Korean J. Environ. Biol. 22(2) : 321~328(2004)

Detecting Points for Ecological Disruptions and Developmental Delay Exposure to DEHP in *Chironomus riparius* (Diptera: Chironomidae)

Inn-Sil Kwak* and Wonchoel Lee

Department of Life Science, Hanyang University, 17 haengdang-dong, Seongdong-gu, Seoul 133-791, Korea

Abstract - The effects of Di (2-ethylhexyl) phthalate (DEHP) on the fourth instar larvae of *Chironomus riparius* were tested in the laboratory. Employing a waterreplacement exposure setup, chironomids were subjected to various concentrations. In the most treatments mortality reached a statistically significant difference from the control conditions. As DEHP concentrations were increased, the rates of emerged adults decreased. Sex ratio was unaffected with little deviation from a 1:1 relationship (except in 1 and 30 μg L^{-1}). The developmental stages was delayed at low concentration (0.3 and 1 μg L^{-1}). Generally the emergent period was different between males and females, and the first emergent day of males was faster than females. The body shape of female adults was larger than males. Differences between males and females were found in body volume, body length and body width. In addition, the body volume showed the significant difference between controls and treatments, and those especially well observed females.

Key words : developmental retardation, *Chironomus riparius*, DEHP, sex ratio, body shape, emergence periods

INTRODUCTION

By far the most frequently reported phthalate, and that found at highest concentrations in the environment, is DEHP. This is to be expected, considering its high usage and greater persistence relative to the shorter chain phthalates. Di (2-ethylhexyl) phthalate (DEHP) is widely used in the production of various plastics, polyvinyl chloride (PVC), inks and industrial oils. Especially, flexible PVC is employed for the production of floor tiles, furnishing, food packaging materials, and a variety of medical devices. The tolerable daily intake (TDI) for human is presumed as 40~140 μg kg^{-1} day^{-1} (Inoue 2000). DEHP produced doserelated delays on surface righting in male offspring (Tanaka 2002) and opposite effects on the sex ratio of offspring of male and female mice (James 2003). DEHP should give rise to awareness about the animal and human exposure to these pollutants suspected to be carcinogenic and estrogenic (Harris *et al.* 1997).

Chemical substances of anthropogenic origin alter hormonal regulation or hormonal functions in humans and animals. In recent years, the most well known are the "xenoestrogens", man-made estrogen-mimicking chemicals, which interfere with functions of the female steroid hormone via interaction with the cellular receptor. In addition, xenoestrogens disturb endocrine functions in wild fish populations, leading to feminization and altered gonadal development (Sumpter 1995; Jobling *et al.* 1996; Van der Kraak *et al.* 1998). For examples, TBT-induced imposex in female gastropods (Matth-

* Corresponding author: Inn-Sil Kwak, Tel: -82-2-2290-0951, Fax: -82-2-2296-7158, E-mail: inkwak@hotmail.com

iesen and Gibbs 1998), abnormal sex ratios in marine harpacticoid copepods (Moore and Stevensen 1994) and chironomids (Watts *et al.* 2001), and the development of ovotestes in male lobsters (Sangalang and Jones 1997) have been reported various endocrine disruptions.

However, endocrine disruption (ED) has become common (Colborn *et al.* 1993; Ankley *et al.* 1998), and it should be required to detect specific responses to EDCs. Therefore, 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, including embryogenesis, gonadal development, molting or metamorphosis, growth and reproduction, all of which are regulated by the endocrine system and potentially susceptible to disruption. Moreover the short generation time of invertebrate species used to extend several generations. In addition, the important criterion for the assessment of EDCs is an understanding of the endocrine system of the test species. *C. riparius,* which has been extensively used in environmental assessment schemes and standardized chronic assays (Hill *et al.* 1993; USEPA 1994; Environment Canada 1997) and has a well studied endocrine system.

The objective of this study was to investigate an indicator or end-point for detecting ED in *C. riparius*. In addition, a quick indicator among morphological characters should be provided for rapid risk assessments.

MATERIALS AND METHODS

1. Rearing experimental animals

Conditions were according to the suggestions for a standard procedure by Streloke and Kopp (1995). Egg masses of *Chironomus riparius* (Chironomidae) were provided by Dr. Mick Hamer, Jealott's Hill Research Station for Zeneca Agrochemicals, Bracknell, UK. Animals were reared in an environmental chamber under long-day conditions with a light: dark cycle of 16: 8 hours and a light intensity of about 500 Lx. Water temperature was constant at $20\pm1°C$ in incubator chamber (Sanyo MIR-553, Japan). Larvae were kept in crystallizing dishes (Schott Duran, Germany) with approximately 500 mL of the culture medium (M4; Elendt and Bias 1990), a sediment layer of 1 cm of fine sand (<63 μm particle size), and aerated continuously after midge larvae were introduced. The larvae were fed finely grounded fish food (Tetra-Werke, Melle, Germany).

2. Test organisms

The test individuals of *C. riparius* were provided by eleventh day larvae after being hatched from control egg masses. The test individuals with clear red color were selected and injected into test vessels by glass pipette. 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, and a sediment layer of 1 cm of fine sand (<63 μm particle size). The test vessels were aerated continuously after midge larvae were introduced. Water loss due to evaporation was low, and if necessary, vessels were refilled with M4. Each vessel was provided 10 mg of ground fish food (0.5 mg/larvae) to avoid excess food affecting the water quality of the test. To prevent escape of adults during test periods, each vessel was covered with 0.5 mm mesh net.

3. Toxicant solutions

Solutions of DEHP for use in the study were prepared from the solid compound (99%, Junsei Chemical Co. Ltd., Japan), which had been dissolved in analytical grade acetone to provide stock concentration of 20 mg L^{-1} active ingredient. Water used for dilution was taken from a water purification system (Human, Pure Power). From this solution aliquots ranging from 30 μL to 300 μL were placed in the test vessels, resulting in nominal test concentrations from 0.3 to 30 mg L^{-1} in the respective treatments. The nominal concentrations of DEHP were as follows: control, 0.3, 1, 10 and 30 mg L^{-1}. Contamination was conducted on the second day when larvae were introduced into the test vessels. The halftime of DEHP is reported to be about 14~21 days. To achieve an exposure to constant substance concentrations through the midges' pupal phase and to avoid water quality changes from excess food, M4 was removed daily and replaced by new M4. The water replacement exposure setup was unaffected by evaporation and daily addition of food suspension.

4. Test end points and data analysis

As endpoints of the toxicity test, the sex ratio of emerged adults and body shapes from each vessel were counted and measured. Subsequently, the experiments were ended if there was no emergence or living larvae or pupae. All data were recorded at daily intervals. Body shapes of emerged adults, such as head capsule length, head capsule width, body length, body width and body volume, were measured by Meta Morph program 6.0 (Universal Imaging Corporation®) under Olympus SZX-ILLB 200. Rates of dead larvae (RDL) and emergence data were arcsine transformed prior to oneway ANOVA in order to identify any statistical differences between treatments (Zar 1984). Also, F-test was employed to observe whether differences of body shape characters exist between male and female adults, and a two-sample t test for two-tailed hypotheses was conducted. In all cases, the significance levels were set at $P \leq 0.05$.

RESULTS

1. Mortality along developmental process

Employing a water-replacement exposure setup, *C. riparius* were subjected to various DEHP concentrations. There was the obvious difference in rates of dead larvae (RDL) found in concentrations (Fig. 1). In most treatments it reached a statistically significant difference from the control group. As seen in Fig. 1, RDL did not increase in a dose-dependent manner along DEHP concentrations.

The RDL was observed 5% at control and 15 to 21% after treatment (Fig. 1). Especially, the RDL at 0.3 μg L^{-1} was more than the RDL at 1, 10 μg L^{-1}; however, the highest RDL was at 30 μg L^{-1}. In addition, the interesting difference was that the RDL for concentrations over 1 μg L^{-1} DEHP was lower than the control and 0.3 μg L^{-1}.

Test individuals who reached the pupal phase rarely died; therefore, generally the RDP (rates of dead pupae) was low (Fig. 1). The RDP occupied ranges of 1~5% of test larvae and the highest RDP was at 1 μg L^{-1}. The REC (rates of emergent accidents) of larvae was less than 3% (Fig. 1). The REC only appeared at 30 μg L^{-1}.

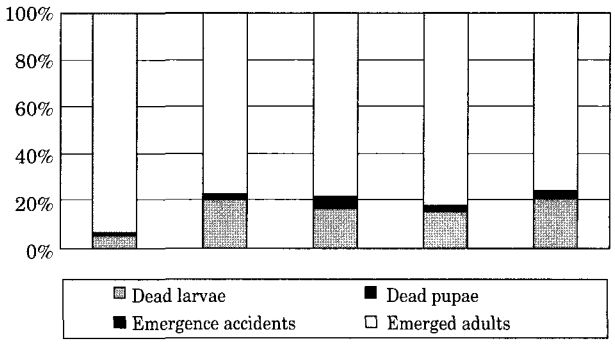

Fig. 1. The rates of dead larvae, dead larvae, emergence accidents and emergence adults of *C. riparius* to various Di (2-ethylhexyl) phthalate concentrations.

2. Survival curve and sex ratio of emerged adults

The end day of survival curve was different with DEHP concentrations (Fig. 2a). Based on the slope value of each treatment, the decreasing of larvae followed this order: control > 30 μg L^{-1} > 10 μg L^{-1} > 0.3 μg L^{-1} > 10 μg L^{-1}. The larvae phase was observed until day 25 in controls, day 27 at 10 μg L^{-1} and 30 μg L^{-1}, day 28 at 0.3 μg L^{-1}, and day 29 at 1 μg L^{-1} treatments. Therefore, the larval stage was forced to delay development in relatively low concentrations, such as 0.3 and 1 μg L^{-1}. The decreasing larvae of the survival curve included dead larvae and developing larvae (pupae or adult).

As DEHP concentrations were increased, the rates of emerged adults decreased (Fig. 3). The sex ratio was unaffected with little deviation from a 1:1 relationship, except in 1 and 30 μg L^{-1} treatments female adults (55~61%) were more than males (39~44%).

3. Emergence periods (EP) of male and female

Generally, the EP was different between male and female adults, and the first emergence day (FED) for males was faster than for females. When the concentration increased, the EP of males was shorter than females and the FED of males was faster than females.

The EP of males was various along the concentrations: day 8~28 in controls, day 9-day 25 in 0.3 μg L^{-1}, day 9-day 22 at 1 μg L^{-1}, day 8-day 27 at 10 μg L^{-1}, and day 7-day 24 at 30 μg L^{-1} (Fig. 4). The EP at high concentrations, such as 30 μg L^{-1}, was faster than at ot-

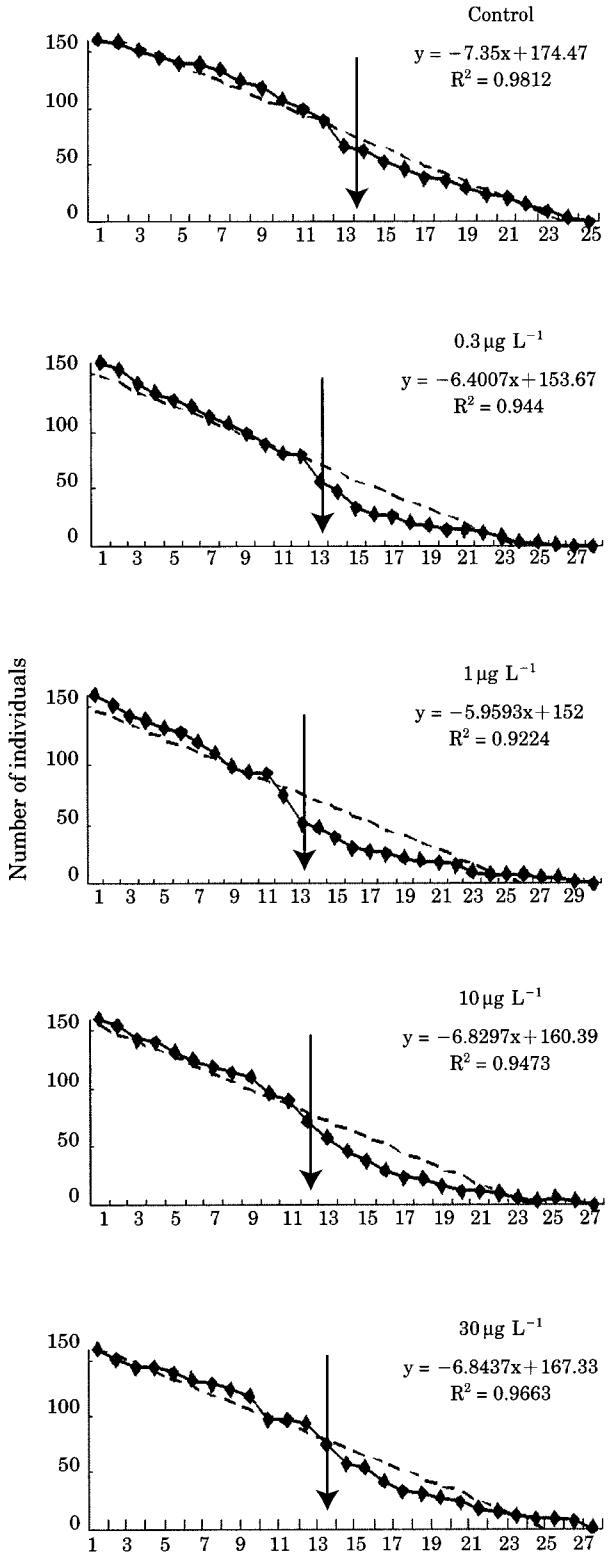

Fig. 2. Survival curves of *C. riparius* at five Di (2-ethylhexyl) phthalate concentrations under water-replacement exposure setup.

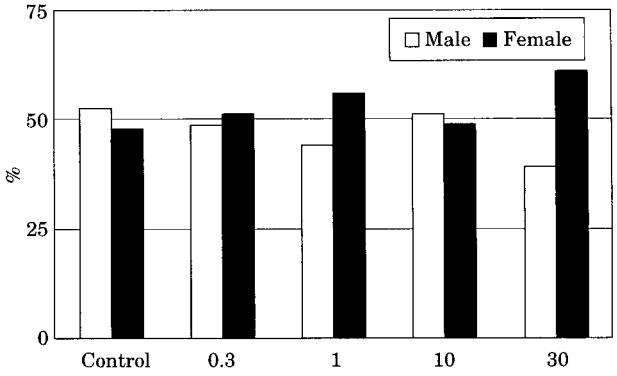

Fig. 3. The percent of emergent males and females of *C. riparius* at five Di (2-ethylhexyl) phthalate concentrations.

her treatments in males. While the females were day 9~24 in controls, day 13~29 at 0.3 μg L^{-1}, day 9~33 at 10 μg L^{-1}, day 9~30 at 10 μg L^{-1}, and day 9~30 at 30 μg L^{-1}. The FED between males and females was obvious differences at 0.3 μg L^{-1}. After treatments, the EP of higher concentrations (17~19 days over 10 μg L^{-1}) was relatively longer than lower concentrations (13~16 days) in male adults. While the EP of females observed 21~24 days over 1 μg L^{-1} and 16 days in 0.3 μg L^{-1}. The different EP between males and females was significant at 1 μg L^{-1}; males was the shortest short days (13 days), but females had the largest days (24 days).

4. Body shape of emergent adults

The body shape of female adults was larger than males (Table 1). Differences between male and female were found in body length (BL), body width (BW), and body volume (BV), but head capsule length (HCL) and head capsule width (HCW) were not different from male to female. In addition, a significant difference between controls and treatments was especially seen in BV in females. Also, a significant difference for BW was found at 0.3 and 10 μg L^{-1} in males.

Statistically, the difference in HCL was not significant with sex and concentrations. However, the HCL of males was 1.270±0.181 mm in controls and decreased along DEHP concentrations but not significant. However, female did not significantly deviate (1.314±0.116 mm in controls, 1.239±0.304~1.311±0.123 mm after treatments). Also, the HCW of males was not signifi-

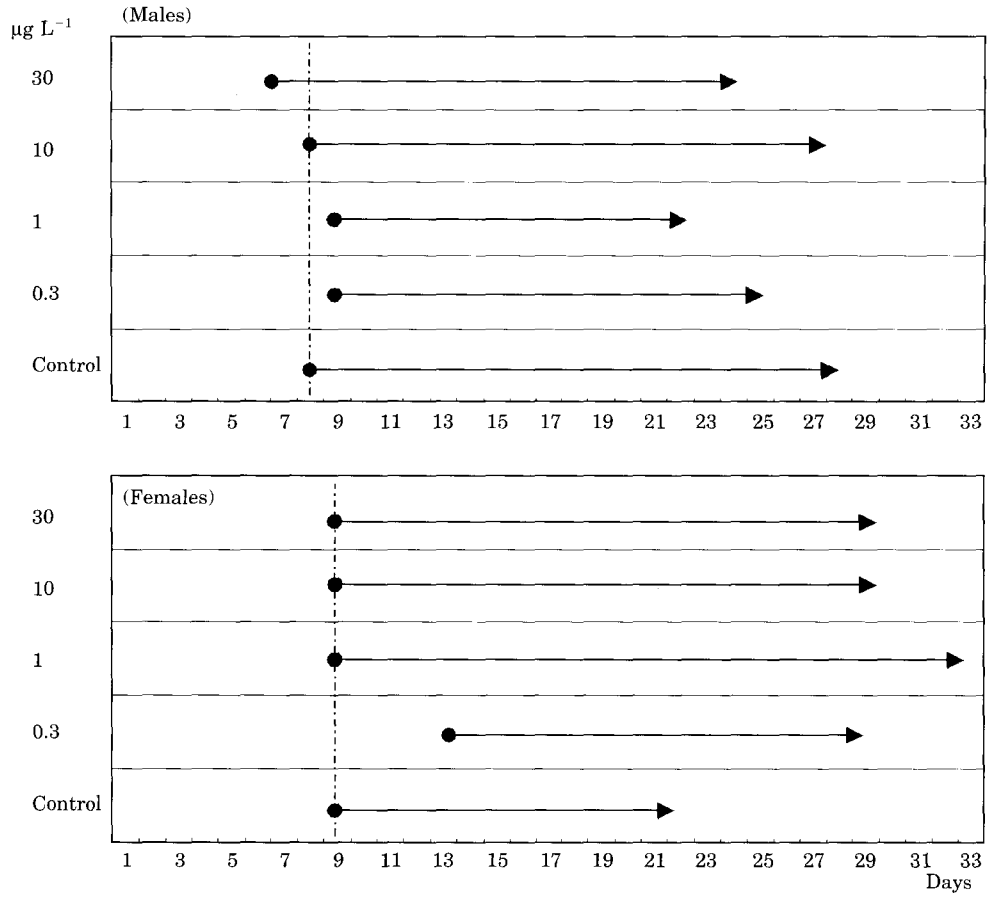

Fig. 4. The period of emergent males and females of *C. riparius* at five Di(2-ethylhexyl) phthalate concentrations.

Table 1. Body shapes of emerged adults such as head capsule length, head capsule width, body length, body width and body volume at five concentrations

		Concentrations (μg L^{-1})									
		Control		0.3		1		10		30	
	Sex	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Head capsule length (mm)	Male	1.270	0.181	1.321	0.153	1.255	0.099	1.128	0.355	1.134	0.305
	Female	1.314	0.116	1.265	0.338	1.311	0.123	1.281	0.212	1.239	0.304
Head capsule width (mm)	Male	0.719	0.118	0.700	0.144	0.745	0.120	0.727	0.080	0.693	0.108
	Female	0.700	0.224	0.784	0.115	0.776	0.144	0.751	0.135	0.787	0.149
Body length (mm)	Male	10.702*	1.335	11.118*	0.343	9.719	3.334	10.503*	0.635	10.017	2.510
	Female	9.068	0.683	9.528	0.731	9.547	1.155	9.634	1.355	9.391	1.144
Body width (mm)	Male	1.051	0.335	0.895*	0.165	0.955*	0.163	0.976*	0.138	0.859*	0.162
	Female	1.221	0.197	1.226	0.370	1.352	0.397	1.464	0.272	1.360	0.290
Body volume (mm^3)	Male	6701.088	1205.870	6693.536*,**	2258.044	6295.854*,**	1593.094	6414.043*,**	907.191	6734.407*,**	915.583
	Female	6854.564	2689.278	8233.291**	2117.276	8231.908**	1743.039	8532.692**	1542.673	8315.541**	1448.077

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 ($P < 0.05$).
SD: Standard Deviation

cantly different among treatments; 0.719±0.118 mm in controls, 0.693 ±0.108~0.745±0.120 mm after treatments. The HCW of females showed a difference between controls (0.700±0.224 mm) and after treatments

($0.751 \pm 0.135 \sim 0.787 \pm 0.149$ mm). The BL did not differ with concentrations but showed a significant difference between male/female in controls, 0.3 and 10 μg L^{-1}. When concentrations increased, generally the BL decreased in males but did not differ with concentrations in females.

The BW differed a significantly between male and female (except in controls), and especially males were smaller than females. Also the highest BW for males was 1.051 ± 0.335 mm in controls, and BW showed a significant decrease after treatments (0.895 ± 0.165 mm $\sim 0.976 \pm 0.138$ mm). The BW of females did not differ significantly among concentration groups. The BV had large significant difference between male and female, and at each concentration (except in controls). In the control, the BV between male and female did not significantly differ; however, females were larger than males. After treatments, the BV of females largely increased but males decreased. The BV of males was observed between 3707.44 ± 951.080 mL and 4047.91 ± 897.660 mL after treatments, 5275.694 ± 1583.130 mL in controls. And the BV of females varied; 5546.729 ± 1819.700 mL in controls and 7822.13 ± 2062.130 mL $\sim 9308.67 \pm 2220.340$ mL after treatments.

DISCUSSION

The fourth larva of *C. riparius* used this study has a sensitive to ecdysteroidal molting hormones for the life cycle developments. Already the life-cycle characteristics of *C. riparius* examined have previously been utilized as effective indicators of general toxic stress in chronic assays (Benoit *et al.* 1997; Sibley *et al.* 1997; Watts and Pascoe 2000). Already chronological postponements of contaminations and effects have been reported (Liess and Schulz 1996).

In present, many researches provided altered sex ratio as the end point for EDCs (Christopher *et al.* 1999; Hahn *et al.* 2001). But many cases, sex ratio showed no consistent dose-dependent manner and not disturbed: bisphenol A and 17 α-ethinylestradiol not altered sex ratio in the first generation but 17 α-ethinylestradiol act as an oestrogenic mode of action affected adult sex ratio in second generation (Watts *et al.* 2001). Another example, *C. tentans* exposure to 4-nonylphenol did not affect emergence, sex ratio, reproduction or egg viability (Baldwin *et al.* 1996; Jobling *et al.* 1996; Kahl *et al.* 1997).

However, due to no consistent chemical effects of DEHP, evaluation of the response criteria as biomarkers of chemical exposure is difficult. It is considered that no consistent DEHP-related effects were attributed adsorption of the test organisms. Nevertheless, this study data provide indications for disruptions that the normal developmental processes in *C. riparius* have been disrupted. For example, relatively low concentrations such as 0.3 μg L^{-1} observed retardation of developments in survival curve (Fig. 2a).

Next step, pupae to adult emergence among life development stages, female adults dominant patterns were observed in high concentrations (1 and 30 μg L^{-1}). However, balances of sex ratio for mating chances were disturbed but DEHP could not affect sexual differentiation act as oestrogenic mode, due to dominance of female adults at high concentrations. Further experimental investigations would be needed both to confirm the result and to establish a possible mode of action for DEHP in *C. riparius*.

In addition, the strongest indications that affected the development of *C. riparius* was the altered emergence periods and body volume in the DEHP study but there was no clear relationship between *C. riparius* and chemical concentrations. The male emerged earlier than the corresponding females and at high concentrations male adults emerged earlier than at high concentrations. Similar results reported that at relative low concentrations adults emerged significantly earlier than control *C. riparius* (Watts *et al.* 2001) but not considered the emergence periods. The emergence periods of male were shorter than corresponding females and at low concentrations (0.3 μg L^{-1}) the first emergence day was clearly delayed (Fig. 4).

The differences of BW and BV among body shape characters were well observed and especially well showed BV in female individuals. The others characters such as HCL and HCW were a little various changes to DEHP. Also, we observed that the female treated with DEHP was fatty and annoyed/or not adapted flying and mating behavior. In this view, the BV or BW should be considered an indicator to detect EDCs/or various chemicals because the advantage of these indicators was an

easy detection in laboratory condition due to their visible size. The other characters, HCL and HCW were not a difference with male/female and concentrations. The HCL and HCW for taxonomical identification were well know stable keys and so far used to determine developmental stages for Chironomidae. These taxonomical characters, the BV and BW, should be suggested a useful indicator for determining or detection for input EDCs.

Nowadays many researches for detection of EDCs were considered lab condition, physiology and toxic-chemical analysis, however, each organism of ecosystem was disturbed and required detection for ecological disruption. The body shape (or morphological characters) was well observed and detected faster than physiological detection for various EDCs. Therefore, a sustainable and stable indicator of body shape characters should be researched and found laboratory and field conditions.

CONCLUSION

The exposure of *C. riparius* to DEHP was not consistent relationship between mortality or sex ratio and concentrations. The retardation of development stages was observed at low concentration. Especially female was clearly delayed and required many days or times for emergence. Generally the emergent female exposure to DEHP appeared a fatty and large body volume. The emergent periods, the first emergent day and body volume could be considered suitable biomarkers (characters) for rapid detection of various EDCs exposure.

ACKNOWLEDGEMENTS

This study was supported by the Korea Research Foundation Grant (KRF-2002 -005-C00022).

REFERENCES

Ankley G, E Mihaich, R Stahl, D Tillitt, T Cloborn, S McMaster, R Millier, J Bantle, P Campbell, N Denslow, R Dickerson, L Formar, M Fry, J Giesy, LE Gray, P Guiney, T Hutcison, S Kennedy, V Kramer, G LeBlanc, M Mayes, A Nirmrod, R Patino, G van der Kraak and T Zacharewski. 1998. Overview of a workshop on screening methods for detecting potential (Anti-) Estrogenic/chemical in wildlife. Environ. Toxicol. Chem. 17:68-87.

Baldwin WS, SE Graham, D Shea and GA LeBlanc. 1997. Metabolicof female *Daphnia magna* by thxenoestrogen 4-nonylphenol. Environ. Toxical. Chem. 16:1905-1911.

Benoit DA, PK Sibley, JL Juenemann and G T Ankley. 1997. *Chironomus tentans* life-cycle test: design and evaluation for use in assessing toxicity of contaminated sediments. Environ. Toxicol. Chem. 16:1165-1176.

Colborn T, FS vom Saal and AS Soto. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Persp. 101:378-384.

DeFur PL, M Crane, CG Ingersoll and L Tattersfield. 1999. Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment. SETAC thchnical publication, Pensacola, Florida, pp. 303.

Environment Canada, 1997. Biological test method: test for survival and growth in sediment using the larvae of freshwater midges (*Chironomus tentans* or *Chironomus riparius).* Report EPS/RM/32, Ottawa, Ontaio, Canada.

Elendt BP and WR Bias. 1990. Trace nutrient deficiency in Daphnia magna cultured in standard medium for toxicity testing; effects of the optimization of culture conditions on life history parameters of *Daphnia magna.* Water Res. 24:1157-1167.

Hahn T, L Matthias and S Ralf. 2001. Effects of the Hormone Mimetid Insecticide Tebufenozide on *Chironomus riparius* Larvae in Two Different Exposure Setups. Toxicol. Environ. Saf. 49:171-78.

Harris CA, P Henttu, MG Parker and JP Sumper. 1997. The Estrogenic Activity of Phthalate Esters in vitro. Environ. Health Persp. 105:802-811.

Hill R, P Matthiesen and F Heimbach. 1993. Guidancd document on sediment toxicity tests and bioassays for freshwater and marine environments. Society of Environmental Toxicology and Chemistry-Europe, Workshop on sediment toxicity assessment. 8-10 Novermber, Renesse, Netherlands.

Ingersoll CG, T Hutchinson, M Crane, S Dodson, T DeWitt, A Gies, MC Huet, CL McKenney, E Oberdorster, D Pascoe, DJ Versteeg and O Warwick. 1999. Laboratory toxicity tests for evaluating potential effects of endocrine-disrupting compounds. In: DeFur, P.L., M Crane, CG Ingersoll, L Tattersfield (Eds.), Endocrine Disrupion in Invertebrates: Endocrinology, Testing and Assessment. SETAC technical publication, Pensacola, Fl-

orida, pp 107-197.

Inoue Y. 2000. The use of vinyl chloride gloves for handling food. Food Sanitation Reserch 50:7-12.

James WH. 2003. Phthalates, hormones and offspring sex ratios. Food Chem. Toxicol. 41:599-600.

Jobling S, D Sheahan, JA Osborne, P Matthiesen JP Sumpter. 1996. Inhibition of testicular growth in rainbow trout (*Onchorhyncus mykiss*) exposed to estrogenic alkylphenolic compounds. Environ. Toxicol. Chem. 15: 194-202.

Kahl MD, EA Makynen, PA Koisan and GT Ankley. 1997. Toxicity of 4-nonylphenol in a life-cycle test with the midge *Chironomus tentans*. Ecotoxicol. Environ. Saf. 38:155-160.

Liess M, R Schulz. 1996. Chronic effects of short-term contamination with the pyrethroid insecticide fenvalerate on the caddisfly *Limnephilus lunatus*. Hydrobiologia 324:99-106.

Matthiesen P and PE Gibbs. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ. Toxicol. Chem. 17:37-43.

Moore CG and JM Stevensen. 1994. Intersexuality in benthic harpacticoid copepods in the first of forth, Scotland. J. Nat. Hist. 28:1213-1230.

Sumpter JP. 1995. Feminized responses in fish to environmental oestrogens. Toxicol. Lett. 82/83:737-742.

Sangalang G and G Jones. 1997. Oocytes in testis and intersex in lobsters (*Homarus americanus*) from Nova Scotian sites: natural or site related phenomenon. Can. Tech. Rep. Fish. Aquat. Sci. 2163, 46.

Streloke M and H Kopp. 1995. Long-term toxicity test with *Chironomus riparius:* development and validation of a new test system, Mitt. A. D. Biol. Bundesanst. 315, Blackwell Wissenschaftsverlag, Berlin/Vienna.

Sibley PK, DA Benoit and GT Ankley. 1997. The significance of growth in *Chironomus tentans* sediment toxicity tests: relationship to reproduction and demographic endpoints. Environ. Toxicol. Chem. 16:336-345.

Tanaka T. 2002. Reproductive and neurobehavioural toxicity study of bis(2-ethylhexyl) phthalate (DEHP) administered to mice in the diet. Food Chem. Toxicol. 40:1499-1506.

USEPA United States Environmental Protection Agency. 1994. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates, EPA/600/R-94/024. Technical report, Washington DC. 133.

Van der Kraak GJ, KR Munkittrick, ME McMaster and DM MacLatchy. 1998. A comparison of bleached kraft pulp mill effLCEnt, 17-oestradiol and -sitosterol effects on reproductive function in fish. In: Kendall R, R Dickerson, W Suk and J Giesy (Eds.), Principles and Processes for Evaluating Endocrine Disruption in Wildlife. SETAC Press, Pennsacola, FL.

Watts MM and D Pascoe. 2000. Comparison of *Chironomus riparius* Meigen and *Chironomus tentans* Fabricius (Diptera: Chironomidae) for assessing the toxicity of sediments. Environ. Toxicol. Chem. 19:1885-1892.

Watts MM, D Pascoe and K Carroll. 2001. Chronic exposure to 17α-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diptera: Chironomidae). Aquatic Toxicol. 55:113-124.

Zar JH. 1984. Biostatistical analysis. Prentice-Hall International Editions, New Jersey.

Manuscript Received: April 5, 2004
Revision Accepted: May 20, 2004
Responsible Editorial Member: Myung Chan Gye (Hanyang Univ.)