Pakistan J. Zool., vol. 46(4), pp. 1153-1159, 2014.

Histological Changes in Zebrafish (*Danio rerio*) Ovaries Following Administration of Bisphenol A

Nazan Deniz Yön* and Cansu Akbulut
Department of Biology, Science and Letters Faculty, Sakarya University, Sakarya, Turkey

Abstract.- Bisphenol A (BPA) is one of the most potent endocrine disrupting chemicals and therefore is classified as environmental estrogen. BPA is found in polycarbonate plastics (baby feeding bottles, carboys, etc.) and inside of epoxy coated cans and it is widely used in industry for making plastics harder. The aim of this study is to investigate histopathological changes after exposure of different doses of bisphenol A in zebrafish (*Danio rerio*) ovaries. The zebrafish are divided into three groups (n=30) according to their different BPA (group I: 3 mg/L BPA; group II: 5 mg/L BPA; group III: control group) concentrations. Histological studies showed severe deterioration of ovarian tissue. The number of the atretic oocytes increased BPA. Structurally distorted and less developed oocytes were also observed. To conclude, the acute doses of BPA slowed down the process of oogenesis in zebrafish.

Key Words: Bisphenol A, zebrafish, oocyte, histology

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are compounds in the external environment that modulate the physiology of the endocrine system and often cause health disorders in a healthy organism or in its next generation (Crews and Mclachlan, 2006; Goldman *et al*., 2010). Effects of endocrine disrupting chemicals are similar to hormones and they can interact with endocrine systems in vertebrates. The presence of endocrine disruptors were detected in industrial wastes and waste waters.

Environmental pollutants, especially endocrine disruptors have side effects on reproductive system. Organic chemicals such as cleaning materials, fungicides, pesticides, herbicide, paints, plastics and solvents have potential to be endocrine disruptors. Endocrine disrupting chemicals can cause lethal effects on human and animals reproductive systems (Fox, 2004; Zhaobin and Jianying, 2008; Cek and Sarıhan, 2010). Duration of exposure time and the dose of endocrine disruptors has direct relationship with the adverse effects. In some vertebrates, differentiation of sex is under the influence of genetic and environmental factors. In fishes sex differentiation is not only due to sex hormones but also depends upon environmental conditions (Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Altunok *et al*., 2008).

BPA (2 bis(4-hydroxiphenyl) propane, BPA) was first synthesized by a Russian chemist Alexander P. Dianin (Dianin, 1891). BPA is one of the most important endocrine disruptors and it is widely used at industry for making plastics harder and it mimics estrogen *in vivo* and *in vitro* (Kim *et al*., 2011). Therefore, it is classified as xenoestrogen. Chemicals, that mimic or antagonize the effects of endogenous hormones could potentially have serious affect not only on the development but also reproductive ability of the animal (NOAA, 2002). BPA is found in polycarbonate plastics (baby feeding bottles, carboys) (Krishnan *et al*., 1993; Cao and Corriveau, 2008) and inside of epoxy coated cans (Kang *et al*., 2006). BPA is one of the highly produced chemicals in the world and is the source of increasing contamination of aquatic systems. BPA levels were reported between 0.02–21 µg/L in different river waters in Netherlands (Kang *et al*., 2007).

Disruption of the physiological functions in fish, either specifically of the hormonal system or of a more generalized toxic nature, will have an effect on the structural organization of more than a single primary target organs. The histological manifestation of this effect is less widely

* Correspondence author: ndy31@hotmail.com
0030-9923/2014/0004-1153 $ 8.00/0

appreciated because such studies usually limit themselves to morphological changes in gonads.

In this study, we have investigated the histopathological effects of different sublethal doses of BPA on ovaries of zebrafish.

MATERIALS AND METHODS

Model organism

The zebrafish *(Danio rerio)*, which lives in India and Pakistan naturally, is a member of the Cyprinidae family. It is widely used as laboratory model, especially in developmental biology. Zebrafish can be stimulated in the laboratory to breed throughout the year and the development from the fertilised egg to the reproducing stage takes only about 3-4 months. Their short generation makes them an ideal candidate for genetic studies and their susceptibility to mutagens, carcinogens, teratogens and toxins makes them ideal as environmental models.

BPA and other chemicals

Bisphenol A (98%) were purchased from Sigma Chemical Company (St. Louis, USA). All additional chemicals used during the experiments were of analytical grade. BPA was dissolved in 1% dimethylsulfoxide (DMSO).

BPA treatment

Zebrafish were fed daily with *Artemia* sp. and TetraMin© Hauptfutter (Tetra Werke, Germany) under standardized conditions (20-L glass aquaria, 28±1°C, light/dark cycle = 14 h/10 h). The fish were acclimatized for 48 h before starting the BPA treatment. The zebrafish were divided into 3 groups: a control group and two experimental groups, which received 1% DMSO and treated with 3 mg/L and 5 mg/L BPA. In each group there were 30 adult individuals which were 1 year old and 4-5 cm length.

Histological process

Fishes were taken out after 24, 48, 72, 96, 120, 144 and 168 h after exposure, anaesthetized in iced water and the ovary was fixed in Bouin's fixative. Fixed tissues were dehydrated and embedded in paraffin wax, sectioned transversely at 5-6 µm thickness, and stained with hematoxylin-eosin and toluidine blue. Presence of stage II oocytes, cortical alveolus stage and yolk vesicles were taken as criteria from mature ovaries (Selman *et al*., 1993).

RESULTS

BPA causes slowing down of oogenesis, increase in the number of atretic follicles and connective tissue in the ovaries, besides discontinuation in growth of developing follicles and start of follicle breakdown.

Control group

Primary oocytes in the control group were oval-shaped cells with oval nucleus and large nucleolus. In the first growth phase, follicle layers were not entirely developed but they were visible, and the diameter of these follicles was small. The nuclei of oocytes in the pre-vitellogenic phase were central. Under light microscope, nucleoli were close to the nuclear membrane. Oocytes in the vitellogenic phase were larger in size according to their baseline sizes. The increase in size was attributed to nutrient accumulation in cytoplasm. (Fig. 1).

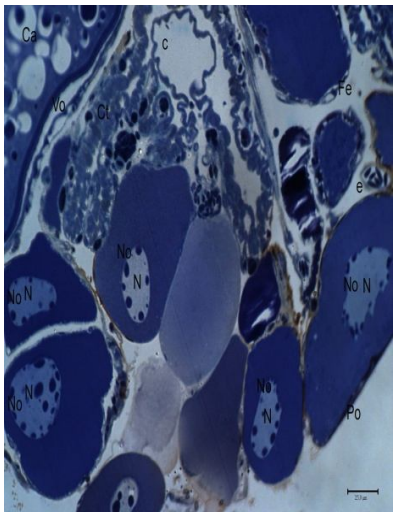

Fig. 1. Control group. General view of development stages. X10 Toluidine blue staining. Po, primary oocyte; CoC, cortical alveolar oocyte; Vo, vitellogenic oocyte; Ca, cortical alveoli; N, nucleus; O, ooplasm; Om, mature oocyte.

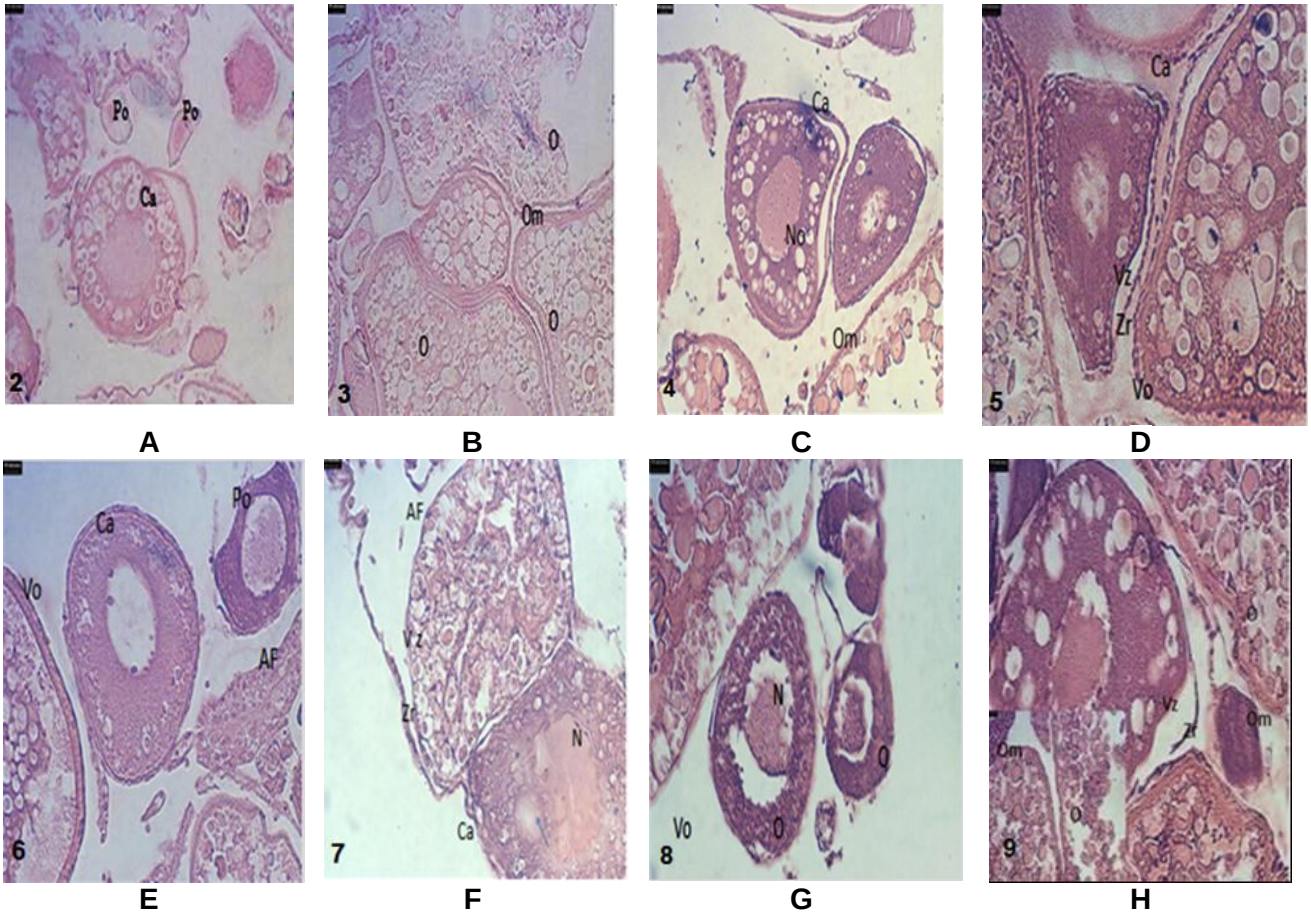

Fig. 2. Histological structure of ovary of zebrafish exposed to BPA 3 mg/L for 7 days. A, B 1st day. number of primary oocytes (Po) is reduced, structural deformities were observed. Cortival alveolar oocytes (Ca) had normal morphology. Reduction at the number of developing oocytes, deformation at the ooplasm (O) and structure of the mature oocytes (Om) were observed. C, 2nd day. Reduction at the number of cortical alveoli (Ca), structutal deformities, irregularities at nucleolus (No), deformation at mature oocyte (Om) cytoplasm was detected. D, 3rd day. Expansion between vitelline membrane (Vz) of cortical alveoli (Ca) and zona radiata (Zr), degeneration at cortical alveoli structure and expansion at alveolar structure of vitellogenic oocyte were detected. E, 4th day. Deformation at the structure of ooplasm of the vitellogenic oocytes (Vo), cortical alveoli (Ca) and primary oocyte (Po), structural deterioration at intercellular space and atretic follicule (AF) were monitored. H, 5th day. Deformation nucleus (N) structure of cortical alveoli (Ca), vacuolisation inner part of oocyte (→), expansion between vitelline membrane (Vz) and zona radiata (Zr) and atretic follicule (AF) were seen. G, 6th day. Severe deformation at oocytes. Expansion at ooplasm (O) and nucleus (N) of developing oocytes; degeneration at vitellogenic oocyte (Vo) ooplasm area were observed; H, 7th day. Vacuolisation at alveolar structure of cortical alveolar oocytes, deformation at mature oocyte (Om) ooplasm and significiantly seperation between zona radiata (ZR) and vitelline membrane (Vz) were detected.
Magnification: A, B, C = 20X; D, E, F, G, H = 40X.
Stain: H & E.

BPA treated group

In 3mg/L BPA groups, disintegration of vesicular structures of mature oocytes and irregularities at cytoplasm were detected (Fig. 2). In addition, in experiment groups, the reduction in the number of primary oocytes has been noticed and it was observed that the development of oocytes were stopped.

Minor changes at the structure of cortical granules were detected (Figs. 2A,C). Compared with the control group, reduction at the number of primary oocytes and structural deformities were

observed (Fig. 2A). Reduction at the number of developing oocytes, deformation at the ooplasm and structure of the mature oocytes and irregularities at nucleolus were monitored (Fig. 2B,C).

In addition, in this group, degeneration of chromatin material, expanse between ooplasm and vitelline membrane and granular apperance of ooplasm were detected (Fig. 2B,D). Deformation at the structure of ooplasm of the vitellogenic oocytes, cortical alveoli and primary oocyte, structural deterioration at intercellular space and atretic follicule were monitored on the 4th day of exposure (Fig. 2E).

In the 5th day of exposure degeneration, fragmentation and autolysis of cortical granules at cortical alveolar oocytes were observed (Fig. 2F-H). Deformation at nucleus structure of cortical alveoli, vacuolisation inner part of oocyte, expansion between vitelline membrane and zona radiata and atretic follicule were seen (Fig. 2F). Expansion at ooplasm and nucleus of developing oocytes; degeneration at vitellogenic oocyte ooplasm area were observed (Fig. 2G). Vacuolisation at alveolar structure of cortical alveolar oocytes, deformation at mature oocyte ooplasm and significiantly seperation of zona radiata from cell surface were monitored (Fig. 2H)

The results of histopathological changes at ovaries of 3mg/L BPA treatment group proved that this chemical handicaps gametogenesis. Because at this group there was an increment at the number of arteric follicule and ovary connective tissue. In addition, degeneration was observed at ooocyte morphology.

In 5mg/L BPA treatment group, degeneration at oocytes of zebrafish ovary was detected. On the first day we observed degeneration at oocyte morphology and increment at the number of atretic follicules (Fig. 3A). On the second day, expanse in oocyte cytoplasm, reduction at the number of primer oocytes and reduction at the number of nutrition granules in cortical alveolar oocyte cytoplasm was observed (Fig. 3B). On the 4th day there were severe degeneration at primary oocytes. In addition, decomposition at nucleoplasm was also detected (Fig. 3C). On the 5th day, expanse at cortical alveolar oocyte cytoplasm occured and there was vacuolisation at cortical alveoli. Also, expanse between zona radiata and follicule epithelium was observed (Fig. 3D). On 6th day, increment at the number of atretic follicules depending on amount and increase of the dose. Distinctly decomposition of vitellogenic structure, structural deformation and reduction at the number of primary oocytes was observed (Fig. 3E). On the 7th day, reduction at the number and deformation at developing oocytes were discerned. Unlike developing follicules we observed increment at the number of atreric follicules (Fig.3F).

DISCUSSION

Environmetal estrogens are the additives that are found in detergants, paints, pesticides and plastics. More than 60% of the total world production of these compounds are collected in sewage and industrial waste waters. These substances have estrogenic effects on fish therefore they cause serious abnormalities in their reproductive and endocrine systems. In our study, as a result of BPA treatment, we observed histological changes at zebrafish ovaries dose depended. Environmental estrogenic this material caused slowing down of oogenesis. In Sumpter's study (1995), reduction at testicular growth rate of trout which was exposed to estrogenic alkaline phenol was observed. In study on BPA effects on *Salmo trutta*, male germ cells were detected between oocytes (Bjerregaard *et al*., 2008). However, such an outcome was recorded in our study. Weber *et al*. (2003) examined gametogenesis and gonad toxicity at zebrafish. In their study, 17α-ethinylestradiol (EEP) ve 4-nonylphenol (NP) carried out to fish and as a result they detected increase at the number of atretic follicule but they observed normal histology at testes, kidney and liver tissues. These findings are compatible with our study. NP and other alkaline phenols cause testicular fibrosis, reduction at the number of primordial germ cells and reduction fixative alkaline phenols cause ectopic oocyte development, increase atretic follicules at ovaries and increase proliferation of interstitial stroma of ovary in females (Tanaka and Grizzle, 2002).In an another study which reveals histological effects of 2,4 D on ovary of zebrafish, atresia and extreme cellular necrosis were visible and increased in the

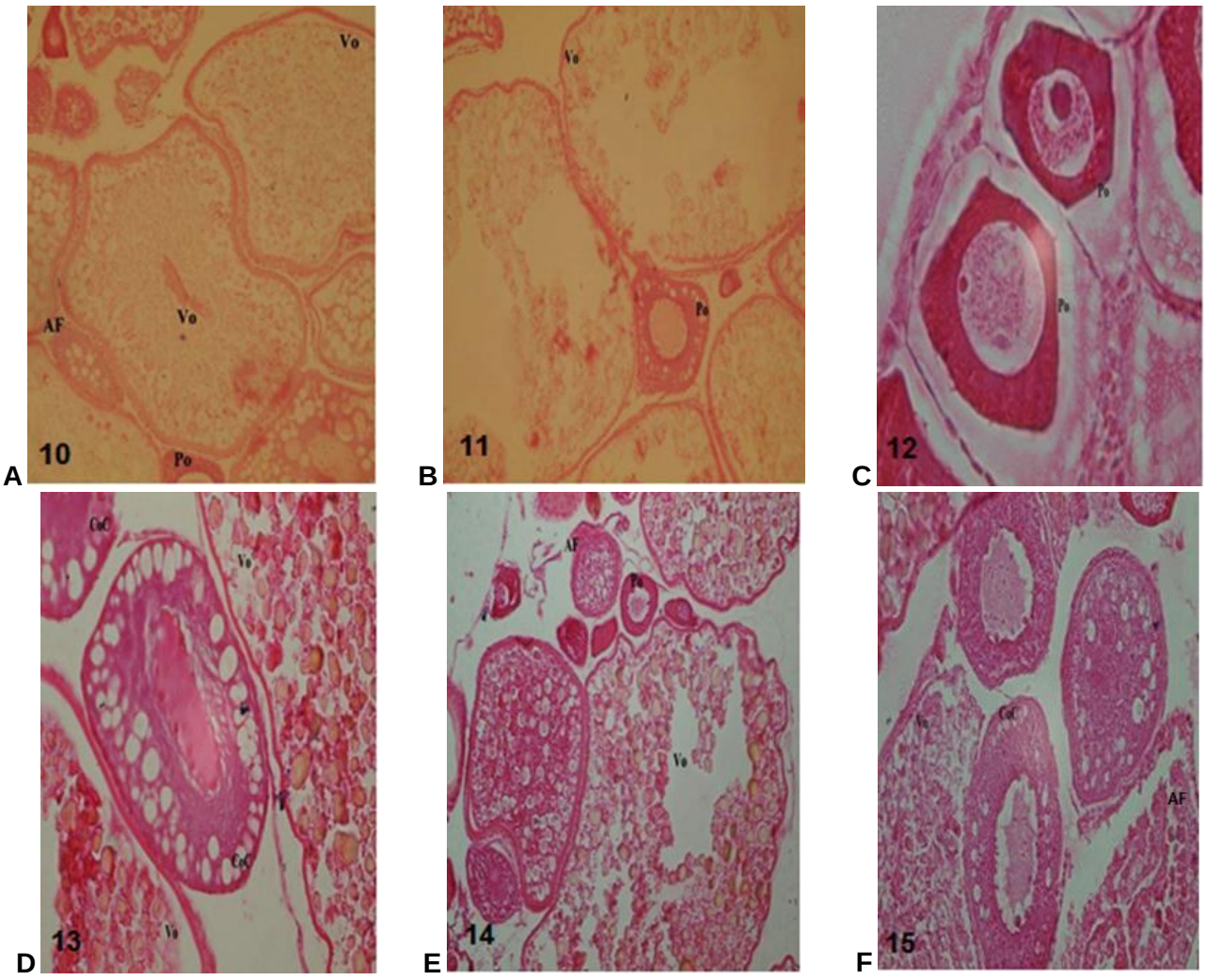

Fig. 3. Histological structure of ovary of zebrafish exposed to BPA 5 mg/L for 7 days; A, 1st day. Degeneration at vitelline oocyte (Vo) morphology and increment at the number of atretic follicules (AF) was observed; B, 2nd day. Expansion in vitellogenic oocyte (Vo) ooplasm, reduction at the number of primary oocyte (Po) and reduction at the number of nutrient granules in cortical alveolar oocyte cytoplasm was detected; C, 4th day. Severe degeneration at primary oocytes (Po) and deterioration at nucleoplasm was seen; D, 5th day. Expansion at cortical alveolar oocyte (CoC), vacuolisation at cortical alveoli and expansion between zona radiata and follicular epithelium at vitellogenic oocyte (Vo) were monitored; E, 6th day. Depending on dose atretic follicule (AF) number was increased. Reduction at the number of primary oocyte (Po) and degeneration at vitellogenic oocyte (Vo) and primary oocyte (Po) structure; F, 7th day. Deterioration and reduction of developing oocytes was observed. On contrary, we detected increment at the number of atretic follicule (AF).
Magnification: All 40x; Stain; H & E.

number of degenerating oocytes. It was deduced that acute doses of 2,4 diclorophenoxyacetic acid decelerates oogenesis in fishes (Koç and Akbulut, 2012). Deltamethrin cause increase in the number of atretic oocytes and it has significant negative effects on oogenesis in zebrafish (Koç *et al.*, 2009). These findings are similar with the result of this study. In Han *et al.* (2011)'s study, effects of β-endosulfan on the growth and reproduction of zebrafish were investigated and the histology of ovaries was normal in fish treated with 10 or 50 ng/L b-endosulfan, as compared with the control or solvent control groups.

Atretic follicles were detected in the 200 ng/L b-endosulfan–treated group. These atretic follicles exhibited characteristic degenerative changes, including decreased vitellogenesis, oocyte membrane folding, and an increase in the number and size of follicular cells. Similarly in our study, decrease in vitellogenesis and increase in atretic follicles were monitored.

Uchida *et al*. (2002) suggested the mechanism of testicular and ovarian differentiation in zebrafish to be induced by oocyte apoptosis. Ovarian follicle apoptosis has been reported in other species (Drummond *et al*., 2000). In the present study, no inflammatory cells were observed in the ovary. Furthermore, surrounding follicular epithelial cells were involved in the phagocytosis of the degenerating oocytes.

BPA is classified as slightly to moderately toxic to fish (Staples *et al*., 1998). Lee *et al*. (2003) investigated the estrogenic effect of bisfenol A on the sex differentiation of Korean rockfish (*Sebastes schlegeli*). They fed the 51-day-old fry with food containing bisphenol A at the dose of 0.05, 0.5, 5, 50 and 100 mg/kg-1 for 29 days. Their results are in accordance with our examination of zebrafish, because we did not observe estrogenic effect of bisphenol A at a concentration of 500 mg/kg-1 on the sex differentiation compared with the control group. When exposed to 59 µg/L BPA for 3 weeks, male Atlantic cod (*Gadus morhua*) had significantly elevated serum vitellogenin concentrations, whereas male turbot (*Scophthalmus maximus*) did not (Larsen *et al*., 2006).

Further examination of the relationships among altered structural integrity, glucose and lipid metabolism, immune response, and regulation of transcription in response to ovarian toxicity will better clarify the mechanisms by which bisphenol A exerts its reproductive toxicity, particularly its low-dose, long-term effects.

As a result of our study, structural deformities at follicles increase were found with dose depended. Bisphenol A cause serious disorders at follicular morphology were detected. In addition, increase in BPA exposure cause decrease in the number of primary oocytes and increase in the number of atretic follicles were also monitored. In conclusion, we can say that BPA hinders oogenesis.

REFERENCES

ALTUNOK, M., KIZAK, V., ÖZDEN, O. AND TÜREL, M., 2008. The Influence of environmental factors on sex determination in fish. *EU Su Urunleri Dergisi*, **3:** 247-251.

BAROILLER, J.F. AND D'COTTA, H., 2001. Environment and Sex Determination in Farmed Fish. *Comp. Biochem. Physiol. Part C.*, **130:** 399-409.

BJERREGAARD, L.B., LINDHOLST, C., KORSGAARD, B. AND BJERREGAARD, P., 2008. Sex hormone concentrations and gonad histology in brown trout (*Salmo trutta*) exposed to 17beta-estradiol and bisphenol A. *Ecotoxicology*, **17:** 252-263,.

CAO, X.L. AND CORRIVEAU, J., 2008. Migration of bisphenol A from polycarbonate baby and water bottles into water under severe conditions. *J. Agric. Fd. Chem.*, **56:** 6378-6381.

CEK, S. AND SARIHAN, F., 2010. The effects of sex steroids of endokrin disrupting chemicals on fishes. *EU Su Urunleri Dergisi*, **1:** 41-46,.

CREWS, D. AND MCLACHLAN, J.A., 2006. Epigenetics, evolution, endocrine disruption, health, and disease. *Endocrinology*, **147:** 4-10.

DEVLIN, R.H. AND NAGAHAMA, Y., 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental ınfluences. *Aquaculture*, **208:** 191-364.

DIANIN, A.P., 1891. Condensation of ketones with phenols. *Z. Russ. fiziko-khim. Obshchestva*, **23:** 492.

DRUMMOND, C.D., BAZZOLI, N., RIZZO, E. AND SATO, Y., 2000. Post-ovulatory follicle: a model for experimental studies of programmed cell death or apoptosis in teleosts. *J. exp. Zool.*, **287:** 176–182.

FOX, J.E., 2004. Chemical communication threatened by endocrine-disrupting chemicals. *Environ. Hlth. Perspect*,, **112:** 648-654.

GOLDMAN, J.M., LAWS, S.C., BALCHAK, S.K., COOPER, R.L. AND KAVLOCK, R.J., 2010. Endocrine-disrupting chemicals: pre-pubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations. *Crit. Rev. Toxicol.*, **30:** 135-196.

HAN, Z., JIAO, S., KONG, D., SHAN, Z. AND ZHANG, X., 2011. Effects of b-endosulfan on the growth and reproduction of zebrafish (*Danio rerio*). *Environ. Toxicol. Chem*., **30:** 1-7.

KANG, H.J., KONDO, F. AND KATAYAMA, Y., 2006. Human exposure to bisphenol A. *Toxicology*, **226:** 79-89.

KANG, J.H., AASI, D. AND KATAYAMA, Y., 2007. Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. *Crit. Rev. Toxicol.*, **37:** 607–625.

KIM, Y.J., YUN, H.J. AND RYU, J.C., 2011. Expression profiling of estrogen responsive genes on bisphenol A, 4-nonylphenol and 17β-estradiol treatment using in house cDNA microarray. *BioChip. J.*, **5:** 86-94.

KOÇ, N.D., MUŞLU, M.N., KAYHAN, F.E. AND ÇOLAK, S., 2009. Histopathological changes in ovaries of zebrafish (*Danio rerio*) following administration of deltamethrin. *Fresen. environ. Bull.*, **18:** 1872- 1878.

KOÇ, N.D. AND AKBULUT, C., 2012. Histological analysis of acute toxicity of 2,4-diclorophenoxy acetic acid in ovary of zebrafish. *Anim. Cells Syst.*, **16:** 400-407.

KRISHNAN, A.V., STATHIS, P., PERMUTH, S.F., TOKES, L. AND FELDMAN, D., 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology*, **132:** 2279-2286.

LARSEN, B.K., BJORNSTAD, A., SUNDT, R.C., TABAN, I.C., PAMPANIN, D.M. AND ANDERSON, O.K., 2006/ Comparison of protein expression in plasma from nonylphenol and bisphenol A-exposed Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) by use of SELDI-TOF. *Aquat. Toxicol.*, **78S:** 25–33.

LEE, W.K., LEE, K.W., KWAK, E.J., YANG, S.W., YANG, K.S., PARK, J.C., JOO, H.S., LEE, W.J. AND LEE, W.B., 2003. Effects of environmental endocrine disruptors on the sex differentiation in Korean rockfish, *Sebastes schlegeli*. *Wat. Sci. Technol.*, **47:** 65-70.

NOAA National Oceanic and Atmospheric Administration, 2002. Endocrine Disruption in Fish An Assessment of Recent Research and Results, Technical Memorandum NOS NCCOS CCMA 149, 2002. http://aquaticcommons.org/2177/1/NOS_TM149.pdf (cited 2013, Jan. 27)

ORN, S., YAMANI, S. AND NORRGREN, L., 2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17α-ethinylestradiol and 17β-trenbolone. *Arch. environ. Contam. Toxicol.*, **51:** 237–243.

SELMAN, K., WALLACE, R.A., SARKA, A. AND QI, X., 1993. Stages of oocyte development in zebrafish, *Brachydanio rerio*. *J. Morphol.*, **218:** 203–224.

STAPLES, C.A., DORN, P.B., KLECKA, G.M. AND O'BLOCK, S.T., 1998. A review of the environmental fate, effects, and exposure of bisphenol A. *Chemosphere*, **36:** 2149-2173.

SUMPTER, J.P., 1995. Feminized responses in fish to environmental estrogens. *Toxicol. Lett.*, **82-83:** 737-742.

TANAKA, J. AND GRIZZLE, J.M., 2002. Effects of nonylphenol on the gonadal differentiation of the hermaphroditic fish, *Rivulus marmoratus*. *Aquat. Toxicol*,, **57:** 117-125.

UCHIDA, D., YAMASHITA, M., KITANO, T. AND IGUCHI, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *J. exp. Biol.*, **205:** 711–718.

WEBER, L.P., HILL, R.L. AND JANZ, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*): II. Histological evaluation of gametogenesis and organ toxicity. *Aquat. Toxicol.*, **63:** 431-446.

ZHAOBIN, Z. AND JIANYING, H.U., 2008. Effects of p,p′ - DDE exposure on gonadal development and gene expression in Japanese medaka (*Oryzias latipes*). *J. environ. Sci.*, **20:** 347-352.

(Received 8 November 2013, revised 14 June 2014)