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Transcriptional and Biochemical Alterations in Zebrafish Eleuthero-Embryos (*Danio rerio*) After Exposure to Synthetic Progestogen Dydrogesterone

Wen-Jun Shi[1] · **Guang-Guo Ying**[1] · **Guo-Yong Huang**[1] · **Yan-Qiu Liang**[1,2] ·
Li-Xin Hu[1] · **Jian-Liang Zhao**[1] · **Jin-Na Zhang**[1]

Abstract Little information has so far been known on the effects of synthetic progestogen dydrogesterone (DDG) in organisms like fish. This study aimed to investigate the effects of DDG on the transcriptional and biochemical alterations in zebrafish eleuthero-embryos. Zebrafish eleuthero-embryos were analyzed for the transcriptional alterations by real-time quantitative PCR (RT-qPCR) and biochemical changes by attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FITR) after 144 h exposure to DDG. The results of qPCR analysis showed that DDG exposure significantly suppressed the transcriptions of target genes involved in hypothalamic–pituitary–thyroid (HPT) axis, while it induced the expression of target genes mRNA belonging to hypothalamic–pituitary–gonad (HPG) axis. In addition, ATR-FTIR spectroscopy analysis showed that the biochemical alterations of protein, nucleic acid and lipid were observed following DDG treatment. The finding from this study suggests that DDG exposure could have potential multiple effects in fish.

Synthetic progestogens are widely used in human and veterinary medicine. For example, progesterone (P4) and dydrogesterone (DDG) were extensively used for contraception and in hormone replacement therapy (Rižner et al. 2011). After use, these progestogens could end up in receiving aquatic environments due to wastewater discharge and agricultural run-off (Chang et al. 2008; Liu et al. 2011, 2012). Various progestogens have been detected in the aquatic environment at several to hundreds ng L^{-1} concentrations (Liu et al. 2012). Among various detected progestogens, DDG was detected in WWTP effluents at concentrations of up to 35.1 ng L^{-1} (Liu et al. 2015). In Chinese swine farms, DDG was found even at concentrations up to 2790 ng L^{-1} in flush water (Liu et al. 2015). However, the potential toxicological effect of DDG on aquatic organisms is still unclear.

Various studies have revealed that some synthetic progestogens such as progesterone (P4), norgestrel (NGT) and drospirenone (DRS) could cause endocrine disruption in the reproductive system of fish (Liang et al. 2015a, b; Runnalls et al. 2013). More importantly, Liang et al. (2015b) showed that NGT and P4 could disrupt sex differentiation by modulating some gene transcripts after a long-term exposure to environmentally relevant concentrations. However, reported toxicological effects of DDG in zebrafish are very limited.

Up to now, decreased fecundity and increased the gonadsomatic index in adult fish has been shown after DDG exposure resulting in the histological alterations in ovaries and testes (Zhao et al. 2015). Moreover, Zhao et al. (2015) also reported the expression of HPG axis related genes were altered both in adult zebrafish and eleuthero-embryos. However, it is not known whether DDG could have effects on the target genes involved in HPT axis and specific pathway in HPG axis. For instance, very little attention has been paid to the Gonadotropin–releasing hormone (GnRH) signaling pathway. As the upstream signaling pathways in

✉ Guang-Guo Ying
   guangguo.ying@gmail.com; guang-guo.ying@gig.ac.cn

[1] State Key Laboratory of Organic Geochemistry, CAS Research Centre of PRD Environmental Pollution and Control, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, 510640 Guangzhou, China

[2] School of Chemistry and Environment, Guangdong Ocean University, 524088 Zhanjiang, China

HPG axis, GnRH signaling pathway regulates the gonadotropin–releasing hormone secretion from the hypothalamus (Pawson and McNeilly 2005).Similarly, HPT axis regulates the thyroid endocrine system, while thyroid hormones are known to be essential for the development and physiological functions in fish (Lorenz et al. 2011; Liang et al. 2015a). In addition, the biochemical alteration is largely unknown after DDG exposure in fish. Thus, a further investigation is clearly needed to understand the potential impact of environmental DDG on transcription and biochemistry of zebrafish.

The aim of this study was to assess the transcriptional and biochemical changes in zebrafish eleuthero-embryos induced by DDG at environmentally relevant concentrations. The qPCR analysis was applied to analyze transcriptional expressions of the typical genes involved in HPT axis and HPG axis. ATR-FITR was used to detect the biochemical changes in zebrafish eleuthero-embryos after DDG treatment. The results from this study can help us comprehensively understand the toxicological effect of DDG on the endocrine system in organisms.

Materials and Methods

Two groups of 8 males and 4 females of zebrafish (*Danio rerio*) were placed separately overnight by a plastic board in a specific spawning aquarium equipped with a mesh bottom. In the following morning, these spawning adults were stimulated by the light for 30 min and the eggs were collected. Then the eggs were rinsed with aerated embryonic rearing water (58.8 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 24.66 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 12.6 mg L^{-1} $NaHCO_3$, and 1.1 mg L^{-1} KCl). The exposure solutions (a total volume of 400 mL) were prepared in embryonic rearing water containing 0.01% DMSO at the required three concentrations (50 ng L^{-1}, 500 ng L^{-1} and 5000 ng L^{-1}). 50 fertilized eleuthero-embryos that had developed normally at the early blastula stage (at 2–4 hpf) were randomly placed into 50 mL glass beakers filled with 30 mL DDG (CAS number: 152–62–5; purity 98%) exposure solutions. The water and solvent controls containing 0.01% DMSO were also included in the eleuthero-embryos exposure experiment. The present experiment included 4 replications for each treatment group. All beakers (n=20) were incubated in a semi-static system at 26 ± 1°C with a 14:10 light/dark cycle for 144 hpf. The exposure solutions for chemical analysis were also kept under the same conditions. Every 24 h, the embryos were observed by inverted biological microscope and dead embryos were removed. The exposure solutions were renewed every 24h and the exposure solutions were collected for target chemical analysis.

At 144 hpf, 5 eleuthero-embryos per replicate (n=4) were randomly collected and transferred to 1.5 mL centrifuge tube filled 400 μL 10% neutral buffered formalin (NBF), then stored at 4°C for biospectroscopy analysis. Similarly, 20 eleuthero-embryos per replicate (n=4) were randomly pooled and transferred to RNAlater, then stored at −80°C for RNA analysis. The sampling time of biospectroscopy analysis and RNA analysis occurred within 30 min after collecting.

The method of DDG analysis was modified from our previous publication (Liu et al. 2014). For detailed information about chemical analysis, please refer to the previous publication. Briefly, the all exposure solutions of eleuthero-embryos experiment from per replicate (n=4) were collected at 144 h as the water samples (total 100 mL) at the beginning of exposure (0 h) and prior to water renewal (24 h). The water samples from two replicates were pooled (total 200 mL for solvent control, 50 ng L^{-1} DDG, 500 ng L^{-1} DDG, 5000 ng L^{-1} DDG). The collected water samples were extracted using CNWBOND LC–C18 SPE cartridges (200 mg, 3 mL) (Germany). The DDG was analyzed by an Agilent 1200 series ultra-high performance liquid chromatography (Agilent, USA) coupled to an Agilent 6460 triple quadrupole mass spectrometry (UHPLC-MS/MS) equipped with electrospray ion source and a Zorbax SB-C18 column (100 mm × 3 mm, 1.8 μm particle size) with its corresponding pre-column filter (2.1 mm, 0.2 μm) (Agilent). The column oven temperature was maintained at 40°C and the injection volume was 5.0 μL. The mobile phase consisted of (A) Milli-Q water containing 5mM ammonium acetate and 0.05% formic acid (v/v) and (B) MeOH. The gradient program of the mobile phase was: 70% B at 0 min, increased to 90% B at 13 min, and decreased back to 70% B at 17 min, at a flow rate of 0.35 mL/min. A post run time was set at 5.0 min for column equilibration prior to next injection. The mass spectrometry was operated under positive ion mode with fragmentor (110, 110 eV) and collision energy (56, 92 eV) for ion pairs 313.2/43.1 and 313.2/77.1, respectively. The limits of detection and quantification for DDG were 0.5 ng/L and 1.8 ng/L, while its the recovery was $101\% \pm 3\%$. Laboratory blanks, reagent blanks and quality control standard solution (50 μg/L each compound) were also performed with the samples during the analysis of each batch to assess potential background values and instrument performance.

RNA isolation, cDNA synthesis and qPCR analysis were performed by following our previous method with slight modifications (Liang et al. 2015a, b). In this study, all primers were designed (batchprimer3) and synthesized in Biotechnology (Shanghai, China). Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to check the specific for the primers (Table 1). A total of twenty-eight genes involved in the HPG axis and HPT axis

Table 1 Primers for quantitative real-time PCR analysis in zebrafish

Gene	Gene Bank no.	Sense primer (5′–3′)	Antisense primer (5′–3′)	Product size (bp)	efficiency
gnrhr4[a]	NM_001098193.1	GGGCGATGAGTGTTGTTCTC	TGCCAGTGTTTGACGAAGC	118	95
adcy5[a]	NM_001171585.2	GTGCTCATCTCGTGGAGGTT	TTCTGCTGGGTAGTGTGTGC	120	97
adcy7[a]	NM_001166272.2	GCGTGTTTTTGAGGGTGAGT	GCAGGCAGTTGTAGTTGCTG	120	99
adcy8[a]	NM_001143752.1	GGACGAGACCCTACAGCATC	AAGTTCCTGAGCGGAGAGTG	124	107
atf4a[a]	NM_213233.1	AGGATGAGGAGAGCTCCGTG	GTCAGCAGGACATCTGACGG	139	96
atf4b[a]	NM_001103192.1	AGGTGAGGTGGTTGTGGAAA	AAATCGTGGGAAAGGTAGGG	111	95
gnrh2[b]	NM_181439.4	GGTCTCACGGCTGGTATCCT	TGCCTCGCAGAGCTTCACT	89	104
gnrh3[b]	NM_182887.2	TGGTCCAGTTGTTGCTGTTAGTT	CCTGAATGTTGCCTCCATTTC	116	99
lhb[b]	NM_205622.2	GGCTGGAAATGGTGTCTTCTT	GGAAAACGGGCTCTTGTAAAC	202	99
fshb[b]	NM_205624.1	GCAGGACTATGCTGGACAATG	CCACGGGGTACACGAAGACT	151	98
cyp11a1[b]	AF527755.1	GAGGGGTGGACTCGGTTACTT	GCAATACGAGCGGCTGAGAT	109	99
star[b]	NM_131663.1	GCCTGAGCAGAAGGGATTTG	CCACCTGGGTTTGTGAAAGTAC	170	98
cyp17a1[b]	NM_212806.3	ATGAGGAGGGTGATGGTTTG	CACGCCAGGAAGAGAAAGAG	118	101
cyp19a1a[b]	NM_131154.2	CGGGACTGCCAGCAACTACT	TGAAGCCCTGGACCTGTGAG	264	103
cyp11b[b]	DQ650710.1	CTGGGCCACACATCGAGAG	AGCGAACGGCAGAAATCC	171	106
hsd3b[b]	AY279108.1	AGCCCATTCTGCCCATCTT	TGCCTCCTCCCAGTCATACC	200	99
Hsd20b[b]	AF298898.1	TGGAGAACAGGCTGAGGTGAC	CGTAGTATCGGCAGAAGAGCAT	81	98
hsd17b1[b]	NM_205584.1	GTCTGATGGGTCCTCTGGAA	TGCCGTGTCTCTTTTTCTTCA	126	97
hsd17b2[b]	NM_001040188.1	AAATCGTGCTTGGATGTGAA	GGACCTCTCCTGCCATACTG	118	100
Hsd17b3[b]	AY551081.1	ACATTCACGGCTGAGGAGTTT	ATGCTGCCATACGTTTGGTC	74	102
trh[c]	NM_001012365.2	TGGAGCCGGAGGTGAAGA	GCAGTGGGGTCCTCTAGCAT	91	107
tshb[c]	NM_181494.2	AGGTTGCCGTGCCTATGTG	GGACCCACCAACTCCTTTATGT	145	99
nis[c]	NM_001089391.1	TGGTTGGTGTGGTGGTCAGTTA	GCATCGCAGGGCTTTTGTT	100	100
tg[c]	DQ278875.1	GCAGAGCCAAGAACATCA AGAAT	GGCGAGTGCTGTAAAGAGTAG AAC	157	104
dio1[c]	NM_001007283.1	GGTGGTGGATGAGATGAACAAC	TCCGATGCCTCCCTGATAGA	106	96
dio2[c]	NM_212789.3	ATTTCTCCTTGCCTCCTCAGTG	GCCACCTCCGAACATCTTTAAG	171	96
tra[c]	NM_131396.1	GGCTCGGAGTGGTTTCTGA	CTTGCGGTGGTTGATGTAGTG	200	96
trb[c]	NM_131340.1 NM_131340.1	AGCGTTGTCAGGAGGAGTTTC	GATTGGATTGCCATCAGTCTTC	222	96
β-actin[c]	AF057040.1	TCTGGCATCACACCTTCTACAAT	TGTTGGCTTTGGGATTCAGG	93	97
rpL13α[c]	NM_212784.1	CCCTTCCCGTGGATCATATC	TTTGCGTGTGGGTTTCAGAC	208	96
ef1-α[c]	BC064291.1	GAGGAAATCACCAAGGAAGTCA	AATCTTCCATCCCTTGAACCAG	147	96

[a] Refer to Kyoto Encyclopedia of Genes and Genomes and Zebrafish International Resource Center

[b, c] Refer to Liang et al. (2015a, b)

were selected, including upstream pathway of HPG axis (*gnrh2, gnrh2, gnrhr4, adcy5, adcy7, adcy8, atf4a, atf4b, lhb* and *fshb*), downstream pathway of HPG axis (*cyp11a1, star, cyp17a1, cyp19a1a, cyp11b, hsd3b, hsd20b, hsd17b1, hsd17b2* and *hsd17b3*) and HPT axis pathway (*trh, tshb, nis, tg, dio1, dio2, tra* and *trb*). The average mRNA expression levels of ribosomal protein L (*RpL13α*), elongation factor 1 alpha (*EF1-α*) and *β-actin* were selected as housekeeping genes to normalize the expression of mRNA for the target genes (Vandesompele et al. 2002). The relative mRNA expression of target genes was calculated with the delta–delta CT method (Livak and Schmittgen 2001). Data from qPCR were analyzed for normality and homogeneity

of variances by using the Kolmogorov–Smirnov and Levene's test, respectively. One-way analysis of variance (ANOVA) was selected to evaluate the significance of differences between the DDG treatment groups and solvent controls group followed by Tukey multiple comparison tests. Data were considered significantly different at $p < 0.05$.

ATR–FTIR was applied to analyze the biochemical alterations in eleuthero-embryo samples following DDG exposure. The 70% ethanol was used to wash the eleuthero-embryos sample three times (German et al. 2006), and then the eleuthero-embryos samples were put on the BaF_2 windows to be air-dried. All

eleuthero-embryos samples were interrogated using a Bruker Vertex 70 FTIR spectrometer with Platinum ATR attachment containing a single reflection diamond crystal (Bruker Optics Ltd., Germany). ATR–FTIR spectra of eleuthero-embryos samples were recorded with a resolution of 4 cm^{-1}, accumulating 64 scans. From each sample, three different scans of identical spectra under the same conditions were performed. These replicates were averaged and then used for further data analysis. IRootLab toolbox was used as pre-treatment of the raw IR spectra by running on MATLAB r2012a (Trevisan et al. 2013). Spectral range (1800–900 cm^{-1}) of biochemical fingerprint region was used to constitute the chemometric analysis through rubber-band baseline correction and normalization to Amide I (1650 cm^{-1}). Furthermore, multivariate analysis (PCA-LDA) was conducted for the dataset. The scores plots and cluster vectors were performed to visualize the data analysis results (Martin et al. 2010).

Results and Discussion

During the exposure experiment, no significant differences of mortality were observed between the solvent control group and treatment groups (data not shown). This indicated that DDG was not acutely toxic to zebrafish at environmentally relevant concentrations, which was consistent with the result of Liang (2015a, b). In addition, the measured three DDG concentrations in the eleuthero-embryos exposure solutions both at 0 h (T_0) and 24 h (T_{24}) were found to be very close to the nominal concentrations with mean concentrations of 49, 527 and 5886 ng L^{-1}, respectively (Table 2).

As the up-stream signaling pathway, GnRH signaling pathway is important for the zebrafish reproductive development, which cross–talks with several signaling pathways. The transcriptional response for target genes involved in GnRH signaling pathway are shown in Fig. 1a. DDG exposure significantly induced the transcription of *adcy8, atf4b2, lhb* and *fshb* at different doses. Even at 49 ng L^{-1},

Table 2 The nominal and measured concentrations of DDG in the exposure experiment

Nominal concentration	Measured concentrations (ng L^{-1})[a]						Mean concentrations
	0 h exposure time			24 h exposure time			
	Tank 1	Tank 2	Average	Tank 1	Tank 2	Average	–
DMSO control	<1.8[b]	<1.8	–	<1.8	<1.8	–	–
50 ng L^{-1}	54.1	54.7	54.4[a]	40.8	46.4	43.6	49.0
500 ng L^{-1}	595.2	514.4	554.8	468.9	528.0	498.5	526.7
5000 ng L^{-1}	6410.7	6824.6	6617.65	4290.0	6017.2	5153.6	5885.6

[a]Measured concentrations are given as mean (n = 2 replicates)
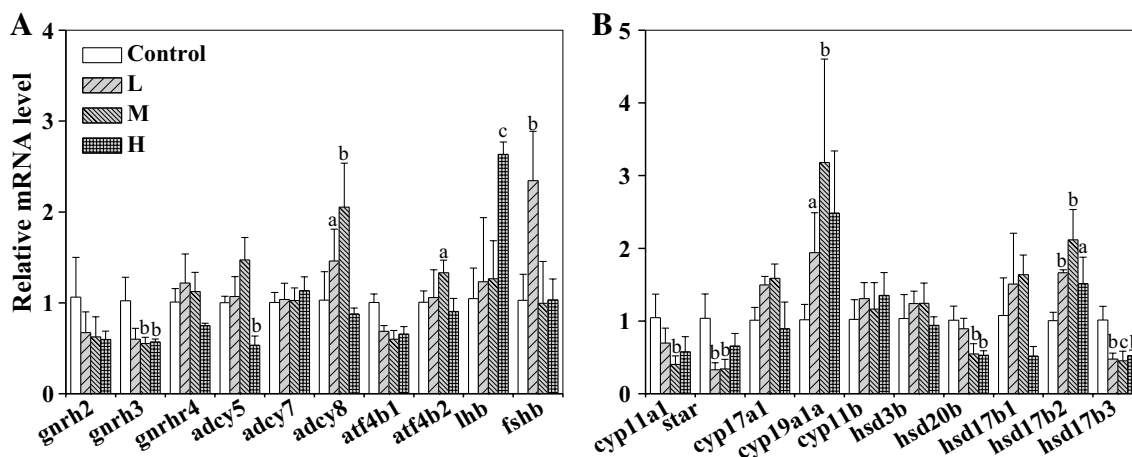
[b]Limit of quantification

Fig. 1 Transcriptional alteration of target genes involved in the GnRH pathway (**a**) and steroidogenic pathways (**b**) in zebrafish eleuthero-embryos compared to the solvent control after DDG exposure for 144 hpf. Data are shown as mean ± SD (n = 4). [a]*p-value* < 0.05, [b]*p-value* < 0.01, and [c]*p-value* < 0.001. Key: *C* solvent control; *L* low dose; *M* medium dose; *H* high dose

the significant up-regulation of *adcy8* and *fshb* transcriptions were observed. The *adcy* could increase the concentration of cyclic adenosine monophosphate (cAMP) (Gur et al. 2002). As the second messenger, the cAMP could interact with cAMP Response Elements Binding protein (*atf4*) to form transcriptional activator (Pawson and McNeilly 2005). Then, the *atf4* participates in regulating the expression of *lhb* and *fshb* (Pawson and McNeilly 2005). In present study, the expression levels of *adcy5, adcy8* and *atf4b2* was similar after DDG treatment, which demonstrated that the DDG exposure affected the transcription of *adcy*, resulting in disturbing the expression of *atf4b2* mRNA. However, the expression levels of *fshb* and *lhb* mRNAs were not completely consistent with transcriptional alterations of *adcy* and *atf4*. Several signaling pathways regulated the GnRH signaling pathway together. Therefore, we believe that other mechanisms existed to interpret the expression of *fshb* and *lhb* mRNA under the DDG exposure.

The enzymes involved in the down-stream signaling pathway catalyze the steroidogenesis via a series of reactions, and these enzymes are recognized as important targets for the actions of various endocrine disrupting chemicals (Sanderson 2006). In present study, DDG exposure significantly increased the transcription of *cyp19a1a* and *hsd17b2*, while suppressed the expression of *cyp11a1, star* and *hsd17b3* (Fig. 1b). It was reported that progestin decreased *star* and *cyp11a1* mRNA levels in male *Xenopus laevis* (Lorenz et al. 2011). Our present results showed that the similar expression level of *cyp11a1* and *star* in zebrafish eleuthero-embryos after DDG treatment. The *cyp11a1* and *star* gene encodes an enzyme that is involved in catalyzing the first step of the steroidogenic pathway, and this enzyme plays a crucial role in the transporting cholesterol into the inner mitochondrial membrane. Thus, the decreasing expression of *cyp11a1* and *star* gene could retard the synthesis of steroids. In addition, the transcripts of the *hsd17b2* and *cyp19a1a* genes were upregulated both at 49 and 527 ng L^{-1}. The over-expression of *hsd17b2* and *cyp19a1a* mRNA resulted in the stimulation of steroidogenesis, which are required during the estrogen biosynthesis. Subsequently, the down-expression of *hsd17b3* influenced the 11-ketoandrostenedione biosynthesis. Thus, the results implied that DDG had potential effects on the production of estrogens after 144h exposure.

It is known that HPT axis controls the thyroid endocrine system and regulates the thyroid hormone synthesis and secretion (Carr and Patiño 2011). However, thyroid hormone also exerts negative feedback control of the generation of TRH and TSH (Dietrich et al. 2012). Negative–feedback loops are widely observed in physiological process (Lechan and Fekete 2004; Nguyena and Kholodenkoa 2016). Previous studies have reported that THs modulate

the expression of TRH and TSH in mammals via a negative feedback mechanism (Chiamolera and Wondisford 2009; Yan et al. 2012). In the present study, there was a very interesting phenomenon about the expression level of target genes mRNA. DDG exposure strong suppressed the transcription of the *tshb, nis* and *tg* at 526.7 or 5885.6 ng L^{-1}, while slight significantly reduced the expression of *tra* and *trb* at the same concentrations. And then, even the over-expression of *trh* was observed at 526.7 ng L^{-1} (Fig. 2). We considered that these results can be explained by negative–feedback loops in zebrafish after 144 h exposure. Briefly, significantly low expression of *tshb, nis* and *tg* might trigger the negative–feedback loops resulting in gradually decrease in the effect of *tra, trb* and *trh* (Fig. 2). As the plasma membrane glycoprotein, *nis* regulates the transport of active iodide into the thyroid follicular cells, which is the vital first step in the synthesis of thyroid hormone (Dohán and Carrasco 2003). Thus, the low transcription of *nis* indicated that the DDG treatment might reduce the extracellular to intracellular transport of the active iodide. In addition, *tg* directly participates in the generation of thyroid hormones by couple with iodinated tyrosines (Noguchi et al. 2010). The down regulation of *tg* suggested that DDG would reduce the thyroid hormone production. These results demonstrated that DDG could significantly influence the transcription of HPT genes and had a high risk for development of zebrafish eleuthero-embryos.

Moreover, the ATR-FTIR spectroscopy in the present study showed some biochemical alterations at Amide I (~1600 cm^{-1}) and DNA/RNA (~1238 cm^{-1}) (Fig. 3). It is generally known that amide I and amide II bands are sensitive to protein conformation (Cakmak et al. 2006) and
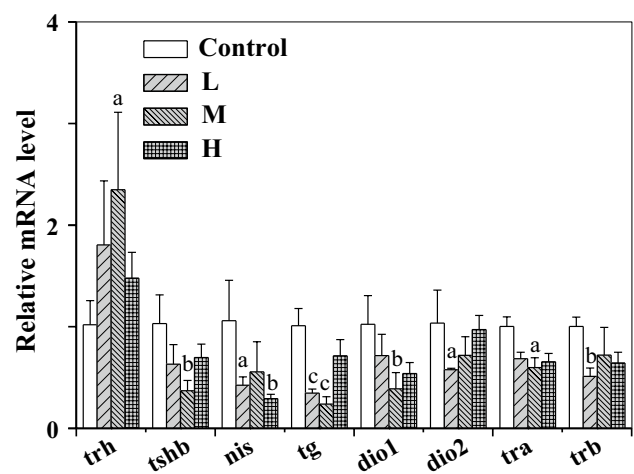

Fig. 2 Transcriptional alteration of HPT axis in zebrafish eleuthero-embryos compared to the solvent control after DDG exposure for 144 hpf. Data are shown as mean ± SD (n = 4). [a]p-value < 0.05, [b]p-value < 0.01, and [c]p-value < 0.001. Key: *C* solvent control; *L* low dose; *M* medium dose; *H* high dose
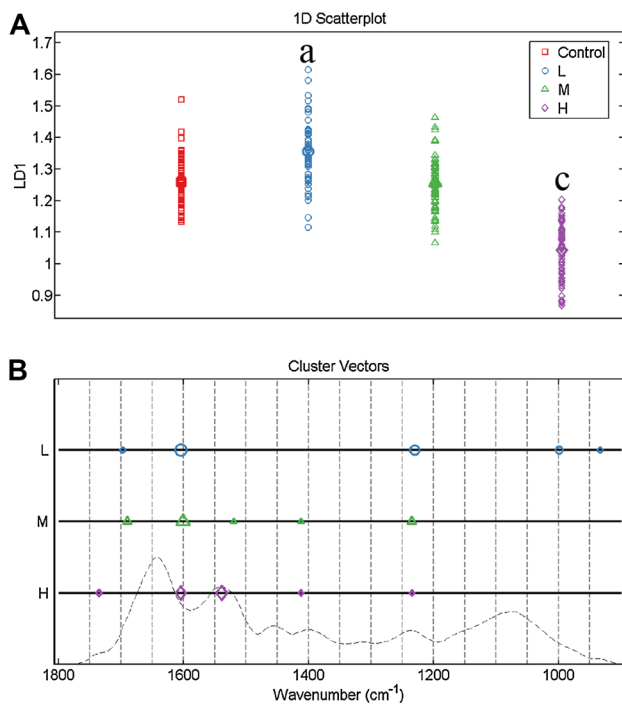
Fig. 3 Analysis of IR spectra in zebrafish eleuthero-embryos after DDG exposure for 144 hpf. **a** One-dimensional cross-calculated PCA-LDA scores plot. **b** The cluster vectors peaks plot marking the wavenumber discriminating effects in zebrafish eleuthero-embryos. The size of the symbol in the cluster vector peaks plot is proportional to the height of the corresponding peaks, which are relative to the extent of biochemical alteration compared to the solvent control. The hint line represents the typical infrared (IR) spectrum of the biochemical-fingerprint region ($1800 - 900$ cm^{-1}). Compared to the solvent control, statistically significant differences are marked with letters ([a]p-value < 0.05, [b]p-value < 0.01, and [c]p-value < 0.001). Key: C solvent control; L low dose; M medium dose; H high dose

are used to detect the protein secondary structures. This shifting and shape changes of the protein secondary structures indicate that DDG treatment significantly induced the change of protein conformation at all concentrations. The band at 1237 cm^{-1} is due to the asymmetric phosphate (PO$_2^-$) stretching modes (Palaniappan and Pramod 2010; Li et al. 2015). The vibrations from PO$_2^-$ groups originate mainly from the phosphodiester groups of nucleic acids. Normally, the frequency of the asymmetric PO$_2^-$ stretching band is at 1220 cm^{-1} when the PO$_2^-$ group is fully hydrogen bonded and at or above 1240 cm^{-1} when it is not hydrogen bonded (Palaniappan and Pramod 2010). The present study revealed that many PO$_2^-$ groups of nucleic acids in zebrafish embryos-larvae were hydrogen bonded. The decreased intensity of the PO$_2^-$ bands after DDG treatment might lead to the decrease in the relative content of the nucleic acids (DNA/RNA). This suggests that DDG exposure may have potential effects on gene expression by affecting the structures of nucleic acids and protein. More

importantly, the alteration of the band around 1750 cm^{-1} showed the changes of lipid (Fig. 3). The change in the intensity of the band around 1743 cm^{-1} indicated the changes in the ratio of triglycerides and cholesterol esters and their saturation states (Cakmak et al. 2006). In present study, the results of transcription of HPG axis demonstrated that DDG has potential effects on the steroidogenesis (Fig. 1b). It is interesting that the down-expression of *star* and *cyp11a1* gens mRNA might influenced the transporting cholesterol into the inner mitochondrial membrane (Fig. 1b). As we know, the lipid is so vital for the normal physiological function, such as the metabolism of the energy and hormone synthesis (Ceylan et al. 2014). Thus the alterations of the lipid structure and the effect of steroid biosynthesis implied that DDG exposure influence the metabolism of lipid at biochemical level and transcription level.

The present study showed the biochemical effect of DDG on zebrafish via FTIR spectroscopy and obtained structural and functional information. The application of IR spectroscopy with computational analysis posed potential ability to identify underlying pathological alterations and offered possibility to investigate the mechanistic effects of environmental contaminants at low-concentration levels (Gajjar et al. 2014). Moreover, ATR-FTIR spectroscopy can be employed in a non-destructive fashion to interrogate the biochemical signature of cells by generation of infrared (IR) spectra (Ahmadzai et al. 2015; Trevisan et al. 2010). Biospectroscopy technique is a novel approach to analyze toxicological effect of environmental contaminants on zebrafish.

In conclusion, the present study demonstrated that DDG exposure could influence the early life stage of zebrafish (*Danio rerio*) at transcriptional level and biochemical level. However, the present study only detected the effect of DDG in eleuthero-embryos and did not investigate the relationship between the transcriptional alterations of target genes and biochemical changes. Therefore, further research should explore the relationship between the transcriptional level and biochemical level after long-term exposure in zebrafish.

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