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Integrated approach of eco-epigenetics and eco-metabolomics on the stress response of bisphenol-A exposure in the aquatic midge *Chironomus riparius*

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ABSTRACT

The stress response mechanisms of Bisphenol A (BPA), an endocrine disrupting compound, remain to be elucidated. In this study, we explored the effects of BPA on the non-biting midge *Chironomus riparius* through basic ecotoxicity assays, DNA damage (comet assay), eco-epigenetics (global DNA and histone methylations) and non-targeted global metabolomics (NMR based) approaches. The reproduction failure, increase in DNA damage, global DNA hyper-methylation, and increased global histone modification (H3K36) status were evident due to BPA exposure at 10% lethal concentration (LC_{10}: 1 mg/L, based on 48 h acute toxicity). Moreover, non-targeted global metabolomics followed by pathway analysis identified alterations of energy metabolism, amino acids, and methionine metabolisms etc. Most importantly, we found a potential cross-talk between altered epigenetics and metabolites, such as, increase in methionine and o-phosphocholine metabolites corresponds with the phenomena of global hyper-methylation in DNA and H3K36 mark. Overall, our results suggests that the crosstalk of global metabolomics and epigenetic modification was fundamental of the underlying mechanisms in BPA-induced stress response in *C. riparius*.

1. Introduction

Bisphenol A (BPA), a monomer of polycarbonate plastics and epoxide resin, is a widly used chemical, having endocrine disrupting property. Being an industrially important chemical material for the manufacture of engineering plastics (e.g., epoxy resins/polycarbonate plastics), food cans (i.e., lacquer coatings), dental composites/sealants etc., BPA pollutes freshwater and marine ecosystems via industrial and municipal effluents, leachates from landfill sites, and litter (Huang et al., 2012). Recently, BPA pollution hotspots were identified, further reflecting the importance of determining the effects of current and predicted levels of BPA pollution on ecosystem health (Little and Seebacher, 2015). BPA disrupts several receptor-mediated pathways including thyroid hormone, estrogen-related, and glucocorticoid receptors in various species, including aquatic organisms (Little and Seebacher, 2015; Canesi and Fabbri, 2015). Animal studies and human epidemiological studies indicate that BPA is an endocrine disrupting chemical (EDC) that has been implicated as a potential carcinogen and epigenotoxicant (Weinhouse et al., 2015). Moreover, BPA exposure has been linked to adult metabolic pathologies, but the pathways through which these disruptions occur is unknown (Veiga-Lopez et al., 2015).

Epigenetics represents how the environment interacts with genes and modulates gene expression/activity without changing the DNA sequence of the cell (Head et al., 2012; Casati et al., 2015; Vandegehuchte and Janssen, 2011). The commonly studied epigenetic mechanisms involved chromatin remodeling (DNA methylation and histone modification) and RNA-mediated modifications (non-coding RNA and microRNA) (Ho and Burggren, 2010). Alterations in epigenomes could cause mitotically or meiotically heritable changes in gene function and pass through subsequent generations (Vandegehuchte and Janssen, 2011) or could result in stable transcriptional changes of health susceptibility to develop diseases (from cancer to mental disorders), which might not necessarily be heritable (Casati et al., 2015). At present, it is widely recognized that exposure to environmental factors (chemical, diets, temperatures etc.) alters gene expressions and affects health by affecting genome and modulating epigenome. Growing evidence indicates the use of epigenetic tools in ecotoxicology; however, the knowledge and applications in ecologically relevant organisms are not as comparable as mammalian model organisms (Suarez-Ulloa et al., 2015).

Metabolomics usually reflects the combined effects of multiple upstream factors, such as transcriptomes, proteomes, and the nutritional environment (Wilmes et al., 2013). Metabolic profiling not only permits candidate biomarkers identification but also would elucidate the

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induced toxicity mechanisms of the compound (Long et al., 2015). Environmental metabolomics, the application of metabolomic techniques to analyze the interactions of organisms with their environment, can be attributed to generate hypotheses involving nontargeted metabolomics of environmental stressors with an unknown mode of action (Lankadurai et al., 2013).

Whether environmental levels of BPA have adverse effects on aquatic organisms remain controversial (Huang et al., 2012) and BPA induced mechanisms of toxicity, specifically epigenotoxicity and metabolomics pathways, in aquatic species remain elusive.

In the present study, we explored the effects of BPA on the crosstalk of eco-epigenetic and metabolomic modification in 4th instar larvae of the non-biting midge *Chironomus riparius* (*C. riparius*). First, acute and chronic toxicity tests were conducted and thereafter, assays for DNA damage, global DNA methylation and histone methylation modifications were performed to elucidate the genetic-epigenetic alteration mechanisms in BPA-treated *C. riparius*. Finally, global metabolomic profiling with nuclear magnetic resonance (NMR) was performed to elucidate not only altered metabolomic pathways but also the role of epigenetics-metabolomics crosstalk in BPA-induced toxicity.

2. Materials and methods

2.1. Test organisms and media

C. riparius were obtained from the Toxicological Research Center of the Korea Institute of Chemical Technology and have been reared in our laboratory for more than 10 years. The larvae were reared on an artificial diet of fish food flakes (Tetramin; Tetrawerke) in glass chambers containing dechlorinated tap water and acid-washed sand, with aeration at 20 ± 1 °C under a 16:8-h light:dark photoperiod. All experiments were performed in US Environmental Protection Agency moderately hard water (EPA-MHW) (US EPA, 2002).

2.2. BPA exposure condition

The effect of BPA (purity > 99%; Sigma-Aldrich Chemical, St. Louis, MO) exposure on groups of 4th instar larvae collected from the rearing aquaria was assessed. At the beginning of the experiment, 1 mL of an acetonic solution of the compounds was placed in the 1 L experimental tanks. Thirty larvae (of sublethal toxicity) were randomly introduced into each test aquarium. The exposure was carried out at a constant temperature (20 ± 1 °C) within a 16:8 h (light:dark) photoperiod in all the experiments. The comet assay, metabolomics assay, global DNA methylation assay and primary screening assay of histone methylation were conducted in sublethal concentration of BPA (48 h at 1 mg/L: LC_{10} BPA).

2.3. Acute toxicity test

A mortality test was conducted using a modified OECD guideline (TG 235) (OECD, 2011). A group of 10 larvae was exposed to four concentrations of BPA (1, 2.5, 5, and 7 mg/L), and the other was the control group. It was determined that acute toxicity occurs after 48 h exposure, and that the condition eventually leads to death. Log-probit (epa probit analysis program version 1.5) data transformation was used to estimate 48 h 10%, 50%, and 90% lethal concentration (LC_{10}, LC_{50}, and LC_{90}) values and the corresponding 95% confidence intervals.

2.4. Chronic toxicity tests (reproduction and development tests)

For the chronic toxicity test, a modified OECD guideline (TG 219) (OECD, 2004) was used. Exposure aquariums were prepared by adding 400 mL of EPA-MHW to 100 mL of acid-washed sand. The thirty 4th instar larvae were introduced and BPA concentration (1 mg/L BPA: concentration of LC_{10}) were spiked to the treatment conditions. Emergence and reproduction were monitored for 25 days until all treated and control organisms were dead. The emerging adults from each vessel were counted and retained with steel-wire mesh until emergence was complete in all treatments. The egg masses oviposited by the emerged adults in the control and treated vessels were counted and used for reproduction parameters. Every 2 days, 50 mg tetramin fish food flakes was supplied to each aquarium. Test solutions were not renewed. All data were recorded at daily intervals.

2.5. DNA damage measurement by the comet assay

Ten larvae of *C. riparius* were collected from the control and the 48-h BPA treatment (at 1 mg/L) for the comet assay as described previously (Park and Choi, 2007). Briefly, 100 µl 1% low melting point (LMP) agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4 °C for 5 min to allow solidification. The samples were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 NaCl, 10% DMSO (only organisms), 10% Triton X-100, pH 10), and subsequently exposed to alkali conditions (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow the DNA to unwind and the alkaliliable sites to be expressed. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min, after which, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place prior to image analysis. Before their analyses, the slides were stained with 50 µl ethidium bromide (5 µg/mL), and then observed under a fluorescence microscope (Nikon, Kanagawa, Japan) equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm at 400 × magnification. Approximately, 50 cells per slide (3 slides per treatment) were examined. DNA damage was expressed as the tail moment using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

2.6. Global DNA methylation

Total DNA from BPA-treated samples (for 48 h at 1 mg/L) was extracted using a DNA extraction kit (NucleoSpin, Macherey-Nagel) and the quantity and quality of DNA were detected in Nano-drop. Next, global DNA methylation assays were performed according to the manufacturer's instructions (MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric), Epigentek Group Inc. NY, USA). Briefly, 100 ng DNA and other provided standards were added to a 5-Methylcytocine (5-mc) coated well and incubated for 90 min at 37 °C. Next, after adding the antibodies (anti-5mc and secondary antibody in provided ELISA buffer), enhancers and stop solutions sequentially, absorbance at OD450 nm was measured and the 5-mc % was calculated from the standard curve. Appropriate negative and positive controls were used to prepare the standard curve.

2.7. Screening assay of global histone methylation modifications

Total histone from the control and BPA-treated samples (for 48 h at 1 mg/L) was extracted using a histone extraction kit (EpiQuik Total Histone Extraction Kit, Epigentek Group Inc. NY, USA) and the quantity was detected in a spectrophotometer. The primary screening assay of histone methylation was carried out in BPA-treated *C. riparius* (EpiModifier™ Epigenetic Histone Modification Profiling, Epigentek Group Inc. NY, USA). The methylated fraction (K4, K9, K27, and K36) of histone H3 was detected using specific antibodies (0.5 µg/mL of each antibody) against histone H3 with these methylated lysine sites, and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of methylated histone H3 is proportional to the OD450 intensity measured. The volume for each sample was 300 µl with the histone concentrations ranging from 0.112 to 0.319 µg/µl.

2.8. NMR metabolomics analysis

The NMR-based metabolomics were performed with BPA-treated *C. riparius* (48 h at 1 mg/L) in ^{1}H NMR experiments using an Agilent 600 spectrometer (Agilent Technologies, CA, USA) operating at 600.17 MHz. All spectra were processed and assigned by Chenomx NMR suite 7.1 professional and the Chenomx 600 MHz library database for the identification of metabolites. The details, including sample preparations and statistical analysis, are provided in Supplementary materials (Section 2). Metabolomics enrichment pathway analysis was performed using MetaboAnalyst software 3.0 (a web service for metabolomics data analysis) with metabolites that were significantly different ($p < 0.05$) from those of the control.

2.9. Statistical analysis

Statistical differences between the control and treated samples were examined with a one-way analysis of variance (ANOVA) using SPSS 12.0KO. All data are reported as the mean standard error of the mean. Toxicological data were assessed for normality using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. The One-way analysis of variance (ANOVA) was performed on all data, and $p < 0.05$ was considered statistically significant by Tukey's honestly significant difference test.

3. Results

3.1. Acute toxic effects of BPA

Based on the results of the 48-h acute toxicity tests, the LCs (LC_{10}, LC_{50}, and LC_{90}) of BPA in the 4th instar larvae of *C. riparius* were calculated (Table S1) as 1.29, 2.64, and 5.44 mg/L, respectively, and the sublethal exposure concentration less than LC_{10}, 1 mg/L for 48 h, was selected for subsequent experiments, including chronic toxicity, global DNA methylation, metabolomics analysis, histone modification, and DNA damage.

3.2. Chronic toxicity of BPA

The pupation and emergence parameters (the developmental toxicity markers) were not significantly affected in *C. riparius* by BPA exposure (Table S2 and Fig. 1A). The time to emergence and its rate were not significantly different. In another study, BPA was reported to cause a significant delay in the emergence of males and females in *C. riparius* between the concentrations ranging from 78 ng/L to 0.75 mg/L at their second generation, but almost no significant differences were exhibited in first generation (Watts et al., 2001). In addition, the effect of BPA on reproduction was determined according to two parameters, namely, the number of eggmasses/treatment and the number of eggs/eggmass. The effect of BPA on the number of eggmasses/treatment was not significant. These data were similar to the previous study (Watts et al., 2001). However, a considerable decrease in the number of eggs/egg mass showed a significant alteration in BPA-induced reproduction potentiality (Fig. 1B).

3.3. DNA damage potentiality of BPA in C. riparius

BPA exposure to *C. riparius* caused DNA damage even at sublethal doses (LC_{10}, 1 mg/L). As shown in Fig. 2, the comet tail moment significantly increased with BPA exposure ($p < 0.049$). Likewise, our previous study reported that the BPA exposure caused DNA damage in *C. riparius* at the concentrations less than 1 mg/L BPA (Park and Choi, 2009). Hence, BPA exhibited a genotoxic effect on *C. riparius*.

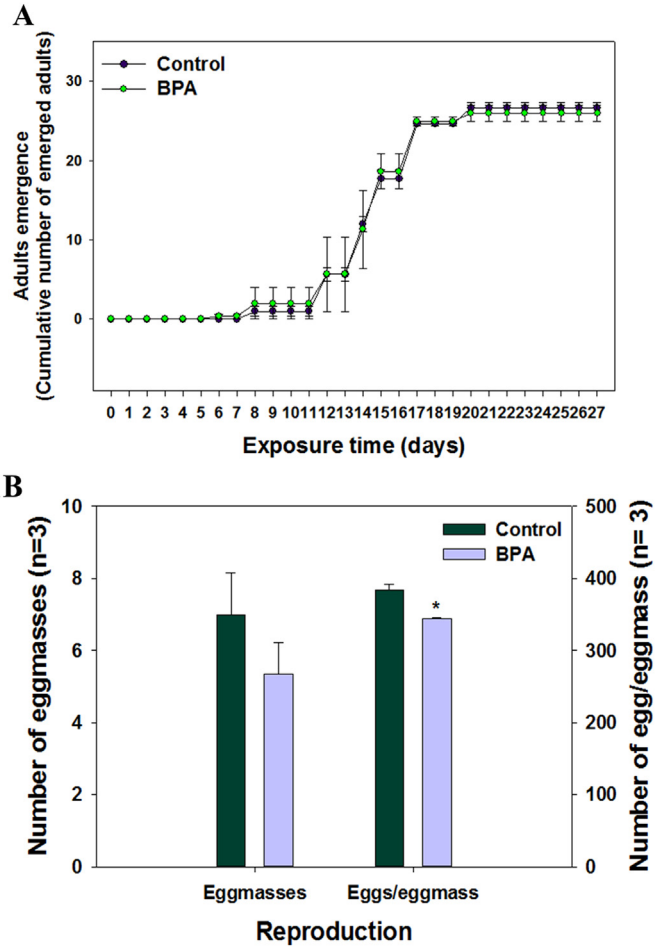

Fig. 1. Cumulative frequency distribution of development (adult emergence (A)) and reproduction parameters (number of eggmasses and number of eggs/eggmass) (B) in bisphenol A (BPA)-exposed 4th instar larvae of *Chironomus riparius* (n = 3, mean ± standard error of means (SEM)). Asterisks indicate significant difference (*$p < 0.05$) compared with the BPA-treatment group on analysis of variance ($p < 0.063$ for eggmasses; $p < 0.042$ for egg/eggmass).

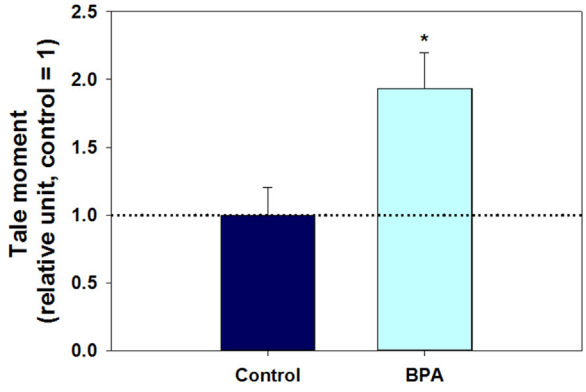

Fig. 2. DNA damage measured in *Chironomus riparius* exposed for 48 h to 10% lethal concentration (LC_{10}) of bisphenol A (BPA) using single-cell gel electrophoresis. The results was expressed as tail moment measured in BPA-exposed *Chironomus riparius* (n = 3, mean ± standard error of mean (SEM) and *$p < 0.05$).

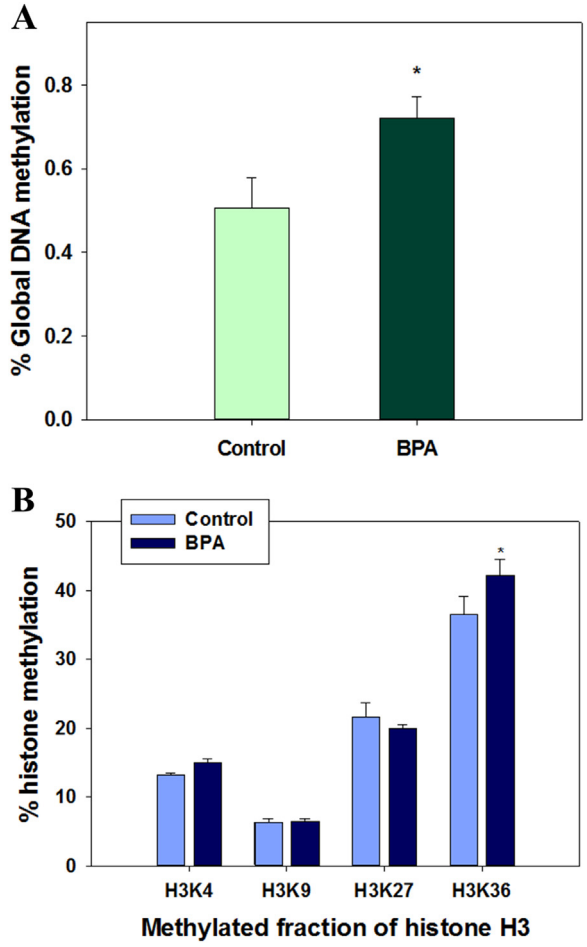

Fig. 3. The changes in global DNA methylation (A) and global histone methylation modification marks (H3K4, H3K9, H3K27 and H3K36) (B) due to bisphenol A (BPA) exposure at 10% lethal concentration (LC_{10}) concentrations in *Chironomus riparius.* (n = 3, mean ± standard error of means (SEM) and *p < 0.05). (n = 3, mean ± standard error of means (SEM) and *p < 0.05).

3.4. Effects of BPA exposure on global DNA methylation of C. riparius

The global methylation status exhibited that DNA methylation was changed upon BPA exposure at LC_{10} (1 mg/L) in *C. riparius*. Hyper-methylation was clearly exhibited in the BPA-treated larvae of *C. riparius* ($p < 0.041$) (Fig. 3A). The BPA-induced alteration in DNA methylation status, global as well as gene specific, were reported in some fish model systems, and mammals including human (Liu et al., 2014; Jiang et al., 2015; Kim et al., 2013); however, no studies were found on the BPA effects in *C. riparius* or related species.

3.5. Alterations of histone methylation modification in BPA treated C. riparius

Alterations in global histone modification status (H3 methylation of H3K4, H3K9, H3K27 and H3K36) were observed in BPA-exposed *C. riparius*. In particular, hyper-methylation in H3K36 (statistically significant, $p < 0.012$) and H3K4 (no statistical significance), demethylation in H3K27 (no statistical significance), and no change in H3K9 were evident (Fig. 3B). Unlike DNA methylation, only a few studies have demonstrated the effects of BPA on histone modifications (Singh and Li, 2012; Trapphoff et al., 2013).

Table 1
List of NMR metabolites in *Chironomus riparius* exposed to bisphenol A (BPA) for 48 h at 10% lethal concentration (LC_{10}) concentration.

Metabolites name	% Change
1,3-Dihydroxyacetone	− 28.46
2-Oxoglutarate	9.44
ADP*	**− 32.08**
ATP**	**78.53**
Acetate	− 24.49
Alanine*	**29.84**
Arginine**	**− 40.59**
Fumarate	− 16.29
Glucose	− 14.34
Glutamate	− 25.16
Glutamine*	**33.42**
Glycine	− 14.54
Isoleucine	− 7.13
Lactate**	**81.96**
Leucine	4.28
Lysine	15.34
Malate	24.57
Methionine	27.50
O-Phosphocholine**	**44.78**
Phenylalanine	− 11.31
Pyruvate	− 8.71
Valine	6.02

(+) indicates up-regulation whereas (-) indicates down regulation. Asterisks are significantly altered metabolites (*: p value < 0.05 and **: p value < 0.01).

3.6. Metabolomics profiling

To elucidate the BPA-induced metabolic alterations in *C. riparius*, non-targeted global metabolomics analysis was performed with NMR. The orthogonal partial least-squares discriminant analysis (OPLS-DA) and the score plots of the OPLS-DA model (Supplementary material, Fig. S1) showed that BPA groups clustered together and were clearly distinct from the control group. The NMR spectrum for the whole body extract of *C. riparius* with identified metabolites was shown in Fig. S2 (Supplementary material). The BPA induced differential metabolites were identified and further analyzed quantitatively, in comparison to the control, using a traditional statistical method (ANOVA), and Table 1 presented the significantly altered metabolites (up- or down-regulated in respect of the control) in both of BPA-treated larvae. Several amino acids and sugars were identified: 1,3-dihydroxyacetone, 2-oxoglutarate, ADP, ATP, acetate, alanine, arginine, fumarate, glucose, glutamate, glutamine, glycine, isoleucine, lactate, leucine, lysine, malate, methionine, O-phosphocholine, phenylalanine, pyruvate and valine.

Furthermore, the biological pathway enrichment analysis was performed in MetaboAnalyst 3.0 software with significantly altered metabolites ($p < 0.05$). The BPA evoke different up regulated metabolic pathways in *C. riparius* (Fig. S3). The main altered pathways were energy metabolism, protein biosynthesis, gluconeogenesis, and methionine pathways. These differentially altered metabolic pathways are possibly the mechanisms underlying tissue-specific effects of BPA.

It is proposed that metabolomics can be used to highlight the effects of low doses of endocrine disruptors by demonstrating the disrupting effects of low doses of BPA on global energy metabolism, brain function in CD-1 mouse pups (Cabaton et al., 2013). In particular, a previous study conducted on zinc-exposed *Chironomus tepperi* showed the usefulness of metabolomic approaches in understanding the mechanism of exposure as well as the identification of suitable candidate biomarkers for biomonitoring purposes (Long et al., 2015).

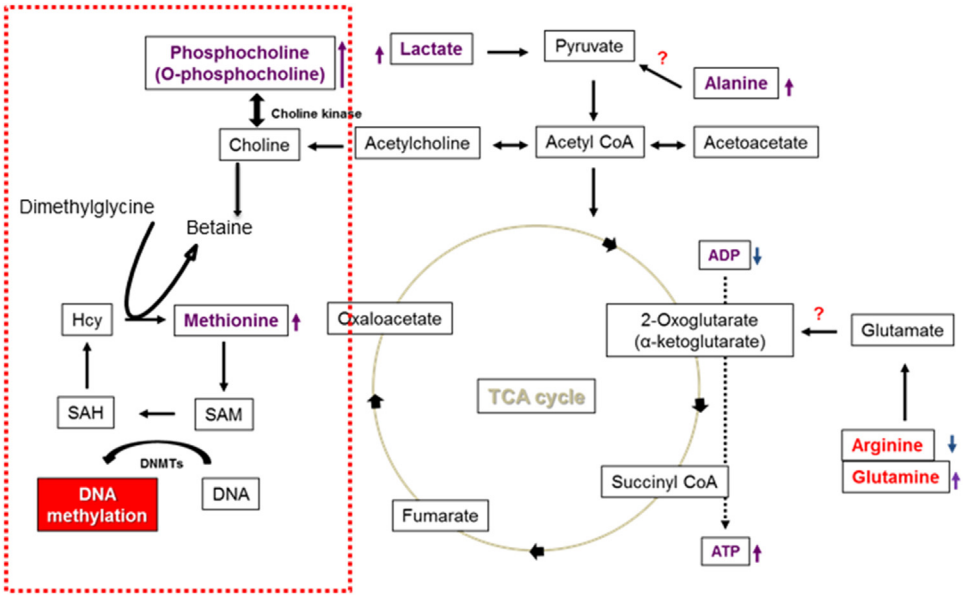

Fig. 4. The schematic presentation of potential crosstalk between epigenetic and metabolomics changes due to bisphenol A (BPA) exposures at 10% lethal concentration (LC_{10}) concentrations in *Chironomus riparius*. The metabolites which showed alteration are presented in color other than black and corresponding arrows for up/down regulation. This figure is modified from Locasale (2013) and Liu et al. (2016).

4. Discussion

The present study demonstrated reproduction failure, DNA damage, and global epigenetic and metabolomic changes in *C. riparius* upon exposure to BPA at an acute sublethal dose (LC_{10}, 1 mg/L). In general, DNA hypermethylation is a gene repressive marker; H3K4 and H3K36 are activation marks, whereas H3K9 and H3K27 are repression marks for gene functions in mammalian systems, which are also mostly conserved in *Drosophila* (Keating and El-Osta, 2015; Herz et al., 2012). Hence, BPA-induced alteration in epigenetic marks possibly caused dysregulation in gene function and therefore affected the stress response and normal physiological status, in particular, the reproductive health of the organism (Trapphoff et al., 2013; Kitraki et al., 2015; Camacho et al., 2015).

Besides the epigenetic modifications, the imbalances of normal metabolic activities were also a critical factors in BPA-induced stress response. The concomitant increase in global ATP level and decrease in ADP level indicated an increase in metabolic activities, and 2-oxoglutarate was not changed, possibly because of a decrease in arginine and glutamate, and increase in glutamine (Table 1 and Fig. 4).

In addition, we assume that crosstalk between global metabolomic and epigenetic modification plays pivotal role in the mechanism underlying alterations in BPA-induced stress response.

4.1. Global epigenetic and metabolomics crosstalk

DNA and histone methylation depends on the availability of methyl groups from S-adenosylmethionine (SAM) and transmethylation of metabolic pathways, which closely interconnect with choline, o-phosphocholine, methionine, betaine, folate, as well as with cysteine, glutathione etc. (Niculescu and Zeisel, 2002; Medici et al., 2014). Although structurally diverse and possessing high substrate specificities, DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs) share a similar reaction mechanism: transferring a methyl group from S-adenosyl methionine (SAM) to the substrate with the formation of the by-product S-adenosyl homocysteine (SAH). The byproduct of these reactions, SAH, is a potent methyltransferase (DNMTs and HMTs) inhibitor. Competition for available SAM may regulate the contrasting events associated with histone methyl transfer or methyl-CpG modification in DNA strand (Keating and El-Osta, 2015). In addition, the accumulation of succinate activates hypermethylation by inhibiting 2-oxoglutarate-dependent DNA demethylase enzymes (Yang and Pollard, 2013).

In our study, we observed changes in global DNA methylation in BPA exposed midges with an increase in global H3K4 methylation, hyper methylation of H3K36 and a demethylation of H3K27. No change in methylation was observed for H3K9. The trend of global hypermethylation of H3K4 and H3K36 may be due to the increase in methionine and o-phosphocholine metabolites (Table 1 and Fig. 4). On the contrary, we found demethylation in H3K27, which could be a result of competition for SAM availability, not the activation of histone demethylase enzymes (as no change was evident for 2-oxoglutarate). In summary, BPA exposure disrupted the balance of methionine production and SAH clearance which caused DNA hyper-methylation, H3K4 and H3K36 hypermethylation, but H3K27 demethylation. Recent findings indicated a strong connection between metabolism and epigenetics in *Drosophila melanogaster*. It is reported that reduced levels of some enzymes involved in methionine metabolism and histone demethylases lead to lethality, along with wing development and cell proliferation defects in *D. melanogaster*. Additionally, disruption of methionine metabolism can directly affect histone methylation levels (Liu et al., 2016).

5. Conclusion

We found that the reproduction failure, increase in DNA damage, global DNA hyper-methylation, increased global histone modification (H3K36) status and alterations of energy metabolism, amino acids, and methionine metabolisms in BPA exposed *C. riparius*. Moreover, potential crosstalk between altered epigenetics and metabolites were also evident. In particular, the epigenetics-metabolomics crosstalk possibly play the pivotal role in BPA-induced stress response in *C. riparius*. Therefore, we believe our study would not only add an additional layer of information for accurate risk assessment of BPA but also would demonstrate a paradigm shift in the ecotoxicity testing by integrated approach of epigenetics and metabolomics.

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Conflict of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.06.084.

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