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

P. García-Fernández^{1,a}, S. Castellanos-Martínez^{1,3,a}, J. Iglesias², J.J. Otero²,
C. Gestal^{1*}.

¹Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas
(IIM-CSIC). Vigo, Spain.

²Instituto Español de Oceanografía, Centro Oceanográfico de Vigo. Subida a Radio
Faro nº 50, 36390. Vigo, Spain.

³Present address: Instituto de Investigaciones Oceanológicas, Universidad Autónoma de
Baja California, Km 103 Carretera Tijuana-Ensenada, C.P 22860, Ensenada BC,
México

*Corresponding Author:

Camino Gestal

Instituto de Investigaciones Marinas (CSIC)

Eduardo Cabello 6

36208 Vigo, Spain.

Tf. +34 986 231930 Ext. 290

e-mail: cgestal@iim.csic.es

^a These authors contributed equally to the work presented in this paper.

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 *V. 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.

Keywords: *Octopus vulgaris*, paralarvae, reference gene, RT-qPCR, development, infection.

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, Iglesias et al. 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 and 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-dehydrogenase (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 and 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, GAPDH 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 to 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 to 34 days *Nannochloropsis* sp. were also used to complement 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 days d.p.h. were challenged by adding *V. lentus* or *V. splendidus* to the water with a final concentration of 3×10^6 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 and 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 alpha 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.

To confirm the effect of the use of different reference genes during the normalization of the gene expression level of a target gene a RT-qPCR for the gene PGRP (PGRP-F: 5'-GAGCTGCTCCACAACCTGC-3' and PGRP-R: 5'-CGACACCATTTCCACCA-3') was performed. This is an immune-related gene known to be up-regulated in octopus paralarvae infected by *V. lentus* (Castellanos-Martínez et al. 2013). The most and less stable reference genes were used to normalize the expression of PGRP. Results were expressed as the mean $\pm$ standard deviation. Fold units were calculated dividing the normalized expression values of infected paralarvae by the normalized expression values of non-infected ones.

3. Results

The overall stability of the reference genes after comparing the three approaches was ranked from the most stable to the less stable gene for each dataset (Fig. 1). The M values calculated by geNorm, from the most to the least stable gene, for sample set A ranked as follow: UBI + EF > 18S > TUB > GADPH > ACT (Table 2.a). For sample set B the resulting ranking

was: ACT + 18S > UBI > EF > TUB > GADPH (Table 2.b). Data obtained were also analyzed using NormFinder, which calculates the stability value of the candidate reference genes according to their minimum variance. Thus, the most stable genes showed, in average, a low stability value. For set A, the most stable genes were UBI > 18S > ACT (Table 2.a) whereas, UBI > 18S > ACT were the most stable pair of genes for set B (Table 2.b). BestKeeper determines the best suited housekeeping genes based on a repeated pair-wise correlation and regression analysis. According to the lowest coefficient of variation (CV) and standard deviation (SD), the most stable genes for set A were (in order of most stable to least stable) TUB > UBI > EF (Table 2.a). For set B, the most stable genes were (in order of most stable to least stable) TUB > UBI > ACT (Table 2.b).

Taking into account the overall results, geNorm and NormFinder agreed that UBI, 18S and EF are the best reference genes to be used for set A (Fig. 1.a, Table 2.a) while UBI, ACT and 18S are the best candidate reference genes for set B (Fig. 1.b, Table 2.b). BestKeeper results support those genes exhibiting the lowest variation in both sample sets.

The number of candidate genes required for normalization is based on the pairwise number variation analysis ($V_{n/n+1}$). According to geNorm 0.15 is the cut-off suggested for such variation and indicates that below of this value, the addition of another normalizing gene is not necessary. The results showed that the theoretical number of genes recommended for using in set A and set B for normalization are five (Fig. 2).

3.1 Reference gene selection: impact over PGRP expression level

PGRP was here selected as stimulus-specific gene to evaluate the impact of the use of the best and worst reference genes identified in this study. Previous studies showed PGRP gene expression level up-regulated at 24 hours post infection with *V. lentus* (Castellanos-Martínez et al., 2013). The expression level of the gene PGRP was normalized using UBI as the most stable reference gene and GADPH as the less stable one. The results of gene expression level showed

that PGRP fold change (expression level infected versus control non-infected) normalized with UBI as reference gene was 1.5 times higher than the obtained using GADPH (Fig. 3).

4. Discussion

In this study, we assessed the variation of six reference genes commonly used in a wide array of organisms to test their validity as reference in octopus paralarvae. According to our results, UBI is a suitable reference for measuring the gene expression in both paralarvae sample sets. This result agrees with that of Sirakov et al. (2009) who identified UBI as an appropriate reference gene to analyze the gene expression in octopus brain samples. Therefore, the gene UBI seems to be the best option as a stable gene for measuring the gene expression in *O. vulgaris* and according to our study, UBI remains stable for analyzing infected and non-infected paralarvae.

Additional stable genes here found are EF, 18S and ACT. All of them have been classically used to assess the gene expression in RT-qPCR. Of these, the EF has been found as valuable reference gene for *Dicetrarchus labrax* (Mitter et al. 2009) and now also in *O. vulgaris* ontology. The 18S is a classical gene used as internal control for RT-qPCR with controversial results. The expression of 18S was found unstable in *Crassostrea gigas* development (Du et al. 2013), however, the same gene remained stable in cells infected by virus (Kuchipudi et al. 2012). In this study, the suitability of 18S as an appropriate reference gene for normalizing data of octopus paralarvae development is supported by its stability tested by different software (Table 2). Similar controversy arises about actin. For example, ACT was the least stable gene in tissues of adult and larvae of *Patinopecten yessoensis* (Feng et al. 2013). In contrast, ACT is valuable as reference to test the gene expression in intestine and kidney of the rock bream fish, *Oplegnathus fasciatus*, after *Vibrio alginolyticus* infection (Zhang et al. 2014); and in muscle, gill and spleen of red drum, *Sciaenops ocellatus*, infected with *Edwardsiella tarda* (Sun and Hu 2015). Now, according to our results, ACT is one of the most stable genes for testing the gene expression in paralarvae infected by *V. splendidus* and *V. lentus*.

The optimal number of reference genes is an additional controversial topic. In the past, a single reference gene was used. Currently, two or three genes are used for data normalization (Edmunds et al. 2014). However, it becomes expensive, impractical and unhelpful when samples are limited (Huggett et al. 2005). In the present study, the pairwise number variation analysis recommend the use of five genes. However, regarding at the $V_{n/n+1}$ values the difference between use three or use more than three reference genes won't have an important effect over the normalization procedure. In consequence three genes could be used for normalizing the gene expression: UBI, 18S and EF in set A and UBI, ACT and 18S in set B. This statement was confirmed by the analysis of the expression profile of a yet known stimulus-specific gene, the PGRP, involved in the recognition of Gram negative bacteria (Castellanos-Martínez et al., 2013). The results show that the selection of the normalizer gene would have an impact over data interpretation leading us to reach different conclusions in terms of significance of the expression level, with a clear effect on the identification of potential biomarkers.

5. Conclusion

In conclusion, we identified as the most stable reference genes UBI, 18S and EF for RT-qPCR normalization during *O. vulgaris* paralarvae development, and UBI, ACT and 18S as reference genes for paralarvae infected by bacteria. The use three, or at least two of these genes is recommended for normalization.

Establishing normalization genes will facilitate further studies that will provide a more reliable study of the gene expression/regulation of *O. vulgaris* during their first days of development and after infections. This will contribute to understand how it is linked with the cephalopod welfare at the beginning of their lives.

Acknowledgements

This work was funded by the Xunta de Galicia 10PXIB402116PR research project, and “AGL20134910-C02-2R” from the Spanish Ministerio de Economía y Competitividad. S.

Castellanos-Martínez thanks CONACyT for her awarded scholarship, and P. García- Fernández thanks Xunta de Galicia for his predoctoral fellowship.

Conflict of interest

The author declares no conflict of interest.

References

- Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 64, 5245–5250. doi: 10.1158/0008-5472.CAN-04-0496
- Bustin, S., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. *J. Mol. Endocrinol.* 29, 23–39. doi: 10.1677/jme.0.0290023
- Bustin, S., Benes, V., Garson, J., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: Minimum Information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622. doi: 10.1373/clinchem.2008.112797
- Castellanos-Martínez, S., Vizcaíno, R., Iglesias, P., Sanchez, F.J. and Gestal, C. 2013. Immune-related gene expression of common octopus (*Octopus vulgaris*) paralarvae during ontogenesis and after bacterial challenge. *Aquaculture 2013. Aquaculture Elsevier Conference: To the Next 40 years of sustainable Global Aquaculture.*
- Castellanos-Martínez, S., Arteta, D., Catarino, S., Gestal, C., 2014. De Novo Transcriptome Sequencing of the *Octopus vulgaris* Hemocytes Using Illumina RNA-Seq Technology:

Response to the Infection by the Gastrointestinal Parasite *Aggregata octopiana*. PLoS One 9, e107873. doi: 10.1371/journal.pone.0107873

Du, Y., Zhang, L., Xu, F., Huang, B., Zhang, G., Li, L., 2013. Validation of housekeeping genes as internal controls for studying gene expression during Pacific oyster (*Crassostrea gigas*) development by quantitative real-time PCR. Fish Shellfish Immunol. 34, 939–945. doi: 10.1016/j.fsi.2012.12.007

Edmunds, R.C., McIntyre, J.K., Luckenbach, J.A., Baldwin, D.H., Incardona, J.P., 2014. Toward enhanced MIQE compliance: reference residual normalization of qPCR gene expression data. J. Biomol. Tech. 25, 54–60. doi:10.7171/jbt.14-2502-003

Feng, L., Yu, Q., Li, X., Ning, X., Wang, J., Zou, J., Zhang, L., Wang, S., Hu, J., Hu, X., Bao, Z., 2013. Identification of reference genes for qRT-PCR analysis in Yesso scallop *Patinopecten yessoensis*. PLoS One 8, 1–10. doi:10.1371/journal.pone.0075609

Gestal, C., Castellanos-Martínez, S., 2015. Understanding the cephalopod immune system based on functional and molecular evidence. Fish Shellfish Immunol. 46, 120–130. doi:10.1016/j.fsi.2015.05.005

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19. doi: 10.1186/gb-2007-8-2-r19

Huggett, J., Dheda, K., Bustin, S., Zumla, A., 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 6, 279–284. doi: 10.1038/sj.gene.6364190

Iglesias, J., Otero, J.J., Moxica, C., Fuentes, L., Sánchez, F.J., 2004. The completed life cycle of the octopus (*Octopus vulgaris*, Cuvier) under culture conditions: Paralarval rearing using Artemia and zoeae, and first data on juvenile growth up to 8 months of age. Aquac. Int. 12, 481–487. doi:10.1023/B:AQUI.0000042142.88449.bc

- Iglesias, J., Fuentes, L., Sánchez, J., Otero, J.J., Moxica, C., Lago, M.J., 2006. First feeding of *Octopus vulgaris* Cuvier, 1797 paralarvae using *Artemia*: Effect of prey size, prey density and feeding frequency. *Aquaculture* 261, 817–822. doi: 10.1016/j.aquaculture.2006.08.002
- Iglesias, J., Pazos, G., Fernández, J., Sánchez, F.J., Otero, J.J., Domingues, P., Lago, M.J., Linares, F., 2014. The effects of using crab zoeae (*Maja brachydactyla*) on growth and biochemical composition of *Octopus vulgaris* (Cuvier 1797) paralarvae. *Aquac. Int.* 22, 1041–1051. doi: 10.1007/s10499-013-9725-7
- Kozera, B., Rapacz, M., 2013. Reference genes in real-time PCR. *J. Appl. Genet.* 54, 391–406. doi: 10.1007/s13353-013-0173-x
- Kuchipudi, S.V., Tellabati, M., Nelli, R.K., White, G.A., Perez, B.B., Sebastian, S., Slomka, M.J., Brookes, S.M., Brown, I.H., Dunham, S.P., Chang, K.C., 2012. 18S rRNA is a reliable normalisation gene for real time PCR based on influenza virus infected cells. *Virol. J.* 9, 230. doi: 10.1186/1743-422X-9-230
- Mather, J.A., Anderson, R.C., 2007. Ethics and invertebrates: a cephalopod perspective. *Dis. Aquat. Organ.* 75, 119–29. doi: 10.3354/dao075119
- Mitter, K., Kotoulas, G., Magoulas, A., Mulero, V., Sepulcre, P., Figueras, A., Novoa, B., Sarropoulou, E., 2009. Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European seabass (*Dicentrarchus labrax*). *Comp Biochem Physiol B Biochem Mol Biol.* 153, 340–347. doi: 10.1016/j.cbpb.2009.04.009
- Moltschaniwskyj, Natalie, A., Hall, K., Lipinski, Marek, R., Marian, J.E.A.R., Nishiguchi, M., Sakai, M., Shulman, D.J., Sinclair, B., Sinn, D.L., Staudinger, M., Van Gelderen, R., Villanueva, Roger, Warnke, K., 2007. Ethical and welfare considerations when using

cephalopods as experimental animals. Rev. Fish Biol. Fish. 17, 455–476. doi:

10.1007/s11160-007-9056-8

Moreira, R., Pereiro, P., Costa, M.M., Figueras, A., Novoa, B., 2015. Evaluation of reference genes of *Mytilus galloprovincialis* and *Ruditapes philippinarum* infected with three bacteria strains for gene expression analysis. Aquat. Living Resour. 27, 147–152. doi: 10.1051/alr/2014015

Naef, A., 1928. Die Cephalopoden. Fauna Flora Golfo Napoli, 35. monogr., part I, vol. 2, 357 pp (first publ. 1923).

Navarro, J.C., Villanueva, R., 2003. The fatty acid composition of *Octopus vulgaris* paralarvae reared with live and inert food: deviation from their natural fatty acid profile. Aquaculture 219, 613–631. doi: 10.1016/S0044-8486(02)00311-3

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45. doi: 10.1093/nar/29.9.e45

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515. doi: 10.1023/B:BILE.0000019559.84305.47

Sirakov, M., Zarrella, I., Borra, M., Rizzo, F., Biffali, E., Arnone, M.I., Fiorito, G., 2009. Selection and validation of a set of reliable reference genes for quantitative RT-PCR studies in the brain of the Cephalopod Mollusc *Octopus vulgaris*. BMC Mol. Biol. 10, 70. doi: 10.1186/1471-2199-10-70

Sun, B., Hu, Y., 2015. Evaluation of potential internal references for quantitative real-time RT-PCR normalization of gene expression in red drum (*Sciaenops ocellatus*). Fish Physiol. Biochem. 41, 695–704 doi: 10.1007/s10695-015-0039-8

- Taylor, S.C., Mrkusich, E.M., 2014. The state of RT-quantitative PCR: Firsthand observations of implementation of minimum information for the publication of quantitative real-time PCR experiments (MIQE). *J. Mol. Microbiol. Biotechnol.* 24, 46–52. doi: 10.1159/000356189
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, Research0034.1–0034.11. doi: 10.1186/gb-2002-3-7-research0034
- Vidal, E.G., Villanueva, R., Andrade, J.P., Gleadall, I.G., Iglesias, J., Koueta, N., Rosas, C., Segawa, S., Grasse, B., Franco-Santos, R.M., Albertin, C.B., Caamal-Monsreal, C., Chimal, M.E., Edsinger-Gonzales, E., Gallardo, P., Le Pabic, C., Pascual, C., Roumbedakis, K., Wood, J., 2014. Cephalopod culture: current status of main biological models and research priorities. *Adv. Mar. Biol.* 67, 1–198. doi: 10.1016/B978-0-12-800287-2.00001-9
- Xavier, J.C., Allcock, A.L., Cherel, Y., Lipinski, M.R., Pierce, G.J., Rodhouse, P.G.K., Rosa, R., Shea, E.K., Strugnell, J.M., Vidal, E.A.G., Villanueva, R., Ziegler, A., 2014. Future challenges in cephalopod research. *J. Mar. Biol. Assoc. United Kingdom FirstView*, 1–17. doi: 10.1017/S0025315414000782
- Zhang, B., Sun, L., Xiao, Z., Hu, Y., 2014. Quantitative real time RT-PCR study of pathogen-induced gene expression in rock bream (*Oplegnathus fasciatus*): internal controls for data normalization. *Mar. Genomics* 15, 75–84. doi: 10.1016/j.margen.2014.03.001
- Zhu, X., Li, X., Chen, W., Chen, J., Lu, W., Chen, L., Fu, D., 2012. Evaluation of new reference genes in papaya for accurate transcript normalization under different experimental conditions. *PLoS One* 7, e44405. doi: 10.1371/journal.pone.0044405

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

Table 2 Summary of the results after evaluation of the gene stability using different software to identify the best reference genes in two dataset: (a) gene expression during paralarvae development and (b) gene expression after bacterial challenge. The three most stable expressed genes detected by each method are highlighted.

Fig. 1 Average position of the six candidate reference genes in both dataset (a: development, and b: infected) in rankings obtained with GeNorm, Normfinder and BestKeeper.

Fig. 2 Determination of the optimal number of reference genes for normalization. The pairwise variation ($V_{n/n+1}$) was calculated between the normalization factors NF_n and NF_{n+1} by geNorm software, which is indicated at the top of the bars.

Fig. 3 Reference gene validation using PGRP as stimuli specific gene. The most stable reference gene (UBI) and the less stable one (GADPH) were used to normalize gene expression data and they show an impact on PGRP fold change value. Error bars represent standard deviation.

Table 1

Primer name	GenBank	Sequence (5'→3')	Product size (bp)	E (%) ^a	R ²
β-actin	AB053937	F: TGTTGCCGCTTTAGTTGTTG R: TTTTGCTCTGGGCTTCATCT	170	93.97	0.999
EF	AY651883	F: GTAGAGATGCACCACGAGTCACTT R: CATACCCACGACGAAGATCCTT	100	105.04	0.999
Ubiquitin	FJ617440	F: AGAAGGTAAAGTTGGCGGTTTTG R: CCAGCTCCACATTCCTCGTT	100	93.50	0.998
α-tubulin	X15845	F: TCCAACGTGCTGTGTGCAT R: AAAGCACGCTTGGCATAACATC	100	110.41	0.998
GADPH	EF634059	F: GGTGGCAGAACTGCTGGT R: GGCACCCTGAAAGCCATA	116	125.40	0.986
18S	FJ617439	F: GCGTTCGCTTCGATGAC R: GCCCTTCCGTCAATTCC	105	80.11	0.999

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

Table 2a and Table 2b

Octopus vulgaris – paralarvae development (a)

	ACT	EF	UBI	TUB	GADPH	18S
geNorm	1.024	0.524	0.524	0.786	0.921	0.611
NormFinder	0.181	0.217	0.124	0.294	0.187	0.171
BestKeeper (Ct CV%)	7.871	3.757	3.572	2.523	4.820	5.642
BestKeeper (Ct±SD)	1.377	0.670	0.762	0.525	0.977	0.591

Octopus vulgaris – paralarvae infected (b)

	ACT	EF	UBI	TUB	GADPH	18S
geNorm	0.609	0.723	0.680	0.902	1.297	0.609
NormFinder	0.249	0.288	0.125	0.412	0.530	0.226
BestKeeper (Ct CV%)	4.139	5.614	2.929	1.511	5.731	7.357
BestKeeper (Ct±SD)	0.651	0.967	0.585	0.310	1.080	1.002

Check your best reference

18S is not ever the best option

↑↑ quality of your RTqPCR data ↑↑
A different reference gene could change
dramatically the final conclusions

Highlights

- The immune status of the *O. vulgaris* paralarvae has an impact over the gene expression stability of some commonly used reference genes.
- Ubiquitin and 18S were optimal as reference genes during *O. vulgaris* paralarvae development.
- Ubiquitin and actin are suggested as reference genes during immune response to a bacterial infection in *O. vulgaris* paralarvae.