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Transcriptional response provides insights into the effect of chronic polystyrene nanoplastic exposure on *Daphnia pulex*

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HIGHLIGHTS

- Chronic nanoplastic exposure inhibited expression of a few key genes in *D. pulex*.
- Chronic nanoplastic exposure increased doublesex gene expression in *D. pulex*.
- Chronic nanoplastic exposure resulted in more male *D. pulex* neonates.

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ABSTRACT

Nanoplastic pollution is widespread and persistent across global water systems and can cause a negative effect on aquatic organisms, especially the zooplankter which is the keystone of the food chain. The present study uses RNA sequencing to assess the global change in gene expression caused by 21 days of exposure to 75 nm polystyrene (PS) nanoplastics on *Daphnia pulex*, a model organism for ecotoxicity. With the threshold value at P value < 0.05 and fold change >2, 244 differentially expressed genes were obtained. Combined with real-time PCR validation of several selected genes, our results indicated that a distinct expression profile of key genes, including downregulated trehalose transporter, trehalose 6-phosphate synthase/phosphatase, chitinase and cathepsin-L as well as upregulated doublesex 1 and doublesex and mab-3 related transcription factor-like protein, contributed to the toxic effects of chronic nanoplastic exposure on *Daphnia*, such as slowed growth, subdued reproductive ability and reproductive pattern shifting. Our study also showed that chronic exposure to nanoplastic changed the sex ratio of *D. pulex* neonates. By integrating the gene expression pattern in an important model organism, this study gained insight into the molecular mechanisms of the toxic effect of chronic PS nanoplastic exposure on *D. pulex*, which may also extend to other nanoplastics or aquatic animals.

1. Introduction

More than 300 million tons of plastic are produced around the world per year and a large proportion of this is disposed into the environment, especially the water environment (Jambeck et al., 2015). Plastic waste accumulates in or around urban areas and gathers in the water system decomposing into smaller fragments, including microplastics (0.1 μm-5 mm) or nanoplastics (<0.1 μm), which are both pervasive and persistent pollutants. With increasing plastic production and advancing plastic technology, micro- or nanoplastic pollution has become more severe with more and more found in rivers, lakes and oceans (Wright et al., 2013; Obbard et al., 2014; Zhao et al., 2014). Both micro- and nanoplastics have excellent stability and can be ingested by pelagic or benthic organisms and then transferred to higher trophic levels and finally reach humans along the food chain (Cole et al., 2013; Biginagwa et al., 2016). Microplastic or nanoplastic toxicity, which mainly causes physical effects, is different from conventional dissolved chemicals or other pollutants. In addition to plastic toxicity, it can carry other organic pollutant, accumulate along the food chains and then,

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further threaten aquatic organisms and the ecosystem (Besseling et al., 2013; Wright et al., 2013; Lönnstedt and Eklöv, 2016). However, comparatively, little is known about the influence caused by nanoplastics. Nanoplastics are widely used in drug delivery, cosmetics, biosensors, and so on (González-Fernández et al., 2018), and can also be formed from the weathering of microplastics. Because of their smaller size and larger surface/volume ratio, nanoplastics can be ingested by organisms more easily than microplastics, which can be selectively avoided by several organisms (Kühn et al., 2015; Aljaibachi and Callaghan, 2018). Furthermore, nanoplastic pollution deserves more attention because of their ability to penetrate and interact with cells (Lard, 2012; Canesi et al., 2017). Nanoplastics are undoubtedly long-lasting in the water system and can thus cause chronic exposure to aquatic organisms (Paik and Kar, 2008). Therefore, it is essential to reveal the effect of chronic nanoplastic exposure on aquatic organisms.

Zooplankter is important for the ecosystem because it is a primary consumer in the food chain. Zooplankter can ingest microplastics or nanoplastics easily (Cole et al., 2013; Liu et al., 2018) and, thus, it is an entrance for plastic toxicity to enter the food chain and aquatic ecosystem. Nanoplastic exposure can alter the life history traits of zooplankter because decreased feeding, reduces survival and fecundity (Lee et al., 2013). For example, long-term nanoplastic exposure can inhibit the body length, decrease growth rate, prolong the time to first eggs and decrease the number of clutches in *Daphnia pulex* (Liu et al., 2019). These effects are believed to be due to the physical harm of nanoplastics, including internal abrasion and blockage (Sul and Costa, 2014), while relative molecular mechanisms are not completely known. On the other hand, as chronic nanoplastic exposure mainly causes physical harm, whether organisms, including the zooplankter, can acclimate to the plastic exposure and produce an adaptive response, is also not completely known. It is important to reveal the molecular mechanisms of chronic nanoplastic exposure toxicity on zooplankter.

The present study aims at detecting the molecular evidence and mechanisms of chronic nanoplastic toxicity on life history traits in *D. pulex*, a model organism of freshwater ecotoxicology with small body size, wide geographical distribution, and high reproduction rate. The flea was exposed to 75 nm polystyrene nanoplastics for 21 days, a duration used in the chronic toxicity assessment guidelines and *Daphnia* reproduction test (Guilhermino et al., 1999; OECD, 2012). Polystyrene (PS), a representative plastic at the micro- or nano-scale, was selected because of its abundance in freshwater or marine debris (Hidalgoruz et al., 2012). Changes in the transcript were detected by high throughput sequencing. We hypothesized that chronic nanoplastic exposure affected important traits, such as growth, lifespan or reproduction, of *D. pulex* by altering key gene expression levels. In addition, the cladoceran may change reproductive patterns when exposed to environmental toxicants, therefore, we also measured the sex ratio of *D. pulex* neonates to detect the possible effect of chronic PS nanoplastic exposure on population structure.

2. Materials and methods

2.1. D. pulex culture

The *D. pulex* used in the present study were obtained from the Laboratory of Zooplankton Adaption and Evolution, East China Normal University (northern subtropical marine monsoon climate, adequate sunshine and rainfall, 121°E, 31°N). Water fleas were cultured in 4-L glass beakers with approximately 3-L algal medium at 20 ± 0.5 °C, 5 mg L^{-1} dissolved oxygen concentration and a 16:8 h light-dark photoperiod, and fed with unicellular green alga *Chlorella pyrenoidosa*. Before the exposure, the female was cultured individually during oviposition and then the neonates (<24 h old) from the third brood were used for chronic PS nanoplastic exposure.

2.2. Polystyrene nanoplastics

Unlabeled PS nanoplastics were obtained from the BaseLine Chromtech Research Centre, Tianjin, China. The primary diameter of the PS nanoplastic particle was determined by transmission electron microscopy (HT-7700, Hitachi Ltd., Japan). The size distribution and ze-ta-potential were detected by dynamic light scattering assessments (Liu et al., 2018).

2.3. Nanoplastic exposure

The toxic exposure experiments followed the ecotoxicology guideline for *Daphnia* (OECD, 2012). Two groups, the control and the exposure treatment, containing 20 individuals each were set as samples for RNA sequencing. A PS exposure concentration of 1 mg L^{-1} 75 nm, which has been proven to significantly affect the survival and stress response of *D. pulex*, was used for exposure treatment with a 40 mL test media in a 50-mL glass beaker according to previous studies (Liu et al., 2018, 2019). The fleas were fed daily during the exposure, which lasted for 21 days. Each treatment was conducted with three replicates.

To detect the sex ratio of the *D. pulex* population, the control and exposure treatment contained 10 individuals each. The neonates born on the 14th and 21st day of the control treatment or nanoplastic exposure was collected for culture. Sex of neonates was distinguished by observing the morphology after 3 days culture and then the sex ratio was calculated. Each treatment was conducted with four replicates.

2.4. RNA isolation and Illumina de novo sequencing

After nanoplastic exposure, samples from each replicate were collected and pooled for total RNA isolation with TRIzol reagent (Invitrogen, USA). The concentration, integrity and RNA integrity number (RIN) of total RNA were measured with Nanodrop 2000 (Thermo Scientific, USA), agarose gel electrophoresis and biological analyzer (Agilent, USA), respectively. Total RNA (1 μg) with a concentration of more than 50 ng μL^{-1} and OD 260/280 between 1.8 and 2.2 was used for the library construction. The mRNA was isolated with the oligo (dT) magnetic bead method (Invitrogen) and was then broken randomly into fragments with an approximate 300 bp length. All the fragments were reverse transcribed to stable double-stranded cDNA with random hexamers added and end repairing. After library enrichment, the library was paired-end 150 bp sequenced with Illumina Hiseq 4000.

2.5. Quality control, transcript assembling and functional annotation

Quality control was conducted using SeqPrep (Param: -q 20 -L 30) and Sickle (v 1.2, Param: -q 20 −I 30) to remove adaptors and reads with low quality, high N ratio, and a short length. Clean reads were assembled for transcripts using Trinity (v 2.5.0, default param). TransRate (v 1.0.3, default param) and CD-HIT were employed to optimize transcripts by trimming low grade or redundant reads. TransRate and BUSCO (v 3.0.2, default param) were used to assess the quality of the transcripts. Unigene was defined as a set of transcripts with the same transcriptome locus.

Unigenes were blasted using six databases: non-redundant protein database (NR), Swiss-Prot, Pfam, Clusters of Orthologous Groups of proteins (COG), Gene Ontology (GO) and Kyoto

Encyclopedia of Genes and Genomes (KEGG) for functional annotation. The threshold value was set at E value < e^{-3}.

2.6. Expression analysis, differentially expressed gene comparation and function enrichments

The expression level of each unigene was calculated as tags number per million tags mapped (TPM) with the software RSEM (v 1.2.31, default param). Principal component analysis (PCA) was used to assess the relevance between samples. For two groups, TPM of each unigene from three replicates were grouped to conduct differentially expressed gene (DEG) intergroup comparation with edgeR (v 3.14.0). False discovery rate (FDR) and expression fold change (FC) of genes were used to determine DEGs. However, no DEG was detected with the high-stringency analysis (FDR < 0.05 and FC > 2). Thus, the threshold value of DEGs screening was changed to *P*-value < 0.05 and fold change (FC) > 2 to scan out potential target genes (Li et al., 2015; Jiang et al., 2016; Jung et al., 2017).

Goatools (v 0.6.5) and Python were used to conduct GO and KEGG enrichment analysis. The threshold value of both GO and KEGG enrichments was set as *P*-adjust < 0.05.

2.7. Validation of RNA sequencing

To validate the DEGs, the expression of several key DEGs was tested by real-time PCR. The design of the primers was based on sequence obtained from the RNAseq and shown in Supplementary file 1. Briefly, real-time PCR was conducted for three replicates using reactions containing 10 μL SYBR mixture (TransGen, Beijing, China), 0.6 μL each primer, 7.6 μL ddH_2O, and 1.2 μL cDNA template in a real-time PCR system (Bio-Rad Laboratories, Hercules, USA). The relative expression level was calculated with the $2^{-\Delta\Delta Ct}$ method and the *18s ribosomal RNA* gene as the internal control gene (Schmittgen and Livak, 2008; Liu et al., 2019).

2.8. Statistics for the sex ratio of neonates and real-time PCR validation

A *t*-test was conducted to compare the sex ratio of *D. pulex* neonates collected from control treatment and PS nanoplastic exposure on the same day. To validate the DEGs, the Mann-Whitney *U* test was conducted to between the relative expression levels of chosen genes from control and exposure groups. The statistic significance was set at $P < 0.05$.

3. Results

3.1. Illumina sequencing, de novo assembly, reads mapping, and functional annotation

Paired-end 150 bp sequencing resulted in 45–56 million reads per sample, and more than 98% clean reads were retained after subsequent quality control (Supplementary file 2). Following de novo transcript assembly, post-optimization and BLAST, 66290 transcripts with an N50 value at 2273 were obtained and were then used to form the reference transcriptome (Supplementary file 2). More than 74% reads per sample can be mapped to the reference and 41,513 unigenes were obtained from all transcripts for subsequent analysis (Supplementary file 2). A BLAST search identified 25,419 unigenes, taking up 61.23% of total unigenes, and these were functionally annotated using six major databases (Table 1).

PCA of overall gene expression data showed that the first principal component (PC1) accounted for 34.45% difference among samples and the second principal component (PC2) accounted for 24.74% difference (Fig. 1A). The Pearson correlation analysis indicated that a correlation higher than 0.7 between every two samples (Fig. 1B). In general, the gene expression pattern between the control group and the PS nanoplastic exposure group was similar (Fig. 1).

3.2. Differentially expressed gene analysis and functional enrichment

Based on the TPM value and our threshold for DE genes, 244 DE genes were screened, 87 of these were upregulated genes and 157 were downregulated (Fig. 2, Supplementary file 3).

In upregulated genes, NADPH oxidase 5 (*NOX5*, $P = 0.001$) and peroxidase (*PX*, $P < 0.001$) were involved in the oxidation-reduction process (Fig. 3A). Metabolism related enzyme genes, such as very-long-chain 3-oxoacyl-CoA reductase (*IFA38*, $P < 0.001$), lipase (*LIPA*, $P = 0.008$), fatty acid hydroxylase domain-containing protein 2 (*FAXDC2*, $P = 0.039$), fatty acid elongase 3-ketoreductase (*IFA38*, $P < 0.001$) and alpha-1,3-fucosyltransferase (FUT-1, $P = 0.008$), were observed as upregulated (Fig. 3A). Importantly, expression of two genes involved in sex differentiation, doublesex 1 (*DMRTA*, $P = 0.016$) and doublesex and mab-3 related transcription factor-like protein 1 (*DMRT1*, $P = 0.018$), increased by more than two folds (Fig. 3A).

Genes associated with substrate metabolism were observed among the downregulated genes (Fig. 3B). These genes included histone-lysine N-methyltransferase (*MLL3*, $P = 0.031$), trehalose transporter (*TRET1*, $P = 0.027$), 4 chitinase (*CHI*) isoforms ($P = 0.003$, 0.008, 0.040 and 0.033, respectively), trehalose 6-phosphate synthase/phosphatase (*TPS*, $P = 0.022$), 4-coumarate–CoA ligase (4CL, $P = 0.040$), ethanolamine kinase (*EKI*, $P = 0.044$), glutathione S transferase (*GST*, $P = 0.026$) and alcohol-forming fatty acyl-CoA reductase (*FAR*, $P = 0.018$), which participates in chitin metabolism, glycan degradation and fatty acid metabolism. (Fig. 3B). Two cathepsin-L isoforms (*CTSL*, $P = 0.003$ and $P = 0.004$, respectively) and the transforming growth factor-beta-induced gene (*BIGH3*, $P = 0.041$), which are all involved in growth regulation, were downregulated (Fig. 3B). The expression of several genes associated with RNA or the translation processes were suppressed after chronic nanoplastic exposure, including ribosomal 40S subunit protein S14A (*RPS14*, $P = 0.022$) and two isoforms of DNA-directed RNA polymerase II subunit (*RPB1*, $P = 0.030$ and 0.044, Fig. 3B).

From gene ontology enrichment analysis, five different levels of biological processes (all *P*-adjust = 0.044), belonging to amino sugar metabolism, were enriched with downregulated hydrolase genes: chitin catabolic process (GO:0006032), glucosamine-containing compound catabolic process (GO:1901072), amino sugar catabolic process (GO:0046348), cellular nitrogen compound metabolic process (GO:0034641), and aminoglycan catabolic process (GO:0006026) (Table 2). In addition, no pathway was significantly enriched with DE genes.

3.3. Validation of RNA sequencing

Expression of *DMRT1*, *DMRTA*, *TPS*, *BIGH3*, one *CHI* isoform, one *CTSL* isoform and several other genes were tested by real-time PCR (Fig. 3C). Real-time PCR indicated that mRNA levels of *DMRT1* and *DMRTA* increased significantly by about three folds in *D. pulex* after 21 days of nanoplastics exposure ($P_{DMRT1} = 0.004$ and $P_{DMRTA} = 0.003$). Alpha-carbonic anhydrase (*CA4*) and *FUT-1* also increased by more than one fold after nanoplastic exposure ($P_{CA} = 0.017$ and $P_{FUT\text{-}1} = 0.043$). Expression of *TPS* and *BIGH3* were inhibited by chronic nanoplastics exposure ($P_{TPS} = 0.008$ and $P_{BIGH3} = 0.001$). The selected isoforms of *CTSL* and *CHI* from the

Table 1
Results of the functional annotation of transcripts.

Database name	Transcript number (percentage)	Unigene number (percentage)
NR	43,334 (65.37%)	25,134 (60.54%)
Swiss-Prot	28,155 (42.47%)	16,656 (40.12%)
Pfam	27,117 (40.91%)	16,228 (39.09%)
COG	5101 (7.70%)	3223 (7.76%)
GO	26,476 (39.94%)	15,485 (37.30%)
KEGG	21,409 (32.3%)	13,175 (31.74%)
Total annotated	43709 (65.94%)	25,419 (61.23%)
Total	66290	41513

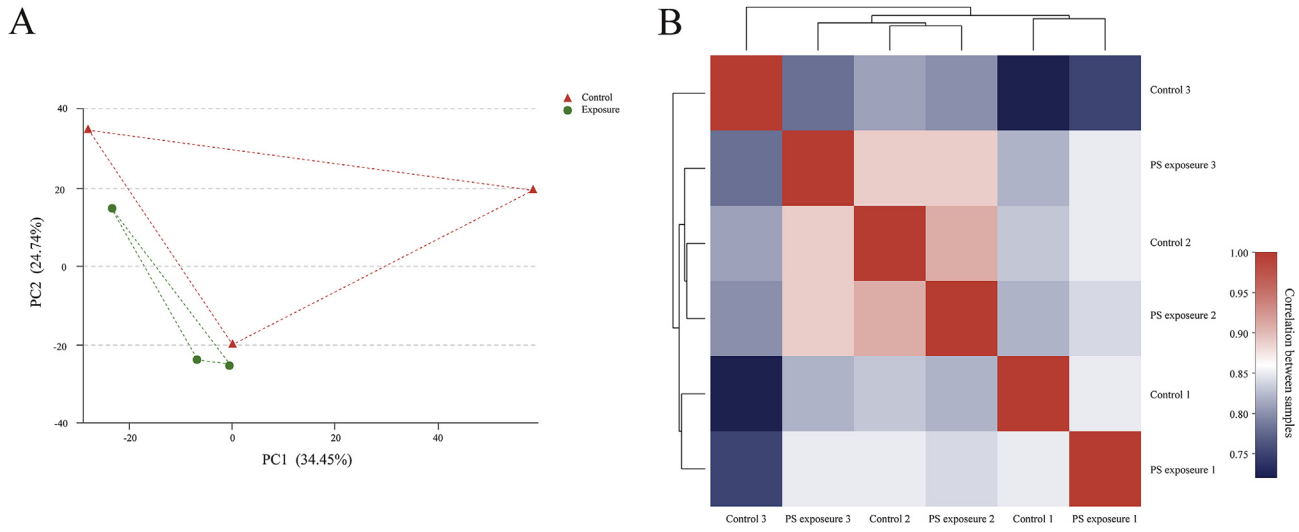

Fig. 1. Principal components analysis (A) and correlation analysis (B) of overall transcriptome expression.

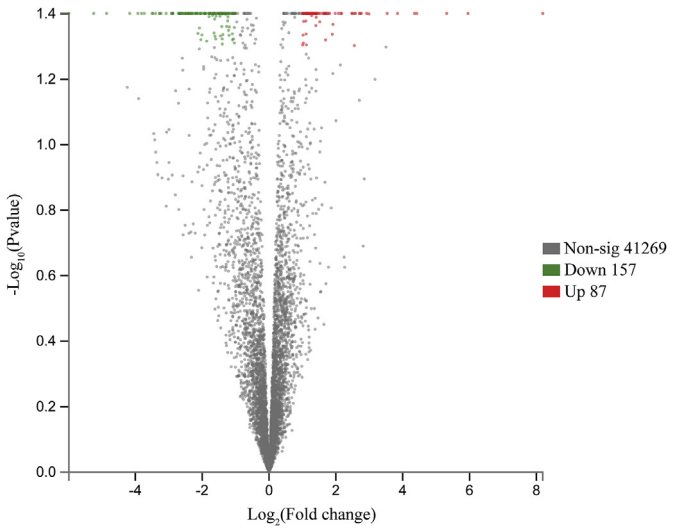

Fig. 2. Counts of non-significant (gray), down-regulated (green), and up-regulated (red) genes from samples of the polystyrene (PS) exposure group. DEGs were selected with the threshold value of P value < 0.05 and fold change >2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DEGs also showed decreased mRNA levels in real-time PCR validation ($P_{CTSL} < 0.001$ and $P_{CHI} < 0.001$). These results confirmed the effect of chronic PS nanoplastic exposure on the transcriptional response in *D. pulex* indicated by RNA sequencing.

3.4. Change in D. pulex sex ratio

After 14 days or 21 days of PS nanoplastic exposure, the proportion of male individuals increased significantly compared to that in the control group ($P_{14days} = 0.02$ and $P_{21days} = 0.009$, Fig. 4).

4. Discussion

Several adverse effects of acute nanoplastics exposure have been revealed, such as oxidative stress induction, growth rate inhibition or decreasing lifespan (Wright et al., 2013; Jeong et al., 2016; Chae and An, 2017). In chronic nanoplastics exposure, the organism could physiologically adapt to exposure conditions and reach a new steady state or the situation may further deteriorate with the accumulation of plastic particles. In the present study, RNA sequencing only found a small number of DEGs, accounting for only about 0.6% of total genes in *D. pulex* after chronic PS nanoplastic exposure. At the same time, PCA and correlation analysis also indicated a similar expression pattern of the transcriptome between samples under control and PS nanoplastic exposure conditions (Fig. 1). These may indicate a moderate adaption of *D. pulex* to the nanoplastic exposure because the nanoplastics mainly cause the physical effect on the organism (Sul and Costa, 2014). Several physiological traits of *D. pulex* are reported to change with the effect of chronic 1 mg L^{-1} 75 nm PS nanoplastics exposure, including growth and reproductive abilities (Liu et al., 2019). In spite of the small number of genes differentially expressed, our results, obtained from both RNA sequencing and real-time PCR, revealed several key genes involved in substrate metabolism, growth regulation, reactive oxygen species (ROS) metabolism and sex

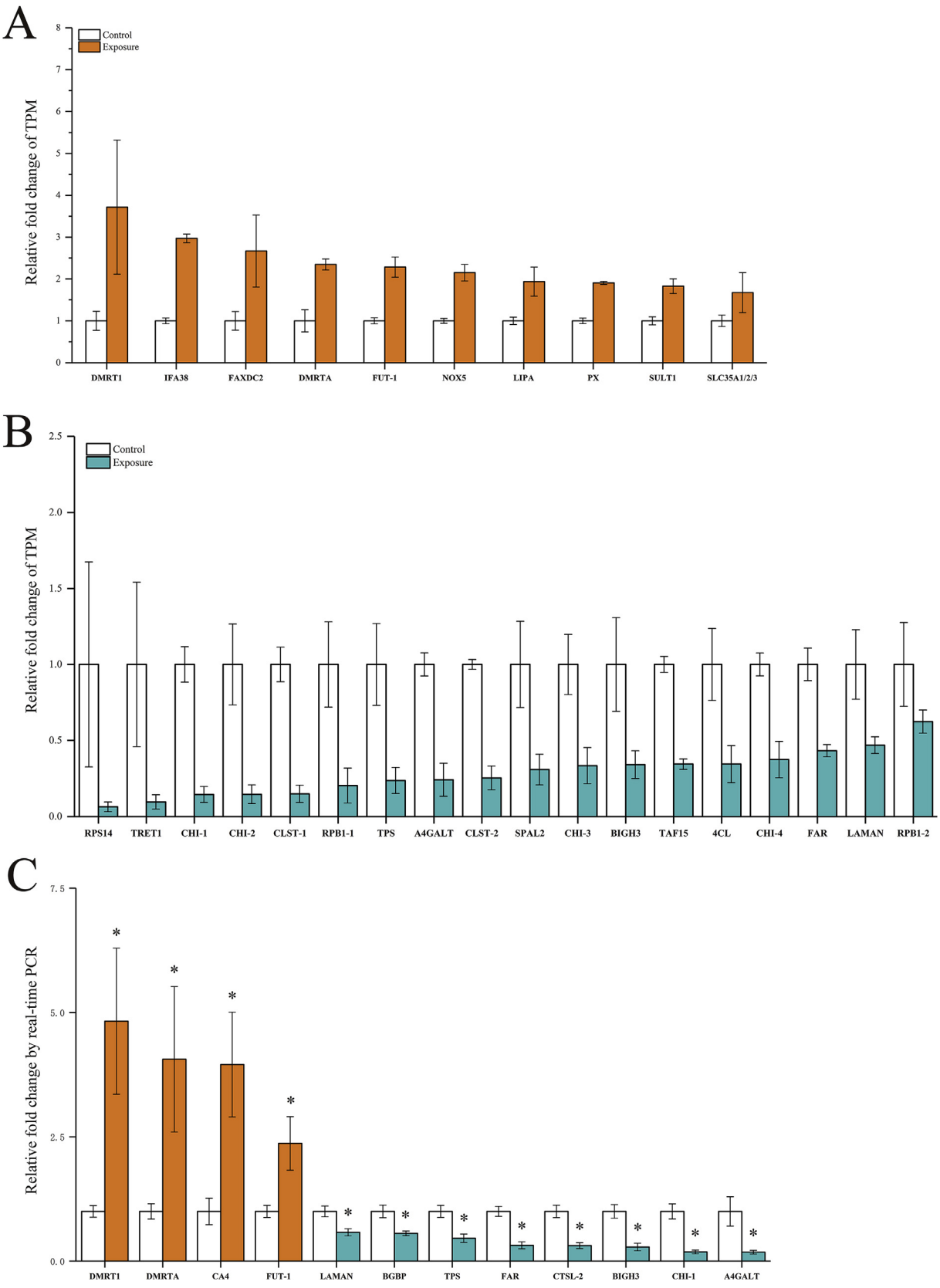

Fig. 3. Relative expression levels of key differentially expressed genes (DEGs) from samples of the polystyrene (PS) exposure group by RNA sequencing (A and B) and real-time PCR (C). Data are presented as mean ± SEM. Relative expression levels of DEGs in the control group are in white, the relative expression levels in the PS exposure group are in orange (upregulated) or green (downregulated). In A and B, the relative expression level of DEGs was calculated with TPM and all genes showed significant change with the threshold of $P < 0.05$ and fold change >2. In C, the star symbol indicated the significant change of gene expression from exposure group compared with that from control group, $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Results of Gene Ontology (GO) enrichment of differentially expressed (DE) genes.

GO ID	GO Term	Term type	DE genes number	*P*-adjust
GO:0006032	chitin catabolic process	Biological process	4	0.044
GO:1901072	glucosamine-containing compound catabolic process	Biological process	4	0.044
GO:0046348	amino sugar catabolic process	Biological process	4	0.044
GO:0034641	cellular nitrogen compound metabolic process	Biological process	1	0.044
GO:0006026	aminoglycan catabolic process	Biological process	4	0.044
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	Molecular function	8	0.011
GO:0016798	hydrolase activity, acting on glycosyl bonds	Molecular function	8	0.021
GO:0004568	chitinase activity	Molecular function	4	0.044

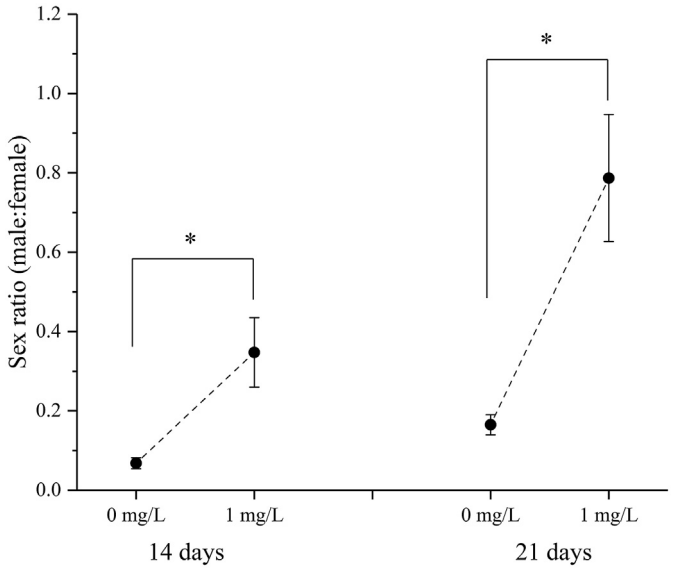

Fig. 4. Changes in the sex ratio of the *Daphnia pulex* population after 14 days or 21 days polystyrene (PS) exposure. Data are presented as mean ± SEM. Significant difference was set at $P < 0.05$.

differentiation that account for the molecular basis of the effect caused by chronic PS nanoplastic exposure on *D. pulex*.

When nanoplastics are ingested by the organism, blockages are caused in the digestive tract, which result in decreased feeding and energy intake (Chae and An, 2017). Furthermore, nanoplastics remain in organisms longer than other sizes of plastics. A study showed that *Mytilus edulis* decreased filtering activity after the intake of 30 nm of PS nanoplastic in the presence of food (Wegner et al., 2012). The decrease in feeding causes starvation in organisms, which then affects energy metabolism. In the present study, 21 days PS nanoplastic exposure inhibited the expression levels of regulated genes involved in trehalose transport and metabolism, *TRET1* and *TPS*. *TRET1* is an important trehalose-specific facilitated transporter and *TPS* can synthesize trehalose with glucose (Takahiro et al., 2007). Inhibition of *TRET1* and *TPS* expression, especially decrease by 50% in *TPS* expression, indicated a decreased content of trehalose in *D. pulex* after chronic PS nanoplastic exposure. Trehalose is an important and widely existing source of sugar in organisms, and works in protecting organisms against the effects of environmental stressors (Tapia and Koshland, 2014). In fact, the trehalose level is closely related with metabolic homeostasis and the inhibition of these two genes indicated that metabolic homeostasis shifted and *D. pulex* was in the state of malnutrition after 21 days nanoplastic exposure (Friedman, 1978). However, this state did not cause a response to starvation as no compensatory response of genes for other glucose metabolisms was observed (Supplementary file 3). This meant that the effect of chronic PS nanoplastic exposure on energy intake was not compensated by physiological regulation and, therefore, growth or reproductive ability of *D. pulex*, which is energy dependent, is further affected by chronic PS nanoplastic exposure.

Inhibition of chitin metabolism by chronic PS nanoplastic exposure is indicated by the decreasing expression of multiple *CHI* isoforms, which also contributes to the decreased growth or reproduction of *D. pulex* (Fig. 3 and Table 2). *CHI* is responsible for the biosynthesis of chitin, which is an important structural component. Therefore, *CHI* is a key enzyme gene for molting, which is an essential process for the growth, development, and reproduction, of *Daphnia* (Qi et al., 2018). Our results provided molecular evidence that chronic PS nanoplastic exposure can interfere with exoskeleton maintenance and molting of *D. pulex*, by affecting the expression of *CHI*. Removing the exoskeleton is necessary for releasing a new brood of neonates, therefore, chronic PS nanoplastic exposure may prolong the time to first eggs and decrease fecundity by inhibiting the expression of chitinase forms (Liu et al., 2019). A similar mechanism has been reported where other toxicants affect the reproduction of *Daphnia* by the direct interference of chitinase activity or expression (Poynton et al., 2007). Interestingly, chitin metabolism, especially *CHI* expression, is affected by trehalose metabolic genes (Zhao et al., 2016). Therefore, we speculate that inhibition of chitinase expression may be the result of energy intake limitation, but not the direct interference of nanoplastics with the expression of chitinase. This mechanism then contributes to the inhibition of growth and reproduction of *D. pulex*.

The exposure also caused a significant inhibition on the expression of growth-related genes in *D. pulex* in addition to inhibiting genes involving in trehalose and chitin metabolism. First, we noticed the down-regulation of *BIGH3* (Fig. 3). *BIGH3*, known also as *TGFBI*, participates in tissue growth of organisms and, in molecular function studies, suppression of *BIGH3* resulted in the maldevelopment of tissues in organisms (Ahlfeld et al., 2016). Therefore, in *D. pulex* exposed to PS nanoplastics, decreased growth can also be attributed to the inhibition of *BIGH3* expression. Another target of inhibition is *CTSL*, a proteolytic enzyme with an important role during organism development or growth (Kane and Gottesman, 1990). In nematodes, *CSTL* is essential for molting and feeding (Sarwar et al., 2002). Interestingly, in a study about the toxic effect of Zn on *D. magna*, inhibited *CTSL* expression was also observed and considered to be a protective mechanism (Vandegehuchte et al., 2010). Similarly, inhibited *CTSL* expression can be an important protective response to chronic PS nanoplastic exposure while resulting in a weaker growth rate.

With chronic PS nanoplastic exposure, long-chain fatty acids metabolism was regulated in the form of down-regulated *FAR* expression upregulated *LIPA* and *FAXDC2* expression. *FAR* encodes the protein that catalyzes the reduction of activated fatty acids to fatty alcohols, which may function as sex or communication pheromones, which is important for mating in *Daphnia* or the avoidance of inbreeding during sexual reproduction (Ken'Ichi et al., 2003;

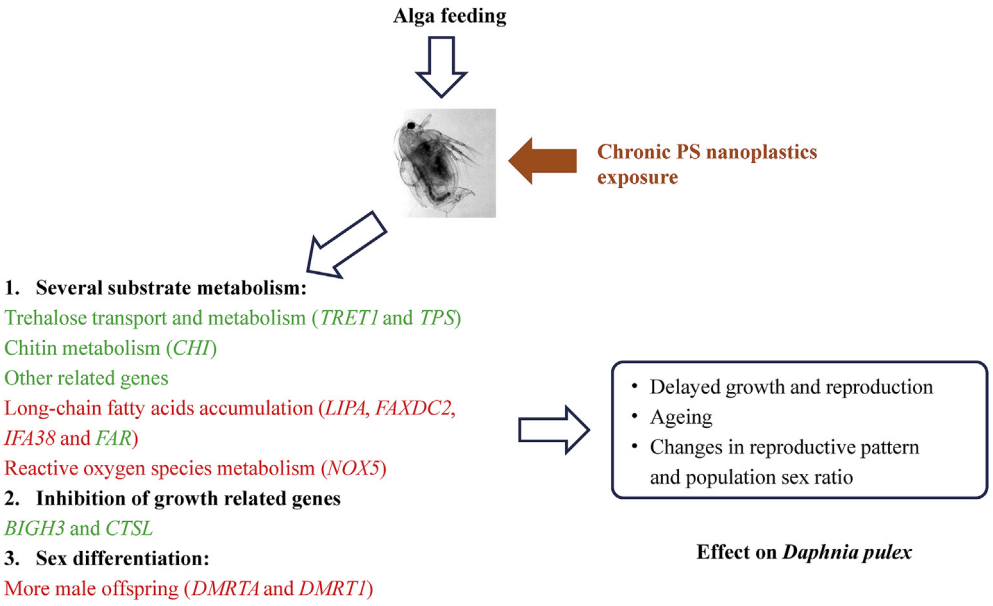

Fig. 5. Summary of the effects of chronic polystyrene (PS) nanoplastic exposure on *Daphnia pulex* from RNAseq results. The red text indicates enhanced functions or key upregulated genes; the green text indicates inhibited functions or key downregulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Winsor and Innes, 2010; Geung-Hwan et al., 2014). Inhibited *FAR* expression may thus affect the mating success between male and female *D. pulex*. *LIPA* is an important enzyme gene which allows for the organism to use triolein as a carbon source and can catalyze the transesterification of fats to alter the composition and properties of fats (Kok et al., 1996; Decagny et al., 1998). Notably, enzymatic transesterification is a more effective manner to use lipid with mild reaction condition and less thermal damage during the reaction (Mukherjee, 2009). Thus, the regulation of *LIPA* could be a potential compensation for *D. pulex* to reduce energy consumption in metabolism. Upregulated *FAXDC2* is related to the enhanced synthesis of sphingolipid (Jin et al., 2016). It has been reported that increased sphingolipids were associated with a decrease in ceramides and in turn, represses reproductive development (Schmidt et al., 2017; Sengupta et al., 2017). Therefore, chronic PS nanoplastic exposure affected the mating and reproduction of *D. pulex* by altering long-chain fatty acid metabolism.

Nanoplastics can puncture cells and thus induce a defense mechanism of cells to maintain homeostasis. *NOX5*, which can produce superoxide anions in response to infectious stimuli, may participate in this process (Leto and Geiszt, 2006). In fact, *NOX5* is also important for growth, reproduction, and the innate immune system (Montezano et al., 2011; Bedard et al., 2012). Thus, chronic nanoplastics exposure may call up a regulation of *NOX5* expression to respond to the stimuli and the adverse effects including inhibition on growth or reproduction. However, enhanced *NOX5* is also related with increased ROS production. Furthermore, it has been reported that after 21 days 1 mg/L PS nanoplastic exposure, expression levels of several antioxidant genes, which encode the enzymes to remove ROS, were suppressed or constant (Liu et al., 2019). Our gene expression profiling also found that chronic PS nanoplastic exposure inhibited the expression of GST, an important antioxidant enzyme. Thus, chronic PS nanoplastic exposure may cause oxidative stress in *D. pulex*, as shown in other organisms (Harman, 1956; Bhattacharya et al., 2010; Chen et al., 2017).

In *Daphnia*, an important response to pollutants is reproductive modes shifting, which is: sexual reproduction changes into asexual reproduction in *Daphnia* exposed to adverse environments (Geung-Hwan et al., 2014). The asexual reproduction produces resting eggs and is important to the persistence of the population. However, this transformation of reproductive modes is usually with a change in the sex ratio of the population and is believed to be gene regulation dependent. Increased *DMRTA* and *DMRT1* expression has been shown to be important in the development of male traits and transformation of reproductive modes from asexual to sexual in *Daphnia* (Kato et al., 2011; Xu et al., 2014). Our results provided direct evidence that 21 days PS nanoplastic exposure activated the expression level of key genes, *DMRTA* and *DMRT1*, in *D. pulex* and then resulted in a change in the sex ratio of neonates (Figs. 3 and 4).

To aid the detection of nanoplastics in the environment and understanding of the mechanism of nanoplastic exposure, we have shown that several important genes involved in metabolism, growth regulation, ROS metabolism, and sex difference have distinct expression patterns in the classic ecotoxicology test organism, *D. pulex*, after 21 days PS nanoplastic exposure and these genes also have potential as candidate biomarkers (Fig. 3). Briefly, the main effect of chronic nanoplastic exposure on *D. pulex* can be attributed to the obstruction of energy intake, with shifting in expression in *TPS* and *TRET1* (Fig. 5). This obstruction may further affect the growth and reproduction of *D. pulex* by regulating the expression of key genes including *CHI*, *FAXDC2*, *FAR*, or *CTSL*. Importantly, chronic nanoplastic exposure can alter the expression of the sex determining genes to promote sexual reproduction and the change in the sex ratio of the neonate (Fig. 5). Our study provides a molecular explanation for the toxic mechanism of chronic exposure to nanoplastics on the freshwater model organism *D. pulex* and guides the direction for ecotoxicological studies on mechanistic and biochemical responses to nanoplastic exposure.

Declaration of interest

All authors stated that there are no competing interests.

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Appendix A. Supplementary data

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