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Modulation in the mRNA expression of ecdysone receptor gene in aquatic midge, *Chironomus riparius* upon exposure to nonylphenol and silver nanoparticles

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ABSTRACT

Chironomus riparius, a non-biting midge (Chironomidae, Diptera), is extensively used as a model organism in aquatic ecotoxicological studies, although little is known about its genome sequences. Ecdysteroids are steroid hormones that play an important role in development, growth, moulting of larva, and reproduction in *Chironomus* spp. The effect of ecdysteroids is mediated by their binding to the ecdysteroid receptor (*EcR*). To study the effect of environmental stressors, nonylphenol and silver nanoparticles (AgNPs), on the modulation of *EcR* mRNA, in this study, full length cDNA of *C. riparius* ecdysone receptor (*CrEcR*) was identified from the Expressed Sequence Tags (ESTs) database and expression of the corresponding mRNA was analyzed following exposure to nonylphenol and AgNPs. The *CrEcR* cDNA was 2548 base pairs (bp) in length, with a 5′ untranslated region (UTR) of 242 bp and a 3′ UTR of 684 bp. The open reading frame contains 1617 nucleotides, encoding 539 amino acids with a predicted molecular weight of 61 kDa and p*I* of 5.89, and revealed the presence of several domains associated with DNA binding, dimerization, ligand binding and transcriptional activation characteristic of steroid receptor family members. It was found that the expression level of *CrEcR* was significantly up-regulated on exposure to nonylphenol and significant up or down regulation was observed on exposure to AgNPs. These finding shows that nonylphenol as well as AgNPs could modulate the ecdysone nuclear receptor and may have significant implications in different developmental stages in *C. riparius*.
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1. Introduction

The endocrine system within invertebrates is well characterized; it coordinates and regulates growth, development, reproduction and other physiological processes and in insects it is controlled by the steroid hormone ecdysone (Thummel, 1995). The active metabolite 20-hydroxyecdysone binds to the ecdysone heterodimeric complex, comprising various nuclear receptors, the ecdysone receptor (*EcR*) and the ultraspiracle protein. The hormone's action is mediated by this complex at the transcriptional level via binding to ecdysteroid response elements (Yao et al., 1993).

Nonylphenol is widely used in many industrial applications, as well as household cleaning products (Ying et al., 2002) and its oestrogenic effects as an endocrine disruptor have been extensively studied (Nimrod and Benson, 1996; Bandiera, 2006; Lye et al., 2008). According to some studies, nonylphenol concentrations in rivers receiving effluents from sewage water could reach the level of 180 μg/L (Blackburn and

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Waldock, 1995; Tyler et al., 1998). In surface sediments of rivers 700–900 μg/kg concentrations of nonylphenol, nonylphenol monoethoxylate, and nonylphenol diethoxylate have been reported (Marcomini and Giger, 1987). The acute and chronic toxicities of nonylphenol were previously investigated in *C. ripaius* (Lee and Choi, 2006), as well as in other aquatic organisms (Nimrod and Benson, 1996; Lussier et al., 2000). In recent years, with the fast growth of nanotechnology, the application of nanomaterials in various products has increased and their release to aquatic environments could cause many adverse effects at various levels in aquatic ecosystems (Handy et al., 2008). Silver nanoparticles (AgNPs) are widely used in many commercial products, and the AgNPs released from these products could ultimately end up in aquatic environment (Benn and Westerhoff, 2008), where they could cause adverse effects to many aquatic organisms (Asharani et al., 2008; Griffitt et al., 2009; Nair et al., 2011).

Chironomus riparius (Chironomidae, Diptera), is widely used in aquatic exotoxicological studies for assessing acute and sub-lethal toxicities of contaminated sediments and for water monitoring due to their widespread occurrence, short life-cycle, ease with which they are reared in the laboratory, physiological tolerance to various environmental conditions and the fact that they undergo a metamorphosis under hormone control (Bettinetti et al., 2002; Crane et al., 2002; Taenzler et al., 2007). The endocrine system is known to regulate different developmental stages such as embryogenesis, growth, metamorphosis, and reproduction in *C. riparius* (Watts et al., 2001). In *Chironomus* low receptor levels were found when the moulting hormone tiers were low, and high receptor levels corresponded to stages with high moulting hormone levels. This is similar to the sequence of hormone and receptor level changes reported in *D. melanogaster* and in vertebrates (Syms et al., 1985; Deak and Laufer, 1995; Deak et al., 1998).

It has been reported that the expression profiles of the ecdysone receptor (*EcR*) genes can be modulated by environmental chemicals thereby disrupting the hormone regulating mechanisms of insects (Zou and Fingerman, 1997; Mu et al., 2005; Rodriguez et al., 2007; Planelló et al., 2008). Nonylphenol exposure in *C. riparius* induced a reduction in the rates of total adult emergence and emergence failures (Lee and Choi, 2006). Recently we observed that exposure to AgNPs could cause significant impairment on development, emergence, male to female ratio and egg production in *C. riparius* (Nair et al., 2011). Therefore in this study, the cDNA of *CrEcR* was selected from the previously developed *C. riparius* ESTs database and characterized. Expression patterns in *C. riparius* fourth instar larvae were analyzed after exposure to different concentrations nonylphenol and AgNPs for different time intervals in order to determine the effects of nonylphenol and AgNPs in the modulation of *CrEcR* in *C. riparius*.

2. Materials and methods

2.1. Animals

C. riparius larvae were obtained from the Toxicological Research Center of the Korea Institute of Chemical Technology (Daejon, South Korea). The larvae were reared on an artificial diet of fish flake food (Tetramin, Tetrawerke, Melle, Germany) in glass chambers containing dechlorinated tap water and acid washed sand, with aeration under a 16–8 h light–dark photoperiod at room temperature (20 ± 1 °C).

2.2. Identification and sequence analysis of CrEcR

Gene sequences were retrieved from the ESTs database and were manually annotated to predict transcription initiation and termination sites using BlastX comparisons of putative amino acid translations deduced using translation tool (http://www.expasy.ch/tools/) and aligned using ClustalW (Thompson et al., 1994). The ligand binding domains were aligned and a phylogenetic tree was constructed by the neighbour-joining method using MEGA4.1 (Tamura et al., 2007).

2.3. Chemical exposure, RNA isolation and cDNA synthesis

Groups of 15 fourth instar larvae were exposed to nonylphenol (Sigma–Aldrich Chemical, St. Louis, MO, USA) (10 and 50 μg/L) and AgNPs (Sigma, USA) (0, 0.2, 0.5 and 1 mg/L) for 0, 12, 24, 48 and 72 h. The exposure concentrations of nonylphenol and AgNPs were selected based on our previous studies (Lee and Choi, 2006; Nair et al., 2011). The control group for nonylphenol was exposed to the solvent acetone (Sigma, USA). Aqueous suspensions of AgNPs were prepared and characterized as described previously (Nair et al., 2011). Three independent treatments and three replications were maintained for each treatment. After the exposure the larvae were collected, immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA from samples was isolated using TRIZOL™ Reagent (Invitrogen Life Technology, USA). One microgram of total RNA was used for making cDNA by reverse transcription, using an oligo dT_{18} primer and RT-Premix (Bioneer, South Korea), in a total reaction volume of 20 μl, following the manufacturer's instructions.

2.4. Expression analysis

The primers were checked for their substrate specificity using RT-PCR using the thermal cycle program consisting of an initial denaturation at 94 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min using 1 μl cDNA with an RT-PCR premix (Bioneer, South Korea) as per the manufacturer's instructions. Primer3 (http://frodo.wi.mit.edu/primer3/) programme was used to design all the PCR primers (*CrEcR* – Forward – 5′-CATCCAATGCAACAGCTTTTACCAGA-3′ and *CrEcR* – Reverse – 5′-TGGCTGTTCGTAACCGTCTTGATA-3′ and *CrActin* – Forward – 5′-GATGAAGATCCTCACCGAACG-3′; *CrActin* – Reverse – 5′-TTCGAGTGAGGTTGATGCAG-3′). To study the gene expression of AgNPs exposed larvae, 1 μl cDNA was used for quantitative real time PCR using a 0.2 μM of the sense and antisense oligonucleotide primers of each gene, 10 μl of 2× IQ SYBR Green Super Mix (Bio-Rad, USA) and 1 μL template cDNA in

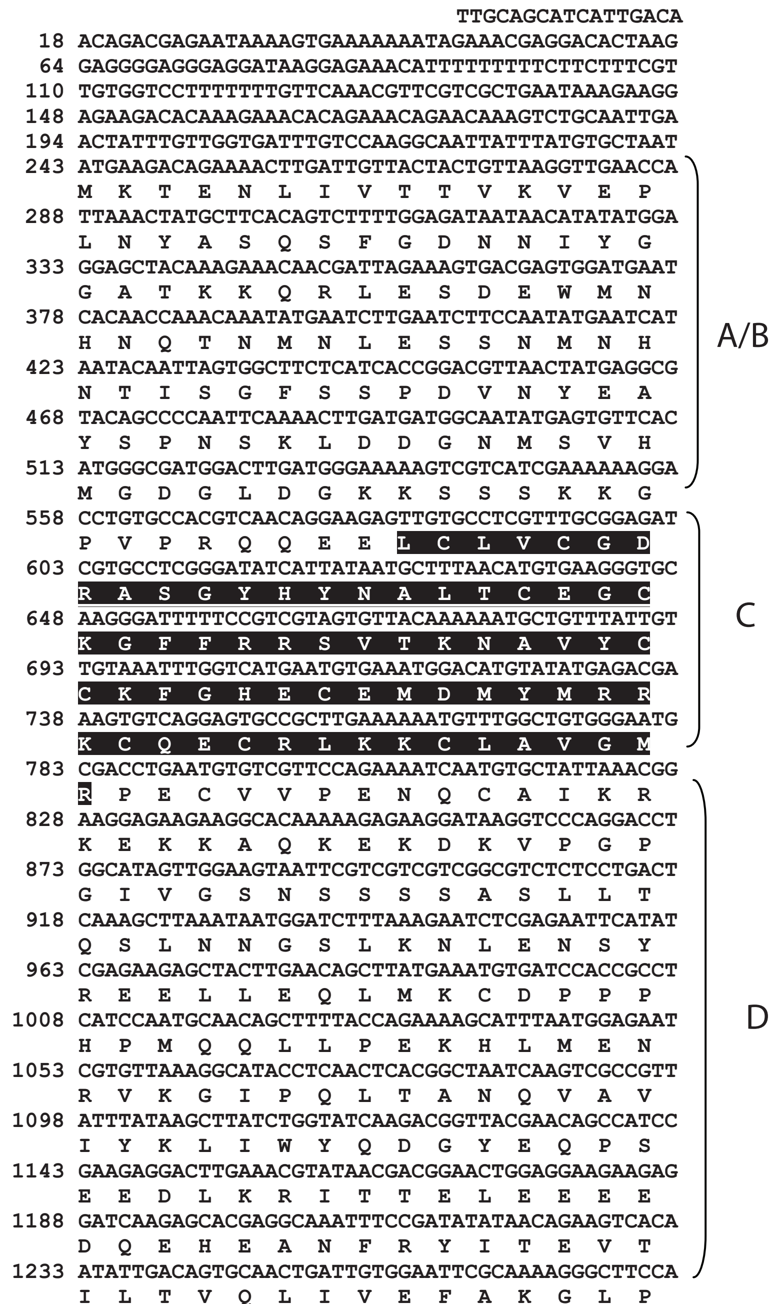

Fig. 1 – Sequence and deduced amino acid sequence of the *C. riparius* ecdysteroid receptor (*CrEcR*) gene. Different domains are marked with different letters and are associated with DNA binding (C domain), dimerization (C–E domains), ligand binding (E domain) and transcriptional activation (A/B and E domains). Dark and light shaded amino acids indicate DNA-binding domain (DBD, C, 164–236 amino acids) and ligand-binding domain (LBD, E, 321–545 amino acids) respectively.

a final volume of 20 μl using reaction conditions as follows: 95 °C/7 min; 44 cycles of 95 °C/15 s, 55 °C/1 min, 72 °C/30 s and final extension at 72 °C/5 min followed by a melting curve analysis with a 0.2 °C increase per cycle using a Chromo4 Real-Time PCR detection system (Bio-Rad, USA). The mRNA level of each gene was normalized to that of the *C. riparius* Actin mRNA and data were shown as the mean ± SE of three replications. The relative fold change of the mRNA expression of a gene, compared to control, was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. *Data analysis*

Statistical differences between the results obtained from different experiments in control and treated larvae were analyzed using ANOVA with SPSS 12.0 KO (SPSS Inc., Chicago, IL,

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1278 GCATTTATTAAAATACCACAAGAAGATCAAATTACTCTCTTAAAG
     A  F  I  K  I  P  Q  E  D  Q  I  T  L  L  K
1323 GCTTGCTCCAGTGAAGTTATGATGTTGCGCATGGCTCGACGATAC
     A  C  S  S  E  V  M  M  L  R  M  A  R  R  Y
1368 GATCACGATTCTGATTCTATATTGTTTGCAAATAATACAGCATAC
     D  H  D  S  D  S  I  L  F  A  N  N  T  A  Y
1413 ACTAAGCAAACGTATCAATTAGCGGGCATGGAAGAGACAATTGAT
     T  K  Q  T  Y  Q  L  A  G  M  E  E  T  I  D
1458 GATTTACTGCACTTTTGTCGACAAATGTATGCATTATCTATTGAT
     D  L  L  H  F  C  R  Q  M  Y  A  L  S  I  D
1503 AATGTCGAATATGCTCTTCTCACGGCCATCGTCATCTTCTCAGAT
     N  V  E  Y  A  L  L  T  A  I  V  I  F  S  D
1548 CGACCTGGTCTTGAAAAGGCTGAAATGGTAGACATAATTCAAAGC
     R  P  G  L  E  K  A  E  M  V  D  I  I  Q  S
1593 TATTATACAGAAACTCTCAAGGTTTATATCGCCAATCGACATGGT
     Y  Y  T  E  T  L  K  V  Y  I  A  N  R  H  G
1638 GGCGAAACAAGATGCAGCGTTCAATTTGCAAAGCTTTTAGGCATT
     G  E  T  R  C  S  V  Q  F  A  K  L  L  G  I
1683 CTTACTGAACTACGAACAATGGGCAATAAAAATTCTGAAATGTGC
     L  T  E  L  R  T  M  G  N  K  N  S  E  M  C
1728 TTTTCATTAAAACTGAGAAACCGAAAACTGCCACGATTCTTAGAA
     F  S  L  K  L  R  N  R  K  L  P  R  F  L  E
1773 GAAGTCTGGGATGTCGGCGATGTTAATAACCAACCGACGGCAACA
     E  V  W  D  V  G  D  V  N  N  Q  P  T  A  T
1818 ACAAATACAGAAAACATCGTTCGGGAACGAATAAATCGAATCTAA
     T  N  T  E  N  I  V  R  E  R  I  N  R  I  *

1863 AGCTATATGACTTCGTATTTTATATATTTACCTTCCCTTAAAAAA
1909 TTTCAAAAGAAAAAAATTATGGAAATAAGTGTAATTACTACATTT
1954 CGGATTTTATGAAACATAACAATATGGAGATAATATATATATATC
1999 TAGAATTACATTTATTTAGGTATAATACATGAAAAAAAGTAACGA
2044 TGAACATGAAAAGTCACATAAAGCATTATTATTCGAAAACAAAAA
2089 AAACATGAGAGAAAAAGATTATTTTGTTGAATTGTCAACAAAAGG
2134 ATGTAAATGGATCAGCCCGATTCGATTCTTGATATATATAAAATC
2179 GCAATGAGTATATATCTAAATAAATAATAATAATAATAATATAAT
2224 TATTACCTACGTACTTTATGAAATATTTTTATATAGTATTAAAAG
2269 AAAGTCACCAATTATAATCTCTAATAGATGATTCAAAGATCCAAA
2314 ACAGATAAACTTATCGATTTGTGCTGCAGATAAGCTATAGAAGAA
2359 ACATTTTTCTAATTCAACAATTATATGTTTAAGGGGTTCTTTACT
2404 TATTACGTGCGCTTTTTAAGGGAAGGGAGGTCTAAAGTCTTTTTT
2449 TTTGTCGTAGCATAATAAGTGAACAGCCTTTAATATTGATAATGT
2494 CATATACATTAACAAACGTCTTTCTCGTAATTTATTCAGATAAAA
2539 AAAAAAAAA
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E/F

Fig. 1 – (*Continued*)

USA) and p values less than 0.05 were considered statistically significant.

3. Results

3.1. Sequence analysis of *CrEcR*

From our ESTs database, we obtained the full length cDNA of *CrEcR* determined by BlastX searches to the NCBI data base (http://www.ncbi.nlm.nih.gov/). The *CrEcR* showed 96% identity at the amino acid level with equivalent gene from *C. tentans*, 88% with *A. mellifera*, above 75% identity with those of *C. quinquefasciatus* and *A. gambiae* and 60% identity with those of *A. aegypti*, *D. melanogaster* and *B. mori*. The *CrEcR* cDNA was 2548 bp in length, with a 5′ untranslated region (UTR) of 242 bp and a 3′ UTR of 684 bp. The open reading frame was 1617 nucleotides in length, encoding 539 amino acids with a predicted molecular weight of 61 kDa and pI of 5.89 (http://cn.expasy.org/cgi-bin/pi_tool) (Fig. 1).

Alignment of the deduced amino acid sequences of *CrEcR*, with steroid receptor families from other species and the pfam (http://pfam.sanger.ac.uk/) analysis, revealed the presence of several domains (A/B, C–F domains) associated with DNA binding (C domain), dimerization (C–E domains), ligand binding (E domain) and transcriptional activation (A/B and E domains) characteristic of steroid receptor family members. The DNA binding (C-region) and ligand-binding domains (E/F-region) showed the presence of highly conserved amino acid residues that are present in arthropods (Fig. 2). The phylogenetic relationship of *CrEcR* was determined by aligning the deduced amino acids from the ligand binding domain with those of different species, showed that *CrEcR* was clustered along with the *EcRs* of insects from the Dipteran order, showing that *CrEcR* belongs to the respective nuclear receptor groups (Fig. 3). The sequences of *CrEcR* have been deposited in NCBI GenBank in the accession no. HP608039.

```
Ae.aegypti              --------MMKRRWSNNGGFTALRMLDDSSSEVTS-------SSAALGMTMSPNSLGSPN 45
C.quinquefasciatus      ------------------------------------------------------------
A.gambiae               ------------------------------------------------------------
D.melanogaster          MLTTSGQQQSKQKLSTLPSHILLQQQLAASAGPSSSVSLSPSSSAALTLHVASANGGARE 60
C.tentnas               -----------------------------------------------------MKTEN----- 5
C.riparius              -----------------------------------------------------MKTEN----- 5

Ae.aegypti              YD-----ELELWSSYEDNAYNGHSVLSNGNNNLGGCGAANNLLMNGIVGNNNLNGMMNMA 100
C.quinquefasciatus      ---------------------------------------------------------MTL 3
A.gambiae               ------------------------------------------MS--EKRNVSREWIILA 15
D.melanogaster          TTSAAAVKDKLRPTPTAIKIEPMPDVISVGTVAGGSSVATVVAPAATTTSNKPNSTAAPS 120
C.tentnas               ---------LIVTTVKVEPLNYASQSFGDNNIYGGATKKQRLESDEWMNHNQTN--MNLE 54
C.riparius              ---------LIVTTVKVEPLNYASQSFGDNNIYGGATKKQRLESDEWMNHNQTN--MNLE 54

Ae.aegypti              SQAVQAN--------ANSIQHIVGNLINGVNPNQT-----LIPPLPSIIQNTLMNTPRSE 147
C.quinquefasciatus      IQSVRA--------------RVTGGMIG-TDPYHL-----RLP----------------- 26
A.gambiae               APSGQGK--------G----HAIG-FADVLLPRRS-----QMHTARYCSFES-------- 49
D.melanogaster          TSAAAANGHLVLVPNKRPRLDVTEDWMSTPSPGSVPSSAPPLSPSPGSQNHSYNMSNGYA 180
C.tentnas               SSNMNHN-----------------TISGFSSPDVN------------------------- 72
C.riparius              SSNMNHN-----------------TISGFSSPDVN------------------------- 72

Ae.aegypti              SVNSI----------SSGREDLSPSSSLN----GYT--DGSDAKKQ--KKGPTPRQQEEL 189
C.quinquefasciatus      -----------------GREDLSPSSSLN----GYSG-DGNDPKKQ--KKGPTPRQQEEL 62
A.gambiae               ---------------VARREDLSPSSSLN----GYTG-DGSEAKKQ--KKGPTPRQQEEL 87
D.melanogaster          SPMSAGSYDPYSPTGKTGRDDLSPSSSLN----GYSANESCDAKKS--KKGPAPRVQEEL 234
C.tentnas               ------------------YEAYSPNSKLDDGNMSVHMGDGLDGKKSSSKKGPVPRQQEEL 114
C.riparius              ------------------YEAYSPNSKLDDGNMSVHMGDGLDGKKSSSKKGPVPRQQEEL 114

                        *  *             *  *               *     *         *  *
Ae.aegypti              CLVCGDRESGYHYNALTCEGCKGFFRRSVTKNAVYCCKFGHACEMDMYMRRKCQECRLKK 249
C.quinquefasciatus      CLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAVYCCKFGHACEMDMYMRRKCQECRLKK 122
A.gambiae               CLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAVYCCKFGHACEMDMYMRRKCQECRLKK 147
D.melanogaster          CLVCGDRASGYHYNALTCEGCKGFFRRSVTKSAVYCCKFGRACEMDMYMRRKCQECRLKK 294
C.tentnas               CLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAVYCCKFGHECEMDMYMRRKCQECRLKK 174
C.riparius              CLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAVYCCKFGHECEMDMYMRRKCQECRLKK 174

Ae.aegypti              CLAVGMRPECVVPENQCAIKRKEKKAQKEKDKVQTN----------ATVSTTN------- 292
C.quinquefasciatus      CLAVGMRPECVVPENQCAIKRKEKKAQKEKDKVQTT----------ATVSTTN------- 165
A.gambiae               CLAVGMRPECVVPENQCAIKRKEKKAQKEKDKVPPNPST-------TTVSTTNS------ 194
D.melanogaster          CLAVGMRPECVVPENQCAMKRREKKAQKEKDKMTTSPSSQH--GGNGSLASGGG------ 346
C.tentnas               CLAVGMRPECVVPENQCAIKRKEKKAQKEKDKVPG--IVGSN-TSSSSLLNQSLNNGSLK 231
C.riparius              CLAVGMRPECVVPENQCAIKRKEKKAQKEKDKVPGPGIVGSNSSSSASLLTQSLNNGSLK 234

Ae.aegypti              ---STYRSEILPILMKCDPPPHQAIPLLPEKLLQENRLRNIPLLTANQMAVIYKLIWYQD 349
C.quinquefasciatus      ---STYKSEILPILMKCDPPPHAAIPLLPEKLLNENRLRNIPPLTANQMAVIYKLIWYQD 222
A.gambiae               ---SSYKSELLPVLMKCESPPTAAIPLLPEKLLNENRQRNIPLLTANQMAVIYKLIWYQD 251
D.melanogaster          ---QDFVKKEILDLMTCEPPQHATIPLLPDEILAKCQARNIPSLTYNQLAVIYKLIWYQD 403
C.tentnas               NLEISYREELLEQLMKCDPPPHPMQQLLPEKLLMENRAKGTPQLTANQVAVIYKLIWYQD 291
C.riparius              NLENSYREELLEQLMKCDPPPHPMQQLLPEKHLMENRVKGIPQLTANQVAVIYKLIWYQD 294

Ae.aegypti              GYEQPSEEDLKRIMIGSPNEEEDQHDVHFRHITEITILTVQLIVEFAKGLPAFTKIPQED 409
C.quinquefasciatus      GYEQPSEEDLKRIMIGSPNEEEDQHDVHFRHITEITILTVQLIVEFAKGLPAFTKIPQED 282
A.gambiae               GYEQPSEEDLKRIMINSPNEEEDPHEIHFRHITEITILTVQLIVEFAKGLPAFTKIPQED 311
D.melanogaster          GYEQPSEEDLRRIMS-QPDENESQTDVSFRHITEITILTVQLIVEFAKGLPAFTKIPQED 462
C.tentnas               GYEQPSEEDLKRITTELEEEEDQEHEANFRYITEVTILTVQLIVEFAKGLPAFIKIPQED 351
C.riparius              GYEQPSEEDLKRITTELEEEEDQEHEANFRYITEVTILTVQLIVEFAKGLPAFIKIPQED 354

                                    ** *   *        *           *
Ae.aegypti              QITLLKACSSEVMMLRMARRYDAATDSILFANNRSYTRDSYRMAGMADTIEDLLHFCRQM 469
C.quinquefasciatus      QITLLKACSSEVMMLRMARRYDAATDSILFANNRSYTRDSYRMAGMADTIEDLLHFCRQM 342
A.gambiae               QITLLKACSSEVMMLRMARRYDAETDSILFANNRSYTRDSYKMAGMADTIEDLLHFCRQM 371
D.melanogaster          QITLLKACSSEVMMLRMARRYDHSSDSIFFANNRSYTRDSYKMAGMADNIEDLLHFCRQM 522
C.tentnas               QITLLKACSSEVMMLRMARRYDHDSDSILFANNTAYTKQTYQLAGMEETIDDLLHFCRQM 411
C.riparius              QITLLKACSSEVMMLRMARRYDHDSDSILFANNTAYTKQTYQLAGMEETIDDLLHFCRQM 414

Ae.aegypti              FSLTVDNVEYALLTAIVIFSDRPGLEQAELVEHIQSYYIDTLRIYILNRHAGDPKCSVIF 529
C.quinquefasciatus      YSLTVDNVEYALLTAIVIFSDRPGLEQAELVEHIQSYYIDTLRIYILNRHGGDHKCSVIF 402
A.gambiae               YTLTVDNVEYALLTAIVIFSDRPGLEKAELVETIQSYYIDTLRVYILNRHGGDPKCSVTF 431
```

Fig. 2 – Amino acid alignment of *EcRs* from *C. riparius* and other dipterans. Deduced amino acid sequences were aligned using ClustalW alignment programme. The DNA binding domain (solid line) and the ligand-binding domain (dotted line) are shown. The cysteine residues are boxed and indicated by asterisks above.

```
D.melanogaster        FSMKVDNVEYALLTAIVIFSDRPGLEKAQLVEAIQSYYIDTLRIYILNRHCGDSMSLVFY 582
C.tentnas             YALSIDNVEYALLTAIVIFSDRPGLEKAEMVDIIQSYYTETLKVYIVNRHGGESRCSVQF 471
C.riparius            YALSIDNVEYALLTAIVIFSDRPGLEKAEMVDIIQSYYTETLKVYIANRHGGETRCSVQF 474
                                          *  *
Ae.aegypti            AKLLSILTELRTLGNQNSEMCFSLKLKNRKLPRFLEEIWDVQDIPPSMQAQMHSHGTQSS 589
C.quinquefasciatus    AKLLSILTELRTLGNQNSEMCFSLKLKNRKLPRFLEEIWDVQDIPPSMQAQIVAQQQQGS 462
A.gambiae             AKLLSILTELRTLGNQNSEMCFSLKLKNRKLPRFLEEIWDVQDIPP-------------- 477
D.melanogaster        AKLLSILTELRTLGNQNAEMCFSLKLKNRKLPKFLEEIWDVHAIPPSVQSHLQITQEENE 642
C.tentnas             AKLLGILTELRTMGNKNSEMCFSLKLRNRKLPRFLEEVWDVGDVNNQTTATTNTEN---- 527
C.riparius            AKLLGILTELRTMGNKNSEMCFSLKLRNRKLPRFLEEVWDVGDVNN-------------- 520

Ae.aegypti            --------SSSSSSSSSSNGSSNGNSSSNSNSSQHGPHPHPHGQ--QLTPNQQQHQQQH 639
C.quinquefasciatus    --------AGVLQLPAIQAANNNAAALSQNSGSSGS----SLHGQ--QMTTP---NNNSS 505
A.gambiae             ------------------------------------------------------------
D.melanogaster        RLERAERMRASVGGAITAGIDCDSASTSAAAAAAQHQPQPQPQPQPSSLTQNDSQHQTQP 702
C.tentnas             --------------IVRERINRN------------------------------------- 536
C.riparius            --------------QPTATTNTENIVRERINRI--------------------------- 539

Ae.aegypti            SQLQQV---------------------------------------HANGSGSGGGSNNNS 660
C.quinquefasciatus    SSQQQQ---------------------------------------SRNGSGVVVVACSTR 526
A.gambiae             ------------------------------------------------------------
D.melanogaster        QLQPQLPPQLQGQLQPQLQPQLQTQLQPQIQPQPQLLPVSAPVPASVTAPGSLSAVSTSS 762
C.tentnas             ------------------------------------------------------------
C.riparius            ------------------------------------------------------------

Ae.aegypti            SSGGVVPGLGMLDQV--------------------------------------------- 675
C.quinquefasciatus    YS---------------------------------------------------------- 528
A.gambiae             ------------------------------------------------------------
D.melanogaster        EYMGGSAAIGPITPATTSSITAAVTASSTTSAVPMGNGVGVGVGVGGNVSMYANAQTAMA 822
C.tentnas             ------------------------------------------------------------
C.riparius            ------------------------------------------------------------

Ae.aegypti            ---------------------------
C.quinquefasciatus    ---------------------------
A.gambiae             ---------------------------
D.melanogaster        LMGVALHSHQEQLIGGVAVKSEHSTTA                                  849
C.tentnas             ---------------------------
C.riparius            ---------------------------
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Fig. 2 – (*Continued*)

3.2. *The effect nonylphenol and AgNPs on gene expression*

The mRNA expression of *CrEcR* gene was investigated after exposure to a known endocrine disrupting chemical, nonylphenol, and it was observed that the expression varied depending on the concentration and duration of the nonylphenol treatment. The mRNA level of *CrEcR* was significantly increased upon exposure to different concentrations of nonylphenol in a dose and time dependent manner. The highest expression of *CrEcR* was observed after expositing the larvae to 50 μg/L of nonylphenol for 48 and 72 h (Fig. 4). The change in the mRNA expression of the *CrEcR* was also evaluated using RT-PCR after exposure to different concentrations of AgNPs for various time intervals. It was noticed that in contrast to the response to nonylphenol exposure, a significant up regulation of *CrEcR* occurred after exposure to AgNPs for 24 h, compared to the control larvae, followed by a significant decrease at 48 and 72 h. Responses in *CrEcR* mRNA levels to AgNP exposure were dose dependent. There was no significant change in the expression of *CrEcR* after exposure to different concentrations of AgNPs for 12 h period. The expression of *CrEcR* mRNA was significantly up regulated by 12 h at 0.2 mg/L and the up regulation was more pronounced at 0.5 and 1 mg/L of AgNPs exposure for 24 h period compared to other treatments. However, a significant down regulation of *CrEcR* was noticed at all concentrations of AgNPs after 48 and 72 h exposure period as compared to the control (Fig. 5).

4. Discussion

Ecdysteroids play an essential role in development, growth, reproduction and embryogenesis in arthropods (Sekimoto et al., 2006; Lafont and Mathieu, 2007). Ecdysteroids are targeted to the ecdysone receptor (*EcR*) and ultraparticles (*USP*) receptor heterodimeric complex (Yao et al., 1993), resulting in activation of downstream genes of ecdysteroid-regulated signaling pathways (King-Jones and Thummel, 2005). *EcR* belong to the nuclear receptor family and have A/B and C (DNA binding), D, E (ligand binding) and F regions and the DNA binding and ligand binding domains are highly conserved among nuclear receptors (Aranda and Pascual, 2001). Complete cDNA sequences for *EcR* have been cloned from *C. tentans* (Imhof et al., 1993) and a number of insects (Kothapalli et al., 1995; Hannan and Hill, 1997; Chung et al., 1998; Riddiford et al., 2000; Nagata et al., 2005; Kim et al., 2005; Kato et al., 2007; Tan and Palli, 2008). From our ESTs database we obtained the full length cDNA of *CrEcR* consisting of all the domains and conserved amino acid residues present in the *EcRs* of

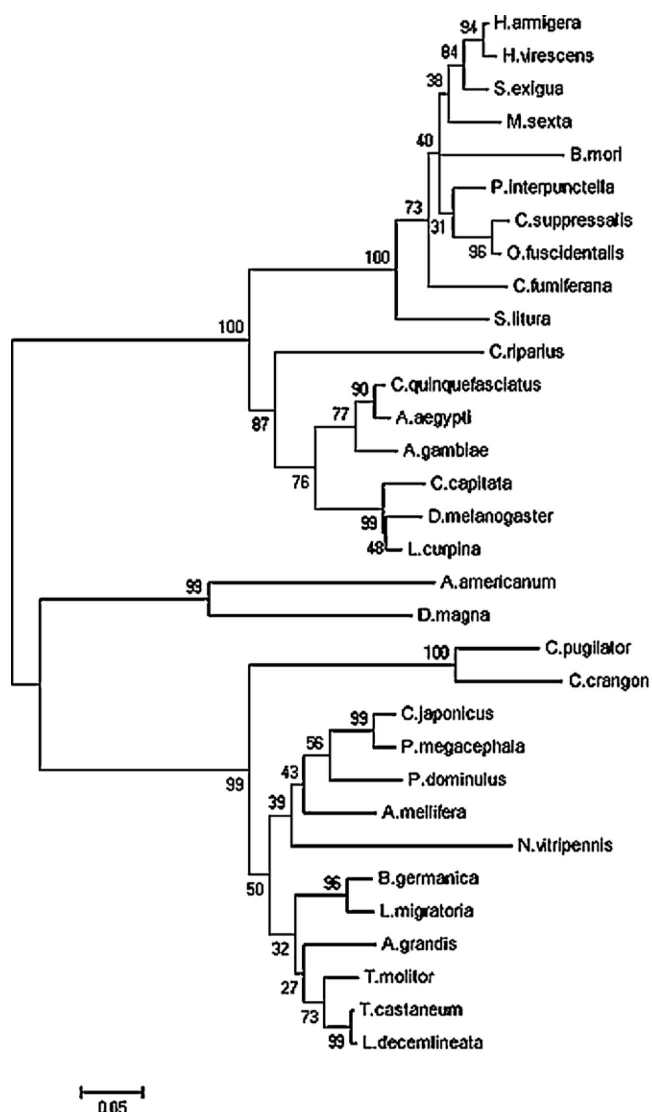

Fig. 3 – Phylogenetic tree of *C. riparius EcR* using amino acid sequences corresponding to the ligand-binding domain in insect and other species. The GenBank accession numbers are given in brackets. (AAB94567 – *A. americanum*, AAC33432 – *C. pugilator*, AAC36491 – *C. fumiferana*, AAD19828 – *L. migratoria*, AAR84611 – *P. interpunctella*, AAZ38145 – *P. dominulus*, ABS00248 – *O. fuscidentalis*, ABX79143 – *S. litura*, ACA30302 – *S. exigua*, ACD74807 – *H. armigera*, ACK57879 – *A. grandis*, ACO44665 – *C. crangon*, BAC11713 – *C. suppressalis*, BAD99296 – *L. decemlineata*, BAE47509 – *P. megacephala*, BAF49030 – *D. magna*, BAF79666 – *C. japonicus*, CAA11907 – *C. capitata*, CAA72296 – *T. molitor*, CAJ01677 – *B. germanica*, EAA00117 – *A. gambiae*, NP_001091685 – *A. mellifera*, NP001107650 – *T. castaneum*, NP001152828 – *N. vitripennis*, NP_724456 – *D. melanogaster*, O18473 – *H. virescens*, O18531 – *L. curpina*, P49880 – *Ae. aegypti*, P49881 – *B. mori*, P49883 – *M. sexta*, XP_001844581 – *C. quinquefasciatus*). The bootstrap values for 1000 replications are given at the nodes.

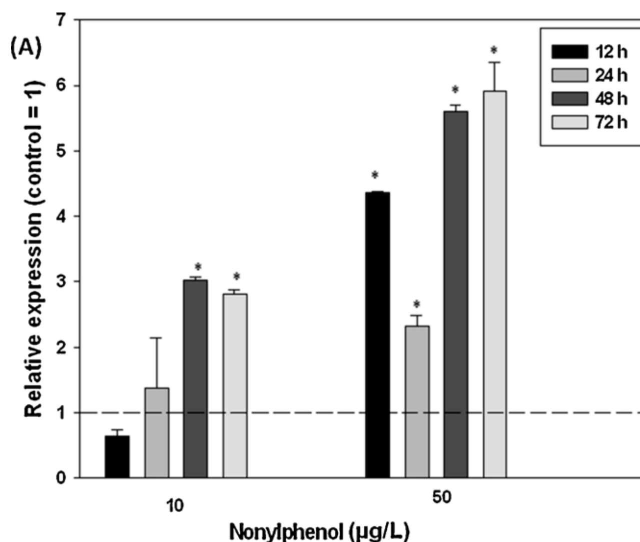

Fig. 4 – Expression levels of *CrEcR* mRNA after exposure of fourth instar larvae to different concentrations (0, 10, 50 μg/L) of nonylphenol for 12, 24, 48 and 72 h. The relative expression level of *CrEcR* mRNA with that of *CrActin* mRNA is shown. Real-time RT-PCR was performed in triplicate for each sample. Differences between treatment and control groups (data shown as means ± SE) were analyzed for statistical significance using ANOVA. An asterisk indicates a statistically significant difference, $p < 0.05$ (*) as compared with the control group.

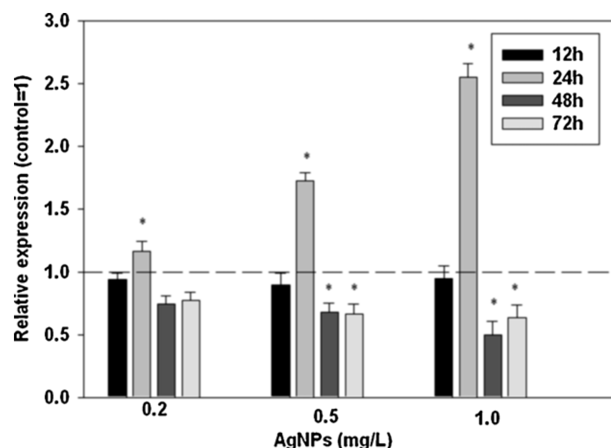

Fig. 5 – Expression levels of *CrEcR* mRNA after exposure of fourth instar larvae to different concentrations (0, 0.2, 0.5 and 1 mg/L) of silver nanoparticles for 12, 24, 48 and 72 h. The relative expression level of *CrEcR* mRNA with that of *CrActin* mRNA is shown. Real-time RT-PCR was performed in triplicate for each sample. Differences between treatment and control groups (data shown as means ± SE) were analyzed for statistical significance using ANOVA. An asterisk indicates a statistically significant difference, $p < 0.05$ (*) as compared with the control group.

other species. Phylogenetic analysis also suggested that the *CrEcR* sequence grouped with that of insects belonging to the Dipteran order and was more closely related to mosquitoes.

It has previously been reported that many environmental pollutants can act as agonists/antagonists in the *EcRs* of aquatic invertebrates (Mu et al., 2005; Janer and Porte, 2007). Testosterone and bisphenol A (BPA) had anti-ecdysteroid activity in *D. magna* (LeBlanc et al., 2000; Mu et al., 2005). Nonylphenol and AgNPs exposure in *C. riparius* also induced a reduction in the rates of total adult emergence and emergence failures (Lee and Choi, 2006; Nair et al., 2011). However, the underlying molecular mechanisms of toxicity of environmental pollutants are not well understood in *C. riparius*. Genomic techniques based on expression analysis of genes are important tools for investigating molecular level effects caused by exposure to environmental pollutants (Snell et al., 2003; Ankley et al., 2006). In an earlier study, Planelló et al. (2008, 2010) reported that exposure to BPA and cadmium chloride up-regulated the expression of *EcR* mRNA in *C. riparius*. Recently, Hwang et al. (2010) reported that in *T. japonicus*, BPA resulted in the transcriptional repression of *EcR* gene. In *A. bhia EcR* mRNA was up-regulated after nonylphenol exposure (Hirano et al., 2008). From our studies, we also found that the mRNA expression level of *CrEcR* was significantly up-regulated on exposure to the known endocrine disrupting chemical, nonylphenol, but up or down regulated on exposure to AgNPs. Recently it was reported that the expression of another nuclear receptor gene, the oestrogen related receptor gene, was significantly up regulated after nonylphenol exposure (Park and Kwak, 2010). Taken together, our findings suggest that nonylphenol has the ability to mimic the effect of the hormone and activate the expression of *CrECR* mRNA in *C. riparius*. We also suggest that AgNPs may interfere with the action of moulting hormones and the modulation of the expression of *CrEcR* mRNA by AgNPs is a first step in understanding its action on *CrEcR* and further studies are required in this direction.

5. Conclusions

In conclusion, the overall results of our present studies suggest that nonylphenol and AgNPs could modulate the transcription of *CrEcR*, which in turn could affect ecdysteroid-regulated pathways, which may ultimately lead to developmental failures in *C. riparius*. However, more detailed studies are required to understand the changes in the ecdysone receptor genes in cells upon exposure to nonylphenol and AgNPs and to understand the interplay between ecdysone responsive genes, and to integrate the effects to the organism level response.

Conflict of interest

The authors have no conflict of interest.

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