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Identification and mRNA expression of two 17β-hydroxysteroid dehydrogenase genes in the marine mussel *Mytilus galloprovincialis* following exposure to endocrine disrupting chemicals

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ABSTRACT

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are multifunctional enzymes involved in the metabolism of steroids, fatty acids, retinoids and bile acid. In this study, two novel types of 17β-HSDs (named as MgHsd17b10 and MgHsd17b12) were cloned from *Mytilus galloprovincialis* by using rapid amplification of cDNA ends (RACE) approaches. Sequence analysis showed that MgHsd17b10 and MgHsd17b12 encoded a polypeptide of 259 and 325 amino acids, respectively. Phylogenetic analysis revealed that MgHsd17b10 and MgHsd17b12 were evolutionarily clustered with other invertebrate 17β-HSD type 10 and 17β-HSD type 12 homologues. The MgHsd17b10 and MgHsd17b12 transcripts could be detected in all examined tissues with higher expression levels in digestive glands and gonad. After exposed to endocrine disrupting chemicals (Bisphenol A or 2,2′,4,4′-tetrabromodiphenyl ether), the expression of MgHsd17b10 and MgHsd17b12 transcripts was both down-regulated in digestive glands. These findings suggest that MgHsd17b10 and MgHsd17b12 perhaps play an important role in the endocrine regulation of *M. galloprovincialis*.

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1. Introduction

17β-Hydroxysteroid dehydrogenases (17β-HSDs) constitute a class of enzymes involved in the last step of steroids synthesis (Labrie et al., 1997). They catalyze the conversion of keto groups (estrone, androstenedione) into hydroxy groups (estradiol, testosterone) or vice versa at position C_{17} of the steroids backbone (Peltoketo et al., 1999), using the cofactors NADPH or NADH in reduction reactions, and $NADP^+$ or NAD^+

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in oxidation reactions (Jörnvall et al., 1995). Most 17β-HSDs belong structurally to the family of short chain dehydrogenase/reductases (SDRs), which are known to be present in bacteria, fungus, plants and animals (Baker, 2001; Lukacik et al., 2006). Recently, 17β-HSDs attract considerable attention due to their regulative functions on steroids metabolism. Presently, fourteen types of 17β-HSDs in mammals and nine types in zebra fish have been characterized according to the chronology of identification (Peltoketo et al., 1999; Mindnich et al., 2004; Mindnich and Adamski, 2009). However, numerous studies suggest that the same enzyme types which share high amino acid sequence conservation in distinct species may execute different functional roles and display completely different tissue distribution (Biswas and Russell, 1997; Su et al., 1999; Moeller and Adamski, 2009; Zhai et al., 2012).

17β-HSD type 10 and 12 are both ancient and multifunctional enzymes due to their broad substrate specificity (Mindnich et al., 2004; Mindnich and Adamski, 2009). 17β-HSD type 10 acts on diverse substrates such as steroids, fatty acids, bile acid, and xenobiotics (He et al., 1999; Yang et al., 2005). It was found that 17β-HSD type 10 catalyzed the deactivation of active steroids such as testosterone (T) and estradiol (E2) (Jazbutyte et al., 2009). Mammalian 17β-HSD type 12 which was first designed as 3-ketoacyl-CoA reductase, is mainly involved in the lipid metabolism (Moon and Horton, 2003). Meanwhile, this enzyme has been proved to catalyze the transformation of estrone (E1) into estradiol (E2) in vertebrates (Luu-The et al., 2006; Mindnich and Adamski, 2009).

Recent alarm regarding exposure of aquatic species to environmental contaminants and their adverse effects on growth, metabolism and breeding has raised serious concern (Kidd et al., 2007). Endocrine disrupting chemicals (EDCs), which constitute a heterogeneous group of either natural (E2) or synthetic compounds (PBDEs, DDTs, BPA, Alkylphenols, Tributyltin), are prevalent over a wide range in the aquatic environment (Ortiz-Zarragoitia and Cajaraville, 2005; Porte et al., 2006; Andrew et al., 2010). The main sources of EDCs in the marine environment are from inland pollution and shipping sewage (Gatidou et al., 2007).

The industrial chemical bisphenol A (BPA) is widely used in the manufacture of plastic products and has been detected extensively in the aquatic environment (Fleisch et al., 2010). It was reported that the concentration of BPA from some Chinese estuaries reached a value of 8.3 μg/L (Jin et al., 2004). Flint and coworkers defined that BPA was already harmful to living organisms even at the environmentally relevant concentrations (12 μg/L or lower) (Flint et al., 2012). Polybrominated diphenyl ethers (PBDEs) are used as brominated flame retardants in polyurethane foams, textiles, and electric appliances amongst others (Sjödin et al., 2008). The congener 2,2′,4,4′-tetrabromodiphenyl ether (BDE47) is one of the dominant PBDE congeners generally found in wildlife and human (Meerts et al., 2001). In China, the concentrations of PBDEs in some wastewater treatment effluent, which finally flows into the sea, can reach a value of 4.42 μg/L (Fang et al., 2012). Previous studies have revealed that BDE-47 exposure (1 μg/L) decreased phospho-protein levels in both sexes of mussels, and induced spawning in male mussels (Aarab et al., 2006). Severe damages caused by BPA and BDE-47 to ovarian follicles and oocytes were also observed in female mussels (Matozzo et al., 2008). In addition, induction of bi-nucleated, fragmented-apoptotic cells and nuclear buds was found in gills of blue mussels exposed to BDE-47 (5 μg/L) (Barsiene et al., 2006).

Bivalve mollusks, such as *Mytilus galloprovincialis*, are studied as sentinels on account of their susceptibility to environmental contaminants (Siah et al., 2003; Ortiz-Zarragoitia and Cajaraville, 2006). Though 17β-HSDs have been extensively characterized in humans and other vertebrate species, information on invertebrate is still fragmentary, especially in mollusks (Mindnich et al., 2004; Zhou et al., 2005; Lima et al., 2013). In this work, we isolated two types of 17β-HSD genes in *M. galloprovincialis* and compared the deduced amino acid sequences with counterparts from other species. We also evaluated the basal expression pattern of these genes in various tissues as well as their temporal expression profiles in digestive glands after exposure to BPA and BDE-47. The data presented here hopefully provide a better understanding of the endocrine regulative function of these two 17β-HSD members in mussels.

2. Materials and methods

2.1. Chemicals

BPA and BDE-47 were purchased from Sigma Aldrich (133027, USA) and ChemService (N-10522-100MG, USA), respectively. A stock solution of BPA or BDE-47 was dissolved and diluted in dimethyl sulfoxide (DMSO) to achieve different final concentrations (1 μg/L, 10 μg/L). The doses of BDE47 and BPA used in the exposure experiments were based on previous research (Aarab et al., 2006; Ortiz-Zarragoitia and Cajaraville, 2006; Flint et al., 2012).

2.2. Animals and treatments

The mussels (shell length: 4–5 cm) were purchased from a local aquaculture farm and placed in aerated seawater (1 L water per mussel) at 20 °C for 8 days before the commencement of the experiment. During the acclimatization period, mussels were fed with *Phaeodactylum tricornutum* and *Platymonas helgolandica*, and the water was completely renewed daily. Following acclimatization, the mussels were randomly divided into six treatments: BPA (1, 10 μg/L), BDE-47 (1, 10 μg/L), solvent control and blank. Three replicates were used per treatment, and each replicate consisted of 40 individuals. As the sex of mussels cannot be determined before dissection, more than 12 mussels (6 males and 6 females) per time period were required. The normal filtered seawater (FSW) and FSW containing 0.02% DMSO (v/v) were used as the blank and solvent control groups, respectively. During the exposure experiment, the mussels were fed for 2 h in FSW to maintain the normal physiological condition and then transferred into renewed exposure solutions daily. After 24, 48 and 96 h of exposure, the digestive glands of 12 individuals (six males and six females) were rapidly dissected from mussels of each treatment, frozen in liquid nitrogen and maintained at −80 °C for subsequent total RNA extraction.

Table 1 – Primers used in this study.

Primer name	Sequence (5′–3′)	Annealing temperature (°C)	Amplification efficiency (%)	Sequence information
Oligo(dT_{17})	GGCCACGCGTCGACTAGTACT(17)	63.2		Primer for cDNA synthesis
MgHsd17b10-F1	TTGTGGGATTAGTTACAGGAGGG	57.6		3′RACE
MgHsd17b10-F2	ACTGATTGGGGAGAATGAACCT	58.2		3′RACE
MgHsd17b12-F1	TGGGCTGTGGTTACTGGTGC	59.4		3′RACE
MgHsd17b12-F2	ACCTGAAATGGTGTCACGGAG	57.3		3′RACE
MgHsd17b10-GSP1	TGACCCTCAAATGCTG	46.2		5′RACE
MgHsd17b10-GSP2	GTCCAACTGCCAATCTTATCAC	63.5		5′RACE
MgHsd17b10-GSP3	TAACTCTAGCACCTTGTCTTAC	59.2		5′RACE
MgHsd17b12-GSP1	ACGCTACATTGATGACC	49.6		5′RACE
MgHsd17b12-GSP2	CTCCAATGTCAAGTCCAGCTAA	62.4		5′RACE
MgHsd17b12-GSP3	GTCCATTCCTTTCTTGGCCAGTTG	66.2		5′RACE
MgHsd17b10-RT-F	GGGTGCCATTGTAGGAATGA	59.7	103.6	Real time PCR
MgHsd17b10-RT-R	TGTGGGCATATTCATCTGGA	60.2		Real time PCR
MgHsd17b12-RT-F	TGGTCATCAATGTAGCGTCTG	59.5	101.4	Real time PCR
MgHsd17b12-RT-R	AGCACTGGCAGCATACTGA	60.6		Real time PCR
28S-RT-F	CCGAGACCGAGGATTTGCC	59.2	98.8	Real time PCR
28S-RT-R	ACCGATTCGCCACTGACCC	59.9		Real time PCR

2.3. *Total RNA extraction and cDNA preparation*

Total RNA was extracted using Trizol reagent according to the manufacture's protocol (Invitrogen, USA). The quality and quantity of total RNA were determined by micro-ultraviolet spectrophotometer (Nanodrop 2000, ThermoFisher, USA) and agarose gel electrophoresis. The first-strand cDNA was synthesized based on Promega M-MLV RT Usage information using the RNase-free DNase I (Thermo, USA) – treated total RNA as template and Oligo(dT_{17}) as primer (Table 1). The reaction conditions were incubated for 5 min at 70 °C, 1–2 min on ice, 50 min at 42 °C and terminated by heating at 65 °C for 15 min.

2.4. *Cloning the full-length cDNA of 17β-HSDs*

Partial cDNA sequences of 17β-HSD type 10 and 12 were obtained from the subtractive cDNA library constructed from the digestive gland of *M. galloprovincialis* (unpublished data). The complete cDNA sequences of MgHsd17b10 and MgHsd17b12 were amplified by ten gene-specific primers combined with anchor and adapter primers (Table 1). The 5′-RACE (5′-rapid amplification of cDNA ends) method was conducted to get the sequence of the 5′ region with the 5′ RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen, USA). The 3′ region was amplified by using nested PCR strategy with a combination of four gene-specific primers (Table 1). The resultant PCR products were sub-cloned into pMD18-T vector (TaKaRa, China) and subjected to DNA sequencing in both directions by the Chinese National Human Genome Center (SinoGenoMax, China).

2.5. *Sequence analysis and phylogenetics*

The obtained partial fragments were assembled into full-length sequences and further analyzed using the BLAST algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequences were predicted with the Expert Protein Analysis System (http://www.expasy.org/). Transmembrane domain was calculated using the TopPred 1.1 software (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred). Alignment of the conserved domains was performed against the conserved domain database for protein classification in NCBI database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple alignments of 17β-HSDs were performed with the software ClustalX2. A phylogenetic tree was constructed by the Neighbor-Joining (NJ) Method with MEGA version 5.0, and tested for reliability over 1000 bootstrap replicates. The evolutionary distances were estimated under the Poisson model (+G). The rate variation among sites was modeled with a gamma distribution (shape parameter = 2.4). All positions containing gaps and missing data were eliminated. There were a total of 147 positions in the final dataset.

2.6. *Quantitative PCR assays*

The differential tissue-specific expression of 17β-HSD type 10 and 17β-HSD type 12 was determined in Applied Biosystem 7500 fast Real-time PCR System by using DyNAmo Color Flash SYBR Green qPCR Kit (Thermo, USA). The PCR amplification was carried out in a total volume of 50 μL, containing 25 μL of 2 × SYBR Green PCR Master Mix, 6 μL of the diluted cDNA, 0.8 μL of each of primers (5 μmol/L), and 17.4 μL of DEPC-treated water. The thermal profile for qRT-PCR was 50 °C for 2 min and 95 °C for 7 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 min. At the end of each PCR reaction, a dissociation curve analysis of amplification products was performed to confirm that only one PCR product was amplified and detected. Multiple tissues, including adductor muscle, gills, hemocytes, mantle, digestive glands and gonad, were examined. The qRT-PCR was performed using 28S rRNA as an internal reference (Cubero-Leon et al., 2012a) and relative mRNA level was calculated with respect to the mRNA level in adductor muscle. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA (1:10 for reference gene and 1:5 for genes of interest). Furthermore, the temporal expression profiles of 17β-HSDs transcript in digestive glands after exposure treatments were also measured by qRT-PCR. The primer information was provided in Table 1. The

relative gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method as previously described (Bustin et al., 2009; Livak and Schmittgen, 2001).

2.7. *Statistical analysis*

Quantitative RT-PCR data were analyzed by SPSS 18.0 software (SPSS Inc., USA). All data were given in terms of relative mRNA expression as means ± S.D. The data were tested for normal distribution using the Kolmogorov–Smirnov test and equal variance was tested using the Levene Median test as assumptions for running ANOVA. A one-way ANOVA was used to compare the expression level of each time point in exposure treatment versus solvent control. The Fisher's Least Significant Difference (LSD) test was used for posthoc test and the significance level was set as $P < 0.05$.

3. Results

3.1. *cDNA cloning and sequence analysis*

The complete coding sequences of two novel types of 17β-hydroxysteroid dehydrogenases were obtained by overlapping the amplified fragments and the original ESTs. The nucleotide sequences of these two mussel 17β-HSDs, designated as MgHsd17b10 (Fig. 1) and MgHsd17b12 (Fig. 2), were deposited in GenBank under the accession numbers KF031120 and KF031121, respectively. The full-length cDNA of MgHsd17b10 (916 bp) contained an ORF of 780 bp, encoding a polypeptide of 259 amino acids, with a predicted molecular weight of 27.47 kDa and a theoretical isoelectric point of 6.52. The amino acid sequence of MgHsd17b10 shared high homology with 17β-HSD type 10 homologues from *Xenopus laevis* (NP001087068, 73% identity), *Strongylocentrotus purpuratus* (XP780014, 71%) and *Danio rerio* (NP001006098, 69%). Conserved domain analysis showed that MgHsd17b10 contained a typical 17β-hydroxysteroid dehydrogenase (HSD10)-like domain, a classical short chain dehydrogenase/reductase (SDR) domain ranging from Lys^{7} to Ala^{259}. ScanProsite analysis showed that this domain region comprised a SDR family signal (Ser^{153}-Ala^{181}). Multiple alignments revealed the presence of a conserved co-factor binding motif (TGXXXGXG), a catalytic triad Ser^{153}-Tyr^{166}-Lys^{170} and an active site motif (YXXXK) with tyrosine acted as catalytic residue in MgHsd17b10 (Fig. 3A). Furthermore, a deduced mitochondrial target signal (Glu^{26}-Gln^{31}) was detected in the N-terminus of MgHsd17b10, suggesting that this protein may be a mitochondrial enzyme.

MgHsd17b12 (1196 bp) possessed an ORF of 978 bp, encoding a polypeptide of 325 amino acids, with a predicted molecular weight of 35.83 kDa and a theoretical isoelectric point of 10.05. Pairwise alignment indicated that MgHsd17b12 had a 51% identity to that of *Nucella lapillus* (AFV95595), 49% to that of *Haliotis diversicolor supertexta* (ADF80270). The deduced peptide of MgHsd17b12 was predicted to contain three transmembrane domains (Ser^{13}-Leu^{33}, Leu^{166}-Val^{186}, Ile^{194}-Ser^{214}) and a SDR domain, ranging from Gln^{59} to Arg^{230}. Amino acid sequence alignment showed that MgHsd17b12 also contained a conserved co-factor binding site motif (TGXXXGXG), a catalytic triad Ser^{198}-Tyr^{211}-Lys^{215} and a putative catalytic site (YXXXK). In addition, there was a typical SDR superfamily signal motif (NNVG) in the deduced amino acid sequence of MgHsd17b12. Moreover, the conserved motif PXXVXT was found in the C-terminus of MgHsd17b12 (Fig. 3B).

3.2. *Phylogenetic analysis*

In order to determine the evolutionary position of MgHsd17b10 and MgHsd17b12, a phylogenetic tree was constructed using the Neighbor-Joining method (Fig. 4). The results indicated that 17β-HSDs were separated into several clades. 17β-HSD type 10 and type 12 were clustered into clade3 and clade2 and seemed to have a close evolutionary relationship with 17β-HSD type 8 and 17β-HSD type 3, respectively. The assignment of MgHsd17b10 and MgHsd17b12 to 17β-HSD type 10 and type 12 were clearly supported by the phylogenetic analysis. It was found that MgHsd17b10 and MgHsd17b12 were first clustered with counterparts from invertebrate to form the invertebrate sub-group and then grouped with the vertebrate sub-clade.

3.3. *Tissue distribution of 17β-HSDs transcripts*

17β-HSDs are involved in a variety of physiological functions and the expression pattern may indicate their possible roles. In this work, the tissue-specific expression of MgHsd17b10 and MgHsd17b12 were investigated by qRT-PCR. Both MgHsd17b10 and MgHsd17b12 were detected in all tissues examined at different levels (Fig. 5). Appreciable expression level of both genes was found in digestive glands and gonad from mussels of both sexes. For MgHsd17b10, the mRNA was expressed with higher level in digestive glands, especially in the female mussels. Meanwhile, a relatively high expression level was detected in gonad and adductor muscle of both sexes as well as hemocytes of female mussels. As for MgHsd17b12, a similar expression pattern was observed. The transcript of MgHsd17b12 was mainly expressed in digestive glands and gonad, followed by adductor muscle. The expression levels of both genes were relatively low in the tissues of mantle and gills.

3.4. *Effects of BPA and BDE-47 exposure on gene transcription*

To better understand the function of 17β-HSD types 10 and 12 and their responses to xenoestrogens, the transcripts of MgHsd17b10 and MgHsd17b12 in digestive glands responded to BPA or BDE-47 exposure was measured by qRT-PCR. In both males and females, the relative expression levels of MgHsd17b10 and MgHsd17b12 were down-regulated after exposure, but the males seemed more susceptible to the chemicals exposure (Fig. 6). For the female mussels, the expression level of both genes was significantly inhibited ($P < 0.05$) at 24 h post-exposure. After that, the expression level of MgHsd17b10 and MgHsd17b12 in females increased gradually to that of the solvent control group at 96 h post-exposure. As concerned to male mussels, a significant decrease in the expression of both genes was also observed at 24 h post-exposure ($P < 0.05$). As exposure time increased, the transcript level of both genes increased gradually. However, the expression was still significantly suppressed at 96 h except the expression level of MgHsd17b10 under 1 μg/L BPA and BDE-47

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  1                                        M  P  I  R  N  L  K  G  L  V
  1 AAGTAAACTTCGCACTTGCAAATTACAAATATGCCGATTAGAAATTTAAAGGGTCTTGTG
 11 G  L  V  T  G  G  A  S  G  L  G  K  A  T  V  E  R  F  V  R
 61 GGATTAGTTACAGGAGGGGCATCAGGACTTGGGAAAGCCACTGTTGAAAGATTTGTAAGA
 31 Q  G  A  R  V  I  L  C  D  L  E  K  S  A  G  K  D  V  A  D
121 CAAGGTGCTAGAGTTATACTATGTGATCTTGAGAAATCAGCTGGTAAAGATGTTGCTGAC
 51 N  L  G  D  N  C  I  F  S  P  T  N  V  T  S  E  A  D  V  K
181 AATTTAGGTGACAACTGTATATTCTCTCCAACAAATGTAACGTCAGAAGCTGATGTGAAA
 71 K  A  L  E  I  A  K  D  K  F  G  K  L  D  V  A  V  N  C  A
241 AAGGCTTTAGAAATAGCCAAAGATAAATTTGGTAAACTTGACGTTGCTGTTAACTGTGCA
 91 G  I  G  I  A  F  K  T  Y  N  F  N  K  K  L  P  H  N  L  E
301 GGAATTGGAATAGCCTTTAAAACATACAACTTCAACAAAAAGCTACCTCATAATCTGGAA
111 D  F  A  E  V  I  T  V  N  T  V  G  S  F  N  V  I  R  L  A
361 GATTTTGCAGAAGTTATAACAGTTAATACTGTTGGCAGCTTCAATGTGATAAGATTGGCA
131 V  G  L  I  G  E  N  E  P  D  A  D  G  Q  R  G  V  V  I  N
421 GTTGGACTGATTGGGGAGAATGAACCTGATGCAGATGGACAGAGGGGTGTGGTCATTAAC
151 T  S  S  V  A  A  F  E  G  Q  M  G  Q  A  A  Y  S  A  S  K
481 ACATCAAGTGTGGCAGCATTTGAGGGTCAGATGGGTCAAGCGGCTTACTCAGCTAGTAAG
171 G  A  I  V  G  M  T  L  P  I  A  R  D  L  A  N  Q  G  I  R
541 GGTGCCATTGTAGGAATGACTTTACCTATAGCCAGAGATTTAGCTAATCAGGGCATCAGA
191 C  C  T  I  A  P  G  L  F  N  T  P  L  L  A  S  L  P  E  K
601 TGTTGTACTATAGCTCCAGGTTTATTTAACACACCTTTACTTGCCAGTTTACCAGAAAAG
211 V  Q  T  Y  L  A  S  T  I  P  F  P  K  R  L  G  D  P  D  E
661 GTTCAGACTTATTTAGCATCAACAATTCCTTTTCCAAAACGCTTGGGCGATCCAGATGAA
231 Y  A  H  M  V  Q  T  I  V  E  N  P  Y  L  N  G  E  V  I  R
721 TATGCCCACATGGTACAAACAATCGTAGAAAACCCATATCTAAATGGTGAGGTCATAAGA
251 V  D  G  S  I  R  M  M  A  *
781 GTGGATGGTTCAATAAGGATGATGGCATAATTTGTAAAGAAAACTGAACAAACTTTGATT
841 AAAAATGTGTATTGTGATATTTTATAATTGAAATGAAGACATAATATTTGTTAAAAAAAA
901 AAAAAAAAAAAAAAAA
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Fig. 1 – The complementary DNA and deduced amino acid sequences of type 10 17β-HSD from *M. galloprovincialis*. Predicted HSD10-like domain is shaded in light gray and the signal is underlined with red line. The conserved co-factor binding motif (TGXXXGXG) and active site motif (YSASK) are in bold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

exposure. Overall, the transcript pattern of both genes showed similar pattern in females and males. In addition, high level of exposure can cause stronger suppression in the expression of both genes, especially in male mussels.

4. Discussion

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are one of the most important enzymes involved in both endocrine and immune system. Although a number of 17β-HSDs have been identified and characterized in vertebrates, the information from invertebrate, especially bivalves is scarce. Here we have characterized two novel 17β-HSDs belonging to 17β-HSD type 10 and 17β-HSD type 12 from *M. galloprovincialis*, and investigated their expression patterns in response to endocrine disrupting chemicals exposure.

As both 17β-HSD type 10 and 12 belonged to the short-chain dehydrogenase/reductase (SDR) family, they shared some structural similarities. Sequence comparisons revealed that MgHsd17b10 and MgHsd17b12 contained a conserved N-terminal co-factor binding site motif (TGXXXGXG), a putative catalytic site (YXXXK) and catalytic triad of Ser-Tyr-Lys. The TGXXXGXG regions which identified as hallmarks of the SDRs family, served as part of the nucleotide binding fold (Filling et al., 2002). The determined triad of Ser-Tyr-Lys residues constituted the active site, and tyrosine was the most conserved residue within the whole family and functioned as the catalytic base (Jörnvall et al., 1995; Filling et al., 2002). Multiple alignments indicated that both the sequences of MgHsd17b10 and MgHsd17b12 contained the conserved signature motifs, which strongly implied that they were new members of the 17β-HSD family.

Previous studies have reported the presence of 17β-HSDs in mollusks. For example, it has been shown that mussel 17β-HSDs were involved in the metabolism of 17β-estradiol or estrone, and also dehydroepiandrosterone or androstenediol in gonad (de Longcamp et al., 1974; Fernandes et al., 2011). Recently, abalone 17β-HSDs were found to modulate the concentration of steroids in gonad (Zhai et al., 2012; Zhou et al.,

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1                           M  A  S  N  V  Q  S  F  L  G  N  Y  S
1    GATTCACTTGCCGGTAGTACCATGGCGTCTAATGTCCAAAGTTTTCTTGGGAATTATTCA
14   D  L  F  A  V  A  G  V  V  S  V  S  Y  I  A  L  K  L  A  L
61   GACCTTTTTGCAGTAGCAGGAGTGGTGTCTGTTTCATACATTGCACTTAAACTCGCCCTG
34   S  I  F  N  G  I  K  V  F  L  L  A  K  P  L  G  L  T  K  N
121  TCAATTTTCAATGGCATCAAGGTCTTTCTGTTAGCAAAACCACTTGGTTTAACTAAGAAT
54   L  K  K  C  G  Q  W  A  V  V  T  G  A  T  D  G  I  G  K  G
181  TTGAAGAAATGTGGACAGTGGGCTGTGGTTACTGGTGCTACAGATGGCATTGGAAAAGGA
74   Y  T  H  Q  L  A  K  K  G  M  D  I  I  L  I  S  R  T  K  S
241  TACACACACCAACTGGCCAAGAAAGGAATGGACATTATTTTGATAAGCAGGACAAAGTCA
94   K  L  E  D  C  A  K  E  I  E  Q  K  F  K  V  K  T  K  I  I
301  AAGCTAGAAGATTGTGCCAAAGAAATAGAACAAAAGTTCAAAGTAAAAACCAAGATAATT
114  V  A  D  F  S  A  G  L  Q  I  Y  D  A  I  R  S  Q  L  A  G
361  GTAGCTGATTTTAGTGCTGGCTTACAAATATATGATGCAATAAGAAGTCAGTTAGCTGGA
134  L  D  I  G  V  L  V  N  N  V  G  M  S  Y  D  F  P  M  Y  F
421  CTTGACATTGGAGTTCTTGTAAATAACGTGGGGATGTCATATGATTTCCCTATGTACTTC
154  L  E  I  P  D  R  E  K  K  I  M  D  L  L  H  V  N  I  T  S
481  TTGGAAATCCCTGATCGAGAAAAGAAGATAATGGACTTACTGCATGTCAATATTACATCT
174  V  T  M  M  T  S  V  V  L  P  E  M  V  S  R  S  R  G  V  V
541  GTAACAATGATGACAAGTGTTGTTTTACCTGAAATGGTGTCACGGAGTAGAGGAGTGGTC
194  I  N  V  A  S  A  S  G  V  N  P  C  P  L  L  T  V  Y  S  A
601  ATCAATGTAGCGTCTGCCTCAGGTGTAAATCCTTGTCCTCTTCTAACTGTGTATTCAGCT
214  S  K  A  F  V  H  Y  F  S  L  C  L  E  R  E  Y  R  D  R  G
661  TCCAAGGCTTTTGTTCATTACTTTTCACTGTGTCTAGAACGAGAATACAGGGACCGTGGT
234  I  T  V  Q  S  V  L  P  Y  F  V  A  T  K  M  S  K  V  K  K
721  ATTACTGTACAGAGTGTATTGCCCTATTTTGTTGCCACCAAAATGTCTAAGGTAAAGAAA
254  G  S  F  W  I  P  Y  P  D  Q  Y  A  A  S  A  L  D  T  V  G
781  GGCAGCTTTTGGATCCCCTACCCTGATCAGTATGCTGCCAGTGCTTTAGATACTGTTGGT
274  F  E  K  A  T  N  G  W  W  S  H  S  I  T  G  W  L  L  D  V
841  TTTGAGAAGGCAACAAATGGATGGTGGTCACATAGTATTACTGGATGGCTGCTGGATGTA
294  I  P  K  Q  T  Q  M  S  I  L  V  R  I  L  L  E  K  K  K  G
901  ATACCCAAGCAGACACAGATGAGCATTTTGGTCAGAATATTGCTAGAGAAGAAAAAGGGA
314  A  P  I  K  R  L  N  G  S  K  T  D  *
961  GCTCCAATAAAAAGATTGAATGGTTCCAAAACAGACTAAATGATACATGTACAATGGGAT
1021 GGTTGTATGGTTTAATCCCAGAAAGTCTCATGCTGAATTCGGGTATGAAGATTTTACAAG
1081 GTGCGAGGAAGAAGGGACTTAGAAAATCTAATGCTCCAAAAAAGGATTAAAATCTTTTGT
1141 TTTTATTTATATTTGATAATAAAAATGATGAAATCATGTAAAAAAAAAAAAAAAAA
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Fig. 2 – The complementary DNA and deduced amino acid sequences of type 12 17β-HSD from *M. galloprovincialis*. Predicted SDR domain is shaded in light gray. The SDR superfamily signal motif (NNVG) is showed with a double underline. The three transmembrane domains are underlined with black line. The conserved co-factor binding motif (TGXXXGXG) and active site motif (YSASK) are in bold.

2011). In addition to gonad, 17β-HSDs were also present in certain extragonadal tissues (Jörnvall et al., 1995). In the present study, both MgHsd17b10 and MgHsd17b12 were expressed in all examined tissues although the levels in some tissues were relatively low. The appreciable expression level of MgHsd17b10 mRNA was mainly found in digestive glands and gonad, which was coincident with the metabolism of steroids in mussels (Janer et al., 2005; Janer and Porte, 2007). 17β-HSD type 10, also known as short chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD), 3-hydroxyacyl-CoA dehydrogenase type II (HADH2), 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD), and amyloid binding alcohol dehydrogenase (ABAD), seems to be the enzymes with the broadest substrate specificity among all 17beta-HSDs. It is therefore not surprising that the enzyme is widely expressed in multiple tissues. In human and other mammals, 17β-HSD type 10 is present in a variety of tissues such as blood, liver, muscle and heart. The same broad distribution pattern was also observed in *Drosophila* (Torroja et al., 1998; He et al., 2001). As concerned to MgHsd17b12, the expression was most robust in digestive glands and gonad from both sexes, consistent with recent study in the mollusk *N. lapillus* and abalone (Lima et al., 2013; Zhou et al., 2011). 17β-HSD type 12, from zebra fish to human, was also ubiquitously expressed (Mindnich and Adamski, 2009; Sakurai et al., 2006).

A

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MgHsd17b10  ..MPIRNLKGLVGLVTGGASGLGKATVERFVRCGARVILCDLEKSACKDVADNLGDNCIFSPTNVTSEADVKKALEIAKD  78
XlHsd17b10  .MAHVRNLKGLVGIVTGGASGLGRATVERFVRCGASAVILDLPKSEGKTVAESLGDTCAFSPTDVTSEVDVKNALELARS  79
OcHsd17b10  MAAACRSVKGLVAVITGGASGLGLATAERLVGCGASAVLLDLPSSDGEAQAKKLGKSCTFAPADVTSEKDVQAALAIAKE  80
BfHsd17b10  .MAAIRSCKGLVAIVTGGASGLGRATVERLVKCGASVVIADLPTSEGESVANTIGGNCVFSPTDVSKEADVLATVAKAKS  79
SpHsd17b10  .MSSVKSVKGLVGLVTGGASGLGKATVERFVRNGARMVIVDLPNSPGEEVAKSLGEDCKFSPGDVTSADDVTRAIDVAKD  79

MgHsd17b10  KFGKLDVAVNCAGIGIAFKTYNFNKKLPHNLEDFAEVITVNTVGSFNVIRLAVGLIGENEFDADGQRGVVINTSSVAAFE  158
XlHsd17b10  KFGRVDIVVNCAGIAVAAKTYNLNKQLPHSLEDFQRVISVNIAGTFNVIRLAAGEMGKNEFDEDGHRGVIINTASVAAFD  159
OcHsd17b10  KFGRVDVAVNCAGIAVAIKTYSLKKNLPHSLEDFQRVINVNLIGTFNVIRLVAGEMSQNEFDQGGQRGVIINTASVAAFE  160
BfHsd17b10  EFGKLNAAINCAGIGVAMKTYNFKKNAPHPLDDFCRVLTVNTVGTFNVIRLSVAEIAQNEFNEDGERGVIINTASVAAFE  159
SpHsd17b10  SFGRLDVAVSCAGIGVAVKTYNFNKDRPHPLEEFCRVLMVNTAGTFNLARLAAGAMGKNEFNVDGERGIIINTASVAAFD  159

MgHsd17b10  GQMGQAAYSASKGAIVGMTLPIARDLANCGIRCCTIAPGLFNTPLLASLPEKVQTYLASTIPFPKRLGDPDEYAHMVQTI  238
XlHsd17b10  GQVGQAAYSASKGGIVGMTLPIARDLAPMGIRVVTIAPGLFATPLLVGLPEKVRTFLAKQVPFPSRLGDPGEYAHLVQSI  239
OcHsd17b10  GQVGQAAYSASKGGIVGMTLPIARDLAPMGIRVMTIAPGLFGTPLLTSLPEKVRNFLASQVPFPSRLGDPAEYAHLAQTI  240
BfHsd17b10  GQCGQVAYSASKGGIVGMTLPIARDLADKGIRVNTIAPGLFLTPLLNSLPDKVRSFLAKMVPYPQRLGHPDEYAHLVQSI  239
SpHsd17b10  GQMGQAAYSASKGAIVGMTLPIARDLASCGIRVCTIAPGLFDTPLLAGLPEKVRTFLARSIPFPSRLAHPDEYAHMVESI  239

MgHsd17b10  VENPYLNGEVIRVDGSIRMVA  259
XlHsd17b10  VENPMLNGEIIRLDGALRMQP  260
OcHsd17b10  IENPFLNGEVIRLDGAIRMQP  261
BfHsd17b10  IENPMLNGEVIRIDGAIRMPP  260
SpHsd17b10  VQNPILNGEVIRLDGALRMQP  260
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B

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MgHsd17b12  ...MASNVQSFLGNYSDLFAVAGVVSVSYIALKLALSIFNGIKVFLLAKPLGLTKNLKKCGQWAVVTGATDGIGKGYTHQ  77
HdsHsd17b12 ...MASAVQSYLGVYSDQFSVLGVLATAYVALKLFRWEYNFLKQHFLGPALGLSVNVKKAGPWAVVTGCTDGIGKAYAEQ  77
NlHsd17b12  MASLATMLCGYFGKSADIFTVIGAVTTAFFAFKATSSVLNGLFSYVLSGALGLSLNLENAGSWAVVTGCTDGIGKAYAEQ  80
MmHsd17b12  ........MESALPAAGFLYWVGAGTVAYLALRISYSLFTALRVWGVGNEAGVGP...GLGEWAVVTGGTDGIGKSYAEE  69
XtHsd17b12  ..MATESLAEVPVPGCNCFWYLGVVAAVWWGLRAAWCLLDGARVWVLGSGAQVGP...RIGKWAVVTGATDGIGKAYAEE  75

MgHsd17b12  LAKKGMDIILISRTKSKLEDCAKEIEQKFKVKTKIIVADFSAGLQIYDAIRSQLAGLDIGVLVNNVGMSYDFPNYFLEIP  157
HdsHsd17b12 LAKKGPNIVLMSRTQSKLDDLAKDLEGRFHIQTRVIAVDFTQ.PEIYSRIEREICGLEIGTLVNNVGMAYELPEFFFNIA  156
NlHsd17b12  LAKRGLNIVLISRTRSKLDELARDIESRFKVKTKVIAADFTR.SDIYDMIPQEIDGLDIGTLVNNVGVGYDHPEFFCQLE  159
MmHsd17b12  LAKRGMKVVLISRSQDKLDQVSSEIKEKFKVETRTIAVDFAS.EDIYDKIKTGLAGLEIGILVNNVGMSYEYPEYFLDVP  148
XtHsd17b12  LAKRGMNIVLISRSPEKLEEVAKQIKEKFKVETKIIAADFGKPTEIYGRIESGIRDLEIGVLVNNVGVSYEHPEYFLEIP  155

MgHsd17b12  DREKKIMDLLHVNITSVTMMTSVVLPEMVSRSRGVVINVASASGVNFCPLLTVYSASKAFVHYFSLQLEREYRDRGITVQ  237
HdsHsd17b12 NREKTVKDMLNCNLSNVMMISIVTPGMIERGRGYVINIASSAGERPMPLLALYSASKVFMDYLTKALCIEYGSKGITFQ  236
NlHsd17b12  NRDQVIMNIINCNSTSVAMMTSLVLPGMVSKKRGVIINIASAAGNRPTPLLTLYSATKSFVDFFSRALCHEYCPKGITVQ  239
MmHsd17b12  DLDNVIKKMINININLSVCKMTQLVLPGMVERSKGAILNISSGSGMFPVPLLTIYSATKTFVDFFSQCLHEEYRSKGIFVQ  228
XtHsd17b12  DLENTLDKMINININTSVCQMTRLVLPGMLGRGRGVILNISSASGMYPVPLLTVYSATKAFVDFFSRGLCAEYRSKGVTVQ  235

MgHsd17b12  SVLPYFVATKMSKVKKGSFWIPYPDQYAASALDTVGFEKATNGWSHSITGWLLDVIPKQTQMSILVRILLEKKKGAPIK  317
HdsHsd17b12 SVRPNFVATKLSGIRRSNPFCPYPDQYVRAALATLGISNATNGYFMTIQSTITALLP.....DFVVFNFLKSARKGALK  311
NlHsd17b12  VVLPYFVATKMSKIRKGGVFAPYPNDFVRSALHTVGLQSRTFGCWSHALQDKVAALLP.....TSVTMQMMEKARAKSLK  314
MmHsd17b12  SVLPYFVATKLAKIRKPTLDKPTPETFVKSAIKTVGLQSRTNGYLIHVLMGWIISN.LPSWIYLKIAMNMNKATRAHYLK  307
XtHsd17b12  SVLPFYVATKLAKIRKPTWDKPSPETYVQSALNTVGLQTQTNGYLPHAIMGWISTSLVPVSTAISLGMKMNKGLRARFLK  315

MgHsd17b12  RLNGSKTD..  325
HdsHsd17b12 RLERRKAKEN  321
NlHsd17b12  KKANKSE...  321
MmHsd17b12  KIKKN.....  312
XtHsd17b12  RAKQK.....  320
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Fig. 3 – Multiple alignments of MgHsd17b10 (A) and MgHsd17b12 (B) with other known 17β-HSD type 10 and 12 orthologues. The conserved co-factor binding motif (TGXXXGXG) and active site motif (YSASK) are marked with (.). The determined triad of Ser-Tyr-Lys residues is indicated by (*). The N-terminal putative mitochondrial target signal of MgHsd17b10 and the C-terminal active site of MgHsd17b12 are indicated by (#). MgHsd17b10 (*Mytilus galloprovincialis*, KF031120); XlHsd17b10 (*Xenopus laevis*, NP001087068); OcHsd17b10 (*Oryctolagus cuniculus*, XP002720063); BfHsd17b10 (*Branchiostoma floridae*, XP002597291); SpHsd17b10 (*Strongylocentrotus purpuratus*, XP780014); MgHsd17b12 (*Mytilus galloprovincialis*, KF031121); HdsHsd17b12 (*Haliotis diversicolor supertexta*, ADF80270); NlHsd17b12 (*Nucella lapillus*, AFV95595) MmHsd17b12 (*Macaca mulatta*, EHH22972); XtHsd17b12 (*Xenopus tropicalis*, NP001017234).

Furthermore, both MgHsd17b10 and MgHsd17b12 were detected in adductor muscle. In vertebrates as well as *Drosophila*, 17β-HSD type 10 is involved in the catalysis of the third step of the mitochondrial β-oxidation, the oxidation of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA, for medium- and short-chain fatty acids (He et al., 2001; Torroja et al., 1998). Study in neogastropods defined that 17β-HSD type 12 might also involved in the lipid metabolism except the steroidogenesis role found in other mollusks (Lima et al., 2013; Zhou et al., 2011). Therefore, the tissue distribution pattern and structure prediction of MgHsd17b10 and MgHsd17b12 (which may be localized in mitochondria) here implied that both 17β-HSDs were perhaps involved in lipid metabolism except their traditional role in endocrine regulative function. Moreover, the transcripts of MgHsd17b10 and MgHsd17b12 exhibited sex-dependent expression pattern in hemocytes. MgHsd17b10 showed higher expression level in female hemocytes, while MgHsd17b12 exhibited higher level in male hemocytes. Recent studies have demonstrated that steroid hormones played an important role in hemocytes. The estradiol in hemocytes can modulate immune functions via active estrogen receptors (Canesi et al., 2004). And 17β-HSDs may be involved in the regulation of steroid metabolism in hemocytes. The different basal gene expression level between male

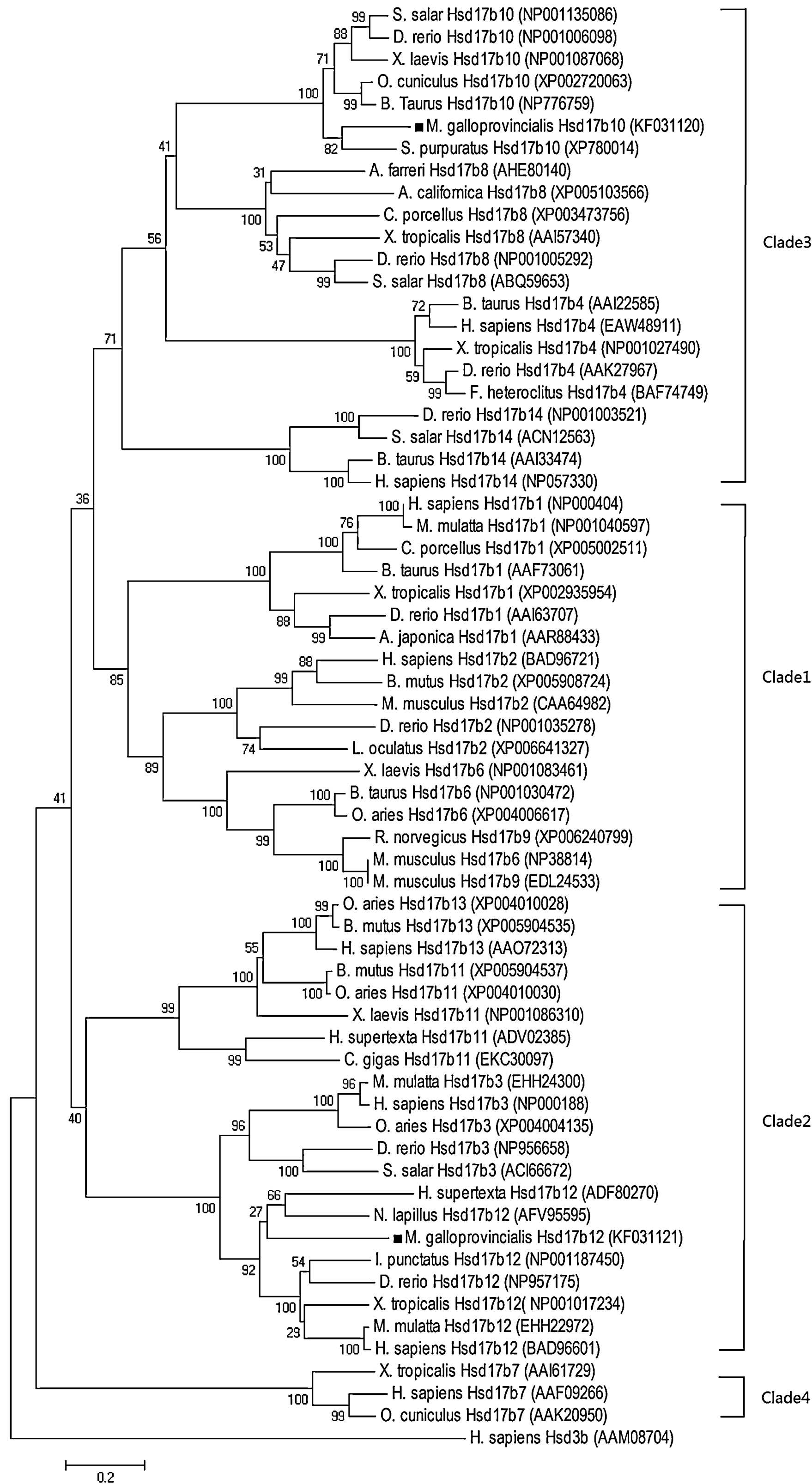

Fig. 4 – Phylogenetic trees constructed by neighbor-joining method based on the sequences of 17β-HSDs among various species. Numbers at the forks indicate the bootstrap values (in %) out of 1000 replicates.

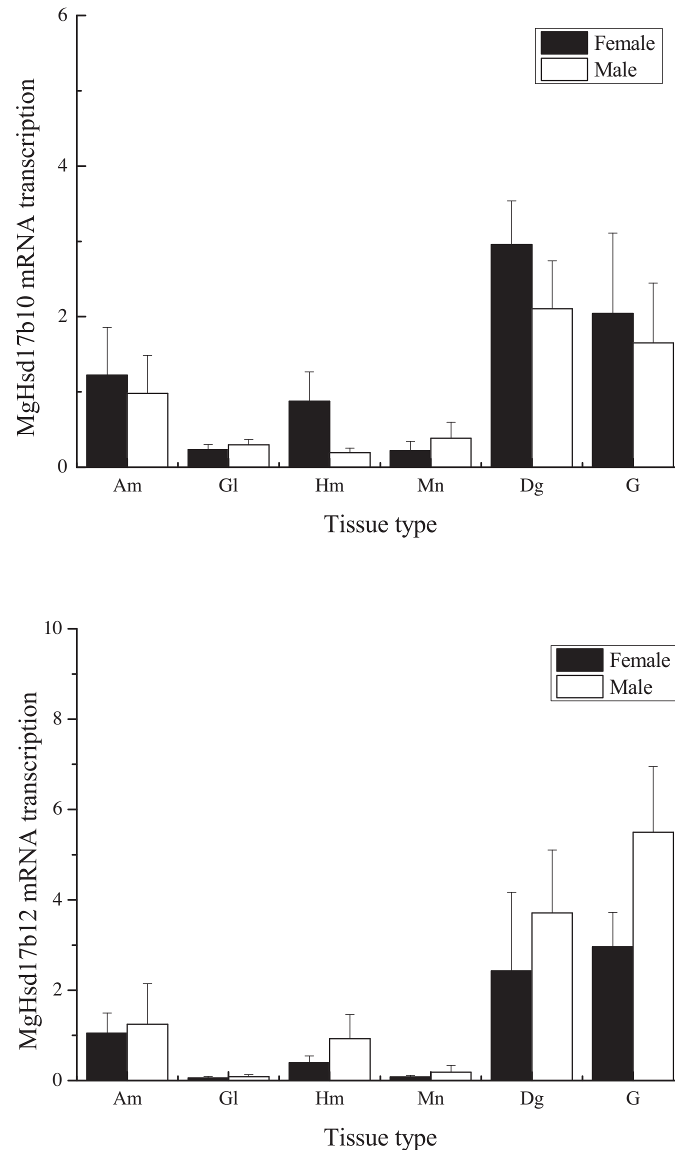

Fig. 5 – Tissue-specific expression of MgHsd17b10 (A) and MgHsd17b12 (B) mRNA measured by quantitative real-time PCR. The mRNA expression level is calculated relative to 28S rRNA expression. Values are mean ± standard deviation (S.D.) (*n* = 5–6). *Abbreviations*: Am, Adductor muscle; Gl, Gill; Hm, Hemocytes; Mn, Mantle; Dg, Digestive Glands; G, Gonad.

and female mussels perhaps implied their different potential role. Similar results were also found in other mollusks (Zhou et al., 2011; Lima et al., 2013).

In order to gain a further insight into the functional role of MgHsd17b10 and MgHsd17b12, the pattern of gene expression in the digestive glands was analyzed following with exposures to BPA and BDE-47. It was found that both BPA and BDE-47 performed strong estrogenic effects on mussels. The mRNA expression levels of 17β-HSD type 10 and 12 in both sexes decreased significantly at 24 h post-exposure and the expression level of both genes in females returned to control at 96 h. These BPA (or BDE-47)-induced modulative effects are consistent with the results obtained from some vertebrates (Karpeta et al., 2011). For example, BPA exposure significantly decreased the expression level of mammal 17β-HSDs (Nakamura et al., 2010). Recent studies showed that exposure to tributyltin (TBT) chloride resulted in a significant decrease of 17β-HSD type 12 mRNA levels in digestive glands of neogastropods (Lima et al., 2013).

Estrogen receptors (ERs), as the primary mediators of estrogen signaling, play a key role in endocrine function and reproductive system in vertebrates. They mediate the endocrine disruption by EDCs that mimic or block estrogen action (Urbatzka et al., 2012). The occurrence of EDCs and their genomic action through binding to the nuclear ER, consequently leading to an up- or down-regulation of specific genes, have been reported in vertebrates (Matthews et al., 2001). Although ER-like sequences have been identified in different species of mollusks (Keay et al., 2006; Oehlmann et al., 2007), information on their function and regulation remains largely unknown. Therefore, whether the gene expression of 17β-HSDs was affected by BPA/BDE-47

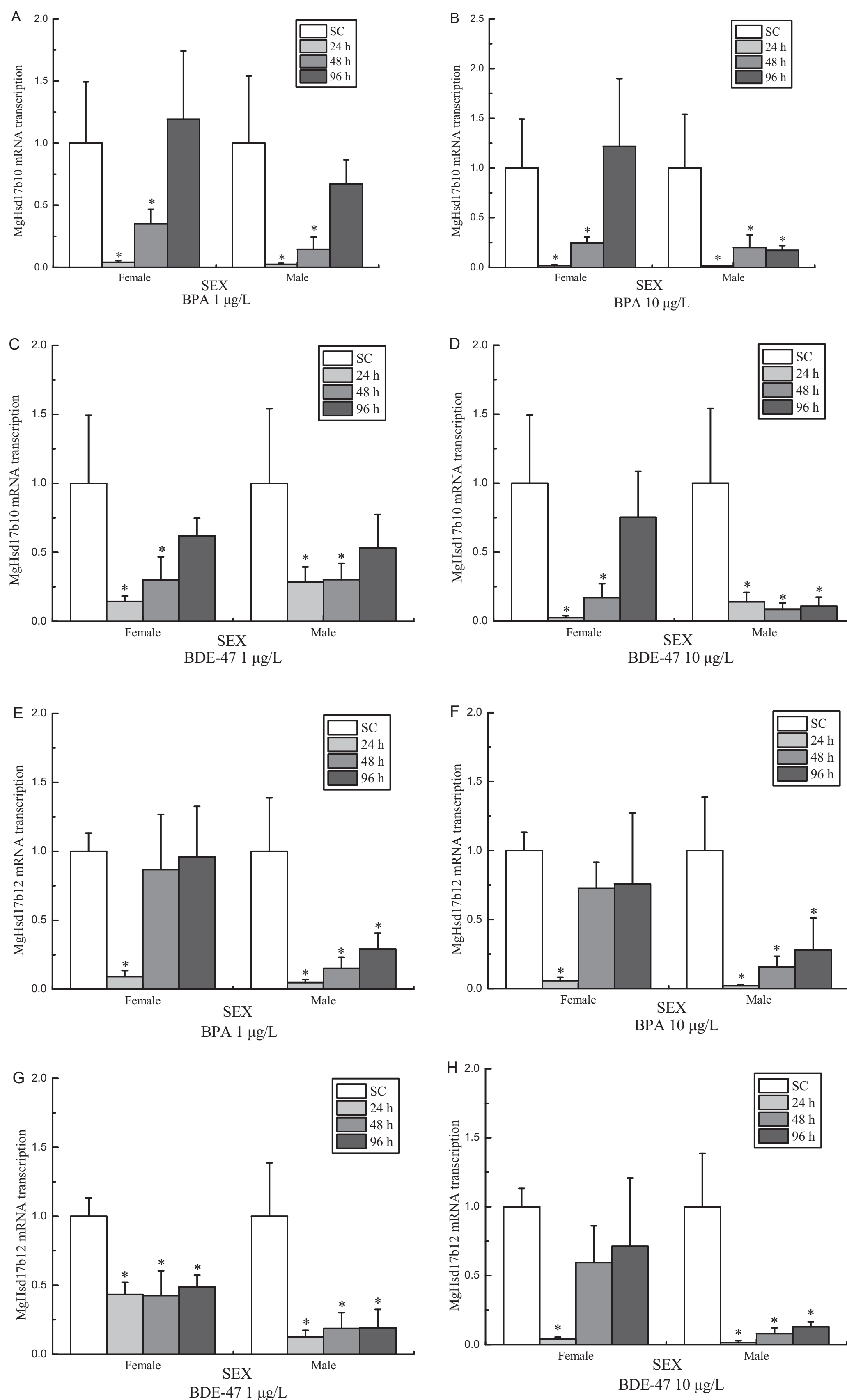

Fig. 6 – The expression levels of MgHsd17b10 (A, B, C, D) and MgHsd17b12 (E, F, G, H) transcript in digestive glands after BPA and BDE-47 exposure. (A, E: 1 μg/L BPA; B, F: 10 μg/L BPA; C, G: 1 μg/L BDE-47; D, H: 10 μg/L BDE-47). Values are mean ± standard deviation (S.D.) (n = 5–6). An asterisk (*) indicates the significant difference (P < 0.05) between the 24 h solvent control (SC) and treatment.

through this nuclear ER-mediated way in mussel remains to be elucidated.

Additionally, non-genomic signaling pathway has been investigated in mussels (Canesi et al., 2004). In mammals, the non-genomic effect is rapid and mediated by membrane associated ER. This effect caused an activation of mitogen activated protein kinases (MAPKs) and phosphatidylinositol 3-kinases, which consequently modulate transcriptional events (Prossnitz et al., 2008). Studies have shown that the responses to estrogens are rapid in mussel hemocytes and ganglia (Stefano et al., 2003). Interestingly, E2, BPA, nonylphenol were shown to distinctly affect the phosphorylation state of different transcription factors in mussel as a consequence of the modulation of kinases/phosphatases (Canesi et al., 2004).

Most steps of the steroidogenic and steroid metabolic pathways described for vertebrates have been demonstrated to occur in invertebrates, especially in mollusks (Janer and Porte, 2007). Some endocrine disruptor chemicals induce alterations in these metabolic pathways and might lead to changes in steroid levels. Recent researches found that several key regulators, such as 17β-HSDs, 3β-HSDs, 5α-reductases (5α-R) were affected by EDCs, including disrupted activities and changed gene expressions (Fernandes et al., 2011; Cubero-Leon et al., 2012b). Overall, the alteration of gene transcription of MgHsd17b10 and MgHsd17b12 after exposure to BPA/BDE-47 may hint to the function of steroid metabolism and steroidogenic in mussel.

Moreover, the expressions of MgHsd17b10 and MgHsd17b12 in male mussels seem be more sensitive to BPA or BDE-47 treatment. Similar results were also found in the expression of vitellogenins (Vtg), which is the major precursor of the egg-yolk proteins. For example, exposure of EDCs induced a significant increase in the transcript of Vtg mRNA in the liver of male zebra fish (Uren-Webster et al., 2010; Urbatzka et al., 2012). Significant increase of Vtg transcript was also observed in male clams post nonylphenol (NP) treatment (Matozzo and Marin, 2005). Testosterone and the androgen receptor (AR) play the key role in the endocrine system of male mollusks (Oehlmann et al., 2007). It is noteworthy that BPA and BDE-47 are potential antiandrogens except their affinity to estrogen receptor (Stange et al., 2012; Stoker et al., 2005). They displayed a noticeable affinity to AR and inhibited AR binding, causing inhibition dihydrotestosterone-induced transcriptional activation (Stoker et al., 2005). In addition, the EDCs also affected aromatase activity in mollusks. The aromatization of androgens into estrogens, which occurs at a very low rate, significantly increased after exposure to a dose of EDCs (Janer et al., 2005). Therefore, further research is required to unravel the mechanism of male-specific sensitivity toward BPA and BDE-47 exposure.

5. Conclusions

In the present study, we identified and characterized two novel types of 17β-HSDs from *M. galloprovincialis*. Multiple alignment results implied they were new members of 17β-HSD type 10 and 17β-HSD type 12 families, respectively. Both 17β-HSD type 10 and 12 were broadly distributed in healthy mussel tissues, and were down-regulated in digestive glands in response to BPA or BDE-47 exposure. The temporal expression profiles indicated that MgHsd17b10 and MgHsd17b12 perhaps played an important role in steroidogenesis and steroid metabolism.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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