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Effects of the Ecdysteroid Agonist Tebufenozide on Freshwater Chironomids

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Ecdysteroid agonist tebufenozide가 담수산 깔다구류에 미친 영향. 곽인실 · 이원철* (한양대학교 생명과학과)

non-steroidal ecdysteroid 길항물질인 tebufenozide를 사용하여 *C. flaviplumus*와 *C. riparius*에 끼친 영향을 실내실험 하였다. 정적인 실험조건 하에서 깔따구류를 다양한 농도로 처리하였다. 대부분의 실험에서 비처리군과 처리군은 통계적으로 유의적인 차이를 보였다. tebufenozide의 농도가 높을수록 치사율은 증가되었으며 이는 탈피과정 또는 변태과정과 연관되었다. 30 μg L^{-1} 이상의 고농도 처리에서 *C. riparius*의 유충 치사율은 *C. flaviplumus*보다 높았다. 발생적인 측면에서 상대적으로 낮은 농도인 10 μg L^{-1} 처리 하에서 성장지연이 보였다. 본 연구에서 탈피를 통하여 성충이 된 비율은 농도처리와 노출된 종에 따라 차이가 있었다.

Key words : development delay, *Chironomus flaviplumus*, *Chironomus riparius*, tebufenozide, endocrine disruption

INTRODUCTION

Chemical substances of anthropogenic origin altered hormonal regulation or hormonal functions in humans and animals. In recent years, the most well known are the "xenoestrogens" which interfere with functions of the female steroid hormone, via interaction with the cellular receptor. In this term "endocrine disruption (ED)" has become common (Colborn *et al*., 1993; Ankley *et al*., 1998). These ED works have conducted on crustaceans in marine and limnic environments with various chemicals after their intended or unintended release into the environment (Baldwin *et al*., 1997; LeBlanc, 1997; Depledge and Billinghurst, 1999; LeBlanc and McLachlan, 2000). The endocrine disrupting chemicals (EDCs) present in surface waters in Europe and the U.S., have been related with wild fish populations, leading to feminization and altered gonadal development (Sumpter, 1995; Jobling *et al*., 1996; Van der Kraak *et al*., 1998). Little work has been directed the possible affects of EDCs in aquatic insects (Kahl *et al*., 1997; Fargasova, 1998).

The test substance, the insecticide tebufenozide (N-tert-butyl-N′-[4-ethyl-benzoyl]-3, 5-dimenthylbenzohydrazide, formerly RH-5992), belongs to insect growth regulators, the benzoyl hydrazines. This substance has been reported to act as agaonists of ecdysteroidal molting hormones at the molecular level and causes a variety of hormonal effects in insects and crustacean arthropods (Wing, 1988; Clare *et al*., 1992; Retnakaran *et al*., 1995; Dhadialla *et al*., 1998). The most toxicity tests on nontarget aquatic arthropods executed with formulations of tebufenozide required high substance concentrations (exceed 361.23 mg L^{-1}) to make a toxicological effect visible (Kreutzweiser *et al*., 1994, 1998; Pauli *et al*., 1999).

The objective of this study is to investigate the

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sensitivity of two midge species for detecting endocrine effects in aquatic insects. And the differences of susceptibility understand the exposure species with various chemicals and the adapted species in the laboratory.

MATERIALS AND METHODS

1. Experimental animals

The test individuals of *Chironomus riparius* (Diptera) were provided the sixth day larvae after hatched from egg masses (Day 6). 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 ± 1°C in incubator chamber (Sanyo MIR-553, Japan). Larvae were kept in crystallizing dishes (Schott Duran, Germany) with approximately 500 mL the culture medium (M4; Elendt and Bias, 1990) and a sediment layer of 1 cm of fine sand (<63 μm particle size). The larvae were fed finely grounded fish food (TetraWerke, Melle, Germany). A long-day photoperiod was provided to the stock cultures (light : dark = 16 h : 8 h). Individuals of *Chironomus flaviplumus* were collected from the sandy or silt zone of Soktae Stream located in a metropolitan city of Korea, where it is one of the dominant species (Chon *et al*., 2000; Park *et al*., 2001; Kwak *et al*., 2002). Laboratory cultures were started with these animals and kept at a temperature of 20 ± 1°C and fed finely grounded fish food (Tetra-Werke, Melle, Germany). And the light-dark condition was 16 h : 8 h and the vessels aerated continuous.

2. Toxicity test procedure

Animals were kept in 300 mL crystallizing dishes (Schott Duran, Germany) filled with 200 mL of M4 water, 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 the M4 medium. Twenty larvae were introduced into each test vessel. Larvae were daily fed a food portion of 1 mg per larva. Contamination was performed on the second day when larvae introduced into the test vessels. Subsequently the experiments were ended if there was no emergence and living larvae or pupae.

Tebufenozide (Sigma-Aldrich Laborchemikalien GmbH, 99.9%) used to prepare a stock solution with a nominal 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 100 μl to 1 ml were placed in the test vessels, resulting in nominal test concentrations from 10 to 100 μg L^{-1} in the respective treatments. The nominal concentrations of tebufenozide were as follows: control, 10 μg L^{-1}, 30 μg L^{-1}, 60 μg L^{-1} and 100 μg L^{-1}. The first contamination was done with the nominal concentration for a test volume of 200 ml in each vessel. The half-time of tebufenozide is reported 40 days persistence (Sundaram, 1997). As endpoints of the toxicity tests the numbers of emerged adults from each vessel were counted, and emergence accidents and dead pupae were observed. All data were recorded at daily intervals. Rates of dead larvae (RDL) and emergence data were arcsine transformed prior to one-way ANOVA in order to identify any statistical differences between treatments (Zar, 1984). In all cases the significance levels were set at $P\leq0.05$.

RESULTS

1. Dead larvae

Employing a static exposure setup, chironomids were subjected to various tebufenozide concentrations. There was the obvious difference in rates of dead larvae (RDL) found in two midge species in the test vessels. After treatments, *C. flaviplumus* found dead 88% to 93% of test individuals but *C. riparius* observed dead 76% to 100% of test organisms over 30 μg L^{-1} treatments. In the most treatments it reached a statistically significant difference from the control group (Fig. 1). At control condition, RDL in *C. flaviplumus* was about 40% and occupied about 45% in *C. riparius*. As can be seen from Fig. 1, RDL in 10 μg L^{-1} treatments were about 60% in *C. flaviplumus* and about 76% in *C. riparius*.

The RDL compared with the both midge species in Day 3 (from Day 1 to Day 3), Day 6 (from Day 4 to Day 6) and Day 10 (from Day 7 to Day 10) (Fig. 2). Due to the difference of the life stages, the RDL of two chironomids showed dissimilar. As the concentration of tebufenozide was incre-

ased, the RDL observed a relatively larger proportion of two midge species. Specially, there was a clear difference in RDL of Day 3 in both species: the RDL of *C. flaviplumus* was from 5% to 13% but *C. riparius* happened from 12% to 45%. The RDL of Day 6 in *C. flaviplumus* showed 35% to 38% in 10 μg L^{-1} and 30 μg L^{-1} treatments, and obviously increased to 77% and 87% in 60 μg L^{-1} and 100 μg L^{-1} treatments, respectively. While, the RDL of Day 6 in *C. riparius* was 21% in 10 μg L^{-1} treatments and happened about 65% over 30 μg L^{-1} treatments. The both species in 10 μg L^{-1} tebufenozide showed the similar RDL of Day 10 (47% or 48%). But RDL of Day 10 for *C. flaviplumus* (63～92%) showed lower lethality than *C. riparius* (88～95%) over 30 μg L^{-1} treatments. When the exposure times are increased, the differences of the RDL in both species were decreased in test vessels.

The last observed day (LOD) of dead larvae in *C. flaviplumus* was Day 15 in control conditions, Day 19 in 10 μg L^{-1}, Day 15 in 30 μg L^{-1} and Day 12 in 60 and 100 μg L^{-1} treatments (Table 1). While the LOD of *C. riparius* found Day 23, Day

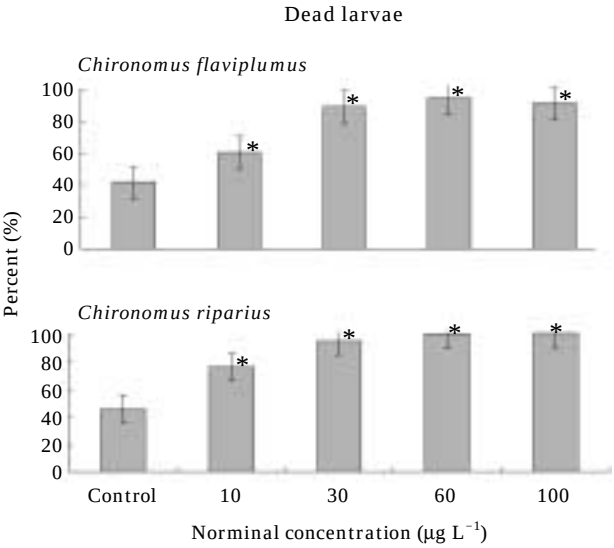

Fig. 1. Rates of dead larvae in *Chironomus flaviplumus* and *Chironomus riparius* after static condition to various concentration of tebufenozide. Error bars indicate ± SE; asterisks denote a significant difference from control ($P < 0.05$).

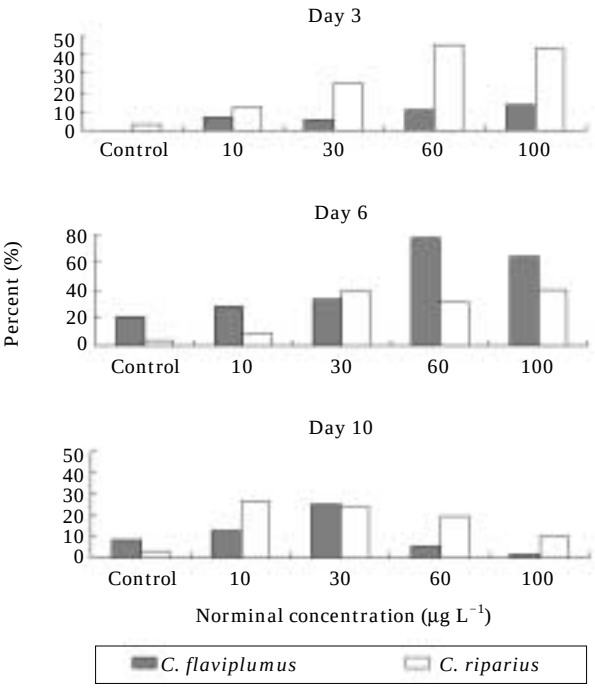

Fig. 2. Rates of dead larvae along the exposure periods in *Chironomus flaviplumus* and *Chironomus riparius* after static condition to various concentration of tebufenozide. Day 3: rates of dead larvae from Day 1 to Day3, Day 6: rates of dead larvae from Day 4 to Day 6, Day 10: rates of dead larvae from Day 7 to Day 10.

Table 1. Rates of dead larvae in *Chironomus flaviplumus* and *Chironomus riparius* in static condition to various concentration of tebufenozide.

Treatment (μg L^{-1})	Rates of dead *Chironomus flaviplumus*				Rates of dead *Chironomus riparius*			
	Day 3	Day 6	Day 10	Last observed day	Day 3	Day 6	Day 10	Last observed day
Control	0	21	29	15	3	7	10	33
10	7	35	48	19	12	21	47	37
30	5	38	63	15	25	65	88	15
60	10	87	92	12	45	76	95	13
100	13	77	78	12	43	83	93	13

Day 3: rates of dead larvae from Day 1 to Day3, Day 6: rates of dead larvae from Day 4 to Day 6, Day 10: rates of dead larvae from Day 7 to Day 10.

Table 2. Rates of daed larvae, dead pupae, emergence accidents and emerged adults in *Chironomus flaviplumus* and *Chironomus riparius* to various concentration of tebufenozide under static condition.

	Rates of dead *Chironomus flaviplumus*					Rates of dead *Chironomus riparius*				
	Treatments (μg L^{-1})					Treatments (μg L^{-1})				
	Control	10	30	60	100	Control	10	30	60	100
Lavae dead	41	60	88	93	90	46	76	95	100	100
Dead pupae	29	20	7	4	8	7	15	5		
Surviving pupae	30	20	5	3	2	47	8			
Emergence accidents	2	0	0	0	0	2	2			
Emerged adults	28	20	5	3	2	46	7			

27, Day 15, Day 13 along the various concentrations (control, 10 μg L^{-1}, 30 μg L^{-1}, 60 and 100 μg L^{-1}), respectively. As mentioned, the LOD of *C. flaviplumus* was relatively shorter than *C. riparius*'s in control conditions and 10 μg L^{-1} tebufenozide. Over 30 μg L^{-1} treatments, however, the LOD of both species was very similar in all test vessels.

2. Adult emergence

The LC_{50} of first-instar in *C. riparius* was 21.14 μg L^{-1} treatments (Hahn *et al*., 2001), consequently the concentrations of tebufenozide, such as 30 μg L^{-1}, 60 μg L^{-1} and 100 μg L^{-1} treatments in this test vessels, was relatively high. *C. flaviplumus* lived in various polluted environments were moved into test conditions and then treated only tebufenozide. And they observed high mortality after treatment conditions. Therefore, the rates of adult through molting process in test vessels were differences along both species (Table 2). Specially, the larvae of *C. riparius* in control condition developed adults 45.8% of test individuals and reached adult only 6.8% in 10 μg L^{-1} tebufenozide, but rarely showed adults in over 30 μg L^{-1} treatments. While 28% of *C. flaviplumus* developed adult in control conditions, 20% of the tested larvae succeeded adults in 10 μg L^{-1} and 5% or less of individuals happen to adults over 30 μg L^{-1} treatments.

DISCUSSION

The aim of this study was to investigate the sensitivity to mimetic insecticide in two species, *C. flaviplumus* and *C. riparius* in aquatic insects. Although the need for researches of invertebrate species and communities has been pointed out recently, these studies are still rare. Also, the most data refer to short-term toxicity testing for acute effects and only data provides 21-day tests of *Daphnia magna* LC_{50} values (EEDB, 1995). This toxicity testing conducted 12-day to 37-day test of the fourth or second instar larvae of chironomids.

The insecticide tebufenozide was chosen as a test substance because of its well-documented hormonal action: it binds to the ecdysone receptor and leads to effects similar to those of the molting hormone 20-hydroxyecdsone (20E) (Wing, 1988; Retnakaran *et al*., 1995; Sundaram *et al*., 1998; Dhadialla *et al*., 1998). And the most important period in insect development was the molting period under strict endocrine system (Nijhout, 1994). Due to the hormonal activity, the high mortality is possible when pupated and emerging midge was especially affected. Nevertheless some researches reported no lethal effects in a variety of aquatic invertebrates below 3.5 mg L^{-1} (Kreutzweiser *et al*., 1994) and observed no toxic effects in tadpoles of four amphibian species below 5 mg L^{-1} treatments (Pauli *et al*., 1999).

As provided by Fig. 1, the mortality of *C. flaviplumus* and *C. riparius* increased in a dose-dependent manner with increasing concentrations of tebufenozide. The experiment could be regarded as valid, as the dead larvae of the control group was a statistically significant difference from the treatment groups (Fig. 1). Also, the mortality of *C. riparius* clearly increased based on exposure times (or days) from Day 1 to Day 3, and that of *C. flaviplumus* relatively slow extended (Fig. 2). *C. flaviplumus* increased abruptly the mortality from Day 4 to Day 6. These differences of the mortality between *C. flaviplumus* and *C. riparius* decreased from Day 7 to Day 10, however, the younger stage of *C. riparius* was continuously dead during test periods. Therefore, the development stages of test organisms were a

important factor for the hormonal disruption researches.

In terms of the pupal stages and emergence accidents, *C. flaviplumus* originated the polluted stream not considered less sensitive than *C. riparius*. In this study the fourth-instar larvae of *C. flaviplumus* were affected the molting process in the rates of dead pupae (Table 2). As discussed before, a large number of of *C. flaviplumus* did not survive the pupal stage and emerged adults. Because the field species not completely accommodated in the laboratory conditions and tebufenozide prevented dopa decarboxylase (help the molt in preparation for sclerotization in the epidermis) at the end of the molt (Hopkins and Kramer, 1992; Retnakaran *et al*., 1995), the fourth-instar larvae of *C. flaviplumus* frequently exhibited dead larvae and rarely showed the pupal stages, and a little pupal individuals arrived adults.

Relatively low concentrations such as 10 μg L^{-1} in this study observed retardation of developments (Last observed day in Table 1). Already chronological postponements of contaminations and effects have been reported (Liess and Schulz, 1996). The fatal effects showed dead larvae in high concentrations and observed the process of emergence in relative low concentration in this study. The fourth-instar larvae of *C. riparius* showed less susceptibility of insecticide than first -instar larvae (Hahn *et al*., 2001). The comparable test species, *C. flaviplumus* was observed the midges' pupal phase expected to be a most hormone-sensitive stage, was more a tolerance to tebufenozide than first instar larvae of *C. riparius*. Most pupae did not observed any phenotypic abnormalities. The effects of tebufenozide in two species found head capsule abnormalities and disruptions of the cuticle-forming process by tebufenozide. Similar abnormalities were reported (Retnakaran *et al*., 1995; Song *et al*., 1997; Hahn *et al*., 2001) and aquatic insects at late nymphal stage caused various morphogenetic anomalies and led to death the final molt (Grenier and Grenier, 1993).

ABSTRACT

The effects of the ecdysteroid agonist tebufenozide on the larvae of *Chironomus flaviplumus* and *Chironomus riparius* were tested in the laboratory. Employing a static exposure setup, chironomids were subjected to various tebufenozide concentrations. In the most treatments it reached a statistically significant difference from the control condition. As the concentration of tebufenozide was increased, a relatively larger proportion of the observed mortality was associated with the metamorphosis and molting process. The larval mortality of *C. riparius* was high in *C. flaviplumus* during over 30 μg L^{-1} treatments. In terms of development, the effects of tebufenozide were delayed growth stage in relatively lower concentration such as 10 μg L^{-1} tebufenozide treatments. The rates of succeed adult through the molting process were various in treated concentrations or/and the species.

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