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Developmental profiles and expression of the DNA methyltransferase genes in the fathead minnow (*Pimephales promelas*) following exposure to di-2-ethylhexyl phthalate

Richard K. Wood · Emma Crowley · Christopher J. Martyniuk

Abstract DNA methylation is an epigenetic regulator of gene expression, and this process has been shown to be disrupted by environmental contaminants. Di-2-(ethylhexyl) phthalate (DEHP) and related phthalate esters have been shown to affect development in early life stages of fish and can alter genomic methylation patterns in vertebrates. The objectives of this study were the following: (1) Describe the expression patterns of the DNA methyltransferase (*dnmt*) genes during early fathead minnow (FHM) development. These genes are critical for methylation and imprinting during development. (2) Determine the effects of DEHP on the development of FHM larvae [1 and 14 days post-hatch (dph)]. (3) Determine the effect of DEHP on *dnmt* expression and global methylation status in larval FHM. FHMs were first collected over a developmental time course [1, 3, 5, 6, and 14 days post-fertilization (dpf)] to investigate the expression patterns of five *dnmt* isoforms. The expression of *dnmt1* and *dnmt7* was relatively high in embryos at 1 dpf but was variable in expression, and these transcripts were later expressed at a lower level (>3 dpf); *dnmt3* was significantly higher in embryos at 1 dpf compared to those at 3 dpf. *Dnmt6* showed more of a constitutive pattern of expression during the first 2 weeks of development, and the mRNA levels of *dnmt8* were higher in embryos at 5 and 6 dpf compared to those at 1 and 3 dpf, corresponding to the hatching period of the embryos. A waterborne exposure to three concentrations of DEHP (1, 10 and 100 µg/L) was conducted on 1-day FHM embryos for 24 h and on larval fish for 2 weeks, ending at 14 dpf. DEHP did not negatively affect survival, hatch rate, or the expression of *dnmt* isoforms in FHMs. There were no differences in global cytosine methylation following DEHP treatments in 14 dpf larvae, suggesting that environmentally relevant levels of DEHP may not affect global methylation at this stage of FHM development. However, additional targeted methylome studies are required to determine whether specific gene promoters are differently methylated following exposure to DEHP. This study offers new insight into the roles of the *dnmt* enzymes during FHM development.

Keywords Phthalates · Toxicology · Survival · DNA methylation

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Introduction

Epigenetics refers to heritable changes in gene expression caused by molecular mechanisms that do not involve a change in the DNA sequence of the organism. Several processes involved in epigenetics are histone modification (e.g., acetylation and methylation), noncoding RNA interactions, and 5-cytosine methylation of DNA (Vandegehuchte and Janssen 2011). Methylation of 5-cytosine in DNA is involved in many aspects of gene regulation, including transcriptional silencing, embryonic development, genomic imprinting, and X-chromosome inactivation (Stein et al. 1982; Razin 1998; Licchesi et al. 2005; Wilkinson et al. 2007; Latham et al. 2008). It has also been hypothesized that DNA methylation increases genomic stability and protection for the cell from possible disruptions such as transposable elements (Yoder et al. 1997; Rizwana and Hahn 1999). It is therefore well documented that DNA methylation is a crucial regulatory mechanism for normal development and physiological function.

In eukaryotes, DNA methylation is a heritable regulatory biochemical process that involves the covalent transfer of a methyl group to the 5-position of cytosine from S-adenosyl-L-methionine to form 5-methylcytosine (Hermann et al. 2004). DNA methylation occurs in all known vertebrate genomes, and the primary site of action is at CpG dinucleotide sites (Tweedie et al. 1997; Jeltsch 2002). DNA methylation is primarily catalyzed by a group of enzymes known as DNA methyltransferases (DNMTs; Jeltsch 2002; Hermann et al. 2004). Currently, three classes of DNA methyltransferases have been characterized in mammals, DNMT1, DNMT2 (though DNMT2 does not appear to be involved in DNA methylation), and DNMT3; moreover, there are three different types of DNMT3 known as DNMT3a, DNMT3b, and DNMT3L (DNA methyltransferase-like protein; Jones and Takai 2001; Jeltsch 2002; Hermann et al. 2004). Homologous genes to the mammalian classes, as well as several other related genes (*dnmt4, dnmt5, dnmt6, dnmt7,* and *dnmt8*), have been identified in zebrafish (Shimoda et al. 2005; Smith et al. 2011). The *dnmt1* gene in zebrafish appears to have a maintenance role similar to the mammalian homologue (Shimoda et al. 2005). The *dnmt6* and *dnmt8* genes appear to be structurally related to DNMT3a, while *dnmt3, dnmt4, dnmt5,* and *dnmt7* are related to DNMT3b. Additional

evidence for these relationships is based upon data demonstrating that *dnmt6* and *dnmt8* show early expression patterns similar to DNMT3a, while *dnmt3, dnmt4, dnmt5,* and *dnmt7* show comparable expression patterns to DNMT3b (Shimoda et al. 2005; Smith et al. 2011; Campos et al. 2012). The various functions of these genes in fish are still under investigation.

Endocrine-disrupting compounds (EDCs) in the environment are ubiquitous chemicals that affect normal functioning of the hormone systems. Environmental contaminants have also been shown to induce changes in DNA methylation patterns, and exposure to several environmental contaminants including tributyltin, triphenyltin, benzo[a]pyrene, and nickel has been shown to affect DNA methylation across multiple taxa (Lee et al. 1998; Wang et al. 2009; Vandegehuchte and Janssen 2011). For research in fish toxicology and physiology, there has been a new focus on epigenetics mechanisms of gene regulation (Williams et al. 2014). A recent study by Olsvik et al. (2014) demonstrated that contaminants that included dioxin (TCDD) and methylmercury affected methylation patterns of ~ 200 genes in the F1 generation, and it is now recognized that this process (i.e., epigenetics) is a primary mechanism by which chemicals exert long-term effects in aquatic organisms (Mirbahai and Chipman 2014).

In addition to the aforementioned chemicals, phthalate esters have also been shown to have effects on DNA methylation. These chemicals are used in plasticizers in polyvinyl chloride (PVC) products such as toys and hospital supplies. Phthalate esters have been detected in the environment in water, sediments, and sewage sludge, on a global scale [Canadian Council of Ministers of the Environment (CCME) 1999; Beauchesne et al. 2008; Crisp et al. 2009; Keil et al. 2011]. The most commonly used phthalate ester is di-2-(ethylhexyl) phthalate (DEHP; Beauchesne et al. 2008). DEHP has been found in surface waters as high as 300 µg/L in Canadian freshwater systems (CCME 1999). Of interest, maternal DEHP exposure has been found to increase the global methylation level and DNMT expression in rat testes and causes testicular malformations that resemble human testicular dysgenesis syndrome (Wu et al. 2010). Another study found that maternal DEHP exposure caused changes in promoter methylation of adults rats in several genes, including mineralocorticoid receptor, estrogen receptors alpha and beta, androgen receptor,

and peroxisome proliferated-activated receptor alpha (Martinez-Arguelles et al. 2009). There is also evidence that exposure to phthalate chemicals can alter insulin signaling in utero via epigenetic mechanisms (Rajesh and Balasubramanian 2014) and reprogram cardiomyocytes, changing the methylation status of specific CpGs in peroxisome proliferator-activated receptor subunits (Schaedlich et al. 2014). In teleost fishes, DEHP has been shown to increase mortality, delay growth, induce oxidative stress, and interfere with normal endocrine and reproductive function (Chikae et al. 2004a, b; Mankidy et al. 2013; Ye et al. 2014). Given the importance of DNA methylation in development, it is plausible that the disruption of methylation processes is one of the mechanisms through which DEHP exhibits adverse effects in aquatic organisms.

The objectives of this study were to first describe the expression patterns of the *dnmt* isoforms in the fathead minnow (FHM; *Pimephales promelas*) in early development. FHMs are a model freshwater toxicological species and are a cyprinid fish found ubiquitously in North America. Due to the large collection of knowledge about their biology, their ability to survive in a range of water types, and their general hardiness and tolerance of handling and experimentation, they have been used extensively in toxicological research for over 50 years (Ankley and Villeneuve 2006). While a number of studies on the FHM transcriptome have been conducted in recent years, there is a paucity of research on the mechanisms of methylation in this species. Therefore, we aimed to address this knowledge gap by first characterizing *dnmt* expression and then determining whether DEHP exposure affects FHM larvae development via epigenetic mechanisms by measuring the expression of the *dnmt* genes and in vivo DNA methylation after exposure.

Materials and methods

Development profiles of the dnmt genes

For generating the developmental profiles of the *dnmt* genes, adult FHMs were taken from a breeding stock at the University of New Brunswick (Saint John) and placed into eight aquariums in 1:2 male/female ratio per tank and a breeding tile was placed in each tank. All animal use protocols were carried out ethically in accordance with Animal Care Committee protocol from the University of New Brunswick, Saint John (approval #2013-3s-08). Each tank was aerated using an air stone, which maintained a mean oxygen content of 86.9 %. The pH was measured daily, and the mean pH was 7.1. Fish remained on a light/dark schedule of 16:8 h, respectively. The experimental room was maintained at approximately 20 °C, while the water was maintained at approximately 25.5 °C. These conditions were maintained for 2 days until breeding was completed (six of the eight aquariums had produced eggs, yielding a total of approximately 360 fertilized embryos) at which point all embryos were pooled and transplanted from the original breeding tiles into petri dishes. The pooled embryos were divided evenly across eight petri dishes, with each petri dish containing 35 embryos. Embryos were maintained at room temperature, also on a 16:8-h light/dark schedule, and water changes occurred every second day. Water changes were performed using a stock tank of water which was maintained at 98.4 % oxygen per liter of water, a pH of 7.4, and a mean room and water temperature both of 20 °C. Mortality rates for embryos were recorded individually as they occurred with mortality rates ranging from 6 to 26 %, with an overall mean mortality rate of 17.5 %.

Embryos were collected and stored at the following time points: 1 day post-fertilization (dpf), 3 dpf, 5 dpf, 1 day post-hatch (6 dpf), and 14 dpf. During the first 3 dpf, the following morphological events occur: completion of embryonic development, post-embryonic development as yolk-sac larvae (yolk-sacs still attached), the switch from endogenous feeding (yolk-sac) to exogenous feeding (food gained via eating), inflation of the swim bladder, development of the anterior dorsal section of the mouth, and development of the gut and intestinal tracts (Kimmel et al. 1995). The first three time points corresponded to the neurula stage, the commencing of pectoral fin blood flow and dorsal swim bladder pigmentation, and pre-hatch (based on work in FHMs by Devlin et al. 1996). The fourth time point (6 dpf) was immediately post-hatch, and the fifth time point was collected after 8 days of larval growth (14 dpf). Pre-hatching development of the FHM *P. promelas* Rafinesque is described in EPA/600/R-96/079 (1996). Micrographs of these stages have been published by us recently in Wood et al. (2015). The embryos were collected using a stainless steel spatula and placed into 2.0 ml vials,

after which a sterile eyedropper was used to remove excess water from the vials. The embryos were placed into the vials in groups of two, while the larvae (6 and 14 dpf samples) were collected individually. Samples were then flash frozen on dry ice and later stored in 1.5 mL microcentrifuge tubes at −80 °C until used in RNA extraction.

DEHP exposures (embryo and larval)

In order to assess the effects of DEHP on pre-hatch embryos and *dnmt* expression, a 48-h exposure was performed. The first 24-h period is critical for methylation, and we aimed to determine whether DEHP affected *dnmt* expression during this sensitive period of development. FHMs were collected after fertilization and all embryos that had progressed past the late cleavage stage were removed. The embryos were evenly distributed into 12 glass petri dishes per treatment group containing 50 mL of water (water control), 0.001 % ethanol solution (carrier control), and nominal concentrations of 1 µg/L DEHP, 10 µg/L DEHP, and 100 µg/L DEHP-treated freshwater. The chorion was left on for all exposures to mimic a natural exposure in the environment. The exposure was carried out for 48 h at ambient temperature, and the chemicals were prepared fresh and renewed at 24 h with a 90 % water change. At 24 h, up to 3 individuals per replicate were collected, flash frozen, and stored at −80 °C for gene expression analysis ($N = 12$). At 48 h, the number of surviving embryos was assessed in each treatment.

A second experiment was performed to assess the effects of DEHP on FHM development over a longer period. This experiment aimed to determine whether exposure to DEHP over the first 2 weeks of development affected global methylation patterns in larvae. The DEHP exposure was conducted in 40 glass petri dishes arranged into 4 groups of 10. The experiment was carried out in a room with a 16:8-h light/dark photoperiod. Four larval [0 days post-hatch (dph)] FHM were randomly placed in each petri plate. This life stage was chosen for the exposure because of research conducted in zebrafish showing that there is high *dnmt* expression in the post-hatch stage (Smith et al. 2011). The larvae were fed twice each day using 0.05 mL of Artemia culture. The 4 treatments were a carrier control ($n = 10$), 1 µg/L DEHP ($n = 10$), 10 µg/L DEHP ($n = 10$), and 100 µg/L DEHP ($n = 10$); these concentrations are all considered environmentally relevant

(CCME 1999). Ethanol was used as a carrier solvent for DEHP. The control group contained only water and the solvent. Each dish contained 50 mL of freshwater at room temperature. The water in each dish was changed every 2 days at 1 PM using a reservoir of freshwater kept at ambient temperature (May 2013), a mean pH of 6.38, and dissolved oxygen (DO_2) level of >95 %. Using stock water, three chemical solutions were prepared fresh every second morning; this included the control water (ethanol added), the D1 water (DEHP dissolved in ethanol added to make a 1.0-µg/L solution), the D2 water (DEHP dissolved in ethanol added to make a 10.0-µg/L solution), and the D3 water (DEHP dissolved in ethanol to make a 100.0-µg/L solution). A 100 % water change was performed for each dish using the reservoir, and the freshly prepared contaminant was added to yield the desired concentration for each treatment group. The ethanol concentration was <0.01 %. Each replicate was checked daily for mortalities (completely white body and/or lack of a heartbeat under a dissection microscope). Mortalities were recorded each morning before the water was changed and the exposure was carried out for fourteen days post-hatch (~19–20 days total post-fertilization).

RNA extraction and cDNA synthesis

RNA was extracted from whole FHM embryo/larval samples (from both the developmental profile and the DEHP exposures) using the Allprep DNA/RNA Mini Kit (Qiagen; Toronto, ON). Samples (whole FHM larvae or 2 embryos) were homogenized in 350 µL Buffer RLT Plus and processed according to the manufacturer's instructions. During the extraction, the DNA spin column was collected and stored at 4 °C for later DNA extraction (Sect. 2.6). The eluted RNA concentration was quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific; Wilmington, DE). RNA samples were tested for RNA integrity with the Agilent RNA 6000 Nano Assay Kit (Agilent Technologies; Santa Clara, CA), and RNA Integrity Numbers (RIN) were generated using the Agilent 2100 Bioanalyzer as per manufacturers' instructions. Samples collected from the DEHP exposures had a mean RIN of 8.8 (±1.9). Complementary DNA (cDNA) was synthesized using DNAse-treated RNA as a template following methods outlined in Chishti et al. (2014). The cDNA product was stored at −20 °C until used in real-time PCRs (Sect. 2.5).

Primer design

The DNA sequences for the DNA methyltransferase genes (*dnmt1*, *dnmt3*, *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8*) were obtained by searching for the nucleotide sequences in the National Center for Biotechnology Information (NCBI) database. As the FHM sequences were not available, the homologous sequences for *Danio rerio* were used. The sequences for the genes in *D. rerio* and at least one other species (e.g., *Carassius auratus*) were aligned using the Clustal Omega program (EMBL-EBI; Cambridge, UK) to identify conserved regions of DNA sequence. Using these conserved regions and regions specific to each isoform, the forward and reverse primers for the gene transcript were designed using Primer3 (Untergrasser et al. 2012). The aforementioned primers were first tested by amplifying the genes using polymerase chain reaction (PCR) and Taq polymerase (Life Technologies; Carlsbad, CA) as per manufacturer's protocols. Following the PCR, a 1 % agarose gel was prepared with SYBR® Safe DNA gel stain to visualize amplicons.

Despite multiple attempts, we were unsuccessful at obtaining sequence information for FHM *dnmt4* and *dnmt5*. However, these transcripts appear to be present in the FHM genome as a survey of a new 8×60 K microarray for FHMs (Garcia Reyero et al. personal communication) suggests that the following genes are present: DNMT1, DNMT3, DNMT3A, DNMT3B, DNMT3L, DNMT4, DNMT5, DNMT6, DNMT7, and DNMT8. The DNMT3A, DNMT3B, and DNMT3L are homologous to the mammalian DNA methyltransferases. Thus, based on zebrafish, dnmt3, 4, 5, and 7 are orthologs of mammalian 3B, and dnmt6 and 8 are orthologs of mammalian 3A. Interesting, DNMT3L also appears to be present in FHMs, and its function is thought to be the recruitment of DNMT3A and B to methylate cytosine residues. We point out that nomenclature for the *dnmt* isoforms conforms to that presented in Campos et al. (2012). All gene-specific primers are listed in Table 1.

Real-time PCR

Primer sets that yielded a single amplicon were tested on a standard curve plate using real-time PCR. Primers that yielded a single melt curve, a $R^2 > 0.97$, and efficiency (*E*) between 90 and 110 % were deemed

Table 1 Gene-specific primers designed to measure gene transcript abundance in FHM embryos and larvae

Identifier	Full name of gene	Forward primer (5'–3')	Reverse primer (5'–3')	E (%)	R^2
Normalizer					
actb	*Beta-actin*	CTCTTCCAGCCATCCTTCCT	ACCGATCCAGACGGAGTATTT	99.0	0.996
rps18	*Ribosomal subunit 18*	CGGTTCTATTTTGTGGGTTTCT	ACCTCCGACTTTCGTTCTTG	106.1	0.998
ef1 alpha	*Elongation factor 1 alpha*	AGCGTAAGGAGGGAAATG	CTCAGAAAGGGACTCGTGGT	110.6	0.988
rps12	*Ribosomal subunit 12*	GCTTGAAACCCGAAGGAC	GTGCCCATTTTGCTTACTTTT	108.8	0.996
DNA methyltransferase					
dnmt1	*DNA methyltransferase 1*	GAAGGTGGAGACAGTTGTCAGAT	CCATTTGAATGTCCATTCTGC	107.4	0.997
dnmt3	*DNA methyltransferase 3*	TGACATGGTGATTGGTGGAAGTC	CCACCACATTCTCAAACATCCAGAA	107.9	0.996
dnmt6	*DNA methyltransferase 6*	TGGTGCACTGAGATGGAGAG	GTGCAAACAAGTGACGGATGA	103.6	0.994
dnmt7	*DNA methyltransferase 7*	AATGTCGTTCAGGTGCATGTT	AGGTGAAATTGGGTCTAAACTTCC	109	0.981
dnmt8	*DNA methyltransferase 8*	GAAGACCCCTGGAACTGCTATA	GAATGCCCAGATCCTTCAGC	102.1	0.993

successful. Real-time PCR (RT-PCR) was performed to measure gene expression of 4 reference genes for normalization and the 5 *dnmt* genes tested. Sample sizes for the developmental profile were the following: 1 dpf = 6, 3 dpf = 8, 5 dpf = 8, 6 dpf = 5, 14 dpf = 7 using Ssofast EvaGreen® RT-PCR mix (Bio-Rad; Hercules, CA) and ~300 nM primer. A seven-point standard curve was generated by diluting 1 μL of pooled cDNA in 9 μL of ultrapure water and then performing a 1:5 dilution series. An eighth point in the curve was a no-template control (NTC) using water. The sample cDNA was diluted 1:20 in ultrapure water prior to real-time PCR using a CFX96 instrument (Bio-Rad) as per details in Chishti et al. (2014). The NRTs and NTCs were evaluated on each plate to rule out genomic DNA contamination. The RT-PCR data were analyzed using the gene study option in the CFX96 software (Bio-Rad). The reference genes were loaded into the gene study in different combinations in order to find the most stable combination; *ef1 alpha* and *actb* yielded the most stable arrangement with $M = 1.10$ and $CV = 0.43$ for the developmental profile and *ef1 alpha*, *rps12,* and rps*18* were most stable for the DEHP exposure experiments ($M = 0.67$; $CV = 0.26$).

Here we point out that many genes commonly used as reference genes in real-time PCR experiments are present in very low amounts before the midblastula transition, and these genes may not be suitable for studies at early time points. This is a limitation for developmental studies in gene expression. Here, *ef1 alpha* did not statistically vary across the five time points; however, *actb* did vary significantly and was lower in expression in the first 24 h compared to all other groups ($p < 0.01$). Differences in Cq values were even more pronounced for *rps12* and *rps18,* and these were not used to normalize data. Noteworthy was that no time point after 24 h differed in terms of the expression level (based on RNA input) for all four control genes tested (i.e., no difference in Cq values), and the differences were confined to comparisons with embryos collected 1 dpf. *Ef1 alpha* has been reported to be one of the most stable housekeeping genes during teleost development (Fernandes et al. 2008; McCurley and Callard 2008; Øvergård et al. 2010). However, *actb* can vary from species to species in terms of stability. Fernandes et al. (2008) showed in halibut that *actb* was one of the most stable across genes across developmental stages, while Overgard et al. (Øvergård

et al. 2010) reported that *actb* varied significantly over time. Similarly, McCurley found *ef1 alpha* to be stable during zebrafish development, while *actb* was not very stable. Thus, species differences can exist in the stability of common reference genes over development, and there is a balance between using a sufficient number of genes to achieve a stable baseline for relative comparisons of genes of interest and variation in their expression from stage to stage. In our study, the combination of *ef1 alpha* and *actb* provided the most stable M value (or baseline) than other combinations of normalizer genes (*ef1 alpha*, *actb*, *rps12, and rps18*). Thus, we believe that we achieved a reasonable stable baseline from which to draw our relative comparisons. We used *ef1 alpha* expression as this gene is considered to be one of the most stable across developmental stages and species compared to several other reference genes. Our $CV = 0.43$ is actually quite good for control genes based on our experience in calculating M values for multiple normalizers (Chishti et al. 2014). However, we caution that the early time points (i.e., <24 h) may show low expression for reference genes in real-time PCR experiments.

The normalized unscaled expression of the genes of interest was analyzed to determine differences in expression between treatment groups. Normalized gene expression was extracted using CFX Manager™ software and gene expression differences determined using the relative ΔΔCq method. All amplicons were verified as correct target genes by Sanger Sequencing at the McGill University and Génome Québec Innovation Centre (Montréal, QB, Canada) (Supplemental Table S1).

DNA extraction and global DNA methylation

DNA purification was conducted using the Allprep DNA/RNA Mini Kit (Qiagen; Toronto, ON) as per manufacturer's protocol. Final DNA concentration was then quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific; Wilmington, DE). Global methylation of the genomic DNA was measured using the MethylFlash™ Methylated DNA Quantification Kit (Epigentek; Farmingdale, NY) without modification. The colorimetric assay plate was read on an iMark Microplate Absorbance Reader (Bio-Rad) at 450 nm. The relative percentages of cytosine methylation as well as the absolute quantity

(ng) of methylated DNA were calculated using formulas described by the manufacturer (Epigentek).

Statistics

All statistical analyses were performed using GraphPad Prism 5.0 (Graphpad Software, Inc., La Jolla, CA). Differences in survival and hatch were tested using a Mantel–Cox log-rank test. The methylation absorbance data (relative quantity as well as absolute), morphometric data, and gene expression data were tested for normality using the Shapiro–Wilks test and for homogeneity of variance using a Levene's test. If the assumptions of normality and homogeneity were met, the data were analyzed using a one-way ANOVA test. If differences were detected, a post hoc analysis using Tukey's HSD test was used to determine which groups differed from the control. If the assumptions of normality and homogeneity were not met, the nonparametric Kruskal–Wallis test (denoted as an H-test statistic) was used and any post hoc differences were tested for using a Dunn's test. The Spearman rank correlation was used to determine whether *dnmt* isoforms were significantly associated with each other in expression during development. A $p < 0.05$ was considered significant.

Results

Developmental expression of *dnmt* genes

The developmental expression profiles for *dnmt1*, *dnmt3*, *dnmt6*, *dnmt7* and *dnmt8* are shown in Fig. 1. *Dnmt3* transcript abundance differed between 1 and 3 days post-fertilization ($H = 10.9$; $p = 0.028$; Fig. 1b), and there was a significant reduction in mRNA levels between these time points. *Dnmt8* transcripts differed significantly ($H = 18.0$; $p = 0.0012$) in abundance between time points, with transcripts being more abundant on days 5 ($p < 0.05$) and 6 ($p < 0.01$) compared to levels at day 1 (Fig. 1e). There were no significant differences in transcript abundance between the 5 time points for *dnmt1* ($H = 7.3$; $p = 0.12$), *dnmt6* ($H = 3.4$; $p = 0.49$), or *dnmt7* ($H = 5.69$; $p = 0.22$) (Fig. 1a, c, d). The Spearman correlation revealed significant ($p < 0.05$) correlations for expression patterns between *dnmt1* and *dnmt3* as well as between *dnmt1* and *dnmt7* (Fig. 1f).

DEHP exposure did not affect survival in embryos or larvae

In the first experiment, a 48-h exposure to one of three concentrations of phthalates did not result in any significant mortality compared to the controls ($H = 6.16$; $p = 0.10$). In the second experiment, larvae survival ranged from 80 to 85 % among the four groups over the 14 days (Fig. 2). Survival of the FHM larvae was not different across the experimental groups ($H = 0.51$; $p = 0.92$).

DEHP exposure did not alter the expression of dnmt or the global methylation

Following a 24-h exposure, no differences were detected among groups for mRNA levels of *dnmt1* ($df = 3$; $H = 1.5$; $p = 0.68$), *dnmt3* ($H = 3.9$; $p = 0.28$), *dnmt6* ($H = 3.5$; $p = 0.33$), *dnmt7* ($H = 3.5$; $p = 0.34$), or *dnmt8* ($H = 0.51$; $p = 0.92$).

Genomic methylation levels ranged from ~ 1.4 to 1.5 % among the four groups in larvae. There were no significant differences in global cytosine methylation levels between the four groups ($H = 1.061$; $p = 0.7865$; Fig. 3).

Discussion

Developmental expression of the *dnmt* isoforms in fathead minnows

Similar to other studies in fish, there was differential expression of the *dnmt* isoforms during FHM development. Cytosine methylation is associated with a number of biochemical and physiological processes. Two notable examples of these are gene silencing and cell differentiation during development (Reik 2007). The zebrafish *dnmt3* (and its paralogues *dnmt4*, *5*, *7*, and *8*) is similar to the mammalian DNMT3 genes that are required for de novo cytosine methylation, while *dnmt1* is a homologue of the mammalian DNMT1 which plays a role in maintenance methylation as well as tissue-specific terminal differentiation (Shimoda et al. 2005; Campos et al. 2012).

We determined that at least 5 *dnmt* isoforms are expressed in FHM embryos; however, it looks as though there are more based upon recent sequencing
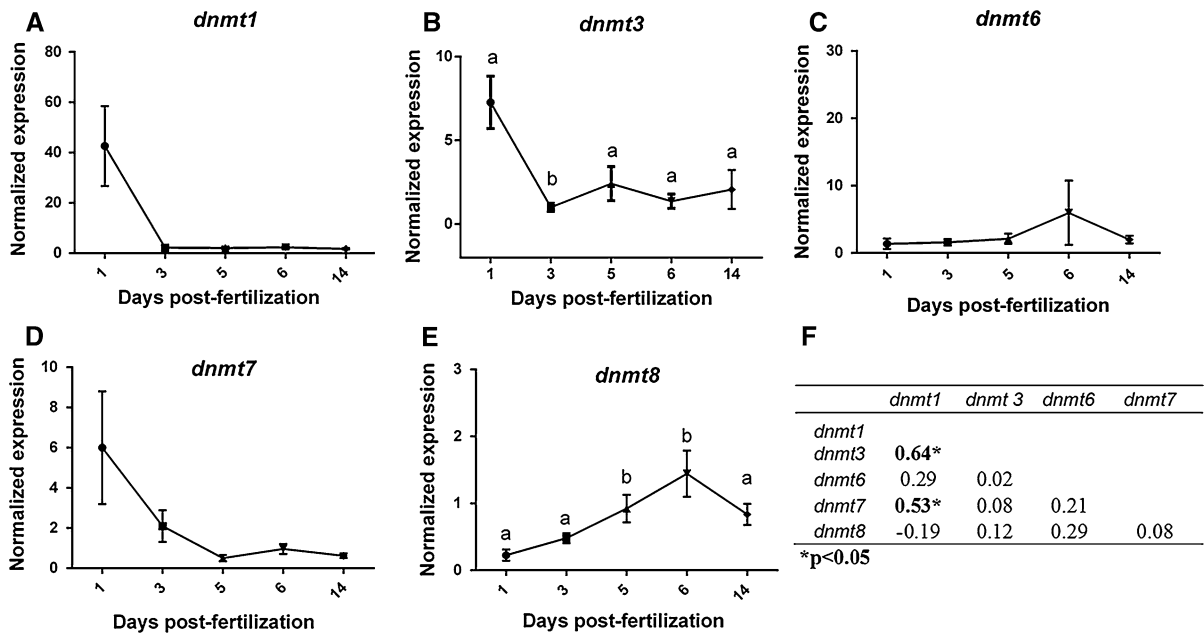
Fig. 1 Normalized gene expression for the dnmt isoforms (**A–E**) across five time points in development. Data are the mean-normalized expression ± standard error of the mean. Time points marked with the *same letter* do not significantly differ from one another. The figure also shows the correlation coefficient for each transcript (**F**)
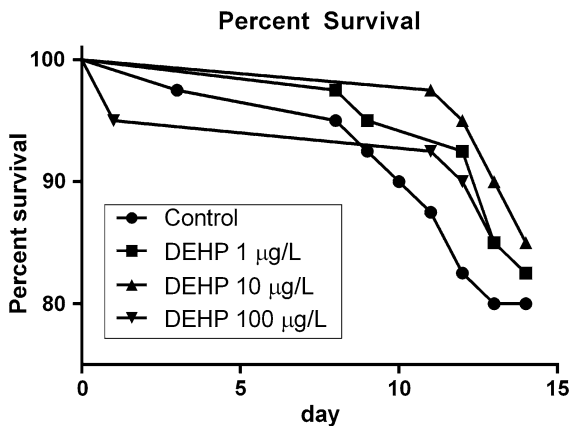

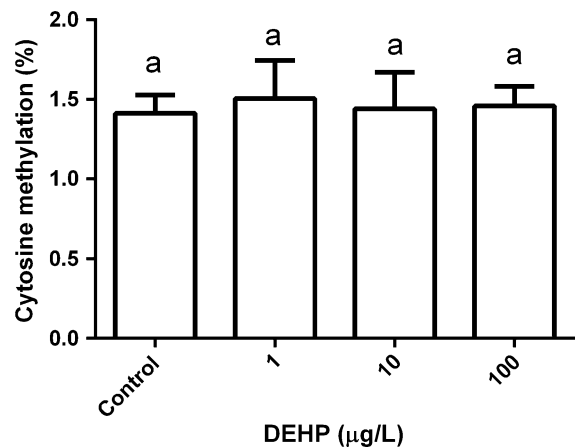**Fig. 2** Percent survival of FHM embryos by day and concentration of DEHP (1, 10, and 100 µg/L) over a 2-week exposure. There was no difference in survival among groups

Fig. 3 Levels of 5-methylcytosine in the four treatment groups of FHM larvae. Results are shown as mean % 5-methylcytosine ± standard error of the mean

of the FHM genome and production of a new high-density (8 × 60 K) microarray platform. The expression levels of *dnmt1*, *dnmt3*, and *dnmt7* were highest at 1 dpf and then decreased by day 3. *Dnmt1* expression was significantly correlated with both *dnmt4* and *dnmt7* expressions. These results are consistent with data generated by Smith et al. (2011). These authors examined the expression of *dnmt7* (among others)

during zebrafish development and found that *dnmt7* mRNA was most abundant during the first 24 h, but by 72 h post-fertilization, mRNA levels had dropped drastically below the detection limit of the bioassay. To better determine the developmental patterns of expression for these genes, it will be necessary to sample at various time points within the first 24 h of development. DNMT7 is known to specifically

methylate the zebrafish *ntl* gene which is crucial to notochord development in fish (Shimoda et al. 2005) and appears to be fish specific (Seritrakul and Gross 2013). *Dnmt3* is highly expressed in developing zebrafish up until 72 h, when it falls below the limit of detection of the assay (Smith et al. 2011) and this is a pattern that we also observed in FHMs. As previously stated, *dnmt3* is related to mammalian Dnmt3b which is involved in de novo methylation during development (Campos et al. 2012). During zebrafish development, there is a complete demethylation and remethylation of the genome within 6 hpf (MacKay et al. 2007). If FHM development follows a temporal pattern similar to that of zebrafish, the higher levels of *dnmt3* and other genes in the mammalian *dnmt3b* family (such as *dnmt4*) observed at 1 dpf may be involved in this reprogramming of the FHM genome. In addition to its role in maintaining methylation patterns, DNMT1 plays a critical role in organ formation in zebrafish development by directing histone methylation by SUV39H1 (Rai et al. 2006) which may explain its higher activity in early embryonic development observed in this study. The correlation between *dnmt1* and *dnmt3* and *dnmt1* and *dnmt7* also suggests that these genes all play a role in early methylation events in FHM within the first 24 h of development.

Dnmt6 and *dnmt8* showed a different pattern of expression compared to *dnmt1*, *3*, *and 7*, consistent with data collected in zebrafish. The mRNA levels of *dnmt8* increased at days 5 and 6 relative to day 1 before dropping again to near-day 1 levels by day 14. There was no difference detected in the expression of *dnmt6* over time points. These genes are phylogenetically related to mammalian DNMT3A and are highly similar to each other in nucleic acid sequence (Campos et al. 2012). The study by Smith et al. (2011) found that *dnmt6* and *dnmt8* mRNA were expressed up until at least 72 h in zebrafish and were ubiquitously expressed throughout different embryo tissues. According to previous research, these genes are important to neural development in fish, being highly expressed in retinal ganglion cells and adult brain tissue (Smith et al. 2011; Seritrakul and Gross 2013). The timeline from fertilization to hatch in this study was ∼24 h longer than that reported for FHMs by the Environmental Protection Agency (EPA 1996). The beginning of the rise in *dnmt6* and *dnmt8* mRNA at approximately 3 days in this study coincides with

several aspects of brain and optic development reported from day 2 to hatch (EPA 1996).

A consideration for future studies with FHMs is the timing used in sampling. The vertebrate genome is thought to be demethylated and progressively methylated to adult levels again following fertilization. Research on zebrafish has demonstrated this, with low levels of genome methylation being detected 1–2 h post-fertilization and subsequently reaching near-adult levels by 6 h post-fertilization (Mhanni and McGowan 2004). Assuming that FHM have a similar rate of post-fertilization methylation, then the 1 dpf measurement of *dnmt* genes offered only a glimpse into an ongoing process which may have ended by the second time point (72 h). Zebrafish also have a faster rate of development and shorter hatching time than FHM which may factor into determining the appropriate window for detection of dynamic changes in *dnmt* expression.

DEHP exposure and survival

There were no effects in embryo or larval survival following DEHP treatment, which is consistent with several studies in teleost fishes. A 21-day exposure by Chikae et al. (2004b) using medaka (*Oryzias latipes*) fry did not detect differences in survival between waterborne DEHP-treated (0.01–10 µg/L) and control groups, nor did a study by DeFoe et al. (1990) which examined juvenile FHM, rainbow trout (*Onchorynchus mykis*), Japanese medaka, and *Daphnia magna* using DEHP concentrations at or above its water solubility. A recent study exposing adult Chinese rare minnow (*Gobiocypris rarus*) to environmentally relevant concentrations (0–117.6 µg/L) of DEHP for 21 days found that there were no mortalities following DEHP exposure (Wang et al. 2013). Similarly, a feeding study with Atlantic salmon (*Salmo salar*) detected no significant differences in mortality between control and treated fish (Norman et al. 2007). Conversely, there are studies that have demonstrated significant mortality due to DEHP exposure. A second study by Chikae et al. (2004a) on Japanese medaka embryos found that low-dose (0.01–1 µg/L) waterborne DEHP exposure caused significantly higher mortalities than the control group. Guppy fry (*Poecilia reticulata*) exposed continuously to a waterborne exposure of 10 µg/L for ∼3 months exhibited higher mortalities than control animals (Zanotelli et al. 2010).

not acutely toxic). However, as other studies in fish suggest, chronic exposures to DEHP may still adversely affect growth and survival.

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