Ocean Sci. J. (2012) 47(4):435-444
http://dx.doi.org/10.1007/s12601-012-0039-x

Article

Available online at http://link.springer.com

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Ocean Science Journal

Assessment of Potential Biomarkers, Metallothionein and Vitellogenin mRNA Expressions in Various Chemically Exposed Benthic *Chironomus riparius* Larvae

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Received 20 August 2012; Revised 12 November 2012; Accepted 3 December 2012

Abstract – The objective of this study was conducted to identify the possibility of using *Chironomus* metallothionein (MT) and vitellogenin (VTG) as biomarkers of stress caused by endocrine-disrupting chemicals (EDCs), heavy metals, herbicides and veterinary antibiotics. We characterized the MT and VTG cDNA in *Chironomus riparius* and evaluated their mRNA expression profiles following exposure to different environmental pollutants. The gene expression analysis showed that the MT mRNA levels increased significantly after long-term exposure to cadmium (Cd), copper (Cu), Lead (Pb), di(2-ethylhexyl) phthalate (DEHP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Moreover, the VTG mRNA expression increased significantly in *C. riparius* larvae exposed to BPA, NP, DEHP, Cd, 2,4-D and fenbendazole. Evaluation of the long-term effects of environmental pollutants revealed up regulation of *Chironomus* MT mRNA in response to DEHP exposure among EDCs, and the level of the VTG mRNA was increased significantly following treatment with Cd and herbicide 2,4-D at all concentrations in a dose-dependent manner. These results indicate that VTG could be used as a potential biomarker of herbicide and Cd as well as EDCs, while MT was a potential biomarker of heavy metals such as Cd, Cu, and Pb in aquatic environments.

Keywords – *Chironomus riparius*, metallothionein, vitellogenin, environmental pollutants, biomarker

1. Introduction

Environmental pollutants from industrial, agricultural, and domestic sources contain a wide variety of natural and synthetic chemicals. Organisms living in aquatic environment are affected by these chemical toxicants (Torres et al. 2008). According to the characteristics of a species, the interactions of toxic elements with living organisms in water may cause biomagnifications along the food chain or bio-concentration of what passes through the water. Furthermore, accumulation of various pollutants in an aquatic environment occurs in the sediment, which is the habitat of the midge larvae (Planelló et al. 2010). It is well known that aquatic sediments can act as sinks for pollutants such as trace metals, with sediment chemical concentrations often being orders of magnitude higher than those encountered in surface waters (Burton 1991). Chironomids are of great interest in ecology because they represent a predominant part of benthic communities in all freshwater systems. Indeed, these organisms play an important role in detritus processing and the recycling of organic matter (Rieradevall et al. 1995). Chironomid species are useful indicators of environmental states in aquatic ecosystems.

Biomarkers have been used for toxic assessment of contamination in aquatic ecosystems (Ladhar-Chaabouni et al. 2012). Gene expression response has also been sensitively induced by characterized or uncharacterized chemical stressors. In some aquatic organisms, metallothionein (MT) and vitellogenin (VTG) genes, which are biomarkers of heavy metals and endocrine disruptor chemicals (Papetti and Rossi 2009; Kim et al. 2011; Velma and Tchounwou 2011), have been assessed. Metallothionein (MT) is a superfamily of cysteine-rich proteins that contribute to metal metabolism, detoxification of heavy metals, and immune responses such as protection against ionizing radiation and antioxidant defense (Wang et al. 2009). MT is a protein that has a low

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molecular weight of ~7 kDa and a high affinity for metals. MT binds with metals and regulates the homeostasis of essential trace metals such as copper and zinc and participates in counteracting the toxic effects of heavy metals such as cadmium, mercury, and silver (Viarengo et al. 1999). Numerous studies have reported that MT is involved in the mechanisms of general responses to stress and the tolerance and the detoxification of heavy metals (Cobbett and Goldsbrough 2002; Choi et al. 2008).

Vitellogenin (VTG) is a large precursor protein of egg yolk vitellin that provides energy reserves in oviparous vertebrates and invertebrates (Shu et al. 2009). In many insect species, VTGs are primarily synthesized in the fat bodies in sex-, tissue-, and stage-specific manners, after which they are secreted into the hemolymph and then sequestered by competent oocytes via receptor-mediated endocytosis (Sappington and Raikhel 1998; Snigirevskaya and Raikhel 2005). Ecdysteroids (20-hydroxyecdysone, 20E, is the most active form) and juvenile hormones assume a gonadotrophic role in adult female insects and regulate vitellogenesis (Engelmann 1986; Bownes 1989; Bownes et al. 1996). Insect VTGs are phospholipoglycoproteins synthesized as ~200 kD precursors derived from a 6-7 kb VTG mRNA transcript (Tufail and Takeda 2008). The number of VTG genes varies among insect species (Tufail and Takeda 2008). Insect VTG is also involved in the regulation of hormonal dynamics and has multiple coordinating effects on the social organization of worker and male honey bees (Guidugli et al. 2005; Nelson et al. 2007). Induction of VTG has been used as a biomarker of exposure to heavy metals and EDCs in aquatic organisms (Cervera et al. 2005; Matozzo et al. 2008; Shu et al. 2009; Hwang et al. 2010). However, there is no information on the sequence of *Chironomus* MT and VTG and the pattern of gene expression by chemical toxicity.

In this study, the expression changes of the *Chironomus* MT and VTG in 4th instar larvae of *Chironomus riparius* Mg. (Diptera: Chironomidae), due to exposure to 8 environmental chemicals with different modes of action - namely, bisphenol A(BPA), 4-nonylphenol (NP), di(2-ethylhexyl) phthalate (DEHP), cadmium chloride (Cd), copper chloride (Cu), lead(II)nitrate (Pb), 2,4-dichlorophenoxyacetic acid (2,4-D), and fenbendazole, were evaluated. Endocrine-disrupting chemicals (EDCs) can potentially interfere with the hormone system and disrupt the reproduction of natural populations of several aquatic organisms (Mills and Chichester 2005; Park and Kwak 2010). Many of the heavy metals released into the environment in recent years can be traced to the wastes associated with electroplating, smelting and mining as well as the intensive use of consumer products such as plastics, pigments and nickel/Cd batteries (Jarup et al. 1998; Zadorozhnaja et al. 2000). Herbicides are agricultural contaminants found in rural ground water. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most successfully and widely used herbicides (Teixeira et al. 2007). The widespread use of pesticides/herbicide has led to increasing contamination of the aquatic environment (Park et al. 2010). Veterinary antibiotics are widely used in many countries to treat disease and protect the health of animals (Park et al. 2009). Benzimidazole anthelmintics have recently received attention due to their high production volumes and potential adverse effects on non-target ecological receptors (Oh et al. 2006; Kreuzig et al. 2007; Escher et al. 2008). For example, the annual sales amount and production of fenbendazole in Korea was reported to be 356 (Kim et al. 2006) and 220 tons (Oh et al. 2006), respectively. These various environmental pollutants have the potential to cause problems in the ecosystem because they are not readily degraded in the environment. Additionally, the toxic effects of these pollutants on aquatic environments are not well understood. To determine the potential for the use of *Chironomus* MT and VTG as biomarkers, we investigated the mRNA expression of MT and VTG genes in *C. riparius* exposed to environmental pollutants such as EDCs, heavy metals, herbicides and veterinary antibiotics.

2. Materials and Methods

Organisms

The rearing conditions utilized in this study followed the methods described by Streloke and Köpp (1995). Briefly, *C. riparius* larvae were obtained from adults reared in the laboratory, with the original strain being provided by the Korea Institute of Toxicology (Daejeon, Korea). All larvae were reared in an environmental chamber under a 16:8 h light: dark cycle and a light intensity of approximately 500 lx. During rearing, the temperature of the water in the incubator chamber was maintained at 20 ± 1 °C (Sanyo, Osaka, Japan). Larvae that hatched from the eggs were maintained in Duran crystallizing dishes (Schott, Mainz, Germany) containing approximately 500 mL of M4 culture medium (Elendt 1990) and a 1 cm sediment layer of fine sand (< 63 μm particle

size). The dishes were continuously aerated after introduction of the midge larvae. Additionally, all dishes received 5 mg of food that had been ground in a blender (0.5 mg $Larva^{-1}$; Tetra-Werke, Melle, Germany) each day.

Exposure conditions

All experimental larvae were acquired by day 11 after hatching from the same control egg masses. All chemicals for experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the chemical treatment, acetone was used as the solvent for bisphenol A (BPA), 4-nonylphenol (NP), and di(2-ethylhexyl) phthalate (DEHP). Water was also used for cadmium chloride (Cd), copper chloride (Cu), and lead(II)nitrate (Pb). The 2,4-dichlorophenoxyacetic acid (2,4-D) and fenbendazole were dissolved in methanol as the solvent. The three nominal concentrations were used for exposure conditions of each pollutant at concentrations that were previously tested in *C. riparius* during toxicity experiments.

For each chemical treatment, 20 of the 4th instar *C. riparius* larvae were transferred to 300-mL crystallizing dishes (Schott, Mainz, Germany) containing 200 mL of M4 media, after which they were treated with one of three concentrations of each chemical for long-term exposure periods (96 h). All experiments were conducted in triplicate using independent samples. The untreated larvae that were used as controls were also measured in triplicate. Exposure was conducted at a constant temperature (20 ± 1 °C) and a 16:8 h light:dark photoperiod was used for all experiments.

MT and VTG mRNA expression analysis

Total RNA was isolated from *C. riparius* larvae with TRIZOL® reagent (Invitrogen, Scotland, UK) in accordance with the manufacturer's instructions. Single-strand cDNA was then synthesized from 3 μg of total RNA using the SuperScriptTM III RT kit (Invitrogen, Carlsbad, CA, *USA*) with a random hexamer primer for reverse transcription in a 20 μl reaction mix. The cDNA samples obtained were then utilized as PCR templates using primers specific for the MT (HQ260607) and VTG genes (HQ260608). The cDNA for the MT and VTG genes was obtained from a *C. riparius* cDNA library constructed in our previous study (Park et al. 2010). Additionally, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (EU999991) for PCR. The sequences of the oligonucleotide primers are provided in Table 1. Quantitative RT-PCR amplification and measurements were conducted using an AB7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. To quantify the cDNA, RT-PCR was conducted using a master mix with a final volume of 25 μl that contained 1μl of cDNA template, 0.2 μM of each primer and 1SYBR® Premix Ex Taq (Takara, Kyoto, Japan). The following PCR conditions were utilized to amplify the genes: 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 30 sec. The quality of the amplification was then assessed with an AB7300 Real Time PCR system to conduct dissociation curve analysis. The cycle threshold (CT) was determined using the AB7300 System SDS software. Fold changes in the expression levels were calculated via the comparative CT method, which was normalized against the GAPDH expression of the same samples. Each test consisted of at least three replicates.

Data analysis

The results are expressed as the means ± SD, unless otherwise stated. MT and VTG mRNA levels in each sample were normalized against the level of GAPDH in the same samples based on standard curves. The normalized levels of the MT and VTG transcripts in each chemical treated group were then compared to those of the solvent controls using ANOVA followed by Tukey's multiple range test. Statistical analyses were conducted using SPSS 12.0KO (SPSS, USA) and a $p < 0.05$ was considered significant.

Table 1. Primers used for the amplification of specific genes

Gene	Primer sequences	Amplified size	GenBank Accession No.
GAPDH	5'-GGTATTTCATTGAATGATCACTTTG-3'	110 bp	EU999991
	5'-TAATCCTTGGATTGCATGTACTTG-3'		
MT	5'-GGGCTGCAAATGTTGTTCACA-3'	130 bp	HQ260607
	5'-GCAGCAGTTCTTGCAGCATTC-3'		
VTG	5'-GACTATAAAGATTGTTCCATGTG-3'	221 bp	HQ260608
	5'-GAGTATGGTGGAGAATCATTAGT-3'		

3. Results

Characterization of the *C. riparius* MT and VTG genes

MT and VTG genes are stress response-associated biomarker genes. In this study, the *C. riparius* MT and VTG genes were first identified from a library of *C. riparius* cDNA. The partial sequences of the *C. riparius* MT and VTG genes were deposited in the GenBank database (accession no. HQ260607 and HQ260608). The nucleotide sequences identified in this study were not significantly homologous with those of any species present in the GenBank database. However, the amino acid sequences of the MT region of *C. riparius* were 56%, 52%, 51% and 45% homologous with those of *Belgica antarctica* (Antarctic midge, ABF72882), *Drosophila melanogaster* (Fruitfly, NP_650882), *Tabanus yao* (Horsefly, ABX80078) and *Anopheles gambiae* (African malaria mosquito, AAX86006), respectively. Additionally, the amino acid sequences of the VTG region of *C. riparius* were 39%, 38% and 37% homologous with those of *Aedes aegypti* (Aegypti mosquito, XP_001652449), *Culex quinquefasciatus* (Southern house mosquito, XP_001865748) and *Tribolium castaneum* (Red flour beetle, XP_001807564), respectively (data not shown). Finally, the sequences of MT and VTG were not highly homologous among species.

The expression of MT and VTG mRNA in response to EDCs exposures

Molecular responses to EDCs toxicity were observed via an investigation of MT and VTG mRNA expression in *C. riparius* exposed to BPA, NP or DEHP with various concentrations for 96 h. The mRNA expression of the MT gene in *C. riparius* larvae subjected to long-term exposure to EDCs is shown in Fig. 1. The response of the MT gene decreased significantly in *C. riparius* exposed to relatively low concentrations, 1 μg L^{-1} NP ($p < 0.05$), although the MT expression did not differ significantly between the control and BPA treated group (Fig. 1). However, the response of the MT mRNA expression was increased significantly at all concentrations after exposure to DEHP alone ($p < 0.05$). A significant increase (two-fold) in *Chironomus* MT was observed in *C. riparius* exposed to a relatively high concentration (50 mg L^{-1}) of DEHP ($p < 0.05$).

VTG mRNA expression in *C. riparius* was increased significantly in response to exposure to all concentrations of BPA, NP or DEHP for 96 h ($p < 0.05$)(Fig. 2). The response of VTG mRNA to BPA exposure was found to occur in a dose-dependent manner. The greatest increase in VTG gene expression was observed in response to exposure to a relatively high concentration (500 μg L^{-1}) of BPA ($p < 0.01$). Additionally, DEHP toxicity against the VTG gene induced its highest expression in *C. riparius* exposed to 5 mg L^{-1}

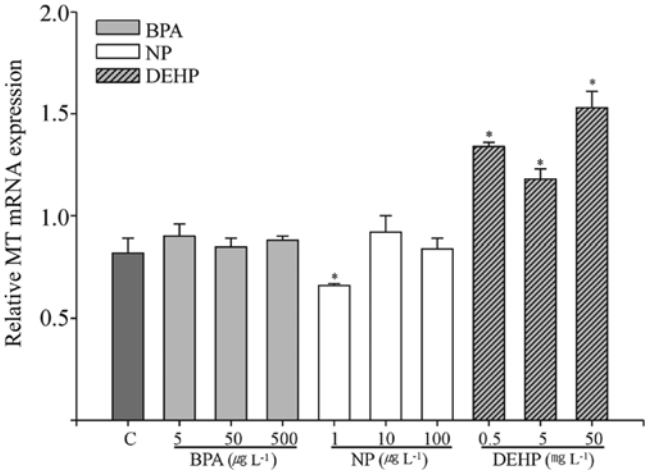

Fig. 1. Expression of the MT mRNA in fourth-instar *C. riparius* larvae exposed to EDCs for 96 h. MT mRNA expression is shown relative to GAPDH expression following normalization. The experiment was conducted in triplicate and the data are expressed as the means ± the standard error of the mean. Differences between chemical treated and solvent control treated samples were considered to be significant at *$p < 0.05$

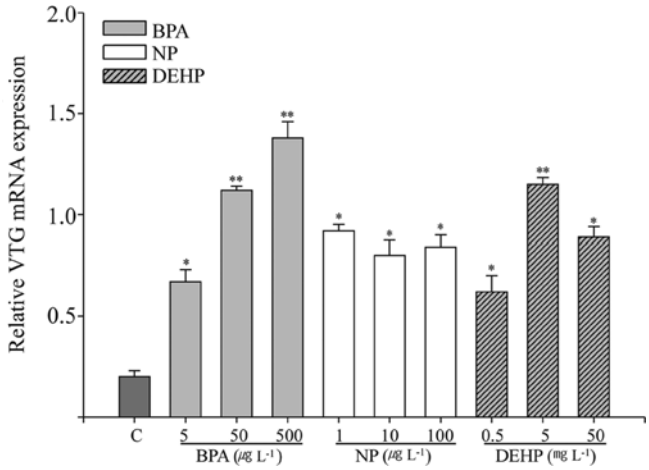

Fig. 2. Expression of the VTG mRNA in fourth-instar *C. riparius* larvae exposed to EDCs for 96 h. VTG mRNA expression is shown relative to GAPDH expression following normalization. The experiment was conducted in triplicate and the data are expressed as the means ± the standard error of the mean. Differences between chemical treated and solvent control treated samples were considered to be significant at *$p < 0.05$. ** indicates $p < 0.01$

DEHP ($p < 0.01$). Thus, all EDC treatments induced VTG mRNA expression in *C. riparius* (Fig. 2).

The expression of MT and VTG mRNA in response to heavy metal exposure

To identify genes involved in the responses to heavy metals toxicity, we observed the MT and VTG mRNA expression in *C. riparius* exposed to various concentrations of Cd, Cu or Pb for 96 h. The expression of MT mRNA was induced significantly in *C. riparius* after exposure to heavy metals (Fig. 3). MT mRNA expression was significantly up-regulated in response to all concentrations of Cd exposure in a dose-dependent manner ($p < 0.05$). A significant increase in MT mRNA of more than ten-fold was observed in response to exposure to a relatively high concentration (20 μg L^{-1}) of Cd ($p < 0.01$). After Cu or Pb treatments, MT mRNA expression was also increased in response to all concentrations (Fig. 3).

VTG mRNA expression was also significantly up-regulated in response to all concentrations of Cd in a dose-dependent manner ($p < 0.05$). The greatest increase in VTG mRNA expression was more seven fold in response to a relatively high concentration (20 μg L^{-1}) of Cd ($p < 0.01$)(Fig. 4). The induced VTG mRNA was not observed in response to exposure to any concentrations of Cu and Pb evaluated in this study. The response of the VTG mRNA was only sensitive to Cd exposure.

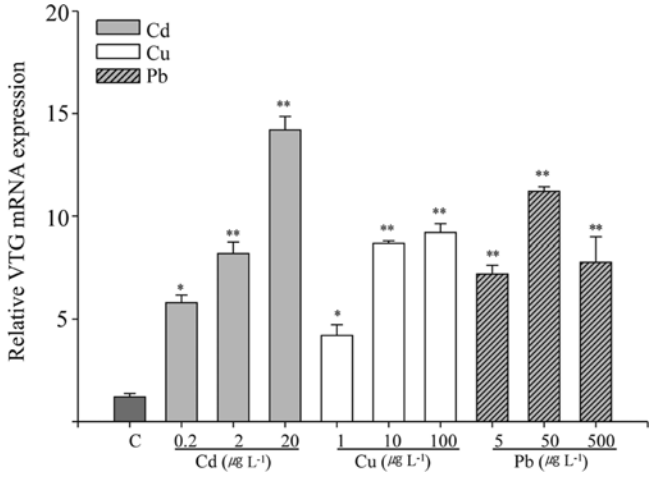

Fig. 3. Expression of the MT mRNA in fourth-instar *C. riparius* larvae exposed to heavy metals for 96 h. MT mRNA expression is shown relative to GAPDH expression following normalization. The experiment was conducted in triplicate and the data are expressed as the means ± the standard error of the mean. Differences between heavy metal treated and non-treated samples (control) were considered to be significant at $*p < 0.05$. ** indicates $p < 0.01$

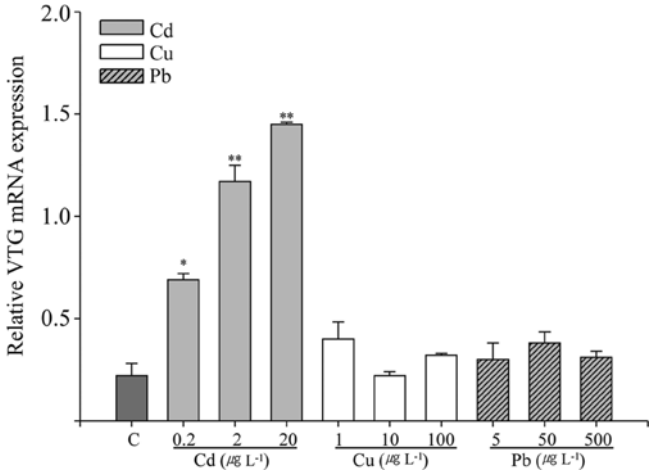

Fig. 4. Expression of the VTG mRNA in fourth-instar *C. riparius* larvae exposed to heavy metals for 96 h. VTG mRNA expression is shown relative to GAPDH expression following normalization. The experiment was conducted in triplicate and the data are expressed as the means ± the standard error of the mean. Differences between heavy metal treated and non-treated samples (control) were considered to be significant at $*p < 0.05$. ** indicates $p < 0.01$

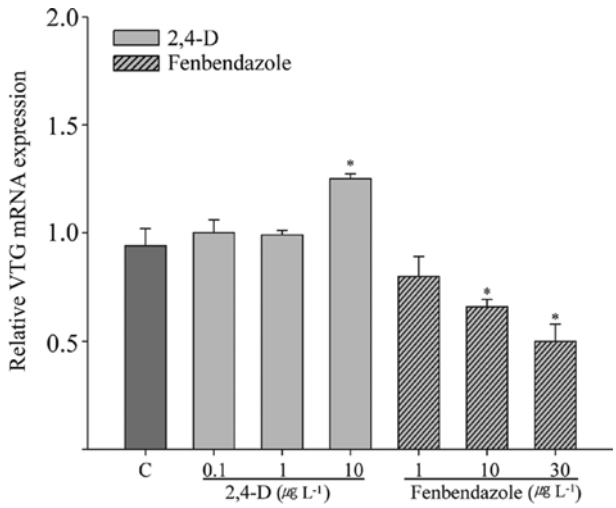

Fig. 5. Expression of the MT mRNA in fourth-instar *C. riparius* larvae exposed to 2,4-D and fenbendazole for 96 h. MT mRNA expression is shown relative to GAPDH expression following normalization. The experiment was conducted in triplicate and the data are expressed as the means ± the standard error of the mean. Differences between chemical treated and solvent control treated samples were considered to be significant at $*p < 0.05$

The expression of MT and VTG mRNA in response to exposure to herbicide and veterinary antibiotics

Changes in MT mRNA expression in response to long-term herbicide and veterinary antibiotics are shown in Fig. 5. MT mRNA expression was only significantly upregulated

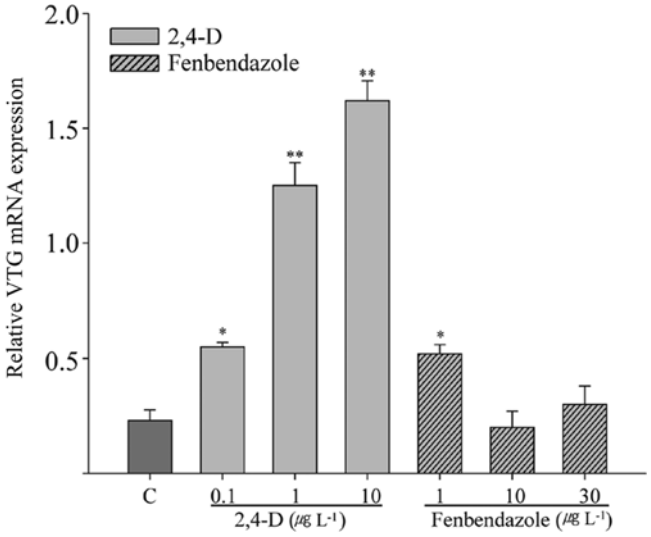

Fig. 6. Expression of the VTG mRNA in fourth-instar *C. riparius* larvae exposed to 2,4-D and fenbendazole for 96 h. VTG mRNA expression is shown relative to GAPDH expression following normalization. The experiment was conducted in triplicate and the data are expressed as the means ± the standard error of the mean. Differences between chemical treated and solvent control treated samples were considered to be significant at $*p < 0.05$. ** indicates $p < 0.01$

in response to relatively high concentrations (10 μg L^{-1}) of 2,4-D ($p < 0.05$). However, the MT mRNA expression decreased significantly in response to the 10 and 30 L^{-1} fenbendazole treatments ($p < 0.05$).

VTG mRNA responses to environmental pollutants were observed in *C. riparius* exposed to various concentrations of 2,4-D and fenbendazole for 96 h (Fig. 6). After 2,4-D exposure, the *Chironomus* VTG mRNA showed a significant increase in a dose-dependent manner. Additionally, the highest increase of more seven fold was observed in response to a relatively high concentration (10 μg L^{-1}) of 2,4-D ($p < 0.01$) (Fig. 6). However, the VTG mRNA response to veterinary antibiotics was only significantly upregulated in response to relatively low concentrations (1 μg L^{-1}) of fenbendazole ($p < 0.05$).

4. Discussion

In this study, we investigated the differential expression of MT and VTG mRNA in *C. riparius* in response to various environmental pollutants. Rapid industrial development has caused the release of diverse and complex forms of chemicals into aquatic environments. MT has been suggested as a biomarker of metal contamination in aquatic environments via its binding to particular metals. Our findings regarding the effects of heavy metal exposure on the mRNA level of MT gene confirm that *Chironomus* MT is a useful biomarker for the detection of long-term effects of heavy metals such as Cd, Cu, and Pb. The induction of the MT gene in response to heavy metals has been previously well characterized in various organisms (Bourdineaud et al. 2006). Cd treatment significantly elevated MT mRNA expression in heavy metal-tolerant *Anopheles gambiae* mosquitoes (Mireji et al. 2010). Cd treatment also significantly induced MT mRNA in a dose-dependent manner in the clam *Mactra veneriformis* (Fang et al. 2010) and killifish *Kryptolebias marmoratus* (Rhee et al. 2009).

MT also reportedly plays an important role in immune response (Cai et al. 1999), antioxidant processes (Viarengo et al. 2000; Cavaletto et al. 2002), and response to estrogenic compounds (Canesi et al. 2007). One of the most notable results was the up-regulation of the MT mRNA in response to DEHP. This increase was observed in response to all concentrations of DEHP evaluated herein (Fig. 1), which suggests that the MT gene plays a functional role in response to long-term DEHP toxicity in the aquatic invertebrate *C. riparius*. DEHP is one of several compounds known to be hepatic tumor promoters and toxins in mice. Hepatic MT was very significantly up-regulated - by as much as eleven fold - in response to MT in an animal model in a dose- and time-related manner (Waalkes and Ward 1989). Additionally, it was previously demonstrated that MTs participate in several cellular functions such as regulation of growth and antioxidative defenses (Mosleh et al. 2005, 2006). MT mRNA levels increased significantly after 2, 4, and 7 days of exposure to different concentrations of the herbicide isoproturon in the aquatic worms *Tubifex tubifex* (Mosleh et al. 2005). In the present study, the herbicide 2,4-D also induced MT mRNA expression in *C. riparius* exposed to a relatively high concentration of 2,4-D (Fig. 5). Moreover, the MT mRNA response in *C. riparius* exposed to veterinary antibiotics was decreased (Fig. 5). These results suggest that decreased MT mRNA expression may be associated with the response to oxidative stress by fenbendazole exposure because the response of HSPs, CYP450 and GST genes was observed in *C. riparius* exposed to fenbendazole (Park et al. 2009). However, additional studies are needed to better understand this result.

Endocrine disruptors or xenoestrogens are exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones

in the body that are responsible for the maintenance of homeostasis (Tian et al. 2009). EDCs can induce VTG in males and immature females among aquatic vertebrates and invertebrates (Matozzo et al. 2008). To our knowledge, there are limited data about VTG induction in insects exposed to EDCs. In the present study, the expression of *Chironomus* VTG mRNA was significantly up-regulated in response to BPA exposure in a dose-dependent manner. NP and DEHP exposure also increased VTG mRNA expression in *C. riparius* (Fig. 2). DEHP was reported to have effects on the female reproductive system of the zebrafish, including interference with the signals involved in oocyte growth, maturation and ovulation, as well as deeply impairing ovarian functions leading to serious consequences on embryo production (Carnevali et al. 2010). The VTG mRNA up-regulation in response to DEHP observed in the present study suggests that DEHP affects oocyte development in the aquatic organism *C. riparius*.

Metallo-estrogens have recently been considered as a new class of potent environmental estrogens (Iacavoli et al. 2009). The heavy metal Cd has recently been identified as a novel EDC (Henson and Chedrese 2004; Planelló et al. 2010). Cd exposure affects the release of pituitary hormones and the gonad production of steroid hormones in vertebrates (Lafuente et al. 2003; Smida et al. 2004). Additionally, reproductive and developmental disorders have frequently been associated with Cd exposure in different organisms, including insects (Gintenreiter et al. 1993; Laskowski 2001). In the terrestrial insect *Oncopeltus fasciatus* Dallas, Cd exposure delays ovarian maturation and inhibits vitellogenesis, probably by reduction in VTG (Cervera et al. 2005). Cd is able to act like steroidal estrogens in vertebrates (Johnson et al. 2003; Henson and Chedrese 2004), and as an endocrine disruptor in invertebrates (Cervera et al. 2005). Therefore, we confirmed the molecular changes that occur in aquatic midge *C. riparius* after exposure to Cd by analyzing the expression patterns of VTG mRNA. Among the heavy metals evaluated in this study, the VTG mRNA was only significantly up-regulated in a dose-dependent manner in response to Cd exposure (Fig. 4).

The aquatic herbicide, 2,4-D, is widely used to selectively control broadleaf and woody plants in various waterways. 2,4-D is one of the oldest and most commonly used herbicides (Xie et al. 2005). VTG mRNA expression increased significantly in *C. ripaius* exposed to 2,4-D in a dose-dependent manner (Fig. 6), which suggests that it has estrogenic effects in aquatic invertebrates and can influence the reproductive system of *C. riparius*. Similarly, 2,4-D caused significant induction of VTG mRNA in rainbow trout (Xie et al. 2005). Once 2,4-D is in water, it is readily degraded to 2,4-dichlorophenol, which is an estrogen receptor ligand (Jobling et al. 1995).

There is no information available regarding the cellular mechanism by which antibiotics toxicity occurs in aquatic organisms. In the present study, the veterinary antibiotic fenbendazole caused significant induction of the VTG mRNA only when administered in relatively low concentrations for long-term periods (Fig. 5). Recently, the veterinary antibiotic sulfathiazole caused a change in the VTG mRNA expression of *Daphnia magna*. The response of the VTG mRNA increased in *D. magna* exposed to sulfathiazole and environmental levels of ultraviolet B irradiation (Kim et al. 2009). VTG serves as a storage protein providing amino acids, carbohydrates, lipids and phosphates to the developing embryo (Matozzo et al. 2008). VTG can also transport trace minerals (Falchuk and Montorzi 2001). Therefore, under oxidative stress, the demand for cofactors would increase, and as would mineral transporters such as VTG. However, further study is necessary to understand the reasons for VTG induction and oxidative stress in response to veterinary antibiotics in aquatic organisms.

5. Conclusion

We investigated the differential expression of MT and VTG mRNA in the aquatic organism *C. riparius*, in response to various pollutants. The induction of MT mRNA expression was observed in response to DEHP and 2,4-D, as well as heavy metals. A significant decrease in expression of the MT mRNA was caused by NP and fenbendazole exposure. The mRNA expression of *Chironomus* VTG increased significantly in response to treatment with Cd, 2,4-D and fenbendazole, as well as EDCs. Induction of VTG mRNA by 2,4-D toxicity appeared to have a similar concentration-dependent pattern of increase. Taken together, the results of this study suggest that VTG has the potential for use as a potential biomarker of EDCs, Cd and 2,4-D exposure in aquatic organisms.

Acknowledgements

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government [NRF-

2012-0004186] and [NRF-2012-0004352].

References

Bourdineaud JP, Baudrimont M, Gonzalez P, Moreau JL (2006) Challenging the model for induction of metallothionein gene expression. Biochimie **88**:1787-1792

Bownes M (1989) The roles of juvenile hormone, ecdysone, and the ovary in the control of Drosophila vitellogenesis. J Insect Physiol **35**:409-413

Bownes M, Ronaldson E, Mauchline D (1996) 20-Hydroxyecdysone, but not juvenile hormone, regulation of yolk protein gene expression can be mapped to cis-acting DNA sequences. Dev Biol **173**:475-489

Burton G (1991) Assessing the toxicity of freshwater sediments. Environ Toxicol Chem **10**:1585-1627

Cai L, Satoh M, Tohyama C, Cherian MG (1999) Metallothionein in radiation exposure: Its induction and protective role. Toxicology **132**:85-98

Canesi L, Lorusso LC, Ciacci C, Betti M, Rocchi M, Pojana G, Marcomini A (2007) Immunomodulation of *Mytilus hemocytes* by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: in vitro and in vivo studies. Aquat Toxicol **81**:36-44

Carnevali O, Tosti L, Speciale C, Peng C, Zhu Y, Maradonna F (2010) DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. PLoS One **5**:e10201

Cavaletto M, Ghezzi A, Burlando B, Evangelisti V, Ceratto N, Viarengo A (2002) Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of *Mytilus galloprovincialis*. Comp Biochem Physiol C Toxicol Pharmacol **131**:447-455

Cervera A, Cristina Maymó A, Martínez-Pardo R, Dolores Garcerá M (2005) Vitellogenesis inhibition in *Oncopeltus fasciatus* females (Heteroptera: Lygaeidae) exposed to cadmium. J Insect Physiol **51**:895-911

Choi YK, Jo PG, Choi CY (2008) Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassostrea gigas*. Comp Biochem Physiol C Toxicol Pharmacol **147**:286-292

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. Ann Rev Plant Biol **53**:159-182

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

Engelmann F (1986) Endocrine regulated insect vitellogenesis: A synthesis. Adv Invertebr Reprod **4**:31-37

Escher BI, Berger C, Bramaz N, Kwon JH, Richter M, Tsinman O, Avdeef A (2008) Membrane-water partitioning, membrane permeability, and baseline toxicity of the parasiticides ivermectin, albendazole, and morantel. Environ Toxicol Chem **27**:909-918

Falchuk KH, Montorzi M (2001) Zinc physiology and biochemistry in oocytes and embryos. Biometals **14**:385-395

Fang Y, Yang H, Wang T, Liu B, Zhao H, Chen M (2010) Metallothionein and superoxide dismutase responses to sublethal cadmium exposure in the clam *Mactra veneriformis*. Comp Biochem Physiol C Toxicol Pharmacol **151**:325-333

Gintenreiter S, Ortel J, Nopp HJ (1993) Effects of different dietary levels of cadmium, lead copper, and zinc on the vitality of the forest pest insect *Lymantria dispar L.* (Lymantriidae, Lepid). Arch Environ Contam Toxicol **25**:62-66

Guidugli KR, Nascimento AM, Amdam GV, Barchuk AR, Omholt S, Simoes ZLP, Hartfelder K (2005) Vitellogenin regulates hormonal dynamics in worker caste of a eusocial insect. FEBS Lett **579**:4961-4965

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

Hwang DS, Lee KW, Han J, Park HG, Lee J, Lee YM, Lee JS (2010) Molecular characterization and expression of vitellogenin (Vg) genes from the cyclopoid copepod, *Paracyclopina nana* exposed to heavy metals. Comp Biochem Physiol C Toxicol Pharmacol **151**:360-368

Iacavoli I, Fontana L, Bergamaschi A (2009) The effects of metals as endocrine disruptors. J Toxicol Environ Health part B **12**:206-223

Jarup L, Berglund M, Elinder CG, Nordberg G, Vahter M (1998) Health effects of cadmium exposure-a review of the literature and risk estimate. Scand J Work Environ Health **24**:1-52

Jobling S, Reynolds T, White R, Parker MG, Sumpter JP (1995) A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect **103**:582-587

Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, Clarke R, Sholler PF, Lirio AA, Foss C, Reiter R, Trock B, Paik S, Martin MB (2003) Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. Nat Med **8**:1081-1084

Kim MH, Jung JY, Park J (2006) Prioritizing pharmaceuticals for aquatic risk assessment in Korea. In: An Approach for Developing Aquatic Environmental Risk Assessment Framework for Pharmaceuticals in Korea. Korea Environment Institute (KEI/2006/RE-05)

Kim J, Park Y, Choi K (2009) Phototoxicity and oxidative stress responses in *Daphnia magna* under exposure to sulfathiazole and environmental level ultraviolet B irradiation. Aquat Toxicol **91**:87-94

Kreuzig R, Blumlein K, Holtge S (2007) Fate of the benzimidazole antiparasitics flubendazole and fenbendazole in manure and manured solis. Clean Soil Air Water **35**:488-494

Lafuente A, Cano P, Esquifino A (2003) Are cadmium effects on

plasma gonadotropins, prolactin, ACTH, GH and TSH levels, dose-dependent? Biometals **16**:243-250

Laskowski R (2001) Why short-term bioassays are not meaningful-effects of a pesticide (imidacloprid) and a metal (cadmium) on pea aphids (*Acyrthosiphon pisum Harris*). Ecotoxicology **10**:177-183

Matozzo V, Gagné F, Marin MG, Ricciardi F, Blaise C (2008) Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. Environ Int **34**:531-545

Mills LJ, Chichester C (2005) Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci Total Environ **343**:1-34

Mireji PO, Keating J, Hassanali A, Impoinvil DE, Mbogo CM, Muturi MN, Nyambaka H, Kenya EU, Githure JI, Beier JC (2010) Expression of metallothionein and alpha-tubulin in heavy metal-tolerant *Anopheles gambiae* sensu stricto (Diptera: Culicidae). Ecotoxicol Environ Saf **73**:46-50

Mosleh YY, Paris-Palacios S, Couderchet M, Biagianti-Risbourg S, Vernet G (2005) Effects of the herbicide isoproturon on metallothioneins, growth, and antioxidative defenses in the aquatic worm *Tubifex tubifex* (Oligochaeta, Tubificidae). Ecotoxicology **14**:559-571

Mosleh YY, Paris-Palacios S, Biagianti-Risbourg S (2006) Metallothioneins induction and antioxidative response in aquatic worms *Tubifex tubifex* (Oligochaeta, Tubificidae) exposed to copper. Chemosphere **64**:121-128

Nelson CM, Ihle KE, Fondrk MK, Page RE, Amdam GV (2007) The vitellogenin gene has multiple coordinating effects on social organization. PLoS Biol **5**:e62

Oh SJ, Park J, Lee MJ, Park SY, Lee JH, Choi K (2006) Ecological hazard assessment of major veterinary benzimidazoles: Acute and chronic toxicities to aquatic microbes and invertebrates. Environ Toxicol Chem **25**:2221-2226

Park K, Bang HW, Park J, Kwak IS (2009) Ecotoxicological multilevel-evaluation of the effects of fenbendazole exposure to *Chironomus riparius* larvae. Chemosphere **77**:359-367

Park K, Kwak IS (2010) Molecular effects of endocrine-disrupting chemicals on the *Chironomus riparius* estrogen-related receptor gene. Chemosphere **79**:934-941

Park K, Park J, Kim J, Kwak IS (2010) Biological and molecular responses of *Chironomus riparius* (Diptera, Chironomidae) to herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Comp Biochem Physiol C Toxicol Pharmacol **151**:439-446

Planelló R, Martínez-Guitarte JL, Morcillo G (2010) Effect of acute exposure to cadmium on the expression of heat-shock and hormone-nuclear receptor genes in the aquatic midge *Chironomus riparius*. Sci Total Environ **408**:1598-1603

Rhee JS, Raisuddin S, Hwang DS, Lee KW, Kim IC, Lee JS (2009) Differential expression of metallothionein (MT) gene by trace metals and endocrine-disrupting chemicals in the hermaphroditic mangrove killifish, *Kryptolebias marmoratus*. Ecotoxicol Environ Saf **72**:206-212

Rieradevall M, García-Berthou E, Prat N (1995) Chironomids in the diet of fish in Lake Banyoles (Catalonia, Spain). In: Cranston P (ed) Chironomids from genes to ecosystems. CSIRO, Australia, pp 335-342

Sappington TW, Raikhel AS (1998) Molecular characteristics of insect vitellogenins and vitellogenin receptors. Insect Biochem Mol Biol **28**:277-300

Shu Y, Zhou J, Tang W, Lu K, Zhou Q, Zhang G (2009) Molecular characterization and expression pattern of *Spodoptera litura* (Lepidoptera: Noctuidae) vitellogenin, and its response to lead stress. J Insect Physiol **55**:608-616

Smida AD, Valderrama XP, Agostini MC, Furlan MA, Chedrese J (2004) Cadmium stimulates transcription of the cytochrome P450 side chain cleavage gene in genetically modified stable porcine granulosa cells. Biol Reprod **70**:25-31

Snigirevskaya ES, Raikhel AS (2005) Receptor-mediated endocytosis of yolk proteins in insect oocytes. In: Raikhel AS, Sappington TW (eds) Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII. Part B. Science Publishers, Inc., Enfield, USA-Plymouth, UK, pp 199-228

Streloke M, Köpp H (1995) Long-term toxicity test with *Chironomus riparius*: Development and validation of a new test system. Blackwell Wissenschaftsverlag, Berlin/Vienna, 315 p

Teixeira MC, Duque P, Sá-Correia I (2007) Environmental genomics: Mechanistic insights into toxicity of and resistance to the herbicide 2, 4-D. Trends Biotechnol **25**:363-370

Torres MA, Barros MP, Campos SC, Pinto E, Rajamani S, Sayre RT, Colepicolo P (2008) Biochemical biomarkers in algae and marine pollution: A review. Ecotoxicol Environ Saf **71**:1-15

Tian H, Ru S, Wang Z, Cai W, Wang W (2009) Estrogenic effects of monocrotophos evaluated by vitellogenin mRNA and protein induction in male goldfish (*Carassius auratus*). Comp Biochem Physiol C Toxicol Pharmacol **150**:231-236

Tufail M, Takeda M (2008) Molecular characteristics of insect vitellogenins. J Insect Physiol **54**:1447-1458

Viarengo A, Burlando B, Cavaletto M, Marchi B, Ponzano E, Blasco J (1999) Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. Am J Physiol **277**:1612-1619

Viarengo A, Burlando B, Ceratto N, Panfoli I (2000) Antioxidant role of metallothioneins: a comparative overview. Cell Mol Biol **46**:407-417

Waalkes MP, Ward JM (1989) Induction of hepatic metallothionein in male B6C3F1 mice exposed to hepatic tumor promoters: Effects of phenobarbital, acetaminophen, sodium barbital, and di(2-ethylhexyl) phthalate. Toxicol Appl Pharmacol **100**:217-226

Wang L, Song L, Ni D, Zhang H, Liu W (2009) Alteration of metallothionein mRNA in bay scallop *Argopecten irradians* under cadmium exposure and bacteria challenge. Comp Biochem

Physiol C Toxicol Pharmacol **149**:50-57

Xie L, Thrippleton K, Irwin MA, Siemering GS, Mekebri A, Crane D, Berry K, Schlenk D (2005) Evaluation of estrogenic activities of aquatic herbicides and surfactants using an rainbow trout vitellogenin assay. Toxicol Sci **87**:391-398

Zadorozhnaja TD, Little RE, Miller RK, Mendel NA, Taylor RJ, Presley BJ, Gladen BC (2000) Concentrations of arsenic, cadmium, copper, lead, mercury, and zinc in human placentas from two cities in Ukraine. J Toxicol Environ Health **61**:255-263