DEHP chronic exposure disturbs the gut microbial community and metabolic homeostasis: Gender-based differences in zebrafish

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Abstract

Background: Di-(2-ethylhexyl) phthalate (DEHP), a predominant phthalate ester (PAE), exhibits various toxic potentials with environmental and human health risks. However, the chronic effects of DEHP exposure on gut microbiota and associated host's health are still largely unexplored. Here, zebrafish were exposed to relative environmental levels (0-100 μg/L) of DEHP from embryos to adult (3.5 months), then the developmental indexes and microbiota as well as the related energy metabolites in gut tissues were checked to unveil the effects during the growth progress.

Results: The results indicated that the composition of gut microbiota was different among male and female adult zebrafish, and DEHP disrupted the diversity and richness of the bacterial community. The histopathological analysis of intestinal tissues revealed the decrease of villus length and tunica muscularis thickness, especially the goblet cells per villi in male fish. Moreover, energy metabolites in gut tissues were actively increased at lower levels groups, which probably contribute to the condition factor of host. For immune-related genes, the expression levels of *il-8* was increased both in female and male fish in the lowest concentration (10 μg/L), but the *tlr-5* and *il-1β* genes were in the different response with significantly decreased in female fish.

Conclusion: Taken all together, those results indicated that chronic exposure to DEHP led to zebrafish obesity with the disturbed bacterial community in the gut and related metabolism pathways. Those findings provided deep insights on the perturbation of gut microbiota, metabolic homeostasis, and gender-based differences by DEHP exposure.

Background

Phthalates (PAEs) are widely used as plasticizers in a great deal of domestic and industrial products to make plastic flexible, which leads to their inevitable release into the environment and exposure to humans and animals [1, 2]. Comprising approximately 50% of the total plasticizer products, di-(2-ethylhexyl) phthalate (DEHP) is the most extensively used PAE and its toxicity has attracted huge attention worldwide [3, 4]. However, the researches mostly focused on the potential risks of DEHP on the neurodevelopment in early life, endocrine disruption, and reproductive toxicity [5–7]. Recently, the environmental contamination and ecological risks of DEHP have been reported in diverse aquatic ecosystems with various levels [8, 9]. DEHP as a small molecule additive compared to the large polymer chains are more prone to dissolve in an aqueous environment and posed risks of exposure of humans, animals, and microbes [10–12]. Therefore, more investigations should be carried out to reveal the comprehensive toxicity of DEHP on aquatic organisms after long-term exposure at environmental levels to aquatic organisms.

Environmental contaminants can disrupt gut microbiota in animals, especially in the aquatic ecosystem [13]. The normal gut microbiota affects specific functions in host nutrient metabolism, xenobiotic and drug metabolism, maintenance of structural integrity of gut mucosal barrier, immunomodulation, and

protection against pathogens [14, 15]. Moreover, the gut microbiota is involved in the regulation of multiple host metabolic functions and energy balance, giving rise to interactive host-microbiota metabolic including energy intake and immune-inflammatory signaling that physiologically connect gut organs [16, 17]. In fish, the intestinal bacterial communities were sensitively shaped by environmental and ecological factors [18]. Therefore, the diversity and richness of bacterial community is considered as an important index to evaluate the toxicity of pollutants, which is detected by the modern biotechnology technique termed as 16S rRNA high throughput sequencing [19, 20].

Zebrafish model possesses numerous advantages involving the short life span, transparent embryos, high similarity to the human genome, and diverse adaptability [21]. Thus, it usually applied as a credible animal model to evaluate the toxicity of various environmental pollutants including DEHP [22]. Moreover, the zebrafish model was recently used to study the functions and impacts of various pollutants on gut microbiota after acute or chronic exposure [23, 24]. Moreover, zebrafish can be employed to study the relationship of microbes, stress response, and gut development [25, 26]. Although the gut desorption of DEHP was reported, the potential disruption to gut microbiota in aquatic organisms is still elusive [27, 28]. Thus, wild type zebrafish were employed in this study to explore the possible toxicity of chronic DEHP environmental relevant exposure on development, gut microbiota, and immune system responses.

The assessment of PAEs showed the ability of them to affect immune and allergic responses, body weight, and intestinal development in experimental animals and in vitro [29, 30]. Previous reports highlighted that the utility and intestinal biology to functionally validate candidate genes identified through genome-wide association studies are unique to the zebrafish model [31]. A large degree of homology has been reported between zebrafish intestines and that of higher vertebrates in terms of cellular composition and function. Moreover, intestine acts as a digestive and immune organ with highly conserved molecular pathways, injury regulating functions, and immune responses. Therefore, the genes expression of intestinal tissues with the changed bacterial community and the content energy materials with the abundance of KEGG pathways could provide more insights to the comprehensive toxicity of DEHP on fish developmental and associated health effects. Therefore, the histological analysis of zebrafish gut tissue is also necessary for evaluating the toxicity of long-term organic pollutants. Moreover, chronic exposure to DEHP induced significant genotoxicity via kinds of pathways to reverse effects including the potential carcinogenic mechanism and potential adverse health outcomes [32, 33]. However, researches proved that long-term exposure to DEHP in early life related to obesity and neuro-behavior and reproductive outcomes [34, 35]. Previous researches proved that the toxicity of DEHP in parents can be transferred to offspring and thus affect the development and growth of animals [36]. Therefore, the effects on tissues after long-term exposure from zebrafish embryos to larvae and then to adult ones are necessarily needed comprehensive investigation.

Therefore, in this study, long-term exposure to DEHP with different concentrations (0, 10, 33, 100 μg/L) was performed using the zebrafish model. Then, the disruption effects on the diversity and richness of gut microbiota in female and male fish were explored by 16S rRNA sequencing analysis. Moreover, DEHP mediated damages on zebrafish gut tissues were detected from histomorphology along with the energy

metabolism levels aspects. The metabolites in zebrafish intestines related to the energy cycle were also measured in this study to investigate the underlying relationships of the gut microbiota and homeostasis. Besides, the expression changes of genes in immune response were deeply explored for investigating the potential effects on zebrafish exposed to DEHP.

Results

Developmental indexes of zebrafish after long-term exposure to DEHP

In this study, the long-term exposure to environmental level concentrations of DEHP at the early life stage induced no serious toxicity on the survival and growth of zebrafish. However, the DEHP exposure from embryos to adult stage significantly altered the basic developmental indexes (Fig. 1). In detail, the average body length of female was significantly increased to 3.056 ± 0.029 cm in the lowest concentration group (10 μg/L) and male to 3.068 ± 0.036 cm in the 33 μg/L group, compared to female (2.881 ± 0.027 cm) and male (2.951 ± 0.022 cm) fish in control (Fig. 1A and 1B). The similar results were observed for body weight that significantly increased to 0.233 ± 0.005 g and 0.210 ± 0.005 g in the DEHP exposed female and male zebrafish in the 10 μg/L group, compared to control female (0.201 ± 0.005 g) and male (0.193 ± 0.004 g), respectively (Fig. 1C and 1D). Moreover, the condition factors (K) of female (71.491 ± 2.035) in the 10 μg/L and male (64.659 ± 2.450) zebrafish in the 33 μg/L was significantly decreased by DEHP exposure, compared to control female (58.267 ± 1.759) and male (57.335 ± 1.562), (Fig. 1E and 1F).

From the organic weight ratio in zebrafish, the intestinal somatic index (ISI) of female and male was increased to 5.300 ± 0.227 and 3.584 ± 0.161 in the DEHP exposed groups, but showed no significant changes compared to female (4.706 ± 0.249) and male (3.334 ± 0.215) in control (Table S2). Similarly, the other index including gonadal somatic index (GSI) and hepatosomatic index (HSI) increased gently but showed no significant changes between DEHP treatment and controls (Table S2).

Diversity and richness of intestinal microbiota in adult zebrafish

The intestinal microbiota of zebrafish in control and the highest concentration of DEHP exposure (100 μg/L) groups were analyzed *via* 16S rRNA sequencing. The result showed that the taxonomic information of total samples was 18 Phyla, 34 Classes, 87 Orders, 144 Families, 247 Genera, 324 Species, and 406 OTUs. According to our previous studies [23, 37], the composition and functions of intestinal microbiota in female and male zebrafish were different, thus the following analysis of each group was departed by gender. The groups were marked by female and male in control (F-0 and M-0), female and male in 100 μg/L DEHP group (F-100 and M-100).

The rarefaction curves of OTUs showed that all samples reached the plateau phase by 26,000 sequences reads (Fig. S1), and the rarefaction curves of the Shannon index approached the plateau from about 4200 sequences per sample (Fig. S2). Generally, we obtained 913,364 high-quality sequences and 400,891,017 bp bases with an average length of 438.917 bp, and the OTUs in each group were optimized

sequence reads for the subsequent analysis. Moreover, the Good's coverage index of samples was higher than 99.9% in all groups, indicating that the range of detected data was high and credible, and the unmeasured part was negligible. The diversity (Shannon, Simpson) and richness (Chao, Ace) indexes were calculated without significant differences by t-test between control and the DEHP exposed groups, but the alteration of diversity and richness of intestinal microbiota still differed in female and male zebrafish. In the F-100 and M-0 group, the Chao (263.55±17.09, 209.74±18.86) and Ace (261.28±20.81, 218.87±14.24) index values were higher than the F-0 and M-100, respectively, indicating the higher diversity in DEHP exposed female and lower diversity in DEHP exposed male. Consistent with other indexes, the lower Shannon (2.35±0.36, 1.87±0.29) value and the higher Simpson index appeared the lower diversity in the F-100 group and M-0 group (shown in Table S3).

Composition of intestinal bacteria in zebrafish with long-term DEHP exposure

The bacterial community of female and male zebrafish intestine in the phylum level was different between control and DEHP treatment groups (shown in Fig. 2). In the taxonomy analysis, the major dominant bacterial phyla in the F-0 group were *Fusobacteria* (35.85%), *Proteobacteria* (29.16%), *Firmicutes* (19.69%), *Bacteroidetes* (6.77%), and *Actinobacteria* (6.02%). In the F-100 group, the abundance of *Fusobacteria* (27.94%), *Bacteroidetes* (4.62%) and *Actinobacteria* (5.23%) were decreased, while other bacterial phyla *Proteobacteria* (32.89%) and *Firmicutes* (27.73%) were increased. Nevertheless, the percent of dominant bacterial phyla in the M-0 group were *Fusobacteria* (48.79%), *Proteobacteria* (19.07%), *Firmicutes* (20.15%), *Bacteroidetes* (7.50%), and *Actinobacteria* (2.04%). In the M-100 group, the abundances of *Fusobacteria* (43.64%), *Firmicutes* (14.04%) and *Actinobacteria* (1.53%) were decreased, but other bacterial phyla *Proteobacteria* (27.64%), and *Bacteroidetes* (12.13%) were increased (Fig. 2). The *Verrucomicrobia* in the F-100 (0.82%) and M-100 (0.80%) group were lower than those in the F-0 and M-0 group (1.90%, 2.30%), respectively. Finally, the richness of minor bacterial phyla as "other" in the F-100 (0.77%) and M-100 (0.21%) zebrafish were increased, respectively compared to the F-0 (0.60%) and M-0 (0.14%) groups.

The intestinal bacteria of zebrafish in control and DEHP groups were identified by the Venn chart according to the level of the family with the special and highly shared family in female and male zebrafish (Fig. S3). Further, the intestinal microbiota was analyzed and the community heatmap of the top 50 genera with the hierarchical clustering was calculated according to the average richness value of the samples to investigate potential effects of DEHP (Fig. 3). The fold-changes at richness and diversity of intestinal bacteria in female and male zebrafish were differed in the phylum level by DEHP exposure (Table S4). Among the most abundant genera, the *Aeromonas*, *Deefgea*, *Shewanella*, *Mycobacterium*, *g_unclassified, f_Rhodobacteraceae*, and *Hyphomicrobium*, as well as lower richness genera *Macellibacteroides* and *Haloferula* in DEHP exposure groups were increased with the fold change 1.29-, 6.73-, 1.17-, 4.85-, 3.53- and 1.59-, 1.77- and 3.28-folds in female fish, and 1.62-, 4.53-, 2.02-, 4.37-, 5.10- and 2.02-, 2.76-, and 13.08-folds in male fish, compared to the control groups. While for the *Cetobacterium*, *Akkermansia*, *g_norank_o_PeM15*, *Bacteroides*, *Rhodobacter*, *Planococcus,* and *Salinicoccus*, the abundance levels were decreased to 0.79-, 0.40-, 0.09-, 0.58-, 0.42-, 0.01- and 0.00- folds

in female fish, and to 0.88-, 0.20-, 0.02-, 0.33-, 0.86-, 0.06- and 0.17- folds in male fish, compared to control group, respectively. Notably, the *Plesiomonas*, *Porphyromonadaceae*, *Hados.Sed.Eubac.3,* and *Enterobacteriaceae* genus were decreased in female fish with 0.71- to 0.37- folds, but showed higher levels with 1.30- to 1.68- folds in male fish after DEHP exposure. In contrast, the abundance of *Erysipelotrichaceae*, *Gemmobacter*, *Meganema*, and *Rubellimicrobium* genus were increased in female fish with 1.25- to 1.60- folds, but showed lower levels with 0.87- to 0.23- folds in male fish after GO exposure. Here, DEHP exposure changed gut bacterial genus differed in female and male zebrafish, such as the *Gordonia* increased with 6.71- folds in male fish and *Legionella* increased with 3.86- folds in female fish without any abundance changes in other gender fish, respectively.

Further, the intestinal bacterial functions in DEHP exposed and control groups were analyzed from the databank of appreciated for Bacteria and Archaea based on the abundance of COG metabolism pathways (Fig. S4). In those functional pathways, A: RNA processing and modification, I: Lipid transport and metabolism, N: Cell motility, and Q: Secondary metabolites biosynthesis, transport, and catabolism showed increased abundance with 1.89-, 1.20-, 1.12- and 1.3- folds in female fish, and with 1.42-, 1.14-, 1.36-, 1.21-folds in male fish, compared to control groups. Notably, the abundance of KEGG metabolic pathways at the first level showed that most pathways including cellular process and metabolism pathways also increased in both female and male fish after DEHP exposure (Fig. S5). Especially, the metabolic pathways at the second level involved in the xenobiotics biodegradation and metabolism, cell motility, endocrine system, metabolism of other amino acids, circulatory system, neurodegenerative diseases, cancers, cell growth and death, metabolism of terpenoids and polyketides, cellular processes and signaling, signal transduction, lipid metabolism signaling, and nervous system potentially increased with 1.27-, 1.12-, 1.10-, 1.13-, 1.23-, 1.19-, 1.05-, 1.13-, 1.19-, 1.01-, 1.01-, 1.15- and 1.10- folds in female fish, and with 1.19-, 1.32-, 1.15-, 1.10-, 1.49-, 1.18-, 1.15-, 1.03-, 1.15-, 1.14-, 1.14-, 1.12- and 1.23- folds in male fish, compared to controls (Fig. 4). Besides, the pathways of the digestive system were increased by 1.42- folds in male fish, and the cardiovascular diseases increased to 1.25- folds in female fish, indicating that several response pathways were differently affected by DEHP exposure.

Histomorphology of intestines in the chronic DEHP exposed zebrafish

For the histological analysis, intestine tissues by HE staining were performed to detect the villus height, villus width, crypt depth, and tunica muscularis thickness in female and male zebrafish (Fig. 5). The intestine tissues were observed normal without serious damages in the female and male controls (Fig. 5A and 5C), as well as the DEHP exposed ones (Fig. 5B and 5D). The average villi height of intestine tissues was normal in the F-0 (112.46 μm) and M-0 (133.54 μm) groups, and the F-100 (127.32μm) and M-100 (112.27 μm) exposed groups (Fig. 5E). The average width of villi in the DEHP exposed female (77.14 μm) and male (90.60 μm) zebrafish intestines were decreased with significant changes, compared to control group female (96.44 μm) and male (113.56 μm) zebrafish (Fig. 5E). Moreover, the crypt depth of intestines was also measured, and the average depth of villi in the F-100 (28.07 μm) and M-100 (28.04 μm) zebrafish intestines were slightly decreased, compared to the F-0 (31.78 μm) and M-0 (30.10 μm) zebrafish. However, the average tunica muscularis thickness of the F-100 and M-100 intestines was 12.81

and 14.28 μm, with the F-100 group appeared significant changes, compared to control (16.12 and 15.19 μm) zebrafish (**Figure 5E**).

For the effects of chronic exposure to DEHP on zebrafish intestinal functions, the goblet cells stained with AB-PAS was performed in this study (Fig. 6). The goblet cells in the female and male zebrafish intestine tissues from the control group (Fig. 6A and C) and DEHP exposed group (Fig. 6B and D) were counted and calculated. The average number of goblet cells per villus in the F-100 and M-100 group (9.5 and 8.1 cells) was decreased, compared to the F-0 and M-0 group (9.9 and 11.5 cells) with the M-100 significant changes (Fig. 6E).

Changes of metabolites content and immune response in zebrafish intestines

The energy metabolites in intestine tissues related to zebrafish body obesity were measured, and the content of TG was significantly increased in the F-10, F-33 and M-33 groups with 0.0769, 0.0905 and 0.0333 mmol/gprot, compared to the F-0 and M-0 groups (0.062 and 0.024 mmol/gprot), respectively (Fig. 7). Similarly, the content of PY was also increased by DEHP in the F-33 and M-10, M-33 groups with 0.0116 and 0.0071, 0.0084 μmol/gprot, compared to the F-0 and M-0 groups (0.0080 and 0.0040 μmol/gprot), respectively (Fig. 7). The content level of FA was increased by DEHP in the low concentration groups, but only the M-10 group with 0.0139 mmol/gprot showed a significant difference, compared to the M-0 group (0.0062 mmol/gprot). Interestingly, the content of Glu was significantly up-regulated to 0.130 and 0.129 mmol/L in the F-10 and F-100 groups, compared to 0.094 mmol/L in the F-0 group. However, the Glu content appeared no changes in the DEHP exposure groups.

The immune response-related genes in gut tissues of zebrafish after chronic exposure to DEHP were checked by RT-PCR (Fig. 8). In the female zebrafish, the relative expression of gene *tlr-5* was inhibited to 0.48-folds in the highest DEHP exposure group, as well as the gene of *il-1β* decreased with rang from 0.37 to 0.69-folds in all the DEHP exposure (10-100 μg/L) groups. The other gene of *nf-κb,* and the male zebrafish appeared no significant changes after DEHP treatment. However, the *il-8* gene in female and male zebrafish showed significantly increased expression in lower DEHP exposure groups (10 and 33 μg/L), compared to the control group.

Discussion

In recent years, DEHP associated diverse toxic implications for ecological and human health have gained huge attention. The elevated levels of DEHP have been reported in freshwater resources, which provides water supply for drinking and domestic utilities, and this could ultimately pose a threat to environmental and human health [38]. In the previous studies, the toxicity of DEHP and its derivative MEHP had mainly focused on the developmental and reproduction effects or organogenesis through various biological pathways in fish [39–41]. However, the eco-toxicological risks of DEHP with environmental concentrations on freshwater fish health including the development and intestinal homeostasis, especially their underlying relationships with the disruption of intestinal bacterial functions were still unclear. In this study, the chronic toxicity of DEHP on zebrafish was investigated and discovered the

obesity developmental indexes of adult stages, which was possibly related to the disruption of gut microbiota and homeostasis between exposure and control groups.

In this study, the body length and body weight of both female and male were increased according to the recorded indexes of adult zebrafish after DEHP exposure groups (10–33 μg/L), compared to control. Moreover, the condition factor (K) stands the critical indicator for the toxicity of pollutants and food intake changes on the fish in fresh and marine water systems [42, 43]. The higher condition factor of female and male zebrafish was calculated to be higher in lower concentration groups, and the organ somatic indexes (ISI, GSI, and HSI) were increased but without significant for signal organ. Thus, we suspected that DEHP exposure may contribute to the increased weight of organs in the whole body. However, it was reported that the long-term exposure to DEHP with concentrations of 0.1–10 μg/L may affect guppy fish development by inhibiting the body growth under higher temperature [44]. Interestingly, other reports showed that exposure to low levels of DEHP may affect organ development, such as by modulating the genes expression related to fatty liver disease with acid metabolism [45]. Here, our results proved that the long-term exposure to DEHP from embryos to adult ones induced zebrafish to be obese both in females and males, and genders appeared with significant alterations in different low-levels treatments.

For energy extraction, lipid metabolism, endocrine functions, and immune system, the influenced developmental indexes possibly connected with the alteration of intestinal microbiota in organisms [46–48]. Previous researches explained that the shifts of intestinal microbiota were sensitively affected by dietary, the gender of the host, drugs, and other environmental factors, but varied with individuals even within the same life-stages [49–51]. As the potential key indicator for animal habit and environments, the gut microbial communities in zebrafish during their growth and development have already been detected in previous studies [52, 53]. In our study, the intestinal microbiota was subsequently investigated to understand the inner relationships of developmental indexes and homeostasis alteration in adult zebrafish. The alpha analysis indexes of richness (OTUs, Chao1, Ace) and diversity (Shannon, Simpson) showed different responses in zebrafish after long-term DEHP exposure. In the F-100 zebrafish, the abundance of major phyla, Fusobacteria, Bacteroidetes, and Actinobacteria were decreased, while other bacterial phyla Proteobacteria and Firmicutes increased. In the M-100 zebrafish, the abundance of Fusobacteria, Firmicutes, and Actinobacteria was decreased, but other bacterial phyla Proteobacteria, and Bacteroidetes were increased. The Fusobacteria and Bacteroidetes were considered beneficial intestinal bacteria by providing vitamins or assist in metabolism in the gut [54]. Moreover, the increased prevalence of Proteobacteria is a potential diagnostic signature of dysbiosis and the risk of diseases [55]. Here, we suspected that the disruption of those phyla in female and male zebrafish probably contributed to the imbalance the metabolic ability after DEHP chronic treatment.

According to the level of genus, the abundances of major bacteria were Aeromonas, Deefgea, Shewanella, Mycobacterium. However, the Rhodobacteraceae in DEHP exposure groups was increased, and Cetobacterium, Akkermansia, Bacteroides, Rhodobacter, Planococcus and Salinicoccus were decreased in female and male fish, compared to control, respectively, indicating the negative effects of DEHP on

zebrafish intestinal homeostasis. Notably, the Plesiomonas, Porphyromonadaceae, and Enterobacteriaceae genera were decreased in female fish, but showed higher in male fish after DEHP exposure. In contrast, the abundance of Erysipelotrichaceae, Gemmobacter, Meganema, Rubellimicrobium, and Legionella genera were increased in female fish, but showed lower levels in male fish after chronic DEHP exposure, respectively. Among those altered genera, some probiotics, such as Cetobacterium and Akkermansia, were the key members essential for organisms, but some of them, such as the genera Shewanella and Mycobacterium were trends to human infections and antibiotic resistance [56, 57]. Under the chronic pressure of DEHP, the increased endotoxins could result in obesity, inflammation, metabolic derangements, and intestinal or other organ damages [58–60]. Thus, the shift of intestinal bacterial community with the changed functions abundance possibly comprehensively led to the developmental abnormality and immune response in the DEHP exposed zebrafish.

Environmental pollutants affect the fish's health by influencing various metabolic pathways, especially on the food intake and the subsequent energy transformation, and finally induce host obesity [61, 62]. Thus, the alteration of gut microbiota exposed to environmental challenges is always related to the diseases in the host or the metabolic functions [63, 64]. In this study, the intestinal microbiota explored by COG and KEGG functional analysis showed similar metabolism pathways between exposure and control zebrafish, but showed various relative abundances. In detail, the pathways of lipid transport or metabolism, cell motility, and catabolism were increased in female and male fish by DEHP treatment. Notably, the KEGG data showed that the cellular process and metabolism pathways were also significantly increased in both female and male fish after DEHP exposure. Especially, the metabolic pathways involved the xenobiotics biodegradation, cell growth and death, cellular processes, signal transduction, and lipid metabolism signaling were increased in female and male fish treated with DEHP.

Scientific evidence supported the idea that obesity and metabolic consequences are strongly related to the changes in both the function and composition of gut microbiota, which exerted an essential role in modulating energy metabolism [65]. Based on human and animal studies, gut microbiota could act as a key modifier for obesity and related metabolic complications [66]. Thus, the critical pathways of metabolism in zebrafish were significantly changed, which may explain the reason of developmental abnormality by the long-term DEHP exposure.

The intestine possesses a large interface between the human organism and the external environment and affects the functions of the epithelial barrier and the gut immune system [67]. Evidence showed that host-microbe interactions shaped the gastrointestinal environment and the microbiota benefited the development and difference of gut tissues [68]. However, enteral nutrition and multiple pathologies are associated with the disruption of gut microbes after exposure to environmental pollutants. In this study, the gut sections with H&E stain of female and male fish showed the average value of villus length and crypt depth without changes by DEHP exposure. However, the villus width of male fish and tunica muscularis thickness of female fish were significantly reduced in the DEHP exposure groups. Besides, the important goblet cells in intestinal tissues analyzed by AB-PAS stain were significantly inhibited in the DEHP exposed male zebrafish. Here, our results indicated that chronic exposure to a low level of DEHP

was harmful to gut developmental indexes by decreasing the villi width and the goblet cells, especially in male fish intestines. We speculated that the alteration of indexes possibly affected DEHP on zebrafish intestine organ as well as its functions, and thus the subsequent metabolic functions were necessary to be explored.

Moreover, healthy microbial communities are important for human metabolome, and especially the harvest of energy and the regulation of body systems outside the digestive tract, and the dysbiosis have been implicated in many disorders, including obesity and inflammation [69, 70]. The gut microbiota is involved in the host energy metabolism through the regulation of body fat storage and serum-free fatty acids (FFA), which are generally elevated in obesity [71]. Among a variety of gut microbiota metabolites, short-chain fatty acids (SCFAs) have received attention because of their important role in maintaining the homeostasis of hosts and the recovery of diseases [72, 73]. In this study, the content of metabolites involved TG, PY, FA, and Glu were significantly increased in female and male zebrafish intestines, especially in the low level (10–33 μg/L) groups. Previous researches proved that the short-chain triglyceride triacetin affected the intestinal mucosa and metabolic substrates in animals [74]. Therefore, consistent with the changes of intestinal bacteria and related metabolic pathways, those results indicated that the potential ability of DEHP disrupted the zebrafish intestinal homeostasis involved in the energy materials metabolism. Here, our findings are in agreement with the recent report, which explored the understanding of phthalate-driven effects on intestinal function and lipid metabolism, implying that the gut-specific gene networks may drive phthalate-exacerbated obesity [75].

The microbes play critical roles not only in the development of gut tissues but also in the regulation of immune-related genes with high sensitivity towards environmental pollutants and diet change [76, 77]. In fish, the stimulation of different cytokines including tlr-5, il-1β, nf-κb, and il-8 played important roles in intestinal defense and inflammation. The innate immune mechanism of protecting the host from pathogens is related to the alteration of normal endogenous microbiota [78, 79]. In this study, long-term exposure to DEHP potentially evoked the expressions of genes related to the immune response in male intestines in the low concentration groups (10–33 μg/L), but significantly inhibited the mRNA level of tlr-5 and il-1β genes in female zebrafish, especially in the highest-level group (100 μg/L). The host immune response to the environmental pollutants is closely related to the resident commensal microbiota. The latest study reported that early-life DEHP exposure altered gut microbiota of newborns and changed their immune responses in later life [80]. Here, the expression of key genes indicated slightly different immune responses between male and female zebrafish by DEHP exposure, which may connect with various alterations of intestinal bacteria.

Taken all together, this study revealed that long-term exposure to DEHP induced developmental effects via increasing the body length and weight of zebrafish, disrupted the abundance of intestinal bacteria at phylum and genus levels, and impaired gut tissues with inflammation responses, especially in male fish. Our study provided evidence that the eco-toxicity of DEHP with environmental levels can disturb the intestinal microbiota and the related functions in freshwater fish, which may increase the risk of organism obesity or metabolic diseases. However, the disruption of intestinal homeostasis and host

health was affected by the changes of the gut bacterial community and alteration of intestinal microbiota by DEHP waterborne exposure, which is still needed further investigations.

Therefore, evidence of intestinal stability finally provided that long-term exposure to DEHP with a low concentration (10–33 μg/L) or a similar environmental endocrine hormone was not safe to animals and humans. Our results provided new insights on the eco-toxicity risks of DEHP, which spurred the host obese by potentially influencing the gut microbiota and metabolic ability. However, there are still important questions that need to be explored to understand the toxicity of low-level DEHP clearly, such as the content of DEHP intake by fish intestines and how the perturbation of bacteria affects the intestinal development and homeostasis.

Conclusion

In conclusion, the long-term exposure to DEHP with low concentrations induced significant effects on fish obesity according to the developmental indexes, and the condition factor as the more sensitive index showed alterations in the 10–33 μg/L group. Consistently, the gut bacterial community as the more sensitive indicator showed the alteration in female and male zebrafish by long-term DEHP exposure, especially the major phylum, such as Fusobacteria, Proteobacteria, Firmicutes, and Bacteroidetes. The exposure caused villi length and width in female and male zebrafish, but it was no obvious damages to gut tissues. However, the metabolic functions analyzed by KEGG pathways were checked to be significantly activated in a low level of DEHP exposed zebrafish intestines. With a higher level of energy metabolites contented in zebrafish intestine tissues, we suspected that the inner mechanism of obesity may be contributed by metabolic disorders. For the immune response in fish, it was observed that the female and male fish possessed slightly different responses to DEHP exposure. Therefore, the comprehensive toxicity of DEHP on organisms and environments after chronic exposure possibly exerted even in the low concentrations. Difference toxicity between female and male zebrafish provided strong evidence for evaluating the pollutants risk based on genders. Moreover, more attention should be paid to the functions of intestinal bacteria, which is sensitively disrupted by the long-term DEHP exposure.

Methods And Materials

Chemicals

The standard of DEHP (99.6%, No: ALR-097N) was purchased from AccuStandard (New Haven, CT, USA) and dissolved in dimethyl sulfoxide (DMSO, CAS: 67-68-5, purity ≥ 99.5%, Sigma-Aldrich, USA). Then the DEHP stock solution was stored at 4^{o}C. 3-Aminobenzoic acid ethyl ester or methane-sulfonate salt (MS-222, 98%) was obtained from Aladdin (Los Angeles, California, USA). The triglyceride assay kit (No. A110-1), the pyruvate assay kit (No. A081), the non-esterified fatty acids kit (No. A042-2), and the glucose assay kit (No. F006) were purchased from Jian Cheng Bioengineering Institute, Nanjing (www.njjcbio.com). All other chemical reagents used in this study were of analytical grade.

Zebrafish maintain and long-term exposure to DEHP

The maintenance and reproduction of zebrafish are referred to as our previous study [22]. Briefly, the wild type zebrafish (*Danio rerio*, AB strain) were maintained in a flow-through system with the condition of temperature (28 ^{o}C ± 0.5) and 14:10 (light: dark) photoperiod, and fed twice every day with the newly hatched brine shrimp (*Artemia nauplii*). Healthy adult zebrafish were placed in the breeding box with a ratio of 2:1 (male: female) overnight, and the spawning was triggered under light and was completed within 30 min.

Then, zebrafish embryos with normal development were selected and randomly distributed into glass beakers or tanks containing different concentrations of DEHP exposure solution (0, 10, 33 and 100 μg/L) with three replicates for each group. The embryos and larvae were cultured in 200 mL to 2 L volume of glass beakers, and the juvenile and adult zebrafish were cultured in 20 L volume of glass tanks. During the experimental process, the embryos/larvae and adult ones were maintained, and the exposure solutions were renewed daily. After exposure for 3.5 months, the adult zebrafish were washed with ultrapure water and anesthetized in 100 mg/L of MS-222, and then dissected them for intestines and other tissues. Then, the samples were immediately frozen into liquid nitrogen, and stored at -80 ^{o}C for the subsequent analysis.

During the dissection, the developmental indices of adult zebrafish including body weight, body length, and organ weight of intestines, gonad, and liver tissues were recorded. The coefficients of condition factor (K, body weight*100/body length^{3}) and the organ weight ratio (organ weight/body weight*100%) of intestines, brain, liver, and gonad tissues were calculated.

The analysis of zebrafish intestinal microbiota by 16S rRNA sequencing

The intestinal microbiota of male and female zebrafish was detected *via* the high-throughput sequencing, and the analysis methods were performed according to previous reports [23, 37]. Briefly, the total genomic DNA of intestinal samples was extracted and purified with FastDNA® SPIN Kit for Soil (Mpbio, USA). The concentration and purity of extracted DNA samples were assessed by a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), and the integrity of DNA was checked by 1% agarose gel electrophoresis.

The concentration of extracted DNA was assessed by a NanoDrop ND-2000 (Thermo Fisher Scientific, USA). Intestinal microbial community composition of zebrafish was detected by Miseq sequencing using adapter primers 338F: (ACTCCTACGGGAGGCAGCA) and 806R: (GGACTACHVGGGTWTCTAAT) for the V3-V4 regions of the 16S rRNA gene [81]. Amplicons for sequencing were amplified as previously described [37]. Briefly, extracted DNA was first amplified with high-fidelity Taq polymerase (Invitrogen, USA). Then equal quantities of three PCR reactions per sample were pooled, purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Finally, the mixture of amplicons from the different samples was sequenced using the MiSeq Illumina platform (Majorbio Biotechnology Co., Ltd., Shanghai, China).

2.5 Taxonomic analysis and community diversity of sequencing reads

Raw pyrosequencing data and related programs were sorted and can be analyzed using the platform of the company Majorbio (http://www.i-sanger.com/). Before analysis, the clustering of operational taxonomic units (OTUs) was retrieved according to our previous description [37, 82]. Briefly, trimmomatic (http://www.usadellab.org/cms/index.php?page=trimmomatic) was used to acquire high-quality tags from raw paired-end reads created by sequencing both directions of the libraries. The maximum number of errors in the barcode was 0 and the number of mismatches in the primer was 2. Reads with more than 10% of bases with a quality score of Q<20, and ambiguous or unassigned characters and adapter contamination were removed. USEARCH (version 7.0, http://www.drive5.com/uparse/) was used to classify the operational taxonomic units (OTUs) based on sequence similarity by cluster cut-off value of 97%. Representative sequences of OTUs were referred to the Silver ribosomal database (Release128, http://www.arb-silva.de) for the bacterial 16S rRNA genes, and the bacterial alpha diversity indexes were calculated by Mothur (version v.1.30.1, http://www.mothur.org/wiki/Calculators).

Firstly, the alpha rarefaction curves showed that the reads number of each sample was sufficient for subsequent analysis. Further to explore the diversity of zebrafish gut bacteria, OTUs were analyzed the number, indicating the abundance in each sample and different diversity index according to the genus level. Among those alpha indexes, the Shannon and Simpson indexes reflected the bacterial community diversity, while the abundance-based coverage estimator (Ace) and Chao1 value and coverage (the Good's coverage) indicated the bacterial community richness. Moreover, the bacterial diversity was evaluated according to the levels of phylum and family with the abundance percentage, while the fold-values were evaluated in the genus level with the abundance percentage of OTUs in each sample of the DEHP group against the abundance percentage in control. For a better understanding of the changes in bacterial diversity and community composition in DEHP exposed zebrafish, the community heat map and the significant differences tested by One-way ANOVA and Welch's t-test were performed between groups [83]. Finally, the COG (Clusters of orthologous groups of proteins) analysis and metabolism pathway levels of zebrafish gut bacteria were predicted based on the 16S rRNA sequences through PICRUSt software with EggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups, http://eggnog.embl.de/) database and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) database, which played key roles in evaluating the potential effects on aquatic animals.

The histomorphology analysis of zebrafish intestines

The histomorphology of intestinal tissues in exposed adult zebrafish was performed, and the tissues were immediately fixed in 4% (w/v) formaldehyde solution for 24 h, then dehydrated in ethanol, and embedded in paraffine. The zebrafish gut slices were stained with hematoxylin and eosin (H&E), and the analysis of villi height and width, crypt width, and tunica muscularis thickness were calculated referred to the previous studies [37, 84]. The intestines analysis was done according to our previous studies. Moreover, the gut slices were stained with Alcian blue-Periodic Acid Schiff (AB-PAS) to check the number

of goblet cells and morphometric parameters at the 200× magnification by photographed with a digital microscope (Nikon, SMZ18). The scale and date were marked with NIS-Elements imaging software (version 4.30) for both anterior-intestine and mid-intestine sections.

The detection of energy metabolism in zebrafish intestines

The triglyceride (TG) content in zebrafish gut tissue was measured according to the commercial kit directions. Briefly, the gut tissues of adult zebrafish were weighted, and the homogenization medium was added by weight (g): volume (mL) 1: 9. Then, the tissues were mechanically ground on ice and then centrifuged for 10 min at 2500 rpm. Finally, the supernatant liquid, standards, and ddH_2O were mixed with the working medium at 37 ^oC for 10 min. After incubation, the OD value was measured at 510 nm wavelengths. The TG content can be calculated by the following formulas:

$$TG\ content=[(OD_{sample}-OD_{blank})/(OD_{standard}-OD_{blank})]*Con_{standard}/Con_{sample}$$

Note:

TG content=mmol/gprot

$Con_{standard}$=2.26 mmol

The pyruvate (PY) content in the supernatant liquid of zebrafish gut tissue was measured according to the kit directions. The standards and ddH_2O as the blank were mixed with working agent II in the kits at 37 ^oC for 10 min, and agent III for 5 min at room temperature. After incubation, the OD value was measured at 505 nm wavelengths. The PY content can be calculated by the following formulas:

$$PY\ content=[(OD_{sample}-OD_{blank})/(OD_{standard}-OD_{blank})]*Con_{standard}/Con_{sample}$$

Note:

PY content=μmol/gprot

$Con_{standard}$=0.2 μmol

The non-esterified fatty acids (FA) content in the supernatant liquid of zebrafish gut tissue was measured according to the kit directions. The standards and ddH_2O as the blank were mixed with a working agent at 37 ^oC for 10 min, and then the OD value was measured at 546 nm wavelengths as the A1 data. After incubation agent II for 5 min at room temperature, the OD value was measured at 546 nm wavelengths as the A2 data. The FA content can be calculated by the following formulas:

$$FA\ content=[(\Delta A_{sample}-\Delta A_{blank})/\ (\Delta A_{standard}-\Delta A_{blank})]*Con_{standard}/Con_{sample}$$

Note:

ΔA=A2-A1

FA content=mmol/gprot

$Con_{standard}$=1.0 mmol

The glucose (Glu) content in the supernatant liquid of zebrafish gut tissue was measured according to the kit manual. The standards and ddH_2O as the blank were mixed with a working agent at 37 ^oC for 15 min, and the OD value was measured at 505 nm wavelengths. The Glu content can be calculated by the following formulas:

Glu content=$[(OD_{sample}-OD_{blank})/(OD_{standard}-OD_{blank})]*Con_{standard}/Con_{sample}$

Note:

Glu content=mmol/L

$Con_{standard}$=5.55 mmol/L

Samples of zebrafish tissues were diluted 1: 9 with 1×PBS

The expression profiling of immune-related genes in zebrafish intestines

For the analysis of gene expression, total RNA was extracted from zebrafish gut tissues using RNAiso Plus reagent (Takara, Japan) according to the manufacturer's instructions. High-quality RNA samples were prepared for cDNA using the Prime Script Reverse Transcription Reagent kit (Takara, China), and quantitative real-time PCR (qRT-PCR) was performed using ABI 7500 system (Applied Biosystems, CA) with SYBR® Premix Ex Taq™ II (Takara, Japan). The primers for selected genes used in this study were according to our previous study [37], and the primer sequences were shown in **Table S1**. Running program initiated from 95 ^oC, incubated for 5 min, and followed by 40 cycles of 95 ^oC for 15 s, 58 ^oC for 30 s, and 72 ^oC for 45 s. Three independent biological replicates were performed in each group, and $2^{-\Delta\Delta CT}$ method was used to quantify relative mRNA expression levels.

Statistical analysis

The statistical analysis was performed using the Kolmogorov-Smirnov test and Leven's test to define the normality of data and the homogeneity of variance. If the data failed to pass the Kolmogorov-Smirnov test, Logarithmic transformation was performed for the homogeneity of variance. The differences between the variables were calculated by t-test and one-way analysis of variance (ANOVA), followed by Dunnett's test using SPSS 20.0 software (SPSS, Chicago, IL, USA). The value of $p<0.05$ was denoted as statistically significant differences, and all values were presented as the means ± standard error (SEM) of the replicates in each group.

Abbreviations

PAEs: Phthalates; DEHP: di-(2-ethylhexyl) phthalate; OTUs: operational taxonomic units; COG: Clusters of orthologous groups of proteins; KEGG: Kyoto Encyclopedia of Genes and Genomes; ISI: intestinal somatic index; GSI: gonadal somatic index; HIS: hepatosomatic index; H&E: hematoxylin and eosin; AB-PAS: Alcian blue-Periodic Acid Schiff; TG: triglyceride; PY: pyruvate; FA: non-esterified fatty acids; Glu: glucose

Declarations

Acknowledgments

Not applicable

Authors' contributions

PPJ and DSP conceptualized and designed this study. PPJ, GYX and YW performed experiments. PPJ, MJ and YBM analyzed experimental results. PPJ and DSP drafted and modified the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The critical part of sequence progress showed in Supplementary **Figure S1**. The rarefaction curve of sequences and in **Figure S2**. The Shannon rarefaction curve of sequences. Additional data of detecting the specific genus and the abundance of mechanism pathways of zebrafish bacterial functions by COG analysis and KEGG analysis also will be available upon request.

Ethics approval and consent to participate

Zebrafish experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" (Eighth Edition, 2011. ILARCLS, National Research Council, Washington, D.C.). The animal protocol was approved by the Animal Care and Use Committee of Chongqing in China and by the Institutional Animal Care and Use Committee of Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences (Approval ID: ZKCQY0063).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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References

1. Shelby MD. NTP-CERHR monograph on the potential human reproductive and developmental effects of di (2-ethylhexyl) phthalate (DEHP). Ntp Cerhr Mon 2006(18):v, vii-7, II-iii-xiii passim.

2. Erythropel HC, Maric M, Nicell JA, Leask RL, Yargeau V. Leaching of the plasticizer di(2-ethylhexyl)phthalate (DEHP) from plastic containers and the question of human exposure. Appl Microbiol Biot 2014;98(24):9967-81.

3. Lyche JL, Gutleb AC, Bergman A, Eriksen GS, Murk AJ, Ropstad E, et al. Reproductive and Developmental Toxicity of Phthalates. J Toxicol Env Heal B 2009;12(4):225-49.

4. Rowdhwal SSS, Chen JX. Toxic Effects of Di-2-ethylhexyl Phthalate: An Overview. Biomed Res Int 2018.

5. Radke EG, Braun JM, Meeker JD, Cooper GS. Phthalate exposure and male reproductive outcomes: A systematic review of the human epidemiological evidence. Environ Int 2018;121:764-93.

6. Zarean M, Keikha M, Poursafa P, Khalighinejad P, Amin M, Kelishadi R. A systematic review on the adverse health effects of di-2-ethylhexyl phthalate. Environ Sci Pollut R 2016;23(24):24642-93.

7. Ma YB, Jia PP, Junaid M, Yang L, Lu CJ, Pei DS. Reproductive effects linked to DNA methylation in male zebrafish chronically exposed to environmentally relevant concentrations of di-(2-ethylhexyl) phthalate. Environ Pollut 2018;237:1050-61.

8. Junaid M, Jia PP, Tang YM, Xiong WX, Huang HY, Strauss PR, et al. Mechanistic toxicity of DEHP at environmentally relevant concentrations (ERCs) and ecological risk assessment in the Three Gorges Reservoir Area, China. Environ Pollut 2018;242:1939-49.

9. Magdouli S, Daghrir R, Brar SK, Drogui P, Tyagi RD. Di 2-ethylhexylphtalate in the aquatic and terrestrial environment: A critical review. J Environ Manage 2013;127:36-49.

10. Huang PC, Tien CJ, Sun YM, Hsieh CY, Lee CC. Occurrence of phthalates in sediment and biota: Relationship to aquatic factors and the biota-sediment accumulation factor. Chemosphere 2008;73(4):539-44.

11. Kim HY. Risk assessment of di(2-ethylhexyl) phthalate in the workplace. Environmental health and toxicology 2016;31:e2016011.

12. Braun JM, Sathyanarayana S, Hauser R. Phthalate exposure and children's health. Curr Opin Pediatr 2013;25(2):247-54.

13. Jin YX, Wu SS, Zeng ZY, Fu ZW. Effects of environmental pollutants on gut microbiota. Environ Pollut 2017;222:1-9.

14. Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World J Gastroentero 2015;21(29):8787-803.

15. Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, et al. The gut microbiota and host health: a new clinical frontier. Gut 2016;65(2):330-9.

16. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-Gut Microbiota Metabolic Interactions. Science 2012;336(6086):1262-7.

17. Nieuwdorp M, Gilijamse PW, Pai N, Kaplan LM. Role of the Microbiome in Energy Regulation and Metabolism. Gastroenterology 2014;146(6):1525-33.

18. Sullam KE, Essinger SD, Lozupone CA, O'Connor MP, Rosen GL, Knight R, et al. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol Ecol 2012;21(13):3363-78.

19. Cenit MC, Matzaraki V, Tigchelaar EF, Zhernakova A. Rapidly expanding knowledge on the role of the gut microbiome in health and disease. Bba-Mol Basis Dis 2014;1842(10):1981-92.

20. Davis DJ, Bryda EC, Gillespie CH, Ericsson AC. 16S rRNA amplicon sequencing dataset for conventionalized and conventionally raised zebrafish larvae. Data in brief 2016;8:938-43.

21. Dai YJ, Jia YF, Chen N, Bian WP, Li QK, Ma YB, et al. Zebrafish as a Model System to Study Toxicology. Environ Toxicol Chem 2014;33(1):11-7.

22. Jia PP, Ma YB, Lu CJ, Mirza Z, Zhang W, Jia YF, et al. The Effects of Disturbance on Hypothalamus-Pituitary-Thyroid (HPT) Axis in Zebrafish Larvae after Exposure to DEHP. Plos One 2016;11(5).

23. Ma YB, Song LY, Lei Y, Jia PP, Lu CJ, Wu JF, et al. Sex dependent effects of silver nanoparticles on the zebrafish gut microbiota. Environ Sci-Nano 2018;5(3):740-51.

24. Cantas L, Sorby JRT, Alestrom P, Sorum H. Culturable Gut Microbiota Diversity in Zebrafish. Zebrafish 2012;9(1):26-37.

25. Cheesman SE, Guillemin K. We know you are in there: Conversing with the indigenous gut microbiota. Res Microbiol 2007;158(1):2-9.

26. Davis DJ, Bryda EC, Gillespie CH, Ericsson AC. Microbial modulation of behavior and stress responses in zebrafish larvae. Behav Brain Res 2016;311:219-27.

27. Coffin S, Lee I, Gan J, Schlenk D. Simulated digestion of polystyrene foam enhances desorption of diethylhexyl phthalate (DEHP) and In vitro estrogenic activity in a size-dependent manner. Environ Pollut 2018;246:452-62.

28. Bakir A, O'Connor IA, Rowland SJ, Hendriks AJ, Thompson RC. Relative importance of microplastics as a pathway for the transfer of hydrophobic organic chemicals to marine life. Environ Pollut 2016;219:56-65.

29. Kimber I, Dearman RJ. An assessment of the ability of phthalates to influence immune and allergic responses. Toxicology 2010;271(3):73-82.

30. Ahmed KS, Kharoubi O, Aoues AEK, Bouchekara M, Khaladi B, Taleb M. Effect of Gestational and Lactational Exposure to DEHP, DINP, and DEP on Intestinal Morphology, Disaccharidases, and Alkaline Phosphatase in Rats during Postnatal Development. Am J Perinat 2018;35(13):1251-9.

31. Zhao X, Pack M. Modeling intestinal disorders using zebrafish. Method Cell Biol 2017;138:241-70.

32. Caldwell JC. DEHP: Genotoxicity and potential carcinogenic mechanisms-A review. Mutat Res-Rev Mutat 2012;751(2):82-157.

33. Minatoya M, Jima SN, Sasaki S, Araki A, Miyashita C, Ikeno T, et al. Effects of prenatal phthalate exposure on thyroid hormone levels, mental and psychomotor development of infants: The Hokkaido Study on Environment and Children's Health. Sci Total Environ 2016;565:1037-43.

34. Wassenaar PNH, Legler J. Systematic review and meta-analysis of early life exposure to di(2-ethylhexyl) phthalate and obesity related outcomes in rodents. Chemosphere 2017;188:174-81.

35. Cruz G, Foster W, Paredes A, Yi KD, Uzumcu M. Long-Term Effects of Early-Life Exposure to Environmental Oestrogens on Ovarian Function: Role of Epigenetics. J Neuroendocrinol 2014;26(9):613-24.

36. Gao N, Hu RX, Huang YJ, Dao L, Zhang CF, Liu YZ, et al. Specific effects of prenatal DEHP exposure on neuroendocrine gene expression in the developing hypothalamus of male rats. Arch Toxicol 2018;92(1):501-12.

37. Jia PP, Sun T, Junaid M, Xiong YH, Wang YQ, Liu L, et al. Chronic exposure to graphene oxide (GO) induced inflammation and differentially disturbed the intestinal microbiota in zebrafish. Environ Sci-Nano 2019;6(8):2452-69.

38. Adeogun AO, Ibor OR, Omiwole RA, Hassan T, Adegbola RA, Adewuyi GO, et al. Occurrence, Species, and Organ Differences in Bioaccumulation Patterns of Phthalate Esters in Municipal Domestic Water Supply Lakes in Ibadan, Nigeria. Journal of toxicology and environmental health Part A 2015;78(12):761-77.

39. Mu XY, Huang Y, Li J, Yang K, Yang WB, Shen GM, et al. New insights into the mechanism of phthalate-induced developmental effects. Environ Pollut 2018;241:674-83.

40. Boran H, Terzi S. Bis(2-ethylhexyl) phthalate induces DNA strand breaks and gene expression alterations in larval zebrafish Danio rerio. Toxicology and industrial health 2019;35(8):520-9.

41. Jacobs HM, Sant KE, Basnet A, Williams LM, Moss JB, Timme-Laragy AR. Embryonic exposure to Mono(2-ethylhexyl) phthalate (MEHP) disrupts pancreatic organogenesis in zebrafish (Danio rerio). Chemosphere 2018;195:498-507.

42. Erasmus VN, Iitembu JA, Hamutenya S, Gamatham J. Evidences of possible influences of methylmercury concentrations on condition factor and maturation of Lophius vomerinus (Cape monkfish). Marine pollution bulletin 2019;146:33-8.

43. Santos KO, Costa-Filho J, Riet J, Spagnol KL, Nornberg BF, Kutter MT, et al. Probiotic expressing heterologous phytase improves the immune system and attenuates inflammatory response in zebrafish fed with a diet rich in soybean meal. Fish & shellfish immunology 2019;93:652-8.

44. Zanotelli VR, Neuhauss SC, Ehrengruber MU. Long-term exposure to bis(2-ethylhexyl)phthalate (DEHP) inhibits growth of guppy fish (Poecilia reticulata). Journal of applied toxicology : JAT 2010;30(1):29-33.

45. Huff M, da Silveira WA, Carnevali O, Renaud L, Hardiman G. Systems Analysis of the Liver Transcriptome in Adult Male Zebrafish Exposed to the Plasticizer (2-Ethylhexyl) Phthalate (DEHP). Scientific reports 2018;8(1):2118.

46. Vatsos IN. Standardizing the microbiota of fish used in research. Lab Anim-Uk 2017;51(4):353-64.

47. Cardinelli CS, Sala PC, Alves CC, Torrinhas RS, Waitzberg DL. Influence of intestinal microbiota on body weight gain: a narrative review of the literature. Obes Surg 2015;25(2):346-53.

48. Fujimura KE, Slusher NA, Cabana MD, Lynch SV. Role of the gut microbiota in defining human health. Expert review of anti-infective therapy 2010;8(4):435-54.

49. Stephens WZ, Burns AR, Stagaman K, Wong S, Rawls JF, Guillemin K, et al. The composition of the zebrafish intestinal microbial community varies across development. Isme J 2016;10(3):644-54.

50. Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Org E, Parks B, et al. Individual diet has sex-dependent effects on vertebrate gut microbiota. Nat Commun 2014;5:4500.

51. Roman P, Cardona D, Sempere L, Carvajal F. Microbiota and organophosphates. Neurotoxicology 2019;75:200-8.

52. Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K, et al. Evidence for a core gut microbiota in the zebrafish. Isme J 2011;5(10):1595-608.

53. Burns AR, Stephens WZ, Stagaman K, Wong S, Rawls JF, Guillemin K, et al. Contribution of neutral processes to the assembly of gut microbial communities in the zebrafish over host development. Isme J 2016;10(3):655-64.

54. Xia JH, Lin G, Fu GH, Wan ZY, Lee M, Wang L, et al. The intestinal microbiome of fish under starvation. Bmc Genomics 2014;15:266.

55. Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in biotechnology 2015;33(9):496-503.

56. Yousfi K, Bekal S, Usongo V, Touati A. Current trends of human infections and antibiotic resistance of the genus Shewanella. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 2017;36(8):1353-62.

57. Wilkes Walburn J, Wemheuer B, Thomas T, Copeland E, O'Connor W, Booth M, et al. Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (Seriola lalandi). Microbial biotechnology 2019;12(2):275-88.

58. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56(7):1761-72.

59. Boutagy NE, McMillan RP, Frisard MI, Hulver MW. Metabolic endotoxemia with obesity: Is it real and is it relevant? Biochimie 2016;124:11-20.

60. Tian X, Yu Z, Feng P, Ye Z, Li R, Liu J, et al. Lactobacillus plantarum TW1-1 Alleviates Diethylhexylphthalate-Induced Testicular Damage in Mice by Modulating Gut Microbiota and Decreasing Inflammation. Frontiers in cellular and infection microbiology 2019;9:221.

61. Meng XL, Li S, Qin CB, Zhu ZX, Hu WP, Yang LP, et al. Intestinal microbiota and lipid metabolism responses in the common carp (Cyprinus carpio L.) following copper exposure. Ecotox Environ Safe 2018;160:257-64.

62. Kindleysides S, Kruger R, Douwes J, Tannock GW, Renall N, Slater J, et al. Predictors Linking Obesity and the Gut Microbiome (the PROMISE Study): Protocol and Recruitment Strategy for a Cross-Sectional Study on Pathways That Affect the Gut Microbiome and Its Impact on Obesity. JMIR research protocols 2019;8(8):e14529.

63. Wong JM, Esfahani A, Singh N, Villa CR, Mirrahimi A, Jenkins DJ, et al. Gut microbiota, diet, and heart disease. Journal of AOAC International 2012;95(1):24-30.

64. Sommer F, Anderson JM, Bharti R, Raes J, Rosenstiel P. The resilience of the intestinal microbiota influences health and disease. Nature reviews Microbiology 2017;15(10):630-8.

65. Avolio E, Gualtieri P, Romano L, Pecorella C, Ferraro S, Di Renzo L, et al. Obesity and body composition in man and woman: associated diseases and new role of gut microbiota. Current medicinal chemistry 2019.

66. Cerdo T, Garcia-Santos JA, M GB, Campoy C. The Role of Probiotics and Prebiotics in the Prevention and Treatment of Obesity. Nutrients 2019;11(3).

67. La Fata G, Weber P, Mohajeri MH. Probiotics and the Gut Immune System: Indirect Regulation. Probiotics and antimicrobial proteins 2018;10(1):11-21.

68. Kaiko GE, Stappenbeck TS. Host-microbe interactions shaping the gastrointestinal environment. Trends in immunology 2014;35(11):538-48.

69. Hall A, Versalovic J. Microbial Metabolism in the Mammalian Gut: Molecular Mechanisms and Clinical Implications. Journal of pediatric gastroenterology and nutrition 2018;66 Suppl 3:S72-S9.

70. Van Treuren W, Dodd D. Microbial Contribution to the Human Metabolome: Implications for Health and Disease. Annual review of pathology 2019.

71. Fernandez-Navarro T, Diaz I, Gutierrez-Diaz I, Rodriguez-Carrio J, Suarez A, de los Reyes-Gavilan CG, et al. Exploring the interactions between serum free fatty acids and fecal microbiota in obesity through a machine learning algorithm. Food Res Int 2019;121:533-41.

72. Feng W, Ao H, Peng C. Gut Microbiota, Short-Chain Fatty Acids, and Herbal Medicines. Frontiers in pharmacology 2018;9:1354.

73. Hernandez MAG, Canfora EE, Jocken JWE, Blaak EE. The Short-Chain Fatty Acid Acetate in Body Weight Control and Insulin Sensitivity. Nutrients 2019;11(8).

74. Lynch JW, Miles JM, Bailey JW. Effects of the short-chain triglyceride triacetin on intestinal mucosa and metabolic substrates in rats. JPEN Journal of parenteral and enteral nutrition 1994;18(3):208-13.

75. Buerger AN, Schmidt J, Chase A, Paixao C, Patel TN, Brumback BA, et al. Examining the responses of the zebrafish (Danio rerio) gastrointestinal system to the suspected obesogen diethylhexyl phthalate. Environ Pollut 2019;245:1086-94.

76. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505(7484):559-63.

77. Maranduba CMD, De Castro SBR, de Souza GT, Rossato C, da Guia FC, Valente MAS, et al. Intestinal microbiota as modulators of the immune system and neuroimmune system: impact on the host health and homeostasis. J Immunol Res 2015;2015:931574.

78. Gomez GD, Balcazar JL. A review on the interactions between gut microbiota and innate immunity of fish. Fems Immunol Med Mic 2008;52(2):145-54.

79. Vasovic M, Gajovic N, Brajkovic D, Jovanovic M, Zdravkovic N, Kanjevac T. The relationship between the immune system and oral manifestations of inflammatory bowel disease: a review. Cent Eur J Immunol 2016;41(3):302-10.

80. Yang YN, Yang YCSH, Lin IH, Chen YY, Lin HY, Wu CY, et al. Phthalate exposure alters gut microbiota composition and IgM vaccine response in human newborns. Food Chem Toxicol 2019;132.

81. Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology 1999;22(3):434-44.

82. Song L, Yang S, Liu H, Xu J. Geographic and environmental sources of variation in bacterial community composition in a large-scale municipal landfill site in China. Appl Microbiol Biot 2017;101(2):761-9.

83. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences 2010;107(33):14691-6.

84. Escaffre AM, Kaushik S, Mambrini M. Morphometric evaluation of changes in the digestive tract of rainbow trout (Oncorhynchus mykiss) due to fish meal replacement with soy protein concentrate. Aquaculture 2007;273(1):127-38.

Figures

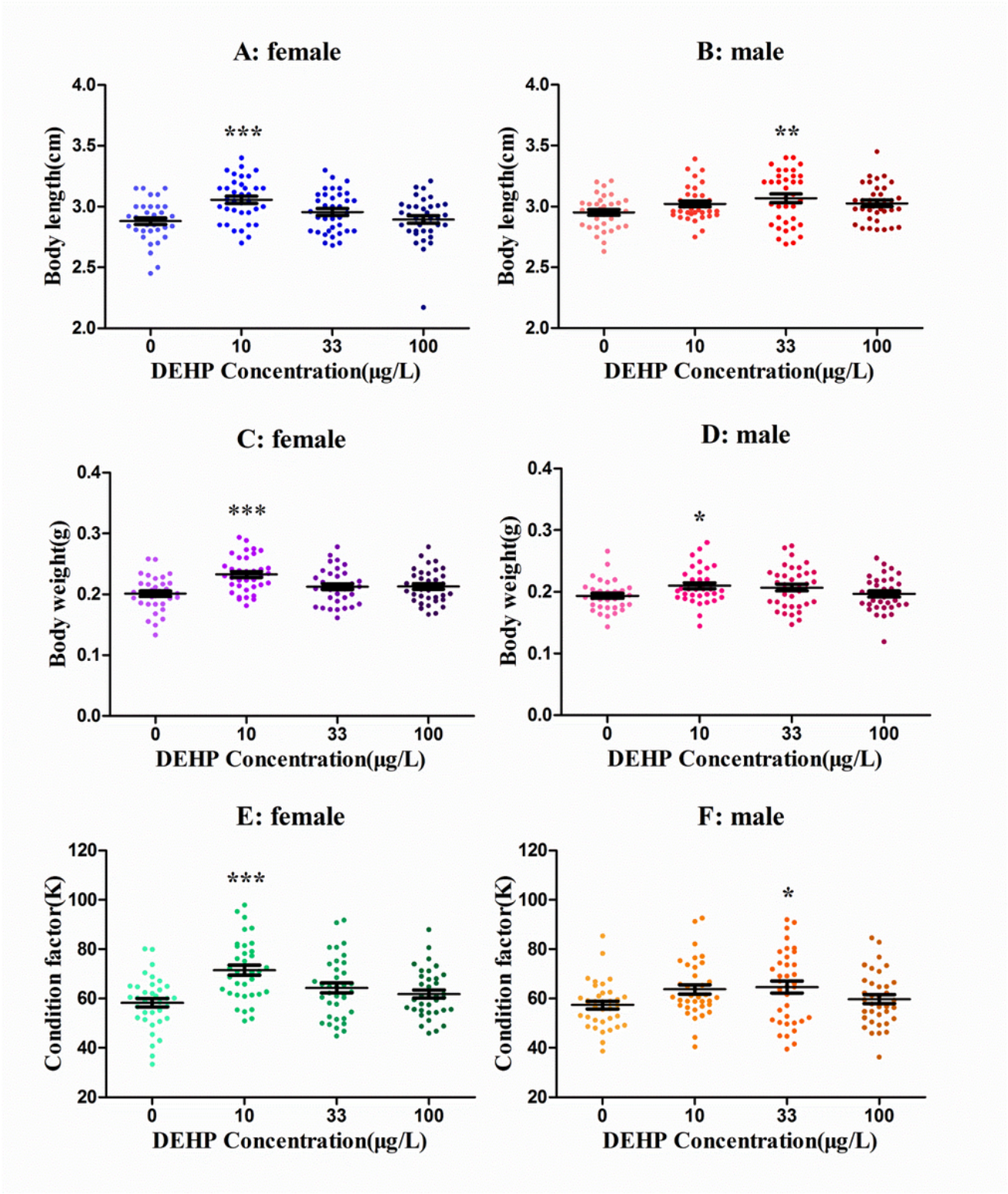

Figure 1

The developmental indexes of zebrafish after long-term DEHP exposure. The body length (cm) of female (A) and male (B) fish, and the body weight (g) of female (C) and male (D) fish were presented. The condition factor (K) of female (E) and male (F) fish were calculated. The symbols *, **, and *** showed p <0.05, 0.01, and 0.001 with significant differences between exposure and control groups.

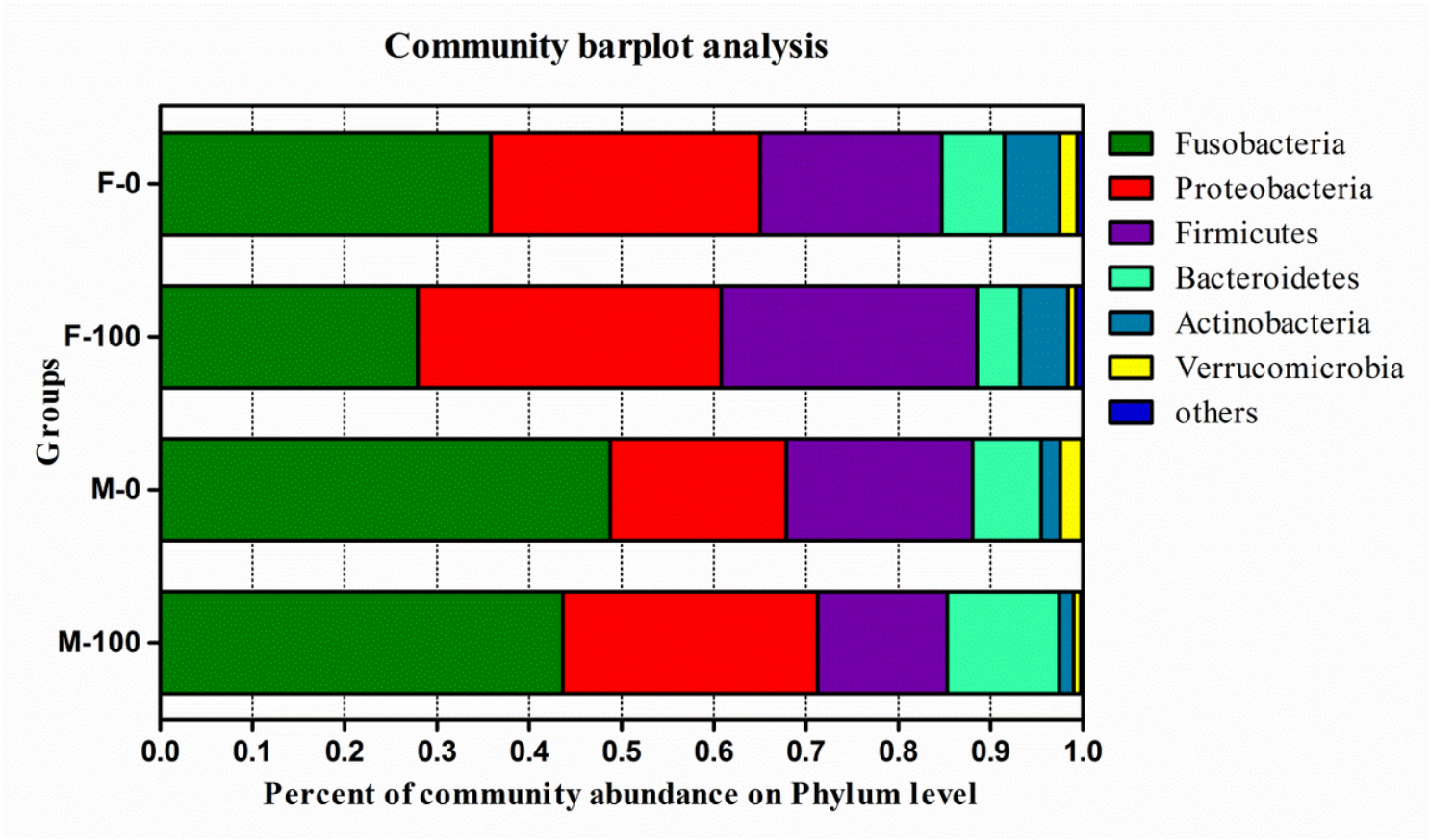

Figure 2

The community abundance of zebrafish intestinal microbiota according to the level of phylum. The average value of three replicate samples was calculated, and the value of phylogenetic groups accounting for < 0.01 was summarized as “others”.

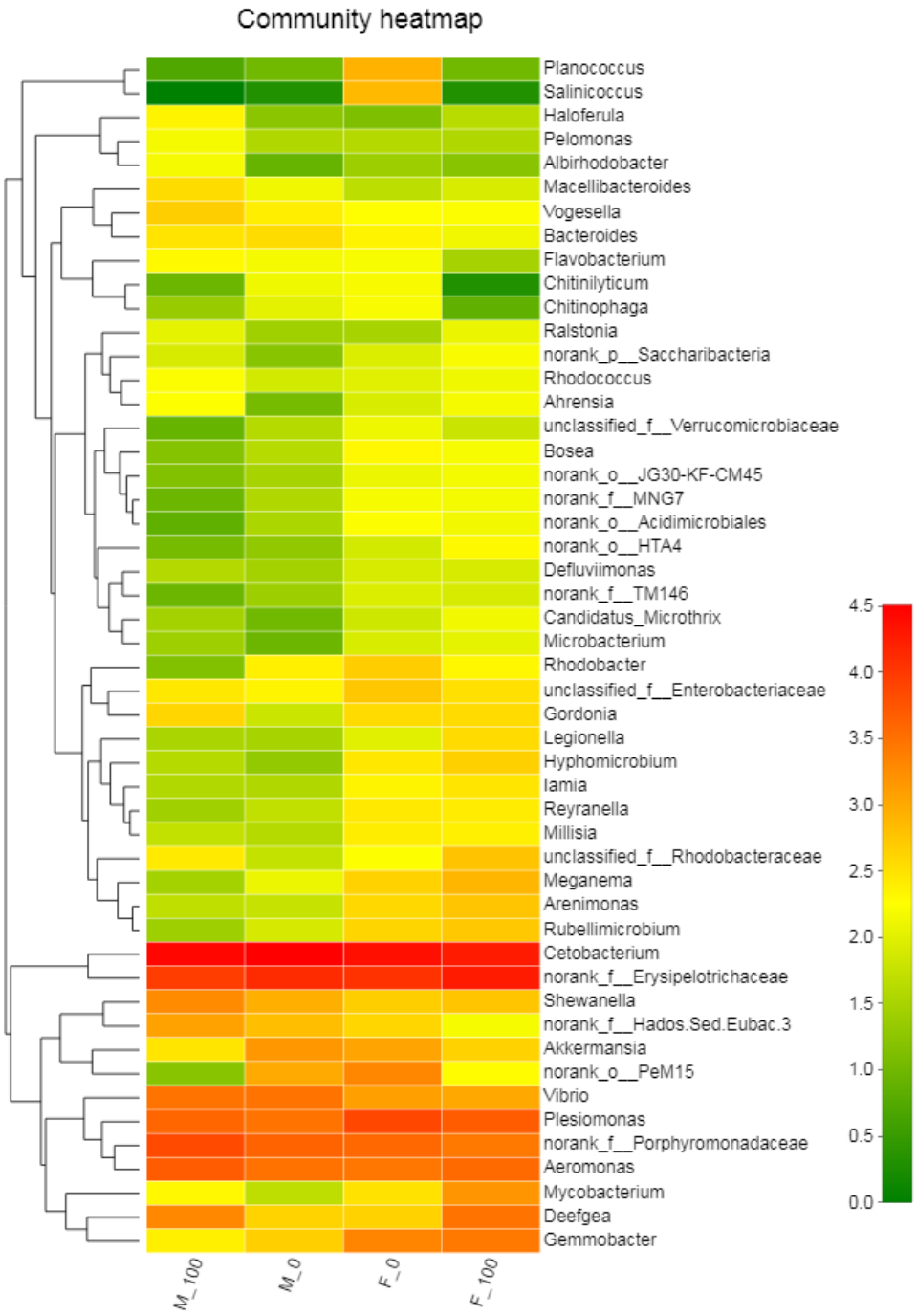

Figure 3

The abundance heatmap of zebrafish intestinal bacteria according to the level of genus. The bacterial hierarchical genera were clustered by the average manner, and the top 50 species shown in the map were calculated by the log value of bacterial abundance percentage in each group.

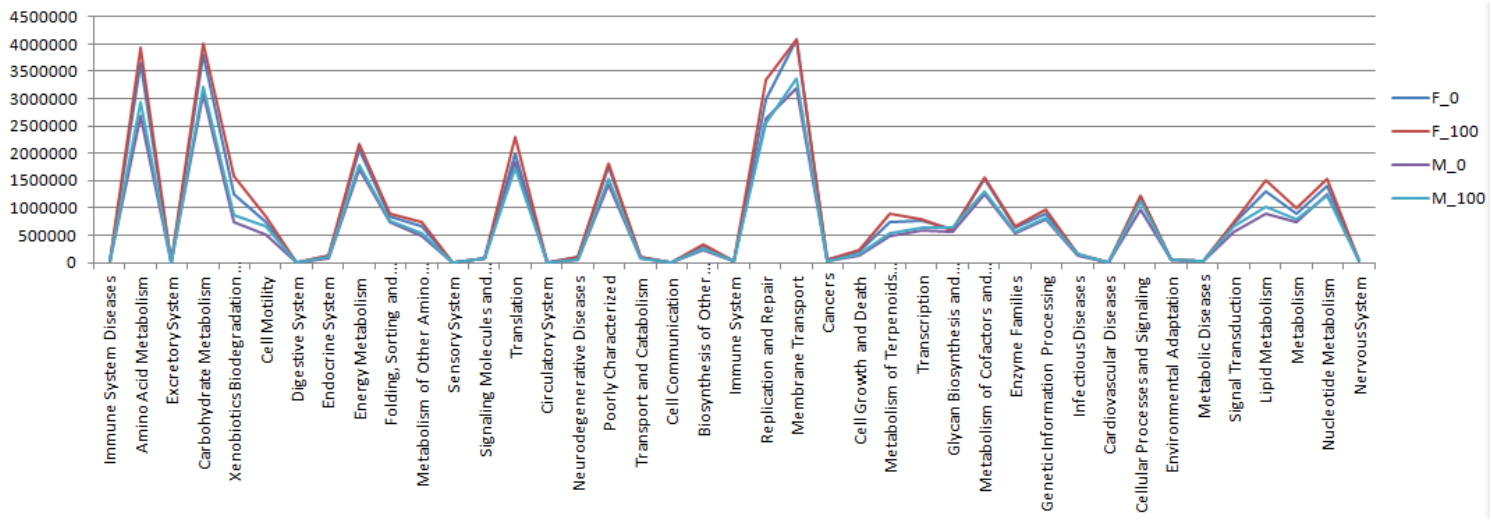

Figure 4

The relative abundance of metabolic pathways at the second level. The values were represented with the average abundance of three replicate samples, and the metabolic pathways showed different levels in female and male zebrafish in DEHP exposure and control groups.

Figure 5

The histomorphological analysis of zebrafish intestine tissues with H&E stain. The histological analysis of gut tissue in female (A) and male (C) zebrafish in the control group, female (B) and male (D) zebrafish in 100 μg/L DEHP treatment group. (E) The height and width of villi, crypt depth, and tunica muscular thickness were measured from gut sections with 200 μm scale bar and 200 × images, and the symbol * indicated $p<0.05$ as the significant changes compared to control.

Figure 6

The goblet cells in zebrafish intestine tissues with AB-PAS stain. The histological analysis of gut tissue in female (A) and male (C) zebrafish in the control group, female (B) and male (D) zebrafish in the 100 μg/L DEHP treatment group. (E) The number of goblet cells was counted from sections with 200 μm scale bar and 200 × images, and the symbol * indicated $p<0.05$ as the significant changes compared to control.

Figure 7

The content of energy metabolites in chronic DEHP exposed zebrafish intestines. The contents of TG, PY, FA, and Glu in female and male zebrafish were measured and the symbols *, **, and *** implied $p<0.05$, 0.01, and 0.001, respectively.

Figure 8

The expression of key genes related to the immune response in DEHP exposed zebrafish. The relative expression of tlr-5, il-1β, nf-κb, and il-8 genes in female and male zebrafish, with the symbols * and ** indicated $p<0.05$ and <0.01 as the significant differences between DEHP treatment and control groups.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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