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Mouthpart Deformity and Developmental Retardation Exposure of *Chironomus plumosus* (Diptera: Chironomidae) to Tebufenozide

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Xenoestrogens are man-made estrogen-mimicking chemicals, which interfere with functions of the female steroid hormone via interaction with the cellular receptors (Sumpter 1995; Jobling et al. 1996). Recently, endocrine disruption has become an increasing problem (Ankley et al. 1998), and it is necessary to develop a technique for the detection of specific responses to endocrine disrupting chemicals (EDCs). Therefore, endocrine specific endpoints have been proposed as the 'gold-standard' for risk assessment (Ingersoll et al. 1999). These tests are generally designed to incorporate sensitive periods during the developmental process, including embryogenesis, gonadal development, molting or metamorphosis, growth and reproduction. All of these are regulated by the endocrine system, and are potentially susceptible to disruption.

The insecticide, tebufenozide (N-tert-butyl-N'-[4-ethyl-benzoyl]-3, 5-dimenthyl-benzohydrazide, formerly RH-5992), belongs to the group known as the insect growth regulators and the benzoylhydrazines have been extensively studied. These substances have been reported to act as agonists of ecdysteroidal molting hormones at the molecular level, and cause a variety of hormonal effects in both insects and crustacean arthropods (Wing 1988; Clare et al. 1992; Dhadialla et al. 1998; Retnakaran et al. 1995). Most toxicity tests on nontarget aquatic arthropods of tebufenozide required high substance concentrations in order to make a toxicological effect visible (Kreutzweiser et al. 1994, 1998; Pauli et al. 1999). *Chironomus riparius* (Chironomidae), which has been extensively used in environmental assessment schemes and standardized chronic assays, and its endocrine system has been intensively studied (USEPA 1994). The experimental organism used in this study, *Chironomus plumosus* belongs to the same genus as C. *riparius*. The objective of this study was to determine a quick, simple, and practical biomarker for the detection of EDCs.

MATERIALS AND METHODS

The culture conditions were set up according to the suggestions for a standard procedure provided by Streloke and Kopp (1995). Egg masses of *C. plumosus* were collected from Jungnang Stream in Seoul, and reared in an incubator chamber (Sanyo MIR-553, Japan) at 20 ± 1 ℃, with a light: dark cycle of 16: 8

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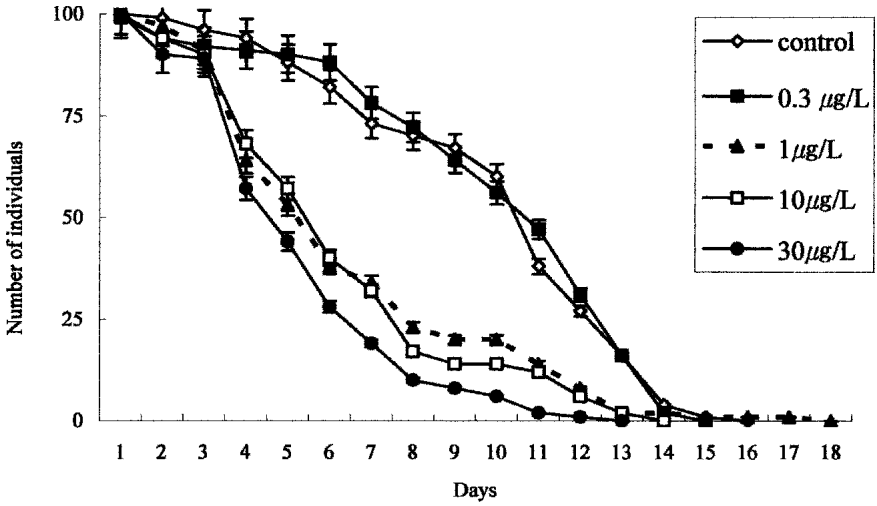

Figure 1. The survival curve of *C. plumosus* exposed to tebufenozide. Larvae phase was observed until day 16 at at the controls, day 15 at 0.3 μg/L, day 18 at 1.0 μg/L, day 14 at 10 μg/L and day 13 at 30 μg/L treatments.

hours and a light intensity of about 500 lx. The test individuals of *C. plumosus* 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, the 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 of 1 cm of fine sand (< 63 μm particle size). The test vessels were continuously aerated the introduction of the midge larvae. Each vessel was provided with 10 mg of ground fish food (Tetra-Werke, Melle, Germany) to avoid excess food affecting the water quality of the test. To prevent adults from escaping during the test periods, each vessel was covered with a 0.5 mm mesh net. Larvae were examined daily to check for survival, molting and emergence. This experiment employed 5 replicates per concentration. Dead larvae were preserved in 7 % formalin and then transferred to a glass microscope slide keeping the head capsule by adding a drop of ethanol. A cover slip, with a drop of Hoyer solution on it, was placed on top of the head capsule. Sufficient pressure was applied to the cover slip to flatten the mentum of the head capsule. Samples were observed under an Olympus SZX-ILLB 200.

Solutions of tebufenozide (99.9%, Sigma-Aldrich Laborchemikalien GmbH) were dissolved in analytical grade acetone to provide stock concentrations of 20 mg/L of active ingredients. From this solution, aliquots ranging from 100 μl to 1 ml were placed in the test vessels, resulting in nominal test concentrations ranging from 10 to 60 μg/L in the respective treatments. The nominal concentrations of tebufenozide were as follows: control, 10, 30 and 60 μg/L. The chemical was added 24 hours after the larvae. The half-time of tebufenozide is reported to be 40

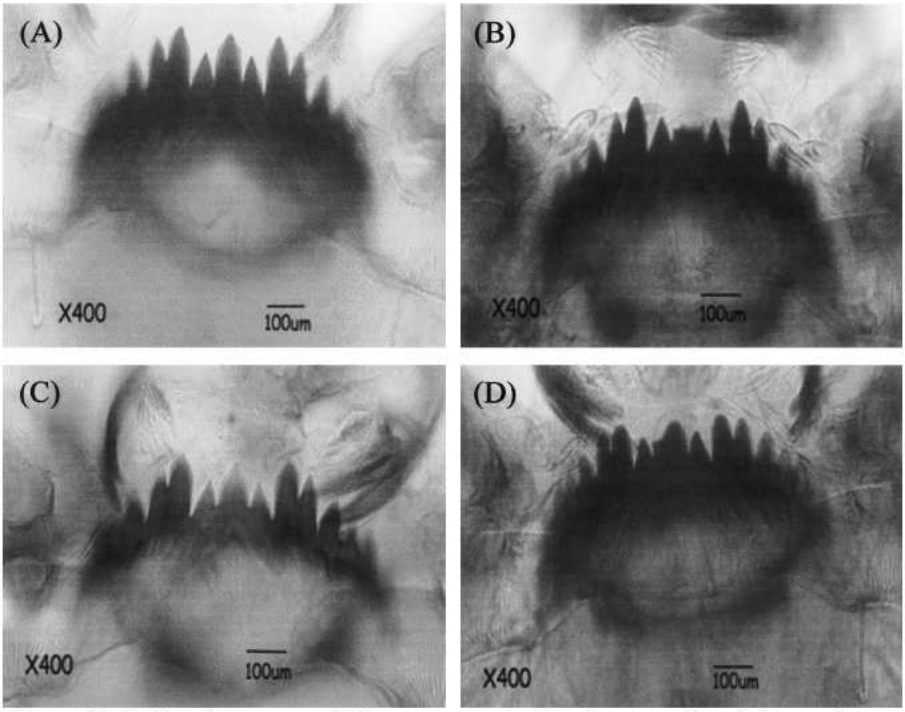

Figure 2. Deformity types of the mentum in *C. plumosus* fourth-instar larvae depicted: (A) control (B) loss of teeth (C) light color (D) smooth and round teeth. In the control, the normal arrangement of teeth is three median lateral teeth (MLT) and lateral teeth (LT).

days of persistence (Sundaram 1997). The F-test was employed to determine whether differences in the rate of mentum deformity existed between the control and treatment groups, and the significance level was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

The fourth-instar larvae of *C. plumosus* are sensitive to ecdysteroidal molting hormones for the life cycle developments. Accordingly, the molting and emergence process were affected by the environments to which the animals were exposed during larval phase. When the larvae were treated with the insecticide tebufenozide, three distinct effects were observed: lethal effects at high concentrations without a dose-dependent manner, developmental retardations at low/intermediate concentrations, and light brown mentum in a dose-dependent manner. *C. plumosus* from a polluted field stream showed a high degree of mortality at concentrations over 1 μg/L tebufenozide (> 93 %). As shown in Figure 1, the exposed larvae in high concentrations ($\geq$ 1 μg/L) abruptly decreased from day 4 to day 9, while those in low concentrations (control and 0.3 μg/L) gradually decreased until day 16 to day 18. The lost of larvae was due either to mortality, or to development into the next stage. The larval stage showed delayed

(48 h) development at relatively low concentrations, such as 1 μg/L (Fig. 1). Developmental retardations of *C. riparius* were showed at 1 ㎎/L of 17α-ethynylestradiol and Bisphenol A (Watts et al. 2003).

The type of deformity observed in the mouthpart mentum is depicted in Figure 2. The deformities noted following exposure to tebufenozide typically involved smooth/round tooth, the loss of ≥1 tooth, and reduced median lateral teeth (MLT). The incidence of mentum deformity was associated with tebufenozide exposure (Table 1). Chemical treatments cause the appearance of a light color and round shape in the mentum that was dose-dependent (Table 1). Also, the incidence of the smooth teeth was observed more frequently in the median lateral teeth (MLT) than in the lateral teeth (LT).

Its simplicity makes the assessment of mouth part deformities a practical potential biomarker for sediment quality. This end-point has already been adopted in routine monitoring programs in Belgium (De Cooman et al. 1998) and interest in using it has also been recently expressed by the UK Environment Agency (Pinder et al. 1999). Chironomid larvae showed deformities after being exposed to copper (Kosalwat and Knight 1987), DDT (Madden et al. 1992), xylene (Janssens de Bisthoven et al. 1997), lead and mercury (Vermeulen et al. 2000), and 4-n-nonylphenol (Meregalli et al. 2000). Some researchers have reported that the greater effect being associated with exposure to lower concentrations has been seen in previous EDC studies (Sheehan and Branham 1978; Patlak 1996; Santillo et al. 1998; Watts et al. 2003). This has resulted in the realization that classical dose-response curves may not adequately characterize the behavior of these chemicals. Recently, some researchers have suggested that such deformities may constitute an endocrine disruption phenomenon (DeFur et al. 1999; Pinder et al. 1999; Vermeulen et al. 2000). The molting process is hormonally regulated by

Table 1. Deformities of the mentum in fourth-instar *C. plumosus*. Asterisks (*) denote a significant difference, H_0: No difference between control and treatments ($P < 0.05$). MLT: median lateral teeth, LT: lateral teeth.

			Tebufenozide (μg/L)				
			0	0.3	1	10	30
Mentum Deformity	MLT	Smooth	1	9	9	5	1
		Loss	0	0	3	1	1
	LT	Smooth	1	0	2	1	0
		Loss	0	0	0	1	0
	MLT/LT	Smooth/Smooth	0	3	5	8	6
		Loss/Smooth	0	0	0	0	7
		Loss/Loss	0	0	0	1	0
		Total Damage	2	12	19	17	15
		%	18.2	66.7*	57.6*	77.3*	78.9*
Mentum pigmentation		Deep brown (%)	100	72.2	63.6	40.9	15.8
		Light brown (%)	0	27.8*	36.4*	59.1*	84.2*

both ecdyson and juvenile hormone (DeFur et al. 1999; Pinder et al. 1999). Therefore, the observed deformities can be interpreted as the result of a physiological disturbance occurring during the development of the buccal structures in the molting process (Janssens de Bisthoven et al. 1992).

Tooth smoothness and loss of tooth were rarely observed in controls. Total damage and the incidence of light brown mentum increased with concentrations. The mentum color was deep brown in the control condition, and changed to light brown, according to the concentration to which the larvae were exposed. As the insecticide, tebufenozide, targets the ecdysone receptor (ER) (Dhadialla et al. 1998), disruption of hormonal processes directly or indirectly controlled by ecdysteroids appears to be a likely mechanism for the effects observed in this study. Also, the enzyme systems involving in the cytochrome P450 monoxygenases may have been affected. Accordingly, endocrine hormone disruption was shown by developmental retardations and the mentum deformity in C. *plumosus*. Although the key enzymes of ecdysteroid metabolism are part of this enzyme system (Lafont and Connat 1989), more than one mode of action can be expected with regard to the effects of tebufenozide in C. *plumosus*. More research is needed in order to understand the effects of EDC exposure on both adults and subsequent generations.

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