RESEARCH ARTICLE

Effect of cadmium exposure on the globin protein expression in 4th instar larvae of *Chironomus riparius* Mg. (Diptera: Chironomidae): An ecotoxicoproteomics approach

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In order to identify *Chironomus* hemoglobin (Hb) as a biomarker of ecotoxicity monitoring; herein, the effects of cadmium chloride (Cd) on Hb parameters were investigated in the 4th instar larvae of *Chironomus riparius*. The expressions of globin mRNA and hemolymph protein, using ecotoxicoproteomic approach, were investigated. Conventional ecotoxicity tests were also conducted to validate the ecotoxicological relevance of the response of *Chironomus* Hb as a biomarker. The proteomic analysis indicated that exposure to Cd lead alteration in the expression of hemolymph protein, with the total expressions of 12 hemolymph protein spots decreasing in response to treatment, with that of two increasing in response to Cd exposure. In addition, all of the spots differentially expressed in response to Cd treatment were identified as globin proteins. The decreased total Hb content observed in the hemolymph of larvae exposed to Cd suggested that the decreased expression of selected globin proteins in response to Cd exposure impacted on Hb synthesis. The overall results suggested that Hb could be a target molecule for exposure to Cd in *C. riparius*, with a proteomic approach appearing to be an ideal tool for the discovery of biomarkers in ecotoxicological research.

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1 Introduction

The aquatic larvae of nonbiting midges (Chironomidae, Diptera), which are distributed globally, are the most abundant group of insects found in freshwater ecosystems. These organisms hold an important position in the aquatic food chain, being a major food source for fish and other vertebrates and invertebrates [1]. In addition, they are sensitive to many pollutants, easy to culture and have a short life cycle, therefore they are used extensively to assess the acute and sublethal toxicities of contaminated sediments and water [2–4]. *Chironomus* are unique, however, because they possess hemoglobin (Hb) during their larval stage, with stage-specific and tissue-specific single-chain globin syntheses existing throughout the four larval stages. The Hb is synthesized in the larval fat body, and then secreted into the hemolymph [5, 6]. Although Hb is widely distributed throughout the animal kingdom, it occurs in only a few invertebrates in Insecta and Crustacea. The Chironomid Hb members of insect respiratory proteins have been extensively studied and found to offer the simplest model for the analysis of Hb. Analysis of the structure of Hb in chironomids may provide key information regarding

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Abbreviations: Cd, cadmium chloride; **Hb,** hemoglobin; **LC,** lethal concentration

the mechanism by which heme proteins maintain their high heterogeneity, which, in turn, may provide insight into many evolutionary questions currently being studied [7, 8]. From an ecotoxicological point of view, freshwater organisms that possess Hb have many interesting characteristics that make them ideal candidates for biomonitoring [7]. Therefore, studies evaluating biomarkers of chemical contamination using Hb-related parameters, which were originally limited to fish erythrocytes [9–12] have recently been expanded to include invertebrate systems [13–17].

Considering the potential of *Chironomus* larvae for biomonitoring, as well as the physiological particularities of *Chironomus* Hb, the respiratory pigment of this invertebrate shows considerable promise as a sensitive biomarker for environmental monitoring. In this study, to identify *Chironomus* Hb as a biomarker for ecotoxicity monitoring, toxicant-induced changes in the Hb of 4th instar *Chironomus riparius* Mg. (Diptera: Chironomidae) larvae were evaluated with respect to changes in their individual globin mRNA and protein expression, as well as in the total Hb content. Cadmium chloride (Cd) was used as a model toxicant due to its abundance in the environment and ecotoxicological importance with respect to *Chironomus* and other aquatic organisms. The expression of individual globin protein was investigated using 1-D (PAGE and IEF) and 2-DE (proteomics analysis). Conventional ecotoxicity tests, such as growth, reproduction, and development as organism/population-relevant endpoints, were also conducted to validate the ecotoxicological relevance of the response of *Chironomus* Hb for use as a biomarker. 2-DE was performed not only to investigate the effect of Cd on multiple globin protein expressions, but also to test the use of proteomics as a tool for biomarker discovery in ecotoxicological research. Changes in protein patterns on exposure to a toxicant may allow identification of new diagnostic biomarkers for indicating toxicant stress at an early biochemical stage [17, 18]. In addition, environmental proteomics (also called as "ecotoxicoproteomics") has recently been applied to laboratory and field studies of organisms, such as, fish and molluscs [18–22]. Considering the importance of *Chironomus* in ecotoxicological studies, a proteomic approach, with highly environmentally relevant species, such as, *C. riparius*, may lead to more sensitive ecotoxicity analysis, which would also advance the *Chironomus* bioassay for ecotoxicological testing.

2 Materials and methods

2.1 Organism and exposure condition

Using an original strain of *C. riparius* provided by the Toxicology Research Center of the Korea Research Institute of Chemical Technology (Daejeon, Korea), 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 16:8 h (light/dark) photoperiod at room temperature (20 ± 1°C).

Hb related parameters were assessed using groups of 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 time after egg hatching to obtain an age-synchronized population. From our previous experiments, 4th instar development of *C. riparius* larvae persisted for 22–38 days after the eggs had hatched [13, 18], and Hb analysis were conducted on the larvae during the middle of the 4th instar developmental stage (*i.e.*, 32 days after the eggs had hatched). For the Cd treatment, 10 of the 4th instar *C. riparius* larvae were transferred into 200 mL beakers, containing 100 mL of dechlorinated tap water, and treated with Cd (Sigma–Aldrich, St. Louis, MO, USA), prepared in water. Exposure was carried at constant temperature (20 ± 1°C), with a 16:8 h (light/dark) photoperiod for all experiments. Three replicates were conducted for each experimental parameter.

2.2 Mortality, growth, reproduction, and development tests

Acute toxicity test was conducted using mortality as an endpoint, as described previously [4, 23]. Growth, reproduction, and development tests were conducted as described previously [4, 24].

2.3 Globin mRNA expression analysis

The cellular fraction of the control and treated larvae were homogenized in 700 μL of TRI reagent (Molecular Research Center, Cincinnati, OH, USA), the RNA was isolated, and RT-PCR was conducted as described previously [25].

2.4 PAGE and IEF

Ten larvae from the control and experimental tanks were pooled, hemolymphs withdrawn by opening the body wall, and the body fluids subjected to electrophoresis. Three independent electrophoreses were performed. Nondenaturing PAGE and IEF were performed on hemolymph protein samples as described previously [25].

2.5 2-DE and in-gel digestion with trypsin and extraction of peptides

Three independent 2-DE replicates were prepared, each with protein samples of ten pooled larvae from the control and experimental tanks. The hemolymphs, withdrawn by opening the body wall, were transferred into Eppendorf cups containing ice-chilled physiological solution (10.3 mM NaCl), and were suspended in 50 mM Tris buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapo-

lis, IN, USA). For 2-DE analysis, pH 5.5–6.7 IPG strips (Amersham Biosciences) were rehydrated in swelling buffer containing the protein lysates (80 μg), 7 M urea, 2 M thiourea, 0.4% w/v DTT, and 4% w/v CHAPS. The 2-D separation was performed on 18% v/v homogenous SDS-polyacrylamide gels. Following fixation of the gels for 1 h in a solution of 40% v/v methanol containing 5% v/v phosphoric acid, the gels were stained with Colloidal CBB G-250 solution (ProteomeTech, Seoul, Korea) for 5 h. The gels were destained in 1% v/v acetic acid for 4 h and then imaged using a GS-710 imaging calibrated densitometer (BioRad, Hercules, CA, USA). Protein spot detection and 2-D pattern matching were carried out using ImageMaster™ 2-D Platinum software (Amersham Biosciences). For comparison of protein spot densities between control and treated samples, more than ten spots throughout all gels were correspondingly landmarked and normalized. The quantified spots of candidate proteins were compared with the aid of histograms. For ensuring the reproducibility of 2-DE experiments, each sample was analyzed in duplicate. The procedure for in-gel digestion of protein spots from CBB stained gels was carried out as described [21].

2.6 Identification of proteins by LC-MS/MS

The resulting tryptic peptides were separated and analyzed using RP capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC-MS/MS) with a slight modification [22]. Both of a $0.1 \times 20\ mm^2$ trapping and a $0.075 \times 130\ mm^2$ resolving column were packed with Vydac 218MS low trifluoroactic acid C18 beads (5 μm in size, 300 Å in pore size; Vydac, Hesperia, CA, USA) and placed in-line. Following the peptides were bound to the trapping column for 10 min at with 5% v/v aqueous ACN containing 0.1% v/v formic acid, then the bound peptides were eluted with a 50-min gradient of 5–80% v/v ACN containing 0.1% v/v formic acid at a flow rate of 0.2 μL/min. For MS/MS, a full mass scan range mode was $m/z = 450$–2000 Da. After determination of the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with relative collision energy of 55%. The individual spectra from MS/MS were processed using the TurboSEQUEST software (Thermo Quest, San Jose, CA, USA). The generated peak list files were used to query either MSDB database or NCBI using the MASCOT program (http://www.matrixscience.com). Modifications of methionine and cysteine, peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 0.8 Da, allowance of missed cleavage at 2, and charge states (+1, +2, and +3) were taken into account. Only significant hits as defined by MASCOT probability analysis were considered initially.

2.7 Protein and Hb content measurement

Group of ten larvae from the control and experimental tanks were pooled, and hemolymphs were withdrawn by opening the body wall. The body fluids were used for hemolymph protein and Hb analysis. Three replicates were conducted. The total Hb contents of the hemolymphs were estimated *via* the cyanometHb procedure [23, 26], using a plasma Hb kit (Sigma–Aldrich Chemical). The protein content was measured using the Bradford method.

2.8 Data analysis

Statistical differences between the control and treated larvae for all analyses were examined with the aid of a parametric *t* test, using SPSS 12.0 KO (SPSS, Chicago, IL, USA).

3 Results and discussion

3.1 Acute toxicity and exposure concentrations

The exposure concentrations were based on the results of acute toxicity testing, which used mortality as an endpoint (Table 1). The 24 h LC50 of Cd in *C. riparius*, as derived from a Probit analysis, was 212.2 mg/L. Therefore, 0.2, 2, and 20 mg/L were used as the sublethal exposure concentrations for evaluation of Hb and the ecotoxicity parameters. These concentrations were higher than those generally found in the field, as *Chironomus* shows high tolerance to environmental stresses, including Cd exposure. However, the present study focused on the identification of Hb-related parameters as potential biomarkers for Cd contamination under laboratory conditions. The *in situ* calibration and validation of the identified biomarkers using environmentally relevant exposure conditions will be addressed in future studies.

Table 1. Estimation of 10%, median, and 90% 24 h LC (LC10, LC50, and LC90, respectively) of Cd ($CdCl_2$) in the 4th instar larvae of *C. riparius*

	24 h LC (mg/L)	95% confidence interval
LC10	137.25	1.208–167.17
LC50	212.23	174.15–27750
LC90	328.16	230.84

3.2 Globin mRNA expression

Hb multiplicity is a well-known phenomenon in chironomid species, and *C. thummi thummi* larvae have been reported to secrete up to 16 Hb and 12 globin polypeptides, with more than 30 cloned globin genes having been sequenced [27–29]. To study the effects of Cd on the expression patterns of the globin transcript, the levels of globin mRNA were assessed in 4th instar larvae of *C. riparius* using five different *Chironomus* Hb ORFs (Fig. 1). Each ORF exhibited different sensitivities to Cd stress, with HbB, HbD, and HbE appearing to increase in response to treatment with 2 and 20 mg/L of Cd,

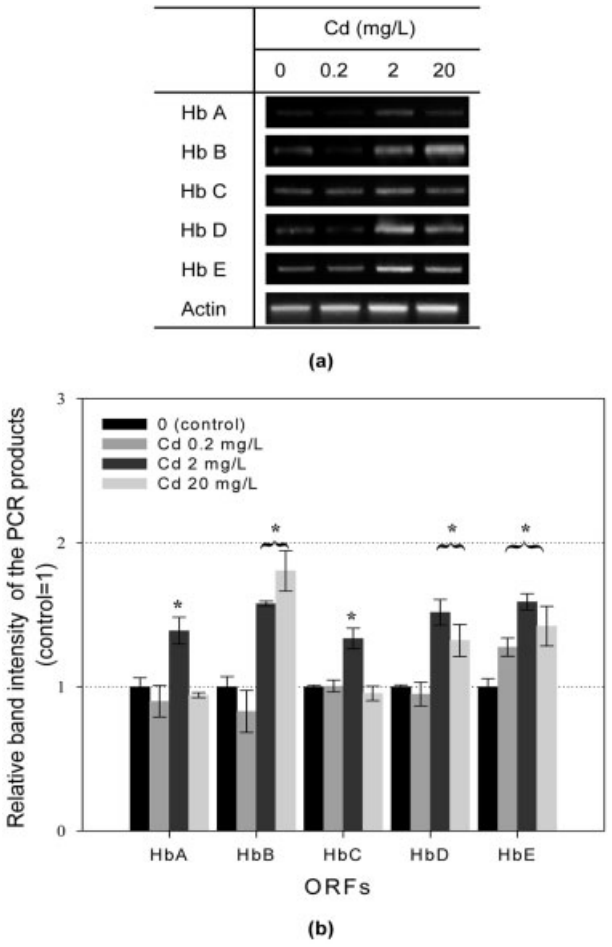

Figure 1. (a) Expression of globin genes in the 4th instar larvae of *C. riparius* exposed to Cd for 24 h. (b) Densitometric values were normalized using actin mRNA ($n = 3$, mean ± SEM, $*p<0.05$).

with the expressions of HbA and HbC showing no changes in response to Cd treatment, but the increases were less than two-fold that of the control. However, in this study, the expression of the globin ORF was assessed using semi-quantitative PCR; exact quantification using real-time PCR may provide more accurate information with respect to the transcriptional regulation of *Chironomus* globin expression. Taken together, the results of the globin mRNA expression obtained in this study suggested that the globin genes were constitutively expressed in the control, with some being overexpressed or upregulated by Cd exposure. Therefore, Hb multiplicity might contribute to their flexibility toward various environmental conditions [7].

3.3 Preliminary characterization of hemolymph protein – PAGE and IEF

The change in the protein expression of *Chironomus* globin in response to Cd exposure was examined using PAGE and IEF (Fig. 2). In our previous study, preliminary characterization of the multiplicity of the *Chironomus* globin protein was conducted [25]. As with other *Chironomus* species [28–32], striking heterogeneity was observed in *C. riparius* Hb. The globin in *C. riparius* was separated into seven and six different components by nondenative PAGE and by nondenative IEF, respectively, with observed molecular weights and p*I*s ranging from 7 to 26 kDa and from 3.5 to 6.2, respectively. In addition, because a red color was visible in the bands prior to staining, these were presumed to be those of globin. However, additional bands in a PAGE gel, found to be positive as a result of silver staining, were not seen prior to staining, indicating they were most likely minor nonglobin proteins associated with *C. riaprius* hemolymph (data not shown).

According to Weber and Vinogradov [8], *Chironomus* Hb exists in monomeric or dimeric forms, with exclusively monomeric Hbs (~17–18 kDa) and no evidence of subunit aggregation found in *C. tentans*; whereas, monomeric and dimeric forms of Hb are found in *C. thummi thummi*. Therefore, the molecular isoform corresponding to 26 kDa observed in *C. ripairus* may be the dimeric form of Hb. Exposure of *C. riparius* larvae to Cd appears to increase the expressions of the two presumed monomeric Hbs (16 and 17 kDa), and one presumed dimeric Hb (26 kDa). However, an investigation of *Chironomus* globin using PAGE and IEF analyses can only provide preliminary characterization; therefore, 2-D analysis was conducted to provide more detailed identification of the individual globins.

3.4 Hemolymph protein expression – proteomics approach

Recently, a similar proteomics approach was conducted on *C. riparius* exposed to Cd [33]; however, those experiments focused on the total larval proteins, not specifically on the hemolymph protein, as in our study. The results of the previous study revealed increases in the proteins involved in energy metabolism, protein fate, production of purines, pyrimidines, nucleosides and nucleotides, cell division, transport and binding, and signal transduction, as well as fatty acid and phospholipid metabolism in the cell, suggesting that Cd affected the entire array of cell functions. In our study, hemolymph proteins displaying altered levels of expression were identified using the TurboSEQUEST program (Fig. 3). When the intensity of the protein spots corresponding to Cd-treated and control larvae were compared, a total of 14 spots were differentially expressed (Fig. 3). As shown in Table 2, the protein spots were identified as globin proteins, such as, globin IV (Cd1,2,9,13), Hb CTTX (Cd3), globin CTT-VIIB-3 precursor (Cd4,5,6,8,10), globin CTT-VIII (Cd7), globin VIIA.1 (Cd11), globin CTP-III (Erythrocruorin III. Cd12), and Ctp HbVIIB-9 (Cd14). A histogram was then used to quantify and compare the intensities of the globin protein expressions in the control and treated organisms (Fig. 4). The expressions of all of the proteins were found to decrease in response to Cd exposure, with the exception of the Cd9 and Cd14 protein spots, which

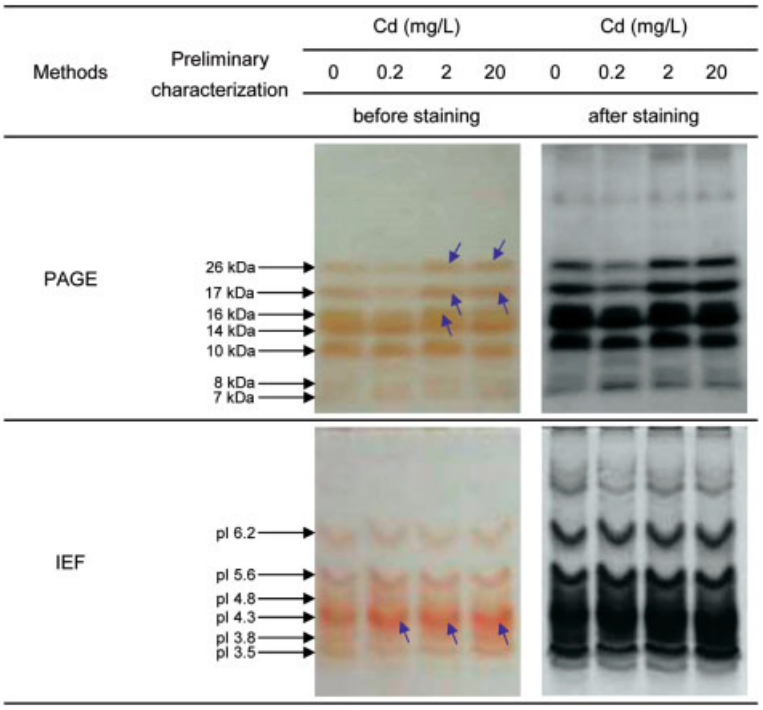

Figure 2. Expression of molecular weight (PAGE) and charge isoforms (IEF) of globin protein in the 4th instar larvae of *C. riparius* exposed to Cd for 24 h.

increased. The peak increase in the Cd14 spot was statistically significant in response to treatment with 2 mg/L of Cd. Furthermore, the expressions of protein spots Cd1, Cd2, Cd3, Cd4, Cd5, Cd6, Cd8, and Cd10, showed similar responses to Cd exposure, with a slight decrease observed in response to treatment with low Cd concentrations (0.2 and 2 mg/L) and a larger decrease in expressions observed in response to the highest treatment concentration (20 mg/L). The expressions of protein spots Cd11 and Cd12 decreased with all three tested concentrations, which was not dependent on the Cd exposure concentration of the organisms. Five spots, namely Cd4, Cd5, Cd6, Cd8, and Cd10, all corresponding to globin CTT-VIIB-3 precursor, showed similar response patterns toward Cd exposure. Conversely, four spots, namely Cd1, Cd2, Cd9, and Cd13, which correspond to globin IV, did not show similar response patterns, which may have been due to the PTM of the proteins. This may imply that these protein spots originated from identical genes, but were regulated at the (post) translational levels. This might also be an explanation for lack of matching between the gene and protein level expressions.

It is believed that the *Chironomus* globin molecules were upregulated at the mRNA level (Fig. 1), but downregulated at the protein level, with the exception of one major spot, which was upregulated (Fig. 4). The expression of each ORF may be related to each globin protein component identified by proteomics, which would imply that the upregulated ORF may be related to the upregulated protein spot (Cd4). The exact relationship between transcriptional and translational regulation is unclear, however, Hb multiplicity may allow this species to better adapt to exogenous stresses, potentially acting as a fine tuning system toward stressful environmental conditions. Therefore, *Chironomus* Hb may have potential for use as a biomarker toward chemical exposure. The main purpose of most proteomic analyses has been to investigate the biochemical pathway mostly using cells or tissues. This study presents a relatively novel application of proteomics, focusing on the holistic usage of this technique to identify biomarkers in an ecotoxicological context, which enabled sensitive monitoring of the environmental quality through the diagnosis of the overall fitness of a truly environmentally relevant organism. Martoja *et al.* [34] pointed out that fat body is a target organ for Cd toxicity. To better understand the regulation of Hb to Cd stress in *C. riparius*, therefore, it would be interesting to investigate the protein expression pattern in the both a fat body, as well as in the hemolymph. Proteomic analysis could be conducted on the entire cellular fraction to compare the protein expression pattern in the hemolymph. Nevertheless, as this study focused on the investigation of altered Hb expression due to Cd exposure, further analysis of the cellular fraction may be possible in the future.

3.5 Total Hb content and hemolymph protein analysis

Physiological studies on *Chironomus* Hb have focused on the unusually high oxygen affinity of Hb (compared to its vertebrate counterparts) and the mechanism by which the oxygen supply is facilitated to larval tissues in a relatively hypoxic

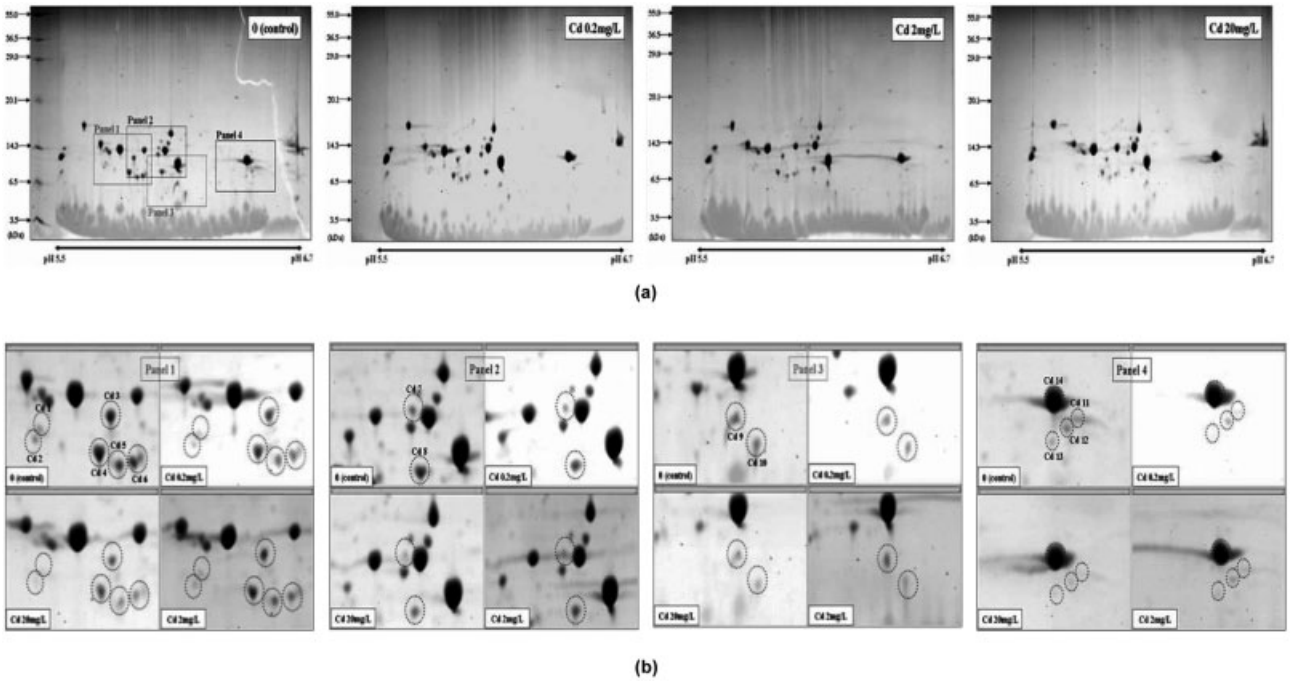

Figure 3. (a) 2-DE images of hemolymph proteins in the 4th instar larvae of *C. riparius* after exposure of a series of concentrations of Cd. (b) Profiling of differentially expressed proteins in *C. riparius* larvae after exposure to $CdCl_2$ using the PDQuest program.

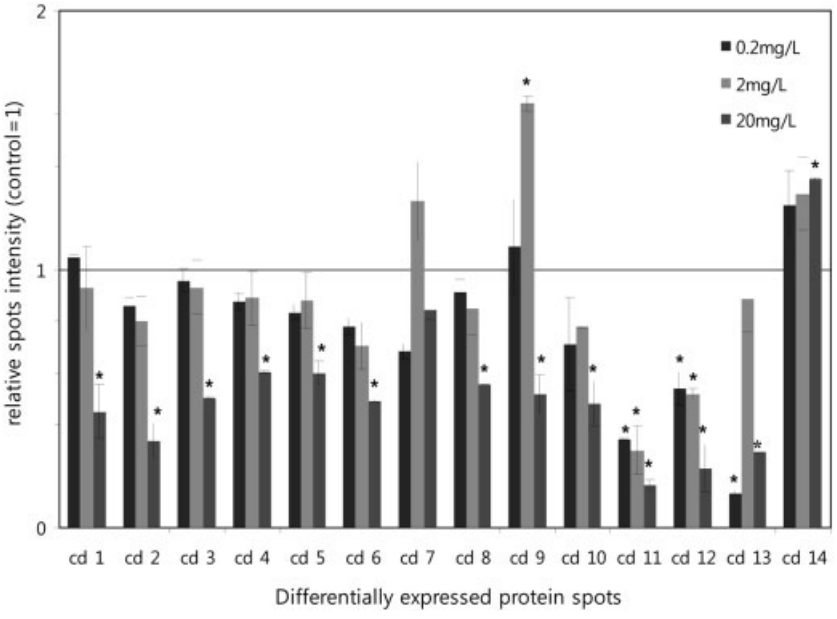

Figure 4. Quantitative histogram of differentially expressed proteins in *C. riparius* larvae after exposure to a series of concentrations of Cd.

benthic habitat. From an evolutionary point of view, it is generally acknowledged that the presence of Hb in invertebrates allows their adaptation to unfavorable environmental conditions, as these pigments help sustain aerobic metabolism under low-oxygen conditions [8]. The induction of Hb synthesis in many invertebrates under stressful conditions (hypoxia, temperature increase, and chemical pollution) demonstrates its role, as do inter- and intraspecific comparisons of animals with and without Hb [35]. *Chironomus* Hb appears to fulfill clear physiological roles in the transportation and storage of oxygen in larvae that burrow into polluted and hypoxic soils. According to Weber [36] and Lindegaard [37], extracellular Hb enhances the exploitation of hypoxic oxygen. To determine if decreases in individual globin

Table 2. Characterization of differentially expressed hemolymph proteins in the 4th instar *C. riparius* larvae following to Cd exposure

Spot no.	Sum of Mascot score	No. of identified peptide	Protein MW/ p*I* value	% Cover-age	Accession no.	Protein name	Identified peptide sequence[a)]	Ref.
Cd 1	298	4	15 794/6.44	38	1009252A	Globin IV	IIGDLPNIDGDVTTFVASHTPR	Ions score 82
Cd 2	290	4	15 794/6.44	38	1009252A	Globin IV	FTQFAGKDLDSIK	Ions score 79
Cd 3	570	6	17 088/5.51	60	0512218A	Hb CTTX	DLEAIKDTADFAVHASR	Ions score 105
Cd 4	496	7	16 973/6.04	55	P12548	Globin CTT-VIIB-3 precursor	TALVAYLSNHVSWGDNVAAAWNK	Ions score 109
Cd 5	454	7	16 973/6.04	70	P12548	Globin CTT-VIIB-3 precursor	DLASIKDTGAFATHATR	Ions score 88
Cd 6	486	6	16 973/6.04	67	P12548	Globin CTT-VIIB-3 precursor	IVSFLSEVIALSGNESNASAVNSLVSK	Ions score 105
Cd 7	422	6	16 888/5.61	50	P02227	Globin CTT-VIII	HNEVDILYAVFK	Ions score 85
Cd 8	508	6	16 973/6.04	55	P12548	Globin CTT-VIIB-3 precursor	TALVAYLSNHVSWGDNVAAAWNK	Ions score 109
Cd 9	398	5	15 794/6.44	38	1009252A	Globin IV	IIGDLPNIDGDVTTFVASHTPR	Ions score 118
Cd 10	236	4	16 973/6.04	32	P12548	Globin CTT-VIIB-3 precursor	GVSAAQFGEFR	Ions score 82
Cd 11	323	5	16 820/6.06	51	AAB58930	Globin VIIA.1	IVGFVSEIIALIGNESNAPAVQTLVGQLAASHK	Ions score 85
Cd 12	495	7	14 774/6.45	52	P22431	Globin CTP-III (erythrocruorin III)	LSADQISTVQASFDK	Ions score 89
Cd 13	346	5	15 794/6.44	38	1009252A	Globin IV	FTQFAGKDLDSIK	Ions score 83
Cd 14	690	6	16 794/6.17	70	CAA39712	Ctp HbVIIB-9	IVSFLSEVIALSGNASNAAAVEGLLNK	Ions score 114

a) Shows one of the representative sequences, which is the highest score of identified peptides in each protein.

Table 3. Protein and Hb contents in the hemolymph of the 4th instar *C. riparius* larvae measured 24 h after exposure to Cd (number = 3, mean ± SEM)

$CdCl_2$ (mg/L)	Protein (μg/mg body dry weight)	Hb (μg/mg body dry weight)	Hb/ Protein
0 (control)	296.1 ± 11.64	284.4 ± 15.02	0.960
0.2	144.5 ± 2.833	140.3 ± 1.384	0.971
2	186.7 ± 6.331	176.3 ± 13.2	0.944
20	167.1 ± 7.545	154.2 ± 2.526	0.922

proteins (Fig. 3) lead to a decrease in actual Hb production, the total Hb and total extracellular protein contents in 4th instar *C. riparius* larvae were measured 24 h after exposure to sublethal concentrations of Cd (Table 3). The Hb/protein ratio was also evaluated in Cd-treated and control samples. In the 4th instar *C. riparius* larvae, the hemolymph protein was found to constitute approximately 50% of the total protein (Table S1 of Supporting Information), with the main property being Hb (more than 95%, Table 3). The hemolymph protein and Hb contents were decreased by 51, 37, and 43% (hemolymph protein) and 50, 38, and 45% (Hb content) in response to treatment with 0.2, 2, and 20 mg/L of Cd, respectively, compared to that of the control. Because Hb is the main property of hemolymph protein, significant alteration was not observed in the Hb/protein ratio after treatment. Taken together, these results indicated that the decreased expression of the selected globin proteins in response to Cd exposure (Fig. 3) appears to impact on Hb synthesis, which was demonstrated by the decreased total Hb concentration in hemolymph (Table 3). Cd may also exert an effect on heme synthase and iron metabolism; however, this was not investigated in this study. It is well known that some metals induce δ-aminolevulinic acid synthase; whereas, Pb, and other metals to a lesser extent, inhibit δ-aminolevulinic acid dehydratase; both of which are involved in porphyrin synthesis [38]. Therefore, the effects of these substances on nonglobin parts, such as, heme, need to be determined for the effects of Cd on *Chironmous* Hb to be better understood. Taken together, our data suggest that the expression of globin protein appears to be directly related to Hb production.

3.6 Ecotoxicological relevance of Hb response

Until now, little attention has focused on the integrated analysis of the molecular responses and higher-level effects within the same study, with the biological links between the mechanisms of action and the complex response patterns still being an item of debate as a result of individual variability [39]. One major obstacle in overcoming the application of proteomics to ecotoxicology is the correlation of the molecular level response to ecologically relevant effects. In this study, to investigate the ecotoxicological relevance of the *Chironomus* Hb response to Cd exposure, organism/population-relevant indicators (growth, reproduction, and development) were investigated (Table 4). Growth indicators, such as ash free body dry weight, decreased by approximately 15% compared to those of the control larvae in response to exposure to 20 mg/L of Cd. Moreover, development indicators, such as pupation and emergence rates, decreased significantly in response to exposure to 2 and 20 mg/L of Cd, with the total adult emergence rate reduced by approximately 61 and 97%, respectively. This decrease in pupation and the emergence of a response to Cd exposure suggests the alteration in these parameters might be the consequence of a serious progression of toxic effects. Such a significant disturbance to pupation and

Table 4. Growth, development, and reproduction parameters investigated in the 4th instar larvae of *C. riparius* exposed to Cd for 24 h (number = 3, mean ± SEM, *$p<0.05$)

$CdCl_2$ (mg/L)	Growth		Development		Reproduction	
	Body fresh weight (mg/larvae)	Ash-free Body weight (mg/larvae)	Pupation (%)	Emergence (%)	Number of egg mass/treatment	Number of egg/egg mass
0 (control)	3.853 ± 0.236	0.045 ± 0.002	82.667 ± 5.696	62 ± 4.163	7	569 ± 37.1
0.2	4.139 ± 0.009	0.051 ± 0.008	84.667 ± 2.906	72.667 ± 6.960	5	481 ± 27.0
2	3.773 ± 0.050	0.043 ± 0.009	56 ± 4.619*	24 ± 6.110**	1	376*
20	3.797 ± 0.036	0.038 ± 0.001*	13.333 ± 2.404***	1.333 ± 0.667***	0	0***

the emergence of a response to exposure to high levels of Cd suggests this metal may provoke serious consequences in *Chironomus* reproduction and, in turn, its population. As shown in Table 4, complete inhibition of egg mass oviposition and a decrease in the average numbers of eggs *per* egg mass occurred in response to Cd exposure, which occurred in a concentration dependant manner, further indicating *Chironomus* populations exposed to Cd may be adversely affected. Taken together, these results indicate that Cd exerted serious chronic toxicity towards the development and reproduction of *C. riparius*.

4 Concluding remarks

In this study, decreases in the expressions of globin protein and the total Hb content were observed in response to exposure to high levels of Cd (2 and 20 mg/L), which occurred concomitantly with the deterioration in physiological levels, such as growth, reproduction and development (Table 4). This phenomenon suggests that the alteration to the expression of globin protein is not a homeostasis response, but is instead a toxic response potentially leading to physiological consequences. Our experiments consisted of the simultaneous observation of different levels of responses, so provides no mechanical evidence of a causal relationship between the protein level and organism/population level responses.

Based on our results, as well as the findings of several previously conducted studies, it is clear that *Chironomus* larvae possessing heme respiratory proteins are pre-adapted to various extreme environmental conditions, including Cd contamination. The flexibility of Hb in response to the environment allows for the best possible adaptation of larvae. This study included a proteomic analysis of the hemolymph protein obtained from *C. riparius* larvae exposed to Cd, which revealed 14 hemolymph proteins differentially expressed in response to Cd toxicity, which were associated with globin proteins. The overall results suggest that the expression of globin may play an important role in Cd ecotoxicity in this species. Moreover, this study presents the potential of proteomics as a tool for the discovery of biomarkers in ecotoxicological research, which is a relatively novel application of proteomics for enabling sensitive monitoring of environmental quality through the diagnosis of the overall fitness of an environmentally relevant organism.

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