Bull. Environ. Contam. Toxicol. (2005) 74:1179–1185

DOI: 10.1007/s00128-005-0705-0

Bulletin of Environmental Contamination and Toxicology

Endpoint for DEHP Exposure Assessment in *Chironomus riparius*

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Received: 23 July 2004/Accepted: 1 March 2005

Di-(2-ethylhexyl)-phthalate (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/day (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).

In recent years, 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). 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 metamorphosis, or growth and reproduction, all of which are regulated by the endocrine system, and are thus potentially susceptible to disruption. In addition, 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. The objective of this study was to determine an endpoint for the detection of ED in C. *riparius*.

MATERIALS AND METHODS

Conditions used in this study were in accordance with suggestions for a standard procedure by Streloke and Kopp (1995). C. *riparius* egg masses were reared in an

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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 ± 1 ℃ in an incubator (Sanyo MIR-553, Japan). The test C. *riparius* were provided by eleventh day larvae after being hatched from control 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 active ingredient. The test solutions were constructed in M4 at ≤ 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 μg/L. 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 9 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

The fourth instar larva of C. *riparius* is sensitive to ecdysteroidal molting hormones in the life cycle developmental stages studied in this work. After most treatments, significant 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 5 % in the control and 15 - 21.25 % after treatment. The RDL at 0.3 μg/L was greater than the RDL at 1 and 10 μg/L. Test individuals

that reached the pupal phase rarely died, and generally the RDP (rates of dead pupae) occupied a range of 1.25 - 5 % compared to the test larvae. The highest RDP occurred at 1 μg/L. The rate of emergent accidents of larvae was less than 2 %. The larval development stage was delayed in response to relatively low concentrations, such as 0.3 and 1 μg/L: Larval phase was observed until day 25 in the controls, day 27 at 10 μg/L and 30 μg/L, day 28 at 0.3 μg/L, and day 29 at 1 μg/L treatments. The sex ratio was unaffected with little deviation from a 1:1 relationship, except in the 1 and 30 μg/L treatments, in which female adults (55-61 %) were more numerous than males (39-44 %) (Figure 1).

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)	n	Dead larvae	Dead pupae	Emergent accidents	Emergent adults
Control	180	5.00	1.25	0.00	93.75
Solvent control	180	5.00	2.50	0.00	92.50
0.3	180	20.00	2.50	0.00	77.50
1	180	16.25	5.00	0.00	78.75
10	180	15.00	2.50	0.00	82.50
30	180	21.25	1.25	1.25	76.25

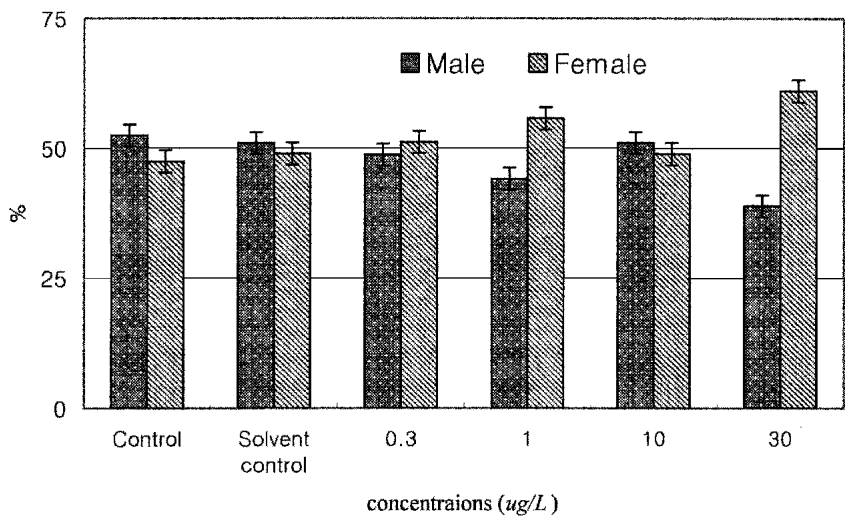

Figure 1. The percentage of emergent males and females of C. *riparius* at five di (2-ethylhexyl) phthalate concentrations.

Some recent research has suggested that altered sex ratios could be used as the endpoint for EDCs (Ingersoll et al. 1999). However, in some other cases, the sex ratio showed no consistent dose-dependent patterns, or remained unchanged (Brown et al. 1996; Watts et al. 2001). 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). In another study, C. *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 DEHP, evaluation of the response criteria for biomarkers of chemical exposure is difficult. Nevertheless, this study provided some indications that the normal developmental processes in C. *riparius* had been disrupted. For example, relatively low concentrations (0.3 and 1 µg/L) induced retardation of development. Since there was a higher percentage of females at the highest tested DEHP concentration (30 µg/L), further testing is needed to determine if DEHP significantly affects sex ratio in C. *riparius* and, if so, to discern a possible mode of action.

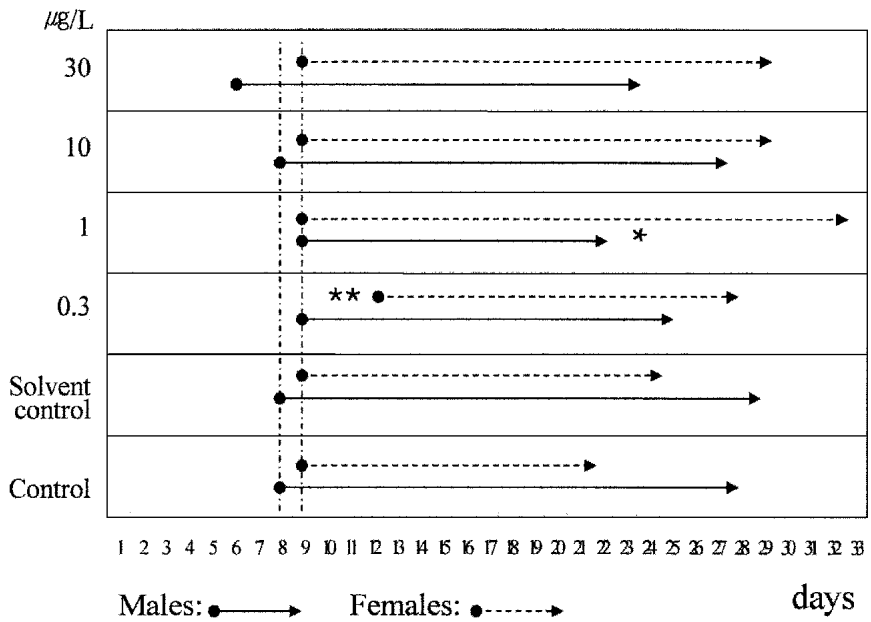

Figure 2. The period of emergent males and females of C. *riparius* at five di (2-ethylhexyl) phthalate concentrations. Asterisks (*) denote a significant difference in the emergence period ($P < 0.05$). Asterisks (**) denote a significant difference in the first emergence day ($P < 0.05$).

Generally, the emergence period (EP) was different between male and female adults, and the first emergence day (FED) for males was earlier in males than in females (Figure 2). When the DEHP concentrations were increased, the EP of males was shorter than that of females and the FED of males was faster than that of females. After treatment, the EP at higher concentrations (17-19 days over 10 µg/L) was relatively longer than that observed at lower concentrations (13-16 days) in male adults. The EP of females, however, was 21-24 days at concentrations over 1 µg/L, and was 16 days at 0.3 µg/L. The EP was significantly

different between males and females at 1 μg/L: the EP in males was 13 days, and in females, it was 24 days. The FED between males and females was most obviously different at a concentration of 0.3 μg/L. The strongest indications that affected the development of C. *riparius* were the altered emergence periods and body volume 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) but the study did not consider emergence periods. The emergence periods of males were shorter than for corresponding females. The FED of females was delayed at low concentrations (0.3 μg/L) (Figure 2).

With regard to body size, female adults tended to be larger than adult males (Table 2). 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 similar between males and females. Additionally, a significant difference between controls and treatments was noted for BV in females. Also, significant differences in BW in males were observed at DEHP concentrations of 0.3 and 10 μg/L. The BW differed significantly between males and females (except in controls), with males becoming smaller than females. The BW of females did not differ significantly among concentration groups. The BV data evidenced a remarkable difference between males and females at each concentration (except in controls). However, females were larger than males in control groups. After treatment, the BV of females largely increased, but that of males decreased. We observed that the treated female appeared fatty. In this view, the BV or BW should be considered to be an indicator for the detection of EDCs. The advantage of these indicators is easy detection in the laboratory, due to the ease of visual verification. The other morphological characteristics, HCL and HCW, were relatively similar between males and females, and by concentration. Also, in this study, we confirmed that the HCL and HCW, used for taxonomical identification, were stable keys for determining developmental stages in the Chironomidae.

Recently, most research regarding the detection of EDCs have focused on lab conditions, physiology, and toxic-chemical analysis. The body shape (or morphological characteristics) is 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. In summary, the exposure of C. *riparius* to DEHP did not result in a consistent relationship between mortality or sex ratio and DEHP concentration. The retardation of development was observed, however, but only at low concentrations. Females especially, clearly exhibited developmental retardation and required greater times to emergence. Generally, the emergent female, after exposure to DEHP, appeared to be fatty, with a large body volume. The emergent periods, the first emergent day, and the body volume could be suggested as suitable bio-markers (characteristics) for the rapid detection of exposure to various EDCs.

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 ($P < 0.05$). Fifty males (45 at 30 μg/L) and fifty females were measured at each concentration. SD: standard deviation.

Concentrations		HCL (mm)		HCW (mm)		BL (mm)		BW (mm)		BV (μℓ)	
(μg/L)		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Control	Mean	1.27	1.31	0.72	0.70	10.70*	9.07	1.05	1.22	67.01	68.54
	SD	0.18	0.12	0.12	0.22	1.33	0.68	0.33	0.20	12.06	26.89
Solvent	Mean	1.28	1.29	0.72	0.71	10.71*	9.15	1.06	1.23	66.37	69.85
control	SD	0.19	0.13	0.11	0.23	0.95	0.69	0.34	0.22	12.51	25.69
0.3	Mean	1.32	1.27	0.70	0.78	11.12*	9.53	0.895*	1.23	66.93*,**	82.33**
	SD	0.15	0.34	0.14	0.11	0.34	0.73	0.16	0.37	22.58	21.17
1	Mean	1.26	1.31	0.74	0.78	9.72	9.55	0.955*	1.35	62.95*,**	82.32**
	SD	0.10	0.12	0.12	0.14	3.33	1.15	0.16	0.40	15.93	17.43
10	Mean	1.13	1.28	0.73	0.75	10.50*	9.63	0.976*	1.46	64.14*,**	85.32**
	SD	0.35	0.21	0.08	0.14	0.64	1.36	0.14	0.27	9.07	15.43
30	Mean	1.13	1.24	0.69	0.79	10.02	9.39	0.859*	1.36	67.34*,**	83.15**
	SD	0.30	0.30	0.11	0.15	2.51	1.14	0.16	0.29	9.16	14.48

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

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