

**SEX DIFFERENTIATION IN SONGSARI,  
ORYZIAS LATIPES EXPOSED TO BISPHENOL A**

Young-Don Lee  
Marine Research Institute, Cheju National University  
3288 Hamduk-ri Chochon-eup, Buk-gun Cheju-do 695-810, Korea  
Tel. +82 064-782-8922/ Fax. +82 064-783-6066  
[leemri@cheju.cheju.ac.kr](mailto:leemri@cheju.cheju.ac.kr)

Oh-Soo Na  
Marine Research Institute, Cheju National University  
3288 Hamduk-ri Chochon-eup, Buk-gun Cheju-do 695-810, Korea  
[naohaqua@hotmail.com](mailto:naohaqua@hotmail.com)

Hyung-Bae Kim  
Department of Fisheries Development, Kangwon Province University  
8-2 Kyohang-ri, Jumunjin-eup, Kangnung-shi, Kangwon-do, 210-800, Korea  
[aldo98@hanmail.net](mailto:aldo98@hanmail.net)

**Abstract**

The effects of bisphenol A(BPA) on gonadal sex differentiation and maturation in *Oryzias latipes* were investigated. Fish were exposed to aqueous solutions of BPA at nominal concentrations of 50, 100 and 200 µg/l from 2 days to 70 days of age. In process of sex differentiation, advanced oocyte development was observed when compared to the testicular growth. Ovaries were composed of the oocyte of the chromatin-nucleolus stage and peri-nucleolus stage in 20 days after hatching. Otherwise testes contained a number of the spermatogonia and spermatocytes in 30 days after hatching. In the process of sex differentiation, gonadal development was not different in the controls and BPA treatment groups until 30 days after hatching. In contrast, 70 days after hatching advanced development of oocytes in the ovary was observed from BPA treatment groups when compared to the controls, and inhibition of development of spermatogenesis in the testis was observed from BPA treatment groups when compared to the controls. In sex ratio of songsari, more females than males were identified in the BPA 50 and 100 µg/l treatments in comparison to the controls and BPA 200 µg/l treatment. The range of BPA effects was dependent on the sex of the songsari, and the concentration of the BPA in the water.

## **Introduction**

An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function (European Commission, 1996). And during the past several years, the environmental endocrine disruptors have been deal with the sensitive concern in scientific, popular and political field. Bisphenol A (BPA) is widely used as the primary product of poly-carbonated plastic and epoxy-wax. Poly-carbonate is at liberty to use as the drink pac and earthen vessel and epoxy-wax apply a primary product to the coating of can, bottle and water pipe (Brotons et al., 1995). Evaluation of reproductive organ development in the male offspring of female wistar rats exposed to BPA in the drinking water has been reported (Diomond et al., 1998). Occurrence of ovo-testis (hermaphroditism) was observed in medaka, *O. latipes* exposed to  $\beta$ -hexachlorocyclohexane and p-nonyphenol (Wester and Canton, 1986; Gray and Metcalfe, 1997). In the present study we histologically investigated on the sex differentiation in songsari exposed from hatched larvae to up to 70 days post-hatch to BPA.

## **Methods and Materials**

### *Chemicals*

The Bisphenol A ethoxylaten (BPA) used in all experiments was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, USA. A stock solution of 100 mg/ml was prepared by dissolving BPA in acetone.

### *Fish*

Songsari used in this study were from a breeding stock that has been maintained at marine research institute of Cheju national university for over 3 months. Fish were maintained in recirculating Cheju Island under-ground water. During breeding, songsari in the culture tank were controlled to a light : dark photoperiod of 16 : 8 h and fed a mixed diet (Ewha oil & fat industry Co. Ltd., Korea) Water temperature of breeding tank ranged from 22 to 24°C. Eggs were collected from females, incubated in 10-L glass tank and checked for hatching.

### *Assay condition*

In chronic exposure experiment, songsari were exposed to 50, 100 and 200 µg/l concentrations of BPA in a static-renewal system. Exposures took place in 1-L glass beaker filled with 900 ml of filtered Cheju Island under-ground water. The water quality parameter for the under-ground water were pH 8.1 and COD 0.8mg/l. The aqueous solutions of BPA were renewed every 72 h for the first month, and every 48 h thereafter, according to the methods described by Gray and Metcalfe (1997).

There were five exposure groups by two times of 40 fry each (total  $n = 400$ ) at the start of the experiment. The five exposure groups were pure spring water and acetone / spring water in the control groups, and 50, 100, and 200 µg/l BPA in the treatment groups. Chronic exposure of songsari to BPA was initiated at 1 or 2 days after-hatching. Nominal BPA concentrations of 50, 100, and 200 µg/l were maintained by adding appropriate volumes (4.5, 9.0 and 18.0 µl) of BPA stock solution to the water in the aquaria. In the acetone (carrier) control, acetone alone (18.0µl) was added. The fish were maintained in a light : dark cycle of 16 : 8 h and were fed a mixed diet of 3-4 times daily for the duration of the experiment.

On the each 10, 20, 30, and 70 days after exposure, 8-30 individuals each were collected and the body weight and body length recorded. The examined total fish was 303 individuals. The length and weight of the fish were measured in 0.1 mm with the dissecting microscope, in 0.01g using an electron balance. The fish were then placed in tissue bottles and fixed in Bouin's fixative.

The fixed songsari were prepared for histological examination using histological procedures (dehydrated in ethyl-alcohol and embedded in paraffin). Fish were embedded whole in paraffin wax and sectioned (5 µm) with a microtome. The sections were stained using Hansen's haematoxylin and 0.5% eosin and examined under a light microscope. Sagittal-sections of the gonads were viewed with a microscope-monitor system. We calculated the proportion of sagittal sectional area of the gonad occupied by each germ cell type. The classification of the stages of oogenesis followed Yamamoto and Yoshioka (1964) and spermatogenesis followed Grier (1976).

#### *Statistics*

The analytical results were calculated by the method of  $\chi^2$ -test and two-way ANOVA.

## Results and discussion

### *Sex differentiation and gonadal development*

*Just after hatching:* 10 individuals were examined. Average body length of these individuals was 4.4 mm. The larvae still possessed the yolk. In sagittal-section, myotome, notochord and gut could be recognized. Between the myotome and the gut, germinal strand was located. The germ cells were visible in the anterior region of the primitive gonad. The germ cells were nearly ovoid shape and average diameter of these cells was 8  $\mu\text{m}$ .

20 days after hatching: In female, the ovaries were composed of the oocytes of the chromatin-nucleolus stage and peri-nucleolus stage. Average diameter of the oocytes of the chromatin-nucleolus stage was 19  $\mu\text{m}$ , and the oocytes of the peri-nucleolus was 25  $\mu\text{m}$ . In male, the testes contained gonial cells alone. The gonial cells were nearly round or ovoid and average diameter of these cells was 7-8  $\mu\text{m}$ .

*30 days after hatching:* The ovaries were composed of the oocytes of the chromatin stage and peri-nucleolus stage increased in numbers. In testis, a number of the spermatogonia and the spermatocytes were observed in the testicular lobule. In the process of the sex differentiation gonadal development was not different in the controls and BPA treatment groups until 30 days after hatching.

*70 days after hatching:* In the controls, the ovaries were composed of the oocytes of the chromatin-nucleolus, peri-nucleolus and yolk vesicle stage. The distribution(%) of its development stage were 13.1 $\pm$ 2.1% and 86.9 $\pm$ 2.1% in the control and 9.5 $\pm$ 2.7%, 86.7 $\pm$ 2.3% and 3.8 $\pm$ 2.7% in the acetone (carrier) control respectively. Otherwise in the BPA treatment groups, the ovaries were composed of the oocytes of the chromatin-nucleolus, peri-nucleolus, yolk vesicle, yolk globule and mature stage. The frequency of oocyte development stage were 8.3 $\pm$ 0.3%, 88.4 $\pm$ 3.0% and 3.2 $\pm$ 2.6% in BPA 50 $\mu\text{g/l}$ , 4.3 $\pm$ 0.1%, 89.6 $\pm$ 2.8%, 3.9 $\pm$ 1.2% and 2.2 $\pm$ 1.6% in BPA 100 $\mu\text{g/l}$  and 3.9 $\pm$ 0.2%, 80.1 $\pm$ 1.5%, 8.4 $\pm$ 3.1%, 5.5 $\pm$ 2.5% and 2.1 $\pm$ 1.9% in BPA 200 $\mu\text{g/l}$  respectively (Fig. 1).

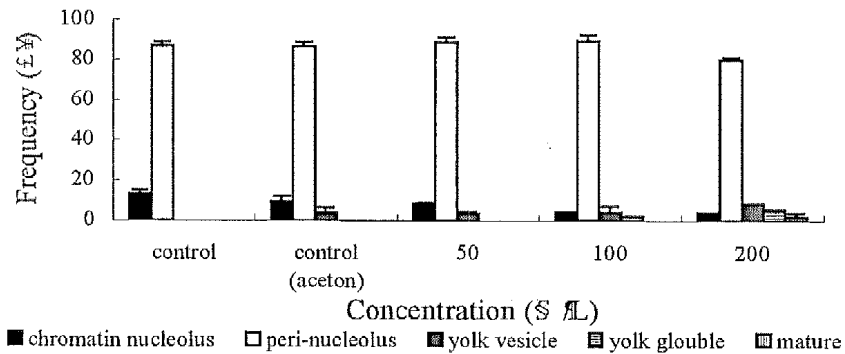


Fig. 1. Frequency of developmental stage of ovary in adult songsari, *Oryzias latipes* exposed to bisphenol A.

The developmental stage of oocyte came to be a yolk vesicle stage in the controls and came to grow yolks stage in the BPA treatment groups. Thus advanced development of oocytes in the ovary was observed from BPA treatment groups when compared to the controls. In sex differentiation of fishes,  $17\beta$ -estradiol also advanced ovarian development in comparison with untreated females as observed in coho salmo (Foyle, 1993) and in pejerrey (Strüssmann et al., 1996).

In testis frequency of spermatogenesis stage is shown in Fig. 2. Frequency of spermatogonium, spermatocyte and spermatid in testis were  $67.9\pm 8.1\%$ ,  $4.0\pm 0.4\%$  and  $22.7\pm 2.4\%$  in the control and  $73.5\pm 3.7\%$ ,  $9.1\pm 5.2\%$  and  $17.4\pm 1.5\%$  in the acetone (carrier) control,  $77.8\pm 10.1\%$ ,  $1.9\pm 0.6\%$  and  $16.7\pm 8.9\%$  in BPA  $50\mu\text{g/l}$  treatment group,  $86.3\pm 10.7\%$ ,  $3.7\pm 0.5\%$  and  $6.0\pm 1.2\%$  in BPA  $100\mu\text{g/l}$  treatment group,  $99.3\pm 0.9\%$ ,  $0.1\pm 0.1\%$  and  $0.5\pm 0.1\%$  in BPA  $200\mu\text{g/l}$  treatment group.

In these results, inhibition of development of spermatogenesis in the testis was observed from BPA treatment groups when compared to the controls. Nonylphenol and  $17\beta$ -estradiol have severe effects on the testis and that the Sertoli cells might be affected in the eelpout (Christiansen et al., 1998). Oestrogen inhibits the differentiation of Leydig cells, Sertoli cells and early formation of the spermatid duct in the European eel (Colombo and Grandi, 1995). In sexually developing fish, the pronounced effects on vitellogenin synthesis caused by exposure to the various estrogenic chemicals were accompanied by concomitant significant decreases in the testicular growth

(Jobling et al., 1996)

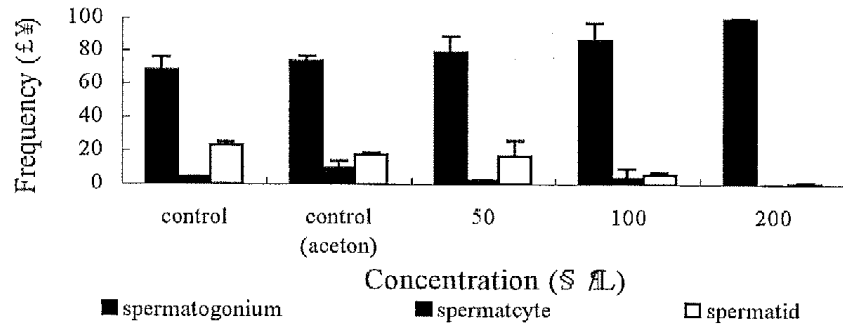


Fig. 2. Frequency of developmental stage of testis in adult songsari, *Oryzias latipes* exposed to bisphenol A

#### Sex ratio and growth

More females than males were identified in the BPA 50µg/l and 100µg/l treatments in comparison to the controls and BPA 200µg/l treatment (Table 1).

Table 1. Numbers of female and male songsari, *O. latipes* in the different groups from 10 to 70 days after hatching

Experimental group	Days after hatching				Total	Sex ratio ( : )
	10 ( : )	20 ( : )	30 ( : )	70 ( : )		
Control	2:2	2:0	8:8	4:2	16:12	1:1
Control (acetone)	1:4	3:1	14:16	6:5	24:26	1:1
BPA 50 ppb	2:3	3:2	19:9	6:3	30:17	2:1
BPA 100 ppb	1:1	3:1	18:9	2:4	24:15	2:1
BPA 200 ppb	2:2	3:0	12:16	4:2	21:20	1:1

$\chi^2$  test analysis indicated that sex ratios of female to male were 2 : 1 in the BPA 50 µg/l and 100 µg/l treatments ( $P > 0.05$ ). Fry of *O. latipes* exposed to 4.0 and 29.4 µg/l 17β-estradiol (Hartley et al., 1998) both exhibited 53% testis-ova or presumptive hermaphroditism, approximately 40% female and 5% male in each dose group. Gray and Metcalfe (1997) reported that fry of *O. latipes*

exposed to 100µg/l of *p*-nonylphenol (NP) induced both the intersex state (i.e., testis-ova) in males as well as sex reversal (i.e., male to female), while exposure to a lower concentration of NP (50µg/l) induced only testis-ova. But present histological analysis of 205 fishes uncovered an intersex individual.

The mean total lengths of songsari at sacrifice are presented in Table 2. Duncan's multiple test indicated that mean total lengths greater for fish in BPA 200 µg/l treatment group in comparison another treatment groups. And BPA treated fish were slightly larger than untreated fish. Fish exposed to estrogenic compounds were larger than the control fish (Gray and Metcalfe, 1997; Moon, 1999).

Table 2. Changes of total length in the different groups at 10, 20, 30 and 70 days after hatching

Exp. Group	Days after hatching								
	10		20		30		70		
	Length (cm)	n	Length (cm)	n	Length (cm)	n	Length (cm)	Weight (g)	n
Control	0.65±0.04 <sup>a</sup>	10	0.83±0.11 <sup>a</sup>	10	1.13±0.14 <sup>b</sup>	30	1.69±0.20 <sup>b</sup>	0.04±0.02 <sup>b</sup>	8
Control (acetone)	0.66±0.04 <sup>a</sup>	10	0.85±0.04 <sup>a</sup>	10	1.15±0.14 <sup>b</sup>	30	1.74±0.22 <sup>b</sup>	0.06±0.03 <sup>ab</sup>	16
BPA 50 ppb	0.65±0.04 <sup>a</sup>	10	0.85±0.14 <sup>a</sup>	10	1.18±0.17 <sup>b</sup>	30	1.76±0.34 <sup>b</sup>	0.06±0.02 <sup>ab</sup>	16
BPA 100 ppb	0.65±0.05 <sup>a</sup>	10	0.88±0.12 <sup>a</sup>	10	1.19±0.12 <sup>b</sup>	30	1.87±0.29 <sup>b</sup>	0.06±0.02 <sup>ab</sup>	9
BPA 200 ppb	0.66±0.05 <sup>a</sup>	10	0.90±0.07 <sup>a</sup>	10	1.23±0.14 <sup>b</sup>	30	2.01±0.31 <sup>a</sup>	0.07±0.03 <sup>a</sup>	14

Values in the same column followed by a different letter are significantly different (P<0.05).

The results indicated that BPA exposed fish enhances ovary development and slows the development of testes. We do not know the mechanism underlying advancement of ovarian development and inhibition of testicular growth by BPA. However based on these results, ovarian development and testicular growth may dependent on concentration of exposed to BPA.

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