# CHARACTERIZATION OF REFERENCE GENES IN RARE MINNOW, *GOBIOCYPRIS RARUS* (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE), IN EARLY POSTEMBRYONIC DEVELOPMENT AND IN RESPONSE TO EDCs TREATMENT

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**Background.** Endocrine disrupting chemicals (EDCs) are natural and anthropogenic compounds discharged into the environment known to disrupt the endocrine system of humans and animals by mimicking functions of steroids in vivo. Many important events occurring during early postembryonic development, in relation to the gene expression attracted our attention. Quantitative real-time PCR (qRT-PCR) is a sensitive and highly reproducible method for gene expression analysis, with gene expression levels quantified by normalization to reference gene. The aim of this study was to select the suitable reference gene after EDCs exposure and during early postembryonic development.

**Materials and methods.** For the study of the fish age effect, juveniles of *Gobiocypris rarus* Ye et Fu, 1983, were obtained at: 18, 22, 26, 30, 34, 38, 42, 46, and 50 days post fertilization (dpf). For mRNA expression analysis of the juvenile fish after EDCs treatment, the juveniles at 31 dpf were exposed to bisphenol A (BPA) (10 nM) and 17α-ethinylestradiol (EE2) (1 nM), respectively dissolved in dimethyl sulfoxide (DMSO) or solvent (0.001% DMSO, v/v) control group for 3 days. $C_q$ values of the reference genes were obtained using qRT-PCR. The stability of these reference genes was analyzed by BestKeeper, geNorm, and NormFinder software, respectively. The expression of each reference gene was calculated using the $2^{-\Delta Cq}$ method. In parallel, the mRNA expressions of *cyp19a1b* were normalized by the single most/least stable reference gene and the combinations of top-ranked reference genes.

**Results.** In this study, six candidate reference genes, *actb*, *ef1a*, *gapdh*, *g6pd*, *tbp*, and *tuba1*, were chosen to analyze their expression stability in relation to fish age and in the juvenile fish exposed to BPA and EE2. During early postembryonic development of *Gobiocypris rarus*, *actb*, *ef1a*, and *gapdh* were identified as the most stably expressed reference genes. In the juvenile fish exposed to BPA and EE2 for three days, *gapdh*, and *actb* were the most stable. However, *g6pd* and *tuba1* were identified as the least stably expressed genes during the early postembryonic development and under BPA and EE2 exposure.

**Conclusion.** The presently reported study suggested that the mRNA expressions of the reference genes could be affected by chemical exposure or different physiological periods. In addition, it was indicated that stable reference gene should be selected to normalize the target gene expression to assure the correctness and accuracy of the experiment results. The last but not the least, we successfully obtained five commonly used reference genes of *Gobiocypris rarus* Ye et Fu, 1983, which can be applied in future studies serving as the stable reference gene and providing a broader range of selecting the stable reference gene.

**Keywords:** BestKeeper; early postembryonic development; EDCs; geNorm; *Gobiocypris rarus*; NormFinder; reference gene

## INTRODUCTION

The quantitative real-time PCR (qRT-PCR) has become one of the most widespread techniques in determining mRNA gene expression level for its high sensitivity, reproducibility, and broad dynamic range. Reference gene acts as endogenous control to correct experimental variations caused by differences in quality or quantity of RNA and differences of reverse transcription reaction between samples when quantifying RNA expression using relative qRT-PCR (Radonić et al. 2004, Huggett

et al. 2005). An inappropriate reference gene can greatly increase experimental noise and subsequently influence the reliability of results. Therefore, the suitable reference gene is critical for RNA expression quantification. The ideal reference gene should meet the following criteria:

• it should be expressed at a constant level among different tissues of an organism;
• it should be constantly transcribed among samples taken from different time points;
• it should not be regulated or influenced by the experimental treatment (Haberhausen et al. 1998, Thellin et al. 1999).

Endocrine disrupting chemicals (EDCs) are natural and anthropogenic compounds discharged into the environment known to disrupt the endocrine system of humans and animals by mimicking functions of steroids in vivo. As a result of exposure to EDCs, a number of adverse effects have been reported, based on symptoms of ill-health including developmental abnormality (Seidman et al. 2009), abnormal sexual development (Jobling et al. 2009), teratogenic effect (Socha et al. 2012), and increased cancer risk (Fernandez and Russo 2010). The aquatic environment is the ultimate sink for most chemicals including natural- and man-made ones (Sumpter 1998) through landfill sites, sewage disposal, etc. (Langston et al. 2005). Fish are directly exposed to a wide variety of EDCs from industrial, agricultural, and municipal effluents. Therefore fish are considered the vertebrates the most endangered by EDCs.

The rare minnow, *Gobiocypris rarus* Ye et Fu, 1983, a Chinese freshwater cyprinid, occurs in the upstream reaches of the Yangze River, Sichuan Province, China. Due to its small size, rapid embryonic development (3 days at 25°C), short generation time (about 4 months), high fertilization- and hatching rate, sensitivity to chemicals, and many other advantages, rare minnow is a promising fish model for development and aquatic toxicology research (Wang 1992, Zha et al. 2007).

In the presently reported study, six commonly used reference genes including β-actin (*actb*), elongation factor 1-alpha (*ef1a*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), glucose-6-phosphate dehydrogenase (*g6pd*), TATA box binding protein (*tbp*), and tubulin alpha 1 (*tuba1*) were selected to systematically define the best set of reference genes in early postembryonic development and under EDCs treatment. The aim of this study was to provide valuable information regarding reference genes for qRT-PCR in the experiments of *G. rarus* development or the exposure in its juvenile the bisphenol A (BPA) and 17α-ethinylestradiol (EE2).

## MATERIALS AND METHODS

**Animals and EDC treatments.** *Gobiocypris rarus* larvae were obtained from fertilized eggs by artificial fertilization methods in our laboratory. The juveniles were maintained in 50-L aquaria with the maximum loading of 1.0 g of fish per L at 25°C on a 14 : 10 dark : light cycle and fed *Artemia* nauplii twice a day. Since there have been no studies of the embryonic development of the rare min-

now, the exact time point of an activation of zygotic transcription can not be determined. We only presume that zygotic transcription could have been activated in the *Gobiocypris rarus* larvae at 18 days post fertilization (dpf). To study mRNA expression in early days of postembryonic development, five juveniles were randomly obtained at: 18, 22, 26, 30, 34, 38, 42, 46, and 50 dpf. BPA and EE2 used in the juvenile EDCs exposure experiments were purchased from Sigma-Aldrich (St. Louis, MO). The EDCs concentrations were chosen according to earlier studies (Kazeto et al. 2004, Hayashi et al. 2007). In order to analyze the mRNA expression in juvenile *G. rarus* treated with EDCs, the 31 dpf *G. rarus* juveniles were exposed to BPA (10 nM) and EE2 (1 nM), respectively dissolved in dimethyl sulfoxide (DMSO) for 3 days as the exposure group or solvent (0.001% DMSO, v/v) for 3 days as the solvent control group. For the exposure experiments, half of the exposure solution was renewed every day. The whole individual juvenile fish were collected randomly from five different fish of each replicate, immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

**RNA extraction and reverse transcription (RT).** Total RNA was extracted from whole frozen juvenile fish using TRIZOL reagent (Invitrogen) and treated with DNase I (Promega) following manufacturer's instructions. RNA concentration and quality were determined by spectrophotometer and electrophoresis gel (1.0%), respectively. An amount of 3 μg RNA was reversely transcribed into cDNA in 20 μL final volume using oligo(dT)$_{18}$ primer and Superscript II reverse transcriptase (Invitrogen).

**Identification cDNAs of reference genes from *Gobiocypris rarus*.** Among six candidate reference genes in the presently reported study, only *G. rarus actb* cDNA sequence is available in GenBank. So the cDNA sequences of remaining five reference genes including *ef1a*, *g6pd*, *gapdh*, *tbp*, and *tuba1* were isolate and sequenced. The primers for every gene cloning were designed based on the corresponding cDNA sequence of *Danio rerio* Hamilton, 1822, using Primer Premier 5.0 software (Table 1). The cDNA fragments were amplified from juvenile *G. rarus* and subcloned into the pMD18-T plasmid (TaKaRa) following manufacturer's protocol. The confirmed recombinant plasmids were sequenced using ABI 3730 automated DNA sequencer (BigDye Terminator Chemistry) by Genscript Corporation (Nanjing, China).

**qRT-PCR and data analysis.** The qRT-PCR primers were designed using Primer Premier 5.0 (Table 2). The qRT-PCR reactions were performed on ABI Prism 7300 thermo cycler (Applied Biosystems Inc.) in a final volume of 25 μL, containing 2 × SYBR Green PCR Master Mix (TaKaRa), 0.4 μM of each primer, and 2 μL RT reaction solution. The thermal condition was initial denaturation at 95°C for 30 s, following 40 cycles of 95°C denaturation for 15 s, 58°C annealing for 31 s. Each individual sample was run in triplicate. Data were collected using ABI Prism 7300 SDS software. Efficiency of PCR reactions was calculated on a standard curve generated by a series of 10-fold diluted

cDNA samples which contained five dilution points and measured in triplicate. The equation of efficiency for each pair primer is E = 10 $^{(-1/slope)}$ (Rasmussen 2001).

The expression of each reference gene was calculated using the $2^{-\Delta Cq}$ method as previously described. The change of transcript abundance of *cyp19a1b* gene was calculated using $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001). The data pertaining to fish age were expressed as mean ± standard deviation (SD) and significant differences were analyzed with one-way ANOVA using SPASS 16.0 (SPASS Inc., Chicago, IL USA). SPASS 16.0 also supplies the calculation of normal distribution. If the data does not satisfy the condition, it was transformed to "log", before the statistics were performed. Groups with the same letter are not significantly different. The different lowercase letter means significant difference; the capital letter the extremely significant difference. All data of the EDC treatments were expressed as mean ± standard deviation and were analyzed by unpaired Student's *t*-test after normalization. The differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.01$.

**Expression stability evaluation of the reference genes.** The stability of these reference genes was analyzed by BestKeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) software. The method implemented in BestKeeper uses the SDs of $C_q$ values across the given set of genes as criteria in which a low SD of the $C_q$ value should be expected for useful reference genes. Ideally, the $C_q$ of a gene with an SD < 1 is considered to be stable (Pfaffl et al. 2004, Stern-Straeter et al. 2009). The calculated gene stability value (*M*) of geNorm program is based on the principle that the expression ratio of two ideal internal reference genes is identical in all samples, regardless of experimental conditions. The *M* value is defined as the average pairwise variation of a certain gene with remaining tested genes. The lowest *M* value indicates that the reference gene is most

stable, whereas the highest M value indicates that the reference gene is least stable. GeNorm sets a threshold of 1.5 for *M* value, below which the expression of reference gene is stable (Vandesompele et al. 2002). NormFinder is another software which uses a model-based approach to evaluate candidate reference genes according to their minimal inter- and intra- group gene expression variation (Andersen et al. 2004). The gene with lowest stability values characterizes the most stable expression.

**Ethical issues.** The presently reported study has been carried out in accordance with the regulations on experimental animals outlined in the "Management Methods of Laboratory Animals" of the Shaanxi Province, China.

**RESULTS**

**Expression profiles of reference genes in *Gobiocypris rarus*.** 1142-bp *ef1a*, 995-bp *gapdh*, 933-bp *g6pd*, 818-bp *tbp*, and 1198-bp *tuba1* cDNA fragments were isolated in *G. rarus* (Table 1). The alignment results of these cDNAs showed high identities with their corresponding genes of teleost species. qRT-PCR primers for the six reference genes were designed based on five new cloned cDNA sequences, and *actb* cDNA sequence of *G. rarus* from GenBank.

The specificity of the amplifications was firstly confirmed by the presence of a single band of expected size for each pair of primers using agarose gel electrophoresis (Fig. 1). The melting temperatures of all amplicons are shown in Fig. 2. Melting curve analysis of PCR products indicated that each pair of primers amplified a single product at the expected melt temperature and no other products appeared from non-specific amplification. PCR reaction efficiency varied from 100.1% (*tuba1*) to 109.9% (*tbp*), and all correlation coefficients were more than 0.99 (Table 2).

The $C_q$ values of the reference genes were found to fluctuate in the range of 13.56 (*actb*) to 20.10 (*g6pd*) (Fig. 3). The lowest $C_q$ values were observed for *actb* in all sets, indicating that its high expression in the given samples.

**Table 1**

PCR primers for the amplification of candidate reference genes in *Gobiocypris rarus*, designed with the software Primer® Premier 5.0

| Gene | Species | Accession number | Primers (5′→3′) | Amplicon size [bp] | Tm [°C] |
|---|---|---|---|---|---|
| *ef1a* | *Danio rerio* | NM_131263 | TTCTGTTACCTGGCAAAGGGGAG<br>GCAGCCTTCTGTGCAGACTTTGT | 1444 | 65 |
| *g6pd* | *Danio rerio* | XM_694076 | ATAGACCATTACCTGGGCAAAG<br>TTGTATGTTCCCTCGTAGCG | 931 | 50 |
| *gapdh* | *Danio rerio* | BC083506 | GTTGGTATTAACGGATTCGGTCGCA<br>TCTACTCCTTGGAGGCCATGTGTGC | 995 | 64 |
| *tbp* | *Danio rerio* | NM_200096 | CTCCCTGCGCTTCTGGAACTATT<br>GATTTCTCCTCTAACCTTGGCACCT | 818 | 64 |
| *tuba1* | *Danio rerio* | NM_194388 | TGAGTGCATCTCTATCCACGTTG<br>CTTGGCGTACATCAGATCAAACTT | 1199 | 60 |

Note: *ef1a* (elongation factor 1-alpha), *gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *g6pd* (glucose-6-phosphate dehydrogenase), *tbp* (TATA box binding protein), and *tuba1* (tubulin alpha 1); The accession numbers of the sequences used to design the primers were listed above.

Meanwhile, *g6pd* showed relatively low expression level. Variations of transcript levels for the reference genes were observed. The individual reference gene showed different expression ranges in all samples. Expression levels during development, represented as mean $C_q$ values, are shown in Table 3. *Gapdh*, *actb*, *tuba1* and *ef1a* exhibited lower expression variation (0.22–0.47 cycles) among six candidate reference genes, while *tbp* and *g6pd* had relatively higher $C_q$ variation (0.94–1.06 cycles). In juvenile fish under BPA and EE2 treatment, *gapdh*, *actb*, and *ef1a* showed the low transcription range (0.14–0.34 cycles), while *tbp*, *tuba1*, and *g6pd* exhibited the relatively high transcription range (0.75–1.27 cycles).

**Expression stability analysis of reference genes and selection of internal control genes.** To identify the most constantly expressed genes in our experimental conditions, $C_q$ values obtained for each reference gene were statistical-ly analyzed using three methods including BestKeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002), and NormFinder (Andersen et al. 2004), designed to determine the expression stability of reference genes.

The BestKeeper analysis results showed the ranking in the following order, from most stable to least, *gapdh > actb > ef1a > tuba1 > tbp > g6pd* during early postembryonic development. The geNorm analysis results showed that the *M* values of *actb*, *ef1a*, *tuba1*, and *gapdh* were the lowest (0.611 to 0.691) in early days of the postembryonic development of *Gobiocypris rarus* from 18 to 50 dpf, while *g6pd* and *tbp* genes had relatively high *M* values, indicating that *actb*, *ef1a*, *tuba1*, and *gapdh* ranked the most stable reference genes but *g6pd* and *tbp* were the most variable reference genes among the six candidate reference genes. The NormFinder program provided the same ranking order as geNorm (Table 4). The com-
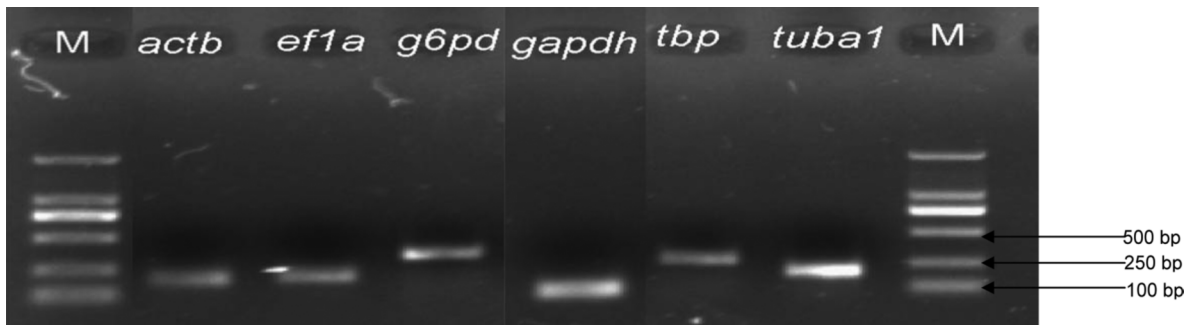


**Fig. 1.** Agarose gel electrophoresis showing specific reverse transcription PCR products of the expected size for each reference gene of *Gobiocypris rarus*
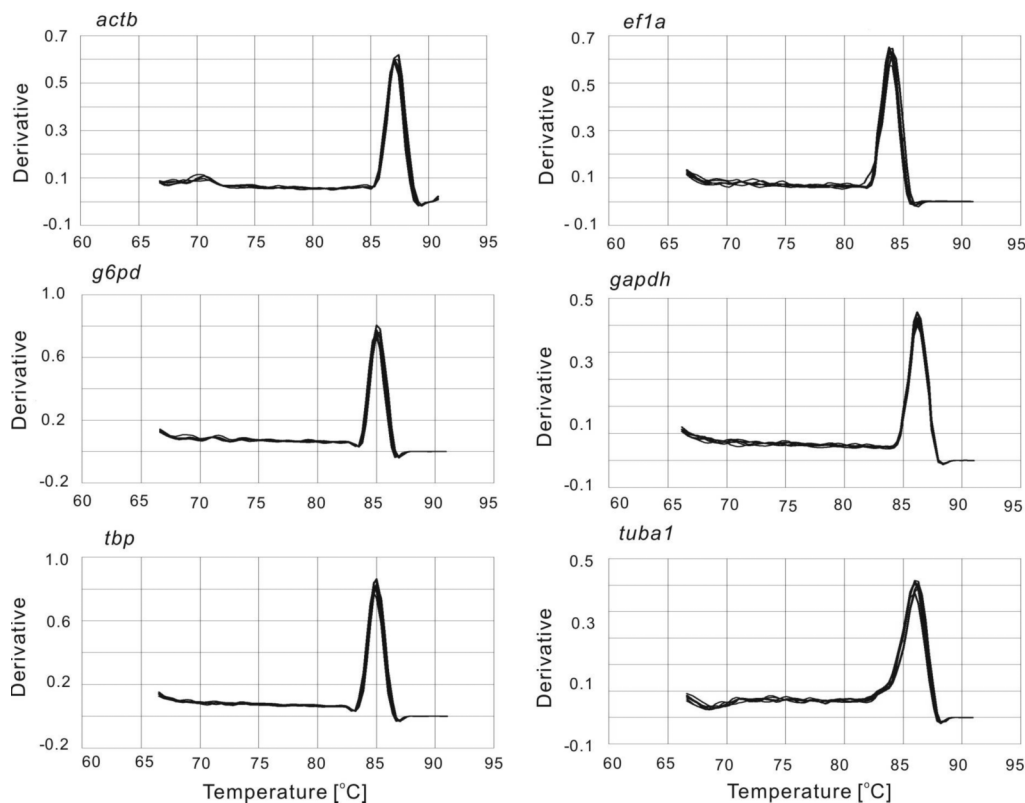


**Fig. 2.** Melting curves generated for all reference genes of *Gobiocypris rarus*

**Table 2**

Details of the primer pairs used in *Gobiocypris rarus* qRT-PCR assays

| Gene | Accession number | Primers (5′→3′) | Amplicon size [bp] | $E$ [%] | $R^2$ |
|------|------------------|-----------------|--------------------|---------|-------|
| *actb* | DQ539421 | GTCCGTGACATCAAAGAG ACCGCAAGATTCCATAC | 195 | 107.4 | 0.990 |
| *ef1a* | HM017974 | ACAAATGCGGTGGAATCG TCAAACTTCCAGAGAGCGATA | 157 | 107.6 | 0.996 |
| *g6pd* | HM017973 | AAGGTGAAGGTGCTGAAGTGC CGTTCCGCCTACACTGAGATT | 302 | 106.4 | 0.999 |
| *gapdh* | HM017972 | GCTGCGAAGGCTGTGGG GCAGGTTTCTCAAGGCGGA | 122 | 108.0 | 0.998 |
| *tbp* | GU567777 | CCTATGACCCCCATCACTCC GCTGCCAATCGGGACTGT | 269 | 109.9 | 1.000 |
| *tuba1* | HM017971 | CCCAGGGCTGTGTTTGTAGA CAGTTTGCGAATCCTGTCCA | 189 | 100.1 | 0.996 |
| *cyp19a1b* | GU220393 | GTAGGTTCCTGTGGATGGG CGTCGGAGGACTTGCTGA | 120 | 105.5 | 0.998 |

Note: *actb* (β-actin), *ef1a* (elongation factor 1-alpha), *gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *g6pd* (glucose-6-phosphate dehydrogenase), *tbp* (TATA box binding protein), and *tuba1* (tubulin alpha 1), and *cyp19a1b* (cytochrome P450 aromatase); $R^2$ = correlation coefficient obtained from the standard curve; $E$ = PCR efficiency represented in percentage.
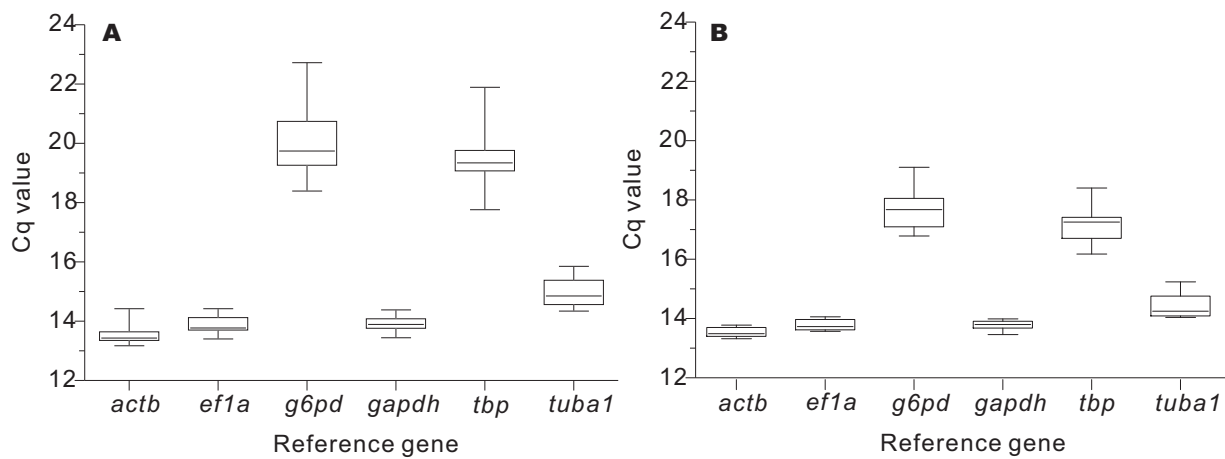


**Fig. 3.** Boxplot representations of $C_q$ values measured in selected days of early postembryonic development and EDCs treatment in *Gobiocypris rarus*; Six reference genes were examined in 45 samples representing the fish development (**A**) and 45 samples of EDCs treatment (**B**); Data are displayed as average raw $C_q$ (not yet normalized) values from individual groups; Boxes show the range of $C_q$ values within each gene; Line in the box indicates the median; The box covers the second and the third quartile (25th through 75th percentile); The extended vertical bars show SD of the mean

**Table 3**

Values of the quantification cycle ($C_q$) of the six reference genes of *Gobiocypris rarus* in different experimental setups

| Gene | Early postembryonic development | | BPA and EE2 treatment | |
|------|---------------------------------|---|------------------------|---|
| | $C_q$ value | $n$ | $C_q$ value | $n$ |
| *actb* | 13.51 ± 0.25 | 45 | 13.46 ± 0.14 | 45 |
| *ef1a* | 13.94 ± 0.48 | 45 | 13.83 ± 0.17 | 45 |
| *g6pd* | 20.06 ± 1.06 | 45 | 18.04 ± 1.27 | 45 |
| *gapdh* | 13.92 ± 0.22 | 45 | 13.85 ± 0.14 | 45 |
| *tbp* | 19.40 ± 0.94 | 45 | 17.30 ± 0.75 | 45 |
| *tuba1* | 14.97 ± 0.47 | 45 | 14.40 ± 0.30 | 45 |

$C_q$ values are the mean ± standard deviation; $n$ = number of samples used in experiment.

prehensive ranking by three algorithms showed the *actb* and *ef1a* as the two highest stable genes and *g6pd* as the least stable gene.

For juvenile fish following 3-day exposure to EE2 and BPA, the ranking from BestKeeper showed the stability of *actb*, *gapdh*, *ef1a*, *tuba1*, *tbp*, and *g6pd* turn. Outputs from the geNorm applet indicated that *gapdh*, *ef1a*, and *actb* are the most suitable normalization factors while NormFinder found that the ranking of most stable three genes is the same. BestKeeper showed that *actb* > *gapdh* > *ef1a* > *tuba1* > *tbp* > *g6pd*. The identical ranking order for expression stability of the remaining four reference genes obtained by these three algorithms was *ef1a* > *actb* > *gapdh* > *tuba1* > *tbp* > *g6pd*. So *g6pd* was consistently ranked as the least stable gene for juvenile *Gobiocypris rarus* exposed to BPA and EE2 (Table 4).

To determine the optimal number of genes required for normalization, geNorm was used to calculate the pairwise variation ($V_n/V_{n+1}$) between sequential normalization factors (NF) ($NF_n$ and $NF_{n+1}$) (Vandesompele et al. 2002). Cut-off value of pairwise variation was suggested to be 0.15, below which the inclusion of an additional reference gene is not required. Pairwise variation values for distinct set of samples showed that the ideal number of reference genes may be different (Fig. 4). The pairwise variation $V_{2/3}$ value was 0.161, higher than the cut-off value 0.15, while $V_{3/4}$ value was 0.107 in early postembryonic development from 18 to 50 dpf which was significantly below the cut-off value. So *actb*, *ef1a*, and *tuba1* genes will be needed for normalization of gene expression during early postembryonic development of *Gobiocypris rarus* from 18 to 50 dpf. Result of pairwise variation analysis showed that the $V_{2/3}$ value is 0.096 in the juvenile fish in EDC exposure experiment which is significantly below the cut-

off value, indicating that the inclusion of two reference genes is necessary. So *gapdh* and *ef1a* are the optimal reference genes in the EE2 and BPA exposure experiment in juvenile fish (Fig. 4).

**mRNA expression of candidate reference genes during early postembryonic development and in response to BPA/EE2 exposure in *Gobiocypris rarus* juvenile.** The expression profiles of *actb*, *ef1a*, *g6pd*, *gapdh*, *tbp*, and *tuba1* in relation to fish age (from 18 to 50 dpf) in *G. rarus* juveniles are shown in Fig. 5A. The mRNA level of *actb* is most uniform at the nine time points from 18 to 50 dpf among six reference genes. The *gapdh* mRNA levels at different time points were relative equal however its level at 50 dpf was significantly higher than those from 18 to 46 dpf. At some time points, the mRNA expression of *ef1a* showed significant alterations (18 to 46 and 50 dpf, 0.54-, 0.61-fold decrease, respectively, $P < 0.05$; 22 to 46 and 50 dpf, 0.49-, 0.53-fold decrease, respectively, $P < 0.05$; 26 to 46 and 50 dpf, 0.38-, 0.45-fold decrease, respectively, $P < 0.05$; 30 to 46 and 50 dpf, 0.55-, 0.63-fold, respectively, $P < 0.05$; 34 to 50 dpf, 0.4-fold decrease, $P < 0.05$; 42 to 50 dpf, 0.4-fold decrease, $P < 0.05$). *Tuba1* was less stable than *ef1a* in relation to fish age (from 18 to 50 dpf). The mRNA level of *g6pd* and *tbp* at the 9 time points had wide range of variations.

The expression profiles of *actb*, *ef1a*, *g6pd*, *gapdh*, *tbp*, and *tuba1* upon BPA (10 nM) and EE2 (1 nM) exposure are shown in Fig. 5B. Three-day exposure of BPA at 10 nM had no effects on mRNA expression of *actb*, *ef1a*, *gapdh*, and *tbp*. However, BPA exposure caused 1.52-fold extremely significant increase of *tuba1* mRNA expression compared to that of solvent control. On the contrary, BPA resulted in 2.05-fold significant decrease of gene expression for *g6pd* compared to control. The expression pattern

**Table 4**

Stability analysis output of BestKeeper, geNorm and NormFinder across the two samples (early postembryonic development and EDCs treatments) studied in *Gobiocypris rarus* juvenile

| Early postembryonic development | | | | BPA and EE2 treatment | | | |
|---|---|---|---|---|---|---|---|
| Comprehensive ranking | BestKeeper | geNorm | NormFinder | Comprehensive ranking | BestKeeper | geNorm | NormFinder |
| *actb* | *gapdh* (0.175) | *actb* (0.611) | *actb* (0.014) | *gapdh* | *actb* (0.094) | *gapdh* (0.548) | *gapdh* (0.010) |
| *ef1a* | *actb* (0.185) | *ef1a* (0.656) | *ef1a* (0.015) | *actb* | *gapdh* (0.106) | *ef1a* (0.550) | *ef1a* (0.011) |
| *gapdh* | *ef1a* (0.305) | *tuba1* (0.659) | *tuba1* (0.020) | *ef1a* | *ef1a* (0.147) | *actb* (0.572) | *actb* (0.012) |
| *tuba1* | *tuba1* (0.395) | *gapdh* (0.691) | *gapdh* (0.023) | *tuba1* | *tuba1* (0.269) | *tuba1* (0.679) | *tbp* (0.015) |
| *tbp* | *tbp* (0.635) | *tbp* (0.988) | *tbp* (0.027) | *tbp* | *tbp* (0.433) | *tbp* (0.732) | *g6pd* (0.018) |
| *g6pd* | *g6pd* (0.855) | *g6pd* (0.996) | *g6pd* (0.061) | *g6pd* | *g6pd* (0.479) | *g6pd* (1.146) | *tuba1* (0.032) |
| Best combination | | *gapdh/actb* (0.29) | *tbp/ef1a* (0.012) | | | *gapdh/actb* (0.17) | *ef1a/gapdh* (0.009) |

Note: BestKeeper output with standard deviations (SD; in parentheses); Ideally the SD should be lower than 1; GeNorm output with expression stability values (*M*) presented in parentheses (The lower the *M* value, the higher is the stability); NormFinder output with stability values for reference genes; A low stability value indicates high stability of the gene.

of reference genes following EE2 exposure is similar to that under BPA exposure. Gene expression of *g6pd* was extremely significantly down-regulated by 1 nM EE2 (2.13-fold, *P* < 0.01), but *tuba1* was extremely significantly up-regulated, significant increase was observed (1.48-fold, *P* < 0.001), while both tested EDCs had no effects on the expression of *actb*, *ef1a*, *gapdh*, and *tbp* (Fig. 5B). Therefore, *g6pd* and *tuba1* should be avoided as the reference gene to normalize expression of target genes following BPA and EE2 exposures.

**Effects of different reference genes on the normalization of *cyp19a1b*.** To demonstrate the significance of suitable reference genes for normalization and to get correct profiling data, we selected *cyp19a1b* as a target gene to measure its mRNA levels in *Gobiocypris rarus* juveniles in relation to fish age and in response to BPA and EE2 exposures.

Normalization to *cyp19a1b* expression during early postembryonic development was performed with four different strategies: one most stable reference gene (*actb*), one least stable reference gene (*g6pd*), combination of two top-ranked reference genes (*actb* and *gapdh*) and combination of three top-ranked reference genes (*gapdh*,

*actb*, and *tuba1*) calculated by NormFinder and geNorm. When the least stable reference gene, *g6pd*, was used in the normalization, the expression profile of *cyp19a1b* was exaggeratedly affected. The *cyp19a1b* expression in the juvenile at 38 dpf was significantly higher than that in the juvenile at 26 and 30 dpf when *g6pd* was used as normalization factor, while the normalization to the most stable reference gene and combination of the most stable reference genes did not cause these significant difference of *cyp19a1b* expression between these two time points (Fig. 6A). The *cyp19a1b* expression of juveniles at 38 dpf normalized to the combination of two top-ranked reference genes (*actb* and *gapdh*) and three top-ranked reference genes (*actb*, *gapdh*, and *tuba1*) was both significantly higher than that in juveniles at 42 dpf, while the normalization to the single most stable reference gene (*actb*) did not result in the significant difference of *cyp19a1b* expression between these two time points (Fig. 6A).

When the expression data were normalized to different reference genes, *cyp19a1b* was, as expected, shown to be diversely regulated by BPA and EE2 (Fig. 6B). *Cyp19a1b* is robustly down-regulated by EE2 treatment
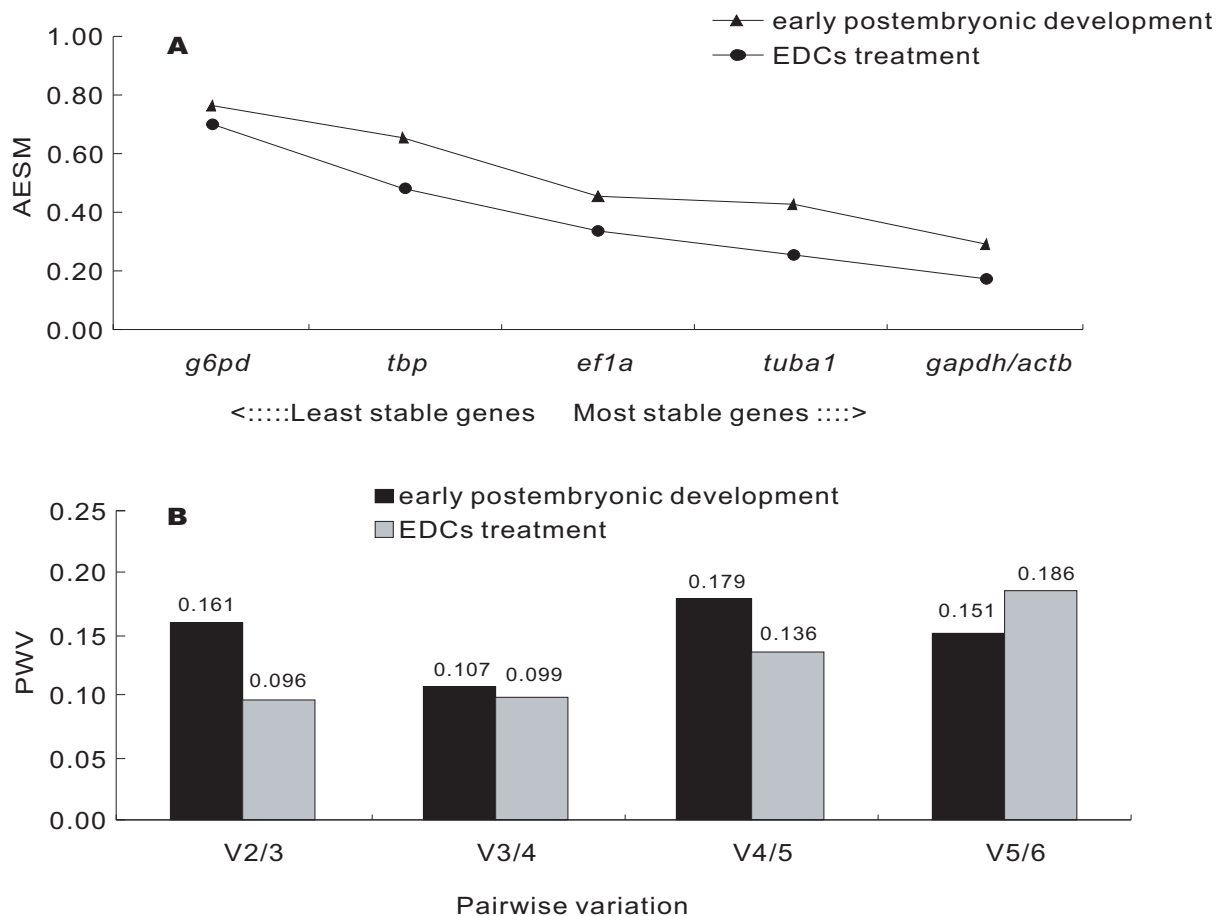


**Fig. 4.** Expression stability, ranking of reference genes and determination of optimal number of reference genes for *Gobiocypris rarus* as calculated by geNorm; Note: AESM: average expression stability (*M*) PWV: pairwise variation V; **A** represents expression ranking based on average expression stability value (*M*) for early postembryonic development and BPA and EE2 treatment; **B** displays optimal number of genes required for the accurate estimation of the target gene mRNA in selected days of postembryonic development and BPA/EE2 treatment, based on the analyses between the normalization factors NF$_n$ and NF$_{n+1}$

normalized to each reference gene. As mRNA expressions of *actb*, *ef1a*, *gapdh*, and *tbp* were not significantly affected by EE2 (Fig. 5B), the inhibitory effects of BPA on the expression level of *cyp19a1b* via normalization of these four reference genes are similar (5.94- to 8.34-fold decrease, Fig. 6B). The effect of EE2 treatment on *g6pd* and *tuba1* expression however, resulted in an exaggerated down-regulation of *cyp19a1b* (4.00- and 11.27-fold). BPA had no apparent effects on *cyp19a1b* expression when the data were normalized to *actb*, *ef1a*, *gapdh*, and *tbp*. But *cyp19a1b* expression was significantly down-regulated for 2.31-fold when normalized to *tuba1* which was strongly up-regulated by BPA (Fig. 6B). To optimize the normalization effect of reference genes, *cyp19a1b* expression were normalized to the combination of *actb* and *gapdh*, the two most stable genes calculated by geNorm (Fig. 6B). The *cyp19a1b* expression under EE2 exposure normalized to the combination *actb* and *gapdh* was lower for 7.18 fold than that in control group.

## DISCUSSION

A wide range of potential reference genes have been evaluated for qRT-PCR studies during the developmental phases of zebrafish, *Danio rerio* (Hamilton, 1822) (see McCurley and Callard 2008); Atlantic halibut *Hippoglossus hippoglossus* (see Fernandes et al. 2008); and Senegalese sole, *Solea senegalensis* Kaup, 1858 (see Infante et al. 2008). In the experiments of fish exposure to EDCs, some candidate reference genes including *18S* rRNA, *actb*, *b2m*, *ef1a*, *gapdh*, *g6pd*, hypoxanthine phosphoribosyltransferase I (*hprt1*), and *tbp* were used to validate their expression stability in zebrafish (McCurley and Callard 2008) and fathead minnow, *Pimephales promelas* Rafinesque, 1820 (see Filby and Tyler 2007). Our study indicated that the majority of reference genes did not have a constant expression level under various conditions. Therefore, the variability of a reference gene alone can impact the relative fold difference of a target gene during qRT-PCR analysis. So it is critical to select a stable refer-
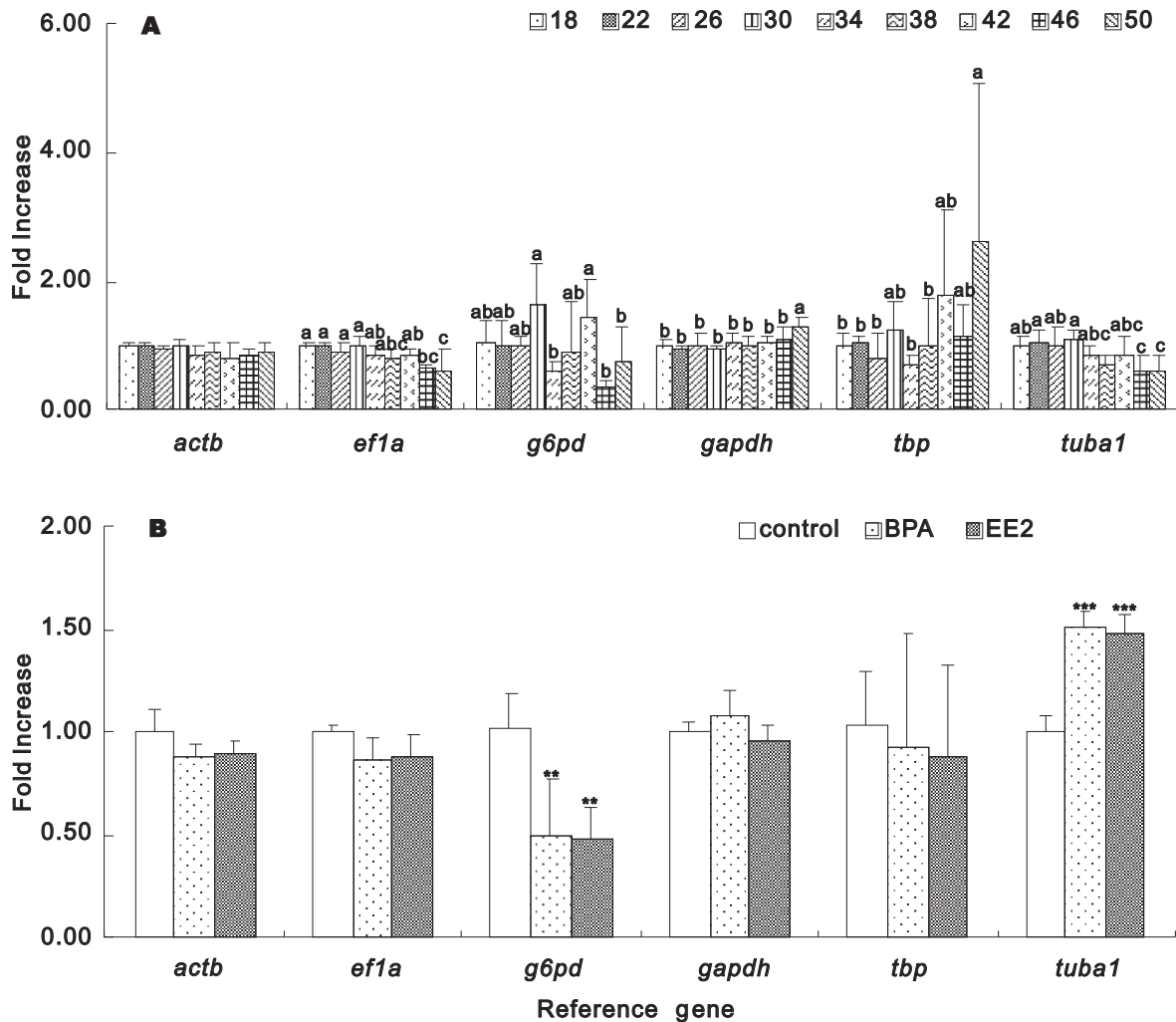


**Fig. 5. A**: The variation of transcripts for reference genes in early postembryonic development of *Gobiocypris rarus*; Groups with the same letter, within selected days of development, are not significantly different; **B**: Effects of BPA and EE2 treatment for 3 days on transcript abundance of reference genes in *G. rarus* juveniles at 34 dpf; Statistically significant differences in gene expression between control and treatment fish are denoted as follows: * *P* < 0.05, ** *P* < 0.01, ***P* < 0.001

ence gene to normalize gene expression under a certain condition. To date, both *gapdh* and *actb* were frequently used as reference genes in studies of *Gobiocypris rarus* without considering any validation of expression stability (Wang 1992, Li et al. 2009, Wang et al. 2012). However, there were some adverse results in previously reported studies. For instance, the most commonly used reference gene *actb* during the developmental phases of *H. hippoglossus* and in the six tissues of the fish injected with nervous necrosis virus was the least stable one among six reference genes (Øvergård et al. 2010). In our study, the expression levels of six candidate reference genes were analyzed by BestKeeper, geNorm and NormFinder programs. Considering the different algorithm methods and analytical procedures, it is not surprising that different ranking orders for some reference genes were generated by the three algorithms. In *G. rarus* juveniles the fish aged from 18 to 50 dpf, *actb*, *ef1a*, and *gapdh* were the most reliable internal controls for accurate normalization. On the other hand, *g6pd* and *tbp* ranked poorly based on the

three algorithms. In the juvenile fish following 3 days exposure to EE2 and BPA, *gapdh* and *actb* was selected as the most suitable combination for accurate normalization of gene expression. And *tbp* and *g6pd* were still the least stable reference genes according to the results of BestKeeper, geNorm, and NormFinder. It means that the most stable reference gene(s) should be identified for a specific species under different experimental setups.

Our data clearly demonstrated that expression level of *g6pd* was significantly down-regulated by BPA and EE2 while *tuba1* gene was significantly up-regulated by these compounds. The BPA and EE2 exposure did not significantly affect the expression of the best three reference genes (*gapdh*, *actb*, and *ef1a*) estimated by the three algorithms. Our results revealed that EE2 has no influence on expression level of *cyp19a1b* except using *g6pd* to normalize, showing that target gene would be significantly over-estimated by inappropriate reference gene. EE2, like nature sex steroids, also has roles in the regulation of the metabolic processes during gonad development and
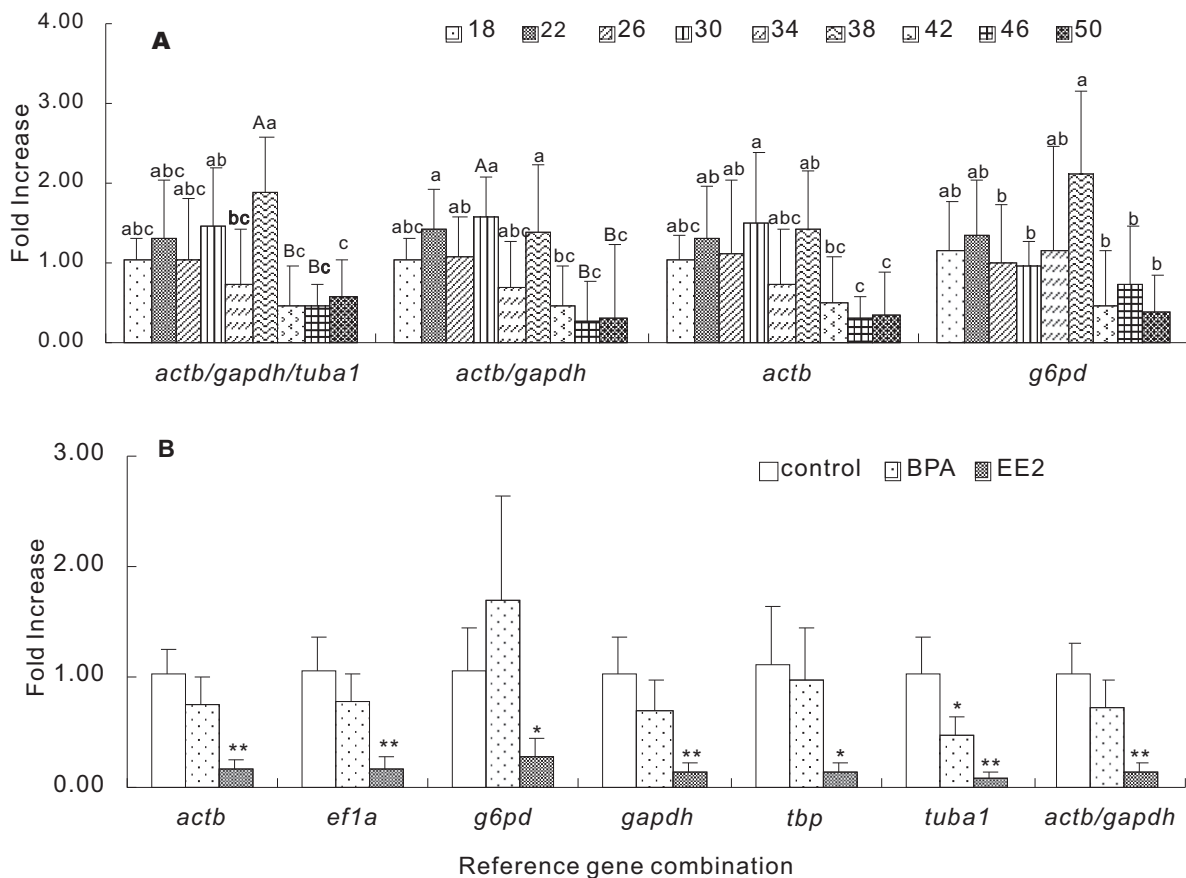


Fig. 6. A: Relative expression of *cyp19a1b* during the early postembryonic development of *Gobiocypris rarus* estimated after using individual reference genes (the combination of three reference genes as suggested by $V_{n/n+1}$ for normalization, the combination of the two most stable genes, *actb* is the most stable gene and *g6pd* is the least stable gene); Groups with the same letter, within individual days of development, are not significantly different; **B**: Relative expression of *cyp19a1b* after BPA (10 nM) and EE2 (1 nM) treatment for 3 days estimated after using individual reference genes and the combination of the two most stable genes according to the $V_{n/n+1}$ (*actb/gapdh*); Statistically significant differences in gene expression between control and treatment fish are denoted as follows: * $P < 0.05$, ** $P < 0.01$

reproduction in fish (Korsgaard and Mommsen 1993, Sunny et al. 2002, Sangiao-Alvarellos et al. 2004). So in the presently reported study, the effect of EE2 on *g6pd* in the juvenile fish might result from its involvement in metabolism. Early studies have shown that *g6pd* is mediated by estrogen at both the transcriptional and translational levels. Estrogen is known to increase the activity of uterine G6PD (Keran and Barker 1976, Rasmussen et al. 1988). On the other hand, testosterone and progesterone have no stimulatory effects on the activity of uterine G6PD (Hilf et al. 1972). BPA and nonylphenol (NP) stimulated high *g6pd* activity in rats MCF-7 cells (Kim et al. 2003). Therefore *g6pd* should be avoided in the normalization of gene expression in *Gobiocypris rarus*. *Tuba1*, encoding cytoskeletal protein, has long been attributed to having major roles in setting up the scaffolding needed to elaborate new neurites and to enable transport of organelles within the neurites. *Tuba1* was identified as being stably expressed across various developmental stages of soybean (Jian et al. 2008) and different tissues of poplar (Brunner et al. 2004). *Tuba1* was recognized as the most stable gene in the four tissues (including spleen, heart, muscle, and gill) in Japanese flounder, *Paralichthys olivaceus* (Temminck et Schlegel, 1846) (see Zheng and Sun 2011). But our results show that the expression of *tuba1* is altered by the EDCs. So it should be avoided to select as reference gene. *Tuba1* is highly active and its transcript is very abundant in the cells. When the cells are infected by virus, the transcription levels of the structural gene is affected depending on the cell-virus system and turned out to be unstable at the mRNA levels in virus infected cells (Radonić et al. 2005). The usage of *tuba1* as the control gene should be avoided because its expression stability is far from acceptable. It was revealed that mouse *tuba1* gene contains partial estrogen responsive elements (EREs) in its promoter region (Gloster et al. 1994). The bioinformatic analysis showed that there are several EREs in the promoter region of *tuba1* gene of *Carassius auratus* (GenBank accession no. AF002654, data not shown). This suggests that *tuba1* could be modulated at a transcriptional level by E2 or estrogenic chemicals.

In this study we demonstrate that *actb*, *ef1a*, and *gapdh* are three suitable genes for the RT-qPCR analyses in relation to fish age and EDCs treatment. In addition, it should be noted that this study could not encompass all experimental conditions or EDC populations used throughout the field of EDC research. With that in mind, it is important to note that prior to collecting and or analyzing RT-qPCR data, the reference genes used for normalization must be validated.

In summary, our study reports the expression level of six commonly used reference genes during rare minnow juvenile development and in the juvenile fish exposed to EE2 and BPA. We demonstrated that *actb*, *ef1a*, and *gapdh* were the most stable reference genes, and *g6pd* and *tbp* were the least stable ones during the early postembryonic development of the *Gobiocypris rarus* juvenile and after exposure to EE2 and BPA.

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