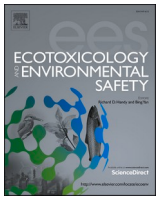
Ecotoxicology and Environmental Safety 258 (2023) 114967

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Insights into the mechanisms of within-species variation in sensitivity to chemicals: A case study using daphnids exposed to CMIT/MIT biocide

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ARTICLE INFO

Edited by Professor Bing Yan

Keywords:
Intraspecific variation
CMIT/MIT
Daphnia
Epigenetics
Proteomics

ABSTRACT

Living organisms adapt to their environment, and this adaptive response to environmental changes is influenced by both genomic and epigenomic components. As adaptation underpins tolerance to stressors, it is crucial to consider biological adaptation in evaluating the adverse outcomes of environmental chemicals, such as biocides. Daphnid studies have revealed differences in sensitivity to environmental chemicals between conspecific populations or clones, as well as between species. This study aimed to identify whether sensitivity to chemicals is subject to intraspecific variation, and whether this sensitivity depends on the genetic and epigenetic backgrounds of the daphnid population. We used an integrative approach to assess the comparative toxicity of a mixture of 5-chloro-2-methyl-4-isothiazoline-3-one and 2-methyl-4-isothiazolin-3-one (CMIT/MIT), a commonly used isothiazolinone biocide, by measuring mortality, reproduction, physiological traits, global DNA methylation, and proteomic expression at the species and strain levels. The results showed that the variation in sensitivity to CMIT/MIT between conspecific strains (*Daphnia pulex*; DPR vs. DPA strains) could exceed that observed between congeneric species (*D. magna* vs. *D. pulex* DPR strain). Under the control conditions, DPR (the strain most sensitive to CMIT/MIT) was characterized by a larger body size, a higher heart rate, and a higher level of global DNA methylation compared to its counterpart (DPA), and proteome profiles differed between the two strains. Particularly, the study identified strain-specific epigenetic and proteomic responses to LC20 of CMIT/MIT, demonstrating putative critical proteins and biological pathways associated with the observed differences in phenotype and sensitivity to CMIT/MIT. Downregulation of certain proteins (e.g., SAM synthase, GSTs, hemoglobin, and cuticle proteins) and DNA hypomethylation can be proposed as key events (KEs) of adverse outcome pathway (AOP) for isothiazolinone toxicity. Our findings indicate that both genetic variations and epigenetic modifications can lead to intraspecific variation in sensitivity to chemicals, and this variation should be considered in the ecological risk assessment framework for chemical substances. We suggest conducting further analysis on methylated gene regions and observing transgenerational effects to verify the role of crosstalk between genetic and epigenetic factors in phenotypic and protein expressions.
Data availability: Proteomic data is available in supplementary materials.

1. Introduction

Living organisms are continuously exposed to a variety of environmental pressures caused by global change and human activities. Chemical pollution, one of the most potent anthropogenic stressors, degrades the ecological integrity of freshwater ecosystems (Malaj et al., 2014) and involves complex mixtures of components, often present at low concentrations and from which the main drivers of toxicity are difficult to assess (Backhaus and Faust, 2012). Among such substances, biocides are of particular concern, as they are effective on target organisms and potentially toxic to other aquatic organisms if released into the environment. Isothiazolinone biocides are heterocyclic compounds and are widely used in various aqueous-based industrial or domestic products to control the growth of microbial organisms (Alvarez-Rivera

Abbreviation: DMI, *Daphnia magna* INERIS clone A; DPR, *Daphnia plex* Rennes; DPA, *Daphnia pulex* Alsace; CMIT/MIT, The mixture of 5-chloro-2-methyl-4-isothiazoline-3-one and 2-methyl-4-isothiazolin-3-one in the ratio of 3:1.

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https://doi.org/10.1016/j.ecoenv.2023.114967
Received 31 January 2023; Received in revised form 24 April 2023; Accepted 28 April 2023
Available online 9 May 2023
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et al., 2012). Among them, the mixture of 5-chloro-2-methyl-4-isothiazoline-3-one and 2-methyl-4-isothiazolin-3-one in the ratio of 3:1 (CMIT/MIT) is broadly used in water-based paints and various types of consumer products such as cosmetics, hair and skin-care products, and cleaning agents (Alvarez-Rivera et al., 2012; Thomsen et al., 2018). Accordingly, CMIT and MIT have received increasing attention due to their widespread use in urban areas, transfer into the environment, and the hazards they pose to aquatic ecosystems (Paijens et al., 2019). However, ecotoxicological information on CMIT/MIT is relatively scarce compared to other environmental biocides. CMIT and MIT have been detected in aquatic environments (Baranowska and Wojciechowska, 2013; Nowak et al., 2020), yet their ecotoxicity is still not realistically evaluated.

Epigenetics, defined as the study of changes in gene function that are mitotically and/or meiotically heritable without a change in DNA sequence (Bird, 2007), plays a pivotal role in gene expression regulatory functions, development, phenotypic plasticity, and genome integrity (Ashe et al., 2021). As triggered by both intrinsic and external signals, epigenetic modifications can be induced by environmental stressors, including chemical toxicants (Chatterjee et al., 2018). When such modifications occur during embryogenesis and affect the stem cells, epigenomic alterations can spread to all cell types of the developing fetus and adult, and in some cases, be transmitted to the subsequent generation(s) through the organism's germ cells along with genetic information (Nilsson et al., 2022; Schmid et al., 2018). Thus, epigenetic mechanisms can contribute to population adaptation in combination with microevolutionary processes involving genetic variation and natural selection (Ashe et al., 2021; Jeremias et al., 2018) Therefore, it is a matter to be considered that adaptive processes, whether genetically or/and epigenetically derived, are incorporated in the assessment of adverse outcomes of environmental contaminants (Brander et al., 2017; Reinikainen et al., 1998).

Daphnids are commonly used as models in ecology and ecotoxicology because of their status as primary consumers and resource in aquatic food webs, their short generation time, and high sensitivity to environmental changes. Furthermore, daphnids are particularly relevant as epigenetic models due to their clonal mode of reproduction, which allows the proper separation of phenotypic variance into genetic and non-genetic components (between- and within-clone variation, respectively) (Jeremias et al., 2018; Wojewodzic and Beaton, 2017). Additionally, many studies reported differences in sensitivity to environmental stressors and chemicals between daphnid species or between conspecific populations or clones (Haap and Köhler, 2009; Reinikainen et al., 1998; Vanvelk et al., 2021). For example, an investigation of seven *D. magna* clones from different geographical regions highlighted genetic divergence in tolerance to cadmium, presumably resulting from local adaptation (Haap and Köhler, 2009).

In the present study, we hypothesized that sensitivity to chemical exposure is subject to within-species variation, and that this sensitivity depends on the genetic and epigenetic backgrounds of the studied population. To test this, we investigated inter and intraspecific variation in daphnid sensitivity to CMIT/MIT using an integrative approach involving phenotypic and molecular responses. We first compared the acute and chronic toxicity of CMIT/MIT to daphnid strains stemming from two different species (*Daphnia magna* vs. *Daphnia pulex*) and populations (two *D. pulex* origins). Then, we focused on *D. pulex* strains and studied intraspecific variation in susceptibility to strain-specific 20% lethal concentration of CMIT/MIT by measuring physiology and global DNA methylation alterations.

2. Materials and methods

2.1. Test organisms

The *D. magna* strain used in this study was initially provided in 2015 by INERIS, France (clone A). The *D. pulex* strains stem from two geographically distant origins: (1) Rennes, north-western France (a natural population of the PEARL outdoor facilities at INRAE U3E), and (2) Alsace, north-eastern France (purchased by INRAE in 2016 from Aqualiment, Niederbronn-les-Bains). The three strains were further reared at INRAE U3E under laboratory standard conditions, as described in Duchet et al. (Duchet et al., 2011). According to their origin, *Daphnia* strains were given the following codes: DMI (*D. magna* INERIS), DPR (*D. pulex* Rennes), and DPA (*D. pulex* Alsace) (Table 1).

2.2. Preparation of chemicals and exposure

CMIT/MIT secondary standard was purchased from Sigma Aldrich (PHR-1597, Sigma-aldrich). Stock solutions were previously prepared with distilled water. Test organisms were then exposed to final concentrations of CMIT/MIT by diluting stock solutions with an M4 medium.

2.3. Mortality and reproduction assay

Acute and chronic toxicity assays were conducted following OECD guidelines TG202 and TG211 with minor modifications. Prior to actual tests, we performed several range-finding tests to obtain the proper exposure concentrations of CMIT/MIT for each strain.

In the acute assay, ten daphnids were placed into each test vessel with a volume of 100 mL M4 medium (three replicates with ten daphnids per condition). Dead and immobilized organisms were counted after 48 h exposure to the following concentrations of CMIT/MIT: 20, 40, 80, 160, 320 μg/L for DMI; 50, 100, 150, 200 μg/L for DPR; 100, 200, 400, 600, 800 μg/L for DPA. We used the percentage of dead and immobilized organisms in each test vessel to estimate strain-specific lethal concentrations (LC_5, LC_{10}, LC_{20}, and LC_{50}).

In order to assess chronic reproductive toxicity, parent animals were individually exposed in test vessels containing 50 mL of chemical solution (10 replicates per condition). The number of neonates was recorded daily for 21 days in *D. magna* and 15 days in *D. pulex* (period adjusted to the time to the 4th brood in each strain). Daphnids were fed on the green algae *Chlorella sp.* ($4{\cdot}10^5$ cells/mL) daily and maintained at a constant temperature of 20 ± 1 ℃ with a 16:8 h light-dark cycle during exposure. The M4 medium was renewed three times a week. Tested concentrations of CMIT/MIT were as follows: 5, 10, 20, 40, 80 μg/L for DMI; 5, 10, 20, 40, 80 μg/L for DPR; 37.5, 75, 150, 300, 600 μg/L for DPA. The number of offspring produced per mother per day was used to estimate strain-specific effective concentrations with respect to reproduction (EC_5, EC_{10}, EC_{20}, and EC_{50}).

2.4. Physiological assay

Heart rate and body size were measured as proxies for physiological status. Fifty animals were exposed from birth to 1 L test solution of CMIT/MIT strain-specific 48 h-LC_{20}s or control conditions. After 9 days of exposure, 10 individuals were isolated on a slide glass and their heartbeat was recorded for 20 s under a microscope (MZ6, Leica) with three measurements for each organism. Body size was measured using the LAS. V4 program and was indicated by adding body length (distance

Table 1
Acute and chronic CMIT/MIT toxicity to different daphnid species/strains.

Species	Strains	Origin	48-hr LC_{20}	21-d EC_{20}
			lower limit < **μg/L** < upper limit	
D. magna	DMI	INERIS clone A	37.00 < **44.80** < 52.70	14.50 < **18.20** < 32.10
D. pulex	DPR	Rennes	34.20 < **53.40** < 69.00	15.66 < **14.30** < 27.00
D. pulex	DPA	Alsace	160.0 < **228.00** < 313.0	11.10 < **16.00** < 22.40

between the anterior extremity of the head and the point of tail spine attachment to the carapace) and tail spine length (distance between the origin and end of the tail spine).

2.5. Global DNA methylation

Global DNA methylation status was quantified colorimetrically by measuring levels of 5-methylcytosine (5-mC). Individuals were exposed to the same conditions as for the physiological assay for nine days, and were then flash frozen in liquid nitrogen (30 animals per condition) and stored at − 80 ℃ until DNA methylation assessment. Total DNA was extracted using a NucleoSpin extraction kit (NucleoSpin, Macherey-Nagel GmbH & Co. KG, Germany), and the quantity and quality of DNA were evaluated with a NanoDrop (NanoReady Touch, Life Real). Next, global DNA methylation assays were executed according to the manufacturer's instructions (MethylFlash global DNA methylation, 5-mC ELISA Easy Kit, Colorimetric, epigentek; P-1030–96) with three biological replicates.

2.6. Proteome profiling and bioinformatics

Proteome profiling was conducted using quantitative mass spectrometry-based proteomics on nine day-aged daphnids exposed from their birth to the 48 h-LC20. A detailed methodology is provided as Supplementary material. Bioinformatic analyses were conducted on proteins found to be differentially regulated between exposed and control groups (fold change > 1.5). GO terms and protein classes were retrieved from Uniprot (https://www.uniprot.org/) and PANTHER (www.pantherdb.org) databases. Protein-Protein Interaction (PPI) and KEGG pathway enrichment analyses were conducted using STRING 11.5 (https://string-db.org/) and visualized using Cytoscape v. 3.8.2.

2.7. Statistical analysis

Statistical analyses were conducted using R version 4.0.4. Statistical significance was assessed by t-tests or Wilcoxon rank-sum tests, depending on whether data fulfilled normal distribution and homogeneity of variances. Significant *P*–values were marked with asterisk and hash symbols (*, # $P < 0.05$; **, ## $P < 0.01$; ***, ### $P < 0.001$).

LCs and ECs were estimated using a Bayesian framework, as implemented in MOSAIC web interface (http://pbil.univ-lyon1.fr/software/mosaic/). As the concentrations used for chronic exposure overlapped with those for acute exposure, we accounted for mortality during the chronic test. Using $MOSAIC_{repro}$, we estimated reproductive toxicity of CMIT/MIT while accounting for mortality during the test. For proteomic data, hierarchical clustering with the complete-link method and heatmaps were generated with R.

3. Results and discussion

3.1. Inter and intraspecific variation in sensitivity to CMIT/MIT

As depicted on Fig. 1A, *D. magna* (clone DMI) appears to be more susceptible to CMIT/MIT than *D. pulex*. However, LC_5, LC_{10}, and LC_{20} values did not differ statistically between this clone and DPR (see 95% confidence intervals) (Table S1). In contrast, the difference in sensitivity was highly significant between the two *D. pulex* strains (DPR and DPA), and much higher than between DMI and DPR. Unlike mortality, CMIT/MIT-induced reproductive impairment did not vary significantly across the three strains, except for the EC_5 values between DMI and DPA (Fig. 1B and Table S2). These results indicate that within-species variation in sensitivity to CMIT/MIT can vastly exceed that observed between two distinct species. This finding is counterintuitive, as we expected more homogeneity within species than between species. Since we observed a clear difference in sensitivity to CMIT/MIT in terms of mortality at strain level, sublethal effects were further assessed using strain-specific 48 h-LC_{20} values (Table 1).

In the case of *D. magna*, our results on acute toxicity (LC_{50} of DMI: 65 µg/L, see Table S1*)* are not in line with the values reported by ECHA (European Chemicals Agency) for CMIT/MIT, i.e. a 48 h-LC50 of 100 µg/L (ECHA RAC, 2016). As both estimations were based on similar test conditions (OECD TG 202), the observed discrepancy implies a strain effect on *D. magna* sensitivity to CMIT/MIT.

3.2. Intraspecific variation in physiological response to CMIT/MIT

CMIT/MIT exposure significantly increased the heart rate and reduced adult body size in DPR, while it did not alter these traits in DMI and DPA (Table 2). It is to be noted that DPA individuals were significantly smaller than DPR, and their heart rate was also lower (under control condition). A few studies on body size-dependent toxicity revealed higher sensitivity to toxic substances, such as zinc and cadmium, in smaller aquatic invertebrates compared to larger ones belonging to the same genus and species (Muyssen et al., 2005; Vesela and Vijverberg, 2007). However, other studies have documented that large-sized cladocerans are more sensitive than smaller species to

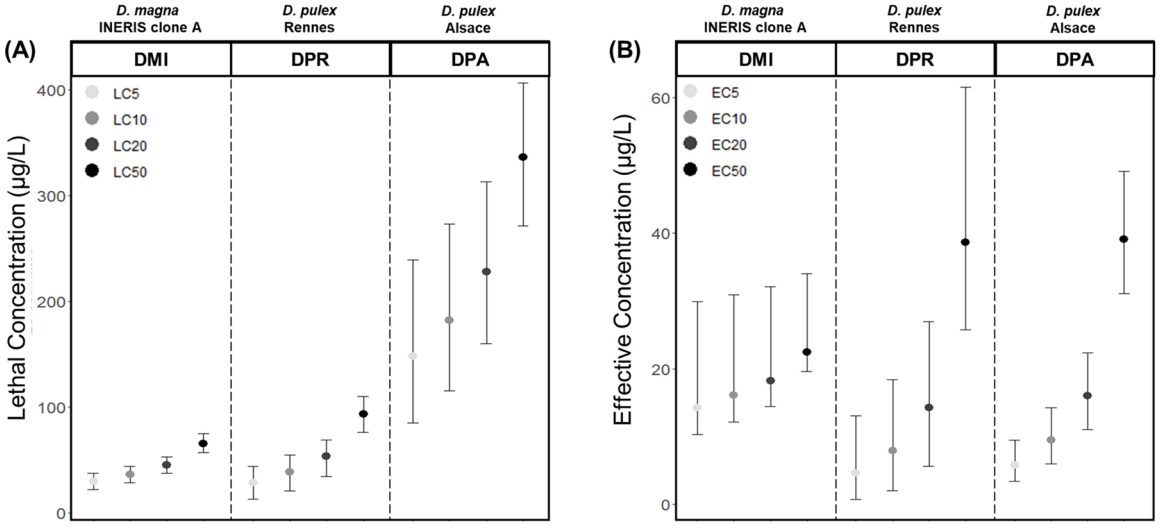

Fig. 1. Comparative (A) lethal concentrations (LCs) and (B) effective concentrations (ECs) of CMIT/MIT in three *Daphnia* strains. LCs and ECs are plotted as dots, with error bars representing 95% confidence intervals (lower limit < µg/L < upper limit).

Table 2
Physiological changes in three *Daphnia* strains exposed to CMIT/MIT. Data are presented as mean ± SE (n = 10) with statistical significance. Asterisks (*) indicate a significant difference between the exposed and control groups. Hash symbols (#) indicate a significant difference of each control compared to the control of DPR (*, # $P < 0.05$; **, ## $P < 0.01$; ***, ### $P < 0.001$).

Strain	DMI (*D. magna* INERIS clone A)		DPR (*D. pulex* Rennes)		DPA (*D. pulex* Alsace)	
Concentration (μg/L)	0 (control)	40 (LC_{20})	0 (control)	50 (LC_{20})	0 (control)	200 (LC_{20})
Body Size (mm)	3.92 ± 0.05###	3.97 ± 0.04	2.61 ± 0.03	2.48 ± 0.04 *	1.87 ± 0.02###	1.87 ± 0.02
Heart rate (beats/20 s)	130.44 ± 5.78 ###	128.10 ± 4.95	179.80 ± 2.75	199.90 ± 1.73 * **	152.70 ± 5.95 ##	154.3 ± 4.69

biocides, including carbaryl, fenthion, and chlorpyrifos (Daam et al., 2008; Hanazato, 2001). Our results showed that despite its smaller intrinsic body size, DPA was more tolerant to CMIT/MIT than DMI and DPR (Fig. 1 and Table 2). This variation may be related to the fact that cladocerans are vulnerable to chemicals after molting, and that large animals molt more times than smaller ones before reproduction (Hanazato, 2001).

3.3. Intraspecific variation in global DNA methylation level under exposure to CMIT/MIT

Low levels of global DNA methylation observed in the three strains under control conditions (1.70% in DMI; 0.66% in DPR; 0.46% in DPA) coincided with previous reports for *Daphnia*. Under control conditions, the level of DNA methylation was significantly higher in *D. magna* than in *D. pulex* (Fig. 2). Under strain-specific LC_{20} exposure conditions, CMIT/MIT significantly affected the level of global DNA methylation of the three strains. We observed significant decreases in DMI and DPR strains and a significant increase in DPA strain (Fig. 2). This magnitude of constitutive variation falls within the range of variation observed at the within-species level in *D. pulex* (0.41–0.70%) (Asselman et al., 2016; Kvist et al., 2018) and *D. magna* (0.52–1.51%) (Asselman et al., 2016; Kvist et al., 2018; Trijau et al., 2018). Even though global DNA methylation change does not identify the significant DNA methylated regions and the concomitant specific gene expression alterations, our preliminary result indicates that the environment can influence intraspecific variation in DNA methylation and epigenetic response to chemical exposure.

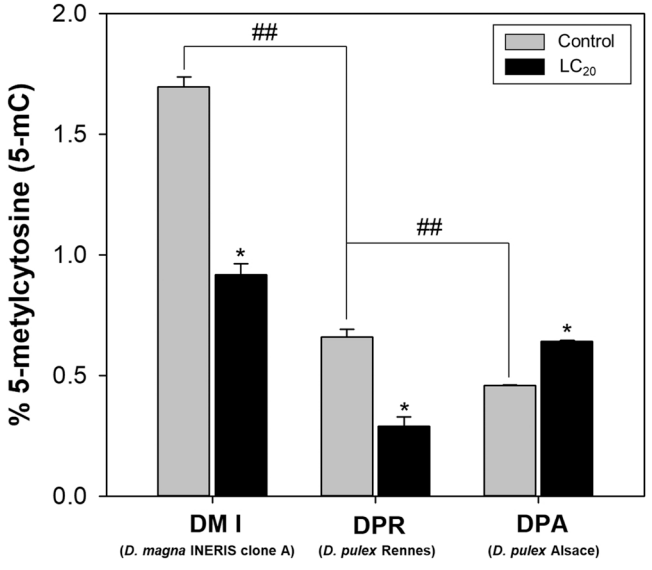

Fig. 2. Alteration of global DNA methylation levels in three *Daphnia* strains exposed to CMIT/MIT. Data are presented as mean ± SE (n = 3) with asterisks (*), indicating a significant difference between the exposed and control groups. The symbol # indicates a significant difference of each control from the control of DPR (*, # $P < 0.05$; **, ## $P < 0.01$; ***, ### $P < 0.001$).

Epigenetic variation between natural populations results from a combination of reversible (environmentally labile) and more persistent (heritable across generations) components. Regarding the daphnid lineages used in the present study, which have been cultured for several generations under laboratory conditions, it is assumed that the observed difference in constitutive DNA methylation status mostly reflects stably inherited differences. Moreover, in daphnids, transgenerational stability of epigenetic marks may be facilitated under apomictic parthenogenesis, as opposed to sexual reproduction where gametogenesis involves epigenetic reprogramming (Kawashima and Berger, 2014).

We also speculate that these differences in DNA methylation might affect the intraspecific variation in intrinsic physiological traits (DPR's smaller body size and higher heart rate than DPA's) and chemicals sensitivity (Table 2). Thorson and colleagues (Thorson et al., 2017) suggested that the differences in the shell morphology among freshwater snail populations from different habitats result from adaptive phenotypic plasticity mediated by epigenetic mechanisms throughout the genome. They found that morphological divergence was related to significant differences in genome-wide DNA methylation patterns between snail populations, with few molecular genetic differences. In addition, plenty of research has confirmed that the heritable epigenome can translate the various environmental signals to phenotypic responses by altering of gene expression profiles (Norouzitallab et al., 2019). This study identified that epigenetic responses can differ between two *D. pulex* strains of different geographical origins and that CMIT/MIT can potentially affect epigenetic processes. From an eco-evolutionary perspective, such a pattern of variation indicates genotype-by-environment interaction, a condition theoretically required for phenotypic plasticity to evolve adaptively (Norouzitallab et al., 2019; Saltz et al., 2018).

3.4. Intraspecific variation in CMIT/MIT-induced proteomic alteration

3.4.1. Differentially expressed proteins (DEPs) profiling

We were able to identify 526 proteins in three *Daphnia* strains. Comparing all protein expression profiles, we observed greater interspecific than intraspecific variation (Figs. S1). There were also differences in protein expression profiles between the *D. pulex* strains and even between the control groups. However, the effect of chemical treatment was more substantial than that of lineage, as reflected by the two sub-clusters shown on the expression of heatmap (Fig. S1). In a total of 526 proteins, 13, 101, and 86 differentially expressed proteins (DEPs) were identified in the DMI, DPR, and DPA strains, respectively, treated with their own CMIT/MIT LC_{20} (Tables S3, S4, and S5). Although the effect on mortality was similar between DMI and DPR, the latter was found to be more sensitive than the former at the proteomic level. The two *D. pulex* strains shared only 16 DEPs, and 85 DPR-specific DEPs and 70 DPA-specific DEPs were identified, indicating that intraspecific expression patterns differed due to CMIT/MIT exposure (Fig. 3A). This

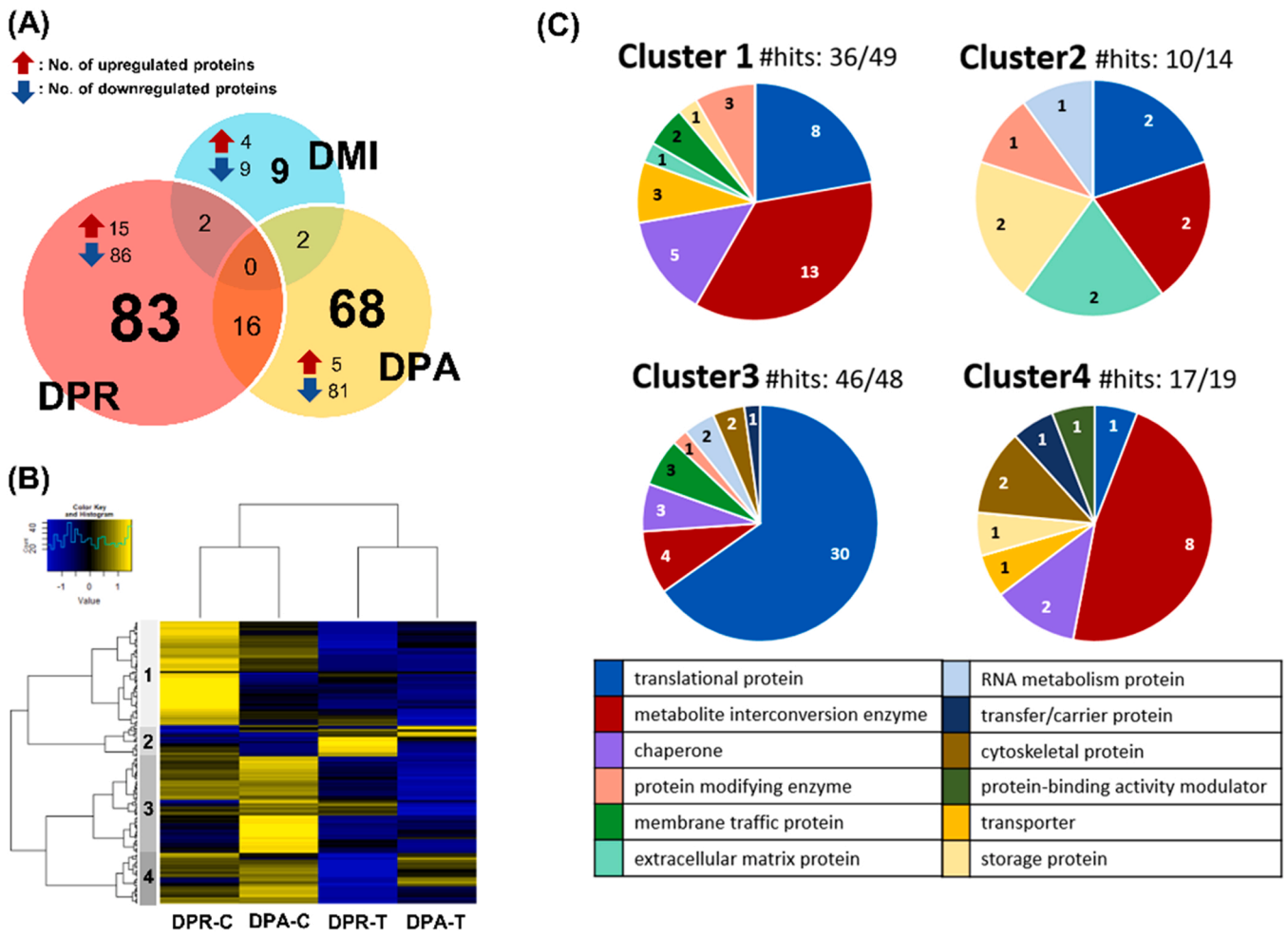

Fig. 3. Analysis of differentially expressed proteins (DEPs) induced by exposure to CMIT/MIT LC_{20} in the two *D. pulex* strains (DPR and DPA). (A) The number of DEPs in DMI, DPR, and DPA strains (> 1.5 fold in CMIT/MIT-treated groups vs. control groups). (B) Hierarchical clustering heatmap of DEPs (protein clustering cutoff = 4; the control groups of each strain are DPR-C and DPA-C; the CMIT/MIT-treated groups of each strain are DPR-T and DPA-T). (C) Pie chart displaying the number of hit PANTHER protein classes (see color legend for interpretation) according to protein clusters (#hits = total number of protein class hits/total number of proteins in each cluster).

result also suggests genotype-by-environment interaction, as do global methylation data.

For further analysis, we excluded the DEPs of DMI from the comparative analysis because of the small number of DEPs and no hits in the PANTHER protein classes. Within *D. pulex*, the variation in CMIT/MIT-altered protein abundance was visualized with a heatmap, showing the main clustering as a function of exposure (control vs. treatment). Four clusters were identified with respect to the proteins, and the classes of proteins included in each cluster were examined (Fig. 3B and C). The proteins in cluster 1 were most exclusively downregulated in DPR exposed to CMIT/MIT, and most of them were metabolite interconversion enzymes (36.1%), translational proteins (22.2%), and chaperones (13.9%). Conversely, cluster 2 comprised 14 proteins whose expression increased specifically in DPR under CMIT/MIT exposure, and included two translational proteins, two metabolite interconversion enzymes, two extracellular matrix proteins, and two chaperones. Cluster 3 grouped proteins downregulated in DPA after CMIT/MIT exposure and, to a lesser extent, in DPR. These were mainly proteins involved in translation (65.2%), metabolite interconversion enzymes (8.7%), membrane traffic proteins (6.5%), and chaperones (6.5%). Lastly, cluster 4 was a group of proteins that were downregulated in both CMIT/MIT-treated DPR and DPA (more markedly in DPR), including metabolite interconversion enzymes (47.1%), cytoskeletal proteins (11.8%), and chaperones (11.8%). Among the DEPs, most downregulated proteins were metabolite interconversion enzymes in DPR and translational proteins in DPA (Figs. 3C and S2).

3.4.2. KEGG pathways enriched in differentially expressed proteins (DEPs)

Few proteins were found upregulated by CMIT/MIT (15 in DPR and 5 in DPA), and no enrichment in any particular pathway could be detected in this group of proteins. Conversely, downregulated proteins were much more numerous and led to several significantly enriched pathways, either common or specific to the two studied strains (Tables S6 and S7). Four KEGG pathways were common to DPR and DPA (i.e., carbon metabolism, biosynthesis of amino acids, protein processing in the endoplasmic reticulum, and ribosome). Pathways specifically enriched in exposed DPR included various energy metabolisms, drug metabolism, cysteine and methionine metabolism, mTOR signaling, and protein export. Downregulated proteins enriched distinguishingly in DPA were involved in two related metabolic pathways, the citrate cycle (TCA cycle) and 2-oxocarboxylic acid metabolism. Therefore, at the proteomic level, the effect of CMIT/MIT appears clearly more important and widespread in DPR than in DPA (Fig. 4). We infer that DPR and DPA-specific patterns of altered protein expression and their associated biological pathway could be causative of the strain difference in susceptibility to CMIT/MIT exposure.

Regarding chemical concentrations, we noted that 50 μg/L of CMIT/MIT had a much stronger impact on DPR protein expression than

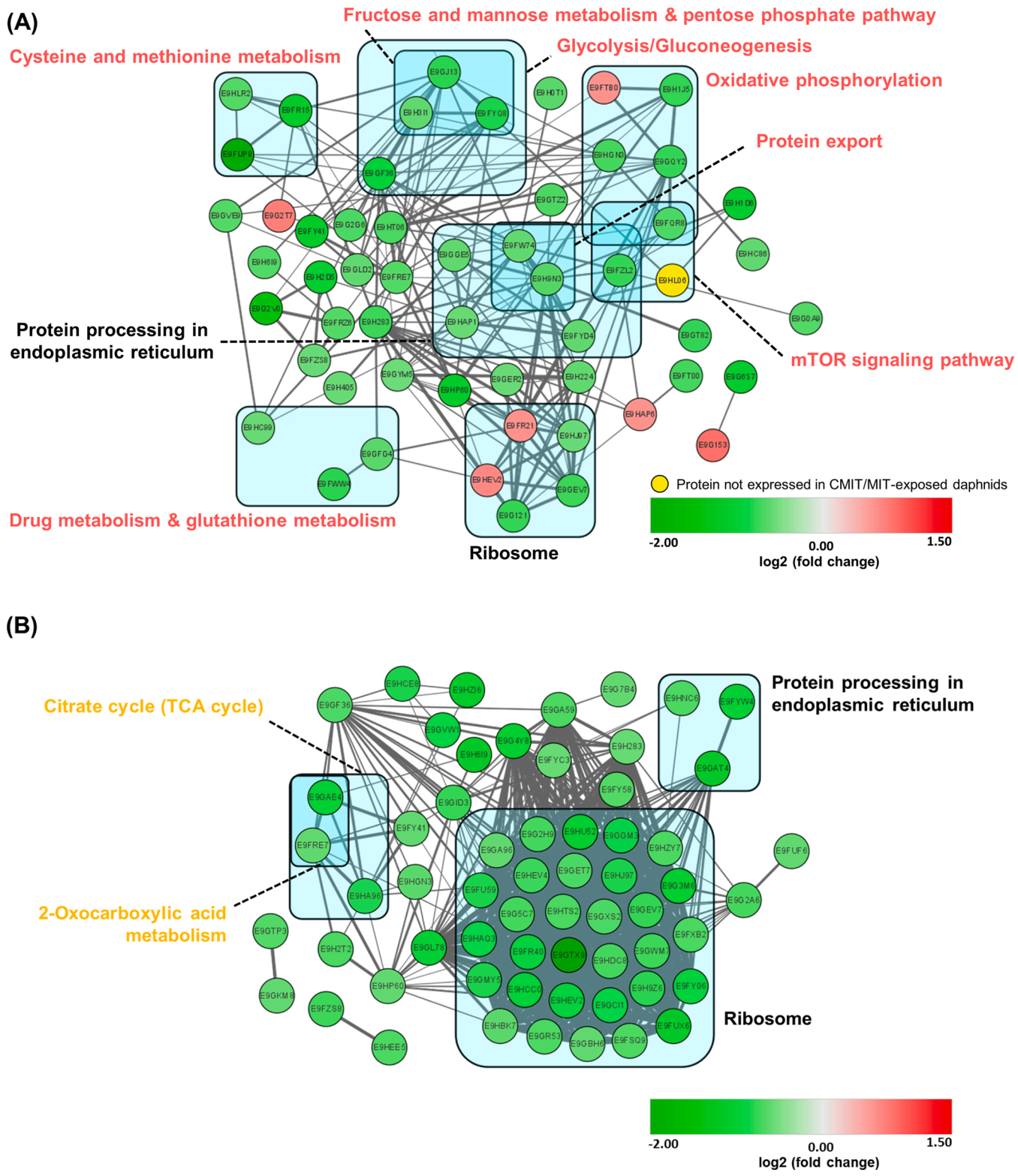

Fig. 4. Graphical representation of (A) DPR and (B) DPA strain-specific KEGG pathway enrichment with differentially expressed proteins (DEPs) as nodes and STRING protein-protein interactions as edges. Edge thickness indicates the strength of data between proteins, and node colors are scaled according to log 2-fold changes in protein expression (CMIT/MIT LC_{20}-treated groups vs. control groups). KEGG pathways were derived from Table S7, except for general pathways, such as metabolic pathways, carbon metabolism, and biosynthesis of amino acids. Colored pathways represent each strain-specific and common pathways (Pink: the DPR-specific pathway; Yellow: the DPA-specific pathway; Black: common pathway between DPR and DPA).

200 μg/L of CMIT/MIT on DPA. Such a differential pattern of pathway enrichment may be associated with the higher sensitivity of DPR to CMIT/MIT exposure, along with physiological changes and alteration of DNA methylation. Finally, isothiazolinone biocides may lead to adverse outcomes through these pathways, considering their mechanisms of action (see below).

3.4.3. Energy and detoxification metabolism-related proteins

Thiols are common active sites on many proteins and enzymes, including dehydrogenase, cysteine, cystine, and glutathione. Isothiazolinone biocides, especially CMIT, can react with protein thiols, and this reactivity causes a loss of cell viability and functions (Williams, 2006). The mechanism of action of these biocides relies on inhibition of respiration and energy generation, followed by irreversible cell damage associated with the destruction of protein thiols and the production of free radicals (Williams, 2006). Glutathione S-transferases (GST) are major phase II detoxification enzymes that catalyze the conjugation of reduced glutathione (GSH) to various electrophiles to protect the cell from oxidative stress. In the CMIT/MIT-treated DPR group, three GSTs were downregulated (E9WW4, E9GFG4, and AOA162Q5W2, see Tables S4 and S6). Slight oxidative stress can induce GST activity; however, severe oxidative stress can suppress GST activity due to the depletion of GSH (Li et al., 2021). In addition, GSTs respond to various toxins and pesticides in a dose-dependent manner in aquatic organisms, suggesting that their expression level is crucial in determining stress sensitivity (Song et al., 2017; Van der Oost et al., 2003). Thus, it is persuasive that the downregulation of many proteins related to general energy metabolisms and drug metabolism in DPR by exposure to LC_{20} of CMIT/MIT was responsible for its particular sensitivity to the chemical mixture (Tables S4, S5, and S6).

3.4.4. DNA methylation-related proteins

In global DNA methylation, DPR and DPA showed an opposed trend after exposure to CMIT/MIT (Fig. 2). E9FUP9 (S-adenosylmethionine synthase), E9FR15 (adenosylhomocysteinase), and E9HLR2 (cystathionine beta-synthase) were downregulated by CMIT/MIT in DPR only, leading to enrichment of cysteine and methionine metabolism pathway (Tables S4, S5 and S6). This result supports the hypothesis that a decrease in S-adenosylmethionine (SAM) production could be responsible for the demethylation of DNA observed in the CMIT/MIT-exposed DPR strain. Indeed, SAM is the major methyl-donor for crucial methylation reactions and methylation of DNA. Moreover, SAM plays a critical role in stress-responsive transcription, as demonstrated in *C. elegans* (Ding et al., 2018), and this may explain the downregulation of stress-related pathways in DPR, such as drug metabolism and protein export, and mTOR signaling (Reiling and Sabatini, 2006). Regarding the higher percent of 5-mC detected in control DPR, three proteins related to cysteine and methionine metabolism-related proteins expressed higher in control DPR than in DPA (Tables 3 and S8). Altogether, these results support that conspecific lineages may differ substantially in their constitutive DNA methylation status and that CMIT/MIT might have induced DNA hypomethylation in DPR due to SAM depletion.

DNA methylation can regulate gene expression, thereby indirectly affecting protein biosynthesis. Although this study did not investigate the direct effect of DNA methylation on the daphnid genome, we observed intraspecific variations in global DNA methylation and proteomic profiles between the control groups of two daphnid strains. This result suggests that these variations could be responsible for the differences in sensitivity to CMIT/MIT.

3.4.5. Cuticle-related proteins

The cuticle proteins and chitin of cladocerans are structural constituents of the cuticle, which can resist environmental stresses and adverse conditions. Several *Daphnia* species (*D. middendorffiana*, *D. pulex*, and *D. cucullata*) exposed to predator stressor shows increased cuticle thickness, hardness, and diameter of the cuticular pillars (Otte et al., 2014). Cuticle proteins are also associated with a hormonally controlled process of ecdysis or molting, which characterizes somatic growth, development, and reproduction in *Daphnia* (Connon et al., 2008). Some studies showed that the somatic growth of daphnids exposed to cadmium correlated with the regulation of cuticle proteins (Chen et al., 2016; Connon et al., 2008). In the present study, A0A162BZC1 (putative endocuticle structural glycoprotein SgAbd-1), A0A0N7ZUK4 (cuticle protein), and A0A164PJ82 (cuticular protein 49Ag) were downregulated in DPR, on the other hand, A0A0P5ZQF6 (eukaryotic translation initiation factor 3 subunit D-like protein) and A0A0N8A729 (cuticle protein) were upregulated in DPA under CMIT/MIT exposure (Tables S4 and S5). Downregulated cuticle proteins by exposure were more highly expressed in unexposed-DPR than in unexposed-DPA (Table S8). These differences in protein expression can underlie intrinsic phenotypic traits, physiological changes in body size, and sensitive response to CMIT/MIT exposure in DPR compared to DPA (Table 2).

Table 3
Putative critical proteins associated with difference in phenotypes and sensitivity to CMIT/MIT between DPR and DPA.

Protein (Uniprot ID)	Fold change		Function (Gene Ontology ID)
	DPR-T /DPR-C[1]	DPA-T /DPA-C[2]	
Putative Endocuticle structural glycoprotein SgAbd-1 (A0A162BZC1)	0.35*	0.98	structural constituent of cuticle (GO:0042302)
Cuticle protein (A0A0N7ZUK4)	0.57*	0.89	
Cuticular protein 49Ag (A0A164PJ82)	0.58*	1.09	
Hemoglobin	0.58*	0.98	oxygen carrier activity (GO:0005344)
Di-domain hemoglobin	1.51*	0.68	
GATOR complex protein SEC13 (E9FZL2)	0.58*	0.71	positive regulation of TOR signaling (GO:0032008)
S-adenosylmethionine synthase (E9FUP9)	0.24*	0.72	methionine adenosyltransferase activity (GO:0004478)
Adenosylhomocysteinase (E9FR15)	0.45*	0.78	adenosylhomocysteinase activity (GO:0004013)
Cystathionine beta-synthase (E9HLR2)	0.63*	0.85	cystathionine beta-synthase activity (GO:0004122)
Glutathione S transferase E10 (A0A162Q5W2)	0.55*	0.89	glutathione metabolic process (GO:0006749)
Glutathione transferase (E9FWW4)	0.57*	0.88	
Glutathione S-transferase (E9GFG4)	0.64*	0.96	

1 Total intensity of protein expression in DPR-T (CMIT/MIT-treated group of DPR) / DPR-C (control group of DPR)
2 Total intensity of protein expression in DPA-T (CMIT/MIT-treated group of DPA) / DPA-C (control group of DPA)
* Fold changes which is more than 1.5, compared to each control group

3.4.6. mTOR signaling-related proteins

The mechanistic target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase that responds to physiological and environmental signals. Among the DEPs involved in the mTOR signaling pathway (Fig. 4 and Table S6), E9FQR8 (V-type proton ATPase subunit C) and E9FZL2 (GATOR complex protein SEC13) are involved in the mTOR complex 2 (mTORC2) signaling pathway, which controls cell division, apoptosis, and cytoskeletal organization. In particular, insulin-like peptide/mTOR pathway regulates the size and ecdysteroidogenic capacity of the insect molting gland (Abuhagr et al., 2014). Liu et al. (2021) proposed the changes in the signaling pathways (including the mTOR signaling) and glutathione metabolism with the consequential growth inhibition as an adverse outcome pathway for nanoplastics in *D. pulex*. Taken together, the downregulation of proteins involved in the

mTOR signaling pathway may cause broader physiological changes and different sensitivity to CMIT/MIT exposure between DPR and DPA strains by regulating various other biological pathways.

3.4.7. Hemoglobin-related proteins

Hemoglobin (Hb) content has been shown to vary between genotypes from the same population of *D. magna* and is linked to oxygen tolerance and thermal acclimation (Cuenca Cambronero et al., 2018). In the present study, A0A0P5EB26 (di-domain hemoglobin) and E9FXJ3 (hemoglobin) were more expressed in unexposed-DPA than in unexposed-DPR individuals (Table S8).

It is impossible with the present dataset to assess whether such a constitutive difference results from adaptation to historical environmental conditions (natural conditions of origin or subsequent long-term rearing conditions in the laboratory). However, it is possible to associate higher Hb expression with the smaller body size and lower heart rate of DPA (Table 2) as a tradeoff relationship due to the consumption of energy for the synthesis of hemoglobin. Indeed, Bäumer et al. (Bäumer et al., 2002) found that Hb-rich *D. magna* individuals had smaller body sizes and lower heart rates than Hb-poor individuals, in perfect line with the relationship between hemoglobin, body size, and heart rate in the present study.

After exposure to CMIT/MIT, A0A0P5EB26 (fold change: 1.51) and E9FXJ3 (fold change: 0.58) were differentially regulated in DPR only (Table S4). Exposure to environmental contaminants such as nonylphenol, chlorpyrifos, paraquat dichloride, and lead nitrate can increase the hemoglobin gene expression of *D. magna* (Ha and Choi, 2009). Although the Hb expression increased at a low concentration (LC_5), the effect reversed gradually at higher concentrations of the chemicals (LC_{20} and LC_{50}) (Le et al., 2010). The effect of CMIT/MIT on Hb expression could be exerted by direct interaction with hemoglobin and/or indirectly result from effects on energy metabolism. Finally, it is worth mentioning that adaptive changes in hemoglobin content may, in turn, impact physiological responses and sensitivity to CMIT/MIT exposure. While deciphering mechanistic processes underpinning such complex relationships is out of the scope of the present study, our results suggest that a global picture implying molecular, physiological, and life-history traits may be necessary to reliably assess the toxicity of emergent contaminants such as CMIT/MIT to non-target organisms.

3.4.8. Others

Vitellogenin-related and myosin-related proteins were downregulated in both *Daphnia* strains under exposure to CMIT/MIT (Tables S3 and S4). Probably because exposure concentrations (LC_{20}) were higher than CMIT/MIT reprotoxic EC_{50} (Tables S1 and S2), vitellogenin proteins (E9GVW7, E9H8Q4, A0A164I1F8, A0A164NU47, and A0A164EIE6) were strongly affected, which might cause the reproductive failure. DPA exposed to a concentration (200 μg/L) close to EC_{90} for reproduction showed the biggest fold changes in vitellogenin proteins. CMIT/MIT exposure also decreased the expression of myosin-related proteins (A0A0P5MYN2, E9FZS8, and A0A162DGC9). Myosin plays vital roles in muscle composition, development, and cellular activities, including cytokinesis, cell polarization, intracellular transport, and signal transduction (Zhang et al., 2019). Especially, myosin heavy chain is abundant in the muscle thick filaments of invertebrates and is ATP-dependent.

4. Conclusion

We investigated the comparative sensitivity to CMIT/MIT among different *Daphnia* species and strains by measuring mortality, reproduction, physiological traits, global DNA methylation, and proteomic expression: 1) Our results showed that intraspecific variation in sensitivity could exceed that observed between two distinct congeneric species. 2) The constitutive global DNA methylation level differed between strains (as estimated under control conditions), and this intraspecific variation may affect the epigenetic response to CMIT/MIT exposure. 3) DPR-specific altered protein expression and the associated pathways, including the cysteine and methionine metabolism, were proposed as possible causes of the higher susceptibility of this strain to CMIT/MIT. In addition, putative critical proteins underlying the intraspecific variation in phenotypes and sensitivity were listed (Table 3) and discussed. Downregulation of these proteins (e.g., SAM synthase, GSTs, hemoglobin, and cuticle proteins) and DNA hypomethylation can be proposed as key events (KEs) of adverse outcome pathway (AOP) for isothiazolinones toxicity. The proposed KEs can be integrated into the AOP in conjunction with the well-known mechanisms of isothiazolinones such as reaction with protein thiols, depletion of thiols from cysteine and GSH, generation of free radicals, and disruption of the metabolic pathways (Silva et al., 2020; Williams, 2006).

Based on the findings of this study, the next hypothesis is that the crosstalk between genetic and epigenetic factors may contribute to the varied phenotypes and sensitivities of daphnids to toxic substances. Further analysis of methylated gene regions using more accurate technology (e.g., bisulfite sequencing) and observation of transgenerational effects are necessary to gain a better understanding of the role of DNA methylation in phenotypic and proteomic expressions under both normal and stressful conditions.

Overall, it is suggested that intraspecific variation should be considered when assessing the ecotoxicity of prevalent biocides by using (at least two) strains or genotypes from distinct populations of origins and/or different adaptive conditions. Furthermore, modified DNA methylation levels could be associated with the differences in sensitivity to chemicals and phenotypic traits. Given that the environment can alter the epigenetic programming of organisms, we propose utilizing epigenetic marks as sensitive biomarkers in ecotoxicological risk assessment. In this context, the application of common garden design is highly recommended since it is the only possibility to decipher reversible and stable components of epigenetic variation.

CRediT authorship contribution statement

Jinhee Choi: Conceptualization, Funding acquisition, Supervision. **Jinhee Choi, Jiwan Kim, Sangkyu Lee**: Investigation. **Jiwan Kim, Marie-Agnes Coutellec**: Methodology. **Jiwan Kim**: Software, Visualization, Writing – original draft. **Jiwan Kim, Sangkyu Lee**: Data curation. **Jinhee Choi, Marie-Agnes Coutellec, Jiwan Kim**: Writing – review & editing. All authors have read and approved the manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jinhee Choi reports financial support was provided by National Research Foundation of Korea. Jiwan Kim reports financial support was provided by National Research Foundation of Korea. Marie-Agnes Coutellec reports financial support was provided by Hubert Curien.

Data availability

Data will be made available on request.

Acknowledgement

We thank Maïra Coke and the INRAE U3E experimental unit for maintaining the studied strains before experimentation. This work was supported by the National Research Foundation of Korea grant (NRF-2020R1A2C3006838) funded by the Ministry of Science and ICT, South Korea. Collaboration between JC and MAC could be initiated thanks to NRF Korea-France collaboration funding (NRF-2016K1A3A1A21005685) and Hubert-Curien STAR funding (PHC STAR 2016–36667TD).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114967.

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