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Transient exposure of methylparaben to zebrafish (*Danio rerio*) embryos altered cortisol level, acetylcholinesterase activity and induced anxiety-like behaviour

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ABSTRACT

Parabens are widely used as antimicrobial preservatives. Recent studies have reported the endocrine disrupting effects of these chemicals, especially methylparaben. Previously, we have reported the alteration in Vtg gene expression upon exposure to environmentally relevant doses of methylparaben in zebrafish (*Danio rerio*) embryos. However, studies reporting neurobehavioural outcomes on exposure to methylparaben are limited. Therefore, this study was aimed at investigating the methylparaben-induced effects on developmental and neurobehavioural endpoints. Zebrafish embryos were exposed to sub-lethal concentrations of methylparaben: 0.1 ppb, 1 ppb, 10 ppb and 100 ppb. Alterations in developmental landmarks such as heart rate and hatching percentage were observed in embryos exposed to 10 ppb and 100 ppb of methylparaben. Results obtained from the novel tank diving test established that anxiety-like behaviour is induced in larvae exposed to 0.1 ppb and 1 ppb of methylparaben. A significant inhibition in the acetylcholinesterase (AChE) activity was also recorded in methylparaben-exposed groups. An increase in cortisol levels was observed in the exposed groups, which further supports the observations made in the novel tank diving test, establishing methylparaben as an anxiogenic agent even at sub-lethal concentrations. The underlying molecular mechanism needs further elucidation to investigate whether the behavioural effects are proximally or distally induced by early developmental exposure to methylparaben.

1. Introduction

Parabens are p-hydroxybenzoic acid esters, widely used as antibacterial (Davidson and Branden, 1981; Darbre and Harvey, 2008) and antifungal (Ito et al., 2015) preservatives in packaged food (Liao et al., 2013), cosmetics (El Hussein et al., 2007) and pharmaceuticals (Soni et al., 2005). Shorter the alkyl side chain length of parabens, higher the ability of the compound to penetrate the skin (Elder, 1984; Hatami et al., 2017). Among parabens, methylparaben showed higher permeation, followed by ethylparaben and propylparaben, while butylparaben exhibited lower permeation through the skin. This difference in permeation is also rendered by variation in solubility and lipophilicity of the parabens (Caon et al., 2010). Studies have found that absorption of parabens is also influenced by the presence of penetration enhancers like ethanol and methanol that are present in cosmetic formulations (Kitagawa et al., 1997). Therefore, the usage of cosmetic products (El Hussein et al., 2007; Seo et al., 2017), apart from oral ingestion accounts for the major exposure route of parabens in humans (Harvey and Darbre, 2004; Darbre and Harvey, 2008). Besides this, human beings are also exposed to parabens *via* inhalation (Lucas et al., 1999; Guo and Kannan, 2013). In humans, parabens are metabolized by carboxylesterases present in subcutaneous fat tissue and keratinocytes. The hydrolyzing ability of differing human skin layers were studied and reported that paraben esterase I, an enzyme of the subcutaneous fat tissue, prefers methylparaben as its substrate, paraben esterase II and III act on butylparaben (Lobermeier et al., 1996). Upon metabolism of parabens in the skin, the primary metabolite is *para*-hydroxybenzoic acid (PHBA) (Boberg et al., 2010), which is then conjugated with glycine, glucuronide and sulfate and excreted in urine (Cashman and Warshaw, 2005; Soni et al., 2005). It was initially conceived that the complete metabolism of parabens takes place in the liver and kidney. However, it has later been reported that a portion of

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parabens remain in body tissues without undergoing esterase hydrolysis (Oishi, 2004). This suggests that chronic exposure to parabens in day-to-day life will result in bioaccumulation and affect the health of an individual. Hence, evaluation of the toxicity of parabens must be carried out in dose ranges covering the human exposure relevant concentrations.

Parabens have been identified as an endocrine disrupting chemical (EDC). Further, their estrogenic nature was characterized with *in vitro* (Routledge et al., 1998; Pedersen et al., 2008) and *in vivo* experiments (Routledge et al., 1998; Okubo et al., 2001; Soni, Carabin and Burdock, 2005; Darbre and Harvey, 2008). Parabens, when tested in estrogen-responsive human cell lines, showed increased estrogenic activity with increase in alkyl side chain length (Byford et al., 2002). A recent study from our lab investigated the estrogenicty of methylparaben by studying the expression of Vtg gene in zebrafish larvae at 96 h post fertilization (hpf) at environment relevant concentrations (Dambal et al., 2017). In another study, our group screened and reported the detectable levels of methylparaben in maternal blood and amniotic fluid, highlighting its capability to cross the placental barrier, further emphasizing the need to evaluate its impact on early development. The level of methylparaben present in amniotic fluid was quantified to be 18.92 ng/mL (Shekhar et al., 2017). Since the role of estrogen in early brain development is well established, it has been hypothesized that early developmental exposure to methylparaben may lead to altered brain organization, manifesting in dysregulated neurobehavioural outcomes. Further, recent literature highlight the role of estrogenic endocrine disrupting chemicals (EEDCs) such as bisphenol-A in inducing anxiety-like behaviour in animal models such as rodents, with profound effects observed when the exposure occurs during early development (Rosenfeld, 2012; Hass et al., 2016). Thus, methylparaben could also potentially affect anxiety-like behaviour in low doses upon exposure during early development. To further investigate this hypothesis, behavioural assays to observe anxiety-like behaviour in zebrafish exposed to methylparaben were performed.

While the lethal concentration of methylparaben upon embryonic exposure to zebrafish has been established and the defects occurring in that dosage range were determined, there still exists a dearth in literature that establishes methylparaben-induced effect on neurobehavioural outcomes at a ‘no-effect’ dose and ‘low-effect’ dose. Most literature concentrates on the reproductive abnormalities caused as a result of exposure to estrogen mimics. However, there has been a continual increase in interest on its effect on the nervous system. The potential of these chemicals to disrupt estradiol action in the brain and alter normal brain development has given us some foresight into studying the effects of these chemicals as neurotoxicants. To fill the gap in knowledge, the following study has been designed to investigate the developmental and neurobehavioural effects of exposure to sub-lethal concentrations of methylparaben in zebrafish.

2. Methodology

2.1. Chemicals

Methylparaben [CAS: 99-76-3, Purity-99%] was purchased from Loba Chemie, Mumbai, India and a stock solution of 1 ppm was prepared using ethanol as the solvent. The stock solution was stored at 4 °C in an amber coloured bottle. Solutions for the four exposure concentrations of methylparaben- 0.1 ppb, 1 ppb, 10 ppb and 100 ppb were prepared by diluting the stock solution in egg water. The final concentration of ethanol in the exposure solutions was lower than 0.02% (v/v). Egg water was prepared as defined in the OECD TG 203 annex-2, for acute fish toxicity test (OECD, 1992), with a final concentration containing: 294.0 mg/L $CaCl_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 64.7 mg/L $NaHCO_3$ and 965.7 mg/L KCl.

2.2. Experimental animals

Zebrafish (wild type- AB) were purchased from local aquarist, further bred and raised in our facility and used for the experiments described. Adult zebrafish (3–4 months old) were maintained in aquaria with carbon-filtered water with the photoperiod of 14:10 h light/dark and acclimatized for period of two weeks before start of the experiment and then separated into groups for spawning, with a male to female ratio of 2:1. Zebrafish eggs were collected in metal mesh-covered spawning trays placed at the bottom of the tanks 1-h after onset of light cycle (For detailed methodology, refer our previous study Dambal et al., 2017). Non-fertilized eggs were separated from fertilized ones using a pipette. The collected embryos were observed under the stereomicroscope at 4 hpf and developmental stages were determined as previously described by Kimmel et al., 1995.

2.3. Embryo toxicity assay

The collected embryos were segregated for each exposure group in 6-well plates, with 10 embryos per well. The control group was maintained in 5 mL of egg water and exposure groups were maintained in egg water with varying concentrations of methylparaben. The zebrafish eggs were maintained in exposure solutions till 6 days post fertilization (dpf) at 26 ± 1 °C. The study was conducted following the guidelines of OECD fish embryo acute toxicity assay (OECD, 2013). All experiments were carried out in triplicate. The exposure solutions were changed daily. The overall exposure paradigm was illustrated in Fig. 1.

2.4. Hatching and heart rate

The hatching and heart rate were observed at 48 hpf using a microscope (10X magnification, Nikon Eclipse LV100D-U, Nikon Inc., Melville, NY, USA) and images were documented. Percentage of hatched embryos, recorded at 48 hpf, was calculated as (number of embryos hatched divided by the number of incubated embryos) × 100. During exposure, dead embryos were removed to avoid contamination.

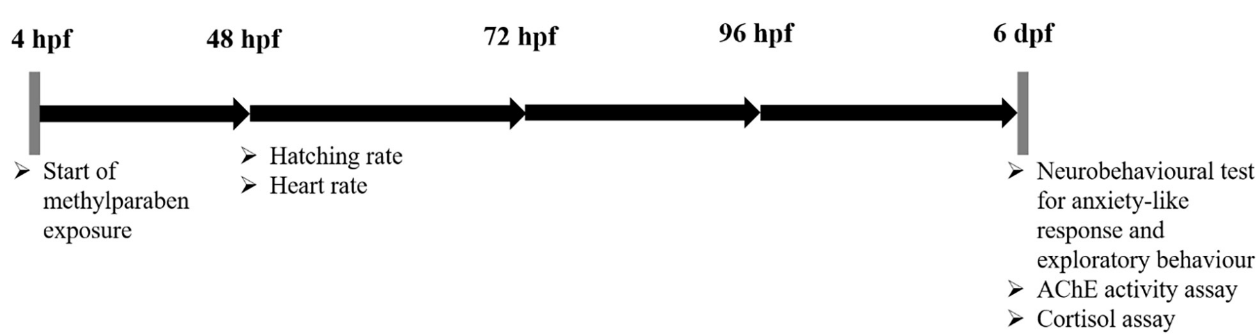

Fig. 1. Treatment paradigm of methylparaben exposure in zebrafish embryos.

Heart rate was recorded for 30 sec in the embryos at 48 hpf.

2.5. Neurobehavioural tests for anxiety and stress response

2.5.1. Novel tank diving test

The novel tank diving test was performed in order to analyze the role of methylparaben in inducing anxiety-like behaviour and alterations in exploratory activity in exposed larvae. The test was conducted following the methods previously reported by Egan et al. (2009). In brief, treated and control larvae were maintained in six-well plates prior to behavioural testing. Novel tank diving was then assayed in 6-day old larvae using a trapezoid tank. Tests were performed during the light phase. Larvae were transferred to novel tanks using hand-nets and observations were made for 6 min. Behavioural parameters observed by two trained observers manually, which includes- latency (time taken) to reach upper half of the tank, total number of transitions to the upper half, total time spent in the upper half, total erratic movements and total freezing bouts. Erratic movement refers to any sudden change in swim direction or velocity, while freezing refers to lack of motion for 1 s or longer. Eight larvae per group were considered for study and the experiments were carried out in triplicate.

2.5.2. Scototaxis (light-dark preference test)

For carrying out this behavioural test, the experimental design was adopted from Steenbergen et al. (2011) to assess the effect of methylparaben on avoidance patterns (natural preference of zebrafish to light or dark compartment) and anxiety-like behaviour. Larvae were maintained and treated as described in 2.5.1. Opaque tanks were divided externally, such that one side of the tank was exposed to light and the other side was darkened. Larvae were transferred using hand-nets into the middle of the dark side and were observed for 5 min. The total time spent in the light and dark sides was recorded. In order to assess the effects of methylparaben on scototaxis, zebrafish larvae were exposed to the respective doses of methylparaben. Eight larvae per group were considered for study and the experiments were carried out in triplicate.

2.6. Acetylcholinesterase (AChE) activity assay

To further supplement the alterations in exploratory behaviour and activity observed in the novel tank diving test, the effect of methylparaben on AChE activity was assessed. Zebrafish larvae at 6 dpf were immobilized with ice water bath and further homogenized in sodium phosphate buffer to prepare extracts for AChE activity following the procedure of Ellman et al. (1961) with modification suggested by Legierse (1998). The homogenate was centrifuged at 12000 rpm for 60 min at 4 °C and the supernatant was collected and stored at −80 °C until assayed. Briefly the aliquots of Tris-HCL buffer, DTNB 5,5́-dithiobis (2-nitrobenzoic acid) (Himedia, Mumbai, India) and supernatant were mixed, incubated for 5 min. S-acetylthiocholine-iodide (Himedia, Mumbai, India) was added to start the reaction. The increase in absorbance was measured at 405 nm using spectrophotometer (Thermo Scientific Multiskan GO). A group of 20 embryos were used for extraction and experiments were carried out in triplicate. The spectrophotometric assay result was represented as enzymatic activity in units per mg of protein.

2.7. Cortisol assay

A set of 20 larvae per group were taken at 6 dpf. The samples were homogenized with ice-cold ethanol using Dounce homogenizer kept in an ice bath. Further, ethyl acetate was added and mixed using a vortex mixer. The homogenate was centrifuged for 10 min at 2000 rpm and the supernatant was collected and vaporized using nitrogen gas (modified from Yeh et al., 2013). For cortisol estimation samples from three sets were pooled. The resultant extract was dissolved in phosphate buffered saline (PBS) and cortisol was estimated using commercially available ELISA kit (Enzo Life Sciences).

2.8. Statistical analysis

Assumptions of normality and equality of variance was met, and One-way ANOVA with Tukey-Kramer's test was performed for comparison between the exposure groups. Statistical significance was set at $p < 0.05$. SPSS 19.0 for Windows (SPSS, Chicago, IL, US) was used for data analyses and GraphPad Prism 5.0 was used for producing all the graphs.

3. Results

3.1. Effect of methylparaben on hatching rate

To evaluate the effect of methylparaben on developmental end points such as hatching rate of zebrafish embryos, hatching percentage was observed at 48 hpf. While all the embryos in the untreated group had hatched, a significant dose-dependent decline in hatching rate was observed in groups treated with lower doses (0.1 ppb and 1 ppb) of methylparaben. Embryos treated with 10 ppb and 100 ppb methylparaben had not emerged from their chorions at 48 hpf, delayed development and hatching was observed in 10 ppb and 100 ppb methylparaben treated embryo compared with the unexposed embryos (Fig. 2).

3.2. Effect of methylparaben on heart rate

Heart rate at 48 hpf was observed to decrease significantly in zebrafish embryos treated with 10 ppb and 100 ppb methylparaben (Fig. 3).

3.3. Effect of methylparaben in novel tank diving test

As a result of delayed hatching and increased incidence of morphological deformities observed in higher exposure groups of 10 ppb and 100 ppb methylparaben, lower exposure groups (0.1 ppb and 1 ppb) alone were considered for behavioural analyses. A dose-dependent decrease in latency to reach the upper portion of the tank was observed in treated larvae. The time spent in the upper half of the tank, however, decreased with increase in methylparaben concentration. Similarly, the total transitions made to the upper half of the tank was

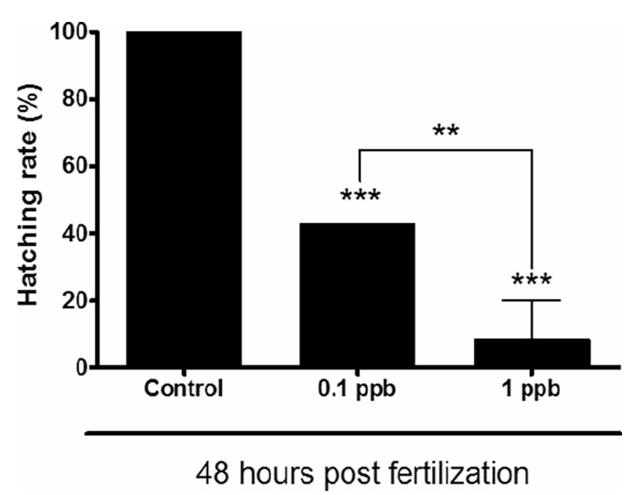

Fig. 2. Effect of methylparaben on hatching rate of zebrafish embryos. Hatching rate of zebrafish exposed to 0.1 ppb and 1 ppb methylparaben and control at 48 hpf. None of the embryos in exposure groups of 10 ppb and 100 ppb had hatched. Data presented as mean ± SD of at least three independent experiments. ** indicates ($p < 0.01$) and *** indicates ($p < 0.001$) significant decrease in hatching rate in exposure groups.

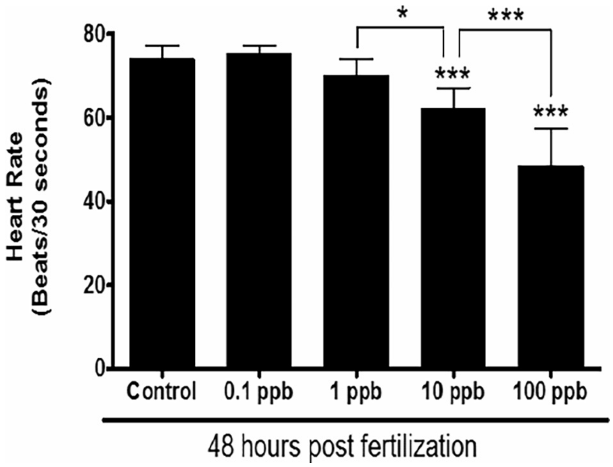

Fig. 3. Effect of methylparaben on heart rate of zebrafish embryos. Heart rate was recorded for 30 s for all exposure groups at 48 hpf. Data presented as mean ± SD of at least three independent experiments. * indicates ($p < 0.05$), ** indicates ($p < 0.01$) and *** indicates ($p < 0.001$) significant dose-dependent decrease in heart rate in exposure groups.

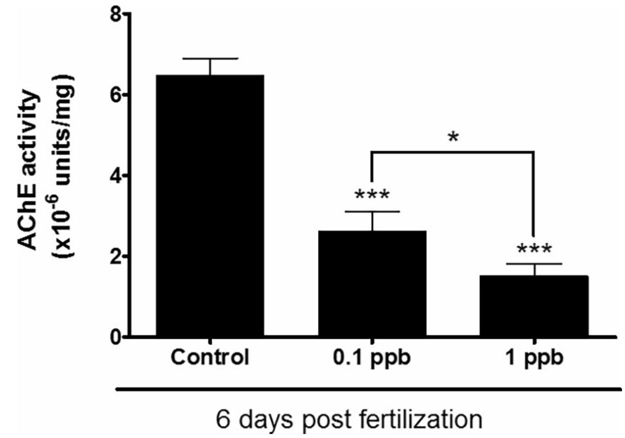

Fig. 5. Effect of methylparaben on AChE activity in zebrafish larvae. Zebrafish larvae exposed to 0.1 ppb and 1 ppb MP, a decrease in AChE activity was observed. Data presented as mean ± SD of at least three independent experiments. * indicates ($p < 0.05$) and *** indicates ($p < 0.001$) significant dose-dependent decrease in AChE activity in exposure groups.

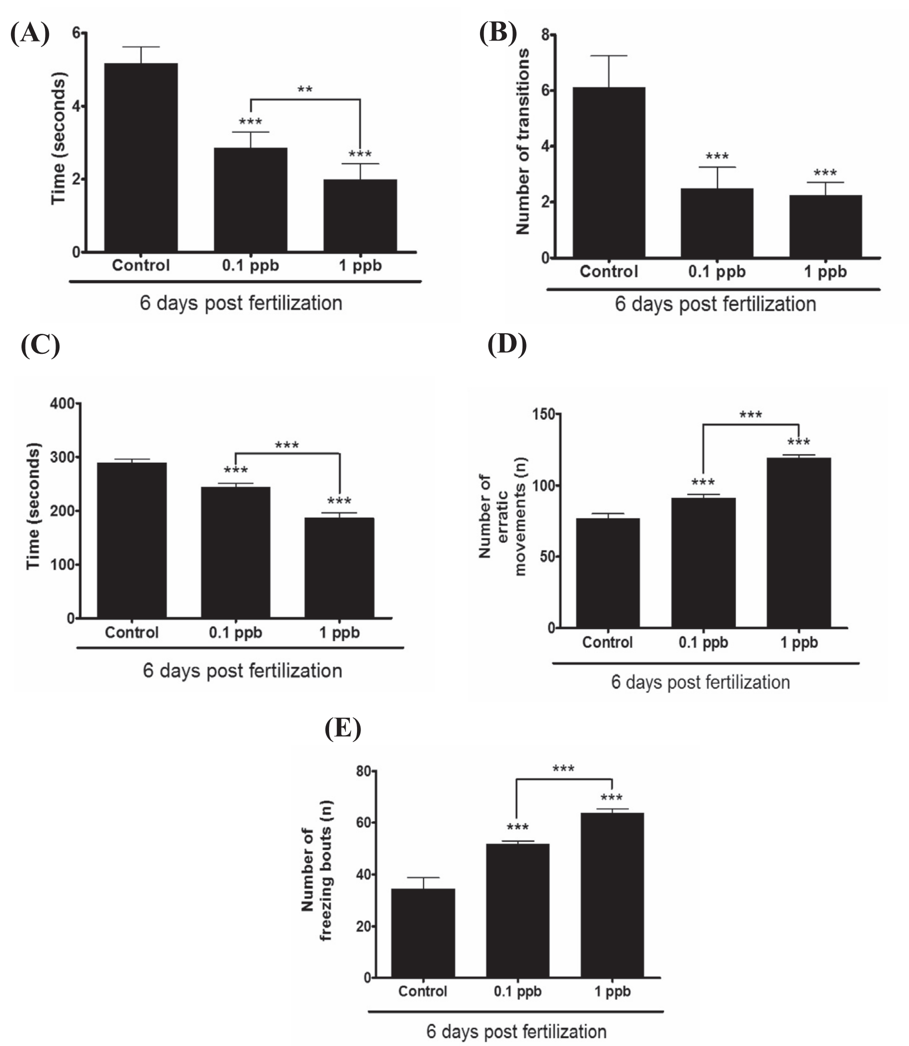

Fig. 4. (A) Latency to reach the upper half. (B) Total transitions to upper half. (C) Total time spent in upper half. (D) Total erratic movements. (E) Total freezing bouts. Data presented as mean ± SD of at least three independent experiments. ** indicates ($p < 0.01$) and *** indicates ($p < 0.001$) significance.

lower in methylparaben-treated larvae. Parameters characteristic of heightened anxiety-like behaviour such as freezing bouts and erratic movements were observed to be significantly higher in methylparaben-treated larvae (Fig. 4A–E).

3.4. Effect of methylparaben in scototaxis (light-dark preference test).

No statistically significant variation in light-dark preference was observed in methylparaben-treated larvae when compared to untreated control group (Supplementary Figs. 1A & B).

3.5. Effect of methylparaben on AChE activity

To assess biochemical parameters associated with the decrease in exploratory activity observed in the novel tank diving test, assessment of AChE activity was performed. It was observed that there was a significant and dose-dependent decrease in AChE activity in groups treated with lower doses of methylparaben (0.1 ppb and 1 ppb) (Fig. 5).

3.6. Effect of methylparaben on cortisol levels

The effect of methylparaben on whole-body cortisol content was assayed at 6 dpf in order to supplement the results obtained in the novel tank diving test, where exposed larvae exhibited a higher degree of anxiety-like behaviour. Whole-body cortisol was observed to increase in a concentration-dependent manner in methylparaben treatment groups (0.1 ppb and 1 ppb) (Fig. 6).

4. Discussion

A significant dose-dependent delay in hatching rate was observed in zebrafish embryos when exposed to methylparaben. This observation is in line with our previous study that showed delayed hatching upon exposure to higher doses of methylparaben (Dambal et al., 2017). Hatching, a key developmental time point occurs between 48 and 72 hpf. Hatching rate is widely used as a developmental endpoint in early-life toxicity testing and a delay in hatching indicates a perturbation of critical developmental pathways required for the embryo's emergence from the chorion. The action of zinc metalloproteases on the chorion and twitching movement of embryos within the chorion result in breaking of the chorion and hatching in zebrafish. The observed delay in hatching upon exposure to methylparaben could be due to factors such as alteration in the function of the hatching protease (chorionase) and inability of the larvae to break the chorion (Hallare et al., 2004). Inhibition of secretion or activity of the chorionase, which degrades the zona interna of the chorion, allowing the zona externa to be broken by osmotic or mechanical processes such as movement of the larvae, could not only result in delayed hatching, but also slow down development (Yamamoto and Yamagami, 1975; Schoots et al., 1982). Similar to our observations, a study reported delayed hatching upon embryonic exposure to estrogenic EDCs bisphenol-A and tetrabromobisphenol-A dimethyl ether (McCormick et al., 2010). Studies also show delayed hatching upon exposure to insecticide deltamethrin and its metabolite 3-phenoxybenzoic acid (Shabnam and Philip, 2018). Triclosan and perfluorooctanesulfonate, used in personal care products and flame retardants respectively, have also been found to delay hatching in zebrafish (Shi et al., 2008; Oliveira et al., 2009).

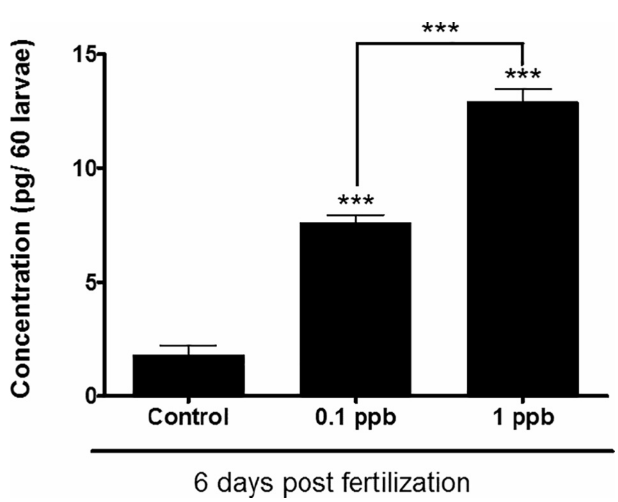

Fig. 6. Effect of methylparaben on cortisol concentration in zebrafish larvae. In zebrafish larvae exposed to 0.1 ppb and 1 ppb methylparaben, an increase in cortisol concentration was observed. Data presented as mean ± SD of at least three independent experiments. *** indicates ($p < 0.001$) significant increase in cortisol levels in exposure groups.

In zebrafish, cardiovascular development begins with the appearance of a heart tube and beating at 22 hpf and is complete by 60 hpf (Stainier et al., 1993; Kimmel et al., 1995, Burggren, Dubansky and Bautista, 2017; Gut et al., 2017). As the concentration of methylparaben increased, a decrease in heart rate was observed in zebrafish larvae at 48 hpf, especially at 100 ppb methylparaben. A study reported the positive correlation between heart rate turbulence, pericardial edema and mortality in zebrafish exposed to environmental chemicals including active pharmaceutical ingredients, aromatic compounds, pesticides, metals or chlorinated anilines for 8 days (Horie et al., 2017). These results highlight the role of high doses of methylparaben in cardiotoxicity during early development in zebrafish.

Our results showed that with the increase in concentration of methylparaben, the larvae exhibited a higher degree of anxiety-like behaviour and decreased exploratory behaviour when introduced into a novel environment, highlighting the role of methylparaben as an anxiogenic agent even at environment relevant concentrations. Similar studies have reported altered novel tank behaviour to establish anxiogenic and anxiolytic effects of several compounds such as nicotine, buspirone, chlordiazepoxide and ethanol exposure in zebrafish (Bencan et al., 2009; Mathur and Guo, 2011). Since our study brought to light alterations in anxiety-like behaviour induced by methylparaben exposure, further *in vitro* and *in vivo* studies using different animal models are recommended to elucidate the molecular mechanisms underlying this behavioural outcome.

Anxiety response in zebrafish is similar to that of humans and hence can be used for the assessment of anxiogenic effects of environmental toxicants, especially at minute doses (Baggio et al., 2017; Collier et al., 2017). These changes in behaviour are also associated with changes in cortisol levels, as observed in the methylparaben-treated larvae, showing a significant increase in levels with the increase in methylparaben concentration. Several studies have reported a correlation between whole-body cortisol levels and behavioural alterations in response to exposure to toxins (Cachat et al., 2010; Maximino et al., 2010; Stewart et al., 2012). A significant increase in cortisol levels in the exposure groups with increasing concentration of methylparaben further supports the observations made in the novel tank diving test that indicated anxiety-like behaviour in exposed larvae. Further, a reduction in AChE activity was also observed in larvae treated with methylparaben, which correlates to the role of methylparaben in affecting movement and exploratory behaviour. Similar studies carried out with environmental toxins such as chlorpyrifos and chemicals like ethanol have also been reported to alter the cholinergic system by dysregulation of AChE activity in freshwater fishes (Agostini et al., 2017; Bernal-Rey et al., 2017). To build on the data obtained in this study, further studies must be carried out to understand the mechanistic role of methylparaben in altering AChE activity and cortisol levels in parallel with neurobehavioural tests.

5. Conclusion

Methylparaben at sub-lethal concentrations altered crucial physiological and developmental landmarks such as heart rate and hatching rate respectively. Further, it has been observed to induce anxiety-like behaviour in treated larvae and decrease exploratory behaviour. A reduction in AChE activity and increase in whole-body cortisol levels further supports the observations made in the novel tank diving test, establishing the role of methylparaben as an anxiogenic agent even at sub-lethal concentrations. To better understand the effects of methylparaben, a mechanistic elucidation of altered pathways involved in neurobehaviour, later translating into adult neurological disorders is required. Further, the long-lasting effects of early-life exposure to such emerging EDCs need to be established, with tests carried out at various developmental time points.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.11.001.

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