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# Selection of reliable reference genes for RT-qPCR studies in *Octopus vulgaris* paralarvae during development and immune-stimulation

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ABSTRACT

The common octopus, *Octopus vulgaris* is a new candidate species for aquaculture. However, rearing of octopus paralarvae is hampered by high mortality and poor growth rates that impede its entire culture. The study of genes involved in the octopus development and immune response capability could help to understand the key of paralarvae survival and thus, to complete the octopus life cycle. Quantitative real-time PCR (RT-qPCR) is the most frequently tool used to quantify the gene expression because of specificity and sensitivity. However, reliability of RT-qPCR requires the selection of appropriate normalization genes whose expression must be stable across the different experimental conditions of the study. Hence, the aim of the present work is to evaluate the stability of six candidate genes: β-actin (ACT), elongation factor 1-α (EF), ubiquitin (UBI), β-tubulin (TUB), glyceraldehyde 3-phosphate dehydrogenase (GADPH) and ribosomal RNA 18 (18S) in order to select the best reference gene. The stability of gene expression was analyzed using geNorm, NormFinder and Bestkeeper, in octopus paralarvae of seven developmental stages (embryo, paralarvae of 0, 10, 15, 20, 30 and 34 days) and paralarvae of 20 days after challenge with *Vibrio lentus* and *Vibrio splendidus*. The results were validated by measuring the expression of PGRP, a stimuli-specific gene. Our results showed UBI, EF and 18S as the most suitable reference genes during development of octopus paralarvae, and UBI, ACT and 18S for bacterial infection. These results provide a basis for further studies exploring molecular mechanism of their development and innate immune defense.



## 1. Introduction

*Octopus vulgaris* have a high potential for aquaculture however, paralarvae rearing faces severe difficulties because of high mortalities at settlement stage. Presumably, poor quality of food supplied to paralarvae does not provide them sufficient essential fatty acids (Navarro and Villanueva, 2003). Consequently, different diets have been tested to obtain suitable food items (Iglesias et al., 2006, 2014). The assessment of the health status and the effect of pathogens have been taken into account as causative of stress in cultured octopuses (Vidal et al., 2014; Xavier et al., 2014) since the latter might affect the paralarvae growth. The signal of stress and immune regulation could be found by the study of genes involved in octopus paralarvae immunity. Therefore, the effort for identifying biomarkers of octopus ability to cope with diseases is undergoing. This information is expected to provide important data about paralarvae survival and thus, overcome the bottleneck of octopus aquaculture (Gestal and Castellanos-Martínez, 2015).

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is widely used to measure gene expression because its high sensitivity, flexibility and efficiency (Bustin, 2002). However, technical variation due to sample-to-sample process, RNA integrity, efficiency of DNAse treatment and cDNA synthesis can occur (Bustin et al., 2009; Taylor and Mrkusich, 2014). Therefore, normalization of data is required and internal controls, or reference genes, are used to minimize the systemic errors and inherent variation (Kozera and Rapacz, 2013).

A suitable reference gene must have a stable expression in the target sample and under different experimental conditions and must not be co-regulated with the target gene. Hence, validation of specific reference gene needs to be tested for each species and experiment before using them (Pfaffl et al., 2004; Kozera and Rapacz, 2013). Genes such as glyceraldehyde-3-phosphate-dehy

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drogenase (GADPH), β-actin (ACT), elongation factor-1 alpha (EF), 18S, α-tubulin (TUB) and ubiquitin (UBI) are among the commonest reference genes used in fish, bivalve, *O. vulgaris* brain, and additional octopus tissues after infection (Mitter et al., 2009; Castellanos-Martínez et al., 2014; Moreira et al., 2015). However, no valid internal reference genes exist for normalization data of *O. vulgaris* paralarvae.

The aim of this study was to evaluate the stability of six genes usually employed as reference genes: ACT, EF, UBI, TUB, GADPH and 18S. The most suitable reference genes to normalize the expression level in octopus paralarvae, at different developmental stages and after bacterial infection, were selected.

## 2. Material and methods

### 2.1. Animal growth and bacterial challenge

Octopus embryos and paralarvae were obtained from a broodstock comprised by individuals of similar sizes maintained in an 8000 L flow-through system tank according to Iglesias et al. (2004). PVC shelters were provided as refuges to induce natural spawning. Individuals were maintained under standard conditions of natural photoperiod, seawater temperature (19–23 °C) and they were fed ad libitum with thawed crabs and fish three days a week. The first 15 days paralarvae were fed with *Artemia* nauplius enriched with *Isochrysis galbana* and complemented with *Maja brachydactyla* zoeas. The next 16–34 days *Nannochloropsis* sp. were also used to compliment the paralarvae feeding. Once the spawn was laid, octopus embryos were collected at stage XV of development following Naef (1928). Sampling collection was followed by recently hatched (day 0) paralarvae. Subsequent samples were collected after 10, 15, 20, 30 and 34 days post hatching (d.p.h.). Three individuals were pooled per sample, fixed in Trizol (Invitrogen) and stored at −80 °C until used.

For bacterial challenge, *Vibrio lentus* and *Vibrio splendidus* LGP32 were grown separately overnight at 24 °C in TSB with a final concentration of 2% NaCl, collected by centrifugation (10,000*g*, 5 min) and re-suspended in PBS. Paralarvae of 20 d.p.h. were challenged by adding *V. lentus* or *V. splendidus* to the water with a final concentration of $3 \times 10^6$ cfu/mL. The untreated specimens were employed as control group. Three individuals were collected at 1 h, 4 h and 24 h post-exposure, fixed in Trizol (Invitrogen) and stored at −80 °C. All the specimens employed were anesthetized in cold water prior to experiments following ethical considerations (Moltschaniwskyj et al., 2007; Mather and Anderson, 2007).

### 2.2. RNA isolation and cDNA synthesis

Two experimental sample sets were built. Set A: development (embryo and paralarvae at 0, 10, 15, 20, 30, 34 d.p.h. non-infected) and set B: infected (paralarvae 20 d.p.h. exposed to *V. lentus* or *V. splendidus*).

Total RNA was extracted from sample sets using TRIzol® (Invitrogen), treated with RNase-Free DNase Set (QIAGEN®) and purified using RNeasy Mini Kit (QIAGEN®). RNA quantity and quality were assessed by using NanoDrop ND2000 spectrophotometer (Thermo Scientific). cDNA was synthesized using 1 μg of total RNA and Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) following the manufacturer's instructions.

### 2.3. Primers design and RT-qPCR

Sequences from GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) were used to design forward and reverse primers of candidate genes (Table 1) using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). β-actin and elongation factor 1-α primers used in brain of *O. vulgaris* (Sirakov et al., 2009) were also included in the analysis to test their validity for octopus paralarvae. Specificity of amplification was confirmed by melting curve and agarose gel electrophoresis. The efficiency was determined based on the slopes of standard curves generated using seven serial five-fold dilutions of sample cDNA and calculated as follows: $E\ (\%) = (10^{(-1/\text{slope})} - 1) \times 100$ (Pfaffl, 2001). The quality control of primer efficiency was used to obtain an efficiency value (E) and determination coefficient ($R^2$). RT-qPCR reactions were performed in a 7500 FAST thermocyclator (Applied Biosystems) sequence detector. Each well contained 1 μL cDNA, 12.5 μL of SYBR green PCR master mix (Thermo Scientific) and 0.5 μL of each diluted primer (10 mM). The final volume of each sample was 25 μL. The standard cycling conditions were: 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed as technical triplicates.

### 2.4. Data analysis

The expression stability of the candidate reference genes was evaluated using geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004). Threshold cycle (Ct) data entry in geNorm and NormFinder were previously transformed to relative quantities (RQ) following Hellemans et al. (2007): $RQ = (1 + E)^{\Delta Ct}$ and $\Delta Ct = \text{lowest Ct value of all samples} - \text{Ct value of the sample}$. Results from the three approaches were subsequently analyzed according to Zhu et al. (2012). This method gives a comprehensive ranking with the overall results obtained by the three algorithms used, assigning a number to each gene (between 1 to the most stable to 6 to the least stable one) according to their position in the ranking obtained by each algorithm (geNorm, Normfinder and BestKeeper). The geometric mean (GM) of the score of each gene was calculated and the genes were ranked again.

**Table 1**
Primer sequences and PCR efficiencies of the candidate reference genes.

| Primer name | GenBank | Sequence (5′ → 3́) | Product size (bp) | E (%)[a] | $R^2$ |
|---|---|---|---|---|---|
| β-Actin | AB053937 | F: TGTTGCCGCTTTAGTTGTTG<br>R: TTTTGCTCTGGGCTTCATCT | 170 | 93.97 | 0.999 |
| EF1-α | AY651883 | F: GTAGAGATGCACCACGAGTCACTT<br>R: CATACCCACGACGAAGATCCTT | 100 | 105.04 | 0.999 |
| Ubiquitin | FJ617440 | F: AGAAGGTTAAGTTGGCGGTTTTG<br>R: CCAGCTCCACATTCCTCGTT | 100 | 93.50 | 0.998 |
| α-Tubulin | X15845 | F: TCCAACGTGCTGTGTGCAT<br>R: AAAGCACGCTTGGCATACATC | 100 | 110.41 | 0.998 |
| GADPH | EF634059 | F: GGTGGCAGAACTGCTGGT<br>R: GGCACCCTGAAAGCCATA | 116 | 125.40 | 0.986 |
| 18S | FJ617439 | F: GCGTTCGCTTCGATGAC<br>R: GCCCTTCCGTCAATTCC | 105 | 80.11 | 0.999 |

[a] $E\ (\%) = (10^{(-1/\text{slope})} - 1) \times 100$.