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Changes of Emergent Period and Body Volume of *Chironomus riparius* Exposure to Di(2-ethyl-hexyl)-phthalate

Inn-Sil Kwak, Ho Young Soh and Wonchoel Lee[1,*]

Bioresources Utilization Program, College of Fisheries and Ocean Sciences, Yosu National University, Yeosu 550-749, Korea
[1]*Department of Life Science, College of Natural Science, Hanyang University, Seoul 133-791, Korea*

Abstract - The exposed strain of *C. riparius* treated with di(ethyl-hexyl)-phthalate (DEHP) did not result in a consistent relationship between mortality or sex ratio and chemical concentrations. And after treating with DEHP, the emergent female from the exposed strain appeared to be fatty with a large body volume comparing with the non-exposed strain. The emergent period (EP) was especially different between the exposed fourth strain and the non-exposed strain; generally the exposed strain was 7~10 days, and non-exposed strain was 17~24 days. Regarding sustainable exposed effects, the EP, the first emergent day (FED), and the body volume (BV) could be suggested as suitable bio-markers for detecting of exposure to various EDCs.

Key words : emergent period, body volume, DEHP, *Chironomus riparius*

INTRODUCTION

In recent years, anthropogenic chemicals that have potential endocrine disruptor activity enter aquatic ecosystems in Western Europe, North America, Japan and France. Man-made estrogen-mimicking chemicals, or xenoestrogens, have been demonstrated to interfere with the functioning of female steroid hormones, via interaction with cellular receptors (Sumpter 1995; Jobling *et al.* 1996). Physiological effects of endocrine disrupting chemicals (EDCs) have been well described for vertebrates and are distinctly different each chemical. For instance, atrazine inhibits ligand binding to both androgen and estrogen receptors (Danzo 1997) and di(ethyl-hexyl)-phthalate (DEHP) inhibits binding to the estrogen receptor and is antiandrogenic (Jobling *et al.* 1996; Harris *et al.* 1997; Moore *et al.* 2001). DEHP is, by a large margin, the most frequently reported phthalate, and also the one found at the highest concentrations in the environment. This is to be expected, considering its widespread usage and greater persistence relative to the shorter chain phthalates. DEHP is widely used in the production of various plastics, polyvinyl chloride (PVC), inks, and industrial oils. Flexible PVC is employed in the production of floor tiles, furnishings, food packaging materials, and a variety of medical devices. The tolerable daily intake (TDI) for humans is presumed to be 40~140 μg kg^{-1} day^{-1} (Inoue 2000). DEHP is a substance which raises questions regarding animal and human exposure to such pollutants, many of which are suspected to be carcinogenic and estrogenic (Harris *et al.* 1997). In mice, DEHP induced dose-related delays on surface righting in male offspring (Tanaka 2002), and exerted opposite effects on the sex ratio of offspring from male and female mice (James 2003).

However, endocrine disruption (ED) has become common (Ankley *et al.* 1998), and endocrine specific endpoints have been proposed as the 'gold-standard' for risk assessment (Ingersoll *et al.* 1999). These tests can be designed to incorporate sensitive periods in the developmental process, such as embryogenesis, gonadal development, molting or meta-

*Corresponding author: Wonchoel Lee, Tel. 02-2220-0951, FAX. 02-2296-7158, E-mail. wlee@hanyang.ac.kr

morphosis, or growth and reproduction, all of which are regulated by the endocrine system, and are thus potentially susceptible to disruption. The most important criterion for the assessment of ED is an understanding of the endocrine system of the test species. *Chironomus riparius* (Chironomidae), is a test species which has been extensively used in environmental assessment schemes and standardized chronic assays (USEPA 1994), and has a well studied endocrine system. Also, both molting and metamorphosis, are regulated by ecdysteroids, and metamorphosis is presumably controlled by compounds similar to the juvenile hormones that are known for insects, *C. riparius*. Many studies have conducted short days or a part of life cycle, and now required chronic and trans-generation effects. Accordingly, this study have conducted that the starting from the fourth larvae stage were designed specifically to incorporate particularly sensitive period to ecdysteroids. The emergent adults have produced the egg mass by mating behavior in the DEHP (10 $\mu g\ L^{-1}$) exposed condition. The objective of this study was to examine impacts of DEHP in the larvae of the fourth generations. And the aim was to determine sensitive or cumulative endpoints for the morphological detection of ED in *C. riparius*.

MATERIALS AND METHODS

The fourth larvae exposure to 10 $\mu g\ L^{-1}$ DEHP was reared in accordance with suggestions for a standard procedure by Streloke and Kopp (1995). The exposed larvae have developed the pupae and emerged adults, and then emerged adults produced the eggs mass through the mating. The produced egg mass moved new vessel and then reared the control condition. *C. riparius* egg masses were reared in an environmental chamber under long-day conditions (light : dark cycle of 16 : 8 hours), at a light intensity of about 500 lx. Water temperature was maintained at $20\pm1°C$ in an incubator (Sanyo MIR-553, Japan). After fourth generations, the test *C. riparius* were provided by eleventh day larvae after being hatched from egg masses. Twenty larvae were introduced into each test vessel. For the toxicity test, animals were kept in 300 mL crystallizing dishes (Schott Duran, Germany) filled with 200 mL of M4 (Elendt and Bias 1990), and a sediment layer consisting of 1 cm of fine sand (<63 μm particle size). The test vessels were continuously aerated after the introduction of midge larvae. Water loss due to evaporation was negligible, but when necessary, vessels were refilled with new M4. Each vessel was provided with 10 mg of ground fish food (Tetra-Werke, Melle, Germany) to ensure that excess food did not affect water quality in the test. To prevent the escape of adults during test periods, each vessel was covered with a 0.5 mm mesh net.

Solutions of DEHP (99%, Junsei Chemical Co. Ltd., Japan) had been dissolved in analytical grade acetone to provide a stock concentration of 20 $mg\ L^{-1}$ active ingredient. The test solutions were constructed in M4 at $\leq 0.2\%$ acetone. This was the final percentage of acetone present in the solvent controls used in the experiment. The nominal concentrations of DEHP were as follows: control, solvent control, 0.3, 1, 10 and 30 $\mu g\ L^{-1}$. The half-time of DEHP has been reported to be about 14~21 days. To achieve an exposure to constant substance concentrations throughout the midges' pupal phase, and to avoid water quality changes from excess food, M4 was removed daily and replaced by new M4. The experiment employed 7 replicates for each concentration. The water replacement exposure setup was unaffected by evaporation and daily food addition. As endpoints for the toxicity test, the sex ratio of emergent adults and body shapes from each vessel were counted and measured. Subsequently, the experiments were halted in the cases in which there was no emergence of pupae or living larvae. All data were recorded at daily intervals. Morphological characteristics of the emergent adults, such as head capsule length, head capsule width, body length, body width and body volume, were evaluated using the Meta Morph 6.0 program (Universal Imaging Corporation®) under an Olympus SZX-ILLB 200. The rates of dead larvae (RDL) and emergence data were arcsine transformed prior to one-way ANOVA, in order to discern any statistical differences between treatments (Zar 1984). Also, the F-test was employed to observe whether differences in morphological characteristics existed between male and female adults, and a two-sample t test for two-tailed hypotheses was conducted. In all cases, the significance level was set at $P\leq 0.05$.

RESULTS AND DISCUSSION

After most treatments, the differences were observed

from the control groups (Table 1). The RDL did not increase in a dose-dependent manner with DEHP concentration. The RDL was 11.67% in the control and 6.67~10.12% after treatment. The RDL at 0.3 μg L^{-1} was highest in all concentrations. Test individuals that reached the pupal phase rarely died (Kwak and Lee 2005), while the RDP (rates of dead pupae) of this temporary exposed strain occupied a range of 1.67~10% compared to the test larvae. The highest RDP occurred at control group, and RDP in relatively high concentrations (>1 μg L^{-1}) showed lower than that of control, solvent control and 0.3 μg L^{-1} groups. The rate of emergent accidents of larvae was less than about 3%. The developmental stage was delayed in response to relatively low concentrations in non-exposed strain (Kwak and Lee 2005), but didn't show retardation in the exposed strain. The sex ratio was unaffected with little deviation from a 1 : 1 relationship, except in the 1 and 30 μg L^{-1} treatments, in which female adults (56~61%) were more numerous than males (39~44%) (Fig. 1).

Recent research has suggested that altered sex ratios could be used as the endpoint for EDCs (Ingersoll *et al.* 1999). Bisphenol A and 17 α-ethinylestradiol had no effects on the sex ratio in the first generation, but 17 α-ethinylestradiol altered adult sex ratios in the second generation (Watts *et al.* 2001). Also, *C. riparius* exposure to DEHP (1 and 30 μg L^{-1}) appeared numerous females (Kwak and Lee 2005). In some other cases, the sex ratio showed no consistent dose-dependent patterns, or remained unchanged (Brown *et al.* 1996; Watts *et al.* 2001). *Chironomus tentans* exposed to 4-nonylphenol were not affected in terms of emergence, sex ratio, reproduction, or egg viability (Baldwin *et al.* 1997; Kahl *et al.* 1997). However, due to the lack of consistent observed chemical effects from EDCs, evaluation of the response criteria for biomarkers of chemical exposure is difficult. The developmental retardation in the non-exposed strain have induced in relatively low concentrations (0.3 and 1 μg L^{-1}; Kwak and Lee 2005), but that in the exposed strain not observed. The other hand, the sex ratio was a similar result; both strains showed numerous female adults at 30 μg L^{-1}.

Generally, the first emergence day (FED) was earlier in males than in females, and the emergence period (EP) was a little different between male and female adults (Fig. 2). After treatment, the FED between males and females was significant different at a concentration of 10 and 30 μg L^{-1}. The EP was shorter 7~10 days in the exposed strain than 17~24 days in non-exposed strain (Kwak and Lee 2005). When the DEHP concentrations were increased, the EP of males was extended from 7 days (≤0.3 μg L^{-1}) to 8~10 days (>0.3 μg L^{-1}), but that of females was a little different

Table 1. The rates (%) of dead larvae, dead pupae, emergent accidents and emergent adults of *C. riparius* in relation to various di (2-ethylhexyl) phthalate concentrations. n: total number of individuals

Concentrations (μg L^{-1})	n	Dead larvae	Dead pupae	Emergent accidents	Emergent adults
Control	140	11.67	10.00	0.00	78.33
Solvent control	140	10.12	7.80	0.00	82.08
0.3	140	16.67	5.00	3.33	75.00
1	140	6.67	3.33	0.00	90.00
10	140	8.33	1.67	1.67	88.33
30	140	10.00	3.33	3.33	83.34

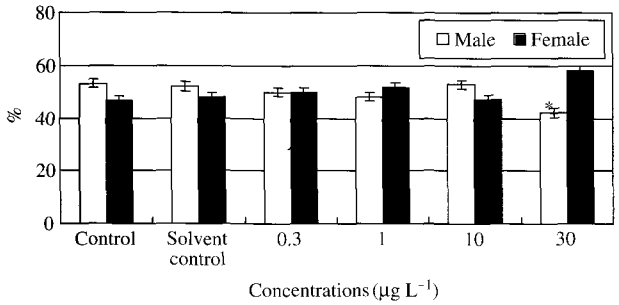

Fig. 1. The percentage of emergent males and females of *C. riparius* at five di (2-ethylhexyl) phthalate concentrations. Asterisks (*) denote a significant difference.

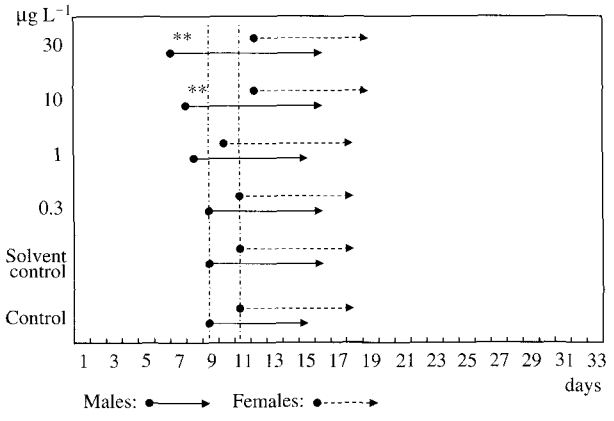

Fig. 2. The period of emergent males and females of *C. riparius* at five di (2-ethylhexyl) phthalate concentrations. Asterisks (*) denote a significant difference in the emergence period ($P<0.05$). Asterisks (**) denote a significant difference in the first emergence day ($P<0.05$).

Table 2. Morphological characteristics of emergent adults, such as head capsule length (HCL), head capsule width (HCW), body length (BL), body width (BW) and body volume (BV), at five different concentrations. Asterisks (*) denote a significant difference, H_0: No difference between male and female ($P<0.05$). Asterisks (**) denote a significant difference, H_0: No difference between control and treatment ($P<0.05$). Forty males and forty females were measured at each concentration. SD: standard deviation

Concentrations ($\mu g\ L^{-1}$)		HCL (mm)		HCW (mm)		BL (mm)		BW (mm)		BV (μL)	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Control	Mean	1.23	1.27	0.75	0.85	11.17	10.51	1.252*	1.98	75.21*	103.33
	SD	0.18	0.34	0.13	0.11	0.63	0.76	0.17	0.13	7.89	7.84
Solvent control	Mean	1.24	1.30	0.77	0.82	11.07	10.65	1.263*	1.95	76.51*	105.45
	SD	0.16	0.35	0.11	0.10	0.59	0.77	0.18	0.14	8.08	8.02
0.3	Mean	1.39	1.39	0.87	0.87	11.19	12.50	1.369*	1.77	84.61*,**	110.99**
	SD	0.13	0.21	0.19	0.17	0.75	2.30	0.11	0.09	8.33	19.60
1	Mean	1.19	1.36	0.669*	0.79	9.92	11.16	1.087*	1.88	59.25*,**	102.14
	SD	0.11	0.16	0.09	0.08	0.62	1.72	0.27	0.17	10.19	15.47
10	Mean	1.221*	1.43	0.76	0.82	9.774*	11.70	1.140*	1.75	61.85*,**	101.96
	SD	0.14	0.09	0.09	0.13	0.81	1.79	0.14	0.26	6.98	17.37
30	Mean	1.28	1.35	0.73	0.72	11.19	11.18	1.346*	1.83	75.81*	105.60**
	SD	0.10	0.14	0.07	0.07	0.52	1.22	0.13	0.20	5.39	12.19

from 8 to 9 days. However, there were similar tendencies: the FED of males was faster than that of females of non-exposed strain, and the EP of males was longer than that of females.

With regard to body size, generally female adults tended to be larger than adult males (Table 2). Differences between male and female were found in body width (BW) and body volume (BV), but body length (BL), head capsule length (HCL) and head capsule width (HCW) were similar between males and females. Also, a significant difference between controls and treatments was noted for BV in males. In addition, significant differences in BV in females were observed at DEHP concentrations of 0.3 and 30 $\mu g\ L^{-1}$. The BV and BW data evidenced a remarkable difference between males and females at each concentration. The BV of female was larger in the exposed strain (101.96 ~ 110.99 μL) than in non-exposed strain (68.54 ~ 85.32 μL; Kwak and Lee 2005). In low concentrations, the BV of male was larger in the exposed strain than in the non-exposed strain. Also, the BW in male was larger in the exposed strain than in the non-exposed strain. Due to the ease of visual verification, the BV or BW should be considered to be an indicator for the detection of EDCs. The other morphological characteristics, HCL and HCW, were relatively similar between males and females, and by concentration. The strongest differences that affected the development of exposed *C. riparius* strain were the shorted emergence periods (EP) and the fatted BV of female in this DEHP study, but there was no clear relationship between *C. riparius* development and chemical concentrations. Similar result reported that *C. riparius* adults emerged significantly earlier than controls when exposed to relatively low concentrations (Watts *et al.* 2001; kwak and Lee 2005), and the emergence period was relatively longer days in non-exposed strain of *C. riparius* (Kwak and Lee 2005). In this study, the EP was not different between male and female, while the FED of males was significant earlier than that of females at 10 and 30 $\mu g\ L^{-1}$ treatments (Fig. 2).

Recently, most researches regarding the detection of EDCs have focused on lab conditions, physiology, and toxic-chemical analysis. In this term, the detection technique for EDCs has accelerated by molecular technique. Nevertheless, these made kits showed confirmed effects through the laboratory test. The body shape and morphological characteristics are easily observed, and could be detected more quickly than physiological verification for various EDCs. Accordingly a sustainable and stable morphological indicator should be determined under both laboratory and field conditions.

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