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International Journal of Current Research
Vol. 9, Issue, 07, pp.53442-53447, July, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLES

EVALUATION OF MORPHOLOGICAL AND BEHAVIORAL VARIATIONS INDUCED IN ADULT ZEBRAFISH (*DANIO RERIO*) ON LONG-TERM EXPOSURE TO ENVIRONMENTAL ESTROGENIC CHEMICALS (INDIVIDUALLY AND IN COMBINATIONS)

Basavaraj B. Goundadkar and *Katti Pancharatna

Department of Zoology, Karnatak University, Dharwad-580 003, India

ARTICLE INFO

Article History:
Received 14th April, 2017
Received in revised form
19th May, 2017
Accepted 05th June, 2017
Published online 22nd July, 2017

Key words:

Environmental estrogenic chemicals,
Combinations,
Pigmentation,
Sensitivity,
Zebrafish (*Daniorerio*).

ABSTRACT

Many chemicals present in the environment have the potential to intervene the endocrine system of the inhabitant organisms causing alterations in their physiology and behavior. In the present study, we made an attempt to compare and evaluate the effects of long-term (75 days) exposure to different environmental estrogenic chemicals (EECs) individually and in combinations on the morphological (body size and mass, pigmentation) and behavioral (feeding activity and sensitivity) aspects of adult zebrafish in the laboratory. Adult (wild) zebrafish (n = 12) procured locally were acclimated to laboratory conditions (temperature: 26 ± 1°C, and photoperiod:11.30 L:12.30 D hrs) for two weeks and exposed semi statically to (i) Ethinyl estradiol-EE2 (3 ng/L) (ii) Diethylstibestrol-DES (3 ng/L) (iii) Atrazine-ATR (3 µg/L) (iv) Bisphenol A-BPA (3µg/L) (v) EE2+ATR (vi) EE2 + BPA (vii) DES + ATR (viii) DES + BPA (ix) EE2 + ATR + BPA (x) DES + ATR + BPA. Fish exposed to Estradiol 17β (3ng/L) served as positive controls and those exposed to conditioned water alone, as negative controls. All the concentrations of EEC used in the experiment were lower than environmentally recorded levels for the respective chemical. Our observations reveal that (i) the rate of mortality was greater in chemical exposed fish especially in those exposed to combination of EEC (ii) fish exposed to combinations of EEC exhibited increased feeding rate and variations in response to external stimuli/sensitivity (iii) in fish exposed to atrazine (individually and in combination with other EEC) pigmentation was greater while it was lesser in those exposed to BPA as compared to controls. Thus, there was a variation in the intensity of effects manifested by a chemical individually and those when exposed in combination with other chemicals. Therefore, effects of EEC on aquatic organisms potentiated in presence of other chemicals which is very relevant in evaluating the impact of water contaminants EEC on aquatic organisms in natural aquatic system.

Citation: Basavaraj B. Goundadkar and Katti Pancharatna, 2017. "Evaluation of Morphological and Behavioral Variations induced in Adult Zebrafish (*Danio rerio*) on Long-term Exposure to Environmental Estrogenic Chemicals (Individually and in Combinations)", *International Journal of Current Research*, 9, (07), 53442-53447.

INTRODUCTION

Aquatic organisms are generally exposed to complex chemical mixtures such as, effluents from industrial, agricultural and domestic sources throughout their life span (Orn *et al*., 2003; Vos *et al*., 2000). Many chemicals which are hazardous and have potential to intervene with the endocrine system of living organisms like synthetic/pharmacological estrogens, phthalates, alkylphenols and other xenoestrogens have been detected in sewage effluents (Tyler *et al*., 1998).Environmental estrogenic chemicals (EECs) not only interfere/modulate/ influence the hormone dependent processes such as reproduction, development and differentiation, homeostasis and behavior of surrounding organisms but, also cause an increase or inhibit the metabolism of naturally occurring steroid hormones and other xenobiotics by activating or antagonizing nuclear hormone receptors (Sassi-Messai *et al*., 2009). Among EECs, those detected with estrogenic activity such as, 17α-ethinylestradiol -EE2 (which is widely used as the active ingredient in contraceptive pills and hormone replacement therapy), Diethylstilbestrol-DES(a synthetic non-steroidal estrogen used in the medical treatmentof breast and prostate cancers and gynecological disorders), Bisphenol A-BPA (a common industrial chemical widely used in the production of polycarbonates, epoxy resins and flame retardants worldwide) and atrazine (a widely used weedicide in agriculture and horticulture) have been routinely detected in sewage effluents as contaminants from anthropogenic sources (Desbrow *et al*., 1998; Larsson *et al*., 1999).

****Corresponding author:*** **Katti Pancharatna,**
Department of Zoology, Karnatak University, Dharwad-580 003, India.

Moreover, environmental estrogens are more stable in aquatic ecosystem than as natural steroids (Yin *et al*., 2002). Contamination and accumulation of these xenoestrogens in the aquatic environment is known toperturb the hormone mediated processes of reproduction and development of non-target species unintentionally. Chronic exposure to both natural andsynthetic estrogens is known to induce immuno-toxic, teratogenic and carcinogenic effects (Roy and Cai, 2002). Foraging is a complex behavior associated with food intake. Fish have a wide variety of feeding habits and patterns, making them good experimental models for the analysis of the feeding behavior. A number of environmental factors through complex homeostatic mechanisms are known to govern feeding behavior (Volkoff and Peter, 2006). Behavioral changesare elicited immediately after the exposure to environmental pollutants and serve as a sensitive indicator for the influence of pollutants on target/non-target organisms, which has lead in the emergence of new branch of environmental science the behavioral eco-toxicology (Gerhardt, 2007). Similarly, sensitivity is a reflex behavior of an organism concerned with the response to external stimuli. Many organic pesticides, pyrethroids, xenoestrogens and aquatic chemical contaminants are known to modulate the sensitivity in vertebrates. The zebra stripes of zebrafish are formed of three types of chromatophores, i.e. xanthophores, iridophores, and melanophores (Parichy and Turner, 2003; Maderspacher and Nüsslein-Volhard, 2003; Patterson and Parichy, 2013; Frohnhofer *et al*., 2013). Some of the EDCs are known to interfere with the pigment formation or with thyroid hormone receptors (Waring, 2005).

Zebrafish (*Danio rerio*) has emerged as model vertebrate in ecotoxicological research (Vascotto *et al*., 1997; Grunwald and Eisen, 2002; Rubinstein, 2003; Amsterdam and Hopkins, 2006). The main benefits of using zebrafish as a model over other vertebrate species are their small body size, ease husbandry and lowmaintenance cost. Developing embryos of zebrafish have been frequently employed in studies to assess the impacts of water contaminants on embryogenesis, hatching, morphological abnormalities, sex differentiation and survival (Gorge and Nagel, 1990; Andersen *et al*., 2003; Brion *et al*., 2004). The present study focus on the long-term effects of low concentrations of ethinylestradiol (EE2), synthetic estradiol (diethylstilbestrol), herbicide (atrazine), and nonylphenol (bisphenol A) individually and in combinations on the morphological (changes in body mass and body length, intensity of pigmentation, and other abnormalities) and behavioral (swimming and feeding activity and sensitivity, i.e. response to stimuli) of adult zebrafish. Appropriate positive controls (estradiol-17 β) and negative controls were used for comparison.

MATERIALS AND METHODS

Animals

Adult (body size: 26 ± 5 mm; body mass: 455 ± 102 mg) zebrafish (bred and raised in fish farm and experimentally naive, wild type) procured from Aquastar, Chennai, were acclimated to the laboratory conditions (temperature, 26 ± 1°C and photoperiod, 11.30L: 12.30D) for two weeks before use. Permission to work on zebrafish was obtained from Animal Ethical Committee, CPCSEA, India, under institutional registration # 639/GO/02/a/CPCSEA). Food was rationed @ 3 pellets per fish and pellets that remained unconsumed were counted prior to adding the next feed (Rajapurohit and Pancharatna, 2007). Fish were maintained in well aerated glass aquaria of size (30 × 25 × 25 cm) containing 10 liters of conditioned water/exposure medium. Physical parameters such as experimental water temperature (24˚C±1˚C), pH (7.0± 0.10) and photointensity (478±5 lux) were recorded twice daily (at 9:00 and 18:00 hours) throughout the experiment. Exposure medium was changed on every alternate day and chemicals were reapplied (semi-static exposure). The physico-chemical parameters like dissolved oxygen (6.8±1 mg/L), BOD (-2.67±1 mg/L), chloride (98.80±1 mg/L), total alkalinity (273.10±1 mg/L), total hardness (233.45±1 mg/L) and ammonia (0.0173±0.0077 mg/L) were recorded every other day till the completion of the experiment to ascertain that the water quality used was within the prescribed range defined for the maintenance of zebrafish in laboratory (Lawrence, 2007; Reed and Jennings, 2010).

Experimental set up

Body weight and body length of each fish were recorded twice, once prior to the beginning of the experiment and second, after the completion of experiment. 12 groups each containing fish(n=12) were exposed to hormones, E2, EE2 and DES(3 ng/L) and chemicals Atrazine and BPA (3 µg/L), individually and in combinations. Medium was changed on every alternate day and hormones/chemicals were reapplied. Feeding activity, sensitivity, pigmentation and mortality rate were observed daily in fishes exposed to treatments and also in controls. The total duration of experiment was 75 days and consisted following treatment groups.

Individual Chemicals

Group I: (Control) Conditioned water
Group II: (Positive controls) 3 ng/L Estradiol (E2)
Group III: Ethinylestradiol 3 ng/L (EE2)
Group IV: Diethylstilbestrol 3 ng/L (DES)
Group V: Atrazine (ATR) 3µg/L
Group VI: Bisphenol A (BPA) 3µg/L

Combinations of two chemicals

Group VII: Ethinylestradiol (3ng/L) + Atrazine (3µg/L)
Group VIII: Ethinylestradiol (3ng/L) + Bisphenol A (3µg/L)
Group IX: Diethylstilbestrol (3ng/L) + Atrazine (3µg/L)
Group X: Diethylstilbestrol (3ng/L) + Bisphenol A (3µg/L)

Combination of three chemicals

Group XI: Ethinylestradiol (3ng/L) + Atrazine (3µg/L) +Bisphenol A (3µg/L)
Group XII: Diethylstilbestrol (3ng/L) + Atrazine (3µg/L) +Bisphenol A (3µg/L)

Hormones and chemicals

All hormones and chemicals except atrazine were procured from Sigma Aldrich, Louis,
USA. Atrazine was purchased from Vantech Chemicals, Hyderabad, India.

Statistics

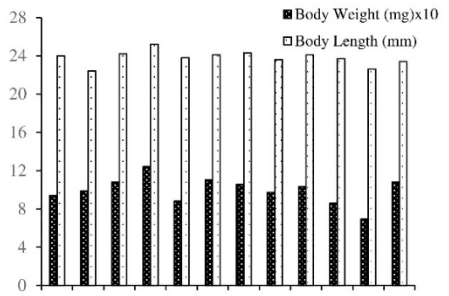
The data was analyzed using non parametric Kruskal-Wallis test. The results were judged significant at 5% level of significance (Steel and Torrie, 1980).

RESULTS

Controls and positive controls

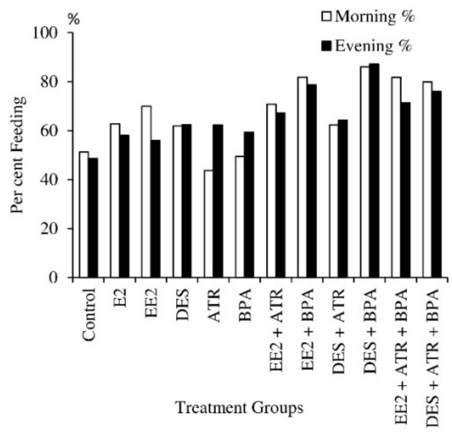
Body weight and body length of all experimental animals are shown in figure 1A. In control group all animals survived until the end of the experiment (Fig. 2C). The pattern of feeding, degree of sensitivity exhibited and pigmentation intensity are shown in Figures 1-3. In estradiol exposed positive controls, pigmentation and sensitivity were slightly lower when compared to controls (Fig. 2D & E). Feeding rate was relatively greater in morning than evening (Fig. 1B), two fishes died in this group during experimental period (Fig. 2C).

A



A. Body weight, (mg) x10 and Body length, (mm) of Zebrafish

B



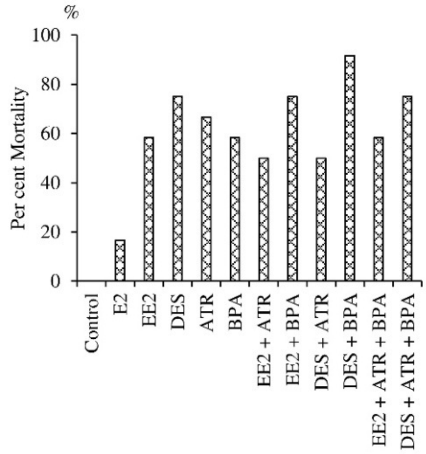
B. Per cent feeding in morning and evening of Zebrafish

Figs. 1A-B. Shows the effects of hormones/chemicals on the body weight, body length and Per cent feeding of Zebrafish

Effects of long-term exposure to individual Chemicals

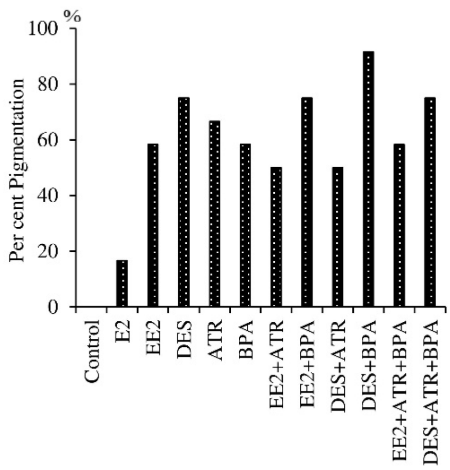
Fish exposed to ethinylestradiol showed body weight and body length, feeding behavior and intensity of pigmentation comparable to controls (Figs.1-3). Mortality rate was higher (n=7) and sensitivity to external stimuli decreased slightly (Fig. 2E). In DES exposed fish, body weight, body length, sensitivity and mortality rate (n=9) were greater (Figs. 1-2) while, feeding rate remained comparable and pigmentation was lower over controls (Figs. 1B & 2D). Fish exposed to atrazine exhibited body weight and body length comparable to controls (Fig. 1A). Mortality (n = 7) was as in EE2 and DES groups but was higher compared to controls and positive controls (Fig.2C). Fish exhibited diurnal feeding behavior i.e. lower feeding rate in morning and increased feeding behavior in the evening (Fig. 1B). Pigmentation was intense but sensitivity remained comparable to controls (Fig. 3).

C



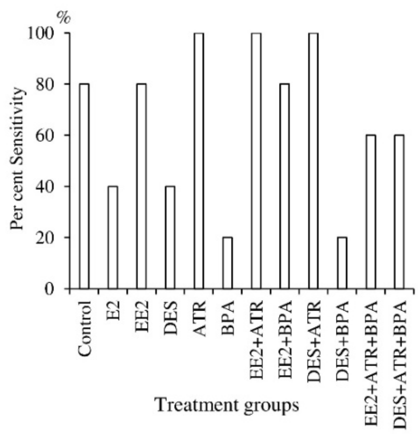
C. Per cent Mortality of zebrafish

D



D. Per cent Pigmentation of Zebrafish

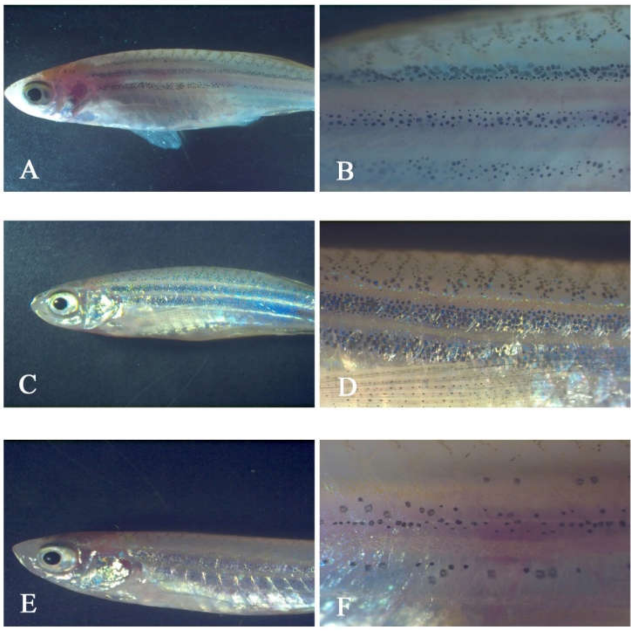
E



E. Per cent Sensitivity of Zebrafish.

Figs. 2C-E. Shows the effects of hormones/chemicals on the Per cent Mortality, Per cent Pigmentation and Per cent Sensitivity

Exposureof fish to Bisphenol A caused variation in feeding pattern as in atrazine exposed group; fish were hypersensitive to the stimuli and exhibited low pigmentation when compared to controls (Figs. 1B, 2D, E & 3). Mortality rate (n = 7) was comparable to other EEC exposed groups (Fig.2C). Body weight and body length remained comparable to controls (Fig. 1A).



A-B. Control group, shows normal distribution of chromatophores, (0.7 X and 2 X)
C-D. Atrazine group, shows increased distribution of chromatophores, (0.7 X and 2 X)
E-F. Bisphenol A group, shows decreased distribution of pigmentation, (0.7 X and 2 X)

Figs. 3A-F; Shows pigmentation in Zebrafish exposed to the chemicals/hormones

Effects of long-term exposure to combinations of EECs

In fish exposed to ethinylestradiol + atrazine the body weight and body length remained comparable to controls (Fig. 1A).Mortality rate (n = 6) was comparable to other groups (Fig. 2C) Feeding rates, sensitivity and pigmentation were greater compared to controls (Figs. 1-3). Exposure to ethinylestradiol + bisphenol A also caused an increase in rate of mortality and feeding behavior (Figs. 1B &2C) while,body weight, body length, pigmentation intensity and sensitivity were comparable to controls (Figs.1-3). In fish exposed to diethylstilbestrol + atrazine (Group IX) body weight and body length remained comparable to controls and other treatment groups (Fig. 1A).Mortality rate(n = 6) was as in other groups (Fig.2C). Fish exhibited normal feeding behavior in both morning and evening regimes (Fig. 1B). Hyperactive movements were frequently observed in this group (Fig. 2E). Pigmentation was lower when compared to controls (Fig. 2D). In fish exposed to diethylstilbestrol + bisphenol A an increase in the feeding behavior was noticed (Fig. 1B). Fish exhibited hyperactive movements (Fig. 2E). Pigmentation was very low compared to controls (Fig. 3). Mortality rate was highest (n = 11) in this group (Fig. 2C). Body weight and body length did not show significant variation compared to controls (Fig. 1A). Exposure to ethinylestradiol + atrazine + bisphenol A (Group XI) showed increase in the feeding behavior (Fig. 1B). Low sensitivity/slower movements (Fig. 2E). Mortality (n=7) was comparable to other treatment groups (Fig. 2C). Pigmentation was lesser than that of controls (Fig. 3). In fish exposed to diethylstilbestrol + atrazine + bisphenol A (Group XII) body weight and body length was comparable to other groups (Fig. 1A). Mortality rate was 80% (Fig.2C). Fish showed normal feeding behavior at both morning and evening (Fig. 1B). Less sensitivity/ movements were observed in this group (Fig. 2E). Pigmentation was higher when compared to controls (Fig. 3).

DISCUSSION

Pollution-induced morphological and behavioral alterations are reported in a wide variety of vertebrate species but, the mechanisms underlying these behavioral abnormalities are not clearly understood (Zala and Penn, 2004). Developmental exposure to endocrine disrupting chemicals like xenoestrogens are reported to alter neuro-development in fish (Kishida *et al*., 2001). Physiological changes such as altered neurotransmitters levels or hormones known to modulate behavior (Scott and Sloman, 2004; Rademacher *et al*., 2003). EECs found in the environment alter the behavior of vertebrates(Colborn *et al*., 1993; Colborn, 1998; Crisp *et al*., 1998). Behavioral responses often occur rapidly after exposure to environmental pollutants and represent a sensitive indicator of the influence of pollutants on non-target organisms (Gerhardt, 2007). Behavioral changes are excellent biomarkers of stress and stressors in animals, given that behavior is the physical manifestation of the animal's internal neuronal, metabolic, and endocrine processes, and at the same time the integrated physiological response to its environment (Clotfelter *et al*. 2004).

In the present study when fish were exposed to same chemicals, individually and in combinations the effects manifested were at different levels both morphologically and physiologically that depicted in the behavioral pattern of exposed fish. Feeding behavior was chosen because previous studies demonstrated that changes in feeding behavior were related to neurotoxin exposure and neurotransmitter concentrations (Gaworecki *et al*., 2009). The changes in feeding pattern was observed in the present study, in all groups either it was more in day and less evening or viva-versa. For instance, in the group exposed to ATR individually, feeding was reduced in the morning and was normal in the evening while ATR when exposed in combination with EE2 the feeding rate was accelerated. EE2 exposed to three spine stickleback (*Gasterosteusaculeatus*) induced hyper active feeding behavior (Bell, 2004).

Exposure to chemicals although for a long-term did not alter body mass and body size significantly (Fig. 1A) The intensity of pigmentation was altered in experimental fish especially in ATR exposed fish that developed darker and concentrated parallel lines throughout the length of the fish, while it was lesser than controls in BPA exposed fish (Fig. 3). Further the effects of ATR remained common when it was treated in combination with other chemicals suggesting that ATR may have induced hyper-pigmentation. Although the mechanism underlying in stimulating hyper-pigmentation by ATR remains unknown, oxidation of tyrosine to melanin is the basis for pigmentation and chemicals that mimic agonistic or antagonistic to tyrosine seem to affect pigmentation pattern (Waring and Haris, 2005). Some xenoestrogens/EECs could also interact with the thyroid, as there is cross-talk between the estrogen and thyroid receptors (Waring and Haris, 2005). Interestingly, bisphenol A and triiodothyronine are found to be unexpected resemblance in their chemical structure (Harvey & Williams, 2002). Further, plasticizers such as nonylphenol, bisphenol-A and butylbenzylphthalate are able to disrupt thyroxine binding to the transport protein transthyretin. The fish exposed to chemicals exhibited hypersensitivity in the present study, especially those exposed to combination of two chemicals compared to controls. Earlier studies also report that many organic pesticides, pyrethroids, xenoestrogens and aquatic chemical contaminants are known to modulate the sensitivity acting on nervous system either being axonic poisons or impairing voltage-gated sodium (Na)channel by binding to the regulatory proteins or inhibiting activity of acetylcholinesterase (AChE) or ATP release (Rao *et al*., 2005). If individuals and populations are exposed to an EDC, it is likely that other environmental pollutants are involved because contamination of environments is rarely due to a single compound. Furthermore, effects of different classes of EECs may be additive or even synergistic (Crews *et al*., 2003)

In conclusion, the present study records an interesting observation that the effects of EEC may vary individually and in combination with other EEC which potentiate the effects of one another which is very relevant in understanding the effects of water pollutants on non-target species in natural ponds which might have been contaminated with different chemicals from diverse sources.

Acknowledgements

This work is supported by Grant No. F40-373/2011 (SR) sanctioned to KP from UGC, New Delhi, India. The work is partially supported by UGC-SAP-DSA-I F.4-18/2015/DSA-I (SAP-II) sanctioned to Dept. of Zoology, KUD. First author gratefully acknowledges grant of BSR-RFSMS Fellowship UGC, New-Delhi. No conflict of interest.

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