Original Research
ISSN (Print): 2455-1244
ISSN (Online): 0971-913X
Journal of Endocrinology and Reproduction, *Vol 23(2), 49-63, December 2019, DOI: 10.18311/jer/2019/27184*

Impact of Bisphenol S as an Endocrine Disruptor in a Freshwater Fish, *Oreochromis mossambicus*

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Abstract

Endocrine Disrupting Chemicals (EDCs) have the potential to alter the hormonal pathways concerned with regulation of the normal homeostatic mechanisms. The adverse effects of EDCs can be observed in areas where pollution is high, particularly in aquatic ecosystems, where persistent environmental chemicals accumulate. In the present study, we investigated the endocrine disrupting effects of Bisphenol S (BPS), if any, in the levels of thyroxine, triiodothyronine, cortisol and sex steroidal [17βestradiol (E2) and testosterone(T)] hormones in juvenile as well as adult *Oreochromis mossambicus.* Drastic changes in serum thyroxine and triiodothyronine levels showed that BPS treatment resulted in disruption of thyroid gland function. Alteration to significant levels in serum cortisol indicated acute stress and impairment of hypothalamic-pituitary-interrenal axis. Significant changes (p<0.05) occurred in the steroidal hormone levels which are biomarkers of endocrine disruption as they affect hypothalamic-pituitary-gonadal axis in fish. Bisphenol S also revealed estrogenic potency by inducing significant alteration in the E2/T ratio. DNA fragmentation, if any, induced by BPS was also analyzed in juvenile fish. There was no significant DNA fragmentation observed in gel electrophoresis, but a significant elevation was seen in percent fragmented DNA in the diphenylamine method. Therefore, it could be concluded that BPS at different sublethal concentrations have a profound impact on endocrine physiology of fish.

Keywords: Bisphenol S, DNA Fragmentation, Endocrine Disruption, Thyroxine, Triiodothyronine

1. Introduction

There is a conclusive agreement among the researchers about the hazards posed by many man-made chemicals in the environment as well as some naturally occurring compounds which have the potential to affect reproductive health, metabolism and growth by disrupting normal endocrine function in wildlife populations, aquaculture species and humans[1]. The chemicals that elicit toxic effects by altering the normal functions of the endocrine system are called Endocrine Disrupting Chemicals (EDCs). Endocrine disruptive actions include alterations in receptor or hormone availability (affecting their synthesis, transport, metabolism and excretion) and also disruption caused by binding to their receptors[2-4].

Aquatic vertebrates, such as fish, are particularly affected by aquatic anthropogenic contaminants and can be exposed lifelong through multiple routes including the skin and gills or through feeding on contaminated sediments or organisms[5]. Owing to the lipophilic properties, generally pollutants in water are readily absorbed into the body of animals and cause bioaccumulation, which in turn get augmented through the food chain by biomagnification. Hence, fish serve as biological markers of environmental pollution[6].

Bisphenol A (BPA) is the monomer of the plastic polycarbonate. Due to Endocrine Disrupting (ED) activity, environmental pollution produced by BPA has raised serious concern[7,8]. As a result, some regulatory agencies, such as the European Commission[9], the US

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Food and Drug Administration[10], and Health Canada[11], have banned the use of BPA in baby bottles during recent years. Given these restrictions and societal pressure, manufacturers have turned to alternative chemicals to produce BPA-free products[12]. One such alternative chemical is bisphenol S (BPS)[13].

Bisphenol-S (4, 4'- sulphony diphenol) is similar to BPA in many properties and uses, especially with respect to aggregation. It can replace BPA in improving the mechanical properties and thermal stability of polymeric products[13]. Bisphenol-S has better resistance against high temperature, sunlight and organic solvents[12]. Since the use of BPS in industry is increasing, some studies have been conducted. For instance, when zebrafish (*Danio rerio*) embryos were exposed long term to different concentrations of BPS, males showed a significant decrease in body length, weight, sperm count, serum thyroid hormones and testosterone while females showed decreased egg ratio and plasma thyroid hormones[14,15]. Unlike BPA, not much of research regarding the effects of BPS as an endocrine disruptor has been carried out in animals. Therefore, in the present study, we explored the endocrinological effects of BPS with regard to thyroxine (T4), triiodothyronine (T3), cortisol, 17β-estradiol (E2) (male and female fish) and testosterone (T) in the serum of juvenile and adult freshwater fish, *Oreochromis mossambicus*. Furthermore, E2 to testosterone ratio (E2/T) was also evaluated to analyze if the value has been deviated in BPS-exposed juvenile and adult male fish. Moreover, DNA fragmentation assay in juvenile fish was also undertaken to scrutinize if DNA damage has occurred on exposure to BPS. This study can be considered as a reliable indicator of genotoxicity in aquatic organisms in polluted water.

2. Materials and Methods

2.1 Experimental Design

2.1.1 Test Chemical

The LC_{50} value of BPS was determined by Probit analysis [Organisation for Economic Co-operation and Development (OECD) Guidelines] and was found to be 180 mg/L BPS for juvenile and 200 mg/L BPS for adult. Deriving from this, three sub-lethal concentrations *viz*., 100, 120 and 140 mg/L for juveniles and 100, 125 and 150 mg/L for adults were selected for treatment.

2.1.2 Fish and Aquaria

The fish, *O. mossambicus,* used in the experiment were obtained from a fish farm in Thiruvananthapuram. Prior to experiment, the fish were acclimatized for 30 days in large cement tanks filled with dechlorinated tap water under laboratory conditions such as natural photoperiod and temperature (26 ± 2°C). They were fed daily on protein feed *ad libitum*.

Prior to experiment, juvenile fish of body weight 7±2 g were divided into 10 groups of ten each in separate glass tanks. The first group of fish were maintained in dechlorinated tap water and served as control. Fish in groups II to X were exposed to 100 mg/L, 120 mg/L and 140 mg/L BPS mixed in the medium (water) for a period of 3, 6 and 9 days, respectively.

Prior to the experiment, adult fish of body weight 45±2 g were divided into 10 groups of eight each in separate glass tanks. The first group of fish served as control and were kept in normal dechlorinated tap water. Fish in groups II to X were exposed to 100 mg/L, 125 mg/L and 150 mg/L BPS mixed in the medium (water) for a period of 4, 8 and 12 days, respectively.

2.1.3 Sampling

The fish were starved for 24 h before sacrifice for getting optimum experimental conditions. After the stipulated periods of BPS exposure, 1 to 2 mL of blood was taken from the caudal vein using a sterile syringe. The blood was transferred to Eppendorf tubes, allowed to clot and centrifuged at 1000 rpm for 5 min. The serum obtained was then collected and stored in a deep freezer (Rotek, Vengola, India) maintained at -20°C until hormone analysis. Fresh liver lobes were dissected out and stored in a deep freezer for DNA fragmentation study.

2.2 Serum Hormone Analysis

The levels of thyroxine (T4), triiodothyronine (T3), cortisol, 17β-estradiol (male and female fish) and testosterone were analyzed by Electro Chemi Luminescence Immuno Assay (ECLIA) kits purchased from Cobas (Roche Diagnosis, Mannheim, USA) as per manufacturer's instructions.

2.2.1 Test Principle for Cortisol Assay

Cortisol assay employs a competitive test principle with monoclonal antibodies specifically directed against cortisol. Ten microlitres of the serum sample was incubated with cortisol-specific biotinylated antibody and a ruthenium complex-labeled cortisol derivative. Depending on the concentration of the analyte in the sample, and the formation of the respective immune complex, the labeled antibody binding site was occupied in part with sample analyte and in part with ruthenylated hapten. After addition of streptavidin-coated micro-particles, the complex bound to the solid phase *via* interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the micro-particles are captured on to the surface of the electrode. Unbound substances were removed with ProCell M. Application of voltage to the electrode then induced chemiluminescent emission which was measured in a photomultiplier. Results were determined by a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided through the reagent barcode[16]. Concentration of cortisol was expressed in ng/mL.

2.2.2 Test Principle for T4 Assay

Fifteen microlitres of the sample and a T4-specific antibody labeled with a ruthenium complex were incubated, and the bound T4 was released from binding proteins in the sample by ANS (8-Anlino-1-Naphthalene Sulfonic Acid). After addition of streptavidin-coated microparticles and biotinylated T4, the still free binding sites of the labeled antibody become occupied, with formation of antibody hapten complex. The entire complex got bound to the solid phase by the interaction of biotin and streptavidin. The reaction mixture was aspirated onto the measuring cell where the micro-particles were magnetically captured on to the surface of the electrode. Unbound substances were then removed with ProCell M. Application of a voltage to the electrode then induced chemiluminescent emission which was measured in a photomultiplier. Results were determined *via* a calibration curve[16]. Serum concentration of T4 was expressed in ng/mL.

2.2.3 Test Principle for T3 Assay

Thyroxine assay employs a competitive test principle with polyclonal antibodies specifically directed against T3. Endogenous T3, released by the action of ANS, competes with the added biotinylated T3 derivative for the binding sites on the antibodies labeled with the ruthenium complex. Thirty microlitres of the sample and T3-specific antibody labeled with ruthenium complex were incubated, and the bound T3 was released from the binding protein in the sample by ANS. After addition of streptavidin-coated micro-particles and biotinylated coated T3, the still free binding sites of the labeled antibody become occupied with formation of an antibody-hapten complex. The entire complex got bound to the solid phase by interaction between biotin and streptavidin. The reaction mixture was aspirated on the measuring cell where the micro-particles were captured on to the surface of the electrode. Unbound substances were removed with ProCell M. Application of voltage to the electrode induced chemiluminescent emission which was measured by a photomultiplier. Results were determined using a calibration curve[16]. Concentration of T3 was expressed in ng/mL.

2.2.4 Test Principle for 17β-Estradiol Assay

Twenty five microlitres of the sample was incubated with two estradiol-specific biotinylated antibodies. Immunocomplexes were formed, the amount of which was dependent upon the analyte concentration in the sample. After the addition of streptavidin-coated micro-particles and an estradiol derivative labeled with a ruthenium complex, the still vacant sites of the biotinylated antibodies became occupied with formation of an antibody-hapten complex. The entire complex became bound to the solid phase *via* interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the micro-particles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell M. Application of a voltage to the electrode then induced chemiluminescent emission which was measured in a photomultiplier. Results were determined by a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided through the reagent barcode[17,18]. Serum level of E2 was expressed in pg/mL.

2.2.5 Test Principle for Testosterone Assay

Twenty microlitres of the sample was incubated with a biotinylated monoclonal testosterone-specific antibody. The binding sites of the labeled antibody came to be

occupied by the sample analyte (depending on its concentration). After the addition of streptavidin-coated micro- particles and a testosterone derivative labeled with a ruthenium complex, the entire complex becomes bound to the solid phase *via* interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the micro-particles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell M. Application of a voltage to the electrode then induced chemiluminescent emission which was measured in a photomultiplier. Results were determined by a calibration curve[18,19]. Serum level of testosterone was expressed in ng/mL.

2.2.6 Agarose Gel Electrophoretic Detection of DNA

Liver DNA isolation and electrophoresis were carried out according to the method of Iwasa, *et al.*[20] with some modifications. Briefly, the homogenized liver tissue was lyzed with buffer containing 10 mM Tris-HCl, 1 mM EDTA (Ethylenediaminetetraacetic acid), 1% SDS (Sodium dodecyl sulfate) and 100 mg/mL Proteinase K and kept in a water bath for 12 h at 37°C. DNA was extracted twice with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). To the aqueous phase, 3M sodium acetate was added and the DNA was precipitated with chilled isopropanol. Following a 70% ethanol wash, the precipitated DNA was resuspended in Tris - EDTA buffer and electrophoresed in 1% agarose containing 5µg/mL ethidium bromide. A 100 bp DNA ladder (G Biosciences, Noida, India) was used for the determination of the sizes of DNA fragments. After separation, the resulting DNA fragments were visible as clearly defined bands. The DNA damage was visualized under Gel-doc (Gestan, Medicare, Germany). The migration distance of the DNA molecule from the top of the gel was used as a measure of DNA damage. It was recognized that the highly fragmented low molecular weight DNA strand would migrate farther than intact high molecular weight DNA strands. Molecular masses of DNA fragments were determined by using standard DNA marker.

2.2.7 Quantitative Estimation of DNA Fragmentation

DNA fragmentation was measured by DNA spectrophotometric method[21]. Intact DNA was separated from fragmented DNA by centrifugal sedimentation followed by precipitation and quantification using DPA (Diphenylamine). To minimize formation of oxidative artifacts during isolation, 2, 2, 6, 6 – tetramethyl pipridinoxyl was added to all solutions and all procedures were performed on ice. Hepatocytes were put in a 1.5 mL centrifuge tube (tube B) and centrifuged at 200 x g for 10min to obtain pellet. The supernatants were transferred to fresh tube (tube S). The obtained pellet was suspended in 1mL Tris-Triton-EDTA (TTE) and centrifuged at 20,000 x g for 10 min. The supernatant was transferred to fresh tubes (tube T) and the resulting pellets were resuspended in TTE buffer. Tri-Chloroacetic Acid (TCA) was added to tubes T, B and S and vortexed vigorously. Tubes were kept overnight at 4°C followed by centrifugation for 10 min. The supernatant was discarded and the pellet was hydrolyzed by the addition of 160 µL of 5% TCA followed by heating at 90°C for 15 min. Color was allowed to develop for about 4 h at 37°C or overnight at room temperature. The optical density (OD) was read at 600 nm.

The percentage of fragmented DNA was calculated by,

% fragmented DNA = (S+T/ S+T+B) x 100 or (T/ T+B) x 100

Where S, T and B are the OD of fragmented DNA in the S, T and B fractions, respectively.

2.3 Statistics

Data analysis was done by ANOVA. The differences in means were tested by using Duncan[22] analysis. Significant level used was $p<0.05$. All the statistical analyses were performed using the software SPSS 22.0 for Windows.

3. Results

3.1 Serum Cortisol Level in Juvenile and Adult Fish

3.1.1 Juvenile Fish

The level of cortisol in the serum of BPS exposed fish slightly decreased in the 3 and 6 day treatment groups and maximum decrease was observed in the 9 day treatment group to 100 mg/L BPS with respect to control. Fish on exposure to 120 mg/L BPS showed no significant change in the 3 and 6 day treatment groups and thereafter resulted in significant increase in cortisol level in the 9 day

exposure group. There was a significant increase in the level of cortisol in 6 and 9 day exposure groups exposed to 140 mg/L BPS (Figure 1A).

3.1.1 Adult Fish

The fish exposed to 100 and 125 mg/L BPS showed significant decrease in cortisol level after all days of exposure with respect to control. The fish exposed to 150 mg/L BPS did not produce any perceptible change in the 4 day treatment group, although the level was found to be increased significantly in the 8 and 12 day exposure groups (Figure 1B).

3.2 Serum T3 Level in Juvenile and Adult Fish

3.2.1 Juvenile Fish

The level of T3 in fish exposed to 100 mg/L BPS showed significant decrease after all days of exposure. Fish on exposure to 120 mg/L BPS, a drastic increase was observed in the 3 day treatment group and thereafter the level was found to be significantly declined in the 6 and 9 day exposure groups. Similarly, there showed a sharp increase in the 3 day treated group to 140 mg/L BPS, but the level was found to be significantly decreased in the 6 and 9 day exposure groups (Figure 2A).

3.2.2 Adult Fish

There was a significant increase in the level of T3 in all treatment groups at 100, 125 and 150 mg/L BPS in a time-dependent manner (Figure 2B).

3.3 Serum T4 level in Juvenile and Adult Fish

3.3.1 Juvenile Fish

The level of T4 was found to be significantly less after exposure to 100 mg/L BPS in all treatment groups. A significant decrease was observed in serum T4 level in fish exposed to 120 mg/L BPS in all exposure groups in a time-dependent manner. Likewise, T4 level was found to be significantly decreased in the 3, 6 and 9 day exposure groups to 140 mg/L BPS (Figure 3A).

3.3.2 Adult Fish

One way ANOVA tests showed that there was a significant increase in T4 level after all days of exposure to 100 mg/L BPS in a time-dependent manner. Exposure to 125 mg/L BPS increased T4 to significant level in the 4 and 8 day exposure groups but the level was found to be drastically increased in the 12 day exposure group. Thyroxine level was found to increase in a time-dependent manner after all days of exposure to 150 mg/L BPS (Figure 3B).

3.4 Serum 17β-Estradiol Level in Juvenile and Adult Fish

3.4.1 Juvenile Male Fish

A time dependent significant increase in E2 level was recorded after all days of exposure to sublethal concentrations to 100, 120 and 140 mg/L BPS exposure in comparison with control (Figure 4A).

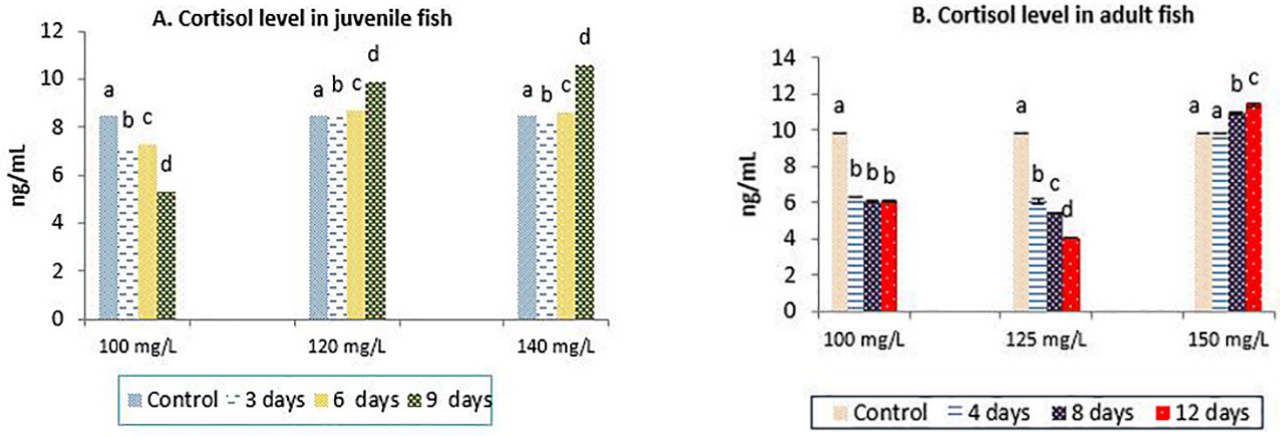

Figure 1. The effect of sublethal concentrations of BPS on Cortisol level in the serum of juvenile (100, 120 and 140 mg/L) and adult (100, 125 and 150 mg/L) *O. mossambicus*. In Figure 1, each bar is mean ± SEM of ten fish and in Fig. B, each bar is mean ± SEM of eight fish. Means with different superscript letters (a,b, c and d) for each parameter is significantly different at $p<0.05$.

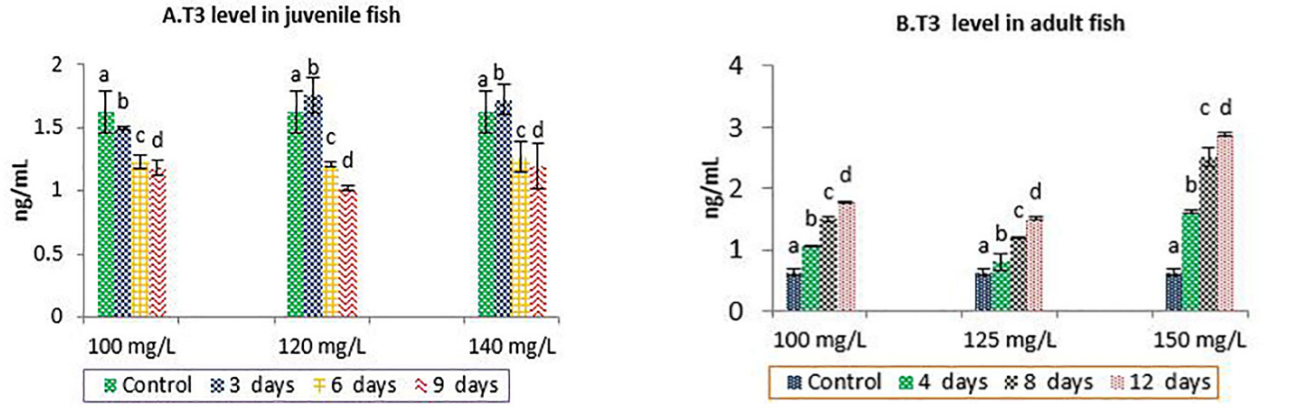

Figure 2. The effect of sublethal concentrations of BPS on T3 level in the serum of juvenile (100, 120 and 140 mg/L) and adult (100, 125 and 150 mg/L) *O. mossambicus*. Other details, as in Figure 1.

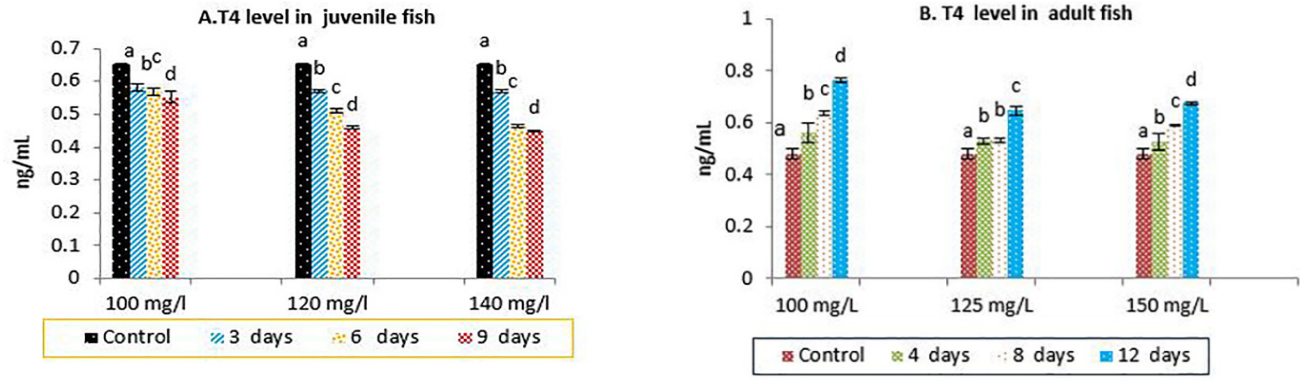

Figure 3. The effect of sublethal concentrations of BPS on T4 level in the serum of juvenile (100, 120 and 140 mg/L) and adult (100, 125 and 150 mg/L) *O. mossambicus*. Other details, as in Figure 1.

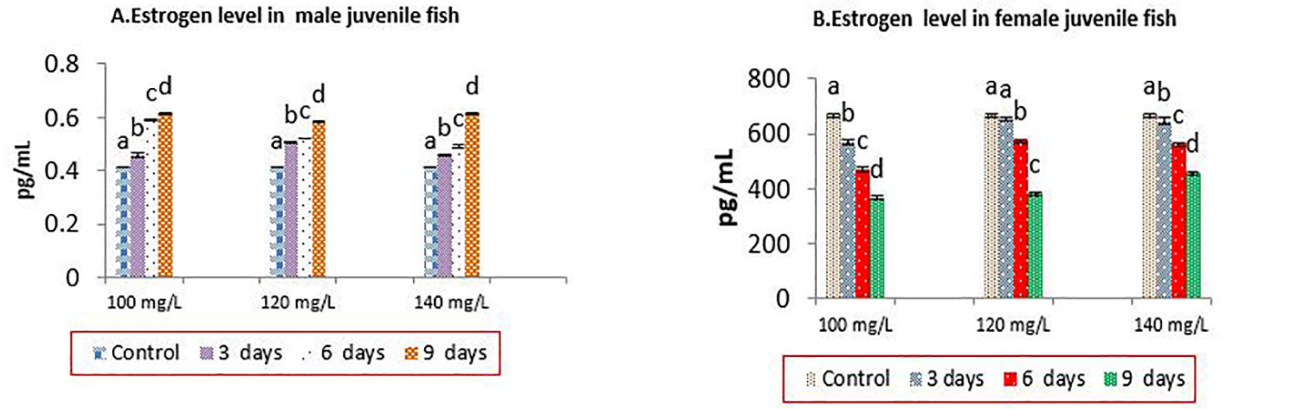

Figure 4. The effect of sublethal concentrations of BPS (100, 120 and 140 mg/L) on E2 level in the serum of juvenile male and female *O. mossambicus*. Each bar is mean ± SEM of ten fish. Means with different superscript letters (a, b, c and d) for each parameter is significantly different at $p<0.05$.

3.4.2 Juvenile Female Fish

In female fish, data showed a time-dependent significant decrease ($p<0.05$) in E2 level over control fish on all exposure concentrations of BPS (100, 120 and 140 mg/L) (Figure 4B).

3.4.3 Adult Male Fish

The male fish, when exposed to 100 mg/L BPS, showed a significant decrease after all periods of exposure in a time-dependent manner. In all experimental fish exposed to 125 and 150 mg/L BPS, there was a significant decrease in E2 level after 3 days exposure, and maximum decrease was observed after 6 and 9 days of exposure (Figure 5A).

3.4.4 Adult Female Fish

In female fish, exposure to sub-lethal concentrations (100, 125 and 150 mg/L) of BPS produced a significant decrease in E2 level at all periods of exposure in a time-dependent manner (Figure 5B).

3.5 Serum Testosterone Level in Juvenile and Adult Fish

3.5.1 Juvenile Fish

The concentration of testosterone significantly decreased in 3 and 6 day exposure to 100 mg/L BPS, though maximum decrease was observed after 9 day. In fish exposed to 120 and 140 mg/L BPS, there was a significant decrease in testosterone level after all periods of exposure (Figure 6A).

3.5.2 Adult Fish

Testosterone level decreased significantly in all treatment groups in a time-dependent manner. Maximum decrease in testosterone level was observed in serum of fish when exposed to 125 and 150 mg/L BPS (Figure 6B).

3.6 Estradiol/Testosterone (E2/T) Ratio in Male Fish

3.6.1 Juvenile Fish

The E2/Tratio was found to be significantly increased after all days of exposure to 100 mg/L BPS. The level was found to be significantly increased in the 3 and 6 day exposure groups to 120 mg/L BPS, while maximum increase was observed in the 9 day exposure group. All experimental fish, exposed to 140 mg/L, showed a significant increase in E2/T ratio in a time dependent manner (Figure 7A).

3.6.2 Adult Fish

There was a significant increase in E2/T ratio in a time-dependent manner after all days of exposure to 100, 125 and 150 mg/L BPS (Figure 7B).

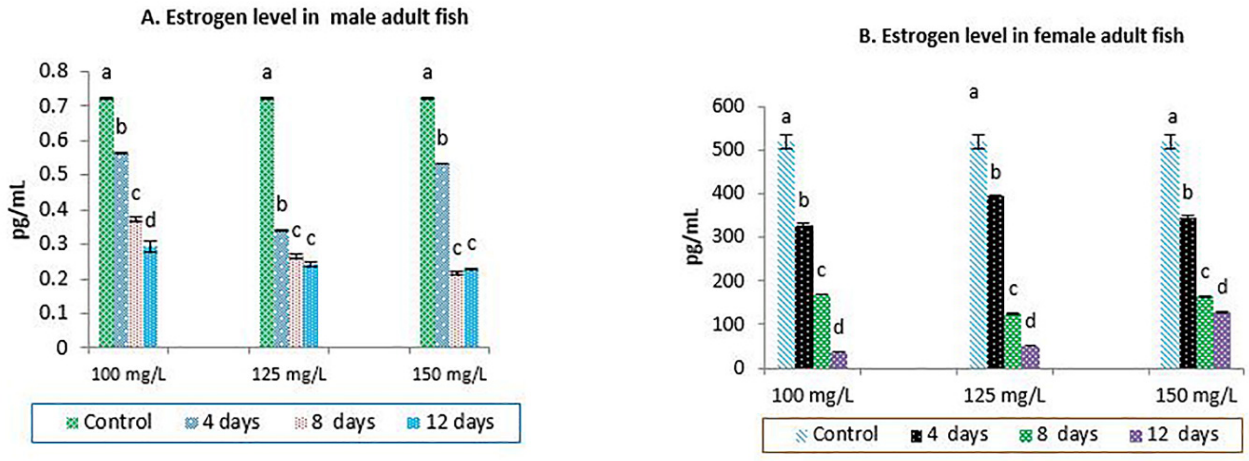

Figure 5. The effect of sublethal concentrations of BPS (100, 125 and 150 mg/L) on E2 level in the serum of adult male and female *O. mossambicus*. Other details, as in Figure 4.

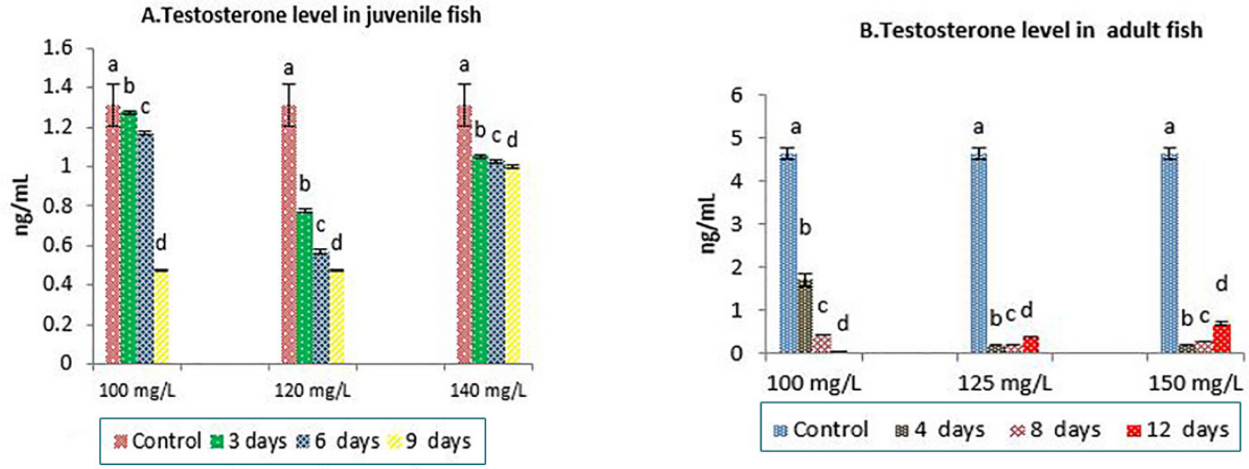

Figure 6. The effect of sublethal concentrations of BPS on testosterone level in the serum of juvenile (100, 120 and 140 mg/L) and adult (100, 125 and 150 mg/L) *O. mossambicus*. Other details, as in Figure 1.

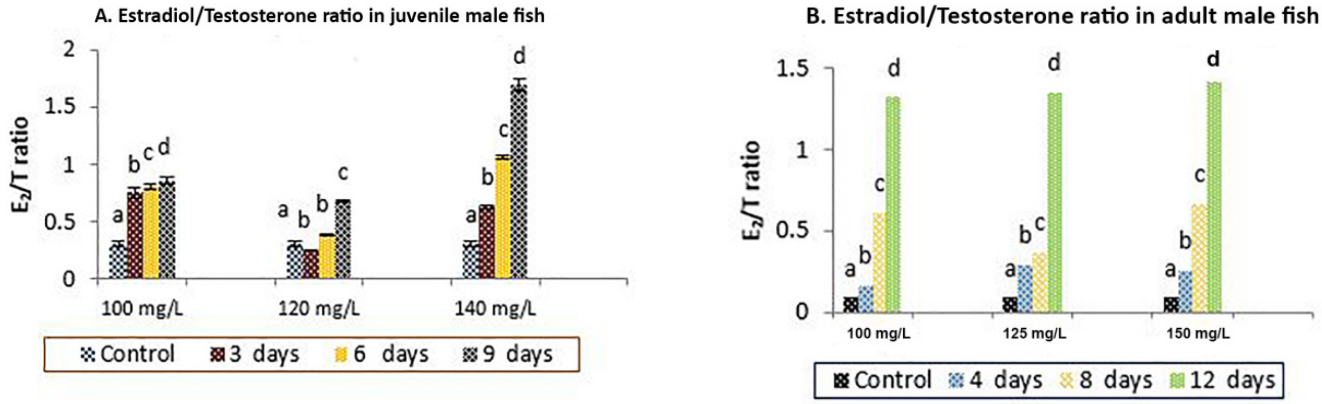

Figure 7. The effect of sublethal concentrations of BPS on E2/T ratio in the serum of juvenile (100, 120 and 140 mg/L) and adult (100, 125 and 150 mg/L) *O. mossambicus*. Other details, as in Figure 1.

3.7 Evaluation of DNA Fragmentation

In the agarose gel electropherogram, DNA fragmentation was not observed because all bands appeared as single high molecular weight intact DNA after all days of exposure to 100, 120 and 140 mg/L of BPS.

A significant increase in % fragmented DNA was observed in the 3 day exposure group to 100, 120 and 140 mg/L of BPS, but the highest increase was found in the 6 and 9 day treatment groups at all sublethal concentrations of BPS (Figure 8).

4. Discussion

Exposures to environmental chemicals during early stages of development can disrupt normal patterns of development of endocrine system and thus dramatically affect the development, metabolism and disease susceptibility later in life. Endocrine disrupting chemicals are hypothesized to interact with the endocrine systems and disturb endocrine homeostasis. Fish are considered especially vulnerable to EDCs due to their multiple uptake routes; directly from water *via* the gills and through the

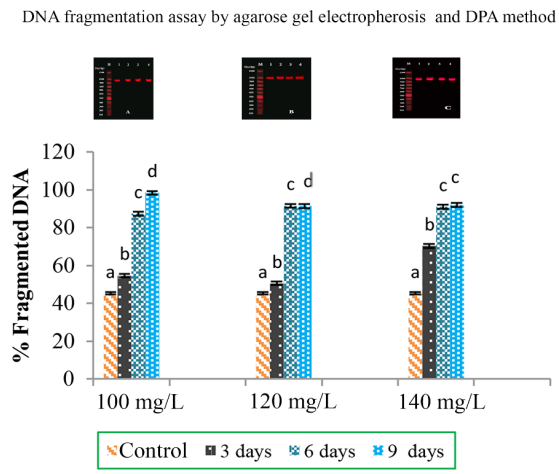

Figure 8. The effect of sublethal concentrations of BPS on DNA damage in the liver of juvenile fish, *O. mossambicus* (100, 120 and 140 mg/L) assessed by agarose gel electrophoresis and DPA method. In electropherogram A (100 mg/L), B (120 mg/L) and C (140mg/L). M - Marker, lane 1 - control, lane 2 - 3 days, lane 3 - 6 days and lane 4-9days. Each bar is mean ± SEM of ten fish. Means with different superscript letters (a, b, c and d) for each parameter is significantly different at $p<0.05$.

skin, drinking and the diet[23,24]. Henceforth, the present study was carried out to assess the endocrinological impacts of BPS as well as its genotoxicity, if any, in *O. mossambicus*.

In the present study, a sharp increase in serum cortisol level was observed in juvenile fish exposed to 120 and 140 mg/L BPS and in adult fish exposed to 150 mg/L BPS. Cortisol (stress hormone) plays a major regulatory role in metabolism and is mediated by the Hypothalamus Pituitary-Interrenal (HPI) axis[25,26]. The major actions of cortisol in fish are the regulation of ion balance in the gills as well as metabolic actions on liver and muscle that direct nutrients to the central nervous system[27]. An elevated plasma cortisol level in the present study is a primary indicator of a stress response in fish[28] which is in accordance with the results of studies on brown trout to Cyanobacterium, *Microcystis aeruginosa*[29] and the European whitefish, *Coregonus lavaretus* induced by subchronic exposure to environmentally relevant densities of *Planktothrix rubescens*[30]. Elevated plasma cortisol levels were also observed in sockeye salmon (*Oncorhynchus nerka*) exposed to copper[31], in salmon trout exposed to chromium[32], in rainbow trout exposed to cadmium[33] and in adults of *Onchorhynchus mykiss* exposed to cadmium[34]. Hence, the results of this study suggest that fish when exposed to BPS suffered physiological stress.

A significant decrease in cortisol level was observed in juvenile fish on exposure to 100 mg/L BPS and in adult fish on exposure to 100 and 125 mg/L BPS. There was a significant decrease in hormonal titer of cortisol upon exposure to curzate and imidacloprid in *O. mossambicus*[35]. Reduction in serum cortisol level was reported in *Clarias gariepinus* exposed to endosulfan[36], in *Sarotherodon mossambicus* under endosulfan toxicity[37] and in *Labeo rohita* fingerlings exposed to endosulfan and dietary pyridoxine[38]. Low level of cortisol indicates interrenal exhaustion or impairment of the HPI axis[39]. The reduced cortisol level in the present study could be considered as an adaptive response by the fish by way of maintaining low metabolic rate upon endocrine disruption[35].

The current study implies that on exposure to BPS, T3 level decreased significantly in juvenile fish. A major role of thyroid hormone in fish is regulation of growth and development, metabolism and osmoregulation, often in association with growth hormone and cortisol[40,41]. Triiodothyronine, the active form of thyroid-stimulating hormone (TSH), appears to be produced largely by peripheral enzymatic monodeiodination of T4 mainly in the liver and other tissues[42]. Ruby et al[43] examined a fall in plasma T3 level in rainbow trout on exposure to cyanide. Exposure of Abu mullet to benzo[a] pyrene resulted in a significant decrease in T3 level[44]. Generally, a reduction of the serum T3 levels is mainly due to a drop in the T4 production and secretion[41]. Similarly, T3 level decreased in the BPS exposed juvenile fish.

In the present investigation, it was found that T3 level increased significantly in treated adult fish. Similar results were found in zebrafish embryo exposed to butachlor and triadimefon[45] and in zebrafish larvae exposed to hexaconazole and tebuconazole, indicating thyroid endocrine disruption[46]. All the results indicated that exposure to BPS may affect the development of thyroid, disrupting thyroid hormones' homeostasis and thereby triggering thyroid endocrine disruption by enhanced level of thyroid hormone[44].

It was observed that T4 level decreased significantly in juvenile fish on exposure to sublethal concentrations of BPS. Decrease in T4 level was observed in *Anguilla anguilla* treated with chromium and copper[47], in

Carassius auratus exposed to extracted microcystins[48], in *Sparus aurata* exposed to diethylstilbestrol, ioxynil and propilthyouracil[49] and in zebrafish larvae treated with pentachlorophenol[50]. A significant decrease in T4 levels also was reported in zebrafish exposed to hexaconazole and tebuconazole (fungicides)[46] and in Atlantic salmon exposed to nonylphenol[51]. Likewise, T4 level was significantly decreased in zebrafish larvae when exposed to BPS[52]. In the present study, decrease in the level of thyroid hormones might be indicative of redirecting metabolic energy away from anabolic process which is essential to carry through life thereby triggering thyroid endocrine disruption[53].

A significant increase in T4 level was found in adult fish on exposure to BPS. Short-term exposures of larval and juvenile turbot (*Scophthalmus maximus*) to water-soluble fractions from oil increased T4 level[54]. Butachlor and triadimefon exposure significantly increased T4 content in zebrafish embryo[45]. Groups of Atlantic salmon smolts, when exposed to low levels of the pesticide atrazine, showed an increase in T4 during stress[55]. Increasing levels of T4 occur due to decrease in the conversion rate of T4 into T3[56].

In the present analysis, E2 level significantly increased in male juvenile fish on exposure to sublethal concentrations of BPS. Estrogens are pervasive steroid compounds that function as hormones mainly in vertebrates. Although E2 is the major estrogen in females, it is also important for normal male reproduction[57]. In fish, detectable levels of E2 have been reported during the reproductive cycle of males of several fish species[57-59]. Estradiol plays crucial roles in the development and functions of male reproductive structures[57]. An elevated E2 level was found in the serum of male *Anabas testudineus* exposed to BPA[60], in male juvenile rainbow troutexposed to 4-nonylphenol[61] and in gold fish (*C. auratus*) exposed to atrazine[62]. Studies exploring contamination with endocrine-disrupting chemicals have demonstrated that a surplus of steroidal estrogens may alter sexual behavior and development and reduce fertility in male fish[63]. The impairment of steroid levels could result either from interactions in the HPG axis or through direct toxic action of BPS on enzymes involved in the steroidogensis processes[64,65] or on the germinal cells capacity of estradiol production[66,67] and these effects were more prominent in males than in females.

The level of 17β-estradiol was found to decrease significantly in female juvenile fish and in male and female adult fish on exposure to BPS. Significant reduction in E2 level was observed in female rainbow trout (*O. mykiss)* on exposure to cyanide[43], *Heteropneustes fossilis* to malathion[68] and male *O. mykiss* on exposure to dimethaote[69]. Decrease of sex steroid hormones may be due to the negative feedback in gonadotrophin secretion that would result in suppression of synthesis of endogenous estrogen by endocrine disrupting chemicals[70]. Decrease in level of estrogen in male and female fish indicates that it may exert reproductive toxicity and estrogenic effects on *O. mossambicus* on exposure to BPS.

Serum testosterone level in male fish of *O. mossambicus* was found to be depleted significantly in both juvenile and adult fish on exposure to BPS. In fish, testosterone, 11KT and 11β-hydroxytestosterone are found in serum and/or testes of several species[71]. Leydig cells are the sites of androgen production in the testis, and the most important androgen produced is testosterone[72]. The effect of E2 was evaluated in the hermaphrodite fish *Kryptolebias marmoratus,* and it was found that plasma testosterone levels in exposed group were significantly lower than in the control groups[73]. Exposure to 4-nonylphenol decreased testosterone level in juvenile turbot (*Psetta maxima)* and *C. gariepinus*[74,75]. Bisphenol S exposure to adult male zebra fish showed significant decrease in testosterone concentration[15]. It may be due to the inhibitory effects of BPS on the Leydig cell function and it might cause feminization of exposed male individuals[70].

In addition to the individual sex steroid hormones, E2 and testosterone, their ratio (E2/T) was also used as an indicator of possible endocrine disruption because a defined estrogen to androgen ratio is necessary for sexual differentiation in developing animals, and alterations of the ratio can result in incomplete or improper gonadal development[70]. The balance between E2 and testosterone hormones determines a fish's phenotype, which includes sex characteristics, differentiation of the brain and behavior, and development of other reproductive organs[76]. There may be an acceptable range of proportions of female to male sex steroid hormones at various stages in a fish life cycle, and the range may be most critical in immature and developing fish.

The values of E2/T ratio were mostly below 1.0 for males, and above 1.0 for females which is in accordance with the study of small mouth bass *Micropterus dolomieu* where, 1.4 was the conservative upper threshold for normal males and 0.8 was the lower threshold for normal

females[77]. In this study, values of the E2/T ratio significantly increased and were found up to 1.5 after exposure to BPS in both juvenile and adult male fish. Folmar *et al.*[70] concluded that the ratio of E2/T appears to be a sensitive marker of abnormal sex steroid concentrations in carp, but has little functional significance because normal ranges have not been established. An increased E2/T ratio was observed in male carps from the more polluted areas and an increase in the estrogen/androgen ratio in male and female Walleye (*Stizostedion vitreum*) collected near a sewage treatment plants-impacted site[78]. Studies in *Anabas testudineus* reported that the value of E2/T ratio increased significantly on exposure to BPA[60]. In short, increased E2 level in male juvenile fish, decreased E2 level in female juvenile fish, male and female adult fish and altered E2/T ratio in juvenile and adult male fish indicated estrogenic potential of BPS.

In the present study, when % fragmented DNA was quantified by DPA method, significant increase in % fragmented DNA was observed in the liver of *O. mossambicus* when exposed to BPS. But on agarose gel electropherogram, no significant change was detected at any concentration, in which all the bands appeared as single high molecular weight intact DNA. Any change to DNA may have long lasting effect but the self-repairing capability of DNA may affect the precise interpretation of relevant bioassays[79]. It has been demonstrated that pollutant exposure does lead to corresponding increase in DNA damage[80]. Exposure to BPA in *A. testudineus* showed a significant increase in percent fragmented DNA[60].

According to Black *et al.*[81], the presence of high molecular weight DNA may not necessarily be a result of the exposure to low impact areas but rather due to the induction of DNA repair mechanisms after the exposure to a highly impacted area. It is assumed that at lower concentration of BPS, the induction of DNA repair mechanisms might have resulted in high molecular weight intact DNA, hence no change was observed on the agarose gel.

Thus, it is evident from the present research that BPS exposure impacts endocrine disruption, altered E2/T ratio and induced genotoxicity in *O. mossambicus*. Serum hormone levels (sex steroid hormones and thyroid hormones) are considered as biomarkers for endocrine disrupting effect of EDCs on fish. Bisphenol analogues may have additional disruptive effects compared to BPA. Therefore, further research efforts should focus on designing chemical substitutes that do not have biological or hormonal activity similar to BPA.

5. Conclusion

This study demonstrates that BPS exposure can induce endocrine disruption in *fish*. The endocrine effects of BPS may be due to its ability to disrupt the synthesis and metabolism of endogenous hormones by altering the hormonal and homeostatic system. Aquatic body contains diverse type of estrogenic compounds which interfere in the steriodogenic pathway and being able to promote severe reproductive impairment in freshwater fish populations. These compounds can act along the HPG axis that govern reproductive processes in fish, and also would potentially impact the DNA.

6. Acknowledgments

We acknowledge the laboratory facilities of University College, Thiruvananthapuram. Financial support in the form of research fellowship from University of Kerala is gratefully acknowledged.

7. References

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