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〈Original article〉

Cross-generational Effect of Bisphenol A on the Harpacticoid Copepod *Tigriopus west*: A Full Life Cycle Toxicity Test

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Abstract - The purpose of this study was to assess cross-generational effects of bisphenol A exposure in benthic copepods, *Tigriopus west*. Nauplii (<24 hours old) were exposed to graded concentrations of bisphenol A, and toxicity end-points such as survival, development, sex ratio, and fecundity were measured. F_1 generations were grown under innoxious conditions, and similarly assessed. Significant differences were observed in development of nauplii and copepodites, between exposed and non-exposed copepods; however, there were no differences in survival of nauplii or copepodites, sex ratio, or brooding rate in parental generation. In contrast, in the F_1 generation, there were significant differences between the control group and exposed group in survival and development of nauplii. Length, width, and biomass of parental and F_1 generations were reduced in the exposed group compared to the control group. In addition, some deformities, such as swelling of the prosome, abnormally shaped egg sac, and dwarfism were observed after exposure to bisphenol A. So, our study demonstrates that a cross-generation toxicity test and monitoring of morphological deformities in harpacticoid copepods, can be useful for development of potential bioindicators for environmental monitoring, and assessment of chemical impact.

Keywords : bisphenol A, *Tigriopus west*, full life cycle test, deformity

INTRODUCTION

Bisphenol A (BPA) is an organic compound with 2 phenol functional groups. Amongst other applications, this monomeric compound is used to make polycarbonate plastic containers and epoxy resins, and it may also be used as an antioxidant in plasticizers for flexible polyvinyl chloride (PVC). BPA is a potential endocrine disruptor and may affect brain development and behavior. Moreover, according to Andersen *et al*. (1999), high concentration of BPA stimulates the maturation of ovaries, as detected by an increase in the fecundity of the planktonic copepod, *Acartia tonsa*; however, BPA and natural steroid estrogens did not inhibit naupliar development in this organism (Andersen *et al*. 2001). Similarly, Marcial *et al*. (2003) reported that BPA did not have extensive effects on the survival, sex ratio, and egg production of the benthic copepod *Tigriopus japonicus*.

The genus *Tigriopus* is easily found in a rock pools on the coast of Korea and Japan, which is a diverse genus at least 15 species occurring worldwide (Karanovic *et al*. 2018; Walter and Boxshall 2018). *Tigriopus* is used in various experiments (Marcial *et al*. 2003; Kwok and Leung 2005; Bang *et al*. 2009; Bang *et al*. 2010) because of they are easy to capture and maintain in a laboratory. Karanovic *et al*. (2018) used morphology and molecular analysis to classify species known as *Tigriopus japonicus* into four distinct clades: *T. japonicus* and *T.* cf. *japonicus* in Japan, *T. west* and *T. east* in Korea.

With the recent issue of the impact of endocrine disruptor

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on the ecosystem, the perception that invertebrates may play an important role as indicators of pollutants (Kwak and Lee 2005; Valavanidis *et al*. 2006; Verslycke *et al*. 2007; Bang *et al*. 2009). This is because invertebrates can quickly and sensitively detect the effects of endocrine disruptors compared to vertebrates, and they represent the entire ecosystem, considering that they are a large part of the food chain that connects to vertebrates (Bechmann 1999; DeFur *et al*. 1999; DeFur 2004; Kusk and Wollenberger 2007).

The genus *Tigriopus* are considered suitable organisms for testing ecological toxicity. Because genus *Tigriopus* are distributed worldwide, can be easily collected from tidal pools on rocky shores, and can be mass collectable in the field. Furthermore, these organisms have a short life-cycle and a high tolerance to temperature, salinity and pH (Ito 1970; Forget-Leray *et al*. 1998; Marcial *et al*. 2003).

The experimental induction of morphological deformities in copepods has only recently been recognized as a simple and effective tool for ecological assessments. In our previous study, urosome deformities and dwarfism were observed in *T. west* under conditions of benzo (*a*)pyrene exposure (Bang *et al*. 2009). In addition, endocrine disruptor toxicity tests have also been used to analyze deformities in larvae of non-biting aquatic midges, *Chironomus* (Kwak and Lee 2005). The primary objective of this study was to measure the effects of BPA on the survival, development and reproduction, as well as morphological deformation, across 2 generations of *T. west*.

MATERIALS AND METHODS

1. Organisms

Benthic harpacticoid copepods *T. west* were collected from Mansungri beach (Yeosu, Korea) using a small hand net (mesh size: 63 μm). The organisms were cultured in 25 psu artificial sea water (Crystal Sea Marine Mix; Crystal Sea®). The animals were bred under a 16 : 8 photoperiod, at 20 ± 1°C water temperature, with dissolved oxygen concentration > 80% and a pH of 8 ± 0.3. These culture conditions followed the procedures described by the International Organization for Standardization (1997).

2. Test Chemicals and Exposure Conditions

The test compound, BPA (Sigma-Aldrich, MO, USA) was dissolved in dimethyl sulfoxide (DMSO); this carrier solvent was used at a maximum concentration of 0.01% (v/v). Test vessels consisted of 6-well cell culture plates (SPL, Korea). The acute and chronic toxicity test was conducted on nauplius and adult specimens following the method described by the ISO14669 standard (ISO 1997). Generally, 35 test organisms were divided into 7 groups and the numbers of living and dead animals were then counted once every 24 h. Toxicity testing consisted of the following treatments: a control, a solvent control containing 0.01% DMSO in sea water, and 5 concentrations of BPA (0.1, 1, 10, 30, and 100 μg L^{-1}). Test solutions were renewed every 3 days by replacing half of the volume with fresh test solution.

3. Biological Endpoint Measurement

Tests were conducted on copepods in stages ranging from the first nauplius stage to adulthood, and their survival, development, brooding, and hatching were evaluated daily. The toxicity of BPA was calculated based on the nauplius survival rate (NSR) and the copepodite survival rate (CSR). Development was assessed based on the copepodite emergence day (CED) and the adult male emergence day (AMED). When copepodites reached the adult stage, the sex ratio (MER), brooding success rate (BSR), and first brooding day (FBD) were measured.

Following the experiment, the remaining copepods were fixed in 70% ethanol and the average length, width, biomass, and occurrence of morphological changes in each group determined using an optical microscope (Olympus BX-51) and a dissecting microscope (Olympus SZX12). Length and width were calculated using an image analysis program (MetaMorph 6.0), while biomass was computed according to the volumetric methods described by Feller and Warwick (1988).

4. Statistical Analyses

Differences in mortality and development between groups were evaluated by one-way ANOVA, followed by Dunnett's test, using the SPSS statistical software program (SPSS 14.0). The sex ratios of the treated and control pop-

ulations were compared using a chi-square contingency test ($p<0.05$).

RESULTS AND DISCUSSION

The ecotoxicological responses of *T. west* to BPA are shown in Table 1. DMSO, which was used as a carrier solvent, does not have any effect on survival, development, sex ratio, or fecundity. Also, F_1 control was omitted because it did not show any difference in the various index of F_0 control.

In the F_0 generation, no significant differences were observed in the survival of nauplius or copepodite (NSR and CSR) stages for any of the treatments when compared to the control, except at 10 μg L^{-1}; similar results have been reported by Marcial *et al*. (2003).

However, in the F_1 generation, the NSR was significantly affected by exposure to BPA, compared to the control, and already relatively low concentrations induced a decline in survival: the lowest NSR occurred at 0.1 μg L^{-1} (70.8%).

This indicated that when *T. west* is exposed to BPA, effects on survival from one generation are transferred to the next. In contrast, Marcial *et al*. (2003) found that BPA did not affect the survival of the F_1 generation.

Contrary to the BPA's impact on the survival of the F_1 nauplius, it has not been shown to have a significant impact on the survival of the F_1 copepodite. This suggests that the impact of BPA on the survival of the benthic copepod is greater in the early stages of growth. In summary, continuous exposure to BPA does not significantly affect the survival of the parent, but the F_1 generation produced by the exposed parent is directly affected from the early stage of growth.

Generally, development (CED and AMED) of the F_0 generation was significantly delayed ($p<0.05$) in response to BPA exposure, as compared to the control, except for the effect of 100 μg L^{-1} on AMED. The longest CED and AMED occurred at 1 μg L^{-1}.

One interesting thing is that the growth rates of nauplius and copepodite (CED and AMED) affected by BPA were the most delayed at 1 μg L^{-1} and that the effect on exposures decreased as concentration increased. Especially for CED at 100 μg L^{-1}, although significantly affected by BPA exposure, it showed the least growth delay effect of all exposure concentrations, and in the case of AMED, no significant effects were found. It is assumed that if *T. west* is exposed to toxic substances more than a certain concentration, than specific genes are expressed to resist the effects of toxicity, thus, the growth of benthic copepods tends to have less impact as concentrations increase. But of course, this hypothesis will need to be confirmed later in additional molecular and endocrinological studies.

In the F_1 generation, the period from birth to the copepodite stage (CED) of all BPA-treated copepods took significantly longer than the control ($p<0.01$). Harpacticoid copepods undergo several molts and 1 metamorphosis through 6 naupliar stages, followed by 6 copepodite stages. Molting and metamorphosis of copepods are regulated by ecdysteroids, and metamorphosis is controlled by compounds similar to the juvenile hormones (JHs), while methyl farnesoate (MF) induces larval metamorphosis (Laufer and Borst 1988; Chang *et al*. 1993). Because of their structural similarities to hormones, endocrine disruptors like BPA have the potential to disrupt molting and metamorphosis. This may explain why development was delayed in our study, as well as in many other studies that have shown that endocrine disruptors confused development and the molting regulation of copepods (Marcial *et al*. 2003; Chandler *et al*. 2004; Bang *et al*. 2009).

The sex ratio of *T. west* with and without BPA exposure is presented in Table 1. The male emergence rate (MER) of copepods was higher at 10 μg L^{-1} in both generations, but none of the treatments had any significant effect on sex ratio; similar results have been reported in other studies (Hutchinson *et al*. 1999; Marcial *et al*. 2003). Hasegawa *et al*. (1993) reported that sexual differentiation in malacostracan crustaceans, such as copepods, is controlled by the androgenic gland hormone (AGH). It may be that androgenic steroids have more influence on sexual differentiation than estrogenic steroids (Marcial *et al*. 2003).

The brooding success rate (BSR) of the control group was 98.1%, with the average first brooding day (FBD) occurring on day 13.6. The BSR ranged from 90.9−100.0% in F_0 and 73.3−100% in the F_1 generation; the BSR of all treatment groups did not change significantly. Similarly, Brown *et al*. (2003) and Marcial *et al*. (2003) found that endocrine disruptors did not affect the fecundity of the benthic copepods.

Table 1. Summary of responses of *Tigriopus west* exposed to different concentrations of bisphenol A

Generation	Concentration (μg L^{-1})	NSR (%)	CSR (%)	CED (day)	AMED (day)	MER (M%)	BSR (%)	FBD (day)
F_0	Control	96.6 ± 9.4	97.9 ± 6.1	5.2 ± 0.7	10.3 ± 0.5	61.5	98.1 ± 10.0	13.6 ± 0.7
	Solvent C.	96.7 ± 7.6	100.0 ± 0.0	5.1 ± 0.6	10.4 ± 1.0	63.3	100.0 ± 0.0	13.8 ± 1.1
	0.1	96.7 ± 8.2	100.0 ± 0.0	6.6 ± 0.8*	12.7 ± 0.9*	58.6	100.0 ± 0.0	15.4 ± 0.5*
	1	83.3 ± 19.7	100.0 ± 0.0	7.0 ± 0.8*	13.4 ± 1.2*	56.0	90.9 ± 22.4	15.7 ± 1.1*
	10	66.7 ± 16.3*	85.0 ± 20.9	6.4 ± 0.9*	12.9 ± 0.8*	70.6	100.0 ± 0.0	15.6 ± 0.6*
	30	79.3 ± 17.9	95.7 ± 13.6	6.7 ± 2.4*	12.6 ± 0.5*	40.9	92.3 ± 13.6	16.9 ± 2.3*
	100	90.3 ± 11.0	92.6 ± 20.4	5.7 ± 1.2*	10.5 ± 0.6	57.7	90.9 ± 40.8	14.8 ± 0.8*
F_1	0.1	70.8 ± 28.9*	80.0 ± 12.7*	6.3 ± 1.2*	11.0 ± 1.8	58.3	100.0 ± 0.0	14.8 ± 2.5
	1	75.0 ± 25.2*	100.0 ± 0.0	6.4 ± 0.7*	10.3 ± 0.9	50.0	73.3 ± 25.8	14.1 ± 2.3
	10	81.0 ± 22.0*	93.3 ± 10.3	5.6 ± 0.5*	10.8 ± 1.8	78.6	100.0 ± 0.0	13.7 ± 1.5
	30	82.8 ± 19.3*	93.3 ± 10.3	6.3 ± 0.9*	10.2 ± 0.9	64.3	100.0 ± 0.0	13.7 ± 1.6
	100	83.3 ± 17.4*	96.7 ± 8.2	6.0 ± 0.2*	10.9 ± 1.1	55.2	100.0 ± 0.0	14.2 ± 1.0

NSR: nauplius survival rate; CSR: copepodite survival rate; CED: copepodite emergence day, i.e. period from birth to copepodite stage; AMED: adult male emergence day, i.e. period from birth to adult male; MER: male emergence rate, i.e. sex ratio; M: male; BSR: brooding success rate; FBD: first brooding day, i.e. period from birth to female borne egg sac; *: significant difference from control by one-way ANOVA, followed by Dunnett's test ($p < 0.05$)

Table 2. Body length, width, and biomass of *Tigriopus west* after exposure to different concentrations of bisphenol A

Generation	Concentration (μg L^{-1})	Female			Male		
		Length (μm)	Width (μm)	Biomass (μg C)	Length (μm)	Width (μm)	Biomass (μg C)
F_0	Control	1034.3 ± 61.9	355.4 ± 13.1	5.9 ± 0.5	858.2 ± 36.9	322.5 ± 11.5	4.0 ± 0.4
	0.1	982.0 ± 50.3	338.9 ± 22.5	5.1 ± 0.9*	851.1 ± 34.3	280.3 ± 10.9*	3.0 ± 0.3*
	1	998.8 ± 52.0	347.1 ± 23.5	5.5 ± 0.9	842.2 ± 8.3	280.4 ± 10.7*	3.0 ± 0.2*
	10	991.0 ± 11.9	346.5 ± 17.2	5.4 ± 0.5	832.2 ± 23.2	278.6 ± 5.6*	2.9 ± 0.2*
	30	970.4 ± 29.3*	347.1 ± 12.5	5.3 ± 0.5*	865.0 ± 49.8	274.3 ± 7.9*	2.9 ± 0.1*
	100	946.5 ± 69.5*	329.8 ± 42.7	4.8 ± 1.5*	853.6 ± 27.2	272.3 ± 20.3*	2.9 ± 0.5*
F_1	0.1	910.3 ± 18.9*	303.0 ± 7.8*	3.8 ± 0.2*	858.7 ± 37.3	313.6 ± 16.4	3.8 ± 0.5
	1	959.6 ± 14.6*	313.8 ± 10.0*	4.3 ± 0.3*	840.4 ± 41.8	289.5 ± 16.9*	3.2 ± 0.5*
	10	916.4 ± 27.7*	321.2 ± 20.5*	4.3 ± 0.6*	858.5 ± 27.7	293.7 ± 9.4*	3.4 ± 0.3*
	30	959.5 ± 38.1*	319.3 ± 13.7*	4.4 ± 0.4*	848.7 ± 21.0	287.6 ± 10.9*	3.2 ± 0.2*
	100	941.1 ± 44.7*	316.5 ± 20.8*	4.3 ± 0.8*	835.6 ± 76.7	286.1 ± 32.8*	3.2 ± 0.8*

*: significant difference from control by one-way ANOVA, followed by Dunnett's test ($p < 0.05$)

However, Chandler *et al*. (2004) demonstrated that the fecundity of copepod *Amphiascus tenuiremis* was affected by exposure to fipronil, and Bang *et al*. (2009) indicated that the sex ratio and sexual maturity of *T. west* were significantly affected by exposure to benzo(*a*)pyrene. Taken together, BPA did not have an extensive effect on sex determination or on fecundity in copepods, although some other endocrine disruptors have been reported to have a negative effect on sex determination and gonadal function.

To estimate the morphological changes in the treatment group, the mean body length, width and biomass were investigated (Table 2). Generally, adult female harpacticoid copepods tended to be longer and bigger than adult males. In this study, mean body length, width, and biomass of the control group of the adult females were 1034.3 μm, 355.4 μm, and 5.9 μg C, respectively, whereas the corresponding mean sizes of the adult males were 858.2 μm, 322.5 μm, and 4.0 μg C, respectively. Thus, the biomass of females was about 1.5 times more than that of adult males, in the control group. The body weight and length of adult female and male were significantly affected by treatment with BPA. In the parental generation, the average body length and width of females exposed to 100 μg L^{-1} BPA was 946.5 μm and 329.8 μm, respectively, while the biomass was 80% of that of the control group. The male length and biomass were significantly reduced in the BPA treatment groups than in the male control group, with the smallest adult male in the group treated with 100 μg L^{-1} of BPA being 853.6 μm in length, 272.3 μm in width, and having a biomass of 2.9 μg C. Moreover, the size of females was significantly reduced

Table 3. Deformities in *Tigriopus west* exposed to bisphenol A

Concentrations (μg L^{-1})	Deformity types			
	Abnormal egg sac	Prosome swelling	Urosome shrink or swelling	Dwarfism
0.1	–	F_0♀(8/10)	–	–
1	F_1♀(1/15)	F_0♀(5/7)	–	–
10	F_0♀(1/5)	F_0♀(3/5)	–	–
30	–	F_0♀(3/10)	–	–
100	F_0♀(2/11)	F_0♀(3/5)	F_1♂(2/13)	F_1♂(2/13)

Data are given as (abnormal/observed individuals)

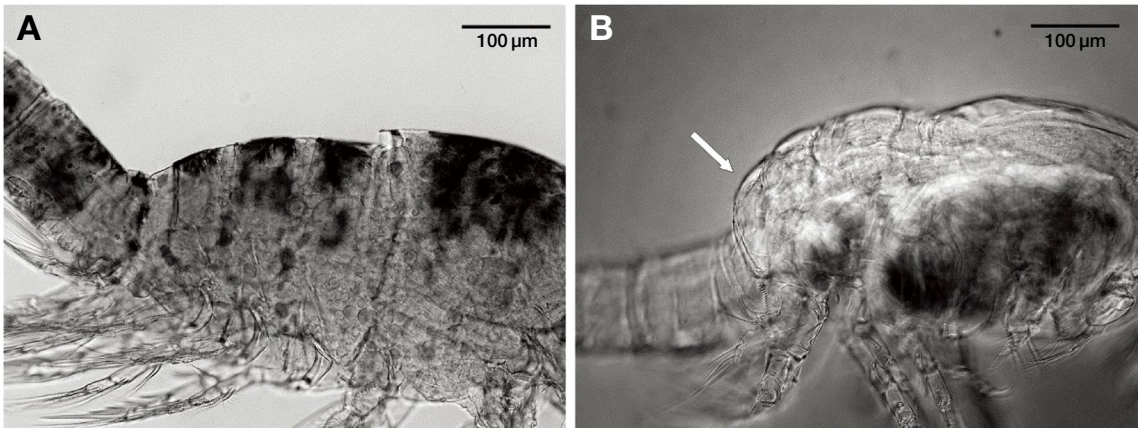

Fig. 1. Prosome deformity in *Tigriopus west* after exposure to bisphenol A. A: normal, B: prosome swelling (arrow).

in the F_1 generation compared to the parental generation, and the smallest adult female was present in the group treated with the lowest concentration of BPA; the mean body length, width and biomass for this 0.1 μg L^{-1} BPA treated group was 910.3 μm, 303.0 μm, and 3.8 μg C. Similarly, endocrine disruptors have been shown to inhibit the development of the copepods *Acartia tonsa* (Andersen *et al*. 2001) and *T. west* (Bang *et al*. 2009).

Moreover, morphological deformities were observed with BPA treatment (Table 3). The most common type of deformity in *T. west* treated with BPA was swelling of the prosome (Fig. 1B), which was present in 59.5%, or 22 of 37 observed individuals. The main feature of this prosome deformity was swelling of the free prosomite in the prosome, giving a hunchback phenotype; these deformed copepods were found at all BPA concentrations. In addition, abnormally shaped egg sacs were observed. Generally, adult female harpacticoid copepods brood egg sacs from which the nauplii hatch at intervals of about 3–4 days. However, when copepods produced deformed egg sacs, the hatching success rate was very low. Furthermore, dwarfism was also observed. Copepods were smallest when exposed to 100 μg L^{-1} BPA in F_1 generation; the length, width, and biomass of these dwarf copepods were about 600 μm, 190 μm, and 1.0 μg C, respectively.

The use of morphological deformities in ecological assessments is a simple and effective tool (Kwak and Lee 2005). Copepods are often used to investigate the effects of endocrine disruption, although there is very little data available concerning chemically induced morphological changes. Bang *et al*. (2009) reported deformity of the urosome and dwarfism in *T. west* in response to benzo(*a*)pyrene. Furthermore, the results of some studies have proposed that intersexuality of aquatic invertebrate was triggered by environmental stress (Moore and Stevenson 1994; Gross *et al*. 2001). Moore and Stevenson (1994) suggested that the high proportion of intersexuality in the harpacticoid copepod *Paramphiascella hyperborea* was caused by sewage pollution.

To summarize, we have here described the cross-gen-

erational effects of exposure to BPA on the harpacticoid copepod *T. west* using a full life cycle toxicity test. The development of nauplii and copepodites were affected by BPA exposure in the parental generation, and significant differences in development and survival of nauplii were still observed in the offspring generation cultured under innoxious conditions. The body sizes of both generations were smaller than that of the relevant control group; moreover, different types in morphological deformities were observed after exposure to BPA. Thus, our study demonstrates that a cross-generation toxicity test and monitoring of morphological deformities in harpacticoid copepods can be useful for the development of potential bioindicators for environmental monitoring and assessment of chemical impacts.

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