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Chemical-induced alteration of hemoglobin expression in the 4th instar larvae of *Chironomus tentans* Mg. (Diptera: Chironomidae)

Mi-Hee Ha, Jinhee Choi *

Faculty of Environmental Engineering, College of Urban Science, University of Seoul, 90 Jeonnong-dong, Dongdaemun-gu, Seoul 130-743, Republic of Korea

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Abstract

In this study, a preliminary characterization of the multiplicity of *Chironomus* hemoglobin (Hb) was conducted on the larvae of *Chironomus tentans* Mg. (Diptera: Chironomidae) by the molecular mass and isoelectric point. In order to identify *Chironomus* Hb as potential biomarker of environmental contamination, alteration of individual Hb by exposure to environmental chemicals, namely, nonylphenol, benzo[a]pyrene, chloropyriphos and cadmium chloride, were evaluated. To validate the ecotoxicological relevance of *Chironomus* Hb as a potential biomarker, ecotoxicity test using growth rate as toxic endpoint was also conducted. This study revealed a striking heterogeneity in *C. tentans* Hb; 10 Hb isoforms were observed in the larvae of 4th instar *C. tentans* by their molecular mass and by their isoelectric point. Chemical-induced alteration of individual Hb expression suggests that *C. tentans* Hb can be a target molecule for chemical exposure. Overall results suggest that the expression pattern of Hb proteins may potentially contribute to the development of a biomarker for ecotoxicity monitoring in *C. tentans*.

Keywords: *Chironomus tentans*; Hemoglobin multiplicity; Environmental contaminants; Ecotoxicity

1. Introduction

The hemoglobin (Hb) of invertebrates is responsible for essentially the same function as those of vertebrates: carrying oxygen from the environment to the respiring tissues. Remarkably, in some invertebrate, the Hb is more than an order of magnitude larger in molecular mass than the tetramers usually found in vertebrates. One of the most well-studied and interesting invertebrate Hb is *Chironomus* Hb, which is synthesized in the larval fat body and is then secreted into the hemolymph (Bergtrom et al., 1976; Saffarini et al., 1991). Chironomids are globally distributed and they are the most abundant group of insects found in fresh water ecosystems, which therefore places them in an important position in the aquatic food chain and thus, they are extensively used in the assessment of the acute and sublethal toxicity of contaminated sediments and water (Matthew et al., 2001; Bettinetti et al., 2002; Crane et al., 2002; Lee and Choi, 2006).

In comparison to the tetrameric mammalian Hb, these insect Hb exist as monomers or dimers (Das and Handique, 1996) and exhibit a high O_2 affinity and a large variation of the Bohr effect (Hundahl et al., 2006). A high degree of polymorphism is one of the most interesting features of *Chironomus* Hb (Osmulski and Leyko, 1986), and the polymorphism of *Chironomus* Hb has been found in many species investigated so far (Fukuda et al., 1993; Kao et al., 1994, 1995; Gruhl et al., 1997; Hankeln et al., 1997). The hemolymph of the larvae of the dipteran insect *Chironomus* contains a complex mixture of free Hb. Although Hb multiplicity is a well-known phenomenon in Chironomid species, few have been reported in *C. tentans.*

The aims of the present study are, first, the characterization of the multiplicity of the Hb in *C. tentans* Mg. (Diptera: Chironomidae) by the molecular mass and by the isoelectric point (p*I*), and second, the evaluation of toxicity of environmental contaminants on this molecule. In this study, the preliminary characterization of the Hb in *C. tentans* was conducted using polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) analyses and the changes in *Chironomus* Hb by exposure to four environmental chemicals with different modes of action were evaluated in protein and mRNA levels. These chemicals were

* Corresponding author. Tel.: +82 2 2210 5622/5643; fax: +82 2 2244 2245.
E-mail address: jinhchoi@uos.ac.kr (J. Choi).

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nonylphenol (NP), benzo[a]pyrene (B[a]P) chlorpyriphos (CP), and cadmium chloride (Cd); NP is used in the polymer industry (EC, 2002), B[a]P is a ubiquitously distributed PAH (Juhasz and Naidu, 2000), CP is an organophosphorous insecticide, and Cd are common heavy metals. Acute toxicity using mortality as an endpoint was conducted to determine the sublethal exposure condition for Hb analysis. Conventional ecotoxicity test, using growth rate as toxic endpoint, was performed to validate the ecotoxicological relevance of *Chironomus* Hb as a potential biomarker for environmental contamination.

2. Materials and methods

2.1. Organisms

Using an original strain provided by the Toxicology Research Center of the Korea Research Institute of Chemical Technology (Daejeon, Korea), *C. tentans* larvae were obtained from adults reared in our laboratory. The larvae, fed fish flake food (Tetramin, Tetrawerke, Melle, Germany), were reared in a 2 L glass chamber, containing dechlorinated tap water and acid-washed sand, with aeration under a 16-h light:8-h dark photoperiod at room temperature ($20 \pm 1\,^{\circ}C$).

2.2. Exposure conditions

Hemoglobin analysis was conducted using groups of the 4th instar larvae collected from the rearing aquaria. All larvae used in the experiment originated from the same egg mass, and were collected at the same period after egg hatching in order to obtain an age-synchronized population. From our previous experiments, 4th instar development of *C. tentans* larvae persisted for 22–38 days after the eggs had hatched (Lee et al., 2006), and the measurements of the cellular and extracellular proteins were conducted on the larvae in the middle of the 4th instar development (i.e. 32 days after the eggs had hatched). For the chemical treatment, 10 of the 4th instar *C. tentans* larvae were transferred into 200 mL beakers containing 100 mL of dechlorinated tap water, and treated with chemicals to assess sublethal exposure. Sublethal exposure concentrations were selected from the results of an acute toxicity test. For each experiment, 0.1 mL of the test solution was added to the experimental beakers prior to the introduction of larvae. Acetone was used as solvent for NP (Sigma–Aldrich Chemical, St. Louis, MO, USA), B[a]P (Sigma–Aldrich) and CP (Sigma–Aldrich) and water was used for Cd (Sigma–Aldrich). Our preliminary test indicated that acetone provoked no significant effects in any of the experiments (data not shown). Exposure was carried out under a constant temperature ($20 \pm 1\,^{\circ}C$), with a 16-h light:8-h dark photoperiod for all experiments. Three replicates experiments were used for each compound. Exposure concentrations were nominal values.

2.3. Acute toxicity test

Groups of 10 larvae were exposed to each compound, whereas other groups were kept as a control. Acute toxicity was determined after 24 h of exposure, using death of individuals as an endpoint. Log-probit transformation of the data was used in order to estimate 24 h LC10, LC50, and LC90 values and the corresponding 95% confidence intervals.

2.4. Non-denaturing polyacrylamide gel electrophoresis

Ten larvae from the control and experimental tanks were pooled, hemolymphs were withdrawn by opening the body wall, and the body fluids were subjected to electrophoresis. Non-denaturing PAGE was performed on hemolymph protein samples to determine the molecular weights of the hemolymph protein. After the electrophoresis, the gels were photographed and stained with Brilliant Blue G (Sigma–Aldrich). Molecular mass were determined in a non-denaturing system, using a non-denatured protein molecular mass marker kit (Sigma–Aldrich Chemical), based on modification to the methods developed by Davis (1964) and Bryan (1977). Briefly, the proteins were subjected to electrophoresis on a set of gels containing various polyacrylamide concentrations (from 5 to 12%) and the electrophoretic mobility (R_f) of the protein in each gel determined relative to the tracking dye; 100 Log ($R_f \times 100$) was plotted against the percentage gel concentration for each marker protein, with the slope of the plot taken as the retardation coefficient (K_R). From these plots, individual slopes (K_R) for each protein were determined, with the logarithm of the negative slope plotted against the logarithm of the molecular mass of each marker protein. This produced a linear plot from which the molecular weight of a *Chironomus* hemolymph protein could be determined. Following determination of the molecular mass of the Hb isoform in the control larvae, using the above-mentioned procedure, 12% gel was used for investigation of chemical effects on individual components of *Chironomus* Hb, due to the ease of identification of the individual Hb bands.

2.5. Non-denaturing electrophoreses of isoelectric focusing

Same procedure as PAGE was used for sample preparation. IEF in non-denaturing systems were used to characterize the *Chironomus* Hb, which allowed separation of the proteins according to their p*I*. As with the PAGE analysis, hemolymphs prepared from control and exposed larvae were used for IEF. The IEF was performed as described previously (Choi and Roche, 2004). Briefly, 12% acrylamide gel was prepared, with ampholyte mix (range of pH 3.5–10; Fluka, Buchs SG, Switzerland), and a pH gradient formed on the gel by ampholyte pre-migration of the gel for 20 min at 300 V. The electrophoresis was performed on non-denatured hemolymph protein samples for 3 h at 300 V. After the electrophoresis, the gels were photographed and stained with Brilliant Blue G (Sigma–Aldrich Chemical), and the p*I* determined from a standard curve prepared from marker proteins (range of p*I* 3.6–9.3; Sigma–Aldrich Chemical).

2.6. Gene expression analysis

The control and treated larvae were homogenized in 700 μL of TRI reagent (Molecular Research Center, Cincinnati, OH), and the RNA was isolated according to the manufacturer's standard protocol. The RNA, resuspended in 50 μL of water treated with diethyl pyrocarbonate (DEPC-H_2O), was quantified with the aid of a spectrophotometer (Thermospectronic, Rochester, NY, USA), and was stored at $-80\,^{\circ}C$ until further use. For the reverse transcription-polymerase chain reaction (RT-PCR), a two-step method, with RT Premix and PCR Premix kits (Bioneer, Seoul, Korea), was employed. Before the RT, 2 μg of total RNA and a random hexamer (Promega, Madison, WI, USA) were denatured at $70\,^{\circ}C$ for 5 min, and then rapidly cooled on ice. These solutions were added to the RT Premix kits, with the RT conducted at $42\,^{\circ}C$ for 60 min and at $94\,^{\circ}C$ for 5 min. These templates were then added to the PCR premix kit, containing the Hb primer, which was designed on the basis of sequences retrieved from GenBank™ (AJ003807; 5′-ATTCGCTGGAAAGGATGTTG-3′, 5′-TATGAGACGAGTGAGGCACG-3′). Actin mRNA (AB070370; 5′-GATGAAGATCCTCACCGAACG-3′, 5′-CCTTACGG ATATCAACGTCGC-3′) was served for normalization of the expression of globin mRNA level. Using a PTC-100 thermal cycler (MJ Research, Lincoln, MA, USA), 30 cycles of PCR were conducted at $95\,^{\circ}C$ for 1 min, $60\,^{\circ}C$ for 1 min, $72\,^{\circ}C$ for 1 min and finally at $72\,^{\circ}C$ for 7 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel (Promega) and visualized with ethidium bromide (Bioneer). All the tests were repeated at least three times, and the relative densities of each band were determined with the aid of an image analyzer, a Gel documentation system (TFX-20.M UV transilluminator) (Vilber Lourmat TFX-20.M, Marne la Vallee, France), with a Kodak 1D 3.6 camera (Kodak EDAS 290, Rochester, NY, USA).

2.7. Larval body weight measurement

Fresh body weight (FBW) was measured using a pool of 10 larvae of 4th instar at the beginning (0 h) and at the end of the exposure period (48 h) to calculate growth rate. Growth rate was then compared between treatments. Weighing was performed to the nearest 0.1 mg.

2.8. Statistics

Median lethal concentrations (LC50s) were derived through Probits analysis. Statistical differences between the control and treated larvae for all analyses were

examined with the aid of a parametric t test, using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA).

3. Results and discussions

3.1. Preliminarily characterization

Hb isoforms were preliminarily characterized by their molecular mass and p*I* in the 4th instar larvae of *C. tentans* (Fig. 1). The Hb of *C. tentans* was separated by non-denaturing PAGE and by IEF into 10 different components. As a red color was visible on the bands of the gel prior to the staining procedure, the properties of the 10 hemolymph proteins were presumed to be those of Hb (Fig. 1). However, two additional bands between 14.5 and 27.2 kDa, and between 27.2 and 33.8 kDa, which gave a positive result with blue staining, were not seen prior to staining, which probably were minor non-Hb proteins associated with *C. tentans* hemolymph. The range of the molecular mass of the 10 observed Hbs varied widely, from 4.76 to 155 kDa. However, seven Hb components, with molecular mass corresponding to 4.76, 6.42, 8.22, 10.0, 12.6, 13.6 and 14.5 kDa, seemed to be the major Hb, according to the examination of band densities, which were easily identified even prior to staining. The range of p*I* was from 2.3 to 9.2. Seven Hb components, with p*I* between 2.3 and 5.0, seem to be the major Hb, according to the examination of their band densities, and were easily identified even prior to staining.

As revealed in the results shown in Fig. 1, as with other *Chironomus* species, there was a striking heterogeneity in the larvae of the 4th instar *C. tentans*, by their molecular mass and by their p*I* (10 Hb isoforms). The pronounced heterogeneity of chironomid Hbs may be adaptive to exogenous and endogenous factors. However, the investigation of *Chironomus* Hb using PAGE and IEF analyses can only provide a preliminary characterization; therefore, 2-dimentional analysis (proteomics) may be needed for a more detailed identification of individual Hb. It appears that in some cases the presence of Hb is connected with an animals' resistance to extreme environments. Indeed, Hb possessing freshwater macroinvertebrates are usually known to be highly tolerant to environmental pollution (Osmulski and Leyko, 1986). A good supply of oxygen may help in the active and rapid removal of toxic compounds, by accelerating metabolic reactions. It is believed that Hb multiplicity in *Chironomus* may be related to their high level of tolerance to pollution. Therefore, the expression pattern of Hb proteins following chemical treatments was investigated.

3.2. Acute toxicity and the exposure condition

Prior to investigating the alteration on chemical-induced Hb protein, LC50s were estimated, in order to assess acute toxicity of four chemicals to *C. tentans* larvae, as well as to set sublethal concentrations levels for Hb analysis. Acute toxicity was studied using 24 h LC10, LC50 and LC90, derived through Probits analysis (Table 1). The 24 h LC50s of NP, B[a]P CP and Cd in *C. tentans* were 1.27, 9.87, 2.12 and 169 mg/L, respectively. The order of acute toxicity (LC50) in *C. tentans* was NP > CP > B[a]P ≫ Cd. In this study, *C. tentans* showed a high level of tolerance to Cd exposure. The LC50 of Cd was about 100–200-fold higher than that of NP or CP, and was almost 20-fold higher than that of B[a]P. The fact that Cd exhibits such a high level of tolerance compared with other compounds tested may imply that these organisms possess efficient defense equipment that prevent Cd-related damage (i.e., the existence of a specific protein that protects against Cd toxicity in *C. tentans*). Based on the results of the acute toxicity test, three sublethal concentrations – corresponding to 1/1000, 1/100, and 1/10 of the 24 h LC50 – were selected for the chemical-induced Hb expression analysis. These concentrations were 1, 10, and 100 μg/L for NP; 0.01, 0.1 and 1 mg/L

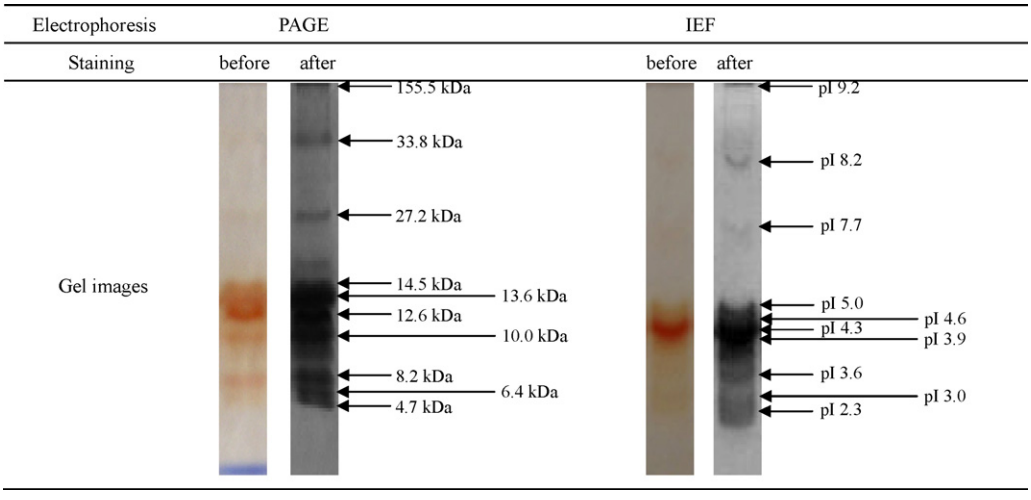

Fig. 1. Analysis of hemoglobin multiplicity in the 4th instar *Chironomus tentans* larvae by the molecular mass and by the isoelectric point. Non-denaturing polyacrylamide gel electrophoresis was performed on hemolymph protein samples, using 5–12% acrylamide gels, to determine the molecular masses of the hemolymph protein. The isoelectric focusing was performed on non-denatured hemolymph protein samples using 12% acrylamide gel. After the electrophoresis, the gels were photographed and stained with Brilliant Blue G. Molecular masses were determined in a non-denaturing system using a non-denatured protein molecular mass marker kit.

Table 1
Estimation of 24 h lethal concentrations in the 4th instar larvae of *Chironomus tentans*

Chemicals	LC10 (mg/L) (95% Confidence interval)	LC50 (mg/L) (95% Confidence interval)	LC90 (mg/L) (95% Confidence interval)
NP	0.736 (0.149–1.046)	1.271 (0.802–2.118)	2.195 (1.529–12.111)
B[a]P	5.982 (1.922–7.808)	9.873 (7.426–14.61)	16.29 (12.11–64.78)
CP	0.657 (0.051–1.219)	2.120 (1.103–5.125)	6.841 (3.444–148.1)
Cd	93.05 (0.172–128.0)	169.5 (119.4–1466)	308.80 (203.5–6843)

LC = lethal concentration; LC10 = 10% lethal concentration; LC50 = median lethal concentration; LC90 = 90% lethal concentration.

for B[a]P; 2, 20, and 200 μg/L for CP; and 0.2, 2, and 20 mg/L for Cd.

3.3. Hb protein and mRNA level expression

The effects of four environmental contaminants on Hb multiplicity were investigated on treated and control larvae using PAGE and IEF (Fig. 2). In PAGE gel, of the 10 Hb isoforms, expressions of Hb, of which molecular mass corresponded to 33.8, 27.2, 13.6 and 12.6 kDa proteins were affected by chemical exposure. The most significant increase was observed in Hb of which molecular mass corresponded to 12.6 kDa protein by NP and B[a]P exposure. The expression of Hb of which molecular mass corresponded to 13.6 and 12.6 kDa decreased in the larvae that had been exposed to the highest levels of CP. In the IEF gel, all four compounds tested induced an expression of Hb whose p*I* corresponded to 8.2 and 7.7 also increased by NP exposure.

Our result shows that chemical exposure induced the expression in selected Hb proteins (Fig. 2). Increased individual Hb protein synthesis, as revealed by PAGE and IEF (Fig. 2), seems to confer a high tolerance to some pollutants in *Chironomus* (Lindegaard, 1995), as no severe disturbance that could affect population fitness (i.e., reproduction and development) was observed at similar levels of exposure in our previous study (data not shown). The different sensitivity of Hb protein expression to chemical exposure suggests that *C. tentans* Hb consists of a mixture of inducible Hb and consecutively expressed forms. Also, Hb multiplicity may allow this animal to better adapt to stressful environmental conditions. These characteristics of *Chironomus* Hb may have a potential for the development of a biomarker for chemical exposure.

Along with the level of protein, the expression pattern of *Chironomus* Hb was also examined in the mRNA level of the 4th instar *C. tentans* larvae to investigate whether chemical exposures also affect the expression of the Hb transcript (Fig. 3). Decreases in globin mRNA level was observed in the larvae that have been exposed to B[a]P and to CP in concentration-dependent manners. Whereas, a high level of NP and Cd exposure induced globin gene expressions in the 4th instar *C. tentans* larvae.

It is speculated that *Chironomus* globin expression is regulated at both protein and mRNA levels, but there is no experimental evidence on the relationship between transcriptional and translation regulations. The expression of globin mRNA may be related to globin protein component identified by PAGE and

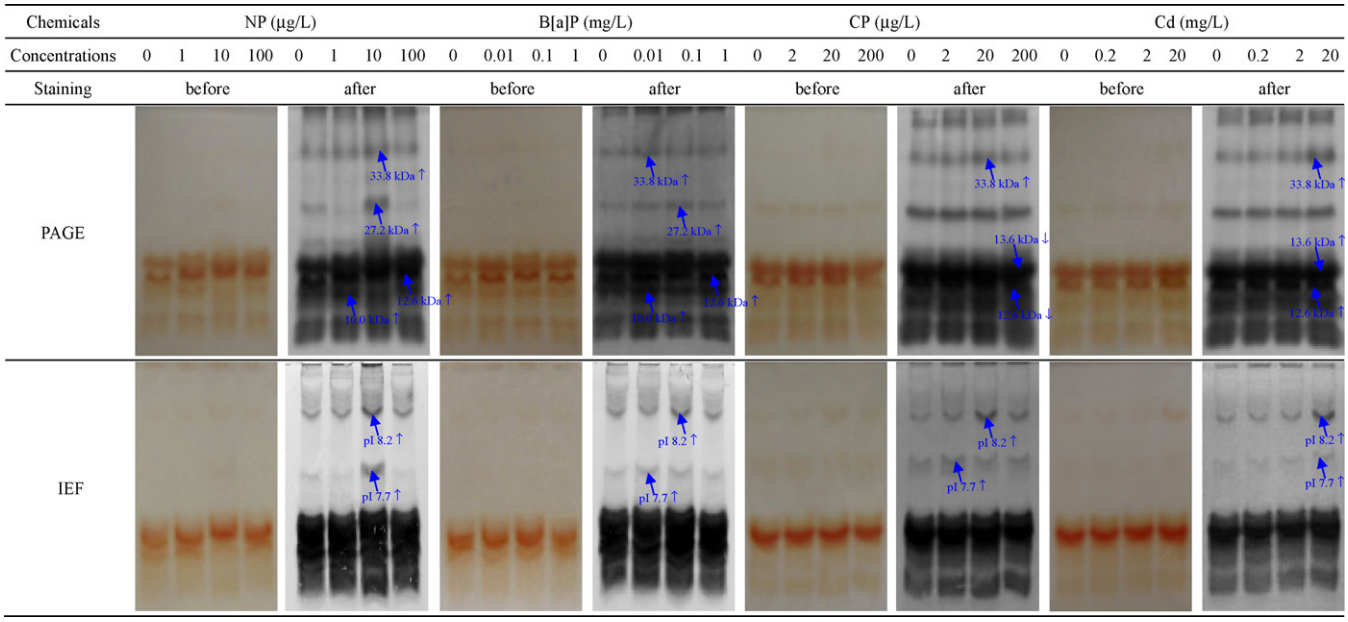

Fig. 2. Analysis of expression of hemoglobin protein by the molecular mass and by the isoelectric point in the 4th larvae of instar *Chironomus tentans* exposed to environmental chemicals for 24 h. Following determination of the molecular mass and isoelectric point of the Hbs in the control larvae, 12% gel was used for investigation of effects of chemicals on individual components of *Chironomus* Hb, due to the ease of identification of the individual Hb bands. After the electrophoresis, the gels were photographed and stained with Brilliant Blue G.

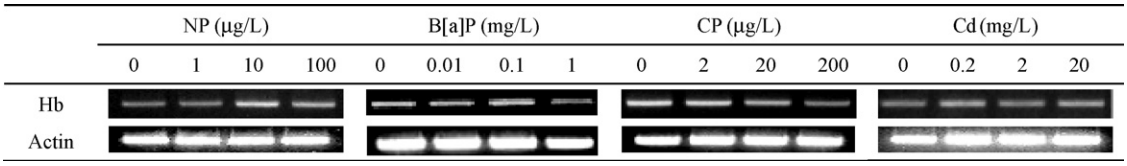

Fig. 3. Expression of hemoglobin genes in the 4th instar larvae of *Chironomus riparius* exposed to environmental chemicals for 24 h. Ten larvae from the control and experimental tanks were pooled and cellular fraction of control and treated larvae were homogenized and the RNA was isolated. Using a PTC-100 thermal cycler, PCR were conducted and the PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide.

IEF. Our results, however, are obtained from the experiment with only one globin open reading frame (ORF) in a semi-quantitative method; if more ORFs with strict quantitative analysis had been conducted, this could probably be better evaluated and explained.

3.4. Organism-level consequence

In order to validate the ecotoxicological relevance of Hb expression, organism-level response was investigated using larval growth rate as an endpoint, on *C. tentans* exposed to four chemicals. As a growth descriptor, changes in the larval body weight 48 h after chemical exposure were presented in Table 2. Generally, the larvae, which had been exposed to chemicals for 48 h, showed a decrease in body weight. The degree of decrease was significant at two highest concentrations of NP and CP. The differences in FBW before and after treatment were 0.99 and 0.69 mg/larvae for 10 and 100 μg/L for NP, respectively; and 1.27 and 1.43 mg/larvae for 20 and 200 μg/L for CP, respectively. Whereas, a slight stimulatory effect was observed at the highest concentration of B[a]P (1 mg/L), as BFW increased after 48 h of exposure. The increase in dry body weights that occurred after B[a]P exposure suggests that this chemical may perturb the physiological processes of *C. tentans*. The increase in dry body weights is difficult to explain, but it could suggest that this chemical may have a growth stimulatory effect. It may also be attributed to a decrease in larval water content, which is eventually related with the alteration of the larval osmoregulation process. However, the ash-free dry body weights should be measured to determine the actual larval growth status. Moreover, our results are only from a 48-h exposure with three concentrations, so to better understand this phenomenon, more concentration levels with longer exposure periods should be tested.

Table 2
The changes in the fresh body weight (FBW) in the 4th instar larvae of *Chironomus tentans* exposed to various environmental chemicals for 48 h

Chemicals	Concentration (μg/L)	FBW (0 h) (mg/Larvae)	FBW (48 h) (mg/Larvae)
NP	0	5.495 ± 0.481	5.195 ± 0.429
	1	5.507 ± 0.524	5.042 ± 0.452
	10	6.413 ± 0.537	5.428 ± 0.404
	100	6.127 ± 0.332	5.435 ± 0.341
B[a]P	0[a]	5.495 ± 0.481	5.195 ± 0.429
	0.01[a]	5.767 ± 0.647	5.459 ± 0.722
	0.1[a]	5.673 ± 0.185	5.472 ± 0.324
	1[a]	5.930 ± 0.612	6.775 ± 0.701
CP	0	5.495 ± 0.481	5.195 ± 0.429
	2	5.000 ± 0.333	4.410 ± 0.391
	20	5.013 ± 0.409	3.744 ± 0.274
	200	6.140 ± 0.404	4.709 ± 0.272
Cd	0[a]	5.495 ± 0.481	5.195 ± 0.429
	0.2[a]	5.867 ± 0.234	5.517 ± 0.309
	2[a]	5.120 ± 0.065	4.653 ± 0.093
	20[a]	5.680 ± 0.425	5.085 ± 0.380

Fresh body weight (FBW) was measured using a pool of 10 larvae at the beginning (0 h) and at the end of the exposure period (48 h).
[a] mg/L.

As chironomids are considered good biological models for the study of the chronic effects of environmental pollutants, the response of several biomarkers to toxicants has already been evaluated (Kosalwat and Knight, 1987; Pascoe et al., 1989; Choi et al., 2000). The effects of xenobiotics on the growth and reproduction of the test organisms are broadly accepted test parameters. The decrease in body weight observed after chemical exposure may induce alteration in the growth of the larval population in the long term, as organism growth may affect population fitness (Liber et al., 1996; Sibley et al., 1997). However, growth rate experiment alone could not provide any physiological meaning or consequences of observed chemical-induced Hb alteration. Direct experimental demonstrations of the wider relationships between the response of Hb and their subsequent physiological and individual consequences may be needed in order to validate the ecotoxicological relevance of chemical-induced alteration on *C. tentans* Hb. There have been few direct experimental demonstrations for the relationship between molecular/biochemical effects and the consequences at higher levels of biological organization (Karouna-Renier and Zehr, 1999; Choi et al., 2002; Lee and Choi, 2006). To define the sublethal hazards of chemicals in this species, we need to characterize the causal relations between biochemical responses, such as Hb alteration, and the effects of these responses at higher biological levels.

4. Conclusion

In conclusion, this study revealed a high level of multiplicity of Hb in the larvae of *C. tentans* and the change in Hb expression by chemical exposure, which suggests that the expression pattern of Hb proteins may potentially contribute to the development of a biomarker for ecotoxicity monitoring. In terms of the potential of *Chironomus* Hb as a biomarker, upstream event of globin protein or gene expression, as well as subsequent physiological consequences need to be elucidated to provide a more meaningful explanation on the function of Hb in chemical exposure

in this organism, and thus to permit better interpretation of the possible role of *Chironomus* Hb in the monitoring of chemical contamination.

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