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# Identification and molecular characterization of doublesex and mab-3-related transcription factor (dmrt) in brackish water flea, *Diaphanosoma celebensis*, exposed to bisphenol analogs

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**Abstract:** Doublesex and mab-3 related transcription factor (dmrt) play crucial roles in sex determination and sex differentiation in vertebrates and invertebrates. Although *dmrt* genes have been identified in vertebrates, little is known about aquatic invertebrates. In this study, two *dmrt* genes, namely, *Dc_dmrt93B* and *Dc_dmrt99B*, were identified from brackish water flea, *Diaphanosoma celebensis*. Transcriptional changes were observed in the *dmrt* genes when the flea was exposed to bisphenol (BP), an endocrine disruptor. Sequence and phylogenetic analyses showed that both *dmrt* genes contained two conserved domains, namely, DM and DMA, closely clustered with those of *Daphnia* spp. Additionally, a significant increase in the *Dc_dmrt99B* mRNA expression level was observed upon exposure to intermediate concentrations of BP (bisphenol A > bisphenol S = bisphenol F, $p < 0.05$), while the expression of *Dc_dmrt93B* mRNA was slightly modulated. These findings imply that the two *dmrt* genes may be involved in sex differentiation of *D. celebensis*. Furthermore, it was found that the ability of BP to modulate *dmrt* genes could affect development and reproduction. This study provides a basis for understanding the function of the *dmrt* genes and the molecular mode of action of BP in small crustaceans.

**Keywords:** bisphenol analogs, crustaceans, *Diaphanosoma celebensis*, *dmrt*

## INTRODUCTION

Bisphenols (BPs) are chemicals with two phenolic hydroxyl functional groups. They are widely used as industrial additives to produce polycarbonate plastics and epoxy resins (Ruan *et al.* 2015; Hu *et al.* 2019). Bisphenol A (BPA) is most often used in the production of food containers, thermal receipts, toys, medical equipment, and electronics (Caballero-Casero *et al.* 2016). However, the use of BPA has been restricted or regulated in many industrial fields because it can cause ROS production, DNA damage, gene mutagenesis; inhibit reproductive development; impair glucose and lipid metabolism; and disrupt the endocrine system (Meli *et al.* 2020). Therefore, BPA analogs such as bisphenol S (BPS) and bisphenol F (BPF) have been developed as alternative substances to replace the use of BPA in a variety of consumer products (Morales *et al.* 2020). BPS is used to make epoxy glues, baby bottles, and artistic organs etc.; BPF to make lacquers and dental sealants (Hu *et al.* 2019; Liu *et al.* 2021). However, it is essential to assess the risks associated with the use of BPA analogs, as several studies have demonstrated that BPA analogs also can cause similar to or higher toxic effects than BPA in genetic, cellular, and reproductive level (Chen *et al.* 2016; Wu *et al.* 2018; Liu

 

*et al.* 2021). Owing to their wide usage, BP analogs are easily detected in the environment. In the Han River in South Korea, BPA (141 ng $L^{-1}$), BPS (41 ng $L^{-1}$), and BPF (633 ng $L^{-1}$) have been detected (Yamazaki *et al.* 2015). In addition, BPA (1520 ng $g^{-1}$ dw), BPS (44.9 ng $g^{-1}$ dw), and BPF (384 ng $g^{-1}$ dw) were detected in sludge from sewage treatment plants in Korea (Lee *et al.* 2015). The decomposition products of BP are discharged into the aquatic ecosystems via industrial wastewater, landfill leachate, urban sewage, and sludge (Caballero-Casero *et al.* 2016; Liu *et al.* 2021). Therefore, studies on the toxicity of BP to aquatic organisms are important.

Reports highlight the detrimental effects of BP analogs on reproduction in aquatic organisms. For example, zebrafish (*Danio rerio*) showed abnormal sex ratios with an excess of females after ingesting food containing BPA (200 mg $kg^{-1}$) (Drastichová *et al.* 2005). Only a few studies exist on the effects of BP on crustaceans. Superfeminization syndrome was found in the freshwater invertebrate *Marisa cornuarietis* after chronic exposure to low BPA concentrations (1 μg $L^{-1}$) (Oehlmann *et al.* 2006). When *D. rerio* was exposed to 10 and 100 μg $L^{-1}$ of BPS, the proportion of females increased by approximately 12% and 20%, respectively, and the spawning rate, hatching rate, and sperm count decreased (Naderia *et al.* 2014). Liu *et al.* (2019) found a gradual decrease in the number of neonates and suppressed reproduction in *Daphnia magna* during single and mixed exposures of BPA, BPF, and BPS for 21 days. Thus, BP may induce sexual disturbances and affect the reproduction of invertebrates (Canesi and Fabbri 2015). However, little information is available on the effects of BP at the molecular level in small crustaceans.

The doublesex and mab-3 related transcription factor (*dmrt*) family is generally known to be involved in development and sex determination in vertebrates (Kopp 2012). In mammals, eight *dmrt* genes (*dmrt1*–*dmrt8*) have been identified; *dmrt1* gene encoding the doublesex (dsx) and mab-3 proteins plays a key role in sex determination and differentiation (Bellefroid *et al.* 2013). In vertebrates, four *dmrt* genes (*dmrt2a/2b*, *dmrt3*, *dmrt4/5* (*dmrt99B*), and *dmrt93B*) are commonly found (Mawaribuchi *et al.* 2019). Three *dmrt* genes (*dmrt11E* (*2a/2b*), *dmrt99B* (*4/5*), and *dmrt93B*) found in Arthropoda have been suggested to be originated from a common ancestor of bilaterian animals (*dmrt2a/2b*, *dmrt4/5*, and *dmrt93B*, respectively). In cladocerans such as *D. magna*, environmental sex determination may be related to the expression of *dmrt93B* only in the testis (Kato *et al.* 2008). On the contrary, *dmrt99B* was suggested to be involved in vitellogenesis due to its higher expression in the ovary. However, the role of *dmrt* genes in aquatic invertebrates, particularly cladocerans, remains largely unknown.

Cladocerans play an important role in the food chain as primary consumers; they are supplied as live food to higher order animals (Marcial and Hagiwara 2007). *Diaphanosoma celebensis*, a euryhaline cladoceran, can inhabit conditions with a wide range of temperature (15–35°C) and salinity (7–32 ppt). It is easy to maintain in a laboratory owing to its short life cycle (4–5 days) and small size in adulthood (Marcial and Hagiwara 2007; Kim *et al.* 2018; Yoo *et al.* 2019). Recently, *D. celebensis* was proposed as a model organism for studies on the effects of BP analogs on molting and reproduction of aquatic organisms (In *et al.* 2019, 2020).

In the present study, two *dmrt* genes were identified in *D. celebensis*. Changes in the *dmrt* gene expression were further investigated after exposure to BP analogs to understand the role of *dmrt* and the effects of BPs on the organism. This study holds implications in understanding the effects of BP on reproduction in aquatic invertebrates.

## MATERIALS AND METHODS

### 1. Experimental organism and culture maintenance

The experimental organism, *D. celebensis* were obtained from the Korea Institute of Ocean Science & Technology (KIOST; South Korea) and maintained in Molecular Toxicology Laboratory at Sangmyung University since 2016. The cultured medium used 15 psu (practical salinity unit) of artificial seawater using filtered Instant Ocean (Aquarium systems, France) with 0.2-μm filters (Whatman, UK) and renewed once a week. The culture conditions were as follows: 25 ± 1°C and a photoperiod of 12 h : 12 h light/dark. *D. celebensis* fed with $1.0–3.0 \times 10^8$ cells $L^{-1}$ of *Chlorella vulgaris* daily as a food source.

### 2. Reagents

All chemical reagents were purchased from Sigma-Aldrich Co. (Saint Louis, USA). The stock solutions were prepared as follows: BPA (3 mg $L^{-1}$), BPS (23 mg $L^{-1}$), and BPF (5 mg $L^{-1}$) in dimethyl sulfoxide (DMSO). All oligonucleotide synthesis and DNA sequencing were performed at Macrogen (Seoul, South Korea) and Bionics (Seoul, South Korea), respectively.

### 3. Exposure test

The *D. celebensis* (4-day-old; 200 individuals per concentration) was exposed to BPA (0.12, 0.6, and 3 mg $L^{-1}$), BPS (0.92, 4.6, and 23 mg $L^{-1}$), BPF (0.2, 1, and 5 mg $L^{-1}$) for 48 h, based on the previous study (In *et al.* 2019). *D. celebensis* were not fed during the exposure period. The DMSO solvent concentration did not exceed 0.05%, where no mortality was observed.

### 4. Total RNA extraction and cDNA synthesis

After exposure to BP analogs, *D. celebensis* were harvested, total RNA was extracted using 500 µL of Trizol reagents, according to manufacturer's instruction. The purity and quantity of the extracted total RNA were checked by gel electrophoresis and nanodrop spectrophotometry (Maestrogen Inc., Taiwan) and stored at −80°C until use. The cDNA was synthesized from 500 ng of the total RNA using Revert Aid First strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA) and kept at −20°C.

### 5. Polymerase chain reaction (PCR)

The cDNA sequences of *dmrt99B* and *dmrt93B* were obtained from the local database of *D. celebensis* transcriptome (Molecular Toxicology Laboratory, Sangmyung University). The sequence was confirmed using Touchdown PCR. All PCR reactions contained 5 µL of 10X reaction buffer, 5 µL of dNTP (2 mM), 4 µL of 10 pmol primer set, 2 µL of cDNA, 0.5 µL of Geneall Taq (5 U $\mu L^{-1}$), and 33.5 µL of PCR-grade water. The PCR primer was designed using the NetPrimer (http://www.premierbiosoft.com/netprimer/) and Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/). Primer sequence information is shown in Table 1. PCR condition was as follows: 15 cycles of 95°C/3 min, 95°C/30 sec, 65.5°C/45 sec, 72°C/1 min 30 sec; 20 cycles of 95°C/30 sec, 54°C/45 sec, 72°C/1 min 30 sec; and finally, a final extension step at 72°C/5 min. The PCR products were confirmed to 1% agarose gel electrophoresis and directly sequenced. Conserved domains/motifs were analyzed by the conserved domain searching of National Center for Biotechnology Information (NCBI).

### 6. Phylogenetic analysis

Deduced amino acid sequences of *D. celebensis dmrt99B* and *dmrt93B* were aligned with those of other species retrieved from GenBank using Clustal X (version 1.83) and visualized using GeneDoc (version 2.6.002). GenBank accession numbers of other species dmrt99B and dmrt93B are indicated in Table 2. A phylogenetic analysis was performed to confirm the systematic location of *D. celebensis* dmrts, and tree

**Table 1.** Primer sets used in this study

| Gene | Oligo name | Sequence (5′→3′) | Amplicon | Remarks |
|---|---|---|---|---|
| *dmrt93B* (MW836827) | Seq_F1 | CGTGTCGGTCATGGAAGC | 749 bp | PCR for sequencing |
| | Seq_R1 | GACCTGGCGAACGACTTC | | |
| | Seq_F2 | GCAGCAGAAAGGTCGGTG | 694 bp | |
| | Seq_R2 | CGGTTTTCTTCAACTTGACATC | | |
| | RT_F | CGTTGAGC GGCAGTAAATC | 98 bp | Real-time RT-PCR |
| | RT_R | GTGCGGATGAGTCAGGTC | | |
| *dmrt99B* (MW836828) | Seq_F1 | ACCGCCGTTCTCCTCTACAA | 479 bp | PCR for sequencing |
| | Seq_R1 | GGGCTTCGTTCTCTTCCTGA | | |
| | Seq_F2 | CTATCAGCGGACACCGAAATG | 864 bp | |
| | Seq_R2 | GTCTTCAGGATGTTGCGTTTG | | |
| | Seq_F3 | CAAACGCAACATCCTGAAGAC | 680 bp | |
| | Seq_R3 | GCGACGGAAATGGTAAACAAAG | | |
| | RT_F | CAAACGCAACATCCTGAAG | 90 bp | Real-time RT-PCR |
| | RT_R | GCTGACGGATTGTGGTAGAC | | |
| *18S rRNA* (AF144210.1) | RT_F | TGGAAGGATTGACAGATTGA | 81 bp | Real-time RT-PCR |
| | RT_R | AAATCGCTCCACCAACTAAG | | |

 

**Table 2.** GenBank accession numbers of the sequences used for phylogenetic analysis

| Protein name | Common name | Species | Accession No. |
|---|---|---|---|
| dmrt93B | Mosquito | *Anopheles gambiae* | XP_321748 |
| | Water flea | *Daphnia magna* | BAG12872 |
| | | *Daphnia pulex* | EFX89054 |
| | Brackish water flea | *Diaphanosoma celebensis* | MW836827 |
| | Fruit fly | *Drosophila melanogaster* | NP_524428 |
| | | *Drosophila pseudoobscura* | XP_001360059 |
| | Beetle | *Tribolium castaneum* | XM_966511.1 |
| dmrt99B | Mosquito | *Anopheles gambiae* | XP_310668 |
| | Water flea | *Daphnia magna* | BAG12873 |
| | | *Daphnia pulex* | EFX84867 |
| | Brackish water flea | *Diaphanosoma celebensis* | MW836828 |
| | Fruit fly | *Drosophila melanogaster* | NP_524549 |
| | | *Drosophila pseudoobscura* | XP_001357766 |
| | Beetle | *Tribolium castaneum* | XP_975675.1 |
| dmrt1 | Human | *Homo sapiens* | NP_068770 |
| | House mouse | *Mus musculus* | NP_056641 |
| dmrt2 | Human | *Homo sapiens* | NP_006548 |
| | House mouse | *Mus musculus* | NP_665830 |
| dmrt3 | Human | *Homo sapiens* | NP_067063 |
| | House mouse | *Mus musculus* | AAN77230 |
| dmrt4 | Human | *Homo sapiens* | NP_071443 |
| | House mouse | *Mus musculus* | NP_783578 |
| dmrt5 | Human | *Homo sapiens* | CAC37946 |
| | House mouse | *Mus musculus* | AAN10254 |

was constructed by 1000 replication bootstraps with neighbor-joining methods using MEGA (version 6.0).

## 7. Quantitative real time - polymerase chain reaction (qRT-PCR)

We performed qRT-PCR to investigate the mRNA expression changes of *dmrt99B* and *dmrt93B* by exposure to BPs using the CFX96$^{TM}$ real-time PCR system. The reactant was 3 μL of cDNA (500 ng), 2 μL of 10 pmol primer set. The PCR product was analyzed on a 1% agarose gel under UV transilluminator to check the single band for confirming the specific amplification of target genes. The PCR efficiency is included in the range of 90−110% (92.9% for *18S*; 102.8% for *dmrt93B*; and 107.7% for *dmrt99B*). PCR cycle condition was as follow: 95°C/10 min, followed by 33 cycles of 95°C/15 sec, 60°C/1 min. Finally, in order to check the amplification of a specific product, the melting curve reaction was increased by 0.5°C every 5 sec from 65°C to 95°C. All the experiments used SYBR master mix (KAPA Bioassay System, USA), and were performed in triplicate. The threshold cycle (Ct) was normalized by *18S rRNA*. The fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

## 8. Statistical analysis

Data from all the experiments were represented as the mean ± standard deviation (S.D.) of three replicates. Comparison of relative mRNA expression level was analyzed using one-way analysis of variance (one-way ANOVA) followed a Tukey's test and *t*-test. The PASW Statistics ver. 18.0 program (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A *p*-value below 0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

### 1. Sequence analysis and phylogenetic relationship of *D. celebensis dmrt93B* and *dmrt99B*

The partial cDNA sequence of *dmrt93B* was 1236 bp in length and consisted of a 33 bp long 5′-untranslated region (UTR) and 1203 bp long open reading frame (ORF) with complete domains that encode a putative polypeptide of 401 amino acids (aa) (Suppl. Fig. 1). The complete cDNA sequence of *dmrt99B* was 2422 bp in length with 5′-UTR of 140 bp, an ORF of 1755 bp encoding a putative polypeptide of 584 aa, and a 3′-UTR of 527 bp (Suppl. Fig. 2). The length of the dmrt93B polypeptide was 321 aa and 400 aa in the rotifer *Brachionus koreanus* (Kim *et al.* 2014) and *D. magna* (Kato *et al.* 2008), respectively. Notably, *dmrt99B* has been previously cloned and characterized in *B. koreanus* (381 aa; Kim *et al.* 2014), *D. magna* (603 aa; Kato *et al.* 2008), and the giant prawn *Macrobrachium rosenbergii* (618 aa; Yu *et al.* 2014).

*D. celebensis* dmrt93B and dmrt99B proteins had two conserved domains; the DM domain and the DMA domain (Suppl. Figs. 1 and 2). Multiple alignments of the deduced amino acid sequences of these domains from *D. celebensis* and other species revealed their highly conserved nature across species (Fig. 1). Additionally, the DM domain was found to contain a nuclear localization signal (NLS; KGHKR, 18–22 aa) and two zinc ($Zn^{2+}$) binding sites (CCHC and HCCC). The DM domain is also called the DM DNA-binding domain and is named from doublesex (dsx) and mab-3. where dsx has one amino-terminal domain and mab-3 has two amino-terminal domains. The dsx DM domain binds to and dimerizes palindromic DNA (Narendra *et al.* 2002). The DMA domain can be identified in the carboxyl-terminal of the DM domain; a DM domain protein with this motif is referred to as a DMRTA protein, and is considered as the DMRTA motif. The function of the DMA domain remains unknown.

In the present study, sequence alignments of the domain amino acids demonstrated that the DM domain was highly conserved whereas the DMA domain exhibited low identity across species. The DM domains of Dc_dmrt93B and Dc_dmrt99B shared 80% identity. Dc_dmrt93B and dmrt99B had the highest identity with those of *D. magna* (100% and 97%, respectively) and *Daphnia pulex* (100% and 100%, respectively) (Suppl. Fig. 3). Considering the DMA domain, Dc_dmrt93B shared a low identity of 34% with Dc_dmrt99B. Dc_dmrt93B showed the highest identity with that of *D. magna* (61% for 93B; 71% for 99B) and *D. pulex* (60% for 93B; 72% for 99B) (Suppl. Fig. 4). Regions other than the DM domain showed low similarity, which may contribute to distinct phylogenetic clusters (Suppl. Fig. 5).

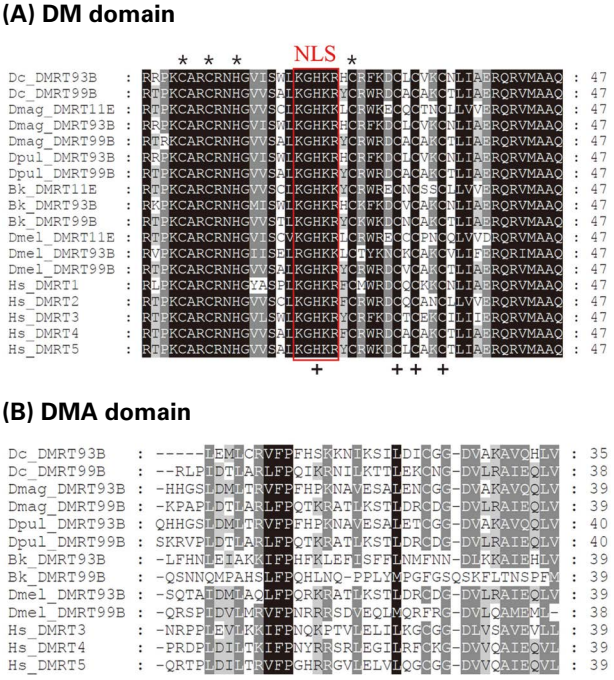


**Fig. 1.** Multiple alignments of the deduced amino acid sequences of (A) DM domain and (B) DMA domain of *Diaphanosoma celebensis dmrt93B* and *dmrt99B* genes using Clustal X and GenDoc. The red box represents the conserved nuclear localization signal (NLS). The asterisk and the cross indicate two conserved zinc ($Zn^{2+}$) binding sites for site I (CCHC) and site II (HCCC), respectively. The shaded region indicates conserved residues. Abbreviations: Dc (*Diaphanosoma celebensis*), Dmag (*Daphina magna*), Dpul (*Daphnia pulex*), Bk (*Brachionus koreanus*), Dmel (*Drosophila melanogaster*), Hs (*Homo sapiens*).

Phylogenetic analysis revealed that the dmrts of *D. celebensis* closely clustered with those of the other invertebrates, particularly *Daphnia* spp. (Fig. 2). As expected, the dmrt93B group was separated from the dmrt99B group in invertebrates, in which dmrts (dmrt1 to dmrt5) of mammals were distinctly clustered from those of invertebrates. Kato *et al.* (2008) also found similar results wherein *Daphnia* dmrt99B and dmrt93B were grouped separately. Each gene was clustered with that of the insects and was distinct from those of the vertebrates. Notably, the Bayesian tree of bilaterian *dmrt* genes depicts the same clustering pattern (Mawaribuchi *et al.* 2019).

 

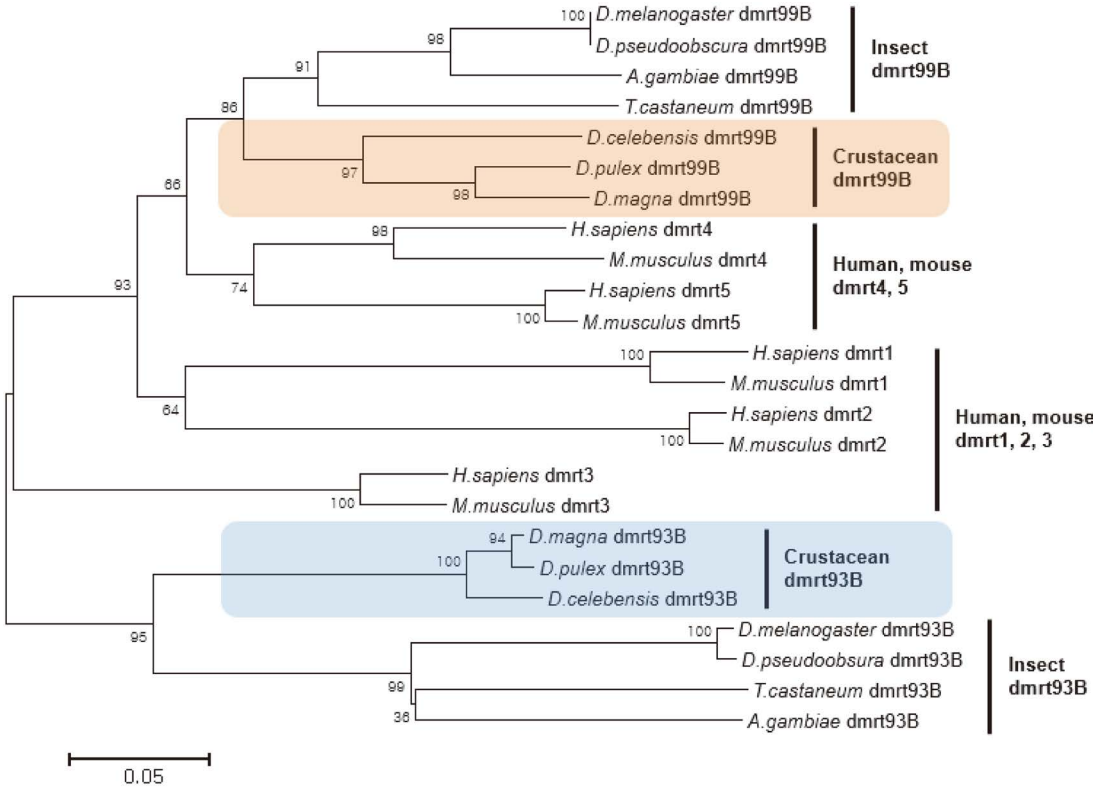


**Fig. 2.** Phylogenetic analysis of the deduced amino acid sequences of *Diaphanosoma celebensis dmrt93B* and *dmrt99B* with those of other species retrieved from GenBank (Table 2). The phylogenetic tree was constructed by the neighbor-joining method using MEGA (version 6.0). Bootstrapping replications of 1,000.

## 2. Transcriptional modulation of *D. celebensis dmrt93B* and *dmrt99B* after exposure to BP analogs

The changes in the *dmrt* genes were investigated at the transcriptional level in *D. celebensis* after 48 h of exposure to three BP (BPA, BPS, and BPF). Both genes showed similar expression patterns after exposure. When compared with the control group, the lowest (BPA 0.12 mg $L^{-1}$, BPS 0.92 mg $L^{-1}$, and BPF 0.2 mg $L^{-1}$) and highest (BPA 3 mg $L^{-1}$, BPS 23 mg $L^{-1}$, and BPF 5 mg $L^{-1}$) concentrations of each chemical did not significantly modulate the expression level of these genes (Fig. 3). However, at their intermediate concentrations (BPA 0.6 mg $L^{-1}$, BPS 4.6 mg $L^{-1}$, and BPF 1 mg $L^{-1}$), the expression of both *dmrt* genes was upregulated ($p < 0.05$); the *Dc-dmrt99B* mRNA level was higher than that of *Dc-dmrt93B*. In particular, the expression of *dmrt99B* was approximately 3.5-fold higher than that of the control group at 0.6 mg $L^{-1}$ of BPA ($p < 0.001$). On the contrary, mRNA levels of both *dmrt* genes were lower ( > 2-fold change, $p < 0.05$) upon exposure to BPS and BPF when compared with those after exposure to BPA. The U- or inverted U-shaped curve obtained at different concentrations of BP analogs indicates non-monotonic dose response effects and is a characteristic of endocrine-disrupting compounds (EDCs), in particular BPA (Vandenberg 2013; Zhang *et al.* 2016). In this case, no response or decreased response are detected at low and high concentration of EDCs, which can be challenging for risk assessment of their effects (Beronius and Vandenberg 2015; Lagarde *et al.* 2015).

The dmrt plays an essential role in the development and differentiation of male testicular in crustaceans such as the Chinese mitten crab *Eriocheir sinensis* (Zhang *et al.* 2010). This family is involved in germline development, embryonic development, and sexual maturation in the river prawn, *Macrobrachium nipponense* (Wang *et al.* 2019). Moreover, the dmrt of *D. magna* contributes to environmental sex determination and reproduction (Kato *et al.* 2008, 2011). However, the role of dmrt in *D. celebensis* remains unknown.

Several studies strongly support the fact that sex different-

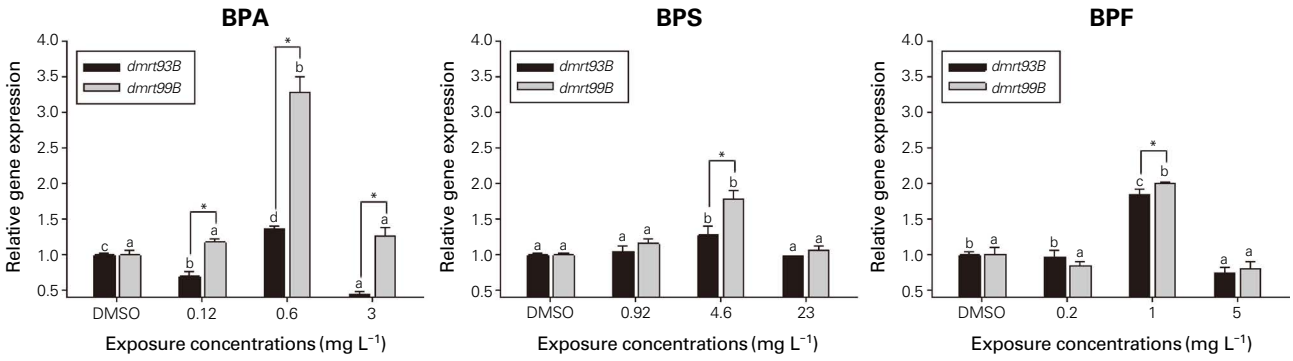


**Fig. 3.** mRNA expression of *dmrt93B* and *dmrt99B* in *Diaphanosoma celebensis* exposed to BPA (0.12, 0.6, and 3 mg $L^{-1}$), BPS (0.92, 4.6, and 23 mg $L^{-1}$), and BPF (0.2, 1, and 5 mg $L^{-1}$) for 48 h. Different letters indicate significant differences among concentrations determined using one-way ANOVA followed by Tukey's test. Asterisks represent significant differences between genes using *t*-test. *p*-value below 0.05 was considered as statistically significant.

iation-related genes are potential molecular targets for EDCs in vertebrates and invertebrates (Iguchi *et al.* 2006; Zhang *et al.* 2008; Rhee *et al.* 2011; Toyota *et al.* 2021). Kim *et al.* (2014) studied the effects of benzo[a]pyrene (a known reproductive toxin) in monogonont rotifer *B. koreanus*. They observed that a decrease in the mRNA levels of *dmrt11E*, *dmrt93B*, and *dmrt99B* may be related to growth retardation and reproduction inhibition. In *D. magna* exposed to fenoxycarb, a juvenile hormone analog, the testis-specific *dmrt93B* mRNA expression was upregulated (Kim *et al.* 2011). Together with our results, these findings suggest that the dmrt family of proteins may be a molecular target for environmental chemicals that interrupt the steroid hormone pathway and result in negative effects on the growth, development, and reproduction of cladocerans.

Kato *et al.* (2008) have suggested a sex-dependent, dimorphic expression of *dmrt93B* and *dmrt99B* in *D. magna*. Here, *dmrt93B* was testis-specific and *dmrt99B* was ovary-specific. In particular, they assumed that *dmrt99B* might be involved in the production of vitellogenin. In the present study, the higher expression of *Dc_dmrt99B* compared to that of *Dc_dmrt93B* upon BP exposure seems to be related to the reproductive strategy of parthenogenesis adopted by *D. celebensis*. In our previous study, *vitellogenine* mRNA levels were upregulated upon exposure to BPA and BPS but reduced in the BPF-exposed group (In *et al.* 2020). These findings suggest that BP may affect reproduction in *D. celebensis* by regulating the *Dc_dmrt99B* expression by different modes. However, the modulation of additional genes and endogenous hormones during development and reproduction needs to be further studied for a better understanding of the molecular mechanisms underlying sex differentiation, and the effect of EDCs such as BP on *D. celebensis*.

In conclusion, we identified the *dmrt93B* and *dmrt99B* genes from the brackish water flea *D. celebensis*. The proteins encoded by these genes contained two conserved domains and were closely clustered with those of *Daphnia* spp. This result indicates their evolutionarily conserved function in sex differentiation of *D. celebensis*. Our findings suggest that BP analogs, in particular BPA, may affect the development and reproduction by modulating *dmrt93B* and *dmrt99B* in different modes in this organism.

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