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Reference gene validation for quantification of gene expression during final oocyte maturation induced by diethylstilbestrol and di-(2-ethylhexyl)-phthalate in common carp

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ABSTRACT

Final oocyte maturation is the key step to successful spawning and fertilization. Quantitative real-time PCR (qPCR) is the technique of election to quantify the abundance of functional genes in such study. Reference gene is essential for correct interpretation of qPCR data. However, an ideal universal reference gene that is stable under all experimental circumstances has not been described. Researchers should validate their reference genes while performing qPCR analysis. The expression of 6 candidate reference genes: *18s rRNA*, *28s rRNA*, *Cathepsin Z*, *Elongation factor 1-α*, *Glyceraldehyde-3-phosphate dehydrogenase* and *β-actin* were investigated during final oocyte maturation induced by different compounds (DES and DEHP) in common carp (*Cyprinus carpio*). Four softwares (Bestkeeper, geNorm, NormFinder and RefFinder) were used to screen the most stable gene in order to evaluate their expression stability. The results revealed that *EF1α* was highly stable expressed when final oocyte maturation was induced by DES, while *gapdh* was the most stable gene when final oocyte maturation was induced by DEHP. Stable expressed reference gene selection is critical for all qPCR analysis to get accurate target gene mRNA expression information.

Introduction

Teleost oocytes, growing within the ovarian follicles, are arrested at the first meiotic prophase after they complete their growth. When environmental conditions such as light, temperature are suitable, the fully grown oocytes become ready for the next process (Devlin and Nagahama, 2002). This process, called final oocyte maturation (FOM), occurs prior to ovulation and is a prerequisite for successful fertilization. The duration of FOM usually lasts very short, generally no more than 24 hr, but it consists of several events including breakdown of the germinal vesicle (GVBD), chromosome condensation, assembly of the meiotic spindle, and formation of the first polar body (Nagahama, 2008). Since 1980s, hormones regulating teleosts FOM have been investigated by a number of researchers, as well as the molecular mechanism of FOM (Goetz, 1983; Kime et al., 1992; Hyttel et al., 1997; Sirard et al., 2007). Several studies also showed that chemicals could impact reproduction through FOM. Tokumoto (2004) investigated the effects of many endocrine-disrupting chemicals (EDCs) by GVBD ratio. It turns out some EDCs may have an influence on fish oocyte maturation like hormone does. Wang et al. (2008) suggests that hypoxia can inhibit FOM. These results indicated that FOM could be a meaningful ecological biomarker to access the effects of environmental stressor on reproduction.

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The pituitary glycoprotein hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) act as high-order controls of the gonads in vertebrates. Gonadotropin control of the growth and function of the gonads is mediated by the gonadotropin receptors, FSH receptor and LH receptor (LHR). Genes encoding these receptors are expressed primarily in the accessory cells (follicular and interstitial cells) of the gonads and directly affect gonadal steroidogenesis (Kwok et al., 2005). In the present study, we used LHR which was cloned before (not published) as the target gene of 6 candidate reference genes for describing FOM.

The determination of variation in transcript abundance is an important element in the uncovering of the underlying processes during FOM and quantitative real time PCR (q-PCR) is a technique of choice. To date, qPCR has become an important method for the detection and quantification of nucleic acids in biological samples due to the advantages of its extreme sensitivity, large dynamic range and outstanding accuracy (Cao et al., 2012). However, the accuracy of the results obtained by this method strongly depends on accurate transcript normalization using stably expressed genes, known as reference genes (Gutierrez et al., 2008). Traditionally, most qPCR studies use housekeeping genes including *18s ribosomal RNA* (*18s*) or *actin* as reference genes. Unfortunately, a lot of studies have shown that these widely used housekeeping genes are not always stably expressed in some circumstances (Stürzenbaum and Kille, 2001; Small et al., 2008; Albershardt et al., 2012; Park and Kwak, 2012). Using unstable reference gene to analyze qPCR results could lead to serious deviation. More and more studies suggest that no single reference gene has been discovered in a universal and invariant level (Huggett et al., 2005; Gutierrez et al., 2008; Guénin et al., 2009). Therefore, reference genes must be carefully validated for each experimental situation (Schmittgen and Zakrajsek, 2000).

Recently, reference gene selection softwares like Bestkeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and RefFinder (Xie et al., 2011) appeared subsequently. These softwares can help researchers find suitable reference genes in different experiments. These four softwares also can give the right number of reference genes needed for accurate quantification (Brinkhof et al., 2006). geNorm calculates and compares gene expression stability (M value) and pairwise variation ($Vn/n+1$) of all candidate gene. Similar to geNorm, NormFinder attempts to identify the optimal reference genes out of a set of reference genes. Bestkeeper assesses the stabilities of candidate reference genes based on the inspection of calculated variation. RefFinder is an online comprehensive software, which is integrated by geNorm, NormFinder, Bestkeeper and delta threshold cycles (Ct) method. It gives comprehensive assessment for the results of the four kinds of analysis methods, avoiding the one-sideness of single analysis method, but it only could be a good reference result unless the amplification efficiency of each primer pair was incorporated.

Endocrine-disrupting chemicals (EDCs) are chemicals that can potentially interfere with endocrine systems which regulate growth, development, reproduction and other physiological processes of animals (Park and Kwak, 2012). Diethylstilbestrol (DES), a nonsteroidal xenoestrogen, could trigger oocyte maturation in fish as an EDC. The morphology (the time course of GVBD) and an intracellular molecular event (the de novo synthesis of cyclin B) induced by DES have been confirmed indistinguishable from those induced by a natural maturation-inducing hormone, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) (Tokumoto, 2004). Phthalates are ubiquitous environmental contaminants because of their use in plastics and other common consumer products. Di-(2-ethylhexyl)-phthalate (DEHP) is the most abundant phthalate and it impairs fertility by acting as an endocrine disruptor, and researchers also found out that it can affect oocyte maturation (Ambruosi et al., 2011).

The present study aimed to identify suitable reference genes for robust analysis of molecular changes during FOM induced by DES or DEHP. Six commonly used reference genes including *18s*, *28s ribosomal RNA* (*28s*), *Cathepsin Z* (*CTSZ*), *Elongation factor 1-α* (*EF1α*), *Glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and *β-actin* were selected and their expression stabilities during FOM under different compoundexposures were analyzed by four statistical algorithms, geNorm, NormFinder, Bestkeeper and RefFinder, respectively. The stability analysis results will provide useful guidelines for the optimal reference gene selection and make it possible to obtain more reliable results of mRNA expression levels of target genes during FOM in common carp (*Cyprinus carpio*).

1. Materials and methods

1.1. Sample preparation

Six gravid adult female common carp (body weight, 500–800 g) was collected from a local aquatic market in Xiamen from December to next February. Fish were killed by decapitation. Ovaries mostly composed of post-vitellogenic follicles were removed and dissected manually with fine forceps. Full grown oocytes with follicles of 0.5–0.7 mm diameter were collected and then incubated in Cortland's solution (Stoeckel and Neves, 1992; Sen et al., 2002) (mmol/L: NaCl 160, KCl 2.55, $CaCl_2$ 1.56, $MgSO_4$ 0.93, $NaHCO_3$ 17.85, NaH_2PO_4 2.97, glucose 5.55) containing streptomycin (100 μg/mL) and penicillin (100 IU/mL) adjusted to pH 7.4. These follicles were divided into different groups based upon different compounds exposed: 0.1, 1, and 5 μmol/L Di-(2-ethylhexyl)-phthalate (DEHP, TCI, Tokyo), 0.1, 1 and 2 μmol/L Diethylstilbestrol (DES, Sigma, USA) (Carnevali et al., 2010; Tokumoto, 2004). Follicles incubated in different compounds were collected at 0, 3, 6, 9 and 12 hr after incubation. The rate of GVBD to total oocytes was determined after fixing them in clearing solution of acetic acid–ethanol–formalin (1:6:3, V/V/V). For each collection, pools of ~20 oocytes per individual were ground in liquid nitrogen and stored at −80°C.

1.2. RNA extraction and cDNA synthesis

Total RNA was extracted from oocytes using trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. Potentially contaminating DNA was eliminated by treatment with DNase I digestion. The quality of RNA was assessed on 1.0% agarose gel electrophoresis and the concentration was determined by A260/280. RNA samples used for studies fulfilled the defined criterions. From each sample, 3 μg of total RNA was reverse transcribed using Reverse

Transcriptase M-MLV (TaKaRa) and random primers according to the manufacturer's instructions. Finally, the synthesized cDNA was stored at –20°C until use.

1.3. Selection of common carp candidate reference genes

Six candidate reference genes were selected for gene expression analysis. The full gene names, functions, and accession numbers were provided in Table 1.

1.4. Primer design

Primer pairs were designed with on-online Primer 3 Plus software (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Untergasser et al., 2007). All primers were synthesized by Shanghai Generay Biotech Co. Ltd., China.

1.5. Quantitative real time polymerase chain reaction

QPCR was performed in 96-well plates with Roche LightCycler 480 real-time PCR instrument. Standard reactions (20 μL) using a SYBR Green-based PCR assay contained 10 μL SYBR Green GoTaq®PCR Master Mix (Promega, USA), 1 μL diluted cDNA template, 0.5 μL each primer (10 μmol/L in stock), and 8 μL sterilized ddH_2O. PCR efficiencies and coefficients of determination (r^2) were established with standard curves generated from a 10-fold dilution series (from 10^1 to 10^5) of cDNA as templates. The qPCR reaction was subjected to the following conditions: an initial step of pre-incubation of 7 min at 95°C, followed amplification step by 45 cycles at 95°C for 10 sec, 60°C for 10 sec and 72°C for 15 sec. At the end of the amplification protocol, melting curves of the amplicons were immediately determined by monitoring fluorescence from 65 to 95°C, with temperature increments of 0.2°C. Each experimental sample was amplified in triplicate.

1.6. Evaluation of expression stability

After extraction of the Ct values with the LightCycler®480 Software 1.5 (Roche Applied Science, UK), stability of the candidate reference genes was evaluated with geNorm, NormFinder, RefFinder and Bestkeeper. In the case of all data, fold-change in relation to the zero hour was calculated using Ct values (as $E^{\Delta Ct(0\text{-}n\ hr)}$). Normalization of the target gene (LHR) was carried out according to all candidate reference genes.

1.7. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using SPSS 20.0 software. One-way ANOVA with different collections as factor was used to compare relative expression levels on log2 transformed fold change. Tukey's post-hoc was used for comparisons against zero hour. The level of significance was less than 0.05.

2. Results

2.1. Amplification performance of primers

The sequences, corresponding amplicon sizes and PCR efficiencies of primers are listed in Table S1. The optimal *Tm* of each primer pair was about (60 ± 1) °C. All primer pairs generated specific amplicons with expected size and no visible primer dimers could be detected in agarose gel following electrophoresis (Fig. 1a). One single peak was obtained in each melting curve analysis, which confirmed the specific amplification of primers (Fig. 1b).

2.2. Expression profile of the reference genes

The *Ct* value of each reference gene was assessed at different incubating time after being exposed to different compounds. All the six candidate reference genes showed different variations (Fig. 2). In all samples, most of these genes including *CTSZ*, *EF1α*, *gapdh* and *β-actin* were expressed with their *Ct* values between 20 to 30 cycles whereas *18s* and *28s* were high expressed with their *Ct* values below 20 cycles (Fig. 2a). The expression levels of *18s*, *28s* and *CTSZ* varied a lot in different exposure groups (Fig. 2b).

2.3. Expression stability of candidate reference genes

Since the six reference genes showed a wide range of expression levels at different incubating time after being exposed to different compounds, it was necessary to apply statistical methods to rank the reference genes and determine the optimal genes needed for accurate normalization in each experiment.

When FOM was induced by DES, the results from geNorm software analysis showed that *gapdh* and *EF1α* had the lowest

Table 1 – Summary of genes used in this study.

Symbol	Name	Accession number	Function
Candidate reference genes			
β-actin	β-Actin	M24113	Cytoskeletal protein
18s	18s ribosomal RNA	FJ710826	Ribosomal subunit
28s	28s ribosomal RNA	JN628435	Ribosomal subunit
EF1α	Elongation factor 1-α	AF485331	Factor for protein translation
gapdh	Glyceraldehyde-3-phosphate dehydrogenase	AJ870982	Glycolytic enzyme
CTSZ	Cathepsin Z	FJ710826	Lysosomal cysteine proteinase
Target gene			
LHR	Luteinizing hormone receptor	Unpublished	G protein-coupled receptor

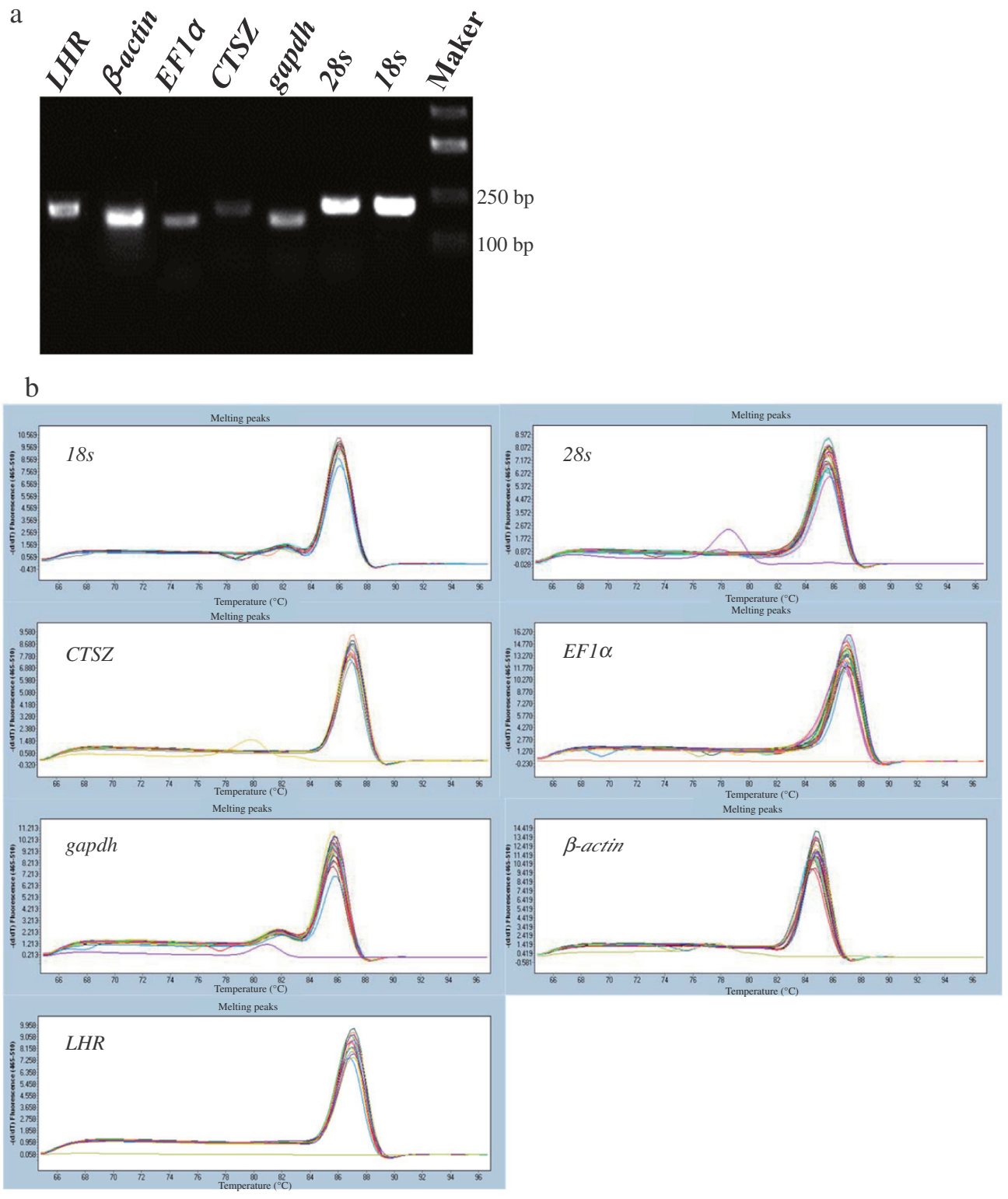

Fig. 1 – Specificity of primers and amplicon size. (a) 1% EB agarose gel electrophoresis of amplified fragments from a single experiment; (b) Melting curves of six reference genes and one target gene. *18s* indicates *18s ribosomal RNA*, *28s* indicates *28s ribosomal RNA*, *CTSZ* indicates *Cathepsin Z*, *EF1α* indicates *elongation factor 1-α*, *gapdh* indicates *glyceraldehyde-3-phosphate dehydrogenase*, *LHR* indicates *luteinizing hormone receptor*.

M values which indicated that they were the most stable genes among all examined 6 genes. In addition, the value of pairwise variation of these two genes (V2/3) was larger than default (0.15), suggesting that there is no need to add the third gene, *gapdh* and *EF1α* could be used simultaneously as best reference gene pair to normalize the expression of the target

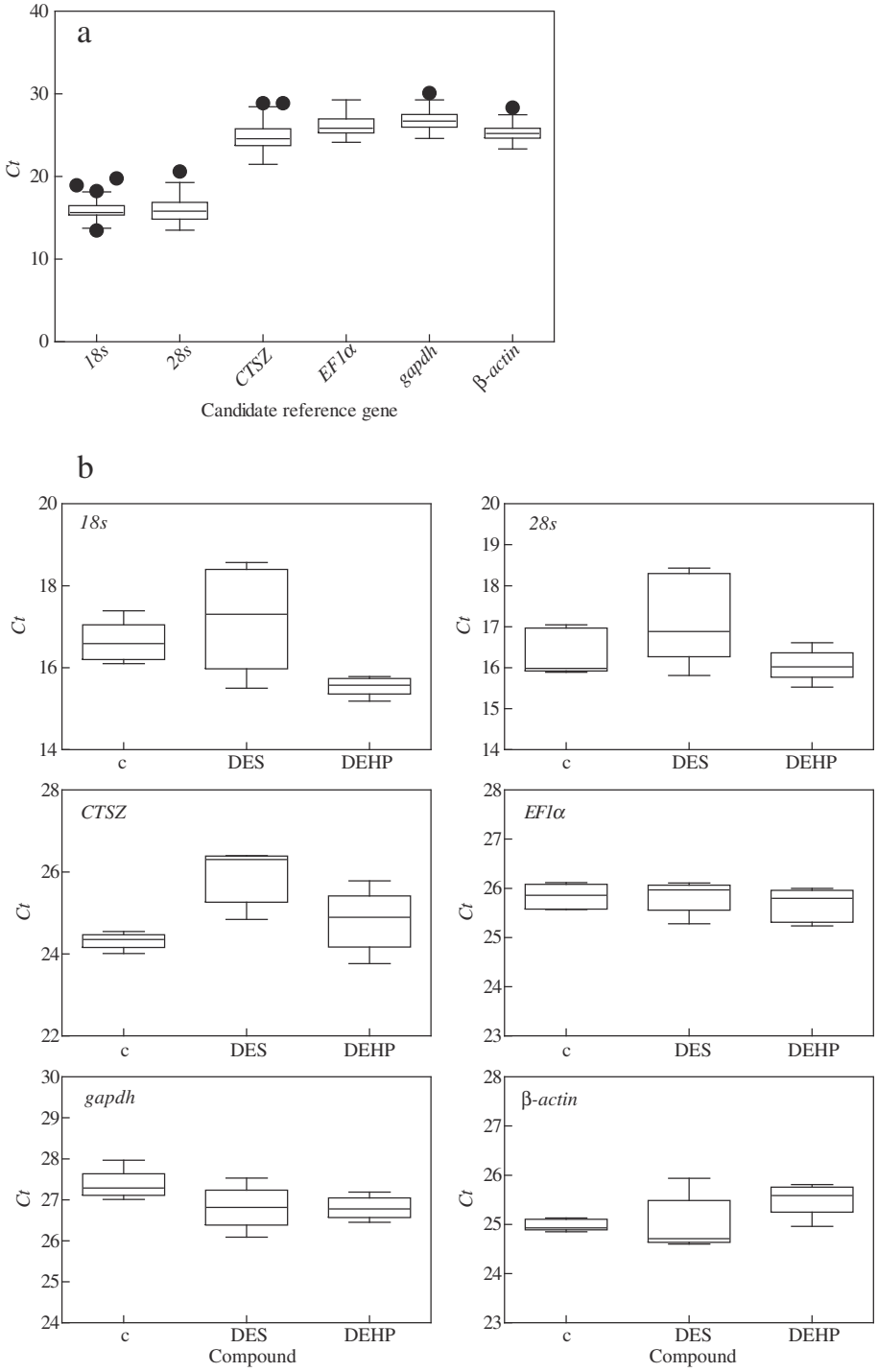

Fig. 2 – Expression variability of references genes. (a) Range of expression of candidate reference genes in common carp (all samples); (b) absolute expression values (Threshold cycles, Ct) of oocytes exposed to different compounds for every candidate reference gene. Ct value (*n* = 15 for per compound) of the qPCR was determined by the LightCycler®480 Software 1.5 (Roche Applied Science, UK). The crosses, boxes and whiskers represent the mean of Ct to one candidate reference gene, interquartile and Tukey range, respectively. c means the control group. DES means the diethylstilbestrol group. DEHP means the di-(2-ethylhexyl)-phthalate group. *18s* indicates *18s ribosomal RNA*, *28s* indicates *28s ribosomal RNA*, *CTSZ* indicates *Cathepsin Z*, *EF1α* indicates *elongation factor 1-α*, *gapdh* indicates *glyceraldehyde-3-phosphate dehydrogenase*, *LHR* indicates *luteinizing hormone receptor*.

genes. The same as DES group, *EF1α* and *gapdh* also were screened as the two best reference genes for DEHP exposure (Fig. S1). In NormFinder analysis, results showed that *EF1α* was suitable alone to be reference gene (Fig. S2a). While in DEHP group, *gapdh* was regarded as the best single reference gene (Fig. S2b). Bestkeeper results showed that *gapdh* had

Table 2 – Ranking of candidate reference genes using geNorm, NormFinder, Bestkeeper and RefFinder analysis when oocytes incubated with DES and DEHP.

Rank	geNorm	NormFinder	Bestkeeper	RefFinder
DES				
1	*EF1α/gapdh*	*EF1α*	*gapdh*	*EF1α*
2		*gapdh*	*β-actin*	*gapdh*
3	*β-actin*	*CTSZ*	*EF1α*	*β-actin*
4	*CTSZ*	*28s*	*CTSZ*	*CTSZ*
5	*28s*	*β-actin*	*28s*	*28s*
6	*18s*	*18s*	*18s*	*18s*
DEHP				
1	*EF1α/gapdh*	*gapdh*	*gapdh*	*gapdh*
2		*EF1α*	*EF1α*	*EF1α*
3	*18s*	*18s*	*18s*	*18s*
4	*28s*	*CTSZ*	*β-actin*	*28s*
5	*CTSZ*	*28s*	*28s*	*CTSZ*
6	*β-actin*	*β-actin*	*CTSZ*	*β-actin*

Candidates are listed from top to bottom in order of decreasing expression stability.

relatively low coefficient variation (CV, Ct%) and standard deviation (SD) values, and the highest Bestkeeper index value (0.929 and 0.906) among the 6 candidate reference genes in both DES and DEHP groups, which indicated that *gapdh* was the best reference gene in the present study (Tables S2–S5). According to RefFinder analysis in Fig. S3, *EF1α* was the most stable gene among the six candidate reference genes when oocytes were incubated with DES, while in DEHP group, *gapdh* showed the highest stability. The results of 4 softwares were summarized in Table 2, RefFinder results could be adopted in the present study.

2.4. *LHR* normalization by different reference genes

The relative expression level of *LHR* was normalized by *18s*, *28s*, *CTSZ*, *EF1α*, *gapdh*, and *β-actin* respectively (Fig. 3). The results indicate that when normalized by different reference genes, the relative expression level of *LHR* turns out to be changeable. That is to say, selecting stable expression reference gene is crucial when we conduct qPCR experiment in variable conditions.

3. Discussion

The selection of the most stable gene or a set of genes under various experimental conditions is very important for obtaining accurate profiling of gene expression with qPCR. Thus far, however, many common reference genes have been used without validation. More and more studies have shown that some of the widely used reference genes such as *18s* and *gapdh* may vary significantly in expression levels under different conditions (Jaffe et al., 1993; Campbell et al., 2006). Hence, to avoid unnecessary errors in qPCR analysis, we have examined the RNA transcription levels of 6 potential reference genes in oocytes of common carp incubated with different compounds.

When FOM was induced by DES, geNorm results showed that *gapdh* and *EF1α* were the two most stable genes among all 6 examined genes. NormFinder and RefFinder analysis also demonstrated that *EF1α* was the most stable gene among the 6

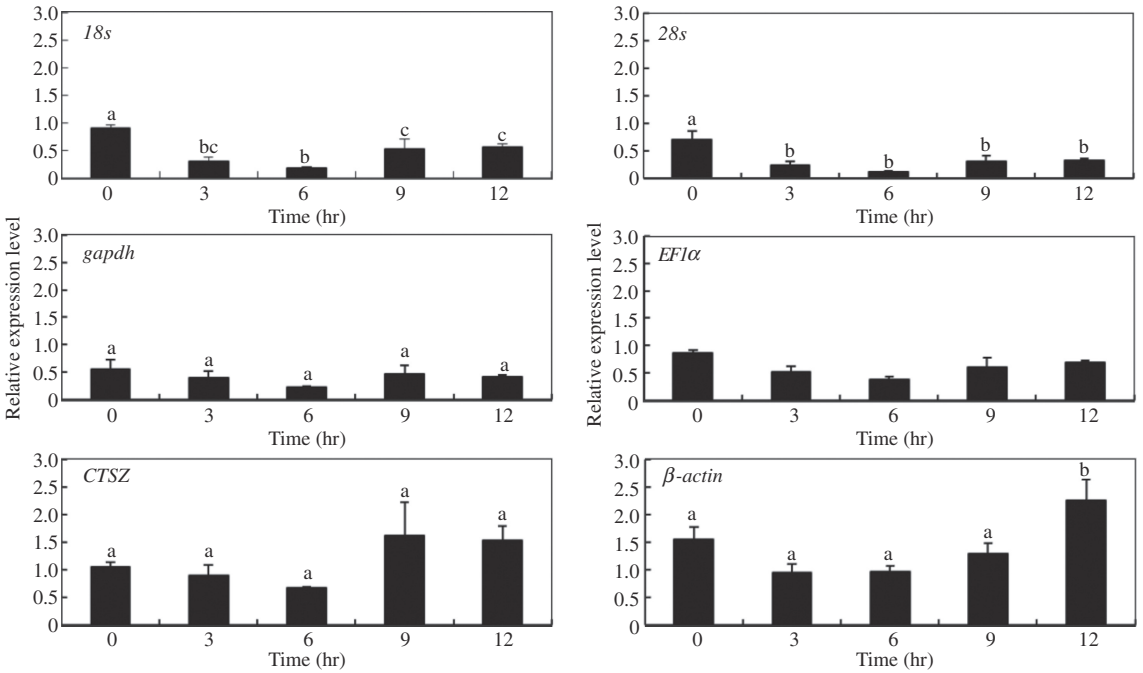

Fig. 3 – *LHR* expression levels normalized by six reference genes during FOM induced by 2 μmol/L DES. Different letters represent statistically significance difference ($p < 0.05$). *18s* indicates *18s ribosomal RNA*, *28s* indicates *28s ribosomal RNA*, *CTSZ* indicates *Cathepsin Z*, *EF1α* indicates *elongation factor 1-α*, *gapdh* indicates *glyceraldehyde-3-phosphate dehydrogenase*, *LHR* indicates *luteinizing hormone receptor*. hr indicates hours.

candidate reference genes. Only Bestkeeper allocated *EF1α* as the third placer in terms of expression stability. According to the results of the 4 softwares, *EF1α* could serve as the reference gene to normalize the expression of target gene during FOM induced by DES in common carp (Table 2). While for DEHP, the same as DES group, *gapdh* and *EF1α* were screened as the two most stable genes by geNorm analysis. Consistently, Bestkeeper, NormFinder and RefFinder softwares also demonstrated that *gapdh* was the most stable gene among the 6 candidate reference genes. Hence, it suggested that *gapdh* could serve as the reference gene to normalize the expression of target gene during FOM induced by DEHP in common carp (Table 2).

In recent years, more and more studies using qPCR as a research tool realizes the importance of the selection of stable expressed reference gene (Andersen et al., 2004; Jorgensen et al., 2006; Fernandes et al., 2008; Bower and Johnston, 2009; Øvergård et al., 2010). Using qPCR, Bower and Johnston (2009) investigated the expression of eight candidate genes to identify suitable reference genes for primary myogenic cell cultures from Atlantic salmon (*Salmo salar* L.). Through the analysis of geNorm, NormFinder and Bestkeeper they achieved the results that any three of *hypoxanthine phosphoribosyl transferase 1*, *EF1α*, *prolylpeptidyl isomerase A* and *RNA polymerase 2* are suitable for the normalization of gene expression data in primary myogenic cultures from Atlantic salmon. The study performed by Infante et al. (2008) found that *ubiquitin*, *ribosomal proteins S4* and *EF1α* are suitable reference genes in larvae developmental stages of *Senegalese sole* and *Hippoglossus stenolepis* by investigating the expression stability of 11 putative reference genes. For selecting the stable reference genes during oogenesis in teleost fish, Deloffre et al. (2012) detected 6 functionally distinct genes, *β-actin*, *cathepsin D* (*CTSD*), *CTSZ*, *EF1α*, *TATA binding protein* and *tubulin A*, their result turns out to be a single gene and up to 3 genes were shown to be insufficient for reliable normalization throughout the whole oogenesis. The combination of the genes *β-actin*, *CTSD*, *EF1α* and *CTSZ* as reference was found to minimize variation and has the most stable expression pattern between maturation stages.

It is important to point out that, in most of the reference gene validation papers, genes such as *ribosome RNA* and *β-actin* which were widely used as reference before are not really stable. Meanwhile, *EF1α*, which is a component of the elongation factor 1 complex responsible for enzymatic delivery of the amino acyl tRNA to the ribosome and for the nuclear export of proteins, shows considerable high stability, whether in primary myogenic cell cultures (Bower and Johnston, 2009), larvae developmental stages (Infante et al., 2008), oogenesis or oocyte maturation (Liang et al., 2015), as well as in the present FOM experiment. However, researchers should keep in mind that a real universal reference gene does not exist, every qPCR experiment should set up a series of candidate reference genes and select the most stable reference gene.

4. Conclusions

In conclusion, the expression stability of 6 candidate reference genes (*18s*, *28s*, *CTSZ*, *EF1α*, *gapdh* and *β-actin*) during FOM induced by DES and DEHP in common carp was studied. Four softwares (Bestkeeper, geNorm, NormFinder and RefFinder) were used to analyze the expression data. The results indicated that *EF1α* was highly stably expressed when FOM was induced by DES, while *gapdh* was the most stable gene when FOM was induced by DEHP. The results could improve accurate analysis of target genes involved in FOM when induced by DES and DEHP in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.12.030.

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