Reference genes to quantify gene expression during oogenesis in a teleost fish

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Understanding the molecular events involved in the acquisition of competence during oogenesis is a key step to determine the secret of ‘high quality’ eggs for aquaculture. Quantitative real time polymerase chain reaction (qPCR) is the technique of election to determine changes in transcript abundance in such studies, but choosing reference genes for normalization, in particular during oogenesis, remains a challenge. In the present study, transcription of 6 functionally distinct genes, β actin (ACTB), cathepsin D (CTSD), cathepsin Z (CTSZ), elongation factor 1 α (EEF1A), TATA binding protein (TBP) and tubulin A (TUBA1A) was assessed as normalizers of bone morphogenetic protein (BMP) and activin membrane-bound inhibitor (BAMBI) gene expression in mRNA from Mozambique tilapia oocytes during oogenesis. Reverse transcription was equally efficient and varies little in all samples. Most of the genes considered for reference were stable during early stages of oogenesis but variations were observed during vitellogenesis. 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 ACTB, CTSD, EEF1A and CTSZ as reference was found to minimize variation and has the most stable expression pattern between maturation stages.

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1. Introduction

Knowledge of the molecular events and the determination of the role of the various factors involved in oogenesis is essential for the development and application of methodologies related to fertility both in the medical and animal production fields. For example, in aquaculture developmental abnormalities attributed to “egg quality” are considered to have an important negative economic impact (e.g., Bonnet et al., 2007; Rideout et al., 2004). However, the extent of the contribution of the constituents of the oocyte to make an egg of good quality and the underlying molecular mechanisms, which are linked to successful fertilization and early development of the zygote remain largely elusive (Gosden, 2002; Lubzens et al., 2010). The determination of variation in transcript abundance is an important element in the uncovering of the underlying processes during oogenesis and quantitative real time polymerase chain reaction (qPCR) is a technique of choice. However, qPCR normally requires normalization to a reference gene with a constant expression and the unique character of oogenesis makes identification of a normalizer(s) particularly challenging. The early stages of embryonic development are driven and controlled by maternal RNAs, which accumulate during oogenesis to produce essential transcription factors, secreted factors and all the machinery for protein synthesis, such as ribosomal RNAs (e.g. 18S ribosomal RNA) (Heasman, 2006; Newport and Kirschner, 1982a, 1982b). As a result of the drastic increase in transcription during oogenesis and modifications in basic cellular processes there is a decrease in the ratio of mRNA/total RNA (Kroupova et al., 2011; Luckenbach et al., 2008b). This invalidates the use of 18S ribosomal RNA to normalize gene expression. Furthermore, normalization of gene expression in Northern blot and polymerase chain reaction (PCR) is often based on a single gene, which is most frequently GAPDH or ACTB (Suzuki et al., 2000), even though these and other commonly used reference genes (e.g., B2m, HPRT1, UBC) have been shown to vary under different experimental conditions (de Jonge et al., 2007; Infante et al., 2008; Lee et al., 2002; Thellin et al., 1999). It is clear that the choice of reference gene(s) may strongly influence results and MIQE (minimum information for publication of quantitative real-time PCR experiments) recommends the use of a minimum of 3 genes for normalization (Bustin et al., 2009). Use of a single gene is acceptable only if the stability of expression under the experimental conditions has been demonstrated (Bustin et al., 2009). Indeed, numerous studies have demonstrated the absence of a universal reference gene and the necessity to look for the optimal normalizer for each experiment or species (Gutierrez et al., 2008; Hugget et al., 2005). The selection of stable genes for normalization can be assisted...
using applications such as Bestkeeper (Pfafli et al., 2004), geNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004), which also indicate the optimal number of reference genes required for robust quantification.

Tilapias are among the most popular aquaculture species (Canonico et al., 2005) and the Mozambique tilapia (Oreochromis mossambicus) is also widely used to produce hybrid tilapia for aquaculture and as an experimental model. It has a heterogametic sex determination system (XX:XY system) with an asynchronous ovarian development and is a good model to study oogenesis (Halikakoty and Biswas, 2005; Srijunngam and Wattanasirikul, 2001).

The present study aimed to identify suitable reference genes for robust analysis of molecular changes during oogenesis. Bone morphogenetic protein (BMP) and activin membrane-bound inhibitor (BAMBI), an inhibitor of BMPs expression (Lankford and Weber, 2010a), was used as target in the analysis. BAMBI is a transmembrane glycoprotein structurally similar to the BMP type 1 receptor but lacking the serine/threonine kinase domain required for signaling. BAMBI appears to be a negative regulator of the BMP signaling pathway through substitution of the type I receptor in the receptor heterocomplex (Sekiya et al., 2004).

Six genes were tested for their adequacy as reference: the cytoskeletal actin beta (ACTB) and tubulin alpha (TUBA1A), the proteases cathepsin D (CTSD) and cathepsin Z (CTSZ aka cathepsin P or X), the member of the translation elongation factor 1 complex, elongation factor 1 alpha (EEF1A), and the transcription factor TATA binding protein (TBP). These genes have been classically used as reference in studies with oocytes and other tissues, with the exception of cathepsins, which in a differential expression study, appeared to be stable during the transition from primary to early secondary oocyte growth in Oncorhynchus kisutch (Baron et al., 2005; Luckenbach et al., 2008b) and therefore seemed appropriate to test.

2. Material and methods

2.1. Animals

Animal maintenance and handling procedures followed the recommendations of the Association of Animal Behaviour (ASAB, 2003) and national regulations. Mozambique tilapia were from a stock maintained at the University of Algarve (Faro, Portugal) at 27 °C and under a 12L:12D photoperiod. They were fed once a day with commercial cichlid food (Nutrafins Basix®; Rolf C. Hagen Inc., Montreal, Canada). Fish were anesthetized in ethyl 3-amino benzoate methanesulfonate salt (MS-222, Sigma-Aldrich, Spain) and killed by decapitation. Ovaries were collected from 5 adult females and were dissected manually under a stereomicroscope (Olympus, SXZ7) in Cortland medium (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM NaH2PO4, 10 mM NaHCO3, 1.5 mM CaCl2, 1.5 mM MgSO4, 5 mM glucose and 15 mM of HEPES). Follicles were divided into 5 groups based upon their developmental stage during oogenesis: perinuclear (PS), cortical alveoli (CAS), early vitellogenic (EVS), vitellogenic (VS) and final maturation (MS). Because yolk reduces the efficiency of RNA extraction, the mature eggs were emptied of yolk by perforation of the membrane. For each stage, pools of -50 eggs per individual were ground in liquid nitrogen and stored at -80 °C.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated using Purezol (Bio-Rad, Hercules, CA) following the manufacturer's instructions. The quality of total RNA was assessed by determining the absorbance ratio of 260/280 and by agarose gel electrophoresis. mRNA was purified using the MicroPoly(A) Purist kit (Ambion/Life Technologies Corporation, Alcobendas, Spain) following the manufacturer's instructions and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). To confirm the absence of inhibitors of the reverse transcription, the ALIEN® RNA transcript control (ALIEN qRT-PCR Inhibitor Alert, Agilent Technologies, Santa Clara, CA) was introduced as an external control into the mRNA samples. A control tube without sample mRNA but with the same quantity of ALIEN® RNA transcript was prepared. After addition of 105 copies of the external control to each tube, first strand cDNA was performed with 50 ng of mRNA and the Iscript™ cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions.

2.3. Cloning of reference genes and BAMBI

Eight micrograms of ovary total RNA from mature oocytes was treated with 2 U of Turbo DNAsase (Ambion/Life Technologies Corporation) for 30 min at 37 °C following the manufacturer's instructions. Three micrograms of treated RNA was used for reverse transcription in a 40 μl reaction volume using 40 U of MMLV reverse transcriptase (Promega, Madison, WI), 8 U of Ribolock RNase inhibitor (Fermentas, St Leon-Rot, Germany), 0.2 mM dNTPs and 10 pmol of oligo(dT) adaptor (5′-CGAGTCGACATCGATCGT(T)10-3′).

To obtain the partial cDNA clones of reference genes and BAMBI, primers (Supplementary Data 1) were designed using conserved regions identified by multiple sequence alignment of orthologues from teleost fish present in GenBank. PCRs (50 μl volume) were performed with -20 ng of cDNA, 2 mM MgCl2, 0.1 mM of dNTPs, 50 pmol of each sense and antisense primers and 0.6 U of DreamTaq™ DNA polymerase (Fermentas). Reactions were carried out in a MyCycler thermocycler (Bio-Rad), using the following conditions: 30 cycles at 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s. PCR products with the expected size were purified, ligated into pGem-T easy plasmid (Promega) and sequenced. For each PCR, negative reactions without cDNA or with mRNA, which was not reverse transcribed, were also performed.

BAMBI full length cDNA was obtained using rapid amplification of cDNA ends (RACE). For the 3′-RACE-PCR, reactions were carried out using an oligo(dT) primed ovary cDNA as template, and amplified using BAMBI-3′-1 primer and adaptor 1. The PCR product was nested with a BAMBI-3′-2 primer to assure the specificity of the product. For the 5′-RACE PCR, a cDNA was synthesized using a forward BAMBI-5′-DNA with the protocol described previously, for the partial cloning. A first PCR was performed with a single reverse primer, BAMBI-5′-nor. A second round of PCR with the reverse primer BAMBI-5′-nor and the complementary primer, BAMBI-5′-opp, amplified a double stranded PCR product. For 3′ and 5′ RACE PCRs, amplicons of the expected size were cloned and sequenced. RACE PCRs were performed using the same conditions as described for partial cloning.

2.4. Quantitative real time polymerase chain reaction

Primers for qPCR (Table 1) were designed using Beacon Designer 7.7 (Premier Biosoft International, Palo Alto, CA) and amplification PCR products using these primers were sequenced to confirm specificity. PCR efficiencies and coefficients of determination (r2) were established with duplicated standard curves generated from a 10-fold dilution series (from 1 ng to 1 fg) of purified PCR fragments as templates using an IQ-5 real-time PCR detection system and SsoFast™ Evagreen® supermix (Bio-Rad).

The final reaction volume was 15 μl and contained 2 μl of cDNA diluted 1/20, 7.5 μl of SsoFast™ Evagreen® supermix and 100 pmol of each reverse and forward primers. The thermocycles consisted of heating at 94 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 10 s. The melt curve was obtained by increasing the temperature from 65 °C to 95 °C in steps of 0.5 °C/cycle. For each maturation stage, 5 independent samples were tested in technical duplicates. The absence of interference with the ALIEN® transcript was tested for each pair of primers. Negative reactions without cDNA were also performed. In
order to test for the presence of potential inhibitors of the polymerase chain reaction, qPCR amplification of the ALIEN transcript in the absence (control) and presence of the sample mRNA was performed following the manufacturer's instructions.

2.5. Evaluation of expression stability

After extraction of the quantification cycle (Cq) values with the IQ software (Bio-Rad), stability of the candidate reference genes was evaluated with the software Bestkeeper (Pfaffl et al., 2004), Normfinder (Andersen et al., 2004), and geNorm (Vandesompele et al., 2002). Results are expressed as the ratio of the target gene expression for each stage relative to expression in the PS stage for the same individuals. Normalization was carried out according to Pfaffl (2001) using the geometric mean of the best combination of selected genes. In the case of non-normalized data, fold-change in relation to the PS stage was calculated using Cq values (as \(E^{\Delta Cq(PS-maturation stage)}\)) in which PS is the Cq value in the perinuclear stage samples and maturation stage is the Cq of the stage being compared with from the same individuals).

2.6. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using SigmaPlot version 12.0 (Systat Software Inc. San Jose, CA). Two-way ANOVA with gene and maturational stage as factors was used to compare relative expression levels on log (2) transformed fold change. Tukey’s post-hoc was used for comparisons against the PS stage. The level of significance was 5%.

3. Results and discussion

3.1. Pattern of expression levels of reference genes

The most common method to normalize gene expression level is to use a reference gene as an internal control. The choice of the reference gene is a crucial element in qPCR analyses and numerous studies have demonstrated the absence of universal reference gene and the necessity to look for the optimal normalizer for each tissue, condition or species (Gutierrez et al., 2008; Hugget et al., 2005). Several studies have dealt with qPCR normalization in fish (Aursnes et al., 2011; Bower and Johnston, 2009; Fernandez et al., 2008; Infante et al., 2008; Jorgensen et al., 2005; Overgard et al., 2010). And while there have been also studies directed at the differential expression of genes during oocyte development in teleosts (Luckenbach et al., 2008b; Tingaud-Sequeira et al., 2009; Von Schalburg et al., 2005), this is the first study which characterized genes with stable expression throughout oocyte growth to be used as normalizers.

The qPCR amplification efficiency for all the genes tested using mRNA extracted from the 5 groups of oocytes ranged between 94.1% and 103.2% and the coefficient of determination (\(r^2\)) of the standard curves varied between 0.994 and 1 (Table 1). Comparison of the qPCR cycles at which template quantification was possible (Cq) revealed that it was not significantly different between Alien RNA alone (20.38 ± 0.128, n = 4) and Alien RNA plus sample mRNA (20.03 ± 0.07, n = 25) indicating that no inhibitors were present in the qPCR. There was no significant variation in ALIEN Cq value among oocyte stage samples with a maximum difference of 2 cycles (Fig. 1a). This was to be expected, as the same amount of ALIEN mRNA was added in each sample, but it also indicates similar efficiency between samples in the various steps from reverse transcription to qPCR.

The transcripts selected as potential reference genes were all relatively abundant in tilapia oocytes and their mean Cq value ranged between 18.8 (ACTB) and 26 (CTSD). EEF1A had the lowest variation in expression level between samples (n = 25) and the Cq values differed by less than 4 cycles among samples. TUBA1A was the most variable gene and differed by more than 8 cycles between some samples (Fig. 1b).

CTSD expression level did not differ significantly between developmental stages (Fig. 1c). In contrast, CTSD and EEF1A expression levels increased significantly from the perinuclear stage to the mature stage, while ACTB, TBP and TUBA1A expression decreased significantly after the late vitellogenic stage. The largest TBP and TUBA1A variation in transcript abundance occurred between immature and mature oocyte stages (Fig. 1c). Thus, it appears that in O. mossambicus all but CTSD are regulated during oocyte development. CTSD is an aspartyl protease with high enzymatic activity during vitellogenesis stage. Coho salmon, O. kisutch, also had stable expression of CTSD during the perinuclear stage and cortical alveolus stage (Luckenbach et al., 2008b). However, rainbow trout, Oncorhynchus mykiss, CTSD mRNA was constitutively expressed during the vitellogenic and final maturation stages, but had a peak of gene expression during early vitellogenesis (Kwon et al., 2001) which suggests some degree of regulation also in that species. Interestingly, human CTSD can have both a constitutive function with multiple start sites and be regulated by estrogen through its TATA box (Cavailles et al., 1993).

CTSZ, a member of the cysteine protease family, increased with the maturational stage, similar to what is observed in rainbow trout, a species in which CTSZ has been implicated in egg quality (Bobe et al., 2008b; Tingaud-Sequeira et al., 2009; Von Schalburg et al., 2005).

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer pairs</th>
<th>Efficiency (%</th>
<th>Coefficient of determination (r^2)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>FN673689</td>
<td>F: TGACCTCACAGACTACCT, R: GCTCTGAACCTCCTCCCA</td>
<td>95.6</td>
<td>0.996</td>
<td>179</td>
</tr>
<tr>
<td>CTSD</td>
<td>FN646079</td>
<td>F: CACGAAACCTGGAAGA, R: TGTGACCATCTCCTGAGA</td>
<td>96.3</td>
<td>0.998</td>
<td>130</td>
</tr>
<tr>
<td>CTSZ</td>
<td>FN673688</td>
<td>F: AGGCTACATCAACACCAT, R: TACTAACCTGGACCTGCTCC</td>
<td>103.2</td>
<td>0.999</td>
<td>156</td>
</tr>
<tr>
<td>EEF1A</td>
<td>FN597061</td>
<td>F: GTTCAGAGGATGAAGAATGG, R: TTCAAGATACAGCTGACAC</td>
<td>95.6</td>
<td>0.996</td>
<td>189</td>
</tr>
<tr>
<td>TBP</td>
<td>FN673692</td>
<td>F: GGCAGCTGTGATGCAAG, R: GATCCAGGAGAACTTCG</td>
<td>96.0</td>
<td>0.997</td>
<td>135</td>
</tr>
<tr>
<td>TUBA1A</td>
<td>FN673693</td>
<td>F: TTCTACTCTTCTCTCATT, R: AACCTAGACTTCTGCGTTA</td>
<td>94.1</td>
<td>0.994</td>
<td>98</td>
</tr>
<tr>
<td>BAMBI</td>
<td>FN543097</td>
<td>F: AGTCAGATCTTCTTCCATT, R: TCTGATTGACATGATCC</td>
<td>100.2</td>
<td>1.000</td>
<td>130</td>
</tr>
</tbody>
</table>
et al., 2004). However, in killifish, *Fundulus heteroclitus*, and in salmon, *CTSZ* expression was relatively stable during the oocyte growth and maturation (Fabra and Cerdà, 2004; Luckenbach et al., 2008b).

The other genes tested in which expression seemed to increase or decrease with increased maturity are involved in major cellular functions. *EEF1A* is a component of the elongation factor 1 complex, responsible for enzymatic delivery of the aminoacyl tRNA to the ribosome and for the nuclear export of proteins. *TBP* is the transcription factor binding the DNA TATA box during the initiation of the transcription. *ACTB* is one of the two non-muscle cytoskeletal actins and *TUBA1A* is a component of the microtubules.

For the genes tested, the largest variation in gene expression occurred during vitellogenesis, a period in which, among other events, vitellogenin and egg shell proteins are incorporated into the oocyte, processed as yolk proteins and the external envelope of the oocyte is formed (Lubzens et al., 2010). The production of these proteins is regulated by estradiol-17β and it is possible that this hormone could be responsible for the changes in the expression patterns of at least some of these genes normally considered to have stable patterns. This is supported by several other studies in which some of the genes that are the objects of this study changed expression in response to estradiol-17β: e.g., *TUBA1A* in human PC12 cell lines transfected with estrogen receptor alpha (Gollapudi and Oblinger, 2001), *CTSZ* in *Sparus aurata* macrophages (Liarte et al., 2011), *ACTB* in mouse uterus (Schroder et al., 2009) and *CTSD* in O. mykiss white muscle (Cleveland and Weber, 2011).

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3.2. Selection of reference genes

The main assumptions are that reference genes should have stable expression and not be co-regulated. Following a preliminary selection, three genes were excluded because their expression was highly variable during oogenesis: GAPDH (glyceraldehyde 3-phosphate dehydrogenase), HPRT1 (hypoxanthine phosphoribosyltransferase 1) and B2m (beta-2 microglobulin).

Several computer programs have been developed to aid in the selection of one or a set of reference genes, which minimize a measure of variation. Bestkeeper calculates the standard deviation (SD) and coefficient of variation (CV) of reference genes and assigns a Bestkeeper index for those with the lowest variation. Pair-wise correlation analyses are performed between possible reference gene pairs, and a Pearson correlation coefficient value close to 1.0 indicates the genes most suitable for normalization (Pfaffl et al., 2004). The SD rank of the candidate reference genes determined by Bestkeeper was: EEF1A (0.64) > CTSZ > ACTB > CTSD > TUBA1A and TBP (Table 2). TUBA1A, TBP and CTSZ had a SD similar to or larger than 1 and were eliminated from the analysis. Bestkeeper stability and correlation analysis of the candidate reference genes showed a strong inter-gene correlation between CTSD and ACTB (0.819), indicating that they had a similar overall expression pattern. CTSD had the highest correlation with the calculated Bestkeeper index (0.919) and was considered the most suitable reference gene by this software (Table 3).

GeNorm calculates the gene expression stability index (M) by pair-wise comparisons based upon the similarity of expression profile, using the geometric mean as the normalization factor. A step-wise analysis is carried out and the gene with the highest M value is eliminated; the analysis restarts until the 2 most stable genes are obtained (Vandesompele et al., 2002). The reference genes from the analysis of oogenesis stages ranked by geNorm according to variability were TBP > TUBA1A > CTSD > EEF1A > ACTB > CTSD (Fig. 2a). The geNorm algorithm also calculates the optimal number of genes for normalization by calculating the pairwise variation Vn/n+1, which measures the effect of adding further reference genes on the normalization factor. Vandesompele et al. (2002) suggested that a Vn/n+1 cut-off value of 0.15 may be considered the limit below which the inclusion of additional genes is not required for normalization. However, in our experimental conditions, Vn/n+1 did not reach the cut-off value, with the lowest value of V3/4 being 0.334 (Fig. 2b). With the addition of a fifth gene (TUBA1A), V4/5 increased to 0.370. We concluded from this analysis that 4 reference genes (ACTB, CTSD, EEF1A and CTSD) are advised for most accurate normalization. As with Bestkeeper, geNorm excluded TUBA1A and TBP as potential reference genes, the M value when the two genes were included being higher than 1.5.

### Table 2
Bestkeeper software: descriptive statistics of the candidate reference genes based on their quantification cycle (Cq) values.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>ACTB</th>
<th>CTSD</th>
<th>CTSD</th>
<th>EEF1A</th>
<th>TBP</th>
<th>TUBA1A</th>
<th>Bestkeeper 3 genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geo mean</td>
<td>18.75</td>
<td>25.98</td>
<td>26.16</td>
<td>20.56</td>
<td>24.15</td>
<td>20.43</td>
<td>21.55</td>
</tr>
<tr>
<td>Ar mean</td>
<td>18.78</td>
<td>26.00</td>
<td>26.19</td>
<td>20.58</td>
<td>24.27</td>
<td>20.50</td>
<td>21.57</td>
</tr>
<tr>
<td>Min Cq</td>
<td>17.70</td>
<td>24.85</td>
<td>23.02</td>
<td>18.53</td>
<td>21.06</td>
<td>18.56</td>
<td>20.54</td>
</tr>
<tr>
<td>Max Cq</td>
<td>21.34</td>
<td>28.42</td>
<td>28.23</td>
<td>22.07</td>
<td>29.97</td>
<td>24.08</td>
<td>23.16</td>
</tr>
<tr>
<td>Std dev (±Cq)</td>
<td>0.85</td>
<td>0.83</td>
<td><strong>0.98</strong></td>
<td>0.64</td>
<td><strong>2.06</strong></td>
<td>1.47</td>
<td>0.55</td>
</tr>
<tr>
<td>CV Cq</td>
<td>4.55</td>
<td>3.19</td>
<td>3.73</td>
<td>2.18</td>
<td>0.98</td>
<td>0.64</td>
<td>0.55</td>
</tr>
<tr>
<td>Min X-fold</td>
<td>−2.93</td>
<td>−2.13</td>
<td>−9.24</td>
<td>−3.91</td>
<td>−8.00</td>
<td>−3.44</td>
<td>−1.99</td>
</tr>
<tr>
<td>Max X-fold</td>
<td>5.71</td>
<td>5.18</td>
<td>4.33</td>
<td>2.77</td>
<td>50.20</td>
<td>11.26</td>
<td>2.98</td>
</tr>
<tr>
<td>Std dev</td>
<td>1.78</td>
<td>1.75</td>
<td>1.93</td>
<td><strong>1.54</strong></td>
<td>4.00</td>
<td>2.68</td>
<td>1.46</td>
</tr>
</tbody>
</table>

Note: TBP and TUBA1A had standard deviations (SD) above 1 (bold) and CTSD had SD close to 1, and were discarded from the calculation of the Bestkeeper index (n = 3). EEF1A was considered the most stable gene (grayed cell); Cq: quantification cycle; Geo mean: geometric mean; Ar mean: arithmetic mean; CV (±Cq): coefficient.

Normfinder selects the best reference gene of a set of candidates by a model-based approach. Moreover, inter-group and intra-group variations are estimated and combined to give a stability value and the gene with the lowest value is selected as the most stable (Andersen et al., 2004). With Normfinder, in contrast to the pair-wise comparison of Bestkeeper and geNorm, the expression of the optimum pair of reference genes compensates for the variation of each other. The most stable genes were ACTB (0.328) and CTSD (0.328) and the least stable was TBP (2.181) (Fig. 2c). Analysis of intra- and inter-group variations selected ACTB as the gene with the lowest variation (0.341, Fig. 2d). In this analysis, Normfinder selected the pair ACTB-CTSD (0.301) as the most stable.

Although, the three software are based on different algorithms, they selected the same best genes for normalization, CTSD and ACTB. However, only geNorm selected the optimal combination and recommended for less variability the use of 4 genes for the normalization.

3.3. Effect of normalization on BAMBI expression

To evaluate the efficiency of the selected reference genes for normalization, the expression of BAMBI during the oocyte development was analyzed. For that purpose a full-length 1197 bp BAMBI cDNA was cloned by RACE-PCR (Supplementary Data 2) from which qPCR primers were designed. The Cq of BAMBI qPCR ranged from 24.86 to 29.63 between oocyte stages, with a mean of 25.95 ± 1.377. The difference between amplification slope of the reference and the target gene (BAMBI) was less than 0.1.

Multiple-gene normalization is advisable when none of the candidate reference genes appear to be suitable. Indeed, in general, variation of normalization with multiple genes is smaller than the variation of normalization with single genes (Andersen et al., 2004). The gene pair associated by Normfinder was selected so as to compensate for the variation of one gene by the variation of the other, while the best pair of geNorm is associated by similarity of expression pattern. In addition, geNorm has also the particularity to determine the best number of genes for accurate normalization. The best pair of genes selected by Normfinder and geNorm (ACTB–CTSD) did not yield a significant modification over single gene normalization (not shown). Stepwise addition of more genes to normalization modified the pattern of statistical significance of the various stages relative to the PS stage (Fig. 3). Thus, the three genes selected for their relative stability during rainbow trout (Kwon et al., 2001) and coho salmon (Luckenbach et al., 2008b) oocyte growth, CTSD and EEF1A were not suitable for single gene normalization in the oogenesis study of O. mossambicus, although when used simultaneously with ACTB they proved to be reliable. Finally, the combination of 4 genes suggested by geNorm appeared to be the most suitable set to evaluate the gene expression during oogenesis. This combination of reference genes had the most stable expression pattern with maximal amplitude of variation between stages of 0.76 Cq, while for the combination of three genes it was 1.03 Cq. The pattern of expression normalized by 4 genes was also statistically indistinguishable from
Data are presented as fold change compared to PS. Different capital letters represent statistical significance. In the present study because of its high variation. The expression pattern without normalization. This was to be expected with adequate normalization, considering that the same concentration of mRNA was used in all samples and cDNA synthesis had a similar efficiency in all reactions (as determined from ALIEN amplification). Although in the current study mRNA was used as starting material, it is to be expected that the same reference genes are equally applicable when total RNA is used instead.

The pattern of BAMBI expression obtained in this study is different from that observed by Lankford and Webber in O. mykiss (Lankford and Weber, 2010b). However, in their study, BAMBI was normalized to EF1A, which was considered to be unreliable for normalization in the present study because of its high variation.

4. Conclusions

When comparing oocytes at different stages none of the individual reference genes tested fulfilled the requirement of expression stability, possibly because of estrogen responsiveness. However, by normalizing the expression of the target gene BAMBI to a combination of 4 reference genes a stable pattern was obtained between maturation stages and variation was minimized. These results will allow appropriate quantitation of gene expression in investigations of oocyte development in O. mossambicus and possibly other fishes and vertebrates.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.06.047.

Competing interests

No competing interests to declare.

Authors’ contributions

LAMD designed the study, carried out experiments, analyzed the results and wrote the manuscript; AA and AIM cloned genes and performed qPCRs. AVMC designed the study and wrote the manuscript. The authors have read and approved the final manuscript.

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