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Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various environmental pollutants: A potential biomarker of freshwater monitoring

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Abstract

To identify a sensitive biomarker of freshwater monitoring, we evaluated pollutant-induced expression of heat shock proteins (HSPs) and hemoglobins (Hbs) genes in the larvae of the aquatic midge *Chironomus tentans* (Diptera, Chironomidae). As pollutants, we examined nonylphenol, bisphenol-A, 17α-ethynyl estradiol, bis(2-ethylhexyl) phthalate, endosulfan, paraquat dichloride, chloropyriphos, fenitrothion, cadmium chloride, lead nitrate, potassium dichromate, benzo[*a*]pyrene and carbon tetrachloride. We also investigated larval growth as a physiological descriptor by measuring changes in the body fresh weight and dry weight after chemical exposure. The response of the HSPs gene expression by chemical exposure was rapid and sensitive to low chemical concentrations but it was not stressor specific. Interestingly, an increase in the expression of HSPs genes was observed not only in a stress inducible form (HSP70), but also in a constitutively (HSC70) expressed form. The expression of Hb genes showed chemical-specific responses: that is, alkyl phenolic compounds increased the expression of hemoglobin genes, whereas pesticides decreased the expression. As expected, molecular-level markers were more sensitive than physiological endpoints, suggesting that gene expression could be developed as an early warning biomarker in this animal. The overall results suggest that the expression of HSP and Hb genes in *Chironomus* could give useful information for diagnosing general health conditions in fresh water ecosystem. The expression of Hb genes, in particular, seems to be a promising biomarker, especially in view of the potential of *Chironomus* larvae as a biomonitoring species and of the physiological particularities of their respiratory pigments.

Keywords: *Chironomus tentans*; Heat shock protein 70; Hemoglobin; Gene expression; Biomarker; Freshwater monitoring

1. Introduction

Environmental contaminants may induce expression of certain genes in an organism. Almost without exception, gene expression is altered in toxicity, as either a direct or indirect result of exposure to toxicants. Depending upon the severity and duration of the contaminant exposure, the expression of certain genes may be linked to short-term toxicological responses that impact on individual fitness (that is, survival and reproduction). Although researchers have widely investigated how toxicants induce molecular-level effects, the study of gene expression and its consequences at a higher level of biological organization in wildlife species has seldom been attempted. Recently, several studies have focused on the responses to chemical stressors at the molecular level in aquatic invertebrates (Yoshimi et al., 2002; Karouna-Renier and Zehr, 2003; Rotchell and Ostrander, 2003; Perceval et al., 2004). Among aquatic invertebrates, the aquatic larvae of

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nonbiting midges (Chironomidae, Diptera) are globally distributed, and they are the most abundant group of insects found in fresh water ecosystems. They hold an important position in the aquatic food chain and are major food source for fish and other vertebrates and invertebrates (Cranston, 1995). Thus, they are used extensively to assess the acute and sublethal toxicity of contaminated sediments and water (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).

With respect to molecular-level responses to chemical stressors, heat shock proteins (HSPs) are the most frequently studied aspect of aquatic invertebrates (Karouna-Renier and Zehr, 2003; Arts et al., 2004; Piano et al., 2004). HSPs are not only induced by heat shock but also by pollutants. As such, they are promising biomarker of exposure and, probably, of effects. Members of the heat shock protein 70 (HSP70) family of molecular chaperones are among the most highly conserved proteins in eukaryotic organisms. The HSP70 gene family contains both stress inducible genes and constitutively (HSC70) expressed genes that share many common structural features. The expression patterns of these proteins are quite different. Inducible heat shock genes are expressed at extremely low levels under normal conditions, but their transcription and translation increase rapidly in response to various stressors (Lindquist and Craig, 1988; Sorger, 1991; Bukau and Horwich, 1998; Juliann and George, 1998). Although researchers have studied the induction and accumulation of HSPs after the invertebrates have been exposed to various pollutants, there has been only limited application of HSPs to environmental biomonitoring (Hahn et al., 2002; Yoshimi et al., 2002; Feng et al., 2003; Karouna-Renier and Zehr, 2003).

Chironomus hemoglobins (Hbs) show many interesting features, such as a high degree of polymorphism, high affinity for oxygen and extracellular localization (Osmulski and Leyko, 1986). From an evolutionary point of view, it is generally admitted that the presence of Hbs in invertebrates reveals the adaptation of these organisms to unfavorable environmental conditions because these pigments help to sustain aerobic metabolism under conditions of low oxygen. *Chironomus* Hbs appear to fulfill clear physiological roles in transporting and storing oxygen in the larvae that burrow in polluted and hypoxic mud (Osmulski and Leyko, 1986). In addition, the extracellular Hbs enhance the good exploitation of hypoxic oxygen (Weber, 1980). Despite such interesting particularity, few toxicological studies have focused on *Chironomus* hemoglobin as a sublethal endpoint (Choi and Roche, 2004). Considering the potential of *Chironomus* larvae as a biomonitoring species, and the physiological particularities of their respiratory pigment, the expression of hemoglobin genes has considerable potential as a biomarker for environmental monitoring in *Chironomus*.

To identify general biomarkers for freshwater monitoring, we investigated the expression of HSP and Hb genes in *Chironomus tentans* larvae. We examined various classes of environmental pollutants, including alkyl phenols, pesticides, and heavy metals, most of which are considered to be endocrine disruptors. Moreover, to verify whether molecular-level effects had consequences at a higher level of biological organization, we investigated the change in larval growth as a physiological descriptor.

2. Materials and methods

2.1. Organisms

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), we obtained *C. tentans* larvae from adults reared in our laboratory. The larvae, which we fed on Tetramin®, were reared under a 14 h to 10 h light-dark photoperiod at room temperature (20 ± 1 °C) in a 2 l glass chamber containing dechlorinated tap water and acid-washed sand with aeration.

2.2. Exposure conditions

We conducted the experiment at a constant temperature of 20 ± 1 °C under light conditions of 14 h to 10 h of light and darkness. By using groups of the fourth instar larvae collected in rearing aquaria, we assessed the effects of heat shock, hypoxia and chemical exposure.

For the heat shock treatment, we exposed the larvae (10 individuals per 100 ml of dechlorinated tap water) to 35 °C for 1, 2, 4, 8, 12 and 24 h. The control organisms were sampled after 4 h at room temperature.

For the hypoxic treatment, we sealed the tanks with parafilm®. We introduced the larvae at 24, 48 and 96 h after the beginning of the experiment and maintained the treatment under each condition for 24 h. The control tanks were left uncovered for the duration of the experiment to ensure normal dissolution of atmospheric oxygen in water. To monitor the concentration of the dissolved oxygen in water in each tank, we used a WTW OXI-96 oximeter (WTM GmbH, Weilheim, Germany). The mean values of the dissolved oxygen concentration were 7.88 (control), 4.55, 1.88 and 0.1 mg/l.

For the chemical treatment, we selected sublethal exposure concentrations from the results of an acute toxicity test (data not shown), after 24 h of exposure three concentrations corresponding to 1/1000, 1/00 and 1/10 of the 24 h LC50 were selected for each compound. We then transferred 10 of the fourth instar *C. tentans* larvae into 200 ml beakers containing 100 ml of dechlorinated tap water, and treated them with chemicals for sublethal exposure. For each experiment, we added 0.1 ml of the test solution into the experimental beakers before introducing the larvae. Acetone was used as solvent for nonylphenol (NP), bisphenol A diglycidyl ether (BPA), 17α-ethynyl estradiol (EE), chloropyriphos (CP), fenitrothion (FT), bis(2-ethylhexyl) phthalate (DEHP), endosulfan (ES), benzo[*a*]pyrene (B[*a*]P) and carbon tetrachloride (CCl_4) and water was used for paraquat dichloride (PQ), cadmium

chloride (Cd), lead(II)nitrate (Pb) and potassium dichromate (Cr). After the physical and chemical treatment, we stored the larvae at −80 °C until further analysis.

2.3. Gene expression analysis

Frozen larvae were homogenized in 700 μl of TRI reagent (Molecular Research Center, Cincinnati, OH) and RNA was isolated according to the manufacturer's standard protocol. The RNA, which was 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 stored at −80 °C until further use. For the reverse transcription-polymerase chain reaction (RT-PCR), we used a two-step method with RT Premix and PCR Premix kits (Bioneer Co., Seoul, Korea). Before RT, 2 μg of total RNA and random hexamer (Promega, Madison, WI, USA) were denatured at 70 °C for 5 min and then rapidly cooled on ice. We added these solutions to the RT Premix kits and conducted the RT at 42 °C for 60 min and at 94 °C for 5 min. Next, we added these templates to the PCR premix kit, which contained the HSP70, HSC70, Hb A, Hb B and actin primers. The primers were designed on the basis of sequences retrieved from GenBank™ (Table 1). Finally, we served actin mRNA for normalization of the HSP and Hb levels.

Using a PTC-100 thermal cycler (MJ Research, Lincoln, MA, USA), we conducted 30 cycles of PCR at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min, and, finally, at 72 °C for 7 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel (Promega, Madison, WI, USA) and visualized with ethidium bromide (Bioneer Co., Seoul, Korea). We replicated all the tests at least three times, and determined the relative densities of each band with the aid of an image analyzer, Gel documentation system (Vilber Lourmat TFX-20. M, Marne la Vallee, France) with a Kodak 1D 3.6 camera (Kodak EDAS 290, Rochester, NY, USA).

Table 1
Sequence of primers used in the amplification of HSP, Hb and actin cDNA

Gene (GenBank accession no.)	Primer sequence
HSP70 (AY163157)	5′CATGTGAACGAGCCAAGAGA3′ 5′TCGAGTTGATCCACCAACAA3′
HSC70 (AF448433)	5′GTCTAAAGCCCCAGCCGT3′ 5′CAAAAATGGTATTTGTTGGATTCAT3′
Hb A (X56272)	5′TTGAGATTCCACGGTTGTGA3′ 5′AAGTTGACATCCTTGCTGCC3′
Hb B (AJ003809)	5′AGATATCCAAGCCCGTTTCC3′ 5′TTGAGATTCCACGGTTGTGA3′
Actin (AB070370)	5′GATGAAGATCCTCACCGAACG3′ 5′CCTTACGGATATCAACGTCGC3′

2.4. Measurement of the body fresh weight and the body dry weight

We measured the body fresh weight and the body dry weight of 10 larvae collected 48 h after the beginning of the exposure. The fresh weight was immediately measured. We evaluated the larval dry weight after placing the larvae at 105 °C for 24 h. The weighing was measured to the nearest 0.1 mg.

2.5. Chemicals

We purchased NP, EE, CP and FT from Riedel–deHaen (Sigma Corp. St. Louis, MO, USA), BPA, DEHP and ES from Fluka (Buchs SG, Switzerland) and PQ, Cd, Pb, Cr, B[*a*]P and CCl_4 from Sigma–Aldrich (Sigma Corp. St. Louis, MO, USA).

2.6. 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) for all analyses.

3. Results

We evaluated how heat shock and hypoxia treatments changed the expression of HSP and Hb genes in the fourth instar larvae of *C. tentans* (Fig. 1). By using the heat shock treatment as a positive control, we observed an increase in HSP70 by thermal stress. The hypoxia treatment induced increases in HSP70 mRNA, whereas the oxygen depletion did not affect the HSC70 levels. The induction of HSP70 occurred as early as 1 h after heat shock and this increase appeared to have risen slightly by the end of experiment (24 h). Heat shock did not seem to affect the Hb gene expression in the fourth instar larvae of *C. tentans*, whereas mild hypoxia (4.55 mg O_2/l) induced slight increase of Hb gene expression.

We also studied how NP, BPA, EE and DEHP affected the HSP and Hb expression in the *C. tentans* larvae (Fig. 2). Four of the compounds we tested induced remarkable increases in both HSP70 and HSC70 gene expression in *C. tentans*. We also observed that there were statistically significant increases for all treatments, except for the HSP70 in 800 μg/l of BPA and the HSC70 in 50 μg/l of DEHP, owing to high experimental variation. Both alkyl phenolic compounds increased the expression of Hb genes, whereas EE and DEHP decreased the expression of Hb genes, especially at low levels of exposure (that is, 8 and 80 μg/l for EE and 0.5 and 5 μg/l for DEHP, respectively).

Fig. 3 shows how pesticide treatment affects the expression of HSP and Hb genes. For the *C. tentans* larvae that had been exposed to pesticides, there was an increase in the HSP70 and HSC70 gene expression. One exception to this phenomenon was FT, for which we observed a

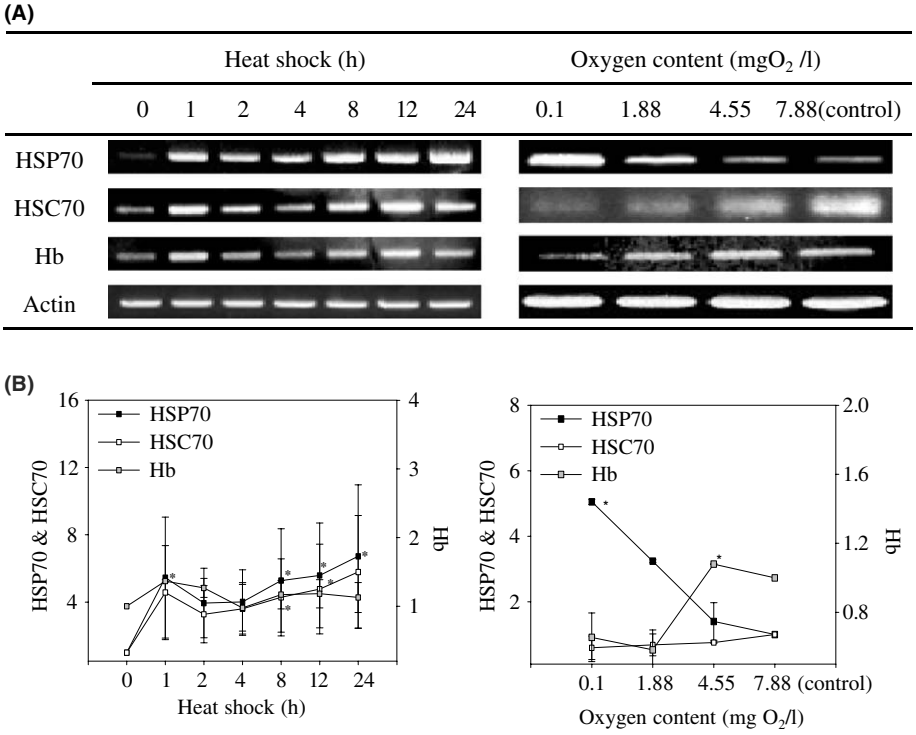

Fig. 1. Effects of heat shock and hypoxia on the expression of HSP and Hb mRNA in the fourth instar larvae of *C. tentans* (A). Densitometric values were normalized using actin mRNA expression (B) ($n = 3$, mean ± SEM, $^{*}p < 0.05$).

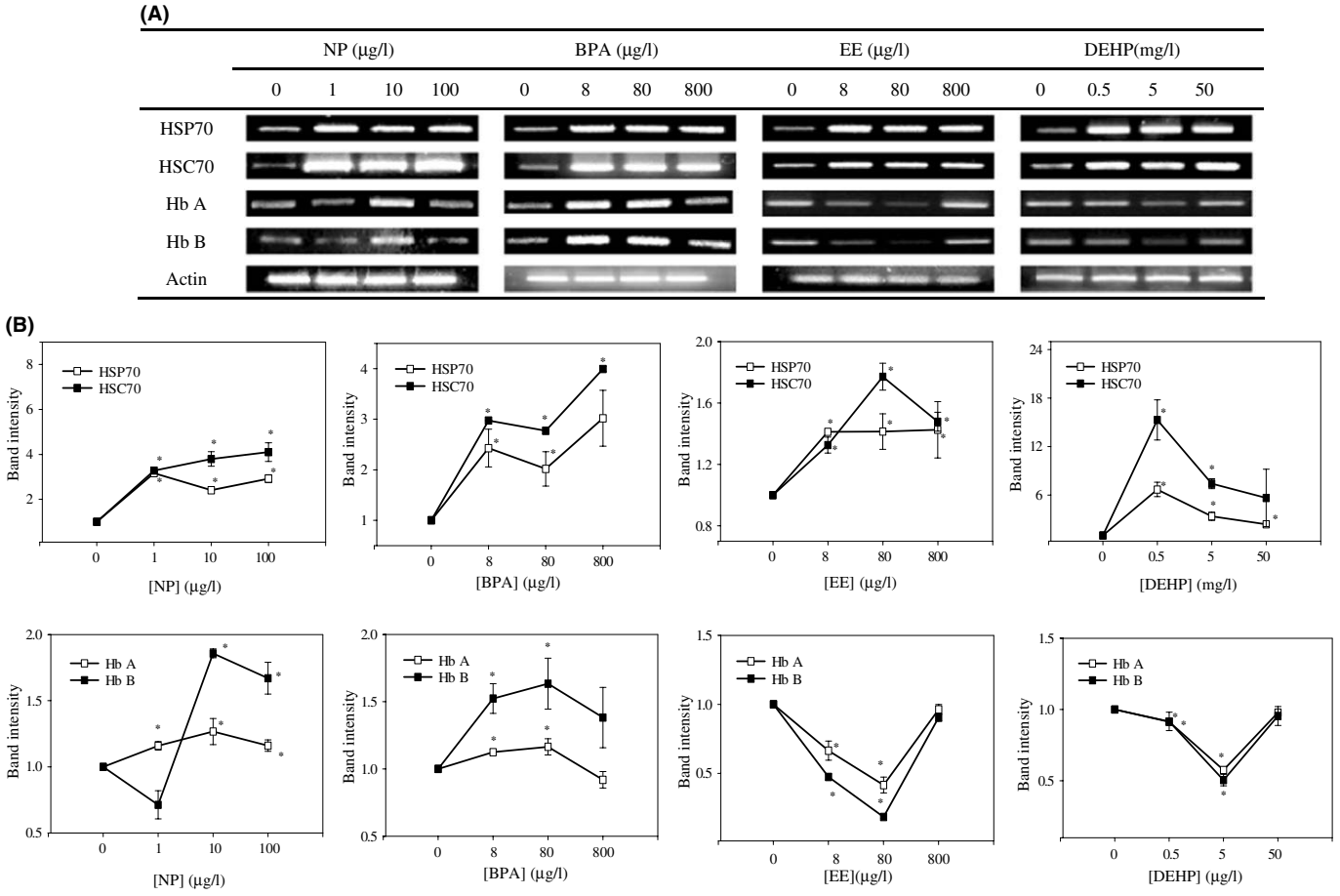

Fig. 2. Expression of HSP and Hb genes in the fourth instar larvae of *C. tentans* exposed to NP, BPA, EE and DEHP for 24 h (A). Densitometric values were normalized using those of actin mRNA (B) ($n = 3$, mean ± SEM, $^{*}p < 0.05$).

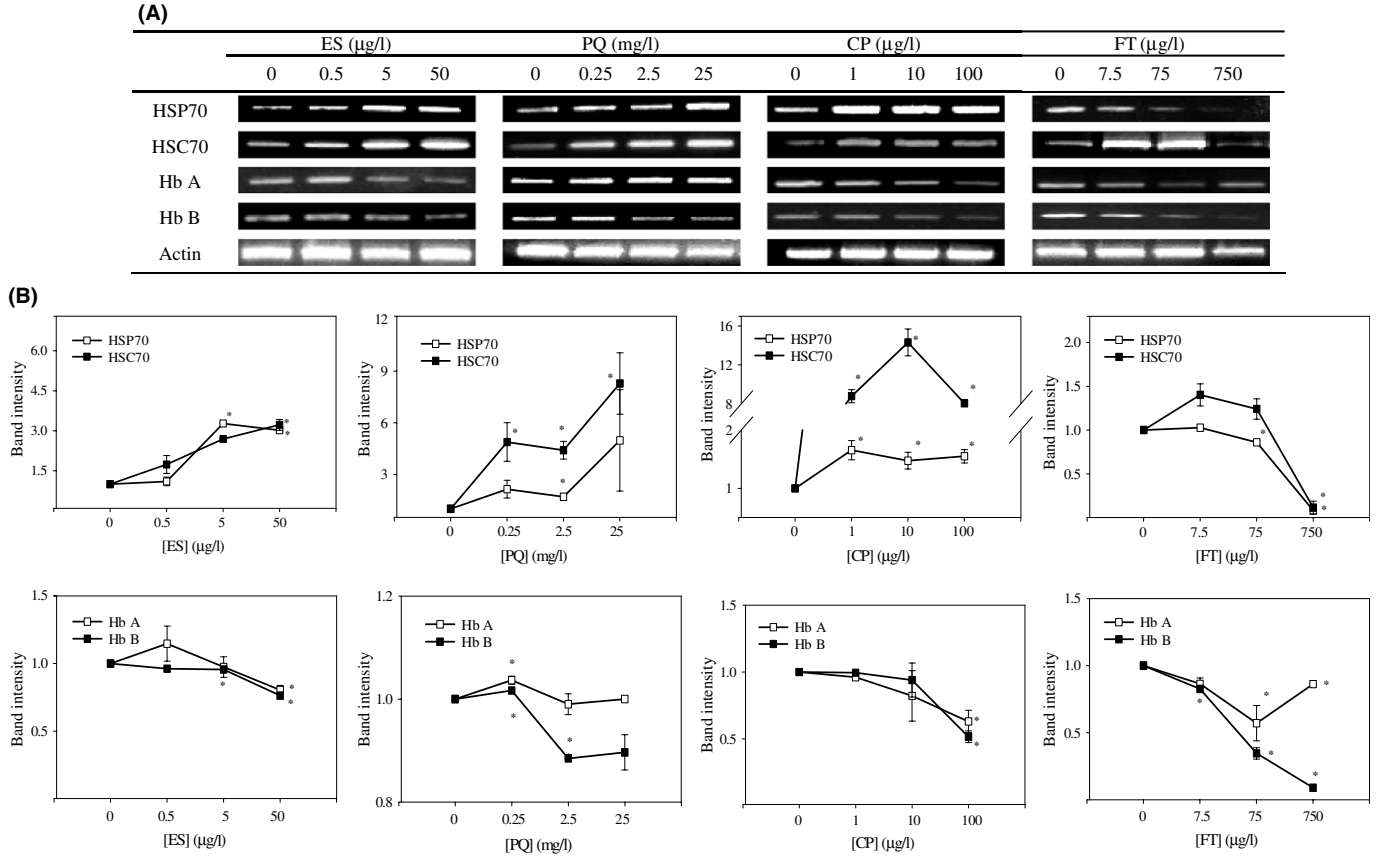

Fig. 3. Expression of HSP and Hb mRNA in the fourth instar larvae of *C. tentans* exposed to pesticides for 24 h (A). Densitometric values were normalized using those of actin mRNA (B) ($n = 3$, mean ± SEM, $^{*}p < 0.05$).

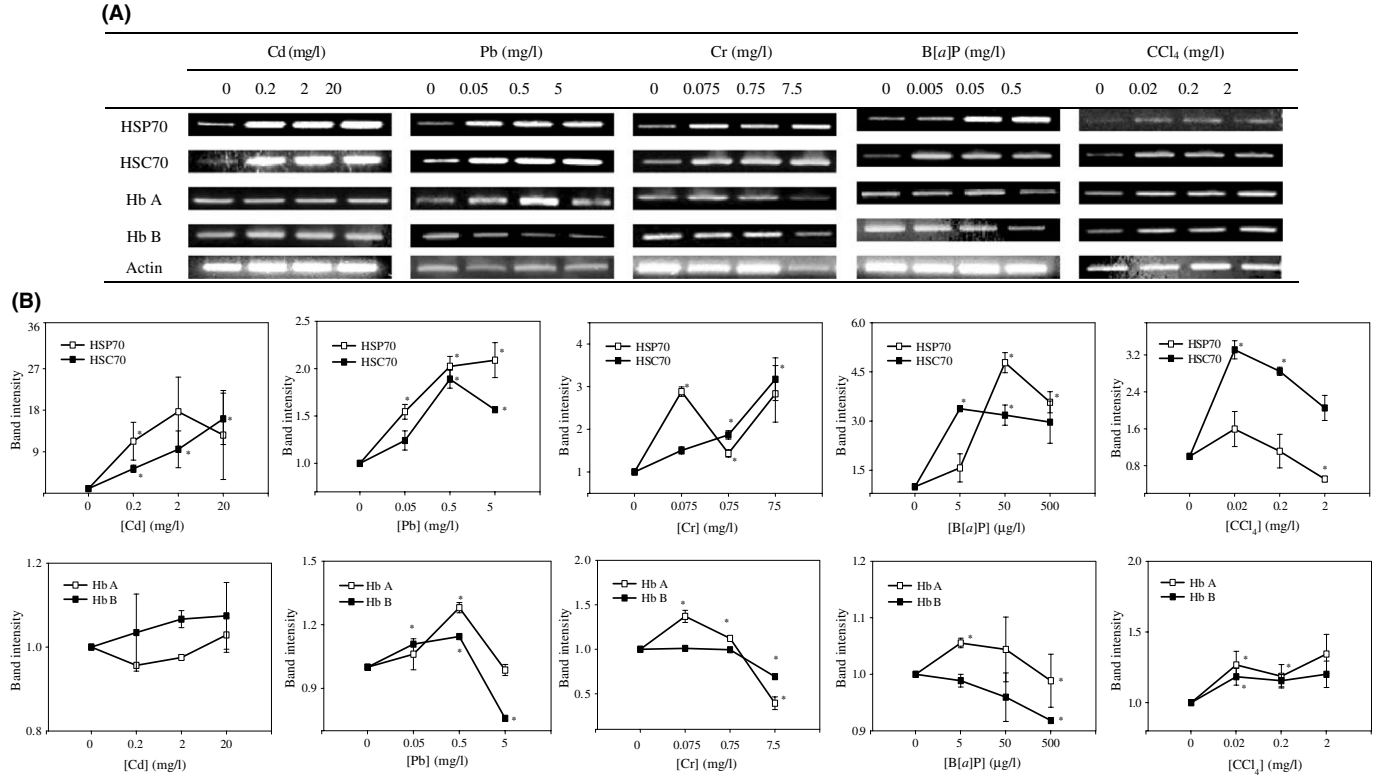

Fig. 4. Expression of HSP and Hb mRNA in the fourth instar larvae of *C. tentans* exposed to heavy B[*a*]P and CCl_4 for 24 h (A). Densitometric values were normalized using those of actin mRNA (B) ($n = 3$, mean ± SEM, $^{*}p < 0.05$).

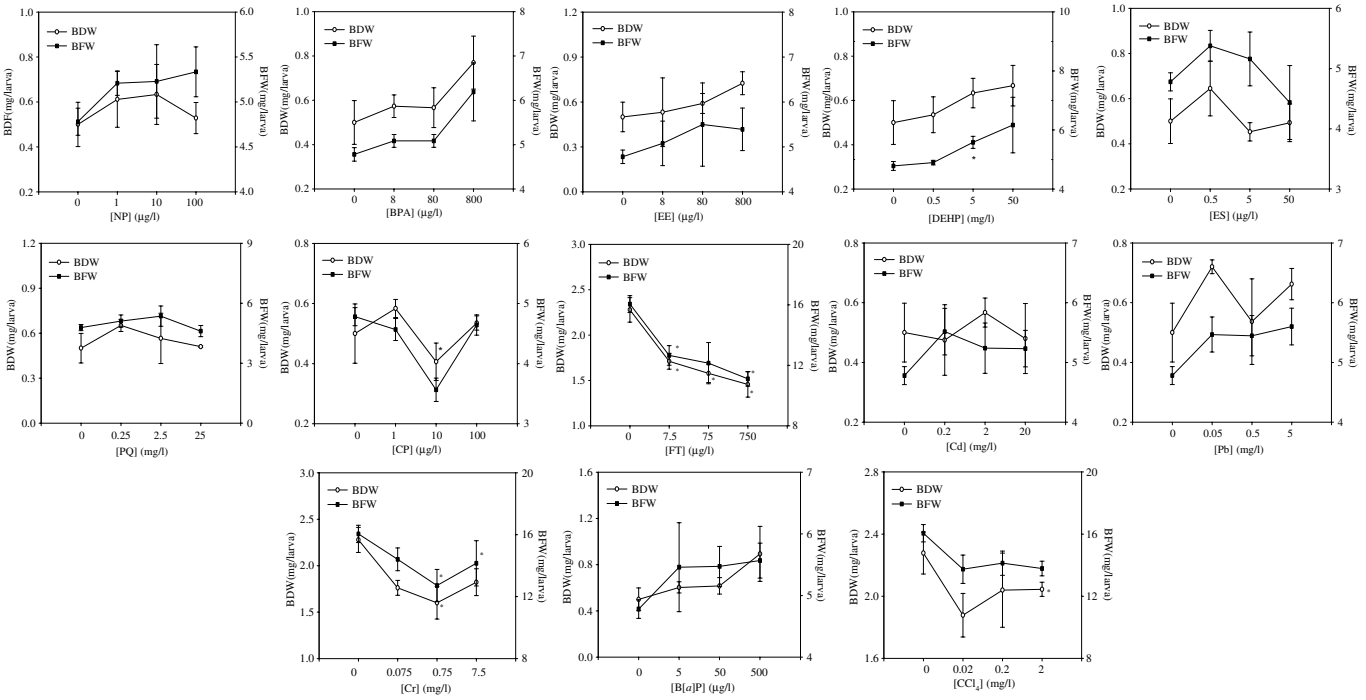

Fig. 5. Body fresh weight and body dry weight in the fourth instar larvae of *C. tentans* exposed to various environmental chemicals for 48 h.

concentration-dependent decrease. We also observed a statistically significant increase in the expression of HSP genes but only at high levels of exposure to ES (that is, 5 and 50 μg/l for HSP70 and 50 μg/l for HSC70). In contrast, with PQ and CP, the increases in the expression of HSP genes were significant at concentrations as low as those that corresponded to 1/1000 of 24 h LC50 (that is, 0.25 mg/l for PQ and 1 μg/l for CP). All four pesticides that we tested induced decreases of Hb gene expression in *C. tentans* larvae. Moreover, we observed a concentration-dependent decrease for the FT exposure.

The HSP and Hb mRNA levels were assessed in *C. tentans* larvae exposed to Cd, Pb, Cr, B[*a*]P and CCl_4 (Fig. 4). The HSP gene expression increased as a result of exposure to metals. In particular, Cd and Cr induced concentration-dependent increases in HSC70 mRNA (that is, there was a 6-, 9- and 16-fold expression at 0.2, 2 and 20 mg/l of exposure to Cd and 1.5, 1.8 and 2.1-fold expression at 0.075, 0.75 and 7.5 mg/l of Cr exposure, respectively). We also found that B[*a*]P induced increases in the expression of both HSP70 and HSC70 mRNA. For exposure to CCl_4, the HSC70 mRNA expression increased, whereas we observed a decrease of HSP70 at the highest concentration of exposure (2 mg/l of CCl_4). In addition, Pb and Cr increased the expression of Hb genes at low levels of exposure (that is, at 0.05 and 0.5 mg/l for Pb, and at 0.075 and 0.75 mg/l for Cr). However, they decreased the expression of Hb genes at high levels of exposure (that is, at 5 mg/l for the Pb, and at 7.5 mg/l for the Cr). The Hb B gene expression showed a concentration-dependent decrease in B[*a*]P exposure, whereas the expression of Hb genes seemed to increase in CCl_4 exposed larvae.

As a growth parameter, we measured the body weight of the *C. tentans* larvae 48 h after treatment (Fig. 5). The body fresh weights of the larvae that had been exposed to DEHP (5 mg/l), FT (7.5 and 750 μg/l) and Cr (750 and 7500 μg/l) differed significantly from those of the control. In terms of body dry weight, we observed a significant change in the larvae treated with CP, FT, Cr and CCl_4, that was decrease in body dry weight, which was observed at high levels of exposure (that is, 10 μg/l for CP, 0.75 mg/l for Cr and 2 mg/l for CCl_4). As Hb gene expression (Fig. 3), a concentration-dependent decrease of body fresh weight and body dry weight was observed in FT exposed larvae.

4. Discussion

We investigated the expression of HSP and Hb genes in *Chironomus* larvae in relation to a wide variety of chemicals. The expression of HSP genes increased by most of chemicals studied, including by NP, which is used in polymer industry (EU, 2002), by BPA, an intermediate in the production of polycarbonate and epoxyresins (Staples et al., 1998), by EE, a synthetic estrogen, (Purdom et al., 1994), DEPH, a plasticizer in polymer products (EU, 2001) by ES, an organochlorine insecticide, by CP, an organophosphorous insecticide, by PQ, an oxygen radical generating herbicide, by Cd, Pb and Cr, commonly found heavy metals, and by BaP, ubiquitously distributed PAH (Juhasz and Naidu, 2000). Moreover, HSP gene expression increased not only by these chemical stressors, but also by physical stressors, such as, heat shock and hypoxia, as well (Fig. 1). Because hypoxic conditions frequently occur in a polluted natural environment, it seems relevant to

investigate the expression of HSP gene as a general biomarker of environmental quality.

Although most of the tested chemicals induced the expression of two HSP genes (HSP70 and HSC70) at sublethal concentrations, FT and CCl_4 induced a decrease in the expression of those genes. The response of HSP expression was rapid and sensitive to low chemical concentrations but not stressor specific. Given the responsiveness to even minor assaults, the expression of HSP70 and HSC70 may prove useful as a molecular indicator of adverse biological conditions, including, for example, the chemical toxicity in the *Chironomus* species. In contrast to HSP70, HSC70 is known to be constitutively expressed and not inducible by environmental stressors (Juliann and George, 1998). Yet we observed that the expression of HSC70 genes increased in response to various forms of chemical exposure. These results suggest that, as with HSP70, HSC70 might be inducible in response to environmental stressors. However, to elucidate the mechanism, further studies are needed with a broad range of chemicals.

Hb expression pattern in *Chironomus* is known to be development stage-dependant (Osmulski and Leyko, 1986; Choi and Roche, 2004). Age-synchronized fully grown fourth instar larvae issued from the same eggmass were used in the experiment to avoid age-dependant Hb content variation. Difference between control and treated larvae, therefore, can be considered as the effect of chemical treatment. The expression of Hb genes shows a chemical-specific response: alkyl phenolic compounds, for instance, induce an increase in the expression of Hb genes, whereas pesticides induce a decrease in the expression of Hb genes. Chemical-induced Hb gene expression could be due to increase in oxygen demand for xenobiotic metabolic process.

While, deceases in Hb gene expression by pesticides are more difficult to explain they might have a repercussion at higher level of biological organization as in FT exposure case. In FT exposure, decreases in body fresh weight and body dry weight occurred concomitantly with decrease in Hb gene expression, which could contribute to support this hypothesis. Except for FT exposure, our results suggest that physiological changes are less sensitive than molecular responses.

It is interesting that larval body weights generally increases as concentrations of NP, BPA, EE and DEHP increase (Fig. 5). These chemicals may have stimulatory effect to the organism at low concentrations. Our results, however, are only from 24 h exposure with three concentrations, if more concentration levels with longer exposure period had been tested, this could probably be better evaluated and explained. Moreover, there have been few direct experimental demonstrations for the relations between molecular/biochemical effects and the consequences at higher levels of biological organization (Karouna-Reneir and Zehr, 1999; Choi et al., 2002). To define the sublethal hazards of chemicals in this animal, we need to characterize the causal relations between molecular/biochemical responses and the effects of these responses at higher biological levels.

It would be ideal for environmental monitoring to have a limited set of specific biomarkers that indicated the exposure and enabled the hazards of all major classes of pollutants to be assessed. Nonspecific biomarkers would also be helpful for accurately and completely assessing the health condition of organisms and the ecosystem (Peakall and Walker, 1994). Therefore, in conjunction with stressor-specific biomarkers, the expression of HSP70 in the *Chironomus* species might be useful for assessing the quality of freshwater.

The overall results suggest that the expression of HSP and Hb genes in the *Chironomus* species could be developed as biomarkers for assessing the general health conditions of freshwater ecosystems. Nonetheless, more particular investigations are still needed for the *Chironomus* Hb. Given the potential of *Chironomus* larvae as a biomonitoring species, and the physiological particularities of their respiratory pigment, we deduce that the expression of Hb genes has considerable potential as a sensitive biomarker for freshwater monitoring with respect to this animal model.

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