Environmental Science & Technology

Article

Cite This: Environ. Sci. Technol. 2019, 53, 8426−8436

pubs.acs.org/est

Transgenerational Proteome Plasticity in Resilience of a Marine Copepod in Response to Environmentally Relevant Concentrations of Microplastics

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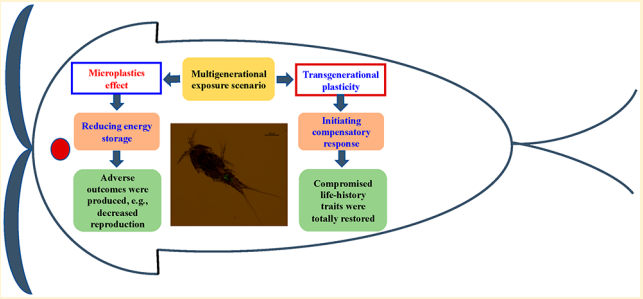
Supporting Information

ABSTRACT: Here, we examined the multigenerational effect of microplastics (6-μm polystyrene beads; with different environmentally relevant concentrations of 0.023 and 0.23 mg/L in seawater) on the marine copepod *Tigriopus japonicus* under two-generation exposure (F0−F1) followed by one-generation recovery (F2) in clean seawater. Also, the seven life-history traits (survival, sex ratio, developmental time of nauplius phase, developmental time to maturation, number of clutches, number of nauplii/clutch, and fecundity) were measured for each generation. Furthermore, to investigate within-generation proteomic response and transgenerational proteome plasticity, proteome profiling was conducted for the F1 and F2 copepods under the control and 0.23 mg/L microplastics treatment. The results showed successful ingestion of microplastics in F0−F1 under both exposure concentrations, while higher concentration (0.23 mg/L) of microplastics resulted in the significant reduction in survival rate, number of nauplii/clutch, and fecundity. However, the affected traits were totally restored in the recovery generation (F2). Proteomic analysis demonstrated that microplastics exposure increased several cellular biosynthesis processes and, in turn, reduced energy storage due to the trade-off, hence compromising survival and reproduction of the treated copepods in F1. Interestingly, the two-generational effect of microplastics in copepods had significant transgenerational proteome plasticity as demonstrated by increased energy metabolism and stress-related defense pathway, which accounts for regaining of the compromised phenotypic traits during recovery (i.e., F2). Overall, this study provides a molecular understanding on the effect of microplastics at a translational level under long-term multigenerational exposure in marine copepods, and also the transgenerational proteome plasticity is likely rendering the robustness of copepods in response to microplastics pollution.



INTRODUCTION

The world's oceans are suffering from conspicuous plastic litter pollution, and it has caused increasing concern at large scale.[1] In 2010 alone, 192 coastal countries generated about 275 million metric tons of plastic waste, of which 4.8 to 12.7 million metric tons ended up in the ocean, and moreover under the business-as-usual scenario, the cumulative quantity of plastic waste inputs into the marine environment is predicted to increase by an order of magnitude by 2025.[2] Under ultraviolet radiation and mechanical abrasion, plastics entering the marine environment will eventually become small pieces ranging from a few μm to 5000 μm (5 mm) in size, termed "microplastics".[3,4] Notably, it has been estimated that at least 14.9 trillion microplastics particles (weighing 93300 t) are floating on the global ocean surface.[5] Microplastics are pervasive and difficult to biodegrade and thus can persist for very long time periods in the ocean, consequently displaying a negative impact on the marine ecosystem.[6]

Microplastics pollution has become a serious environmental problem for the global marine ecosystem, recalling that microplastics particles are ubiquitous in a marine environment and, more importantly, can be ingested by a multitude of marine biota in the laboratory/environment including zooplankton, copepods, crustaceans, bivalves, polychaetes, and fish.[3,7−15] Consumption of microplastics can give rise to adverse health effects such as excessive inflammation,[7,9] oxidative stress,[13,15] energetic depletion,[9] reduced feeding,[10] decreased survival,[8] and reproductive suppression.[12,14] To summarize, microplastics pollution has broadly exerted a range of detrimental impacts on marine animals and therefore compromised their fitness in the ocean; nevertheless, most microplastics exposure studies are partially focused on short-

Received: April 25, 2019
Accepted: June 17, 2019
Published: June 17, 2019

DOI: 10.1021/acs.est.9b02525
Environ. Sci. Technol. 2019, 53, 8426−8436

term or single-generation effects under far more than environmentally realistic-concentration exposure.[7,10−13] Furthermore, little information is available for giving a mechanistic understanding concerning microplastics effects in marine organisms. Correspondingly, any molecular investigation concerning microsplastics effects on marine animals under the long-term multigenerational exposure to environmentally realistic concentrations is particularly and urgently important, and it is required to have a better ecological assessment for microplastics pollution in a marine environment.

In the field, it is very common for marine biota to be faced with environmental stressors including microplastics pollution for many generations. Marine organisms can respond to environmental stressors by modifying the expression of traits during development and also changing traits across multiple generations.[16] "Within-generation effect" and "transgenerational plasticity" (i.e., across-generation plasticity) have been demonstrated in marine biota in several examples of previous research.[17−19] It is important that transgenerational plasticity occurs whenever the parent environment directly gives rise to, without altering DNA sequence, significant changes in shaping the reaction norms of offspring.[19] Many works have shown that transgenerational plasticity can potentially render the offspring generation having increased tolerance in response to environmental stressors such as contaminants and food shortage.[17,19,20] Perhaps transgenerational plasticity will play an important role in marine organisms' response in response to microplatics pollution. However, no previous study has been undertaken to examine the transgenerational effect of microplastics exposure on marine organisms in view of mechanistic research.

Toxicoproteomics, a relatively new approach that applies the global proteomic technologies to toxicology, enables us to detect key proteins/processes affected by environmental stressors including microplastics pollution.[21] Many studies have used proteomic-based techniques to explore the molecular-level responses of marine organisms in response to environmental stressors in a marine environment.[22−24] For example, in the sentinel copepod *Tigriopus japonicus* for marine stress ecology, tandem mass tag (TMT)-based proteomics was applied to investigate the response mechanism in response to cadmium pollution,[23] revealing that this metal treatment has several toxic effects as demonstrated by decreased nutrient absorption, dysfunction in cellular redox homeostasis, metabolic deregulation, and oxidative stress, giving rise to growth retardation and reproduction limitation in the copepod.

In this study, we selected the marine copepod *T. japonicus* as a model species to examine the effect of environmentally relevant level microplastics pollution (concentrations of 0.023 and 0.23 mg/L[15,25] in seawater solutions) under two-generation exposure (F0−F1). To explore the copepod's transgenerational plasticity in response to microplastics, all the animals were subsequently recovered in clean seawater for one generation (F2). Seven important life-history traits including survival rate, development time of nauplius phase, development time to maturation, number of clutches, number of nauplii/clutch, and fecundity were examined for each generation. In addition, the proteome profiles were analyzed for the F1 and F2 copepods under the control and 0.23 mg/L microplastics treatment. Thus, our work was primarily aimed to test the hypotheses as follows: (1) microplastics will adversely affect the important life traits of the copepods and probably decrease their fitness in a marine environment; (2) the copepods will display transgenerational plasticity in response to microplastics exposure; and (3) the trade-off strategy will probably play a crucial role in the response mechanism of the copepods in response to microplastics pollution.

MATERIALS AND METHODS

Copepod Maintenance. The copepod *T. japonicus*, collected from the rocky pools of the intertidal zone in Xiamen Bay, People's Republic of China (N 24°25.73′, E 118°6.34′) in 2007, has been maintained in the laboratory at 22 °C with a 12:12 h light:dark cycle. Copepods were fed with an equal mixture of three microalgae, *Isochrysis galbana*, *Platymonas subcordiformis*, and *Thalassiosira pseudonana*, at a density of 5×10^7 cells/L. All seawater used in the experiments was filtered through 0.22-μm polycarbonate membrane, and the seawater characteristics were described as dissolved oxygen 6.2−6.7 mg/L, salinity 29−30 practical salinity unit (PSU), and pH 8.0−8.1. Exposure experiments were performed in temperature-controlled incubators at 22 ± 1 °C with a 12:12 h light:dark cycle.

Polystyrene Microbead Ingestion. To measure whether the copepods could ingest microplastics, a three-day exposure was employed. In this ingestion test, we used fluorescently labeled (with an excitation of 441 nm/emission 486 nm) polystyrene microbeads (2.7% solids suspension; Polyscience, Inc., Warrington, PA, USA) with a diameter of 6 μm (similar in size to live food organisms) as our representative microplastics (Supporting Information (SI) Table S1). Its size distribution was analyzed using Beckman Coulter Z2 (Beckman Coulter, Inc., Pasadena, CA, USA), and the microbeads' diameter was calculated as 5.846 ± 0.329 μm (Figure S1). Also, the polymer type was confirmed using Raman spectroscopy (i-Raman Plus, B&W Tek, Newark, USA); the detailed information is provided in SI Text S1 and Figure S1. Fluorescently labeled polystyrene beads were added in the seawater to achieve nominal concentrations of 0.023 mg/L (194 particles/mL) and 0.23 mg/L (1937 particles/mL), both of which were environmentally realistic.[15,25] The testing solutions were sonicated for 30 min before the experiment and daily renewed (90% of the total working volume). *T. japonicus* adults (50 copepods) were subjected to 0.023 and 0.23 mg/L microplastics treatments, respectively, in the polycarbonate bottles with 150 mL of seawater solution (three replicates per concentration treatment). The microalgal culture was provided as prey for the copepods at a density of approximately 5×10^7 cells/L during the exposure.

During the exposure, 50 copepods were collected each day to observe whether microplastics had been ingested by the treated animals under a fluorescent microscope (M165FC, Leica, Wetzlar, Germany; 7.3−120 × magnification), and then the sampled copepods were digested immediately using the method provided by Dehaut.[26] Briefly, 10% KOH solution (Aladdin, Shanghai, China) was used to digest the copepods at 60 °C for 24 h. Afterward, the number of beads was counted under a fluorescent microscope to quantify microplastics concentration in the copepods. Each microplastics concentration treatment had nine polycarbonate bottles (50 copepods per container) and three containers of which were randomly sampled for daily copepod digestion. The testing solutions in each container were also collected daily for counting the beads in the seawater. In addition, to examine whether the polycarbonate bottle can absorb microbeads during multi-

DOI: 10.1021/acs.est.9b02525
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generational exposure, another three-day testing was employed as described above for the ingestion testing, but, in this case, no copepods were added. During the testing, the seawaters were daily collected for microbead concentration counting. The detailed procedures were provided in SI Text S2.

Two-Generation Exposure. In two-generation exposure, we used unlabeled, plain polystyrene beads with a diameter of 6 μm (2.5% solids suspension; Polyscience). The size distribution was analyzed using Beckman Coulter Z2 (Figure S1), and its diameter was 5.791 ± 0.727 μm. Meanwhile, its polymer type was confirmed using Raman spectroscopy (SI Text S1 and Figure S1). The microplastics concentrations used in this case were the same as those for the three-day ingestion testing. Briefly, the 6-μm plain microbeads were tested at the concentrations of 0.023 mg/L (194 particles/mL) and 0.23 mg/L (1937 particles/mL), together with a control without microplastics addition. The multigenerational exposure was carried out according to our previous work.[23] Fifty newly hatched nauplii (<24 h) were maintained in a polycarbonate bottle with 150 mL of seawater solution in triplicate (i.e., a total of 150 nauplii per concentration treatment). These nauplii were maintained under the above-mentioned conditions until adult females produced egg sacs. We renewed the testing solutions (50% the working volume for the nauplius stage and 90% for the copepodite stage) daily and supplied the algae at a density of approximately 5×10^7 cells/L as a dietary source for the copepods during exposure. Seven life-history traits including survival (%), sex ratio, developmental time of nauplius phase, developmental time to maturation, number of clutches, number of nauplii/clutch, and fecundity were examined for each individual copepod in each generation.

The survival rate (%) and sex ratio were calculated after exposure. The development period was observed daily under a stereomicroscope to measure the time of development from nauplii to copepodite (i.e., the nauplius phase) and from nauplii to adults bearing egg sacs (maturation). To measure the number of clutches, number of nauplii/clutch, and fecundity (offspring production), six females bearing an egg sac per replicate were individually maintained in a new 6-well plate with 8 mL of working solution. These females were reared under the aforementioned conditions for 12 d. The resulting nauplii and unhatched clutches were counted under a stereomicroscope and removed from the test group.

For the second generation (F1), 50 nauplii produced by the first generation (F0) were randomly transferred to the polycarbonate bottles with 150 mL of working solution. The experimental conditions were the same as those for the F0 testing, and the multigenerational exposure was conducted until the F1 nauplii developed to maturation.

Recovery. After two-generation exposure (F0−F1), the nauplii produced by F1 were restored in clean seawater until they developed to maturation, which corresponds to recovery for one generation (F2). The experimental protocol was the same as the one used for the two-generation exposure of F0−F1. Briefly, 50 nauplii (<24 h) were reared in polycarbonate bottles with three replicates. Also, the seven life traits were examined for each individual copepod during the recovery generation (F2).

Proteomic Analysis. To analyze the proteome profiles in the F1 and F2 adult copepods, a two-generation exposure and recovery testing were simultaneously performed. Briefly, the copepods were exposed to the control and 0.23 mg/L microplastics treatment for two generations (F0−F1) and subsequently recovered in clean seawater for one generation (i.e., F2). The relatively high but still environmentally realistic microplastics treatment (0.23 mg/L) was selected for proteomics profiling in the present study, as the copepod's life traits were significantly affected by this concentration exposure. The detailed experimental procedure was the same as the protocol described above, but in this case, 200 copepods were reared in polycarbonate bottles with 450 mL of working solution, and each treatment was repeated in triplicate. After exposure, 200 copepods per replicate were pooled for each concentration treatment and immediately stored at −80 °C for proteomic analysis (three biological replicates per treatment).

Protein Labeling and Strong Cation Exchange (SCE) Fractionation. Proteins were extracted, quantified, and labeled using the TMT-6plex kit (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Protein samples were labeled with the TMT tags as follows: tags 126, 127, and 128 were designated to label the three replicates for the control in F1 ("F1-C") with tags 129, 130, and 131 for the microplastics treatment in F1 ("F1-M"). The F2 generation was labeled in the same way. Two runs of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were conducted for F1 and F2, respectively. All six samples were pooled and dried using vacuum centrifugation. The peptide mixtures were fractionated using SCE chromatography. The detailed procedures are provided in SI Text S3.

LC-MS/MS Analysis and Database Searching. A Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer coupled with an EASY-nLC 1000 UPLC system (ThermoFisher Scientific) was used to analyze the peptides. The peptide sequences obtained were searched against the NCBI_*Tigriopus* proteome (1746 sequences) and *T. japonicus* transcriptome (i.e., a total of 46369 sequences combined from a previous work[27] and our published data[28]). The detailed protocols are provided in SI Text S4.

Bioinformatic Analysis. Differentially expressed proteins (DEPs) were identified only when the folding change was greater than 1.30 (up-regulated) or less than 0.77 (down-regulated), based on a 95% confidence level calculated from pairwise analysis of three replicates.[29] DEPs were annotated into three categories based on Gene Ontology (GO) terms: biological process, cellular component, and molecular function. Protein domain function was defined by InterProScan based on the protein sequence alignment method (http://www.ebi.ac.uk/interpro/). The protein pathway was annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Enrichment analysis was conducted for GO terms, protein domain, and the KEGG pathway using the Database for Annotation, Visualization and Integrated Discovery. The statistically significant enhancement was determined by the Fisher exact test at p value being <0.05. Hierarchical clustering analysis was conducted for the DEPs based on the significant enrichments using Gene Cluster 3.0 software.

Biochemical Parameter Determination. To confirm the proteomic results, we investigated several biochemical parameters in the F2 copepods. We examined the enzymatic activities of trypsin, cellulase, and carboxylesterase following the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The detail procedures are provided in SI Text S5.

Statistical Analysis. All data were expressed as mean ± standard deviation (n = 3). All statistical analysis was conducted using the SPSS 19.0 software. One-way ANOVA

DOI: 10.1021/acs.est.9b02525
Environ. Sci. Technol. 2019, 53, 8426−8436

and the Fisher least significant difference test were used to evaluate whether the means differed significantly among different treatments. Significant differences were indicated at $p < 0.05$.

RESULTS

Polystyrene Bead Ingestion. The microplastics beads were observed in the guts of all treated copepods (Figure 1),

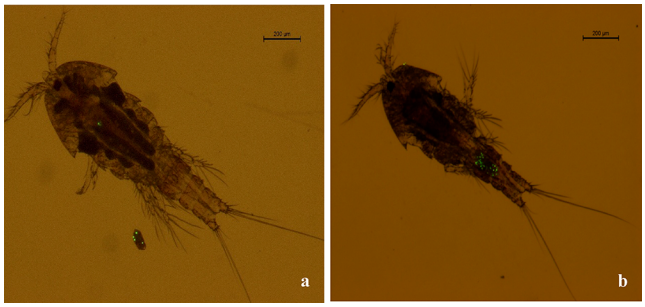

Figure 1. Fluorescently labeled microbeads of 6-μm diameter could be ingested by adult *Tigriopus japonicus* exposed to two microplastics concentrations (0.023 (a) and 0.23 mg/L (b)).

suggesting that the copepods could ingest microplastics. Microplastics concentrations in the copepods under both 0.023 and 0.23 mg/L exposures were not changed significantly within 3 days, resulting in 1.71−2.39 and 34.53−41.62 particles/copepod, respectively (Table 1); thus, microplastics

Table 1. Microplastics Accumulation in the Copepod *Tigriopus japonicus* under Three-Day Exposure to Two Microplastics Concentrations (0.023 and 0.23 mg/L)[a]

	microplastics accumulation (particles/copepod)		
treatment(mg/L)	day 1[a]	day 2[a]	day 3[a]
0.023	1.98 ± 0.45[a]	1.71 ± 0.36[a]	2.39 ± 0.69[a]
0.23	41.62 ± 9.41[b]	38.40 ± 1.97[b]	34.53 ± 7.37[b]

[a]Data are described as mean ± standard deviation ($n = 3$). Different letters indicate a significant difference among different microplastics treatments at $p < 0.05$.

concentrations probably reflected its accumulation by the treated copepods under two-generation exposure. In addition, the measured contents of polystyrene beads in seawater were comparable to nominal concentrations for both microplastics treatments during the three-day exposure (Table S2); the average differences were less than 10% in all cases. The polycarbonate bottle hardly absorbed the microbeads during exposure (Table S3). Overall, we can conclude that the nominal concentrations corresponded to the real exposure conditions during two-generation exposure. Under both microplastics exposures, the treated copepods consumed approximately 40% microplastics in seawater (Table S2); however, most of them are likely present in fecal particles.

Two-Generation Exposure. We investigated seven life-history traits of *T. japonicus* during two-generation exposure. The low microplastics treatment (0.023 mg/L) negligibly affected the seven life traits in *T. japonicus* for F0−F1. As for the high treatment (0.23 mg/L), microplastics did not significantly affect sex ratio, number of clutches, nauplius phase, and development time to maturation during F0−F1 (Figures S2 and S3); however, the high microplastics exposure significantly decreased ($p < 0.05$) survival rate, number of nauplii/clutch, and total fecundity for both generations (Figures 2 and 3).

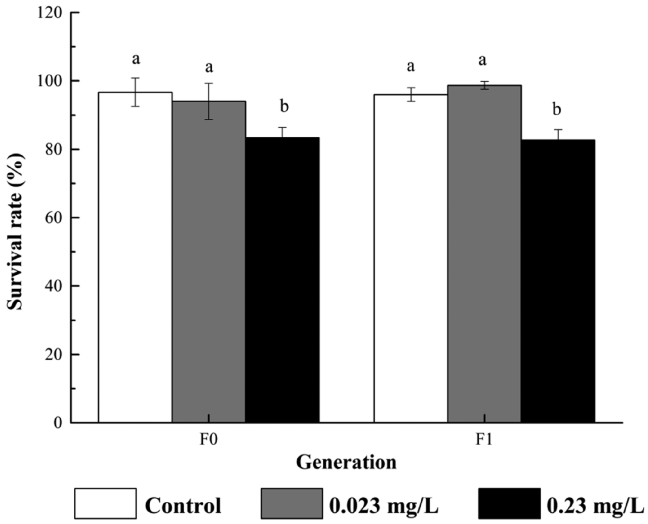

Figure 2. Survival rate in *Tigriopus japonicus* of F0 and F1 exposed to different microplastics treatments (control, 0.023, and 0.23 mg/L). Data are described as mean ± standard deviation ($n = 3$). Different letters indicate a significant difference among different treatments at $p < 0.05$.

Recovery. When all the copepods were transferred in clean seawater for one generation, seven traits were not significantly different among treatments (Table 2). The affected traits were completely regained during one generation recovery, highlighting that microplastics (grand) parental exposure did not display a transgenerational effect on the copepods at the phenotypic level.

Proteome Profiles. The two LC-MS/MS runs produced 239629 and 240631 peptide spectra, corresponded to 3568 and 3237 proteins, respectively (Table S4). Subsequently, 4058 proteins were identified from the combined data, and an additional 2747 overlapped (Figure S4a). Due to microplastics treatment, the DEPs were quantified as 35 and 374, respectively, for F1 and F2 (Tables S5−8); also there were few common proteins in the DEPs between F1 and F2 (Figure S4b). Our proteome profiling displayed high reproducibility because the average relative standard deviation of quantified proteins (which was analyzed for three replicates under each case) was less than 0.1 (Figure S5). The DEPs in each case were mainly classified into a metabolic process, cellular process, and single-organism process (Figure S6).

To understand the functional differences in up-regulated and down-regulated proteins, we analyzed the quantified proteins separately for GO terms, protein domain, and KEGG pathway enrichment-based clustering analysis (Figures 4 and 5; the detailed information is also provided in Tables S9 and S10).

For the second generation (F1), all the enriched processes were constructed by up-regulated proteins. In GO terms, several processes including translation (GO:0006412), peptide biosynthetic process (GO:0043043), gene expression (GO:0010467), cytoplasmic part (GO:0044444), ribosome (GO:0005840), GTPase activity (GO:0003924), and others were increased by microplastics treatment. As for the KEGG pathway and protein domain, the ribosome (dme03010), C-type lectin-like/link domain (IPR016186), and galactose

DOI: 10.1021/acs.est.9b02525
Environ. Sci. Technol. 2019, 53, 8426−8436

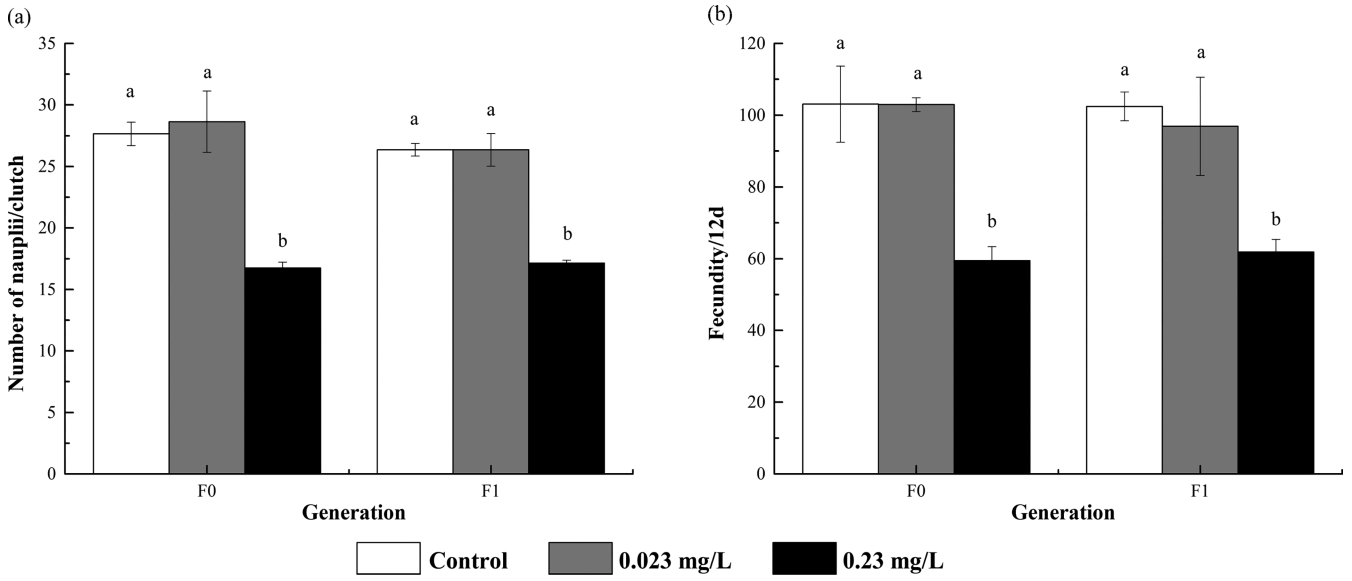

Figure 3. Number of nauplii/clutch (a) and fecundity (b) in *Tigriopus japonicus* of F0 and F1 exposed to different microplastics treatments (control, 0.023, and 0.23 mg/L). Data are described as mean ± standard deviation (n = 3). Different letters indicate a significant difference among different treatments at $p < 0.05$.

Table 2. Seven Life-History Traits in *Tigriopus japonicus* of the Recovery Generation (F2) after Premultigenerational Exposure to Different Microplastics Concentrations (Control, 0.023, and 0.23 mg/L)[a]

	life history traits						
treatment (mg/L)	survival (%)[a]	nauplius phase (d)[a]	development time (d)[a]	number of nauplii/clutch[a]	fecundity[a]	number of clutches[a]	sex ratio[a]
control	95.33 ± 1.15[a]	6.21 ± 0.47[a]	15.94 ± 0.36[a]	26.44 ± 0.27[a]	92.0 ± 5.3[a]	3.56 ± 0.10[a]	0.82 ± 0.15[a]
0.023	92.00 ± 5.29[a]	6.51 ± 0.23[a]	15.98 ± 0.09[a]	26.52 ± 0.25[a]	92.0 ± 5.3[a]	3.67 ± 0.34[a]	0.97 ± 0.34[a]
0.23	90.67 ± 3.06[a]	6.48 ± 0.03[a]	15.62 ± 0.20[a]	26.19 ± 0.13[a]	92.0 ± 5.3[a]	3.50 ± 0.29[a]	0.79 ± 0.09[a]

[a]Data are described as mean ± standard deviation (n = 3). Different letters indicate a significant difference among different microplastics treatments at $p < 0.05$.

oxidase/kelch, beta-propeller (IPR011043) were significantly up-regulated ($p < 0.05$) under microplastics exposure.

In the recovery generation (F2), many important cellular processes were enriched by up-regulated proteins that were related to the biological process, cellular component, molecular function, KEGG pathway, and protein domain. For example, the cell redox homeostasis (GO:0045454), carbohydrate metabolic process (GO:0005975), glucosamine-containing compound metabolic process (GO:1901071), chitin metabolic process (GO:0006030), chromosome (GO:0005694), extracellular region (GO:0005576), structural constituent of cuticle (GO:0042302), structural molecule activity (GO:0005198), hydrolase activity (hydrolyzing O-glycosyl compounds) (GO:0004553), chitin binding (GO:0008061), amino sugar and nucleotide sugar metabolism (dme00520), glycosaminoglycan degradation (dme00531), lysosome (dme04142), chitin binding domain (IPR002557), histone-fold (IPR009072), C-type lectin-like/link domain (IPR016186), carboxylesterase (type B) (IPR002018), glycoside hydrolase (catalytic domain) (IPR013781), and others were significantly increased ($p < 0.05$) by transgenerational exposure to microplastics. In the case of down-regulated proteins, many important processes enriched involved in the categories of the biological process, cellular component, molecular function, KEGG pathway, and protein domain; that is to say, the response to oxidative stress (GO:0006979), proteolysis (GO:0006508), nucleotide biosynthetic process (GO:0009165), microtubule (GO:0005874), antioxidant activity (GO:0016209), endopeptidase activity (GO:0004175), serine-type endopeptidase activity (GO:0004252), longevity regulating pathway-multiple species (dme04213), pyrimidine metabolism (dme00240), HSP20-like chaperone (IPR008978), EGF-like domain (IPR000742), serine proteases (trypsin domain) (IPR001254), and so on, were significantly decreased ($p < 0.05$) by microplastics pre-exposure. Thus, although the copepods' phenotypic traits were totally recovered in clean water, the proteomic analysis demonstrated significant transgenerational plasticity.

Biochemical Parameters. Proteomic data was verified using a biochemical analysis of several enzymes in the F2 copepods (Figure S7); as in terms of both changes in direction and magnitude, the enzymatic activities from biochemical analysis were accordant with our proteome data. For example, the protein expression and enzymatic activity of cellulase were increased by 1.33 and 1.69 times, respectively, in the copepods of F2 pre-exposed to high microplastics (0.23 mg/L) concentration.

DISCUSSION

Despite the numerous amount of research on microplastics effects in marine animals,[8−10,12−15,30,31] very few works have been performed to explain the molecular mechanism of how a marine animal will modulate in response to microplastics stress, particularly in the case of long-term multigenerational exposure to environmentally relevant concentrations. It should

DOI: 10.1021/acs.est.9b02525
Environ. Sci. Technol. 2019, 53, 8426−8436

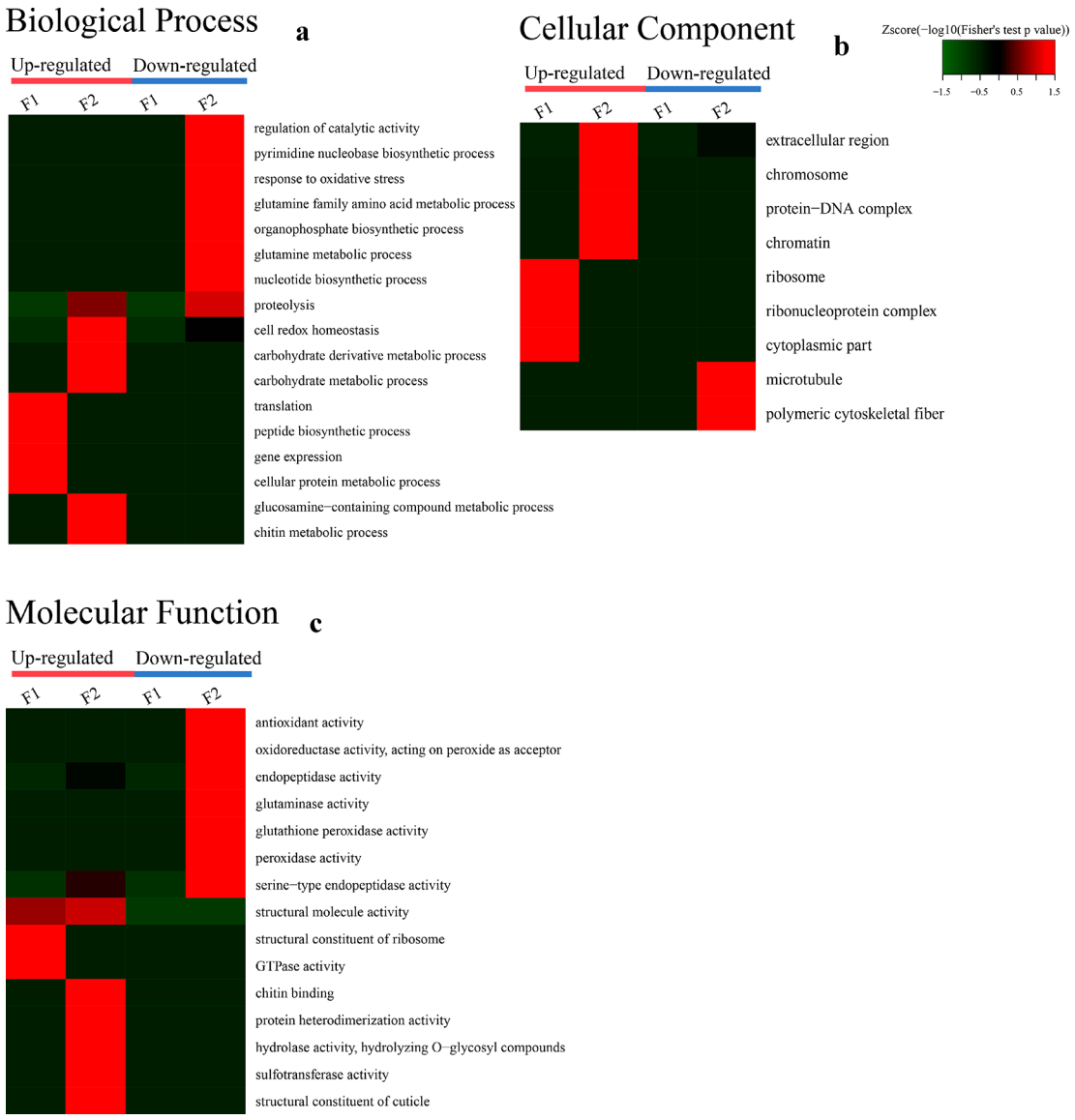

Figure 4. Hierarchical clustering analysis was conducted for differentially expressed proteins (DEPs) in F1 and F2 according to GO terms-based enrichment: (a) biological process, (b) cellular component, and (c) molecular function. The DEPs were divided into two groups (i.e., up-regulated and down-regulated) for both generations. The *p* values were transformed into Zscores for hierarchical clustering analysis. Zscore is shown in the color legend, and the red color indicates the significantly enriched terms.

be underlined that there has been only one previous study to investigate microplastics effects on the copepod *T. japonicus* under two-generation exposure;[8] however, in that previous study, two critical factors required more attention: first, the tested exposure concentrations are far exceeding environmentally realistic levels; second, the transgenerational response to microplastics treatment remains unknown as there is no recovery testing, and more importantly, the related molecular mechanism concerning microplastics effects is also not undertaken.[8] In our research, using the important ecological copepod *T. japonicus* as a model species, we first combined comparative proteome profiling with physiological observation (e.g., survival rate and reproductive performance) to investigate microplastics effects (i.e., within-generation effect and transgenerational plasticity) on a marine animal under multigenerational exposure to environmentally relevant levels. Particularly, our study used GO terms, protein domain, and KEGG pathway-based enrichment analyses to examine the critical processes/pathways involved in the copepod's response to microplastics exposure; thus, the present work provides a mechanistic understanding of how a marine copepod will respond to microplastic stress (Figure 6). After two-generation exposure, microplastics significantly affected the copepod's proteome as demonstrated by an increased expression of several cellular biosynthesis processes, and it might probably lead to an energy shortage in the exposed copepods due to the trade-off; as a result, the proteome change has been translated to the population-level response, i.e., decreased survival and compromised reproduction. After one generation of recovery, the survival and reproduction were completely regained, and then no transgenerational effect was observed at the phenotypic level. Nevertheless, the copepod's proteome displayed strong transgenerational plasticity so that many processes related to energy metabolism and stress defense were up-regulated. The interesting thing is that, on account of microplastics treatment, the proteome alteration in F1 (i.e.,

DOI: 10.1021/acs.est.9b02525
Environ. Sci. Technol. 2019, 53, 8426−8436

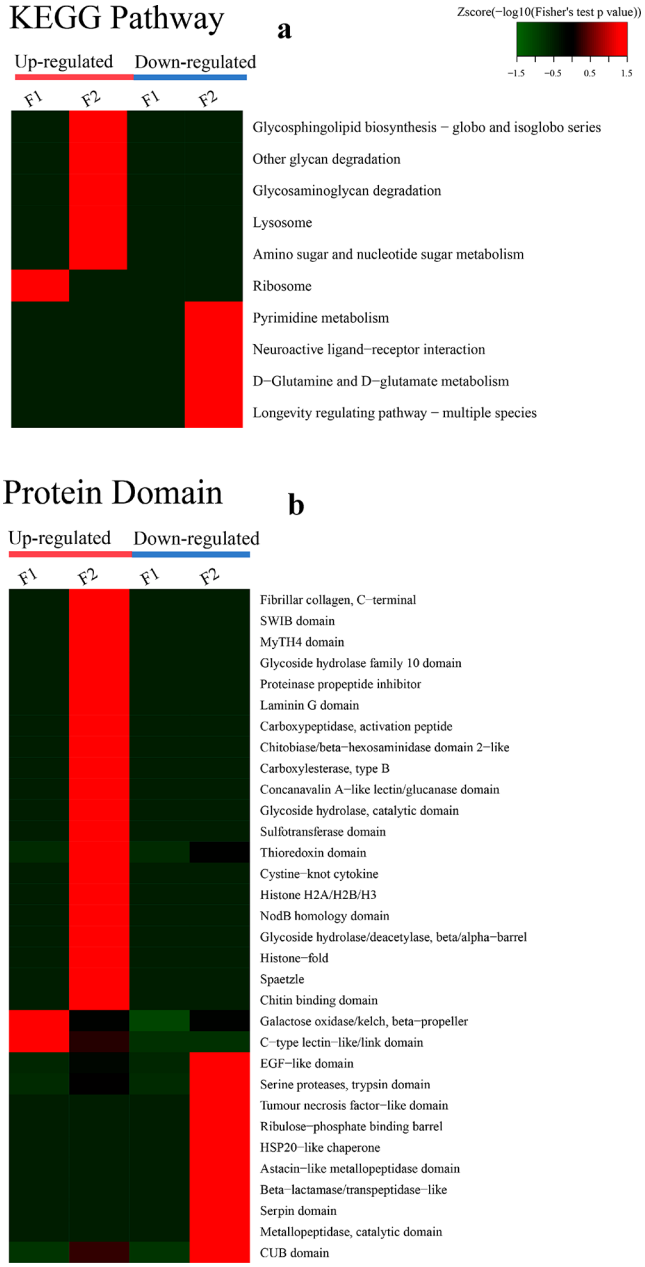

Figure 5. Hierarchical clustering analysis was conducted for differentially expressed proteins (DEPs) in F1 and F2 according to KEGG (a) and protein domain (b) based enrichment. The DEPs were divided into two groups (i.e., up-regulated and down-regulated) for both generations. The *p* values were transformed into Zscores for hierarchical clustering analysis. Zscore is shown in the color legend, and the red color indicates the significantly enriched terms.

within-generation response) was far less than that for the recovery generation of F2 (transgenerational plasticity), as the DEPs were quantified as 35 and 374, respectively, for F1 and F2. Taken together, transgenerational proteome plasticity had probably played an important role in the copepods to regulate in response to microplastics pollution.

Multigenerational Effects. In this study, microplastics concentrations in the treated copepods were approximately 2 and 38 particles/individual for the 0.023 and 0.23 mg/L treatments, respectively; therefore, the copepods could ingest microplastics, which is in accordance with several previous works.[8,10,12] As for the 0.023 mg/L treatment, all the life traits

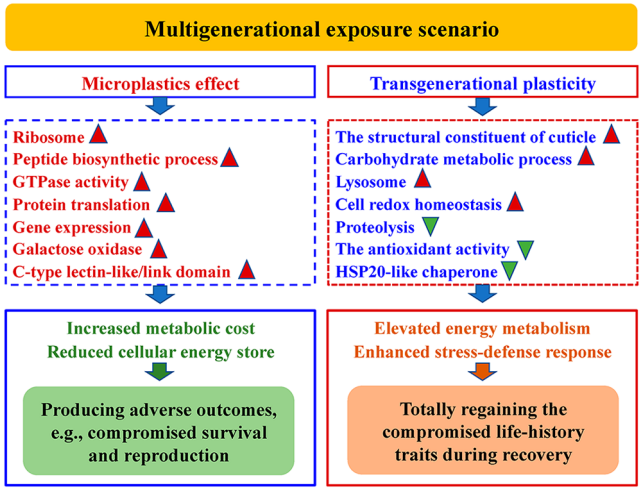

Figure 6. Schematic illustration demonstrating how the copepod had modulated in response to microplastics exposure. Note that microplastics effects had eventually increased metabolic cost and reduced energy storage, as a consequence, due to the energetic trade-off, producing adverse outcomes at the population level as exemplified by compromised survival and reproduction in the copepods of F1. However, the transgenerational proteome plasticity, as demonstrated by increased energy metabolism and stress-related defense pathway, had accounted for regaining of the compromised phenotypic traits during the recovery (i.e., F2).

were negligibly influenced by microplastics. However, the 0.23 mg/L microplastics exposure significantly decreased survival rate, number of nauplii per clutch, and total fecundity ($p < 0.05$), although sex ratio, nauplius phase, developmental time to maturation, and number of clutches were not impacted under such treatment. Similarly, many reports have revealed that microplastics exposure displays adverse effects on survival and reproduction in marine animals.[5,8,12−14,32] For example, in adult *T. japonicus*, 6 μm microplastics beads have significantly decreased fecundity after microplastics exposure.[8] Recent research on the marine copepod *Calanus helgolandicus* has also reported that polystyrene microplastics exposure reduces survival and reproduction of the copepods.[12] Thus, these studies have shown the negative effects of microplastics on marine copepod's survival and reproduction to the decreased energy intake by impeded feeding activity under exposure,[5,12] as feeding is fundamental to the energetic requirements of copepods. Our three-day microbead testing indicated that *T. japonicus* had ingested >2000 microplastics particles/copepod/day for the 0.23 mg/L microplastics treatment. Correspondingly, reduced feeding had likely occurred in the copepods under this level exposure, and as a result, their survival and reproduction were significantly compromised. Alternatively, the copepods might have expended more energy into enhanced maintenance costs under microplastics stress, as all the enriched processes in the exposed copepods of F1 were significantly increased by microplastics exposure in our proteomic analysis, and it can be metabolic cost and less quota was consequently provided for survival and reproduction in the treated animals; thus, the survival and reproduction were significantly decreased by microplastics exposure in this work.

For the recovery generation (F2), all the life traits were totally restored in the copepods after pre-exposure to microplastics, suggesting that two-geneneration microplastics exposure did not exert a transgenerational effect on the

DOI: 10.1021/acs.est.9b02525
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phenotypic traits in the copepod *T. japonicus*. Due to its habitats such as tidy pool and sea bottom frequently with fluctuating environments, this copepod species shows high phenotypic plasticity in response to environmental stressors, which is in line with previous works.[23,33,34] For instance, in the copepod *T. japonicus*, they can regain from the negative effects of mercury multigenerational exposure after one generation recovery,[32] which is ascribed to its high phenotypic plasticity. Two-generation exposure to microplastics did not transfer a parental effect on the physiological traits in the next generation (F2), but it displayed significant transgenerational proteome plasticity, as many processes related to energy metabolism and stress defense were strikingly increased for the recovery generation of F2 (please see the detailed information as follows).

Proteome Alteration Reveals Plastic Response in Copepods against Microplastics Exposure. The negative effects of microplastics on the physiological traits in marine copepods have broadly been reported by many previous studies,[5,12,13,35] nevertheless, none of which has provided a detailed molecular explanation to the impacts as mentioned above. In our study, the copepod proteome was significantly altered by microplastics; thus, many critical processes were significantly affected by this stressor, and it should give us a mechanistic understanding of a microplastics adverse effect on marine animals.

Within-Generation Response. After two-generation exposure, microplastics significantly increased some important cellular processes in the exposed copepods of F1. Notably, the ribosome (four proteins), peptide biosynthetic process (four proteins), gene expression (four proteins), and protein translation (four proteins) were enhanced. All the above cellular processes were enriched by the same four proteins, i.e., 60S ribosomal protein L3, ribosomal protein S19, 40S ribosomal protein S28-like, and 40S ribosomal protein S14, putative, and they participate in protein translation. Obviously, protein biosynthesis was strikingly promoted in the treated copepods after two-generation exposure to microplastics. Interestingly, the C-type lectin-like/link domain (three proteins) was significantly up-regulated by microplastics exposure. The C-type lectin domain containing proteins have Ca^{2+}-dependent binding of carbohydrates and have been implicated in immune response as demonstrated by antimicrobial host defense in aquatic crustaceans.[36,37] Thus, the up-regulation of several cellular processes, e.g., protein biosynthesis and immune defense, enables the copepods to deal with elevated maintenance cost in response to microplastics exposure. Particularly, three proteins, proteasome activator complex subunit 4 (1.923 fold), microsomal GST3 (0.764 fold), and putative NADP-dependent alcohol hydrogenase (0.753 fold), were significantly modified, but these proteins were not significantly enriched. Proteasome activator complex subunit 4 is an associated component of the proteasome which can degrade unneeded or damaged proteins in the cells. Glutathione *S*-transferase is responsible for detoxifying damaged products under stress. The alcohol dehydrogenases contain a group of isozymes that catalyze the oxidation of alcohols to aldehydes and ketones and thus take part in cellular oxidation−reduction processes. The regulated expression of these three proteins could be supportive evidence that microplastics had probably produced oxidative stress in the copepod *T. japonicus*; and also, oxidative stress can compel the exposed animals to be confronted with metabolic cost. To summarize, on account of the trade-off, less energy was distributed into survival and reproduction in the treated copepods, and as a result, both of them were significantly decreased during two-generation exposure to microplastics.

Transgenerational Plasticity. In the case of the recovery generation (F2), we found significant proteome change in the copepods pre-exposed to two-generation microplastics exposure, even though their life traits were completely restored. To correspond with this, transgenerational proteome plasticity was demonstrated here and probably played an important role in the copepods' acclimatory response in response to microplastics. First, several cellular processes enriched, e.g., the antioxidant activity (six proteins), endopeptidase activity (11 proteins), nucleotide biosynthetic process (five proteins), proteolysis (12 proteins), longevity regulating pathway-multiple species (seven proteins), and HSP20-like chaperone (five proteins), were significantly decreased by transgenerational exposure in response to microplastics. The antioxidant activity enables the cells to cope with oxidative stress, and HSP20-like chaperone is able to enhance cellular tolerance under stressors; thus, both of them are involved in defense response against stress. Proteolysis (i.e., protein degradation) and nucleotide biosynthetic process are related to protein and nucleic acid metabolism. As a consequence, a down-regulation of the aforementioned processes demonstrated that being pre-exposed to microplastics for two-generation exposure had indeed compromised the copepods' fitness of F2 at the proteome level.

Although transgenerational exposure to microplastics had caused a significant decrease of several cellular processes in the F2 copepods, more enriched processes were remarkably increased for this generation; it was probably initiated to improve the copepods' robustness in response to microplastics effects and, as a result, totally regained the phenotypic traits in the recovery generation. For example, the structural constituent of cuticle (11 proteins), chitin binding (seven proteins), structural molecule activity (16 proteins), cell redox homeostasis (four proteins), glucosamine-containing compound metabolic process (eight proteins), carbohydrate metabolic process (13 proteins), amino sugar and nucleotide sugar metabolism (five proteins), lysosome (four proteins), C-type lectin-like/link domain (four proteins), and others were significantly up-regulated. The structural constituent of cuticle and structural molecule activity were jointly enriched by 11 cuticular proteins such as putative cuticle protein, cuticle protein 6, flexible cuticle protein 12, and so on; also other specific proteins are involved in ribosome or collagen assembly. In addition, both chitin binding and the glucosamine-containing compound metabolic process were related to the chitin metabolic process. For copepods, one remarkable characteristic is that cuticle proteins are structural proteins, together with chitin, comprising their exoskeleton.[38] Several previous studies have shown a differential expression of cuticular proteins in marine copepods in response to stress[38−40] and has been related to impacts on growth and reproduction.[38] Interestingly, like the C-type lectins, an increased expression of cuticle-related genes was also reported to contribute to antimicrobial host defense in the estuarine copepod *Eurytemora affinis*,[41] supporting our finding that cuticle integrity plays a role in regaining the phenotypic traits for the recovery generation. Particularly, the lysosome pathway was significantly increased in copepods under transgenerational

DOI: 10.1021/acs.est.9b02525
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exposure to microplastics. Owing to its removal of oxidatively damaged organelles and proteins in the cells, the lysosome pathway has provided marine animals with a vital defense strategy to fight against oxidative stress.[42,43] Recalling that microplastics exposure had probably resulted in oxidative stress and consequently damaged several important macromolecules including proteins and enzymes in the treated copepods of F1, the increased lysosomal pathway (which was supported by elevated energy production mainly due to the increased carbohydrate metabolic process), together with enhanced cell redox homeostasis, was probably used by the copepods to resist microplastics stress. Taken together, an increased expression of many crucial cellular processes (e.g., cuticle formation, energy metabolism, and stress-defense response) may account for regaining the compromised life traits in marine copepods during recovery.

Within- versus Across-Generation Effects. In our work, during two-generation exposure, survival and reproduction of the copepods were obviously decreased by microplastics; however, the affected phenotypic traits were regained for the F2 copepods when microplastics was ceased. Thus, the copepods displayed a stronger within-generation phenotypic response in contrast to an across-generation effect (i.e., transgenerational effect); thus, the copepod species responded more to the microplastics environment experienced by themselves rather than their (grand) parents, and it is logical as microplastics pollution has already been a ubiquitous problem for a long time in marine environments. More interesting is that, however, the copepod species showed a different proteomic response from that at the phenotypic level; namely, we found a small within-generation proteomic response (35 DEPs) upon initial exposure to microplastics followed by a pronounced transgenerational proteome plasticity (i.e., 374 DEPs). Moreover, few responsive proteins, as well as functional processes, were shared by the two generations of F1 and F2. As a result, at these proteomic and phenotypic levels, the copepods exhibit strong patterns of within-generation response or transgenerational plasticity but not both in response to microplastics exposure, and this contrasting effect is also demonstrated in several previous works.[16,44,45] For example, in the water flea *Daphnia ambigua*, they prefer to have strong transgenerational transcriptional plasticity but weak within-generational response in response to predator cues.[16] In the present study, the copepod had initiated transgenerational proteome plasticity as demonstrated by an increased expression of cuticle formation, energy metabolism, and stress-defense response, that is, compensatory proteomic reaction, probably taking charge of restoring the affected phenotypic traits during recovery. Also within-generation proteome response was mainly reflected by increased protein biosynthesis and enhanced immune defense, giving rise to an energy shortage due to elevated maintenance cost under microplastics exposure; then the survival and reproduction were significantly decreased by microplastics exposure as the energetic trade-off is working in this case. Taken together, the within-generational effect and transgenerational plasticity appear to take different roles in the marine copepod's response in response to microplastics exposure; however, the exact causation really needs further investigation.

Implications. Our shotgun-based quantitative proteomic analysis demonstrated that the environmentally relevant-concentration microplastics could significantly cause within-generation proteome response and, in turn, probably increase metabolic cost and reduce cellular energy store, hence displaying an adverse effect on survival and reproduction of the treated copepods on account of the energetic trade-off. Thus, microplastics exposure at environmentally realistic concentrations had produced a biochemical response due to its accumulation in the exposed copepods, and this could link to a population-level adverse outcome, i.e., compromised survival and reproduction (Figure 6). The more interesting thing is that, although the decreased phenotypic traits were completely regained for the recovery generation of F2, a significantly transgenerational plasticity was observed at the proteomic level. Also, transgenerational proteome plasticity had enabled the copepods to show resilience in response to microplastics (Figure 6), suggesting that it is likely taking an important part in the copepods' acclimatizing response to microplastics pollution; however, it deserves more study in the near future.

Recalling that the average microplastics bioaccumulation in *T. japonicus* was approximately 2 particles/copepod and 38 particles/copepod under the exposures to 0.023 mg/L and 0.23 mg/L, it is in contrast to the field.[46,47] For example, the bioaccumulated concentration of microplastics is reported as 0.13 ± 0.16 pieces/individual for natural zooplanktonic copepods in the East China Sea, whereas the particle size ranges from 20.3 μm (pellets) to 295.2 μm (fibers, contributing 54.6% to the ingested microplastics),[47] which is far larger than that of microplastics (6 μm) used in our study. We are speculating that the bioaccumulation level of 6 μm microplastics in the copepods in our work can likely be observed in the environment, given that our study has significant environmental-relevance. Meanwhile, because of the perpetual fragmentation of microplastics in a marine environment, microplastics abundance will augment as they become smaller.[3] Above all, aged microplastics tend to have more adverse effects on marine organisms when compared with virgin microplastics, primarily due to aged microplastics acting as both a source and sink for toxic chemicals (e.g., heavy metals and persistent organic pollutants) in a marine environment.[48,49] Consequently, the negative biological effect of microplastics has been underestimated by now in most cases. In the future, more microplastics stress research should be performed for a multitude of marine biota under environmentally relevant conditions, e.g., the long-term multigenerational exposure to costressors of microplastics and toxic pollutants at environmental-realistic levels; it helps to accurately assess the ecological risk of microplastics pollution in a marine environment. In addition, the copepods were supplied with excess food during microplastics exposure in our study, and the negative effect of microplastics on their survival and reproduction could probably be exacerbated under pessimistic food scenarios, e.g., food shortage likely to be encountered in the future ocean. To correspond with this, it deserves further study to investigate how food conditions, particularly limited food provision, will modulate individual marine copepod responses to microplastics pollution.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02525.

DOI: 10.1021/acs.est.9b02525
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Additional details for a three-day exposure testing, proteomic analysis, and biochemical parameter determination, as well as related data (PDF)

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The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (no. 41876117), the Fundamental Research Funds for the Central Universities (no. 20720190094), and the Natural Science Foundation of Fujian Province of China (no. 2017J01081).

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