involved in proteolysis appeared in both up- and down-regulated, but the genes were unique. In the same way to the P class, the sodium ion transport [Nach] was represented in both up- and down-regulated, but showing unique genes associated with each.

Discussion

Researchers and environmental agencies often study the effects of microcystin on aquatic organisms to better understand the potential ecological impacts of harmful algal blooms and to develop strategies for managing and mitigating the risks associated with these toxins. Cyanobacteria can be regarded as a key taxon in many aquatic ecosystems. Especially given that increased nutrient inputs into lakes has led to more frequent cyanobacterial blooms (Dokulil and Teubner 2000) and those high cyanobacterial abundances negatively affect herbivorous zooplankton such as *Daphnia*. The strong impact of cyanobacteria on freshwater ecosystem services is in part due to the very low food quality of cyanobacteria for herbivorous zooplankton (von Elert et al. 2003), but it can at least partly also be assigned to cyanobacterial secondary metabolites, many of which are known to have detrimental effects on zooplankton (Rohrlack et al. 1999).

Bioinformatics tools were used to analyze the biological functions, pathways, and processes associated with the genes differentially expressed in response to MC-LR. This can provide insights into the molecular mechanisms of toxin response. In this study, we discussed the implications of the gene expression changes and their relevance to toxin response mechanisms, and our RNA-seq analysis provides a foundation for further research into molecular and cellular responses to cyanotoxin exposure using gene expression profiling. This may include investigating specific pathways, regulatory elements, or potential therapeutic targets. By pursuing these avenues, we can profile the expression of significant genes responding to cyanotoxin exposure in *Daphnia* or other organisms, contributing to a better understanding of how these organisms adapt to and cope with environmental stressors. Specifically, a novel biomarker may be developed in *Daphnia galeata* to detect organic pollution in groundwater.

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