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Full Length Research Paper

Ribosomal protein S3 gene expression of *Chironomus riparius* under cadmium, copper and lead stress

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Heavy metals are of interest because they are often present at significant levels in the environment and can have severe effects on the aquatic environment. To examine the effects of oxidative stress induced by heavy metals on chironomids, the full-length cDNA of ribosomal protein S3 (RpS3) from *Chironomus riparius* was determined using molecular cloning. The basal expression of the RpS3 gene was not affected by changes in environmental rearing conditions such as temperature, media, and sediment type. However, RpS3 gene expression in *C. riparius* increased significantly in response to exposure to cadmium, copper, and lead, regardless of the exposure level. These results suggest that expression of the RpS3 gene, which is associated with the DNA repair process, may be used as an indicator of oxidative stress induced by heavy metals during environmental risk assessment.

Key words: *Chironomus riparius*, ribosomal protein S3, cadmium, copper; lead, environmental risk assessment.

INTRODUCTION

Environmental pollutants such as heavy metals pose serious risks to many aquatic organisms via changing neurophysiologic, biochemical and behavioral parameters (Scott and Sloman, 2004). Heavy metals from natural and anthropogenic sources accumulate in aquatic sediments in the form of trace elements, where they pose a threat to sediment biotic communities (Muntau and Baudo, 1992; Cheng 2003; Besser et al., 2008). Additionally, heavy metals such as cadmium, copper, lead, chromium, nickel, arsenic and mercury are frequently detected as groundwater contaminants (Clements and Kiffney, 1994). Aquatic species take up and accumulate both essential and nonessential trace elements from sediments. The 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). Therefore, understanding the responses of aquatic organisms to heavy metal toxicity is important to monitoring water quality.

For the purpose of this study, cadmium was chosen as the contaminant of interest because it is widely recognized as an environmental pollutant (Aoki et al., 1984) and is highly toxic, affecting a wide range of physiological processes such as plasma membrane transport and the transcription of genes (Maroni et al., 1986). Exposure to cadmium via the 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 pollution appears in the aquatic environment as a result of mine washing or agricultural leaching. Although copper is an essential trace element involved in biological functions such as iron absorption, hemopoiesis, and fermentation (Skalicka et al., 2005), it is also one of the most toxic heavy metals (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 generating free radicals that ultimately cause morphological alterations and change certain physiological processes.

Due to its high abundance and physical characteristics, such as ductility and high density, lead poses a major threat to genomic processes in vertebrates (Mortada et

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al., 2004). In aquatic habitats, lead can be highly toxic for aquatic species since it can disrupt metabolic pathways and cause ionoregulatory damage (Rogers et al., 2005). Despite significant reductions in its use in paint production and as a fuel additive, lead continues to enter the environment through anthropogenic means, thereby retaining its status as a priority pollutant (USEPA, 2006). Lead acts as a Ca^{2+} antagonist (Busselberg et al., 1991; Rogers and Wood, 2004). At the physiological level, lead accumulates in cellular organelles (Qian and Tiffany-Castiglioni, 2003), impairing the properties of some calcium-dependent proteins such as heat shock proteins (HSPs) with lethal consequences on reproductive behaviors (Feder and Hofmann, 1999). The principal effects of chronic lead exposure on aquatic organisms are presumably hematological (Hodson et al., 1978), neurological (Davies et al., 1976) and renal (Patel et al., 2006) impairment.

Ribosomal protein genes are essential for cellular development (Chen and Ioannou, 1999). The four rRNAs (28S, 18S, 5.8S and 5S) are used in the ribosome machinery for protein biosynthesis. Because ribosomes are protein-rRNA complexes, this also implies that there is a high degree of amino acid sequence conservation between equivalent ribosomal proteins in different species (Draper and Reynaldo, 1999). Ribosomal proteins have the complex task of coordinating ribosome structure and protein biosynthesis to maintain cell homeostasis and survival. Some ribosomal proteins have secondary functions independent of their involvement in protein biosynthesis. A number of these proteins function as cell proliferation regulators, and in some instances, as inducers of cell death (Chen and Ioannou, 1999). Among the ribosomal proteins, ribosomal protein S3 (RpS3) is unusual in that it has multiple functions, including an apurinic/apyrimidinic (AP) lyase activity that participates in the repair of DNA damage. RpS3 shuttles between the cytoplasm and the nucleus to function in both compartments. As RpS3 has a nuclear localization signal in the N-terminal region, it is believed that its translation function operates in the cytosol, while its repair function operates in the nucleus (Lee et al., 2002). These activities repair the DNA damage caused by oxidizing agents and ionizing radiation. In addition to their role in ribosomal functions, many ribosomal proteins have secondary functions in replication, transcription, RNA processing, DNA repair, and malignant transformation (Wool, 1996). More than 80 different types of ribosomal proteins have been identified in eukaryotes, but only a few genes have been sequenced in *Chironomus* (Govinda et al., 2000; Martínez-Guitarte et al., 2007).

Toxins and other stressors can cause changes in gene expression, which have proven useful as biomarkers. For example, the metallothionein gene in *Drosophila* species was induced by a number of heavy metals, including zinc, cadmium, copper, silver, and mercury (Maroni et al., 1986). Metallothionein, heat shock proteins, and glutathione-S-transferase are involved in regulating the interactive effects of metal/metalloid mixtures at low dose levels (Wang and Fowler, 2008). However, few environmental studies of the *Chironomus* family have been conducted at the molecular level (Martínez-Guitartea et al., 2007; Park and Kwak, 2010), even though many studies have been conducted to evaluate their general biological responses to heavy metals (Martinez et al., 2001, 2003; Nowak et al., 2007). This is probably because there is little sequence information available regarding environmentally responsive genes. In *C. riparius*, characterized ribosomal proteins were only four genes of RpL8 (Govinda et al., 2000), RpL11 and RpL13 (Martínez-Guitartea et al., 2007), and RpL15 (Nair and Choi, 2011).

Chironomids are an ecologically diverse family of dipterans that are probably the most widespread 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). Chironomids 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 for evaluation of sediment and water toxicity (Ibrahim et al., 1998). Indeed, morphological abnormalities have been observed in *Chironomus* larvae exposed to heavy metals and endocrine disrupting chemicals (Martinez et al., 2001, 2003; Kwak and Lee, 2005; Park and Kwak, 2008; Park et al., 2010). Thus, Chironomids are a good aquatic model for assessment of the toxicity of freshwater that has been contaminated with heavy metals.

In the present study, the RpS3 gene from *C. riparius* was characterized to determine the effects of oxidative stress induced heavy metals on chironomids. Comparative phylogenetic and molecular studies were conducted to analyze the homologies within insects. RpS3 expression was analyzed by means of real-time RT-PCR during different stages of life-cycle development and under various environmental conditions. Additionally, changes in RpS3 expression in response to exposure to various concentrations of cadmium, copper and lead were evaluated.

MATERIALS AND METHODS

Organisms

C. riparius were reared using the methodologies outlined by Streloke and Köpp (1995). We obtained *C. riparius* larvae from adults reared in the laboratory. An 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 h and a light intensity of about 500 l×. The water in the incubator chamber was maintained at a constant temperature of 20±1°C (Sanyo, Osaka, Japan). After the larvae emerged from eggs, they were kept in Duran crystallizing dishes

(Schott, Mainz, Germany) with approximately 500 ml of M4 culture medium (Elendt, 1990) and a sediment layer of composed of 1 cm of fine sand (< 63 µm particle size) under continuous aeration. All dishes received 5 mg of food that had been ground in a blender daily (0.5 mg Larva^{-1}; Tetra-Werke, Melle, Germany), which resulted in food not being provided in a limited condition (Pery et al., 2002).

Exposure conditions

All experimental larvae were acquired by the eleventh day after hatching from the same control egg masses. The larvae were then exposed to water enriched with cadmium ($CdCl_2$), copper ($CuSO_4$) and lead ($Pb(NO_3)_2$)(Sigma-Aldrich Co., St Louis, USA). The nominal metal concentrations were based on available data regarding the toxicity values for *C. riparius* and actual concentrations in the Anam River (Janssens et al., 1998, 2001; Milani et al., 2003; Igwilo et al., 2006). The nominal concentrations for cadmium were 3.27 and 100 µgL^{-1}, while they were 1.10 and 100 µgL^{-1} for copper and 5. 50 and 100 µgL^{-1} for lead. All treatments were prepared from stock solutions of 0.1 gL^{-1} Cu^{2+}, 0.1 gL^{-1} Cd^{2+} and 0.5 gL^{-1} Pb^{2+}. Additionally, we characterize RpS3 expression variability under several environmental conditions during development. To accomplish this, the embryos were reared at 18°C (Martínez-Guitarte et al., 2007), using dechlorinated tap water as culture media (Nair and Choi, 2011) or using an artificial sediment (150 to 300 µm particle size) (Dias et al., 2008).

Thirteen fourth-instar *C. riparius* larvae were transferred into 300 mL Duran crystallizing dishes (Schott, Mainz, Germany) filled with 200 ml of M4 media and then treated them with one of the three aforementioned concentrations of copper, cadmium or lead. All organisms were exposed to the treatment for 24 h and all experiments were conducted in triplicate using independent samples (for example, three boxes each containing 3 µg L^{-1} of cadmium for 24 h). Each group of thirteen larvae was then utilized for subsequent analyses. Untreated larvae that were used as a control were also evaluated in triplicate. Exposure was conducted under a constant temperature (20±1°C) and a photoperiod of 16:8 h light:dark for all experiments.

RpS3 gene characterization

To amplify sequences of the RpS3 gene from *C. riparius*, polymerase chain reaction (PCR) was conducted using primers specific for higher Diptera (*Aedes aegypti* and *Anopheles gambiae* in Figure 1) consensus sequences. Multiple sequence alignments were then conducted using ClustalW (Thompson et al., 1994). The specific primers used were 5'TTGAARGGYCGCGTGTTTGAAGTT3' and 5' GAATTGCAYGGAGATGG WGGC3' for RpS3. 'R' represents mixture of A and G, 'W' represents a mixture of A and T, and 'Y' represents a mixture of T and C. The PCR mixture, which had a total volume of 50 µl, contained 1× Taq DNA polymerase buffer (SolGent, Daejeon, Korea), 200 µM dNTP, 2 units of Taq DNA polymerase (SolGent, Daejeon, Korea), and primers at a concentration of 20 µM. The template was composed of *C. riparius* DNA or cDNA. DNA was extracted from fourth-instar larvae using an AccuPrep® Genomic DNA extraction kit (Bioneer, Daejeon, Korea). The RNA extraction and cDNA synthesis were conducted as described in section 2.5. PCR was conducted by subjecting the samples to the following conditions: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and 7 min at 72°C using a MyCycler™ thermal cycler (Bio-Rad, Hercules, USA). The reaction products, which consisted of 551bp of DNA or 480 bp of cDNA, were then cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, USA), after which they were sequenced using an ABI 3700 Genetic analyzer. To acquire the full-length RpS3 cDNA, we used a GeneRacer kit (Invitrogen, Carlsbad, USA) according to the manufacturers' instructions.

Phylogenetic analysis

Translation of the RpS3 cDNA sequence was conducted using a web tool available online (nucleic acid to amino acid translation) (http://www.biochem.ucl.ac.uk/cgi-bin/ mcdonald/cgina2aa.pl). The amino acid sequences were aligned with those of other organisms using Clustal X version 1.8 and then displayed using the GeneDoc Program (ver 2.6.001). A phylogenetic tree was constructed by neighbor-joining analyses using software available online (TreeTop) (Brodsky et al., 1993). Bootstrap values were calculated based on 1000 replicates.

Gene expression analysis

Total RNA was isolated from *C. riparius* fourth instar larvae (ten animals) using TRIZOL® reagent (Invitrogen, Scotland, UK) according to the manufacturers' instructions. Single-strand cDNA was synthesized from 4 µg of total RNA using random hexamer primer for reverse transcription in a 20 µl reaction mix using the SuperScriptTM III RT kit (Invitrogen, Scotland, UK). The cDNAs obtained were used as templates for PCR reactions with gene-specific primers for RpS3. In addition, PCR was conducted using primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The sequences of the oligonucleotide primers were: RpS3 forward 5'-GATGTCAAGACAACTGATGGA-3'; RpS3 reverse 5'-CACCATGGCATGCCTTCT CGA-3'; GAPDH forward 5'-GGTATTTCATTGAATG ATCACTTTG-3'; GAPDH reverse 5'-TAATCCTTGGATTGCATGTACTTG -3' (GenBank accession no. EU999991). The relative expression level of genes was measured using real-time RT-PCR, which was conducted on an iCyclerIQ thermocycler (Bio-Rad, Hercules, USA) with SYBR Green (Bio-Rad, Hercules, USA). The PCR program consisted of 1 cycle of 94°C for 3 min followed by 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The data were then analyzed using the delta-delta C_t method in microsoft excel. Each test consisted of at least three replicates and the values were normalized using GAPDH as an internal control. The relative amount of RpS3 under several environmental conditions was calculated in compared to RpS3 expression in the normal rearing condition as a control.

Data analysis

All results are expressed as the mean ± SD, unless otherwise stated. The level of RpS3 mRNA in each sample was normalized against its own level of GAPDH based on standard curves. The levels of the RpS3 transcripts within each metal-treated group relative to the non-exposed control were estimated using normalized values. Differences in the RpS3 mRNA levels among groups were assessed by ANOVA followed by Tukey's multiple range test using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

RESULTS

Identification of RpS3 gene and phylogenetic analysis

Partial sequences of the *C. riparius* RpS3 gene were

A

Species	Protein (%)	Accession number
C. riparius	100	EU683898
C. sonorensis	91	AAU06483
A. aegypti	89	XP_001651594
A. gambiae	88	XP_313275
P. americana	87	AAW57773
T. balearica	87	CAJ17219
B. mandarina	86	ABQ42714
A. mellifera	85	XP_396741
D. melanogaster	85	NP_524618

B

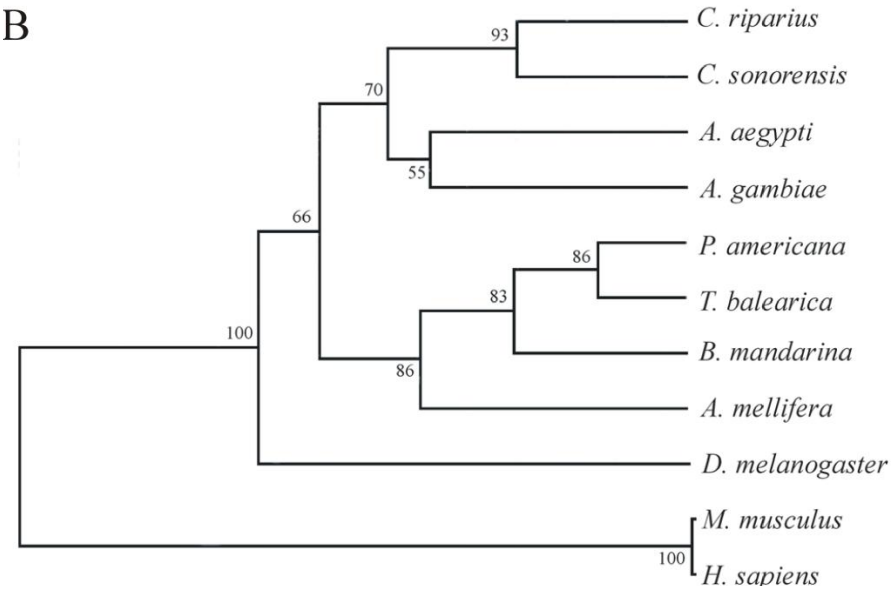

Figure 1. Phylogenetic trees of RpS3 genes constructed by neighbor-joining analysis (bootstrap value 1000). (A) Percentage of sequence similarity among RpS3 genes. (B) The numbers at the nodes are the percentage bootstrap values. Amino acid sequences were aligned using Clustal X (ver 1.8).

amplified by PCR using primers designed from higher diptera consensus sequences. The full-length of cDNA from *C. riparius* was then determined using molecular cloning and rapid amplification of cDNA ends (RACE). The complete cDNA sequence of *C. riparius* RpS3 gene was 813 bp and encoded a deduced amino acid sequence of 271. The complete coding sequences of *C. riparius* RpS3 were deposited in GenBank under accession No. EU683898. The genomic structures for RpS3 constitute two exons and one intron of 71 bp between the first 341 bp and second 472 bp exons. The sequence of the entire nucleotide region of RpS3 identified in this study was found to be 78% homologous with that of the mosquito, *Armigeres subalbatus* (EU205451), 74% homologous with that of the mosquito, *Anopheles gambiae* (BX058714), and 74% with that of the roundworm, *Brugia malayi* (XM_001896856). Although the homology of the DNA sequence was low, the deduced amino acid sequence was approximately 88% identical to that of other insect RpS3 proteins (Figure 1).

To evaluate the relationship of *C. riparius* RpS3 with homologues from other insects, we conducted a phylogenetic analysis (Figure 1). *C. riparius* RpS3 clustered with mosquitoes, such as *Culicoides sonorensis*, *Aedes aegypti* and *A. gambiae*, while RpS3 from a honey bee (*Apis mellifera*) formed another cluster with that of an American cockroach (*Periplaneta Americana*),

a beetle (*Timarcha balearica*), and the wild silkmoth (*Bombyx mandarina*) (Figure 1). Overall, these results indicated that the RpS3 sequence from *Chironomus* is most closely related to those of mosquitoes, such as *C. sonorensis* (Figure 1).

RpS3 gene expression under several environmental conditions

To characterize RpS3 expression variability, its expression was analyzed under several environmental conditions during development (Figure 2). Specifically, the effects of temperature, media, and sediment type on RpS3 expression were evaluated. No significant differences were observed in the expression of the RpS3 gene during different developmental stages in response to the altered rearing conditions ($p > 0.05$) (Figure 2). Additionally, the RpS3 gene was stably expressed during different developmental stages under various environmental conditions.

RpS3 expression in response to heavy metals exposure

To examine the possible environmental regulation of RpS3 expression, real time RT-PCR analysis was conducted to evaluate the expression levels of transcripts under different conditions of cellular stress. Specifically, we analyzed the response of the RpS3 gene of *C. riparius* exposed to cadmium, copper and lead at three different concentrations (Figure 3). RpS3 gene expression increased significantly after cadmium exposure, regardless of the treatment dose. The response of RpS3 was greatest in *C. riparius* exposed to 100 μgL^{-1} cadmium for 24 h. After copper exposure, RpS3 gene expression significantly increased across all copper concentrations in a dose dependent fashion. Expression of the RpS3 gene also increased more than two-fold in *C. riparius* that were exposed to lead (Figure 3). Indeed, expression of the RpS3 gene was significantly higher in *C. riparius* that were exposed to lead than in those exposed to cadmium or copper ($p < 0.01$). There were no significant differences in expression observed among non-treated groups ($p > 0.05$).

DISCUSSION

Chironomids, benthic macro-invertebrates, are used extensively to assess the acute and sublethal 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). However, few studies have been conducted to evaluate the response of chironomids to chemical toxicity at the molecular level (Park et al., 2009; Park and Kwak 2010; Nair and Choi, 2011). In this study, RpS3 cDNA from *C. riparius* was characterized, and transcript level of expression under several environmental conditions and different heavy metal conditions was analysed.

The *C. riparius* RpS3 cDNA encodes 271 aa with a theoretical pI of 5.14. The molecular phylogenetic relationships between *C. riparius* RpS3 and other insects showed that the RpS3 of *C. riparius* was most closely related to the RpS3 genes of mosquitoes, especially *Culicoides* species (Figure 1). This is reasonable because *C. riparius* is closer, in evolutionary terms, to *Culicoides* than to *Drosophila*, and both *Chironomus* and *Culicoides* belong to the suborder, Nematocera. A molecular phylogenetic study revealed that the *C. riparius* L11 and L13 genes were more closely related to those of *Anopheles*, belonging to the suborder Nematocera (Martínez-Guitarte et al., 2007). Recently, a study of expressed sequence tags from *C. tentans* (belonging to the subgenus *Camptochironomus*) also found that the greatest similarity (59%) was to that of sequence tags from *A. gambiae*, while only 24% similarity with the tags from *Drosophila melanogaster* was observed (Arvestad et al., 2005).

The RpS3 protein has been shown to be remarkably versatile in its ability to influence both ribosomal function and DNA repair transactions. Recent studies have shown that RpS3 is an integral part of the organization of the pre-40S subunit in yeast (Schafer et al., 2006). Beyond its role in ribosomal maturation, numerous studies have shown that RpS3 is involved in the repair of DNA damage. The RpS3 protein of *D. melanogaster* possesses various DNA repair activities, including the capacity to incise at apurinic/apyrimidinic (AP) sites and 8-oxo-7,8-dihydroguanine (8-oxoG) residues (Cappelli et al., 2003). Unlike *Drosophila* RpS3, the human RpS3 protein lacks the ability to liberate 8-oxoG from damaged DNA substrates; nevertheless, it possesses a remarkably high binding affinity for 8-oxoG (Hegde et al., 2004). In addition to its role in DNA repair, RpS3 is also involved in apoptosis (Jang et al., 2004). These functions of RpS3 indicate that RpS3 may be useful as an indicator of mutagenic agents or pollutants that cause oxidative stress.

Heavy metal ions, lead and cadmium are well known carcinogens with different natural origins (Rojas et al., 1999; Huff et al., 2007). Cadmium might contribute to increased risk for tumor formation via its inhibition of excision and mismatch DNA repair processes (Giaginis et al., 2006). Metal ion-DNA interactions are important in nature because they can alter the structure and function of genetic material. Pb^{2+} and Cd^{2+} bind to dsDNA, which results in different modifications of the dsDNA structure. Pb^{2+} interacts with dsDNA preferentially at adenine-containing segments, leading to oxidative damage and the formation of 2, 8-dihydroxyadenine, which is the oxidation product of adenine residues and a biomarker of

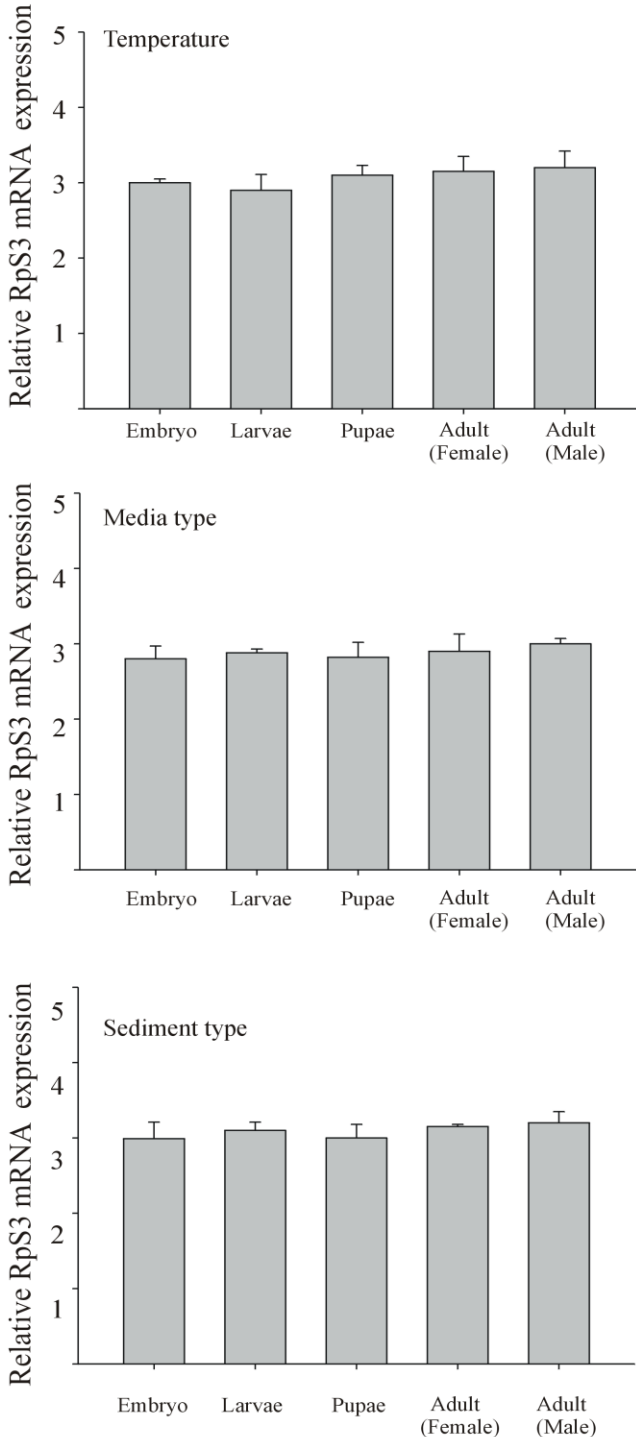

Figure 2. Analysis of RpS3 gene expression under several environmental conditions. RpS3 gene expression during life-history development at 18°C (upper), on media with dechlorinated tap water (middle) or on artificial sediment (150 to 300 µm particle sizes) (lower). The raw values were normalized against GAPDH, and the data were then used to calculate the relative expression levels. The relative amount of RpS3 under several environmental conditions was calculated in compared to RpS3 expression in the normal rearing condition as a control. The experiment was performed in triplicate and the values shown represent the mean ± standard error of the mean.

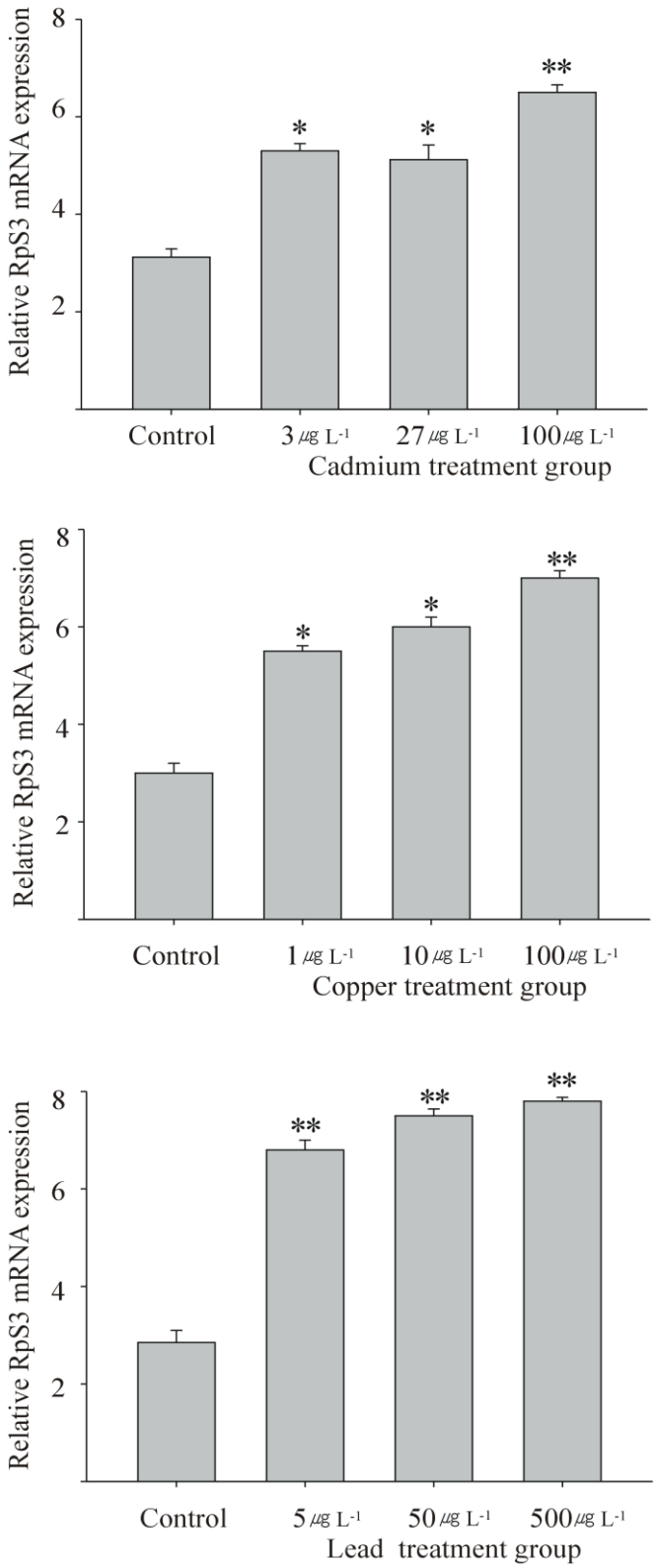

Figure 3. Expression of the RpS3 gene in the fourth-instar larvae of *Chironomus riparius* exposed to cadmium, copper and lead. mRNA expression is shown as relative to GAPDH expression after normalization. The experiment was performed in triplicate and the values shown represent the mean ± standard error of the mean (*indicates statistical significance at $p < 0.05$; ** indicates $p < 0.01$*).

DNA oxidative damage. The interaction with Cd^{2+} only causes conformational changes, which leads to destabilization of the double helix and can enable the action of other oxidative agents on DNA (Oliveira et al., 2008). Copper, which is a redox-active trace metal ion, induces an increase in oxidative stress that results in DNA damage and activation of p53-dependent cell death (Du et al., 2008). Additionally, copper is required as a co-factor for many enzymes that catalyze oxidation/reduction reactions, including those involved in electron transport (cytochrome c oxidase) (Prá et al., 2008), antioxidant enzymes (Cu/Zn superoxide dismutase and ceruloplasmin) (Linder, 2001), melanin and collagen biosynthetic pathways (tyrosinase and lysyl oxidase, respectively) (Lutsenko et al., 2007) and hormones (dopamine-monooxygenase and a-amidating monooxygenase) (Prá et al., 2008). The exposure of *C. riparius* to copper has been found to induce a delay in larval growth in both sexes and a reduction in the lifespan of males (Servia et al., 2006). Additionally, a significantly higher frequency of functional alterations, specifically decondensed centromeres and telomeres, and a reduction in the activity of Balbiani rings, was observed in *C. riparius* that were treated with copper. Finally, a dose dependent relationship was observed between copper concentration and the frequency of chromosomal aberrations (Michailova et al., 2006).

Conclusion

Previous studies have also shown that the expressions of ribosomal protein genes could be altered by heavy metal stress. The RPL23aB and RPL2 transcript level were decreased rapidly in soybean by heavy metal stress of copper (Ludwig and Tenhaken, 2001; McIntosh and Bonham-Smith, 2005). In contrast, it has been shown that other ribosomal protein genes, such as that of S9, was up-regulated in response to cadmium and repressed by zinc in molluscs (Achard et al., 2006). Recent report described that reduction of ribosomal protein L15 transcript occurred in *C. riparius* as a result of exposure to cadmium (Nair and Choi, 2011). However, the expressions of ribosomal protein genes L8 (Govinda et al., 2000), L11 and L13 (Martínez-Guitarte al., 2007) were unaffected after exposure to cadmium. To determine the possible environmental regulation of RpS3 expression, we analyzed responses to cadmium, copper and lead exposure. After cadmium and copper exposure, *C. riparius* RpS3 gene expression increased significantly, regardless of the exposure conditions (Figure 3). Additionally, expression of the RpS3 gene was significantly higher in *C. riparius* that were exposed to lead than in those exposed to cadmium or copper of relative low concentrations (Figure 3). The level of RpS3 expression was induced by heavy metal exposures, regardless of different heavy metal treatments. The response to Pb^{2+} occurred as a result of its interaction with segments of dsDNA, which led to oxidative damage. These results suggest that the expression of RpS3 in *C. riparius* could be employed as an indicator of mutagenic agents, such as heavy metals that cause oxidative stress. Furthermore, to determine if RpS3 is useful as a biomarker, its expression should be investigated under field-conditions through characterization of RpS3 sequence information in wild species of *Chironomus*.

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