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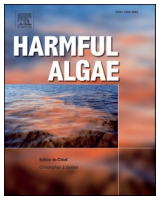

Can a shift in dominant species of *Microcystis* alter growth and reproduction of waterfleas?

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ABSTRACT

The bloom-forming species *Microcystis wesenbergii* and *M. aeruginosa* occur in many lakes globally, and may exhibit alternating blooms both spatially and temporally. As environmental changes increase, cyanobacteria bloom in more and more lakes and are often dominated by *M. wesenbergii*. The adverse impact of *M. aeruginosa* on co-existing organisms including zooplanktonic species has been well-studied, whereas studies of *M. wesenbergii* are limited. To compare effects of these two species on zooplankton, we explored effects of exudates from different strains of microcystin-producing *M. aeruginosa* (Ma905 and Ma526) and non-microcystin-producing *M. wesenbergii* (Mw908 and Mw929), on reproduction by the model zooplankter *Daphnia magna* in both chronic and acute exposure experiments. Specifically, we tested physiological, biochemical, molecular and transcriptomic characteristics of *D. magna* exposed to *Microcystis* exudates. We observed that body length and egg and offspring number of the daphnid increased in all treatments. Among the four strains tested, Ma526 enhanced the size of the first brood, as well as total egg and offspring number. *Microcystis* exudates stimulated expression of specific genes that induced ecdysone, juvenile hormone, triacylglycerol and vitellogenin biosynthesis, which, in turn, enhanced egg and offspring production of *D. magna*. Even though all strains of *Microcystis* affected growth and reproduction, large numbers of downregulated genes involving many essential pathways indicated that the Ma905 strain might contemporaneously induce damage in *D. magna*. Our study highlights the necessity of including *M. wesenbergii* into the ecological risk evaluation of cyanobacteria blooms, and emphasizes that consequences to zooplankton may not be clear-cut when assessments are based upon production of microcystins alone.

1. Introduction

Cyanobacteria blooms formed by genera including *Microcystis, Anabaena* (or *Dolichospermum*), *Aphanizomenon* and *Cylindrospermopsis* are intensifying worldwide and pose a major threat to aquatic ecosystem functioning (Hu et al., 2021; Qin et al., 2023; Zou et al., 2021). Species of the genus *Microcystis* are usually competitively dominant over other species owing to their buoyancy regulation, efficient nitrogen uptake, toxin(s) production and general resistance to predators (Harke et al., 2016; Oberholster et al., 2009; Sidelev et al., 2020). Although less well-known than its congener *M. aeruginosa, M. wesenbergii* is broadly distributed in Chinese lakes (Huo et al., 2021; Zhu et al., 2015), the Laurentian Great Lakes (Murphy et al., 2003), Japan (Ozawa et al., 2005), and throughout Europe (Via-Ordorika et al., 2004). Dominant species contributing to *Microcystis* blooms may shift as a result of adaptation to changing environmental conditions (Yang et al., 2023b; Yue et al., 2014; Zhai et al., 2013; Zhang et al., 2020). For example, *M. wesenbergii* and *M. aeruginosa* may exhibit alternating blooms in

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different seasons or depths in the water column associated with varying competitive abilities (Zhu et al., 2015). In Lake Taihu, China, *M. wesenbergii* dominated when water temperatures exceeded 27 °C, while *M. aeruginosa* dominated between 22 °C and 25 °C (Otten and Paerl, 2011). In Lake Dianchi, another eutrophic lake in China, *M. aeruginosa* has been largely replaced by *M. wesenbergii* coincident with a strong reduction in nutrient input to the lake (Shan et al., 2019). Consequences of such a shift in dominant bloom-forming species to co-occurring zooplankton are not clear.

Many *M. aeruginosa* strains are capable of producing microcystin and other toxins (Chen et al., 2021; Harke et al., 2016). This species is well-known globally for its adverse effects on aquatic species such as zooplankton (Lu et al., 2021; Nandini et al., 2021; Vo et al., 2020; Xu et al., 2023), fish (Cai et al., 2022; Du et al., 2019; Shahmohamadloo et al., 2021; Torres et al., 2023), and macrophytes (Jámbrik et al., 2011; Saqrane et al., 2007; Xu et al., 2016; Xu et al., 2015). On the other hand, *M. wesenbergii* does not produce microcystins (Huo et al., 2021; Xu et al., 2008), though it does produce other compounds, some of which may be toxic (Pires et al., 2011). Impacts of *M. wesenbergii* on co-occurring species have rarely been addressed, though African clawed frog (*Xenopus laevis*) embryos exposed to this cyanobacterium experienced elevated malformations (Dvořáková et al., 2002). Review of publications reveals a clear weakness in publications related to blooms of *M. wesenbergii* relative to those of *M. aeruginosa* (see in Fig. 1).

The relationship between cyanobacteria and zooplankton has become an important topic, particularly that associated with phytoplankton species successions (Sun et al., 2012). Toxic cyanobacteria are commonly believed to strongly inhibit some groups of zooplankton while others develop resistance and may co-exist with cyanobacteria (Sarnelle and Wilson, 2005). Previously, we observed that a *M. aeruginosa* strain (Ma905) isolated from Lake Dianchi increased egg production of *D. magna* when exposed in culture to field densities (2×10^4 and 2×10^5 cells/mL) of *Microcystis* through alteration of hormone and vitellogenin synthesis (Xu et al., 2019). Field data also showed a positive correlation between densities of cladocerans and *Microcystis* in Lake Dianchi in 2019–2020 (Li et al., unpublished data). Given that *M. wesenbergii* appears to be displacing *M. aeruginosa* in Lake Dianchi (Jiang et al., 2023; Li et al., 2020) and in other lakes (Imai et al., 2009; Yang et al., 2023b), a need exists to determine whether this shift has consequences for the zooplankton community.

Here we investigated the reproductive response of *D. magna* exposed to exudates of both *M. aeruginosa* and *M. wesenbergii*. Specifically, our comparative study explores the effects of each of two strains of two *Microcystis* species on *Daphnia*. Among these strains, one of *M. aeruginosa* (Ma905) and one of *M. wesenbergii* (Mw908) originated from Lake Dianchi. Although the non-toxic strain Mw908 was reported to be more sensitive to environmental changes than toxic strain Ma905 (Jia et al., 2024), ecological and adaptive differences between them remain relatively unknown. In this study, we hypothesized that when present at same cell density, toxicity of *M. aeruginosa* would exceed that of *M. wesenbergii* with respect to impact on *D. magna* reproduction, while less toxic species/strains would have lower physiological effects on this animal than highly toxic ones. We assessed potential toxicity using a suite of indicators including changes in reproduction characteristics, triacylglycerol (TAG) and vitellogenin (VTG) biosynthesis, production of hormones involved in VTG regulation, and transcriptome and expression of genes involved in reproduction in *D. magna*.

2. Material and methods

2.1. Algae cultivation and Microcystis exudates preparation

Cultures of *Microcystis aeruginosa* (FACHB-905 and FACHB-526, isolated from Lake Dianchi in Kunming and Lake Donghu in Wuhan, respectively). *Microcystis wesenbergii* strains FACHB-908 and FACHB-929 were isolated from Lake Dianchi in Kunming and Japan, respectively. FACHB-929 was initially obtained from exchange with National Institute of Environmental Research, Japan. *Chlorella vulgaris* (FACHB-32) was obtained from the Freshwater Algae Culture Collection of the Institution of Hydrobiology (FACHB-Collection) at the Chinese Academy of Sciences. *Microcystis* strains were grown axenically in a modified HGZ-145 nutrient solution, while *C. vulgaris* was cultured with COMBO medium at 25 ± 1 °C under 50 mmol quanta m^{-2} s^{-1} light with a 12:12h light–dark cycle and shaken twice daily manually (Xu et al., 2019).

Cultures of four *Microcystis* strains in the exponential growth stage were harvested after about three days at a density of approximately 4×10^5 cells /ml based on previous studies (Xu et al., 2019), and approximating *Microcystis* bloom density in Lake Dianchi (with the peak of 4.21×10^4 cells /mL in winter and the peak of 1.1×10^6 cells /mL in summer; (Ma et al., 2015a; Wu et al., 2016). Cultures were clarified by centrifugation at 6,000 *g* for 10 min at 4 °C following which supernatant was filtered via a glass fiber filter (0.22 μm; Xinya company, Shanghai) to obtain *Microcystis* exudate solutions. Exudates of each strain were freshly prepared and maintained at 4 °C prior to the start of exposure experiments (Xu et al., 2019). Prior to experimentation, we tested for the presence of microcystin in exudates of these *Microcystis* strains by testing MC-LR contents in these solutions, and confirmed that *M. wesenbergii* did not produce microcystins (see Table S1).

2.2. Daphnia magna cultivation

D. magna was originally sourced from Guangdong Laboratory

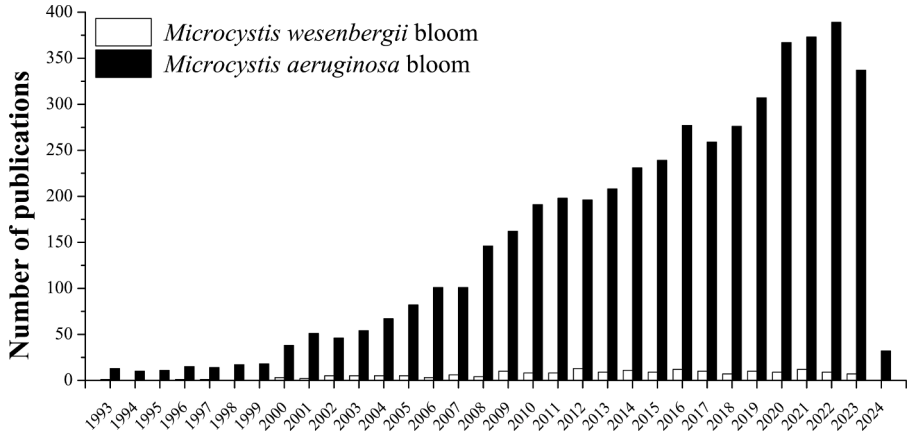

Fig. 1. Publications on *Microcystis aeruginosa* and *Microcystis wesenbergii* blooms over the past 30 years. Database search (Web of Science, Thomson Reuters) performed on 22 February 2024. Y axis: total number of publications by boolean search topic: *Microcystis aeruginosa* AND bloom or *Microcystis wesenbergii* AND bloom.

Animals Monitoring Institute (Guangzhou, China) and cultured in a constant 14:10 h light-dark cycle artificial climate chamber at 25 °C in lab. Cultures were maintained in glass beakers with 50 mL COMBO medium per animal and fed 5×10^5 cells /mL of *C. vulgaris* daily, with renewal of the medium twice a week. About 6 hours before the toxicity test, healthy mature adults were collected to produce the newborn (<24 h). Since females are entirely parthenogenetic, we utilized third generation offspring (<24 h).

Chronic and acute toxicity tests were performed by exposing *D. magna* to COMBO medium with *Microcystis* exudates of four strains, with a cell density of 4×10^5 cells/ml. The experimental groups were set as follows: 1) a negative control without any treatment (CK); 2) *M. aeruginosa* 905 strain (Ma905); 3) *M. aeruginosa* 526 strain (Ma526); 4) *M. wesenbergii* 908 strain (Mw908) and 5) *M. wesenbergii* 929 strain (Mw929).

2.3. Chronic exposure test

In the chronic exposure test, *D. magna* was exposed to the above solutions for 21 days. Exposure to 100 mL glass beakers with 50 mL of solution and one larva in each beaker was repeated 12 times. No more than two replicate animals died in the control group or the treatment groups during the 21-d exposure experiment, thus only live individuals (ten replicates for each treatment) were used in statistical analyses. Daphnids were fed daily (5×10^5 cells/mL^{-1} of *C. vulgaris*) and the test solution was refreshed daily. The survival, growth, development and reproduction of *D. magna* was monitored daily and the following parameters recorded: cumulative molts per individual, time and number of primiparous eggs and young, total eggs and larvae production each individual, and mortality of offspring. At the end of the experiment, we measured body length (from the top of the head to the base of the tail spine) of each female under the microscope with a micrometer.

2.4. Acute exposure test

Acute exposure for 96 h was performed in 500 mL glass beakers with 200 larvae per beaker and 400 mL culture solution (a mixture of *Microcystis* exudates and COMBO medium) for 12 replicates. *D. magna* was fed daily (5×10^5 cells mL^{-1} of *C. vulgaris*) and the test solution was refreshed daily. At the end of experiment, samples of four replicates of each treatment were collected to analyze the level of TAG, VTG, ecdysone and juvenile hormone (JH) in *D. magna* by using commercial ELISA kits (Jiangsu Kete, China) with a microtiter plate reader (Lab Systems Multiskan® MS, Finland) while the rest of the eight replicates were used for total RNA extraction.

2.5. Total RNA isolation and RNA-seq assay

Total RNA was isolated from daphnids samples in all treatment groups with RNAiso plus reagent (Takara, Japan) following the manufacturer's instructions. After isolation, RNA quality was tested by 1% agarose gel electrophoresis, and its concentration and purity measured using a SpectraMax® QuickDrop™ (Molecular Devices, USA); finally, RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) with the RNA Nano 6000 Assay Kit (Agilent Technologies). Construction and sequencing of daphnia cDNA libraries were performed at Novogene Company (Beijing, China; http://www.novogene.com).

Raw reads in fastq format were processed using inhouse Perl scripts. Clean reads were obtained by removing those containing adapters or poly-N, as well as those of low-quality, and used in subsequent analyses. The Q20, Q30, and GC contents of clean data were also calculated. Reference genome and gene model annotation files were downloaded from the latest published *Daphnia magna* genome (http://wfleabase.org/genome/Daphnia_magna/openaccess/). The index of the reference genome was constructed using bowtie 2.2.3 (Langmead and Salzberg, 2012), and paired-end clean reads were aligned to the reference genome using TopHat 2.0.12, which generated a database of splice junctions based on the gene model annotation file, providing better mapping results than other non-splice mapping tools.

The expression level of each gene was estimated by the number of fragments per kilobase of transcript sequence per million base pairs (FPKM), which was calculated based on the gene length and number of mapped reads (Trapnell et al., 2010). Differential expression analysis was performed using the DESeq R package (1.10.1). DESeq provides statistical routines for determining differential expression in digital gene expression data using based on a negative binomial distribution model. The resulting P values were adjusted using the Benjamini and Hochberg approach to control false discovery rate. Genes with an adjusted P - value <0.05 found by DESeq were assigned as differentially expressed. KOBAS 2.0 was used to test the statistical enrichment of differentially expressed genes (DEGs) in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Xie et al., 2011). The total number of genes involved in a pathway was counted, and then the P- value was calculated using a hypergeometric distribution (Mao et al., 2005).

2.6. Quantitative real-time PCR

Eight genes involved in reproduction were used to analyze gene expression of *D. magna* exposed to *Microcystis* exudates. β-actin gene was used as an internal standard. Primers of most genes were designed according to previous studies (Liu et al., 2017) while others were designed with Prime 5 software (see Table S2). Availability of these primers and the cDNA obtained was checked by conventional PCR before qPCR experiments. qPCR experiments were carried out using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) with an ABI QuantStudio™ 7 Flex analyzer (ABI, USA) to amplify genes. Gene expression results were calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). Each reaction was conducted in triplicate to ensure reproducibility of results.

2.7. Statistical analysis

Statistical analysis was performed with SPSS Statistics 25.0 software after normality and homoscedasticity of data were checked. One-way analysis of variance (ANOVA) and LSD post-hoc tests were used to evaluate the significance of differences between exposure treatments and the control. Two factor (species and strain) repeated-measures ANOVA and subsequent post-hoc tests were used to evaluate the significance of differences among treatments. Main effect analysis was used to compare the contribution of *Microcystis* species and strain to all tested parameter responses by *D. magna*. The limit of statistical significance was set as $P < 0.05$. Experimental data was reported as mean ± standard error.

3. Results

3.1. Growth and reproduction in D. magna exposed to Microcystis exudates

Primiparous eggs of daphnids in all treatments occurred on the 5th day. The number of primiparous eggs in Ma526 treatment was 25 % greater than that in the control (Fig. 2A and Table S3, $P < 0.05$). However, there was no difference between any other Microcystis treatment and the control ($P > 0.05$). Overall, the number of primiparous eggs was affected by *Microcystis* species but not by strain (Table 1).

The total number of eggs produced by *D. magna* over 21 days increased significantly in both *M. aeruginosa* MaE treatments well as in the Mw908 treatment by 17 %, 13 % and 16 %, respectively (Fig. 2B and Table S3, $P < 0.05$), though the change in the Mw929 treatment was not significant ($P > 0.05$). Neither main effect - strain and species - of *Microcystis,* nor the interaction of the two, affected total number of eggs

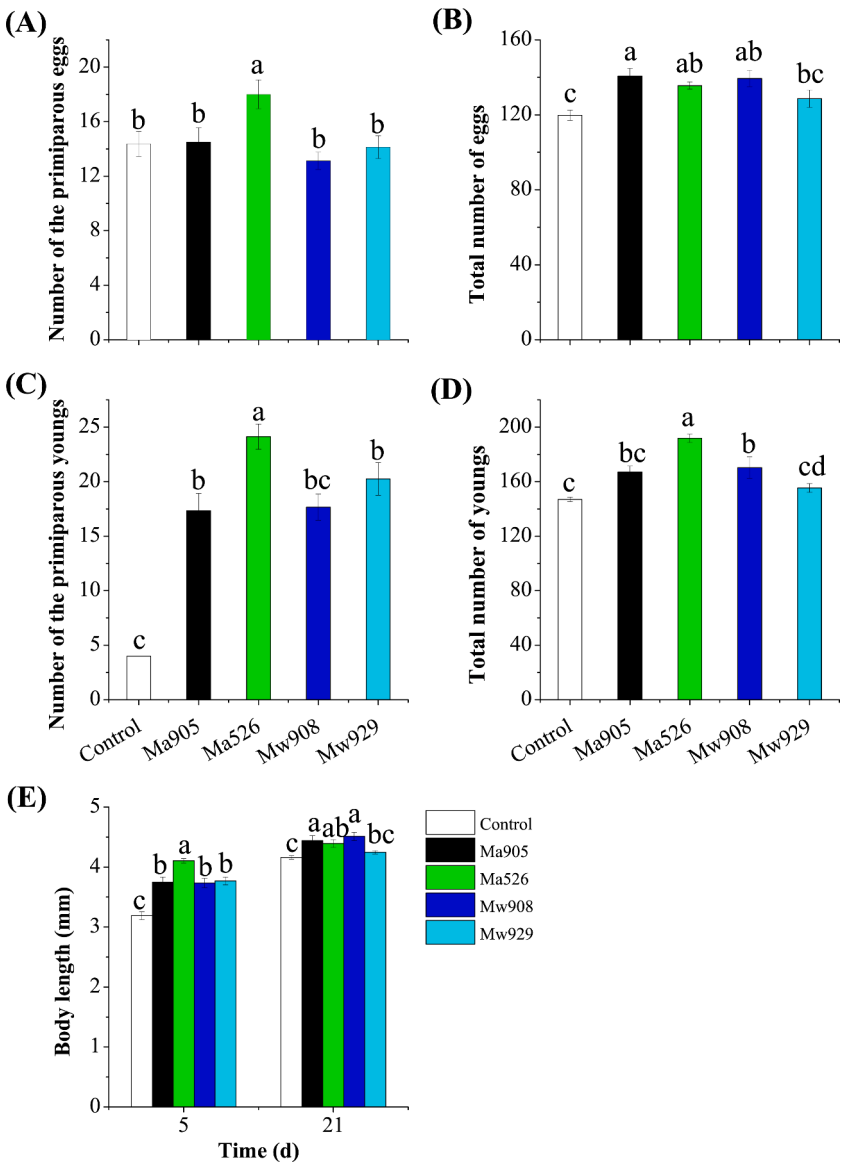

Fig. 2. Mean (+/- SE) growth and reproduction of *Daphnia magna* during 21-day exposure to *Microcystis* exudates (Ma905 and Ma526: treatment with exudates of *M. aeruginosa* FACHB-905 and FACHB-526 strains, respectively; Mw908 and Mw929: treatment with exudates of *M. wesenbergii* FACHB-908 and FACHB-929 strains, respectively; Control = not exposed to *Microcystis* exudates. (A) Number of the primiparous eggs produced per female; (B) Total number of total eggs produced per female after 21 days; (C) Total number of the primiparous neonates produced per female; (D) Total number of offspring produced per female after 21 days; (E) Body length of female adults at day 5 and 21. Different letters above bars indicate a significant difference between treatments using one-way ANOVA (LSD, $P < 0.05$).

produced (Table 1, $P > 0.05$).

The number of primiparous young in all *Microcystis* exudate treatments was significantly higher than that of the control (Fig. 2C, $P < 0.05$). However, there was no difference between Ma905 and either *M. wesenbergii* treatment ($P > 0.05$). The species and strain of *Microcystis* but not the interaction of the two had pronounced effects on number of primiparous young of *D. magna* (Table 1).

The total number of young produced by daphnids in Ma905 and Ma526 and Mw908 treatments were each significantly higher than that in the control after 21 days. The Ma526 treatment responded most, with total larvae produced up 30 % (Fig. 2D and Table S3, $P < 0.05$). Species of *Microcystis* and the interaction of species and strain of *Microcystis* had significant effects on total number of young produced by *D. magna*, though strain had no effect (Table 1).

Body length of adult *Daphnia* was significantly longer after both 5- and 21-day exposure to both *Microcystis* species (Fig. 2E, $P < 0.05$), except for the Mw929 group on the 21st day ($P > 0.05$). Body length in the Ma526 treatment increased by 28.7 % after 5 days, the largest observed increase. The largest increase after 21 days switched to the Ma905 and Mw908 treatments, which experienced increases of 6.7 % and 8.3 %, respectively (Table S3). We observed significant main effects of both strain and species of *Microcystis* as well as their interaction on body length at day 5. But by day 21, only *Microcystis* strain had noticeable impacts on body length of *D. magna* (Table 1).

3.2. Physiological parameters of *D. magna* exposed to *Microcystis* exudates

The concentration of TAG in *D. magna* increased significantly in all exposure treatments (Fig. 3A, $P < 0.05$) excepting the Mw929 group ($P > 0.05$). The Ma905 treatment had significantly higher TAG levels than all other treatments and 43 % more than the control (Table S3). We also observed significant positive correlations between the concentration of TAG and total eggs produced, and between the concentration of TAG and body length on day 21 (Fig. 4).

Concentrations of VTG and ecdysone were significantly higher in all

Table 1
Results of two-way ANOVA analysis performed on all tested parameters of *D. magna* treated with different *Microcystis* exudates.

Source Parameters	Strain				Species				Strain × Species			
	df	Mean square	F	P [a]	df	Mean square	F	P [a]	df	Mean square	F	P [a]
Number of primiparous eggs	1	40.500	4.109	NS	1	55.125	16.162	**	1	12.500	1.268	NS
Total number of eggs	1	496.125	3.855	NS	1	136.125	1.143	NS	1	66.125	0.514	NS
Number of primiparous young	1	427.781	8.077	*	1	830.281	8.940	*	1	116.281	2.196	NS
Total number of young	1	190.125	0.999	NS	1	2211.125	10.647	**	1	3120.500	16.397	**
Body length at the 5th day	1	307966.513	10.581	**	1	244070.057	5.220	*	1	208166.423	7.152	*
Body length at the 21th day	1	197124.468	4.930	*	1	12096.031	0.502	NS	1	92839.995	2.322	NS
TAG	1	4030.444	338.573	***	1	327.498	17.603	*	1	292.753	24.592	**
Vitellogenin	1	4.543	8.204	*	1	117.010	552.239	***	1	90.212	162.891	***
Ecdysone	1	1.311	94.534	**	1	2.828	91.909	**	1	0.280	20.209	*
Juvenile hormone	1	21.778	25.851	**	1	81.146	60.581	**	1	258.433	306.771	***
Vtg1	1	0.012	0.162	NS	1	0.058	0.974	NS	1	0.260	3.547	NS
Vtg2	1	0.015	0.355	NS	1	0.086	2.976	NS	1	0.755	18.129	*
EcR	1	0.011	0.154	NS	1	0.809	41.411	**	1	1.020	14.767	*
USP	1	0.055	0.748	NS	1	0.169	1.656	NS	1	2.557	34.557	**
SRC	1	0.003	0.066	NS	1	0.411	8.268	*	1	1.118	22.949	**
Met	1	2.366	44.774	**	1	0.000	0.000	NS	1	0.421	7.972	*
CYP314	1	0.022	0.195	NS	1	0.091	1.098	NS	1	0.503	4.569	NS
17β-HSD	1	0.286	1.175	NS	1	3.161	76.754	**	1	0.037	0.153	NS

[a] By analysis of variance.
* $P < 0.05$;
** $P < 0.01$;
*** $P < 0.001$; NS, $P \geq 0.05$.

exposure treatments versus those in the control after 96 h exposure. For example, we observed elevated values for maximum VTG and ecdysone concentration in Mw908 treatment by 69.0 % and 31.5 %, respectively (Fig. 3B~C and Table S3, $P < 0.05$). We also observed a positive correlation between ecdysone concentration and VTG concentration after 96-hour exposure (Fig. 4).

The concentration of juvenile hormone was significantly elevated in Ma905, Mw908 and Mw929 treatments relative to the control (Fig. 3D, $P < 0.05$). Juvenile hormone of the Mw929 treatment achieved the highest observed concentration of all treatments, achieving a level 45 % higher than the control (Table S3).

Microcystis strain and species and their interaction had pronounced effects on concentration of TAG, VTG, ecdysone and juvenile hormone of *D. magna* (Table 1).

3.3. Transcriptomic Response of *D. magna* to *Microcystis* exudates

Exudates of Ma905 caused the largest change in differentially expressed genes (DEGs, 2576) in *D. magna*, of which 1126 and 1450 genes were respectively up-regulated and down-regulated relative to the control. The number of DEGs induced by exudates of the two *M. wesenbergii* strains was also higher than those of *M. aeruginosa* strains. Mw908 differentially expressed 1556 genes, 810 up-expressed and 746 down-expressed genes. A total of 1844 genes were differentially expressed in the Mw929 treatment, of which 943 genes were up-regulated and 901 genes down-regulated. The number of DEGs induced was lowest for Ma526, 510 in total, of which 269 were up-expressed and 241 down-expressed (Fig. S1A).

In total, 109 significant GO terms were induced by exposure to *Microcystis* exudates (Fig. 5). Among these, 3, 3, 19 and 13 significantly enriched upregulated GO terms were observed in Ma905, Ma526, Mw908 and Mw929 treatments, respectively, while there were 72, 4, 9 and 2 significantly enriched GO terms for comparable downregulated DEGs (Table S4). Only a small fraction of significantly enriched GO terms was shared by different treatments. For example, seven GO terms were shared by Mw908- and Mw929- upregulated DEGs, while only three were shared by Mw908- upregulated DEGs and Ma905-downregulated DEGs (Fig. 5). Three significantly upregulated GO terms were associated with chitin synthesis. Two of them, chitin binding and chitin metabolic process, were shared between the Mw908-responsive and Mw929-responsive DEGs, whereas one GO term (structural constituent of cuticle), including 61 DEGs, was limited to the Mw908 treatment. Three significantly upregulated GO terms involved in lipid transport and localization were limited to Mw908-responsive DEGs. A large number of downregulated DEGs were enriched in most GO terms (e.g., oxidoreductase activity and oxidation-reduction process) of the Ma905 treatment, while very few significant GO terms were observed in Ma526-responsive DEGs (Fig. 5). We observed that Chitin GO terms was significantly and positively correlated with VTG concentration, ecdysone concentration and lipid GO terms (Fig. 4).

According to the significantly enriched KEGG pathway, DEGs of *D. magna* exhibited the most pronounced response to Ma905 exudate treatment versus other treatments (Fig. 6). The upregulation of autophagy - animal pathway and down-regulated expression of ribosome, protein processing in endoplasmic reticulum, RNA transport, and oxidative phosphorylation were the major pathways affected by Ma905 exudate. Ma526 exudates upregulated expression of metabolic pathways including oxidative phosphorylation, metabolism of xenobiotics by cytochrome P450, and drug metabolism - cytochrome P450 in *D. magna*. Mw908 exudates upregulated expression of oxidative phosphorylation of *D. magna* and downregulated expression of spliceosome and glycosphingolipid biosynthesis - lacto and neolacto series pathways. Mw929 induced only one upregulated metabolic pathway in *D. magna* - oxidative phosphorylation. Oxidative phosphorylation was significantly differentially expressed in the four treatments, though it was down-regulated in Ma905 and up-regulated in the other treatments (Fig. 6).

3.4. Gene expression of *D. magna* exposed to *Microcystis* exudates

We observed no significant difference in relative mRNA expression of *Vtg1* in *D. magna* after 96 h among the control and the *Microcystis* treatments (Fig. 7A, $P > 0.05$). Strain and species of *Microcystis* as well as their interaction did not have significant effects on relative expression of *Vtg1* and *CYP314* (Table 1). We observed significant negative correlations between *Vtg1* expression and juvenile hormone concentration (Fig. 4). Expression of *Vtg2* and *USP* increased in all treatments, except for Mw929, though only the Ma526 treatment and the Mw908 treatment were significantly different from the control (Fig. 7B and 7D, $P < 0.05$). The interaction of *Microcystis* strain and species had significant effects on relative expression of *Vtg2* and *USP* (Table 1). We also observed positive correlations between relative mRNA expression of *Vtg2* and total offspring produced after 21 days in the chronic exposure experiment,

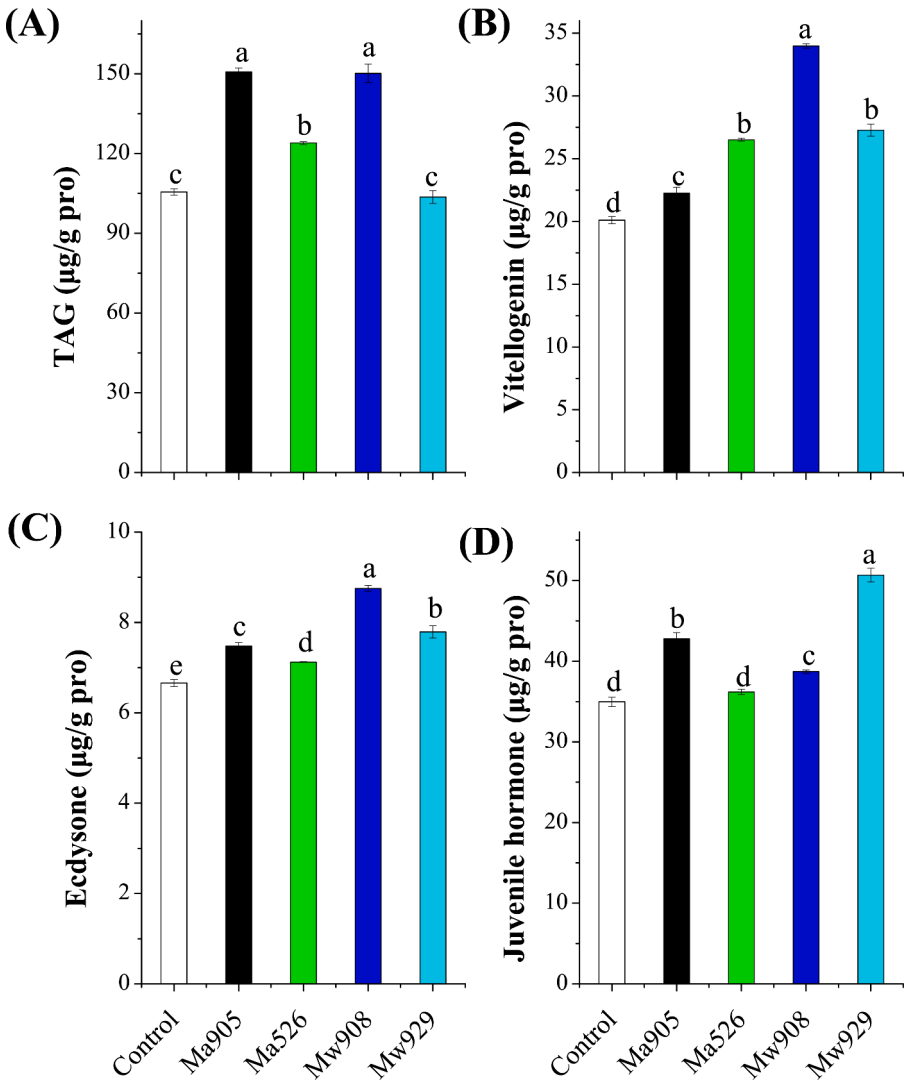

Fig. 3. Mean (+/- SE) physiological parameters of *Daphnia magna* after 96-hours exposure to *Microcystis* exudates. (A) Concentration of triacylglycerol (TAG); (B) Concentration of vitellogenin; (C) Concentration of ecdysone; (D) Concentration of juvenile hormone. Significant differences between treatments are indicated by different letters.

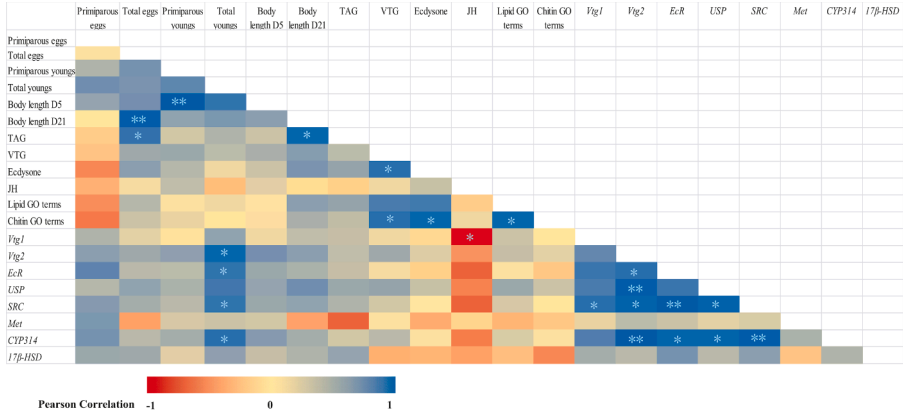

Fig. 4. Heatmap of Pearson's product moment correlations between tested parameters using all treatments and control data. Asterisk indicates a significant correlation between the two parameters (*, $P < 0.05$; **, $P< 0.01$).

and between expression of *USP* and *Vtg2* after 96 h in the acute trails (Fig. 4). Only the Ma526 treatment significantly up-regulated expression of *EcR* and *SRC* relative to the control (Fig. 7C and E, $P < 0.05$). *Microcystis* species and the interaction of strain and species significantly affected relative expression of *EcR* and *SRC* (Table 1). *EcR* expression was positively correlated to total offspring produced and the expression

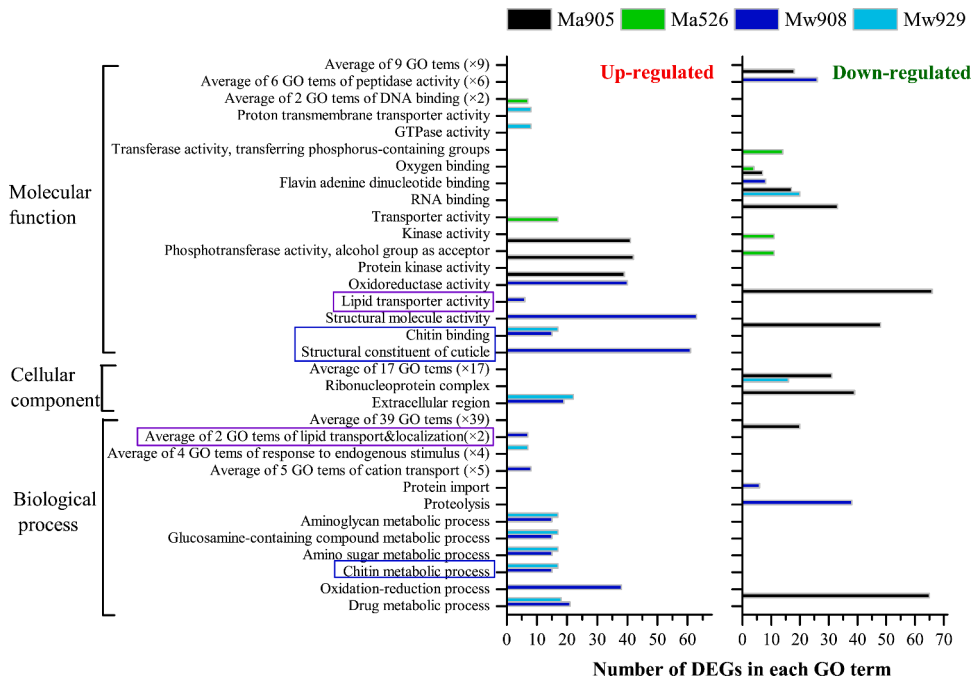

Fig. 5. GO significant enrichment analysis from upregulated and downregulated *Microcystis* exudate-responsive DEGs (109 GO terms in total). GO terms framed by purple and blue colors represent significant enrichment DEGs in lipid metabolism and chitin synthesis, respectively.

of *Vtg2*. We also observed that expression of *SRC* was significantly and positively correlated with total offspring produced and expression of *Vtg1, Vtg2*, and *EcR* (Fig. 4).

We observed no significant difference in expression of *Met* in *D. magna* between the control and any *Microcystis* MaE treatment (Fig. 7F, $P > 0.05$), though Ma905 and Mw908 were significantly lower than the Ma526 treatment ($P < 0.05$). We observed pronounced effects of both *Microcystis* strain and strain*species interaction on relative expression of *Met* (Table 1). Expression of *CYP314* increased in Ma526 and Mw908 treatments, though only the former differed from the Mw929 treatment (Fig. 7G, $P < 0.05$). *CYP314* expression was positively correlated with total offspring produced, and expression of *Vtg2, ECR, USP* and *SRC* (Fig. 4). The relative expression of *17β-HSD* was significantly higher in the two *M. aeruginosa* exudates treatments than in the control group (Fig. 7H, $P < 0.05$). We also found upregulation of *17β-HSD* in the Ma905 treatment in RNA-seq analysis (Fig. 7H). Overall, only *Microcystis* species affected expression of *17β-HSD* (Table 1).

4. Discussion

Previous studies observed that *M. aeruginosa* induced reproductive disorder in aquatic animals, especially daphnids (Isanta-Navarro et al., 2021; Xu et al., 2019). As it was unclear whether impact would vary by *Microcystis* species or strain, here we compared daphnids' response across *Microcystis* species and strains. We observed that except for the Mw929 treatment, body length and egg and offspring number of *D. magna* all increased if exposed to different *Microcystis* strains; additionally, most of the tested physiological and molecular parameters responded positively to exudates of those strains. Given that significant correlations exist among most of the tested parameters of *D. magna* affected by *Microcystis* exudates (e.g., total eggs with body length and triacilglicerol (TAG) production; Fig. 4), as well as previous observations using only strain Ma905 (Xu et al., 2019), we may hypothesize on general mechanisms by which *Microcystis* exudates obtained from cultures at a density of 4×10^5 cells /ml promote reproduction in *D. magna*. *Microcystis* exudates enhanced activity of *17β-HSD* by stimulating expression of its coding gene. Subsequently, this enzyme promoted biosynthesis of ecdysone. Production of JH was also enhanced. In turn, increased ecdysone and JH had greater efficiency binding to their receptors, which collectively induced vitellogenesis and (TAG) accumulation. The ecdysone receptor (*EcR*) activated ecdysone production by forming a heterodimer with ultraspiracle (*USP*) while JH bound to its two receptors (*SRC* & *Met*). At the same time, the expression of genes *Vtg1* and/or *Vtg2* were also enhanced by *Microcystis* exudates exposure, resulting in higher VTG production. The massive VTG increase could, in turn, provide nutritional support for developing eggs. Consequently, affected adult *D. magna* would be expected to produce more offspring. As more lipids (e.g. TAG) accumulated, more energy would be available for egg production and greater body length. In addition, increased ecdysone might help chitin synthesis, which is essential for body elongation (Gong et al., 2022; Wang et al., 2023; Xiong et al., 2022).

We observed that *CYP314, Vtg1, Met* genes expression were not affected by exposure to exudates of any strain (Fig. 7). These findings are consistent with previous observation with Ma905 exudates cultured at the same cell density (Xu et al., 2019). However, *CYP314* had a significant positive correlation with offspring production as well as expression of the genes *Vtg2, EcR, USP* and *SRC*, which can be explained by its function in *D. magna*'s molting and reproduction by converting ecdysone to 20E, an active form (Baldwin et al., 2009; Rewitz and Gilbert, 2008; Rewitz et al., 2007). Expression of *Vtg1* may response to lower doses because significantly increased expression of this gene was observed in *D. magna* when exposed to the lower concentration of Ma905 exudates (Xu et al., 2019). We propose that the gene *Met* might act in similar mode as *Vtg1*, though we haven't tested it with *Microcystis* exudates at low cell density.

Besides enhancement of hormone and vitellogenesis production, we also detected TAG accumulation in *D. magna* after 96h exposure to *Microcystis* exudates, just before females ovulated. TAG content was significantly increased in all treatments except for Mw929. Principal energy-providing fats in animals are stored as TAG, and lipids including TAG accumulate in large quantities for use in ovulation and early embryogenesis (Buszczak et al., 2002; Dunning et al., 2010; Parra-Peralbo and Culi, 2011; Tennessen et al., 2014; Wood et al., 2008). Crustaceans store energy mainly as TAG, which is used for survival and reproduction, and lipid storage is positively correlated with egg number (Tessier and Goulden, 1982). Specifically, female *D. magna* individuals

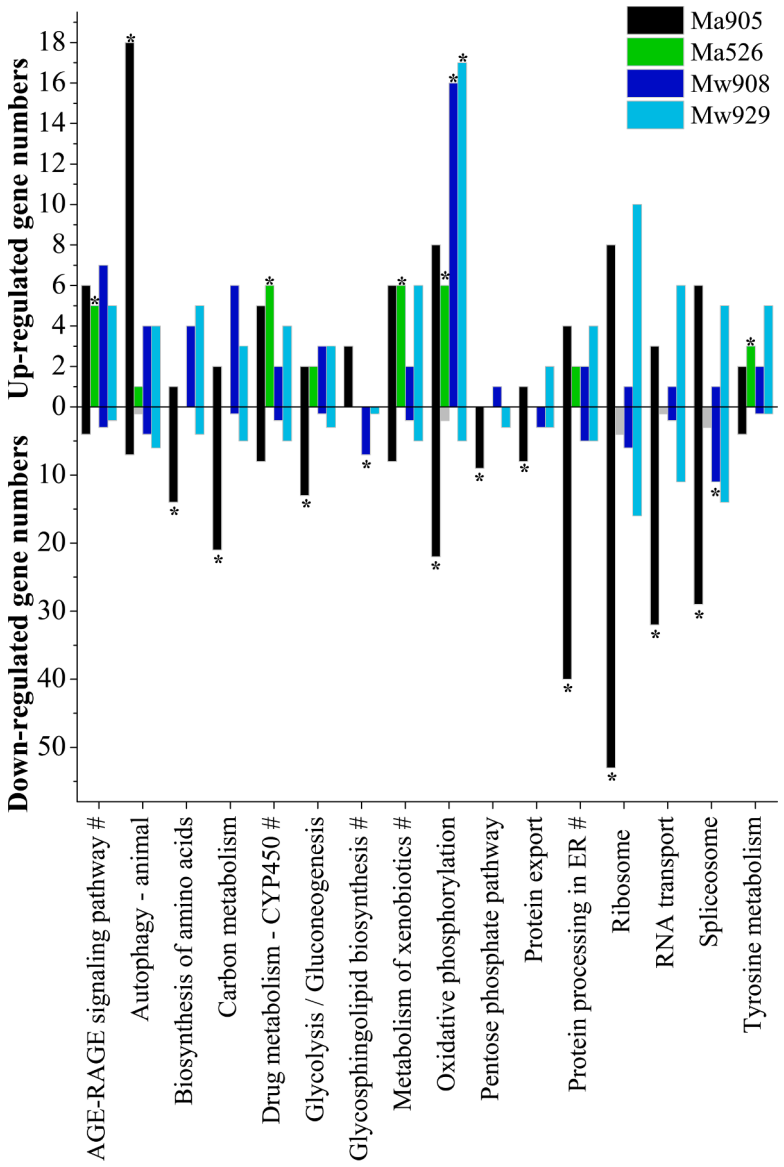

Fig. 6. Significantly enriched pathways in *Daphnia magna* related to up/downregulated DEGs responsible for different *Microcystis* exudate exposures (x-axis pathway names followed by hash symbol are omitted from AGE-RAGE signaling pathway in diabetic complications, Drug metabolism - cytochrome P450, Glycosphingolipid biosynthesis - lacto and neolacto series, Metabolism of xenobiotics by cytochrome P450, Protein processing in endoplasmic reticulum, respectively. Asterisks above/below bars indicate significantly enriched pathways using a corrected *P* value).

accumulated a large amount of glycerolipids and glycerophospholipids, especially TAG, from their algal diet for egg formation (Fuertes et al., 2018). Reproductive female daphnids feeding on algae accumulate fatty acids and TAG, which are then used during molting and egg formation (Goulden and Place, 1990). Consistent with this, we observed significant positive correlations between daphnia TAG content and both total eggs and body length (Fig. 4). These results highlight the importance of TAG to the reproductive process of *D. magna*.

Among all *Microcystis* strains, Mw908 was the most capable at stimulating most of the tested parameters and it had the most positive responses in transcriptomic level, especially with regard to enriched GO terms involved in chitin and lipid synthesis. Mw929 had two significantly enriched GO terms for chitin synthesis containing 34 DEGs. Our study provides abundant evidence that *Microcystis* exudates may affect growth and reproduction independent of species and strain, acting in a general approach with slight differences.

M. wesenbergii, a microcystin-free species, caused similar effects on *D. magna* as the microcystin-producing species *M. aeruginosa*. Although we were not able to identify the specific compounds in *Microcystis* exudates responsible for observed effects, we confirmed that effects caused by different strains were independent of microcystin production. Smutná et al. (2014) also indicated that unknown metabolites other than microcystins were likely responsible for toxic effects on *D. magna* reproduction. Also, toxicity to cladocerans of substances than MCs released from *M. aeruginosa* has been observed (Carmichael, 1992; Semyalo et al., 2009; Yasuno et al., 1998).

Mw908 and Ma905 strains in this study were isolated from Lake Dianchi and performed very similar to each other in affecting reproduction of *D. magna*. These findings suggest that replacement of dominant species in blooms from *M. aeruginosa* to *M. wesenbergii* in this lake may have similar effects on co-existing zooplankton. Despite its inability to produce microcystins, *M. wesenbergii*, being a mucilaginous alga, may be a bad food source for aquatic organisms (Boegehold et al., 2019; Marten, 2007). Mucilage may resist predator digestion of *M. wesenbergii* (Ma et al., 2015b; Reynolds, 2007). For example, *Chironomus pallidivittatus* was harmed by toxins produced by Ma905, while the

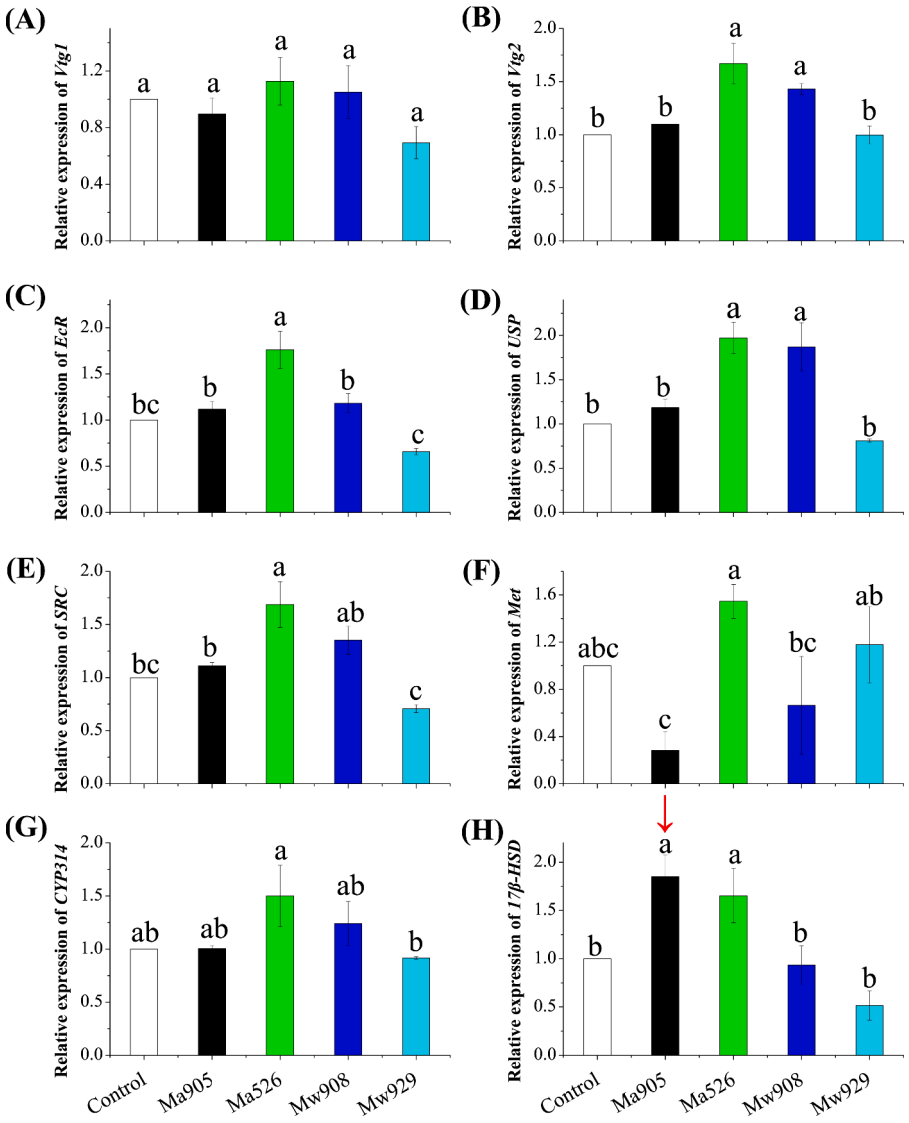

Fig. 7. Mean (+/- S.E.) relative expression of mRNA in *Daphnia magna* genes related to reproduction ((A) *Vtg1*, (B) *Vtg2,* (C) *EcR*, (D) *USP*, (E) *SRC*, (F) *Met*, (G) *CYP314* and (H) *17β-HSD*) after a 96-hours exposure to MaE *Microcystis* exudates exposure (Control group, not exposed; Ma905 and Ma526: treatment with exudates from *M. aeruginosa* FACHB-905 and FACHB-526 strains, respectively; Mw908 and Mw929: treatment with exudates from *M. wesenbergii* FACHB-908 and FACHB-929 strains, respectively. Different letters above bars indicate a significant difference between treatments using one-way ANOVA (LSD, $P < 0.05$). Red arrow indicates upregulation of *17β-HSD* in RNA-seq analysis.

non-microcystin-producing strain Mw908 was indigestible to larvae (Cai et al., 2021). Yang et al. (2023a) found that *M. wesenbergii* and *M. aeruginosa* isolated from Lake Taihu shared common and specific characteristics for colony formation at different growth phase, which contributed to their respective competitive advantage. Therefore, both microcystin-producing and microcystin-free cyanobacterial species/-strains have important ecological consequences for co-occurring zooplankton.

Our study demonstrated that both species and strains of *Microcystis* release bioactive compounds that induce reproductive changes in *Daphnia*. It provides insights into potential risks of cyanobacterial blooms via reproductive disorders based upon *in vivo* trials. However, the role of cyanobacteria in reproductive disorders under natural conditions warrants further attention. In particular, studies on the chemical nature, modes of action, and consequences of chemical exposure to co-occurring species are needed. In addition, physiological factors such as target animal species' tolerance and population biology, and roles of physical drivers must be considered when investigating effects of cyanobacterial blooms.

5. Conclusion

In this study, we assessed the effects of exudates from four strains of *Microcystis* on the reproductive, physiological, and molecular characteristics of *D. magna*. *Microcystis* exudates significantly promoted growth and reproductive output in *D. magna*. *Microcystis* exudates enhanced *D. magna* reproduction by stimulating biosynthesis of ecdysone, JH and TAG, and by increasing VTG biosynthesis via up-regulation of expression of specific genes. Overall, *M. wesenbergii* appears to have a similar impact on reproduction of co-existing zooplankton as *M. aeruginosa*. Therefore, a shift in dominant blooming species from *M. aeruginosa* to *M. wesenbergii* in lakes may not remove the potential disturbance in reproduction of co-existing animals.

CRediT authorship contribution statement

Tao Wang: Writing – original draft, Validation, Methodology, Data curation. **Daochun Xu:** Writing – original draft, Visualization, Validation, Data curation. **Xuexiu Chang:** Writing – review & editing,

Supervision, Funding acquisition, Conceptualization. **Hugh J. MacIsaac:** Writing – review & editing, Formal analysis. **Jingjing Li:** Software, Resources, Methodology. **Jun Xu:** Methodology. **Jinlong Zhang:** Visualization, Validation. **Hongyan Zhang:** Validation. **Yuan Zhou:** Validation, Methodology. **Runbing Xu:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Runbing Xu reports financial support was provided by National Natural Science Foundation of China. Xuexiu Chang reports financial support was provided by National Natural Science Foundation of China. Runbing Xu reports financial support was provided by Yunnan Applied Basic Research Project. Xuexiu Chang reports financial support was provided by Yunnan Applied Basic Research Project. Xuexiu Chang reports financial support was provided by Yunnan Provincial Department of Education. Xuexiu Chang reports financial support was provided by Natural Sciences and Engineering Research Council of Canada. Hugh J. MacIsaac reports financial support was provided by Natural Sciences and Engineering Research Council of Canada. Hugh J. MacIsaac reports financial support was provided by Canada Research Chair. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2024.102657.

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