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Characterization and Expression of *Chironomus riparius* Alcohol Dehydrogenase Gene under Heavy Metal Stress

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중금속 노출에 따른 리파리 깔다구에서의 ADH 유전자의 발현 및 특성

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ABSTRACT

Metal pollution of aquatic ecosystems is a problem of economic and health importance. Information regarding molecular responses to metal exposure is sorely needed in order to identify potential biomarkers. To determine the effects of heavy metals on chironomids, the full-length cDNA of alcohol dehydrogenase (ADH3) from Chironomus riparius was determined through molecular cloning and rapid amplification of cDNA ends (RACE). The expression of ADH3 was analyzed under various cadmium and copper concentrations. A comparative and phylogenetic study among different orders of insects and vertebrates was carried out through analysis of sequence databases. The complete cDNA sequence of the ADH3 gene was 1134 bp in length. The sequence of *C. riparius* ADH3 shows a low degree of amino acid identity (around 70%) with homologous sequences in other insects. After exposure of *C. riparius* to various concentrations of copper, ADH3 gene expression significantly decreased within 1 hour. The ADH3 gene expression was also suppressed in *C. riparius* after cadmium exposure for 24 hour. However, the effect of cadmium on ADH3 gene expression was transient in *C. riparius*. The results show that the suppression of ADH3 gene by copper exposure could be used as a possible biomarker in aquatic environmental monitoring and imply differential toxicity to copper and cadmium in *C. riparius* larvae.

Key words : *Chironomus riparius*, alcohol dehydrogenase, cadmium, copper, biomarker, aquatic biomonitoring

INTRODUCTION

Water contamination is one of the most serious consequences of chemicals emanating from hazardous waste sites. Heavy metals in the form of trace elements from natural and anthropogenic sources accumulate in aquatic sediments and pose a threat to sediment biotic communities (Muntau and Baudo, 1992; Cheng, 2003; Besser *et al*., 2008). Heavy metals, including copper, lead, and cadmium, are frequ-

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ently detected as groundwater contaminants (Clements and Kiffney, 1994). Aquatic organisms take up and accumulate both essential and nonessential trace elements from the sediments. Heavy metals not assimilated into the aquatic organisms, or not easily degraded or excreted, are transferred to higher trophic level organisms (Reynoldson, 1987; Tessier and Campbell, 1987; Landrum and Robbins, 1990; Eimers *et al*., 2002).

Copper pollution occurs in the aquatic environment due to mine washing or agricultural leaching. Although copper is an essential trace element in biological functions such as iron absorption, hemopoiesis, and fermentation (Skalicka *et al*., 2005), it is also one of the most toxic heavy metals identified to date (To'th *et al*., 1996). Organs of aquatic animals may accumulate copper (Rojik *et al*., 1983; B'alint *et al*., 1997), which can lead to redox reactions that generate free radicals that can ultimately cause morphological alterations and change certain physiological processes.

Cadmium is also a contaminant of interest because it is widely recognized as an environmental pollutant (Aoki *et al*., 1984) that is highly toxic, affecting a wide range of physiological processes such as plasma membrane transport and gene transcription (Maroni *et al*., 1986). Exposure to cadmium via air and food can also lead to renal tubular dysfunction (Korenekova *et al*., 2002) or reproductive complications (Massanyi *et al*., 1996; Lukac *et al*., 2003; Henson and Chedrese, 2004).

Copper and cadmium are toxic heavy metals. Copper or cadmium exposure is more toxic at low concentrations than lead or zinc exposure in *Tanytarsus dissimilis* (Chironomidae) (Anderson *et al*., 1980). However, the detoxification routes of cadmium and copper are quite divergent. In mussels, copper is rapidly extruded through the vacuolar-lysosomal system bound to a copper thionein with a half-life of about 6 days, while cadmium accumulates in the cytosol where it binds to a cadmium thionein with a half-life of more than 7 months (Viarengo *et al*., 1985; Viarengo, 1989). Odonate larvae are tolerant of high concentrations of cadmium and lead, exhibiting no significant decrease in survival, but are more sensitive to copper exposure, demonstrating significantly decreased survival following exposure to low copper concentrations (Tollett *et al*., 2008). Copper is also more toxic to *Perinereis nuntia* than cadmium at the same concentration and induces morphological changes at a much lower concentration than cadmium (Won *et al*., 2008).

Toxicants and other stressors can cause changes in gene expression, which have proven useful as biomarkers. For example, the metallothionein gene in *Drosophila* species is induced by a number of heavy metals such as zinc, cadmium, copper, silver, and mercury (Maroni *et al*., 1986). Metallothionein, heat shock proteins, and glutathione-S-transferase are involved in regulating interactive effects of metal/metalloid mixtures at low dose levels (Wang and Fowler, 2008). A molecular approach, based on amplification of gene transcripts by means of reverse transcription polymerase chain reaction (RT-PCR), has been introduced to evaluate relative expression levels following heavy metal exposure (Lemoine *et al*., 2000; Lemoine and Laulier 2003; Rebelo *et al*., 2003; Tom *et al*., 2004). An improvement of this technique is real-time quantitative PCR, which is currently among the most sensitive and reliable methods for detecting gene expression levels, particularly for low-abundance mRNAs (Orlando *et al*., 1998). However, there are few environmental studies at the molecular genetic level for the *Chironomus* family, although there have been many studies on the general biological effects of heavy metals (Martinez *et al*., 2001, 2003; Nowak *et al*., 2007). This is probably because there is little sequence information available on environmentally responsive genes in this family.

Alcohol dehydrogenases (ADH) constitute a large family of related enzymes and isozymes. Known ADHs can be divided into three main groups based on the metal cofactors required for catalysis: no metal requirement, requirement of iron for activity, and requirement of zinc as a cofactor. The latter group represents a functionally heterogeneous group of proteins including representative prokaryotic, fungal plant

and animal ADHs. Diverse mechanisms regulate ADH gene expression, and extensive variation is seen with respect to tissue-specific expression and developmental regulation (Funkenstein and Jakowlew, 1996). Dimeric zinc-containing alcohol dehydrogenases belong to the protein super family of medium-chain dehydrogenases/reductases and consist of a complex system with different forms and extensive multiplicity. ADHs are able to catalyze the reversible oxidation of a wide variety of xenobiotic and endogenous alcohols to the corresponding aldehydes (Dasmahapatra *et al*., 2001). ADH3 is a glutathione-dependent formaldehyde dehydrogenase that can oxidize ethanol at high concentrations. ADH3 is also the ancestral ADH and has been identified in all species analyzed (Danielsson and Jornvall, 1992; Duester *et al*., 1999). In a recent study, the ADHs were identified as some of the differentially expressed proteins in *Phanerochaete chrysosporium* under cadmium and copper stress (Ozcan *et al*., 2007).

Chironomids are an ecologically diverse family of dipterans and probably the most ubiquitous of all aquatic macroinvertebrates. This is due to their physiological tolerance of various environmental conditions, such as extreme salinity or temperature, extreme pH levels, and reduced levels of dissolved oxygen (Anderson, 1977). They are increasingly used in toxicity experiments because of their widespread distribution, short life-cycle, ability to be reared in the laboratory, and their easily identifiable life-cycle stages (Anderson, 1977). Given that they are benthic macroinvertebrates, chronomids can also be used to evaluate sediment and water toxicity (Ibrahim *et al*., 1998). Morphological abnormalities were observed in *Chironomus* larvae exposed to heavy metals and endocrine-disrupting chemicals (Martinez *et al*., 2001, 2003; Kwak and Lee, 2005). Thus, chironomids are a good aquatic model for assessing the toxicity of heavy metal-contaminated freshwater.

In this study, the ADH3 gene from *C. riparius* was characterized. Comparative molecular and phylogenetic studies were carried out to analyze homologies among insects and vertebrates. ADH3 expression was analyzed by means of real-time RT-PCR during different life-cycle stages and also under various concentrations of copper and cadmium to determine the effects of heavy metal exposure on *C. riparius* ADH3 gene expression.

MATERIALS AND METHODS

1. Organisms

Rearing conditions followed methodologies outlined by Streloke and Köpp (1995). *C. riparius* larvae were obtained from adults reared in the laboratory. The original strain was provided by the Korea Institute of Toxicology (Daejeon, Korea). The larvae were reared in an environmental chamber under long-day conditions with a light : dark cycle of 16 : 8 hours and a light intensity of about 500 lx. Water temperature was constant at $20 \pm 1^{\circ}C$ in the incubator chamber (Sanyo, Osaka, Japan). Larvae hatched from eggs were kept in Duran crystallizing dishes (Schott, Mainz, Germany) with approximately 500 mL of M4 culture medium (Elendt, 1990) and a sediment layer of 1 cm of fine sand ($< 63\,\mu m$ particle size), and were aerated continuously after midge larvae were introduced. All dishes received 5 mg of food daily (0.5 mg $Larva^{-1}$; Tetra-Werke, Melle, Germany), which had been ground in a blender, to provide unlimited food conditions (Pery *et al*., 2002).

2. Exposure conditions

All experimental larvae were acquired by the eleventh day after hatching from the same control egg masses. Larvae were exposed to water enriched with copper ($CuSO_4$) and cadmium ($CdCl_2$) (Sigma-Aldrich Co., St Louis, MO, USA). The nominal metal concentrations were based on data regarding toxicity values for *C. riparius* and real concentrations found in the Anam River (Janssens de Bisthoven *et al*., 1998; Janssens de Bisthoven *et al*., 2001; Milani *et al*., 2003; Igwilo *et al*., 2006). The nominal concentrations for copper were 1, 10, 60, and 100 $\mu g\ L^{-1}$ and for cadmium 3, 9, 27, 100 $\mu g\ L^{-1}$. Stock solutions were 0.1 g

L^{-1} Cu^{2+} and Cd^{2+}.

Thirteen fourth-instar *C. riparius* larvae were transferred into 300-mL crystallizing dishes (Schott, Mainz, Germany) filled with 200 mL of M4 media, and treated with four concentrations of copper or cadmium. Exposure periods were 1, 9, and 24 hours. All experiments were conducted in triplicate using independent samples (e.g., three boxes each containing 3 μg L^{-1} of cadmium or 1 μg L^{-1} of copper for 1 hour). Each group contained thirteen larvae and ten of these were utilized for subsequent analyses. Untreated larvae for control were also measured in triplicate. Exposure was carried out under constant temperature (20 ± 1°C), while a photoperiod of 16 : 8 hours light:dark was used for all experiments.

3. ADH3 gene characterization

Sequences of the ADH3 gene from *C. riparius* were amplified by Polymerase Chain Reaction (PCR) using primers designed from higher Diptera (*Aedes aegypti* and *Anopheles gambiae* in Fig. 2) consensus sequences. Multiple sequence alignments were performed by ClustalW (Thompson *et al*., 1994). The primers were 5′ TCAACATTCAGTGARTAYACAGTTG-3′ and 5′-AATGARGCATTYSATTTGATG CATG-3′ for the ADH3 gene. 'R' represents a mixture of A and G, 'S' a mixture of G and C, and 'Y' a mixture of T and C. The 50-μL PCR mix contained 1 × Taq polymerase buffer, 200 μM dNTP, 2 units of Taq polymerase, and 20 μM primers. The PCR reaction was performed under the following conditions: 5 min at 94°C, 38 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and 7 min at 72°C. The 633 bp amplified DNA was cloned into the T-vector (Invitrogen, Carlsbad, USA) and sequenced with an ABI 3700 Genetic analyzer. To acquire full-length ADH3 cDNA, we used the GeneRacer kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

4. Phylogenetic analysis

Amino acid sequences were aligned with those of other organisms using Clustal X version 1.8 and displayed with GeneDoc Program (ver 2.6.001). The phylogenetic tree was constructed by neighbor-joining analyses using TreeTop (Brodsky *et al*., 1993). Bootstrap values were calculated with 1000 replicates.

5. Gene expression analysis

Total RNA was isolated from *C. riparius* embryos (two egg masses), fourth-instar larvae, pupae and adults (ten individuals for each stage) with TRIZOL® reagent (Invitrogen, Scotland, UK) according to the manufacturer's instructions. Single-strand cDNA was synthesized from 4 μg of total RNA using random hexamer primer for reverse transcription in the 20-μL reaction mix using the SuperScript™ III RT kit (Invitrogen, Scotland, UK). The cDNAs obtained were used as templates for PCR reactions with gene-specific primers for ADH3. In addition, PCR was conducted using primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a stable internal control (Vandesompele *et al*., 2002). The sequences of the oligonucleotide primers were: ADH3 forward 5′-GGATGTGGAATTCCAACTGGA-3′; ADH3 reverse 5′-TTCTTGGATTGTCTTGTCACC-3′; GAPDH forward 5′- GGTATTTCATTGAAT GATCACTTTG-3′; GAPDH reverse 5′-TAATCC-TTGGATTGCATGTACTTG-3′ (GenBank accession no. **EU999991**). The relative expression levels of the genes were measured by real-time RT-PCR using SYBR Green and iCyclerIQ (Bio-Rad, Hercules, USA). Each test consisted of at least three replicates and values were normalized to GAPDH as an internal control.

6. Data analysis

Results are expressed as mean ± SD unless otherwise stated. The level of ADH3 mRNA in each sample was normalized against the level of GAPDH based on standard curves. Levels of ADH3 transcripts in metal-treated groups relative to non-exposed control were estimated from normalized values. The differences in ADH3 mRNA levels among groups were

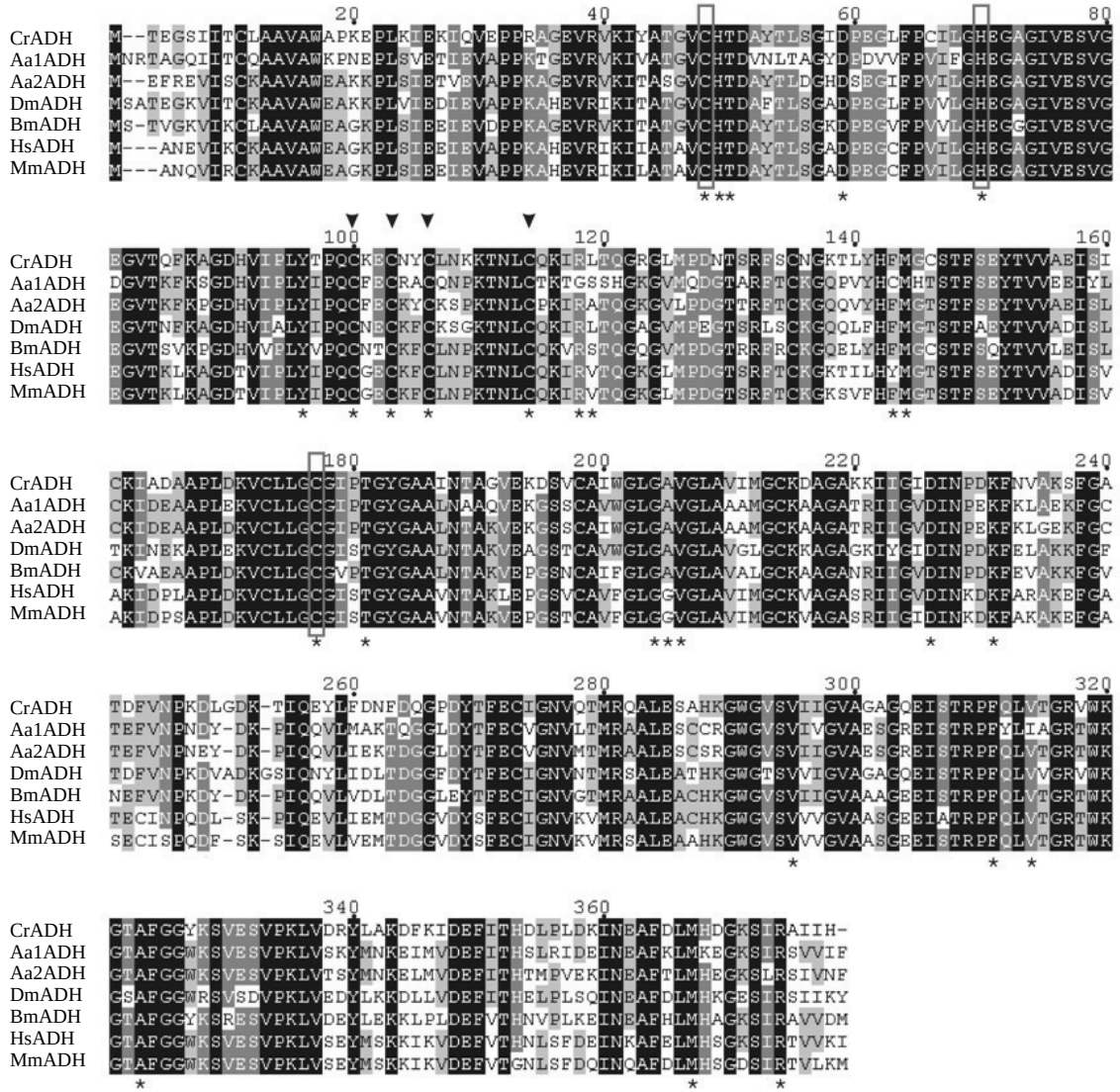

Fig. 1. Multiple sequence alignment of the deduced *Chironomus riparius* ADH3 gene with other insect, human and mouse ADHs. Numbering of the amino acid residues is with respect to the *C. riparius* ADH. Critical amino acids that are part of the substrate or coenzyme-binding domain are denoted by asterisks (Eklund *et al*., 1990; Höög *et al*., 1992; Hurley *et al*., 1994). All ligands to the catalytic (red rectangles) and noncatalytic zinc atoms (Arrow heads) are strictly conserved for all classes of the ADH enzymes. The *Chironomus riparius* ADH3 has all the amino acid residues typical for class III ADH enzymes. CrADH: *Chironomus riparius* alcohol dehydrogenase 3 (**EU683897**), Aa1ADH: *Aedes aegypti* alcohol dehydrogenase (**XP_001657839**), Aa2ADH: *Aedes aegypti* alcohol dehydrogenase (**XP_001657840**), DmADH: *Drosophila melanogaster* alcohol dehydrogenase class III (accession no. **NP_524310**), BmADH: *Bombyx mori* alcohol dehydrogenase (accession no. **NP_001040507**), HsADH: *Homo sapiens* alcohol dehydrogenase 5 class III (accession no. **AAV38636**), Mm: *Mus musculus* alcohol dehydrogenase 5 class III (accession no. **AAC52763**).

assessed by ANOVA followed by Tukey's multiple range test using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

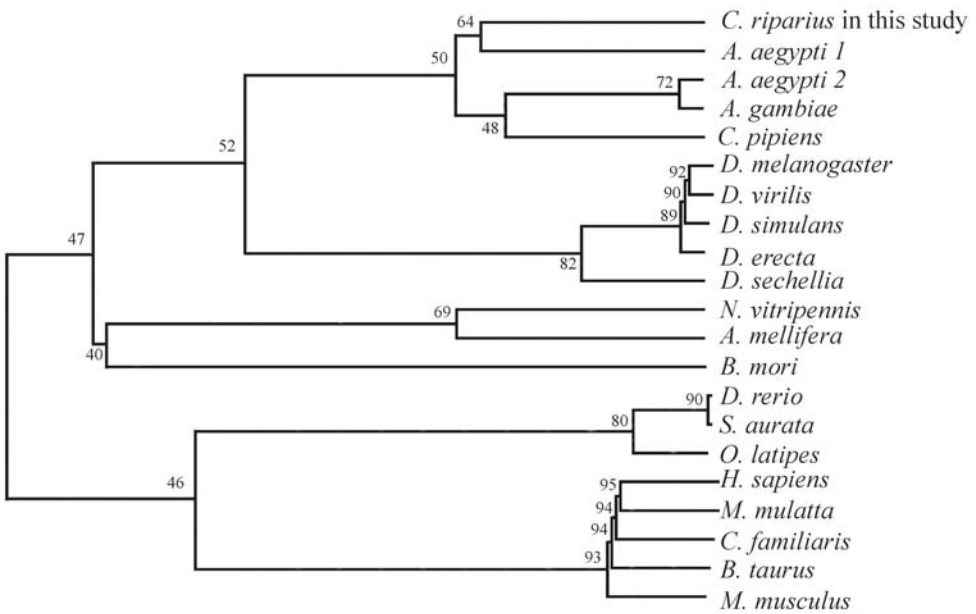

Fig. 2. Phylogenetic trees of the ADH3 gene constructed by neighbor-joining analysis (bootstrap value 100). The numbers at the nodes are the percentage bootstrap values. Amino acid sequences were aligned using Clustal X ver 1.8. The ADH3 sequences were retrieved from GenBank: *Chironomus riparius* in this study (accession no. **EU683897**), *Aedes aegypti 1* (accession no. **XP_001657839**), *Aedes aegypti 2* (accession no. **XP_001657840**), *Anopheles gambiae str. PEST* (accession no. **XP_314472**), *Culex pipiens quinquefasciatus* (accession no. **XP_001850438**), *Drosophila melanogaster* (accession no. **NP_524310**), *Drosophila virilis* (accession no. **XP_002053682**), *Drosophila simulans* (accession no. **XP_002103930**), *Drosophila erecta* (accession no. **XP_001980625**), *Drosophila sechellia* (accession no. **XP_002031693**), *Nasonia vitripennis* (accession no. **XP_001602754**), *Apis mellifera* (accession no. **XP_393266**), *Bombyx mori* (accession no. **NP_001040507**), *Danio rerio* (accession no. **NP_571924**), *Sparus aurata* (accession no. **P79896**), *Oryzias latipes* (accession no. **NP_001098256**), *Homo sapiens* (accession no. **AAV38636**), *Macaca mulatta* (accession no. **XP_001088376**), *Canis familiaris* (accession no. **XP_852213**), *Bos taurus* (accession no. **NP_001029421**), *Mus musculus* (accession no. **AAC52763**).

RESULTS

1. Identification of an ADH3 gene and phylogenetic analysis

Partial sequences of the *C. riparius* ADH3 gene were amplified by PCR using primers designed from higher Diptera consensus sequences. The full-length cDNA from *C. riparius* was acquired using molecular cloning and rapid amplification of cDNA ends (RACE). The complete cDNA sequence of the *C. riparius* ADH3 gene was 1,134 bp. The deduced amino acid sequence for this gene comprised 378 amino acids. The complete coding sequence of *C. riparius* ADH3 was deposited in GenBank (accession no. **EU683897**). There were no matched nucleotide sequences with any species. However, the ADH3 amino acid region had 74%, 73%, and 71% identity to fruit fly, domestic silkworm, and yellow fever mosquito, respectively (Fig. 1). The *C. riparius* ADH3 was related to the class III-type ADH enzymes from human (ADH5) and mouse (ADH5). All ligands to both the catalytic and noncatalytic zinc atoms were strictly conserved among species (Fig. 1). The amino acid residues distinguishing class III enzymes from the ethanol-active classes are mainly located in the substrate pocket of the enzyme, and affect size, shape, and pola-

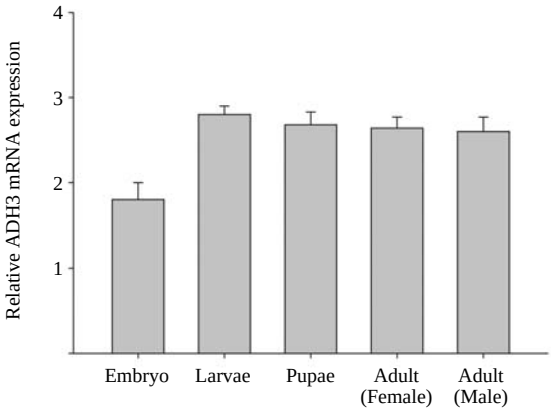

Fig. 3. Analysis of ADH3 gene expression during development. The raw values were normalized to GAPDH, and the data used to calculate relative expression levels. Relative ADH3 mRNA expression on y-axis indicates the mRNA ratios of ADH3/GAPDH. The experiment was performed in triplicate (mean ± standard error of the mean).

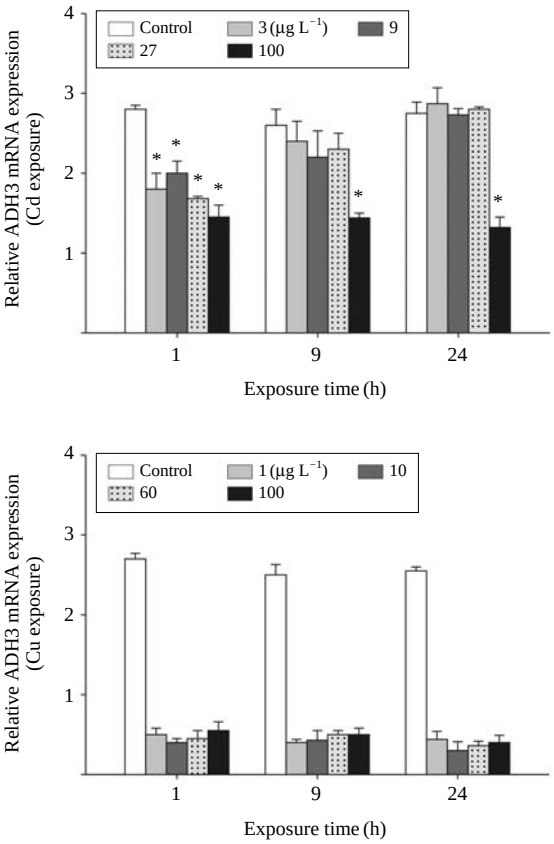

Fig. 4. Expression of ADH3 gene in fourth-instar larvae of *C. riparius* exposed to cadmium and copper. mRNA expression is shown relative to GAPDH expression after normalization. The experiment was performed in triplicate (mean ± standard error of the mean, $*p < 0.05$ in cadmium exposure experiment, all data are $p < 0.01$ in copper exposure experiment). Differences were considered significant at $p < 0.05$.

rity of the substrate cleft, and hence substrate-specificity and accessibility (Eklund *et al*., 1990; Höög *et al*., 1992; Hurley *et al*., 1994). Two amino acids (Asp-59 and Arg-118) were determined to be points of contact with the substrate S-hydroxymethylglutathione in the substrate pocket of class III enzymes, and both are conserved in the *C. riparius* ADH3 enzyme. The *C. riparius* ADH3 has all the amino acids in the substrate pocket typical of a class III ADH enzyme (Fig. 1).

Fig. 2. shows the phylogenetic relationships between the *C. riparius* ADH3 gene and those of other species. Results showed that the ADH3 sequence from *Chironomus* is closely related to that of other insects including mosquitoes. On the phylogenetic tree, ADH3 from *D. melanogaster* formed clusters with ADH3 from other *Drosophila* species. *C. riparius* ADH3 formed large clusters with ADH3 genes from other insects, while vertebrate ADHs formed another cluster with mammals and fish.

2. Analysis of ADH3 gene expression during development

Real-time RT-PCR experiments were carried out to analyze ADH3 expression during different developmental stages of *C. riparius*. Changes were observed in the level of ADH3 transcript throughout the different life-cycle stages. Fig. 3 shows that ADH3 exhibited a high level of expression during all developmental stages, except the embryonic stage.

3. ADH3 expression following cadmium or copper exposure

ADH3 expression in *C. riparius* exposed to cad-

mium and copper was examined by real time RT-PCR. ADH3 expression decreased in *C. riparius* exposed to all concentrations of cadmium, 3, 9, 27 and 100 μg L^{-1}, after 1 hour (Fig. 4). However, the decrease in ADH3 gene expression was only significant in *C. riparius* exposed to 100 μg L^{-1} cadmium for 24 hours ($p < 0.05$). There were no differences between non-treated control and samples from 3, 9 and 27 μg L^{-1} cadmium treatment for 9 or 24 hours ($p > 0.05$). The change in ADH3 gene expression was transient in *C. riparius* after cadmium exposure.

Expression of ADH3 was also examined following copper exposure (Fig. 4). ADH3 expression was significantly affected after exposure to all four concentrations of copper: 1, 10, 60 and 100 μg L^{-1} ($p < 0.01$). ADH3 gene expression significantly decreased in *C. riparius* within 1 hour after copper exposure. In addition, ADH3 gene expression was significantly reduced in *C. riparius* expression after exposure to copper for 24 hours. The response of the ADH3 gene was persistent in *C. riparius* after copper exposure.

DISCUSSION

Among aquatic invertebrates, the aquatic larvae of Chironomidae 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 a major food source for fish and other vertebrates and invertebrates (Cranston, 1995). Thus, they are used extensively to assess the acute and sub-lethal toxicity of contaminated sediments and water (Kahl *et al*., 1997; Matthew and David, 1998; Matthew *et al*., 2001; Bettinetti *et al*., 2002; Choi *et al*., 2002; Crane *et al*., 2002). However, there have been few studies of the molecular response to heavy metal stress and of the gene sequence information in the *Chironomus* family. In the present study, partial sequence information of a gene was obtained from *C. riparius* DNA by PCR with consensus primers. The primers were designed using mixed bases and analysis of homologue regions among Diptera species using multiple alignment programs. Full sequence information was then obtained by RACE and molecular cloning based on the partial sequence. These approaches allowed simple and successful acquisition of sequence information of interesting genes in this species for which little sequence information is available. The complete coding sequence of *C. riparius* ADH3 was deposited in GenBank (accession no. **ACD61704**). The *C. riparius* ADH3 cDNA encode 378 aa with a theoretical pI of 5.12 and a calculated molecular weight of 93 kDa. The expression of ADH3 was high in all developmental stages, except the embryonic stage. There was no significant difference in ADH3 expression between males and females ($p > 0.05$).

Copper and cadmium are widely recognized as environmental pollutants and are toxic heavy metals (Maroni *et al*., 1986; To'th *et al*., 1996). In the aquatic environment, the range of detected cadmium was 2 ~ 5 μg L^{-1} in the Anam River in Nigeria (Igwilo *et al*., 2006) and dissolved copper and cadmium concentrations in seawater ranged from 1.71 to 3.49 and 1.65 to 2.01 μg L^{-1}, respectively in China (Wan *et al*., 2008). However, differential toxicity of copper and cadmium was reported in *Perinereis nuntia* and Dragonfly larvae (Odonata). Copper was more toxic than cadmium at the same concentration to *Perinereis nuntia* and Dragonfly larvae (Odonata) (Tollett *et al*., 2008; Won *et al*., 2008). *C. riparius* was the most sensitive species to copper in water-only exposure experiments (Milani *et al*., 2003).

ADHs generally catalyze the reversible oxidation of a wide variety of xenobiotic and endogenous alcohols to the corresponding aldehydes (Dasmahapatra *et al*., 2001). A recent study identified ADH3 as one of the proteins that is differentially expressed under cadmium and copper stress in *Phanerochaete chrysosporium* (Ozcan *et al*., 2007). The most strongly induced genes were ADHs, glucose-6-phosphate isomerase, flavonol/cinnamoyl-CoA reductase, H1-transporting two-sector ATPase, ribosomal protein S7, ribosomal protein S21e, and elongation factor EF-1 alpha subunit. ADHs seemed to function to counter-

act copper toxicity because of their expression was induced under only copper stress (Ozcan *et al*., 2007). Furthermore, in this study, the response of the *C. riparius* ADH3 gene to cadmium was transient, while that to copper was persistent. The expression of ADH3 gene was suppressed in *C. riparius* larvae exposed to low concentrations of copper (1 μg L^{-1}), but to only relatively high concentrations of cadmium (100 μg L^{-1}). The abundance of *C. tentans* alpha-tubulin 1 (CTT-UB1) gene was also increased in larvae by exposure to 100 μg L^{-1} cadmium, while it was decreased or unchanged in larvae by exposure to 10 μg L^{-1} cadmium (Mattingly *et al*., 2001).

These results imply differential ADH3 toxicity of copper and cadmium in *C. riparius* larvae. These findings also suggest that expression of ADH3 in *Chironomus* species could be employed as a potential biomarker for monitoring heavy metal pollutants such as copper. Therefore, with future identification of sequences and expression profiles of genes in *C. riparius*, this *Chironomus* species will enrich our knowledge concerning the role of environmentally responsive genes in detoxification following heavy metal stress.

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REFERENCES

Anderson RL. Chironomidae toxicity tests-biological background and procedures. In: Buikema, AL and Cairns J, (eds.). Aquatic Invertebrate Bioassays, American Society for Testing and Materials (ASTM), 1977; pp. 209.

Anderson RL, Walbridge CT and Fiandt JT. Survival and growth of Tanytarsus dissimilis (Chironomidae) exposed to copper, cadmium, zinc, and lead, Arch Environ Contam Toxicol 1980; 9: 329-335.

Aoki Y, Suzuki KT and Kubota K. Accumulation of cadmium and induction of its binding protein in the digestive tract of fleshfly (*Sarcophaga peregrina*) larvae, Comp Biochem Physiol 1984; 77: 279-282.

Bálint T, Ferenczy J, Kátai F, Kiss I, Kráczer L, Kufcsák O, Láng G, Polyhos C, Szabó I, Szegletes T and Nemcsók J. Similarities and differences between the massive eel (Anguilla Anguilla L.) devastations that occurred in lake Balaton in 1991 and 1995, Ecotoxicol Environ Saf 1997; 37: 17-23.

Besser JM, Brumbaugh WG, Ivey CD, Ingersoll CG and Moran PW. Biological and chemical characterization of metal bioavailability in sedments from Lake Roosevelt, Columbia River, Washington, USA, Arch Environ Contam Toxicol 2008; 54: 557-570.

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: 201-207.

Brodsky LI, Drachev AL, Leontovich AM and Feranchuk SI. A novel method of multiple alignment of biopolymer sequences, Biosystems 1993; 30: 65-79.

Cheng S. Heavy metal pollution in China: origin, pattern and control, Environ Sci Pollut Res Int 2003; 3: 192-198.

Choi J, Caquet T and Roche H. Multilevel effects of sublethal fenitrothion exposure in *Chironomus riparius* Mg. (Diptera, Chironomidae) larvae, Environ Toxicol Chem 2003; 21: 2725-2730.

Clements WH and Kiffney PM. Integrated laboratory and field approach for assessing impacts of heavy metals at the Arkansas River, Colorado, Environ Toxicol Chem 1994; 13: 397-404.

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: 361-369.

Cranston PS. The chironomidae-The biology and ecology of non-bitting midges. Chapman & Hall, London, UK, 1995.

Danielsson O and Jornvall H. "Enzymogenesis": Classical liver alcohol dehydrogenase origin from the glutathione dependent formaldehyde dehydrogenase line, Proc Natl Acad Sci USA 1992; 89: 9247-9251.

Dasmahapatra AK, Doucet HL, Bhattacharyya C and Carvan M.J.3rd. Developmental expression of alcohol dehydrogenase (ADH3) in zebrafish (Danio rerio), Biochem Biophys Res Commun 2001; 286: 1082-1086.

Duester G, Farres J, Felder MR, Holmes RS, Hoog JO, Pares X, Plapp BV, Yin SJ and Jornvall H. Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family, Biochem Pharmacol 1999; 58: 389-395.

Eimers MC, Evans RD and Welboum PM. Partitioning and

bioaccumulation of cadmium in artificial sediment systems: application of a stable isotope tracer technique, Chemosphere 2002; 46: 543-551.

Eklund H, Müller-Wille P, Horjales E, Futer O, Holmquist B, Vallee BL, Höög JO, Kaiser R and Jörnvall H. Comparison of three classes of human liver alcohol dehydronase, Emphasis on different substrate binding pockets, Eur J Biochem 1990; 193: 303-310.

Elendt BP. Selenium deficiency in Crustacea; An ultrastructural approach to antennal damage in *Daphnia magna* Straus, Protoplasma 1990; 154: 25-33.

Funkenstein B and Jakowlew SB. Molecular cloning of fish alcohol dehydrogenase, Gene 1996; 174: 159-164.

Henson MC and Chedrese PJ. Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction, Exp Biol Med 2004; 229: 383-392.

Höög JO, Eklund H and Jörnvall H. A single-residue exchange gives human recombinant beta beta alcohol dehydrogenase gamma gamma isozyme properties, Eur J Biochem 1992; 205: 519-526.

Hurley TD, Bosron WF, Stone CL and Amzel LM. Structures of three human beta alcohol dehydrogenase variants, Correlations with their functional differences, J Mol Biol 1994; 239: 415-429.

Ibrahim H, Kheir R, Helmi S, Lewis J and Crane M. 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: 448-455.

Igwilo IO, Afonne OJ, Maduabuchi UJ and Orisakwe OE. Toxicological study of the Anam River in Otuocha, Anambra State, Nigeria, Arch Environ Occup Health 2006; 61: 205-208.

Janssens de Bisthoven L, Postma J, Vermeulen A, Goemans G and Ollevier F. Morphological deformities in *Chironomus riparius* meigen larvae after exposure to cadmium over several generations, Water Air Soil Pollut 2001; 129: 167-179.

Janssens de Bisthoven L, Vermeulen A and Ollevier F. Experimental induction of morphological deformities in *Chiromonus riparius* larvae by chronic exposure to copper and lead, Arch Environ Contam Toxicol 1998; 35: 249-256.

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: 155-160.

Korenekova B, Skalicka M and Nad P. Cadmium exposure of cattle after long-term emission from polluted area, Trace Elem Electrolytes 2002; 19: 97-99.

Kwak IS and Lee W. Mouthpart deformity and developmental retardation exposure of *Chironomus plumosus* (Diptera: Chiromonidae) to tebufenozide, Bull Environ Contam Toxicol 2005; 75: 859-865.

Landrum PF and Robbins JA. Bioavailability of sediment associated contaminants to benthic invertebrates. In: Baudo R, Giesy J, Muntau H (eds.), Sediments: chemistry and toxicity of in-place pollutants, Lewis Publishers, Inc., Ann Arbor, MI 1990; pp. 237-263.

Lemoine S and Laulier M. Potential use of the levels of the mRNA of a specific metallothionein isoform (MT-20) in mussel (Mytilus edulis) as a biomarker of cadmium contamination, Mar Pollut Bull 2003; 46: 1450-1455.

Lemoine S, Bigot Y, Sellos D, Cosson RP and Laulier M. Metallothionein isoforms in *Mytilus edulis* (Mollusca, Bivalvia): complementary DNA characterization and quantification of expression in different organs after exposure to cadmium, zinc, and cooper, Mar Biotechnol 2000; 2: 195-203.

Lukac N, Massanyi P, Toman R and Trandzik J. Effect of cadmium on spermatozoa motility, Sovremena poljoprivreda 2003; 3-4: 215-217.

Maroni G, Lastowski-Perry D, Otto E and Watson D. Effects of heavy metals on *Drosophila* larvae and a metallothionein cDNA, Environ Health Perspect 1986; 65: 108-116.

Martinez EA, Moore BC, Schaumloffel J and Dasgupta N. Induction of morphological deformities in *Chironomus tentans* exposed to zinc- and lead-spiked sediments, Environ Toxicol Chem 2001; 20: 2475-2481.

Martinez EA, Moore BC, Schaumloffrl J and Dasgupta N. Morphological abnormalities in *Chironomus tentans* exposed to cadmium-and copper-spiked sediments, Ecotoxicol Environ Saf 2003; 55: 204-212.

Massanyi P, Lukac N and Trandzik J. In vitro inhibition of the motility of bovine spermatozoa by cadmium chloride, J Environ Sci Health Part A 1996; 31: 52-55.

Mattingly KS, Beaty BJ, Mackie RS, McGaw M, Carlson JO and Raymskeller A. Molecular cloning and characterization of a metal responsive *Chironomus tentans* alpha tubulin cDNA, Aquat Toxicol 2001; 54: 249-260.

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

Matthew MW, David P and Kathleen C. Chronic exposure to 17aethinylestradiol and bisphenol A effects on deve-

lopment and reproduction in the freshwater invertebrate *Chironomus riparius* (Diphtera: Chironomidae), Aquat Toxicol 2001; 55: 113-124.

Milani D, Reynoldson TB, Borgmann U and Kolasa J. The relative sensitivity of four benthic invertebrates to metals in spiked-sediment exposures and application to contaminated field sediment, Environ Toxicol Chem 2003; 22: 845-854.

Muntau H and Baudo R. Sources of cadmium, its distribution and turnover in the freshwater environment, IARC Sci Publ 1992; 118: 133-148.

Nowak C, Jost D, Vogt C, Oetken M, Schwenk K and Oehlmann J. Consequences of inbreeding and reduced genetic variation on tolerance to cadmium stress in the midge *Chironomus riparius*, Aquat Toxicol 2007; 85: 278-284.

Orlando C, Pinzani P and Pazzagli M. Developments in quantitative PCR, Clin Chem Lab Med 1998; 36: 255-269.

Ozcan S, Yildirim V, Kaya L, Albrecht D, Becher D, Hecker M and Ozcengiz G. Phanerochaete chrysosporium soluble proteome as a prelude for the analysis of heavy metal stress response, Proteomics 2007; 7: 1249-1260.

Pery ARR, Mons R, Flammarion P, Lagadic L and Garric J. A modeling approach to link food availability, growth, emergence, and reproduction for the midge *Chironomus riparius*, Environ Toxicol Chem 2002; 21: 2507-2513.

Rebelo MF, Pfeiffer W, Da Silva H and Morales MO. Cloning and detection of metallothionein mRNA by RT-PCR in *mangrove oyster* (Crassostrea rhizophorae), Aquat Toxicol 2003; 64: 359-362.

Reynoldson TB. Interactions between sediment contaminants and benthic organisms, Hydrobiologia 1987; 149: 53-66.

Rojik I, Nemcs'ok J and Boross L. Morphological and biochemical studies on liver, kidney and gill of fishes affected by pesticides, Acta Biol Hun 1983; 34: 81-92.

Skalicka M, Korenekova B and Nad P. Copper in livestock from polluted area, Bull Environ Contam Toxicol 2005; 74: 740-744.

Streloke M and Köpp H. Long-term toxicity test with *Chironomus riparius*: development and validation of a new test system, Mitt. A. D. Biol. Bundesanst. 315, Blackwell Wissenschaftsverlag, Berlin/Vienna, 1995.

Tessier A and Campbell PGC. Partitioning of trace metals in sediments: relationships with bioavailability, Hydrobiologia 1987; 149: 43-52.

Thompson JD, Higgins DG and Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment throught sequence weighting, positions-specific gap penalties and weight matrix choice, Nucl Acids Res 1994; 22: 4673-4680.

Tollett VD, Benvenutti EL, Deer LA and Rice TM. Differential toxicity to Cd, Pb, and Cu in dragonfly larvae (Insecta: Odonata), Arch Environ Contam Toxicol 2008; Apr. 18 Epub ahead of print.

Tom M, Chen N, Segev M, Herut B and Rinkevich B. Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarker, Mar Pollut Bull 2004; 48: 705-710.

To'th L, Juha'sz M, Varga T, Csikkel-Szolnoki A and Nemcso'k J. Some effect of $CuSO_4$ on carp, J Environ Sci Health B 1996; 31: 627-635.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaginh of multiple internal control genes, Genome Biol 2002; 3: research 0034.

Viarengo A. Heavy metals in marine invertebrates, mechanisms of regulation and toxicity at cell level, Aqu Sci 1989; 1: 295-317.

Viarengo A, Palermo S, Zanicchi G, Capelli R, Vaissiere R and Orunesu M. Role of metallothioneins in Cu and Cd accumulation and elimination in the gill and digestive gland cells of Mytilus galloprovincialis Lam, Mar Environ Res 1985; 1: 23-36.

Wan L, Wang N, Li Q, Sun B, Zhou Z, Xue K, Ma Z, Tian J and Song L. Distribution of dissolved metals in seawater of Jinzhou Bay, China, Environ Toxicol Chem 2008; 27: 43-48.

Wang G and Fowler BA. Roles of biomarkers in evaluating interactions among mixtures of lead, cadmium and arsenic, Toxicol Appl Pharmacol 2008; Jan. 32 Epub ahead of print.

Won EJ, Raisuddin S and Shin KH. Evaluation of induction of metallothionein-like proteins (MTLPs) in the polychaetes for biomonitoring of heavy metal pollution in marine sediments, Mar Pollut Bull 2008; Apr. 4 Epub ahead of print.