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Effects of cadmium chloride and nonylphenol on the expression of StAR-related lipid transfer domain containing protein (*START1*) gene in aquatic midge, *Chironomus riparius*

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ABSTRACT

We identified and characterized a partial cDNA of StAR-related lipid transfer domain containing protein gene from *Chironomus riparius* (*CrSTART1*) having homology with human MLN64 and *Drosophila melanogaster START1* (*DmSTART1*) and evaluated the effects of cadmium chloride (Cd) and nonylphenol (NP) on its expression. Pfam analysis identified the presence of two StAR-related lipid transfer (START) domains in *CrSTART1* having several conserved amino acid residues, characteristic of the MLN64 and *DmSTART1*. The mRNA expression of *CrSTART1* was observed in all developmental stages. The modulation in the mRNA expression of *CrSTART1* was investigated after exposure to different concentrations Cd (0, 2, 10, and 20 mg/L) and NP (0, 10, 50, and 100 μg/L) for different time intervals in fourth instar larvae of *C. riparius*. Significant downregulation of *CrSTART1* mRNA was observed after exposure to 2, 10 and 20 mg/L of Cd for 24, 48 and 72 h. Significant upregulation of *CrSTART1* was observed after exposure to 10 and 50 μg/L of NP for 24, and 48 h period. At 100 μg/L of NP significant upregulation of *CrSTART1* was observed after 12 h and downregulated after 24, 48 and 72 h.

1. Introduction

Aquatic environments are increasingly contaminated by the release of anthropogenic and industrial wastes and the endocrine systems of aquatic organisms are adversely affected by these different environmental pollutants (Arukwe and Goksoyr, 1998; Leblond and Hontela, 1999). Cadmium (Cd) is a toxic heavy metal and widespread environmental pollutant, and its accumulation occurs in river sediments via various routes. The concentration of Cd is much higher in sediments than in water samples (Korte, 1983; Jung et al., 2005). The endocrine disruption caused by Cd has been reported (Henson and Chedrese, 2004). Nonylphenol (NP) is a degradation product of alkylphenol polyethoxylates which represent an important class of nonionic surfactants widely used in many cleaning detergent formulations and plastic products for both industrial and domestic uses (Ahel et al., 1994a,b; Ying et al., 2002). High concentrations of NP (180–330 μg/L) have been reported in river surface sediments (Marcomini and Giger, 1987; Blackburn and Waldock, 1995; Tyler et al., 1998). The estrogenic effects of NP as an endocrine disruptor have been extensively studied (Nimrod and Benson, 1996; Bandiera, 2006; Lye et al., 2008).

Studies on the effects of environmental pollutants on sediment-dwelling organisms are important for examining their ecotoxicological impacts, because the life cycle stages of many aquatic organisms are associated with this zone. *Chironomus riparius* (Chironomidae, Diptera) holds an important position in the aquatic food chain and is widely used in aquatic exotoxicological studies for assessing acute and sub-lethal toxicities of contaminated sediments because of their widespread occurrence, short life-cycle, easy of rearing in the laboratory, physiological tolerance to various environmental conditions and undergoes a metamorphosis under hormone control (Bettinetti et al., 2002; Crane et al., 2002; Taenzler et al., 2007). *Chironomus* is used as a test organism for investigations of the potential endocrine disrupting effects of chemicals in aquatic invertebrates (Segner et al., 2003; OECD, 2006; Taenzler et al., 2007). Several previous studies have shown that chronic exposure to Cd and NP results in developmental failures such as reduction in larval survival, growth and developmental rate, affecting both the time of emergence and egg production in *C. riparius* (Pascoe et al., 1989; Postma and Davis, 1995; Lee and Choi, 2006).

Steroid hormones regulate the development; elicit molting and metamorphosis in Arthropods (crustaceans and insects) (Riddiford, 1993; Subramoniam, 2000). Regardless of tissue origin, cholesterol is used as the precursor for all steroid hormone syntheses (King et al., 2002; Arukwe, 2005). In insects, cholesterol is obtained from diet (Kircher, 1982). The trafficking mechanism of the steroid

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precursors still remains to be clearly elucidated in insects, but may use similar mechanisms as those of steroidogenesis in vertebrates (Rees, 1995; Gilbert and Warren, 2005; Lafont et al., 2005). The delivery of cholesterol from the outer to the inner mitochondrial membrane is mediated by the sterol carrier protein and is the rate-limiting step in steroidogenesis. This is mediated by the steroidogenic acute regulatory protein (StAR) and is a target for the regulation of steroidogenesis (Stocco, 2001). StAR mediates, by means of the StAR-related lipid transfer (START) domain, the transfer of cholesterol across the mitochondrial membrane to the side-chain cleavage P450 enzyme for the synthesis of steroid hormones (Stocco, 2001). Recently, a gene encoding a protein with the START domain, *START1*, has been characterized in *Drosophila melanogaster* at both the transcript and protein levels (Roth et al., 2004). However, *START1* is not homologous to StAR itself, but contains only the START domain, and is homologous to the human Metastatic Lymph Node 64 (MLN64) gene, which is known to be involved in cholesterol trafficking and promoting steroidogenesis in humans (Watari et al., 1997; Alpy et al., 2001; Zhang et al., 2002).

It has been widely accepted that the use of the expression of the genes involved in many biological processes offer high sensitivity and mechanistic value in the diagnosis of environmental contamination, as the mRNA levels represent a snapshot of the cells activity at a given time (Snell et al., 2003; Ankley et al., 2006). Modulation of genes involved in steriodogenic pathways are being considered as important target for endocrine disrupting chemicals (Sanderson, 2006; Arukwe, 2008; Peretz et al., 2011; Sandhu and Vijayan, 2011). Cadmium and NP are known to modulate genes involved in steroidogenesis, including the StAR gene, leading to impaired steroidogenesis in aquatic organisms (Arukwe, 2005; Kortner and Arukwe, 2007; Kortner et al., 2009; Sandhu and Vijayan, 2011). Therefore, studying the effects of Cd and NP on the transcriptional modulation of the genes involved in the biosynthesis of steroid hormones in ecotoxicologically important species *C. riparius* merits further investigation which may serve as a sensitive endpoint for evaluating the endocrine-modulating effects of these chemicals. Therefore, in this study we identified and characterized a partial cDNA of *C. riparius START1* (*CrSTART1*) homologous to the human MLN64 and *D. melanogaster START1* gene from the previously generated *C. riparius* Expressed Sequence Tags (ESTs) database (Nair et al., 2011). The mRNA expression profile of *CrSTART1* is studied in different developmental stages (egg, larva, pupa, male and female), as well as in response to different concentrations and time periods of Cd and NP exposure using real-time PCR method.

2. Materials and methods

2.1. Animals and chemical exposure

C. riparius (Insecta: Chironomidae) larvae were obtained from Korea Institute of Toxicology (Daejeon, South Korea) and maintained at 21 °C with a photoperiod of 16 h light:8 h dark in 2 L glass chamber, containing dechlorinated tap water and acid washed sand with aeration. The larvae were fed with finely ground fish flake every 2 days (Tetramin, Tetrawerke, Melle, Germany). For chemical exposure 15 fourth instar larvae of *C. riparius* were exposed to sub lethal concentrations of cadmium chloride ($CdCl_2$: 0, 2, 10 and 20 mg/L—Sigma-Aldrich Chemical, St. Louis, MO, USA), and 4-nonylphenol (NP: 0, 10, 50 and 100 μg/L—Sigma-Aldrich-analytical grade) for 12, 24, 48 and 72 h based on the toxicity study conducted earlier in our laboratory (Lee and Choi, 2006; Choi and Ha, 2009). All the exposure and controls were done in triplicates consisting 15 fourth instar larvae in each exposure set in beakers containing 100 mL dechlorinated tap water. For $CdCl_2$ exposure the controls were maintained without any treatment along with the treated larvae. The controls for nonylphenol were exposed with the solvent acetone (concentration 1 mL acetone in 1000 mL dechlorinated tap water: Sigma-Aldrich) which induced no effects on *C. riparius* as per previous reports (Kwak and Lee, 2005). After the exposure the larvae were collected, frozen in liquid nitrogen and stored at −80 °C.

2.2. Identification and sequence analysis of CrSTART1

The partial cDNA sequence for *C. riparius START1* gene was retrieved from the Expressed Sequence Tags (ESTs) database, developed by GSFLX pyrosequencing, using BlastX comparisons to NCBI database. The putative amino acid translations were deduced from cDNA using translation tool (http://www.expasy.ch/tools/) and aligned using ClustalW available in internet (http://align.genome.jp/). The protein motifs were identified using Pfam program (http://motif.genome.ad.jp).

2.3. RNA isolation, cDNA synthesis and quantitative Real-time RT-PCR

Total RNA was isolated using Trizol™ (Invitrogen, USA) from chemical exposed and control larvae as per manufacturer's instructions, cleaned using Nucleospin RNA-Clean up (Macherey-Nagel, Germany) procedure and the quality of RNA preparation was verified by agarose electrophoresis and absorbance spectrophotometry (A260/A280 > 1.8). One microgram of total RNA was reverse transcribed to cDNA with oligo dT_{20} primer using iScript™ select cDNA synthesis kit (Bi-Rad, USA) as per the manufacturer's instructions in a total reaction volume of 20 μL. The primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) (Table 1) and were tested on a representative *C. riparius* cDNA preparation using reaction conditions with 94 °C for 4 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C or 30 s and 72 °C for 10 min using PTC 100 thermal cycler (MJ Research, Lincoln, MA, USA) with the PCR mix (Bioneer, South Korea) according to the manufacturers' manual. The RT-PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide to verify the amplicon length and to assure that only one product is amplified.

To study *CrSTART1* gene transcript expression after exposure to different concentrations and durations of Cd, and NP quantitative real-time RT-PCR was performed. Each reaction included 1 μL of template cDNA, 0.2 μM of corresponding forward and reverse primers, 10 μL of 2× IQ SYBR Green Super Mix (Bio-Rad) in a final reaction volume of 20 μL. The RT-PCR reactions were run with an initial denaturing at 95 °C for 7 min followed by 44 cycles of 95 °C for 15 s, 55 °C for 1 min and 72 °C for 0.15 s and a melting curve analysis was done. Amplification and detection were performed using a CFX96™ Real-Time PCR detection system (Bio-Rad) and accompanying software (CFX Manager Software) according to the manufacturer's instructions. The qRT-PCR was done using samples from three independent exposure and control sets. Each test consisted of three replicates. The expression level of *CrSTART1* gene under exposure to different concentration and time intervals of Cd, and NP was calculated relative to expression levels of the *Chironomus* β-actin (GenBank No: AB070370) mRNA used as an internal standard to normalize the expression levels.

2.4. Data analysis

Cycle threshold (C_t) values were converted to relative gene expression levels by $2^{-\Delta\Delta CT}$ method using the gene expression analysis software in CFX96 PCR machine (Bio-Rad). The data were checked for

Table 1
List of primers used for Real Time PCR analysis.

Primer	Sequence (5′–3′)	Amplicon length (bp)
CrSTART1-F	AGGCCTTGAAACAGTGCAGAAAGC	100
CrSTART1-R	ACGTCCCACTGATCGTGAGCA	
Cβ-Actin-F	GATGAAGATCCTCACCGAACG	145
Cβ-Actin-R	TTCGAGTGAGGTTGATGCAG	

of homogeneity of variance before analysis. Statistical differences between the results obtained from different experiments in control and treated larvae were analyzed using one-way ANOVA with SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA). Dunnett's post-doc test was done to determine the effect of different exposures on gene expressions. A probability level of $P<0.05$ was considered significant.

3. Results

3.1. Sequence analysis of CrSTART1

In this study, a partial cDNA encoding *C. riparius START1* gene was identified from a *C. riparius* ESTs database and the mRNA expression was studied during distinct stages of development and following exposure to Cd and NP. The partial cDNA of *CrSTART1* was 1183 base pair (bp) in length and the Pfam searches identified two lipid-binding START domains in the *CrSTART1* gene showing high homology with equivalent genes from *Aedes aegypti*, *Anopheles gambiae*, *D. melanogaster* and human MLN64 (Hs-MLN64). Several identical amino acid residues important for forming the tunnel were conserved between *CrSTART1* and *START1* from other species and Hs-MLN64 (Fig. 1). The sequence of the cDNA described in this study is deposited in GenBank with the accession number JL641905.

3.2. Expression of CrSTART1

The expression of *CrSTART1* was studied during all developmental stages using qRT-PCR and was found to be expressed during all stages of development (Fig. 2). Further, the modulation of the *CrSTART1* gene expression was studied in fourth instar *C. riparius* larvae after exposures to different concentrations and durations of Cd and NP using real-time PCR method. The quantification of the *CrSTART1* mRNA showed no significant change after exposure to 2, 10 and 20 mg/L of Cd for 12 h compared to the control. However, significant downregulation of *CrSTART1* mRNA was observed after exposure to 2, 10 and 20 mg/L of Cd for 24, 48 and 72 h (Fig. 3).

The mRNA expression level of *CrSTART1* on exposure to different concentrations of NP was significantly upregulated at various time intervals except few exposure conditions. The effects of NP reported in the present study were based on nominal exposure concentrations. No significant change in the expression of *CrSTART1* was observed after exposure to 10 μg/L of for 12 and 72 h. Similarly, no significant change in the expression of *CrSTART1* was observed after exposure to 50 μg/L of NP for 12 h. However, significant upregulation of *CrSTART1* mRNA was noticed after exposures to 10 μg/L of NP for 24 and 48 h. There was significant upregulation after exposure to 50 μg/L of NP for 24, 48 and 72 h as compared to the control. The mRNA expression level of *CrSTART1* was significantly upregulated after exposure to 100 μg/L of NP for 12 h and showed significant downregulation after 24, 48 and 72 h time durations as compared to the control (Fig. 4).

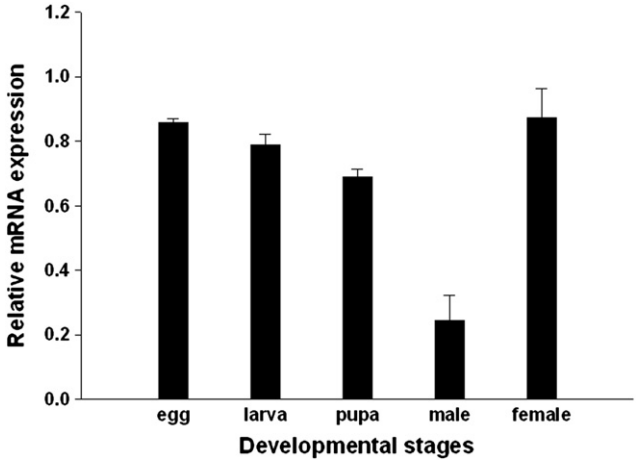

Fig. 2. Relative mRNA expression of *C. riparius START1* at different developmental stages (egg-two egg mass, fourth instar larvae, pupa, male and female adults-five animals for each stage). The mRNA expression of *CrSTART1* was quantified using real time PCR and normalized using *Chironomus* β-actin gene. All values represent the mean ± standard error of the mean.

4. Discussion

In this study, we identified and characterized the *CrSTART1* gene, having high homology with equivalent genes from human MLN64 and insects *START1* for the first time. Based on previous studies with *D. melanogaster START1* (Bose et al., 2000; Alpy et al., 2001; Zhang et al., 2002), and the presence of characteristic STAR domains and conserved amino acid residues it could be hypothesized that the *CrSTART1* is also involved in the trafficking of cholesterol and plays an important role in steriodogenesis in *C. riparius*. As in the case of the *D. melanogaster START1* gene (Roth et al., 2004), we could find only one gene carrying the START domain, *CrSTART1*, from our *C. riparius* ESTs database.

Exposure to heavy metals and endocrine disrupting chemicals (EDCs) could disturb the hormone cycles in aquatic organisms, which

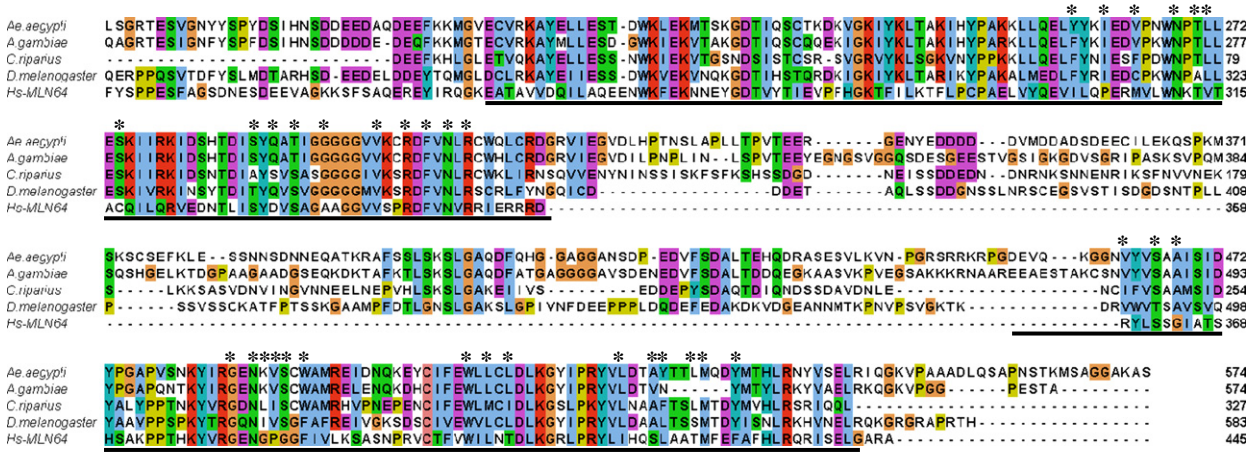

Fig. 1. Protein sequence of the partial cDNA of *C. riparius START1*. Sequence alignment with different dipteran homologues of *A. aegypti* (GenBank accession no: AY947545), *D. melanogaster* (AAF47232), *A. gambiae* (EAA03945) and MLN64 from *Homo sapiens* (Hs-MLN64; Q14849). The sequences were aligned using CLUSTALW. Asterisks (*) denotes identical amino acid residues between the species and Hs-MLN64 playing a role in forming the tunnel. The solid bar underline denotes the putative START domains.

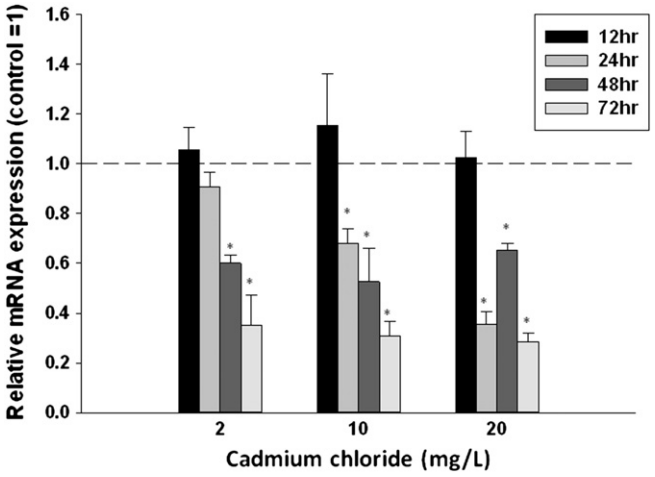

Fig. 3. Effects of different concentrations and durations of cadmium chloride exposure on *START1* mRNA expression of *Chironomus riparius*. The mRNA expression of *CrSTART1* was quantified using real time PCR and normalized using *Chironomus* β-actin gene. Three replications containing groups of 15 fourth instar larvae in each replicate were exposed to different concentrations of cadmium chloride. All values represent the mean ± standard error of the mean. An asterisk indicates a statistically significant difference, $p<0.05$, compared with the control (control = 1), analyzed using ANOVA.

in turn may affect their metabolism, development, reproduction and growth. Many studies have been reported on the adverse effects of EDCs on aquatic organisms in aquatic environments (Soin and Smagghe, 2007; Jackson and Sutton, 2008). The development, metamorphosis and reproduction of chironomids occur under hormone control, and disruption of the hormone balance, such as reduction in ecdysteroid synthesis and subsequent developmental impairments, after exposure to the endocrine disruptor bis (tri-n-butyltin) oxide, has been reported in *C. riparius* (Hahn and Schulz, 2002). Several previous studies have shown that exposure to Cd and NP reduced developmental rate and also affected the time of emergence and egg production in *C. riparius* (Pascoe et al., 1989; Postma and Davis, 1995; Lee and Choi, 2006; Vogt et al., 2007). Gene expression analysis is being increasingly used in the diagnosis of environmental contamination, as it might be more sensitive and is less time-consuming than conventional toxicology endpoints and offers mechanistic values and provides a more comprehensive insight into toxicity (Ankley et al., 2006; Steinberg et al., 2008; Planelló et al., 2011; Nair and Choi, in press). Therefore, in the present study, we analyzed the expression of *CrSTART1* mRNA after exposure to different concentrations of Cd and NP for different time periods in *C. riparius* since these chemicals are known to modulate StAR gene which is involved in the trafficking of cholesterol to the inner mitochondrial membrane, a rate limiting step in steroidogenesis (Arukwe, 2005; Kortner and Arukwe, 2007; Sandhu and Vijayan, 2011). The concentrations of the chemicals used in the present study have been reported from the environment (Korte, 1983; Marcomini and Giger, 1987; Blackburn and Waldock, 1995; Tyler et al., 1998; Jung et al., 2005).

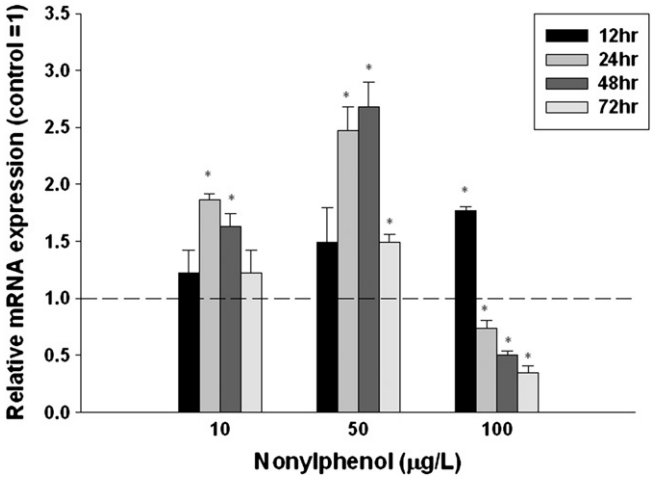

Fig. 4. Effects of different concentrations and durations of nonylphenol exposure on START1 mRNA expression of *Chironomus riparius*. The mRNA expression of CrSTART1 was quantified using real time PCR and normalized using Chironomus β-actin gene. Three replications containing groups of 15 fourth instar larvae in each replicate were exposed to different concentrations of cadmium chloride. All values represent the mean ± standard error of the mean. An asterisk indicates a statistically significant difference, $p<0.05$ compared with the control (control = 1), analyzed using ANOVA.

In the present study, we observed that except for 12 h Cd caused significant downregulation of *CrSTART1* mRNA at all concentrations and exposure periods tested. Our results are in accordance with previous reports where significant down regulation of genes involved in steroidogenesis has been reported after exposure to Cd in aquatic organisms. Sandhu and Vijayan (2011) have reported that StAR gene expression was significantly reduced in rainbow trout (*Oncorhynchus mykiss*) after exposure to Cd. There are also several reports from mammalian systems that Cd causes downregulation of genes involved in steroidogenesis including the StAR gene (Smida et al., 2004; Siu et al., 2009; Pillai et al., 2010). The exact reason for the downregulation of StAR mRNA is not clear and different hypothesis has been put forward to explain this using different experimental systems. There are several previous studies showing that Cd induced oxidative stress is one of the major mechanisms for the downregulation of genes involved in steriodogenesis. For example, it has been reported that reactive oxygen species (ROS) inhibits steroidogenesis by interfering with cholesterol transport to mitochondria. Stocco et al. (2001) reported that ROS could inhibit steroidogenesis during cholesterol transport by suppressing StAR protein expression in MA-10 tumor Leydig cells. Cadmium induced oxidative stress caused downregulation of the StAR protein and mRNA expression levels resulting in altered ovarian steroidogenesis in PND 56 female rats (Pillai et al., 2010). Another reason might be the inhibition of signaling pathways by Cd^{2+} ions, since the Cd^{2+} ion has similarities with Ca^{2+} ions, and could substitute for them in physiological processes (Siu et al., 2009). For example, Sandhu and Vijayan (2011) reported that Cd mediated downregulation of StAR gene expression and suppression of steroidogenesis in rainbow trout occurs upstream of cAMP production, since the cAMP analog (8-Bromo-cAMP) abolished the suppression of steroidogenic genes. StAR mRNA levels increased continuously in MA-10 mouse Leydig tumor cells when stimulated with cAMP (Clark et al., 1997). However, further studies are required to confirm the exact mechanism of Cd induced suppression of *START1* gene in *C. riparius*.

Significant upregulation of StAR mRNA after exposure to NP has also been reported from *Atlantic salmon* (Arukwe, 2005). In yet another study, modulation of StAR mRNA was observed in a time and concentration specific manner after exposure to NP in Atlantic cod (*Gadus morhua*) previtellogenic oocyte cultures. The StAR mRNA expression was increased after 7 day exposure to different concentrations of NP. After 14 days StAR mRNA expression was increased at lower concentrations tested and decreased at higher concentrations. These results suggest that NP modulates steroidogenesis by targeting the StAR gene in *Gadus morhua* (Kortner and Arukwe, 2007). In our present study also it was observed that *CrSTART1* expression is significantly upregulated following exposure to lower concentrations of NP tested (10–50 µg/L) and higher concentration (100 µg/L) decreased *CrSTART1* mRNA level after 24–72 h exposure.

The transcriptional regulation of StAR gene has been discussed earlier (Reinhart et al., 1999; Stocco, 2001). The reasons for the modulation of *CrSTART1* by NP in this study could be explained based on previous studies. We speculate that higher concentrations of NP tested in this study might have interfered with the production of ecdysteroid hormone which in turn downregulated the expression of

CrSTART1. For example, *Americamysis bahia* exposed NP inhibited 20-hydroxyecdysone levels (Hirano et al., 2009). Previous studies in *D. melanogaster* showed a weak expression of *START1* gene in *ecd-1* mutant having reduced ecdysone level. Moreover, the presence of ecdysone responsive elements in *DmSTART1* also suggested that its regulation depends on ecdysone (Woodard et al., 1994; Stocco et al., 2001; Roth et al., 2004). It should also be noted that, the heavy metal Cd reported to have inhibitory effect on ecdysone secretion in crustaceans (Rodríguez et al., 2007).

It has been reported from previous studies that, several xenobiotics and pharmaceuticals such as atrazine, Dimethoate, and Roundup™, herbicide letrozole and ketoconazole are known to modulate StAR and other proteins involved in steroidogenesis (Danzo, 1997; Miranda et al., 1998; Walsh et al., 2000a,b; Assikis and Buzdar, 2002; Hayes et al., 2002; Assikis and Simons, 2004; Hegelund et al., 2004). Our present study confirms that steroidogenesis is one of the possible targets of the endocrine disruptor NP and Cd in *C. riparius*. The changes in the mRNA levels of *CrSTART1* suggest that NP and Cd exposure causes impaired steroidogenesis in *C. riparius*. Thus, the modulation of mRNA of *CrSTART1* might be a sensitive diagnostic tool for short-term exposure to endocrine disrupting chemicals. However, to clearly understand the relationship between the effects of environmental pollutants on the expression of *CrSTART1*, and steroidogenesis in *C. riparius*, further detailed molecular studies using other genes involved steroidogenesis and steroid hormone measurements needs to be conducted.

5. Conclusion

A large number of anthropogenic chemicals that are released into the environment may disrupt endocrine homeostasis in humans, animals and aquatic organisms. Invertebrates occupying key positions in the aquatic food webs, such as *C. riparius*, as sentinel organisms in endocrine-disruptor research, may provide information on the hazardous effects of environmental pollutants. It is evident from the present study that the *CrSTART1* gene is an important target for environmental pollutants; therefore, the identification and characterization of the *START1* gene from *C. riparius*, and its transcriptional modulation by Cd and NP, would be a promising sign in understanding the toxicity of environmental pollutants in the steroidogenesis of this important species used in ecotoxicological studies.

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