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Lethal and Sublethal Toxicities of Bisphenol A and Ethynyl Estradiol Exposure on the 4th Instar Larvae of *Chironomus riparius* (Diptera, Chironomidae)

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비스페놀 에이와 에티닐에스트라디올에 노출된 키로노무스 4기 유충의 치사, 아치사 독성연구

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요 약

본 연구에서는 내분비계장애물질로 잘 알려진 Bisphenol A와 Ethynyl estradiol (이하 BPA, EE)이 *Chironomus riparius*에 미치는 영향을 알아보고자 하였다. 수생생태계에서 *Chironomus*의 생태학적 중요성을 고려하여 연구한 반수치사농도(LC50), 효소 활성, 성장/발달 등의 생화학적, 생리학적 독성에 대한 연구는 이들 물질에 대한 잠재적인 생태학적 중요성을 이해하는데 있어 유용한 정보를 제공할 수 있을 것이다. 연구 결과 BPA와 EE에 노출된 후 *C. riparius*에서 CAT와 GST 활성이 증가하였고, EE에 고농도로 노출된 후 성충까지 충분히 성장하지 못했음을 확인하였다. 이러한 결과로부터 *C. riparius*에서 BPA, EE에 대한 중요한 독성정보를 얻을 수 있었다. 그러므로 앞으로 BPA와 EE의 생화학적 효과와 *C. riparius*의 개체수준 이상에서의 생리학적 실험을 통한 검증이 필요할 것이다.

Key words : *Chironomus riparius*, Bisphenol A, ethynyl estradiol, endocrine disruptors, toxicity

INTRODUCTION

The water contaminants bisphenol A (BPA) and ethynyl estradiol (EE), well known endocrine disruptors frequently found in freshwater, have recently attracted attention. BPA is an intermediate in the production of polycarbonate and epoxyresins (Staples *et al*., 1998), and EE is a synthetic estrogen used as a female contraceptive (Purdom *et al*., 1994). They are known to elicit estrogenic responses in fish via interaction with the cellular receptor, and have been reported to be present in surface waters at concentrations well beyond those known to cause endocrine disruption (Larsson *et al*., 1999). In recent years, the association of altered hormonal regulation in humans and wildlife with their exposure to endocrine-dis-

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rupting chemicals has led to increasing public and scientific concern (Colborn *et al*., 1996).

The aquatic larvae of nonbiting midges (*Chironomidae, Diptera*) are globally distributed, and these nonbiting midges are the insects most often found in freshwater ecosystems. They play an important role in the aquatic food chain and are a major food source of fish and other vertebrates, as well as invertebrates (Cranston, 1995). They are easy to culture, are sensitive to many pollutants, and have a short life cycle (Ingersoll and Nelson, 1990). As such, they are widely used in ecotoxicity tests (Kahl *et al*., 1997; Matthew and David, 1998; Choi *et al*., 2000, 2002; Matthew *et al*., 2001; Bettinetti *et al*., 2002; Crane *et al*., 2002). Taking into account the frequent presence of BPA and EE in surface waters and the ecological importance of *Chironomus* larvae in freshwater, this study on the effects of BPA and EE on *C. riparius* can provide information that may prove to be valuable for the biomonitoring or risk assessment of the aquatic ecosystem.

In this study, to investigate the effects of BPA and EE on *C. riparius*, LC50 was determined and was chosen as the acute toxicity indicator and the biochemical/physiological parameters of *C. riparius* were investigated after their sublethal exposure to BPA and EE. Among the biochemical parameters, the chemical effects of the two compounds on enzyme activities were focused on. Catalase (CAT), peroxidase (PX), and glutathione peroxidase (GPX) were studied as oxidative stress markers. Glutathione S-transferase (GST) and acetylcholinesterase (AChE) were measured as detoxification and neurotoxicity indicators, respectively. As for the physiological responses, growth and development were chosen and studied. The dry body weights of the larvae were measured and were chosen as the growth indicators, whereas successes in pupation and in adult emergence as well as the total emergence times were examined as descriptors of development. The adult sex ratio was also studied to identify any potential difference between male and female susceptibility to these compounds.

MATERIALS AND METHODS

Organisms

Using an original strain provided by the Toxicology Research Center of the Korea Research Institute of Chemical Technology (Daejeon, Korea), *Chironomus riparius* larvae were obtained from adults reared in the laboratory. The larvae, which were fed Tetramin®, were reared within a 16 hr to 8 hr light-dark photoperiod, at room temperature (20 ± 1°C), in a 2 L glass chamber containing dechlorinated tap water and aerated acid-washed sand.

Exposure conditions

The effects of BPA and EE exposure on the groups of the 4th instar larvae collected from the rearing aquaria were assessed. At the beginning of the experiment, 1 mL of an acetonic solution of the compounds was placed in the experimental 1 L tanks. The exposure was carried out at a constant temperature (20 ± 1°C) and within a photoperiod of 16 : 8 hrs (light : dark) in all the experiments. The enzyme activities and growth indicators in three sublethal concentrations (1, 10, and 100 μg/L) of the compounds were studied. Triplicate beakers were designated for 48 hrs growth measurements, each ten 4th instar larvae were added at random to each test aquarium. And fifty 4th instar larvae were randomly introduced into each test aquarium in same concentrations at the adult emergence rate test during twenty days.

Acute-toxicity test

A group of each ten larvae was exposed to 1, 10 and 100 μg/L of BPA and EE, and the others were made control groups. It was determined that acute toxicity occurs after 24 hr exposure, and that the condition eventually leads to death. Log probit data transformation was used to estimate the 24 hr LC50 values and the corresponding 95% confidence intervals (data not shown).

Enzyme activities

A total of ten larvae were collected from the control and experimental tanks 24 hrs after these were treated with the compound solution. The larvae were then pooled for enzyme activity measurements. They were homogenized in 2.5 mL Tris EDTA buffer (40 mM, pH 7.8; Sigma Aldrich, Saint Quentin Fallavier, France) with the use of a Potter Elvehjem homogenizer. Crude homogenate was centrifuged for 15 min at 500 g (4˚C), and supernatant was centrifuged for 30 min. at 12,000 g (4˚C). The resulting supernatant (a post-mitochondrial fraction) was used to measure the enzyme activities. The rate of H_2O_2 disappearance (measured at 240 nm) was used to quantify the catalase activity (Beers and Sizer, 1952). The total peroxidase activity was measured through a guaiacol test (George P, 1953). The method described by Paglia and Valentine (Paglia and Valentine, 1967) was employed to determine the GSH-Px activity, whereas the GST activity was assessed spectrophotometrically through the measurement of glutathione-CDNB (1-chloro-2, 4-dinitrobenzene) conjugate production (Habig *et al*., 1974). The AChE activity was measured with the use of the method introduced by Elmann *et al*. (1961). The enzyme activities were calculated relative to the protein contents of the extracts, which were measured with the use of the Bradford method (Bradford, 1976).

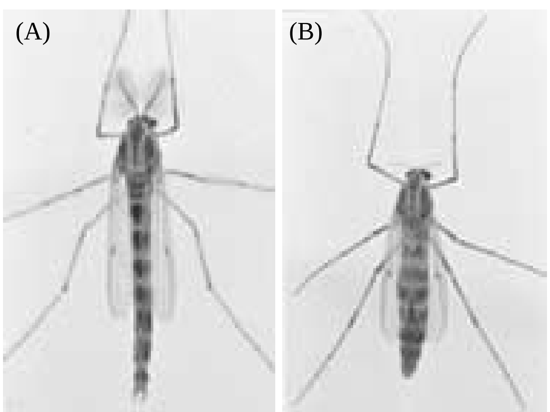

Fig. 1. The images of male (A), female (B) on *C. riparius*.

Water content and dry body weight measurement

The water contents and dry body weights of the ten larvae collected after 48 hrs of exposure to the compounds were measured. The fresh weights were immediately measured. The dry body weights of the larvae were also measured after they were exposed to a temperature of 105˚C for 24 hrs, and the water contents were calculated from the respective dry and fresh weights of the larvae. The weights were rounded off to the nearest 0.1 mg.

Adult emergence rate

For the measurement of the adult emergence rate, fifty of the 4th instar larvae were introduced at the beginning of the experiment. The emerging adults were retained with the use of wood cages covered with steel-wire mesh until the emergence had been completed in the control and experimental aquaria. As the endpoints of the toxicity tests, the numbers of the emerged adults from each vessel were counted and their sexes were determined. The two sexes could be easily distinguished from each other based on the forms and lengths of their antennae and abdominal terminalia (Fig. 1). Upon emergence, each chironomid was sex determined on the basis of the presence of plumose or hairy antennae in males (Day *et al*., 1994). In addition, the dead pupae were counted and the length of time it took for the emergence to be completed was also taken note of. Every two days, 50 mg of Tetramin fishfood flakes was supplied to each aquarium. The test solutions were not renewed. All the data were recorded at daily intervals.

Chemicals

BPA and EE were purchased from Sigma (Sigma Corp., St. Louis, MO, USA).

Data analysis

Statistical differences between the control and treated larvae were examined with the aid of a parametric *t* test using SPSS 12.0KO (SPSS Inc., Chicago, Il, USA). An alpha level of 0.05 was used to determine significance in all statistical analyses.

RESULTS AND DISCUSSION

This study was designed as a short-term experiment under controlled laboratory conditions, using nominal concentrations of BPA and EE, to demonstrate these compounds' toxic effects on *C. riparius*. Taking into account the ecological importance of *Chironomus* in the aquatic ecosystem, this study on the effects of BPA and EE, emerging water contaminants, on *C. riparius* can provide information that may prove to be useful in determining the potential ecological consequences of these compounds.

As for the biochemical parameters, the enzyme activities in the 4th instar larvae of *C. riparius* that were exposed to BPA or EE for 24 hrs were measured (Fig. 2). The CAT activity increased all the concentrations of EE. The PX activity decreased at 10 μg/L of BPA exposure and increased at 10 and 100 μg/L of EE exposure. No significant change was observed in the GPX and AChE activities. The GST activity increased after exposure to 1 and 10 μg/L BPA and after exposure to 10 and 100 μg/L EE.

Chironomus seems to have an efficient biochemical defense mechanism, which may contribute to the organism's tolerance of various environmental stresses, including pollution. Previous studies have shown that enzymatic radical scavengers, including superoxide dismutase (SOD), CAT, PX, and GPX could be developed as non-stressor-specific biomarkers in *C. riparius* larvae (Choi *et al*., 1999, 2000). Various biochemical parameters measured in *C. riparius*

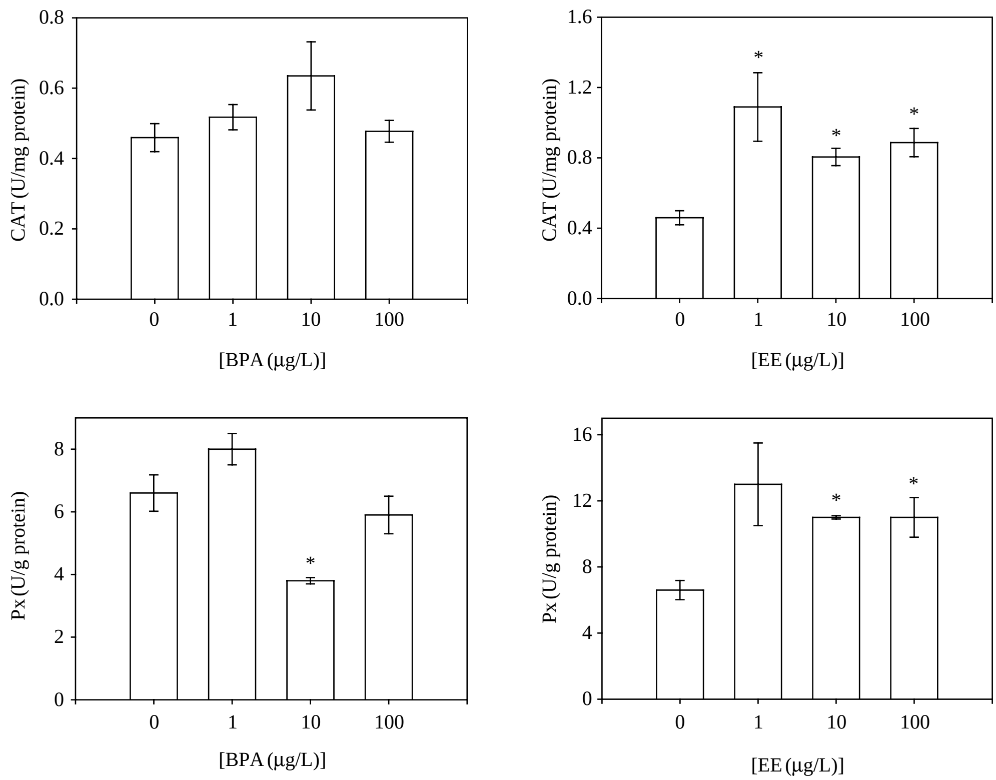

Fig. 2. Enzymes activities measured in the 4th instar larvae of *C. riparius* exposed to sublethal concentration of BPA and EE for 24 h (n=3, mean ± SEM, *$p < 0.05$).

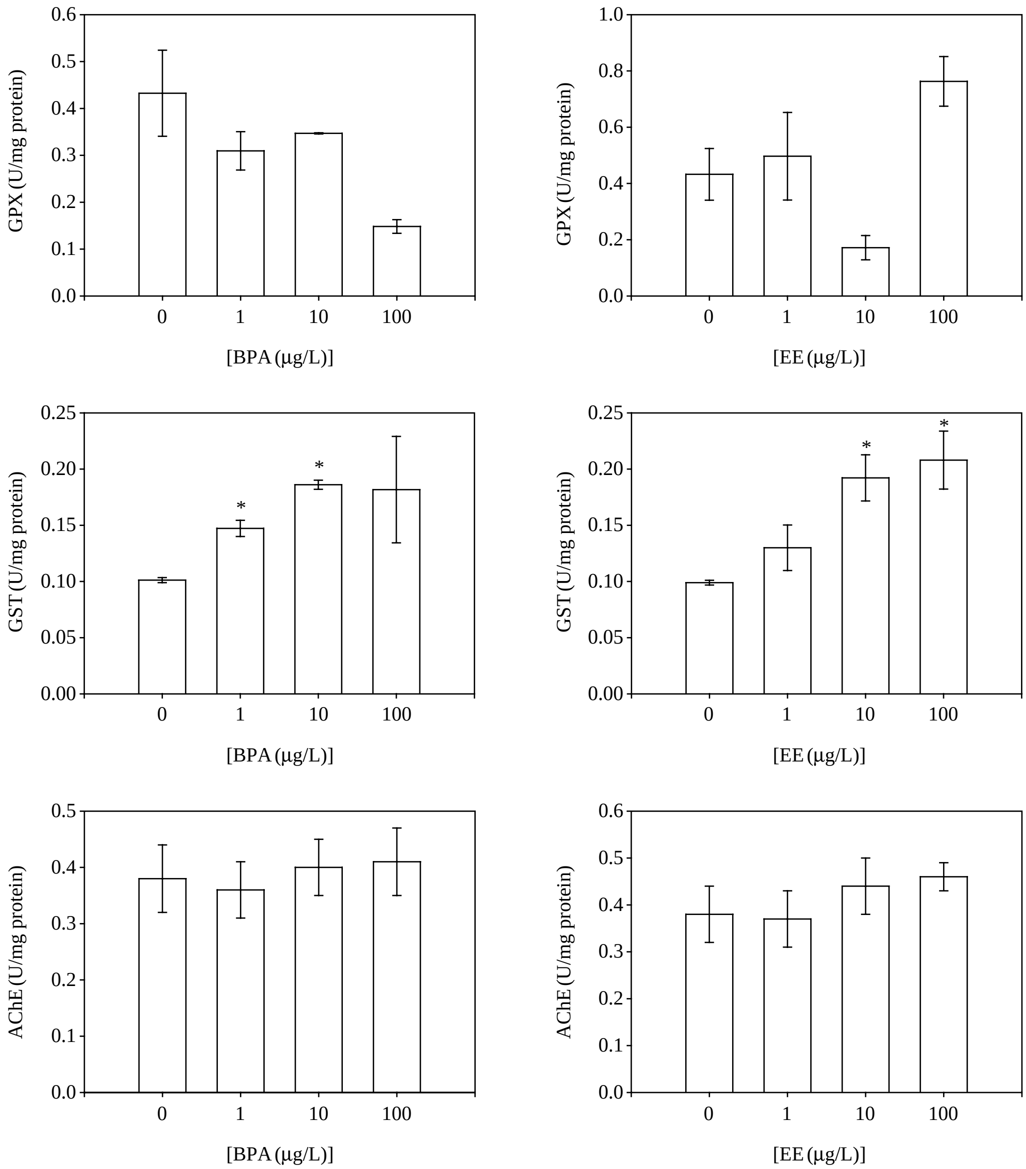

Fig. 2. Continued.

larvae, such as AChE, Cu,Zn-SOD, GST, electron transport system (ETS), and energy yielding substrates, have already demonstrated a high sensitivity to environmental pollutants (Choi *et al*., 2000, 2001; Olsen *et al*., 2001). In this study, five enzymes were investigated as biochemical parameters, and among them, the CAT activities seemed the most sensitive in both compounds. The homeostatic responses of CAT seem to have little impact at higher levels of biological organization. An increase in CAT at all of EE concentrations occurred concomitantly with PX activation, which may reflect an adverse effect of the exposure, since perturbations of the physiological parameters (i.e., increase in emergence failure) were observed at these concentrations. But decrease in PX except for 1 μg/L that may be considered a home-

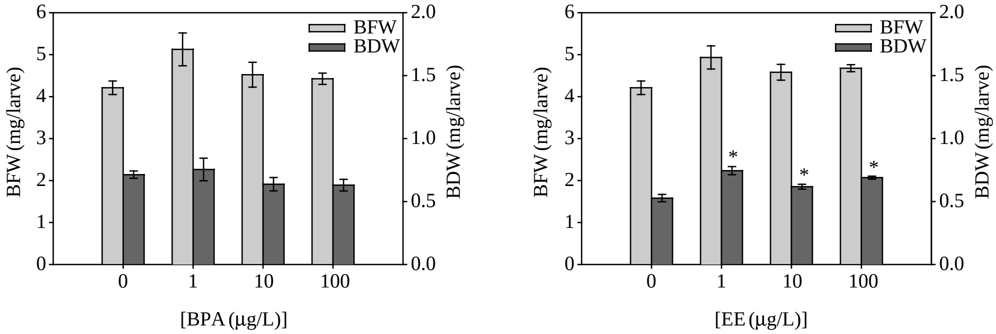

Fig. 3. Dry and fresh body weights measured in the 4th instar larvae of *C. riparius* exposed to sublethal concentration of BPA and EE for 24 h (n=3, mean±SEM, $*p<0.05$).

ostasis-maintaining process rather than an indicator of the permanent adverse effects of these compounds. The increased GST activity after BPA and EE exposure can be attributed to the possible involvement of the GSH conjugation pathway in the detoxification/metabolism of these compounds. The AChE activity did not change after BPA and EE exposure, which suggests that neurotoxicity may not be important in the toxicity of these compounds in *C. riparius* larvae. The LC50 of BPA and EE on *C. riparius* was determined to be around 6～9 mg/L while the sublethal effects (we are not shown), especially the biochemical effects, produced with the 10^7 fold lower concentrations of 24 hr LC50, suggest that the biochemical parameters that were studied, such as the CAT activity, may have considerable potential as an early warning signal of chemical stress in *C. riparius*.

As growth indicators, the dry body weights and fresh body weights of the *C. riparius* larvae were measured after a 48 hr exposure to BPA or EE (Fig. 3). An increase in dry body weights was observed after exposure to 1, 10, and 100 μg/L of EE. No significant change was observed in fresh body weights.

The increase in dry-body weights and emergence failure that occurred after EE exposure, we suggest that this chemical may perturb the physiological processes of *C. riparius*. The increase in dry body weights is difficult to explain, but it may be attributed to a decrease in larval water content, which is eventually related with the alteration of the larval osmoregulation process. The ash free dry body weights must be measured to determine the actual larval growth status.

BPA, EE induced pupation, and emergence failure in *C. riparius* were studied (Fig. 4). EE seemed to induce emergence failure since, compared to the control group, a significant increase in the emergence failure rate was observed after exposure to high concentrations of EE. A decrease in the emergence rate, an indicator of animal development, after exposure to the highest concentration of EE suggests that the alteration of this parameter may be considered the consequence of a serious progression of the toxic effect.

Among the emerged adults, males and females were identified to verify whether there is any difference between the two in terms of their susceptibility to BPA or EE exposure. No significant difference was found from group to group, except after exposure to 1 μg/L BPA, where a high male/female ratio was observed compared to the control group. The overall means did not appear to show any dose related effect on the emergence of the sex ratio. The high male/female ratio after exposure to 1 μg/L BPA can be attributed to the greater vulnerability of female adults to BPA exposure compared to the male adults. More conclusive evidences are needed, though, to support this hypothesis since this phenomenon was not observed after exposure to higher concentrations

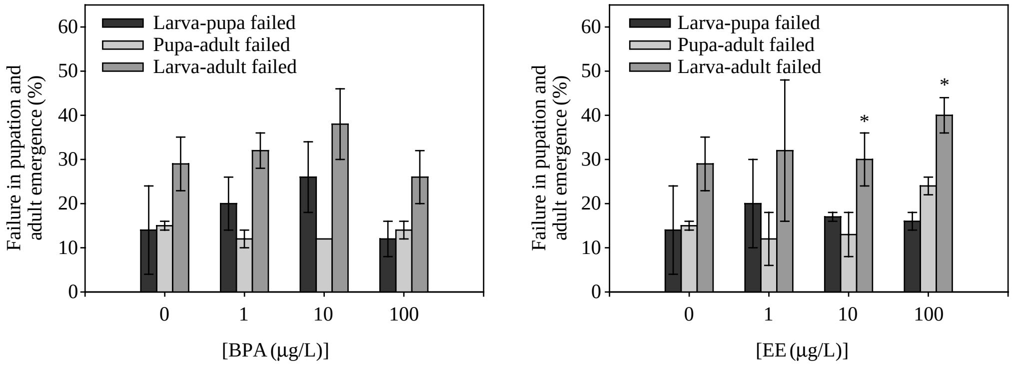

Fig. 4. Failure in pupation and adult emergence measured in sublethal concentration of BPA and EE exposed *C.riparius* (means ± SEM, *$p < 0.05$). Results are expressed as percentage of the total number of larvae introduced at the beginning of the experiment.

of BPA.

Watts *et al.* (2001) showed that a chronic sediment test of BPA and EE on *C. riparius* revealed no consistent relationship between their effects on development and reproduction on one hand and chemical concentration on the other. The results of the present study showed CAT and GST activation after BPA and EE exposure, and increased emergence failure after EE exposure. These data, however, are not sufficient to conclude that there is a correlation or casual relationship between them. Thus, direct experimental demonstrations of the wider relationships between the biochemical effects of BPA and EE exposure and their consequences at higher levels of biological organization, which is an ongoing project of these authors' laboratory, are needed to fully understand the effects of these compounds on *C. riparius* in particular and on the aquatic ecosystem in general. The characterization of the causal relationships between the biomarker responses of *C. riparius* and the effects of their BPA and EE exposure at higher biological levels will help define the sublethal hazards of chemicals in this animal.

In freshwater ecosystems, a complex mixture of pollutants frequently causes chemical pollution. This makes it considerably more difficult to predict the pollutants' effects and emphasizes the need for studies on multiple biological endpoints to be conducted to identify pertinent biomarkers. The simultaneous measurement of various biological parameters will allow data to be obtained at different levels of biological organization and may help bring about a full understanding of the effects of a toxicant on organisms.

CONCLUSIONS

In this study, to investigate the short-term effects of BPA and EE on *C. riparius*, the biochemical and physiological parameters of *C. riparius* were investigated after their sublethal exposure to BPA and EE under controlled laboratory conditions. Taking into account the ecological importance of *Chironomus* in the aquatic ecosystem, this study on the effects of BPA and EE, emerging water contaminants, on *C. riparius* can provide information that may prove to be useful in bringing about a full understanding of the potential ecological consequences of these compounds. The results of the present study showed CAT and GST activation after BPA and EE exposure as well as increased emergence failure after EE exposure. These data, however, are not sufficient to establish any correlation or casual relationship between BPA and EE on one hand and *C. riparius* on the other. Thus, further research is required to come up

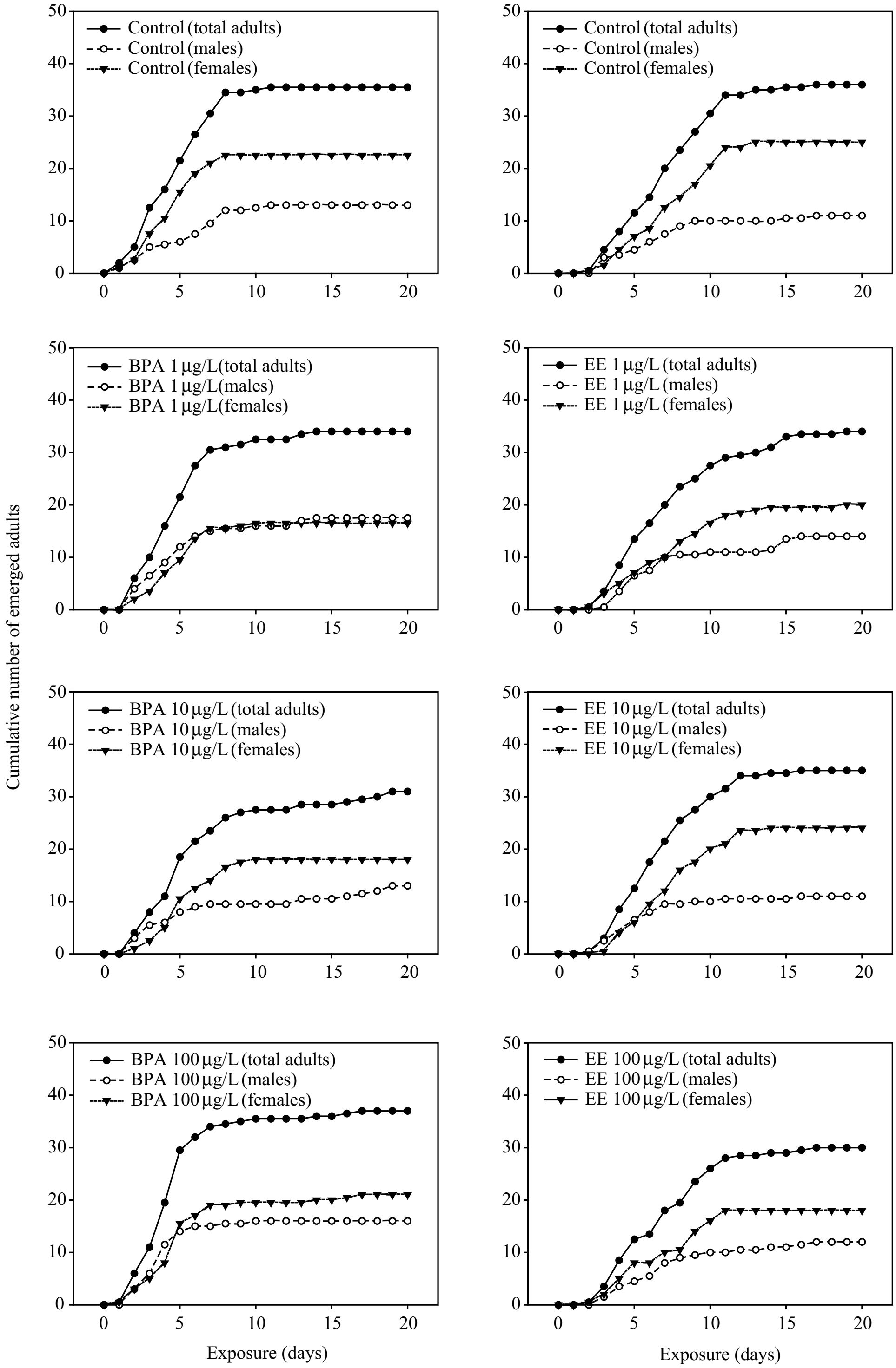

Fig. 5. Cumulative emergence measured in BPA and EE exposed *C.riparius*. Results are expressed as percentage of the total number of larvae introduced at the beginning of the experiment.

with direct experimental demonstrations of the wider relationship between the biochemical effects of the two compounds on *C. riparius* and their consequences at higher levels of biological organization.

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REFERENCES

Bettinetti R, Cuccato D, Galassi S and Provini A. Toxicity of 4-nonylphenol in spiked sediment to three population of *Chironomus riparius*, Chemosphere 2002; 46(2): 201-207.

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding, Anal Biochem 1976; 72: 248-254.

Choi J, Roche H and Caquet T. Effects of physical (hypoxia, hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme activities in *Chironomus riparius* Mg. (Diptera, Chironomidae) larvae: potential biomarkers, Environ Toxicol Chem 2000; 19(2): 495-500.

Choi J, Caquet T and Roche H. Multilevel effects of sublethal fenitrothion exposure in *Chironomus riparius* Mg. (Diptera, Chironomidae) larvae, Environ Toxicol Chem 2002; 21(12): 2725-2730.

Colborn T, Myers JP and Dummanoski D. Our Stolen Future, Little Brown and Co, Boston. 1996.

Crane M, Sildanchandra W, Kheir R and Callaghan A. Relationship between biomarker activity and developmental endpoints in *Chironomus riparius* Meigen exposed to an organophosphate insecticide, Ecotoxicol Environ Saf 2002; 53(3): 361-369.

Day K and Scott I. Use of Acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphorus pesticides, Aquat Toxicol 1990; 18(2): 101-113.

Day KE, Kirby R and Reynoldson TB. Sexual dimorphism in *Chironomus riparius* (Meigen): impact on interpretation of growth in whole-sediment toxicity tests, Environ Toxicol Chem 1994; 13: 35-39.

Detra R and Collins W. The relationship of parathion concentration, exposure time, cholinesterase inhibition and symptoms of toxicity in midge larvae (Chironomidae: Diptera), Environ Toxicol Chem 1991; 10: 1089-1095.

Ellman GL, Courtney KD, Andres V Jr and Feather-stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem Pharmacol 1961; 7: 88-95.

George P. Intermediate Compound formation with peroxidase and strong oxidizing agents, J Biol Chem 1953; 201(1): 413-426.

Habig WH, Pabst MJ and Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acides formation, J Biol Chem 1974; 249(22): 7130-7139.

Ibrahim H, Kheir R, Helmi S, Lewis J and Crane M. The effects of organophosphorus, carbamate, pyrethroid and organochlorine pesticides and a heavy metal on survival and cholinesterase activity of *Chironomus riparius* (Meigen), Bull Environ Contam Toxicol 1998; 60(3): 448-455.

Ingersoll C and Nelson MK. Testing sediment toxicity with *Hyalella azteca* (amphipod) and *Chironomus riparius* (Diptera). In: Landis W, Van der Schalie W (Eds.), Aquatic Toxicology and Risk Assessment. American Society for Testing and Materials, Philadelphia, 1990; 13: 93-109.

Kahl MD, Makynen EA, Kosian PA and Ankley GT. Toxicity of 4-nonylphenol in a life-cycle test with the midge *Chironomus tentans*, Ecotoxicol Environ Saf 1997; 38 (2): 155-160.

Karnak RE and Collins WJ. The susceptibility to selected insecticides and acetylcholinesterase activity in a laboratory colony of midge larvae, *Chironomus tentans* (Diptera: Chironomidae), Bull Environ Contam Toxicol 1974; 12(1): 62-69.

Kosalwat P and Knight AW. Chronic toxicity of copper to a partial life cycle of the midge, *Chironomus decorus*, Arch Environ Contam Toxicol 1987; 16: 283-290.

Lasson DGJ, Adofsson-Erici M, Parkkonen J, Petterson M, Berg AH, Olsson PE and Forlin L. Ethinylestradiol-an undesired fish contraceptive, Aquat Toxicol 1999; 45(2-3): 91-97.

Matthew MW and David P. Selection of an appropriate life cycle stage of *Chironomus riparius* meigen for use in chronic sediment toxicity testing, Chemosphere 1998; 36(6): 1405-1413.

Matthew MW, David P and Kathleen C. Chronic exposure to 17α-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diphtera: Chironomidae), Aquat Toxicol 2001; 55(1-2): 113-124.

Paglia DE and Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathion peroxidase, J Lab Clin Med 1967; 70(1): 158-169.

Pascoe D, William KA and Green DWJ. Chronic toxicity of cadmium to Chironomus riparius Meigen-effects upon larval development and adult emergence, Hydrobiologia 1989; 175(2): 109-115.

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR and Sumpter J. Estrogenic effects of effluents from sewage treatment works, Chem Ecol 1994; 8: 275-285.

Staples CA, Dorn PB, Klecka GM, O'Block ST and Haris LR. A review of the environmental fate, effects and exposures of bisphenol A, Chemosphere 1998; 36(10): 2149-2173.

Taylor EJ, Blockwell SJ, Maund SJ and Pascoe D. Effects of lindane on the life-cycle of a freshwater macroinvertebrate *Chironomus riparius* Meigen (Insecta: Diptera), Arch Environ Contam Toxicol 1993; 24: 145-150.