Chinese Journal of Oceanology and Limnology
Vol. 31 No. 2, P. 421-430, 2013
**http://dx.doi.org/10.1007/s00343-013-2221-0**

# Identification of normalization factors for quantitative real-time RT-PCR analysis of gene expression in Pacific abalone *Haliotis discus hannai**

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Received Sep. 13, 2012; accepted in principle Oct. 24, 2012; accepted for publication Dec. 31, 2012


**Abstract** Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) is widely used in studies of gene expression. In most of these studies, housekeeping genes are used as internal references without validation. To identify appropriate reference genes for qRT-PCR in Pacific abalone *Haliotis discus hannai*, we examined the transcription stability of six housekeeping genes in abalone tissues in the presence and absence of bacterial infection. For this purpose, abalone were infected with the bacterial pathogen *Vibrio anguillarum* for 12 h and 48 h. The mRNA levels of the housekeeping genes in five tissues (digestive glands, foot muscle, gill, hemocyte, and mantle) were determined by qRT-PCR. The PCR data was subsequently analyzed with the geNorm and NormFinder algorithms. The results show that in the absence of bacterial infection, elongation factor-1-alpha and beta-actin were the most stably expressed genes in all tissues, and thus are suitable as cross-tissue type normalization factors. However, we did not identify any universal reference genes post infection because the most stable genes varied between tissue types. Furthermore, for most tissues, the optimal reference genes identified by both algorithms at 12 h and 48 h post-infection differed. These results indicate that bacterial infection induced significant changes in the expression of abalone housekeeping genes in a manner that is dependent on tissue type and duration of infection. As a result, different normalization factors must be used for different tissues at different infection points.

**Keyword**: *Haliotis discus hannai*; housekeeping gene; normalization factor; quantitative real time RT-PCR; reference gene

## 1 INTRODUCTION

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) is a molecular technology based on traditional PCR. In qRT-PCR, RNA from a target sample is transcribed via reverse transcriptase into complementary DNA (cDNA); the cDNA is amplified by PCR, and the amplified products are detected and measured during each cycle of PCR process. The exponential amplification enables qRT-PCR to detect RNA when the concentration of the latter is extremely low (Heid et al., 1996; Bustin, 2002). Since mRNA levels are direct indicators of gene expression at the transcription level, qRT-PCR is a commonly used methodology in the study of gene expression in a diverse range of organisms (Van Guilder et al., 2008).

qRT-PCR quantifies RNA in two ways, relative and absolute quantification; the former being most often used. Relative quantification employs a co-amplified internal control or a reference, to which the data of multiple target samples are normalized, and each sample yields a relative expression level as a ratio of the target to the internal reference. Therefore, the reference gene is a critical parameter that can influence qRT-PCR results (Radonic et al., 2004; Huggett et al.,

* Supported by the Knowledge Innovation Program of Chinese Academy of Sciences (No. KSCX2-EW-G-12B), the National High Technology Research and Development Program of China (863 Program) (No. 2012AA10A412), and the Knowledge Innovation Program of the Chinese Academy of Sciences (No. KZCX2-EW-Q213)
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2005). Depending on the study, the reference gene may vary but should in principle exhibit stable expression in all target samples under the experimental conditions. For the majority of reported qRT-PCR studies, housekeeping genes (most often β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) and ribosomal RNAs are used as internal references (Thellin et al., 1999; Suzuki et al., 2000). These genes have been used traditionally in conventional RNA-measuring methods such as northern blot; however, their appropriateness, as qRT-PCR references has in most cases not been validated. In fact, an increasing number of studies have shown that the expression of some of the commonly used housekeeping genes varies with tissue type and is regulated by development and environmental input (Mansur et al., 1993; Schmittgen and Zakrajsek, 2000; Tricarico et al., 2002; Bas et al., 2004; Dheda et al., 2004; Ruan et al., 2007). These observations indicate a necessity for investigation of the expression stability of house-keeping genes under the conditions of the study before employing them as normalization factors.

Pacific abalone *Haliotis discus hannai* and small abalone *H. diversicolor* are herbivorous marine gastropods and have been used as a human food source in the Asia-Pacific region for many years. In China, abalone farming has become an important sector of the aquaculture industry. However, in recent years, abalone culture has faced increasing problems associated with microbial diseases (Nie and Wang, 2004; Zhang et al., 2004). qRT-PCR has been used to study the effect of pathogen infection on the expression of genes in abalone with, most often, invalidated housekeeping genes as internal standards.

In this study, we examined the transcriptional stability of commonly used housekeeping genes in various abalone tissues in the presence and absence of infection with a pathogenic strain of Vibrios, a known threat to cultured abalone (Liu et al., 2000; Nie and Wang, 2004; Macey and Coyne, 2005; Cai et al., 2007; Iehata et al., 2009). In our study, *Vibrio anguillarum* was used as the infecting organism because it is often used in qRT-PCR analysis of gene expression in abalone in response to pathogenic infection (Cheng et al., 2007; Hong et al., 2008; Ding et al., 2011; Kemp and Coyne, 2011; Dang et al., 2012). The expression stability of the housekeeping genes was analyzed with two independent normalization algorithms, geNorm and NormFinder, widely used for normalization of qRT-PCR data (Olsvik et al., 2008; Øvergård et al., 2010; Penna et al., 2011). GeNorm determines the expression stability measure (M) of each gene in a group of control genes as the average pairwise variation (V) for that gene with all other examined control genes, thereby identifying the most stable control genes and the optimal number of genes required for reliable normalization (Vandesompele et al., 2002). NormFinder identifies stably expressed genes among a set of candidate normalization genes by ranking them according to their expression stability (Andersen et al., 2004).

## 2 MATERIAL AND METHOD

### 2.1 Sample preparation

The bacterial pathogen *Vibrio anguillarum* C312 (Zheng et al., 2010) was cultured in Luria-Bertani broth (LB) medium at 28°C to an $OD_{600}$ of 1. The cells were centrifuged, washed with phosphate-buffered saline (PBS), and resuspended in PBS to $1\times10^8$ cells/mL. Abalone (*Haliotis discus hannai*) (average 40 mm) were divided randomly into two groups (8 animals/group), and 100 μL of *V. anguillarum* suspension was administered to 8 animals via muscle tissue. The control group consisted of 8 animals, they were not infected. At 12 h and 48 h post-infection, digestive glands, foot muscle, gill, hemocyte, and mantle tissues were collected aseptically. To verify bacterial infection, the collected tissues were homogenized in PBS, and 100 μL homogenates were plated on LB agar plates. After incubation at 28°C for 48 h, all colonies on the plates were examined, which confirmed that C312 was recovered from C312-infected animals but not from the control animals.

### 2.2 cDNA synthesis

Total RNA was extracted from the tissue samples using RNAiso Plus (TaKaRa, Dalian, China) and treated with RNase-free DNase I (MBI Fermentas, Canada). The quality of the RNA was examined by NanoDrop2000 (Thermo Scientific, USA) and gel electrophoresis. The purified RNA was adjusted to 0.1 μg/μL with nuclease-free water. One microgram of total RNA was used for cDNA synthesis with the RevertAid™ Reverse Transcriptase (MBI Fermentas) according to the manufacturer's instructions.

### 2.3 PCR efficiency

Six housekeeping genes, 18S ribosomal RNA (18S rRNA), β-actin (ACTB), elongation factor-1-α

**Table 1 Housekeeping genes used in this study**

| Symbol | Name | Function | Accession number |
|---|---|---|---|
| 18S rRNA | 18S ribosomal RNA | Ribosomal subunit | AY319437 |
| ACTB | Actin | Cytoskeletal protein | AY380809 |
| EF1A | Elongation factor-1-a | Protein synthesis | JX002677 |
| eRF1 | Eukaryotic release factor 1 | Protein synthesis | JX002678 |
| RPL5 | Ribosomal protein L5 | Structural component of the large 60S ribosomal subunit | JX002679 |
| TUBA | α-Tubulin | Cytoskeletal protein | JX002680 |

**Table 2 Primers and PCR amplification efficiencies**

| Gene | Primer sequence (5'→3') | Product size (bp) | Efficiency (*E*) (%) | Correlation coefficient ($R^2$) |
|---|---|---|---|---|
| 18S rRNA | TCACTCCACCAACTAAGAACGG<br>ACTCAACACGGGAAAACTCACTG | 111 | 99 | 0.997 |
| ACTB | GGTATCCTCACCCTCAAGTACCC<br>GGGTCATCTTTTCACGGTTGG | 175 | 99 | 0.987 |
| EF1A | TGCTGTCTGATCGTTGCCT<br>GCTGTCCATCTTGTTGATTCCA | 138 | 98 | 0.996 |
| eRF1 | ATTTCCGTCGTGTCTACCTGTT<br>ATGCTTGTTCCATTTCCTCTCG | 155 | 101 | 0.998 |
| RPL5 | GCTTTCCGTGCTTACCTGG<br>GCCTTGAACTCGCTGCTCT | 154 | 98 | 0.998 |
| TUBA | CGACTCCTTCAACACCTTCTTCA<br>TTTGCGGCATCTTCCTTTCC | 165 | 106 | 0.998 |

(EF1A), eukaryotic release factor 1 (eRF1), ribosomal protein L5 (RPL5), and α-tubulin (TUBA) (Table 1) were examined in this study. PCR primers (Table 2) for each gene were designed with AlleleID 6.0 software. PCR efficiency (*E*) and correlation coefficients ($R^2$) were determined based on the slope of the standard curves generated using serial 10-fold dilutions of sample cDNA. The efficiency was calculated as follows:

$E$ (%)=$(10^{-1/\text{slope}}-1)\times 100$ (Kubista et al., 2006).

The acceptable *E* value was defined as between 90%–110%.

### 2.4 qRT-PCR and data analysis

qRT-PCR was carried out in an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara). The reaction was performed in triplicate in a total volume of 20 μL containing 10 μL SYBR Premix buffer, 1 μL cDNA, 0.2 μL each of the primers, and 8.6 μL PCR-grade water. The PCR program was 94°C for 2 min, followed by 40 cycles at 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s. A negative control without cDNA was included in each assay. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. The PCR products were electrophoresed on 2% agarose gels. PCR data was analyzed using geNorm (version 3.5) and NormFinder algorithms (Vandesompele et al., 2002; Andersen et al., 2004).

### 2.5 Statistical analysis

Statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). All PCR assays were performed in triplicate with samples from four animals (one sample per animal). The statistical significance of differences between mean values was determined by analysis of variance (ANOVA), and significance was defined as $P<0.05$.

## 3 RESULT AND DISCUSSION

### 3.1 qRT-PCR amplification of the selected housekeeping genes

Of the genes selected, ACTB has been previously used as a qRT-PCR reference in studies of abalone (Cheng et al., 2007; Jiang and Wu, 2007; Wang et al., 2008; Zhou and Cai, 2010; Li et al., 2012). Other genes have been used in studies of shellfish such as the soft-shell clams (*Mya arenaria*), Chinese white

**Table 3 $C_t$ values of the housekeeping genes expressed in the tissues of uninfected abalone (control) and abalone infected with *Vibrio anguillarum* for 12 h or 48 h**

| | | Digestive glands | Foot muscle | Gill | Hemocyte | Mantle |
|---|---|---|---|---|---|---|
| 18S rRNA | Control | 11.4±0.6 | 11.8±0.7 | 11.7±0.8 | 12.5±0.4 | 11.7±0.5 |
| | 12 h | 12.1±0.3 | 13.9±0.7 | 11.8±1.1 | 12.9±0.5 | 12.0±0.9 |
| | 48 h | 11.2±0.7 | 12.3±0.3 | 11.1±0.5 | 12.1±0.2 | 12.7±0.3 |
| ACTB | Control | 20.0±0.4 | 21.4±1.1 | 21.2±0.3 | 20.6±0.8 | 21.4±0.9 |
| | 12 h | 20.2±0.7 | 21.8±0.2 | 20.9±0.8 | 19.7±0.5 | 21.7±0.6 |
| | 48 h | 20.4±0.4 | 21.3±0.7 | 20.0±0.8 | 19.5±1.1 | 21.8±0.6 |
| EF1A | Control | 19.5±0.8 | 20.4±0.4 | 20.4±0.9 | 21.2±0.5 | 20.4±1.1 |
| | 12 h | 19.8±0.7 | 21.7±1.5 | 20.2±0.4 | 21.1±0.5 | 22.1±1.7 |
| | 48 h | 20.4±0.5 | 20.8±0.4 | 19.2±0.4 | 21.2±0.3 | 22.1±0.1 |
| eRF1 | Control | 23.8±1.1 | 26.6±0.6 | 26.0±0.4 | 27.0±0.6 | 26.6±1.0 |
| | 12 h | 25.2±0.3 | 26.2±0.6 | 24.1±0.9 | 25.2±0.6 | 26.8±0.8 |
| | 48 h | 24.0±0.6 | 26.5±0.3 | 24.3±0.3 | 26.3±0.2 | 27.0±0.4 |
| RPL5 | Control | 21.7±0.6 | 23.7±0.6 | 23.4±0.9 | 23.5±0.4 | 22.9±0.2 |
| | 12 h | 20.1±0.5 | 22.8±0.5 | 21.6±0.7 | 22.2±0.8 | 24.4±0.2 |
| | 48 h | 21.2±1.1 | 22.4±0.4 | 21.1±1.2 | 24.0±0.5 | 23.5±1.0 |
| TUBA | Control | 23.1±1.1 | 24.1±0.4 | 24.4±0.5 | 25.3±0.5 | 24.3±1.4 |
| | 12 h | 24.5±0.8 | 25.4±0.5 | 24.6±0.9 | 24.5±0.5 | 24.6±0.3 |
| | 48 h | 24.3±0.3 | 24.8±0.6 | 22.7±0.8 | 24.8±0.2 | 24.8±1.2 |

Data are presented as means±SE ($n$= 4).

shrimps (*Fenneropenaeus chinensis*), Pacific oysters (*Crassostrea gigas*) and blue mussels (*Mytilus galloprovincialis*) (Araya et al., 2008; Dhar et al., 2009; Zhang et al., 2011; Gerdol et al., 2012). In our study, PCR specificities were confirmed by melting curve analysis and agarose gel electrophoresis, which confirmed that the PCR products were of the expected sizes (Table 2). The PCR efficiency ($E$) and correlation coefficients ($R^2$) were determined with the slopes of the standard curves generated from serial dilutions of the samples. The $E$ values of the six genes ranged from 98% to 106%, and the $R^2$ values ranged from 0.987 to 0.998 (Table 2).

### 3.2 Expression stability of the housekeeping genes in the absence of bacterial infection

The mRNA levels of the housekeeping genes in the digestive gland, foot muscle, gill, hemocyte, and mantle tissues were determined by qRT-PCR (Table 3). GeNorm analysis showed that in the absence of bacterial infection, TUBA and EF1A exhibited the lowest M values while eRF1 exhibited the highest (Fig.1a). The M value is a measurement of the expressional variation of a gene in comparison with all other candidate genes, and a higher M value

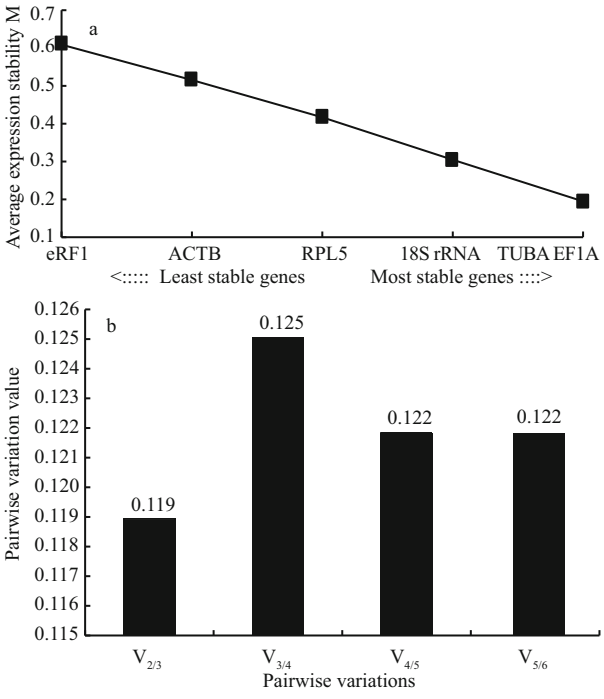


**Fig.1 GeNorm analysis of expression stability in the abalone housekeeping genes before bacterial infection**

The mRNA levels of the housekeeping genes were determined by qRT-PCR, and the data were analyzed with geNorm to calculate (a) the expression stability measure M of each gene and, (b) the optimal number of reference genes required for accurate normalization.

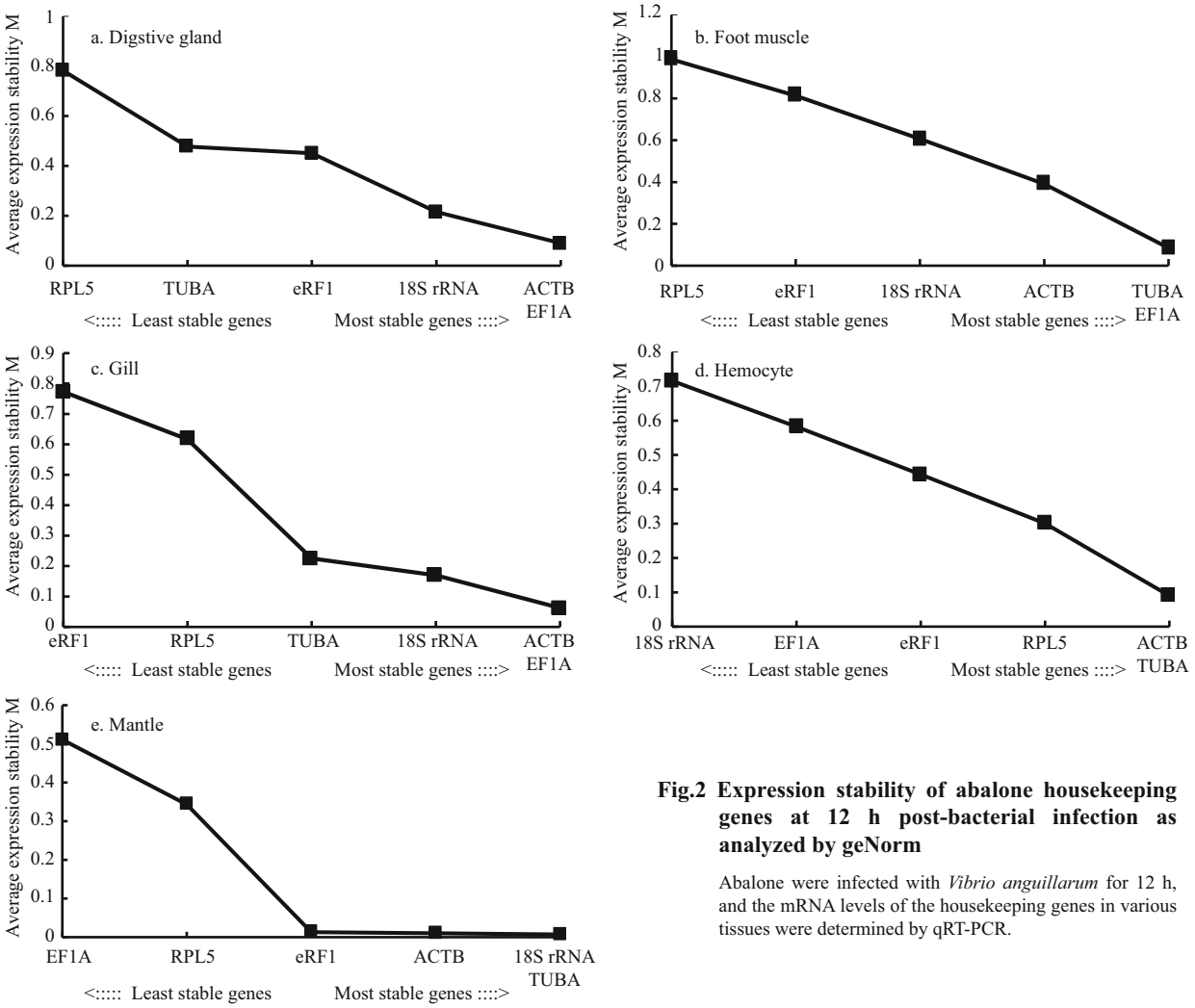


**Fig.2 Expression stability of abalone housekeeping genes at 12 h post-bacterial infection as analyzed by geNorm**

Abalone were infected with *Vibrio anguillarum* for 12 h, and the mRNA levels of the housekeeping genes in various tissues were determined by qRT-PCR.

indicates lower expression stability. Therefore, the stability ranking order based on the M value was EF1A/TUBA, 18S rRNA, RPL5, ACTB, and eRF1. The V values represent variations between two sequential normalization factors containing an increasing number of genes. A large variation means that the added gene has a significant effect and should preferably be included for calculation of a reliable normalization factor (Vandesompele et al., 2002). In our study, all the pairwise variation values, including that of $V_{2/3}$, were less than 0.15 (Fig.1b). Since 0.15 is the proposed cut-off value of geNorm, below which the inclusion of an additional reference gene is not required (Vandesompele et al., 2002), EF1A and TUBA sufficed as normalization factors. NormFinder analysis identified EF1A and TUBA as the most stable genes, followed in order of decreasing stability by RPL5, eRF1, 18S rRNA, and ACTB (Table 4). Thus, NormFinder and geNorm pinpointed the same gene pair, EF1A/TUBA, as the most stable genes across the tissue types examined, suggesting that EF1A/TUBA are suitable internal references for studies of gene expression in abalone tissues under normal physiological conditions.

### 3.3 Expression stability of the housekeeping genes following bacterial infection

Previous studies of marine invertebrates have indicated that housekeeping genes vary in expression level as a function of experimental conditions such as stress signals and development; as a result, optimal reference genes change with experimental settings (Araya et al., 2008; Dhar et al., 2009; Miyazaki et al., 2010; Morga et al., 2010; Wan et al., 2011). Likewise, in our study, we found that the most stable genes vary in different abalone tissues following *V. anguillarum* infection. At 12 h post-infection, both geNorm and NormFinder identified ACTB/EF1A and ACTB/TUBA as the most stable gene pairs in the gill and hemocyte tissues, respectively (Fig.2 and Table 5).

**Table 4 Expression stability of the housekeeping genes in abalone tissues without bacterial infection as calculated by NormFinder**

| Gene | EF1A | TUBA | RPL5 | eRF1 | 18S rRNA | ACTB |
|---|---|---|---|---|---|---|
| Stability | 0.030 | 0.056 | 0.073 | 0.135 | 0.179 | 0.183 |

**Table 5 Ranking of candidate reference genes at 12 h post-infection by NormFinder**

| Ranking order | Digestive glands | Foot muscle | Gill | Hemocyte | Mantle |
|---|---|---|---|---|---|
| 1 | 18S rRNA (0.299) | ACTB (0.089) | ACTB (0.029) | ACTB (0.114) | TUBA (0.072) |
| 2 | EF1A (0.277) | EF1A (0.166) | EF1A (0.118) | TUBA (0.138) | ACTB (0.095) |
| 3 | ACTB (0.304) | TUBA (0.170) | 18S rRNA (0.228) | RPL5 (0.140) | eRF1 (0.129) |
| 4 | eRF1 (0.490) | eRF1 (0.215) | RPL5 (0.142) | EF1A (0.144) | 18S rRNA (0.150) |
| 5 | TUBA (0.545) | 18S rRNA (0.230) | TUBA (0.155) | eRF1 (0.145) | EF1A (0.160) |
| 6 | RPL5 (0.835) | RPL5 (0.299) | eRF1 (0.168) | 18S rRNA (0.246) | RPL5 (0.183) |

The numbers in brackets indicate stability values.

For the digestive gland, foot muscle and mantle tissues, geNorm ranked ACTB/EF1A, TUBA/EF1A, and TUBA/18S rRNA, respectively, as the most stable gene pairs, while NormFinder ranked 18S rRNA/EF1A, ACTB/EF1A, and TUBA/ACTB, respectively, as the most stable gene pairs. Thus, for foot muscle and mantle tissues, the optimal gene pairs identified by the two algorisms overlapped by one gene, while for the digestive glands, the optimal gene pairs identified by geNorm and NormFinder differed. Pairwise variation analysis showed that for the digestive gland, gill, hemocyte, and mantle tissues the $V_{2/3}$ values were 0.093, 0.074, 0.135, and 0.004, respectively (Fig.3), suggesting that for each of these tissues the two most stable genes might serve as normalization factors because they were all less than 0.15. The foot muscle tissue exhibited a $V_{2/3}$ value larger than 0.15 (0.181), therefore, inclusion of the third most stable gene, ACTB, is necessary for accurate normalization.

At 48 h post-infection, the most stably expressed gene pairs in the digestive gland, foot muscle, gill, hemocyte, and mantle tissues ranked by geNorm analysis were ACTB/TUBA, EF1A/18S rRNA, ACTB/EF1A, 18S rRNA/TUBA, and eRF1/ACTB, respectively (Fig.4), while those ranked by

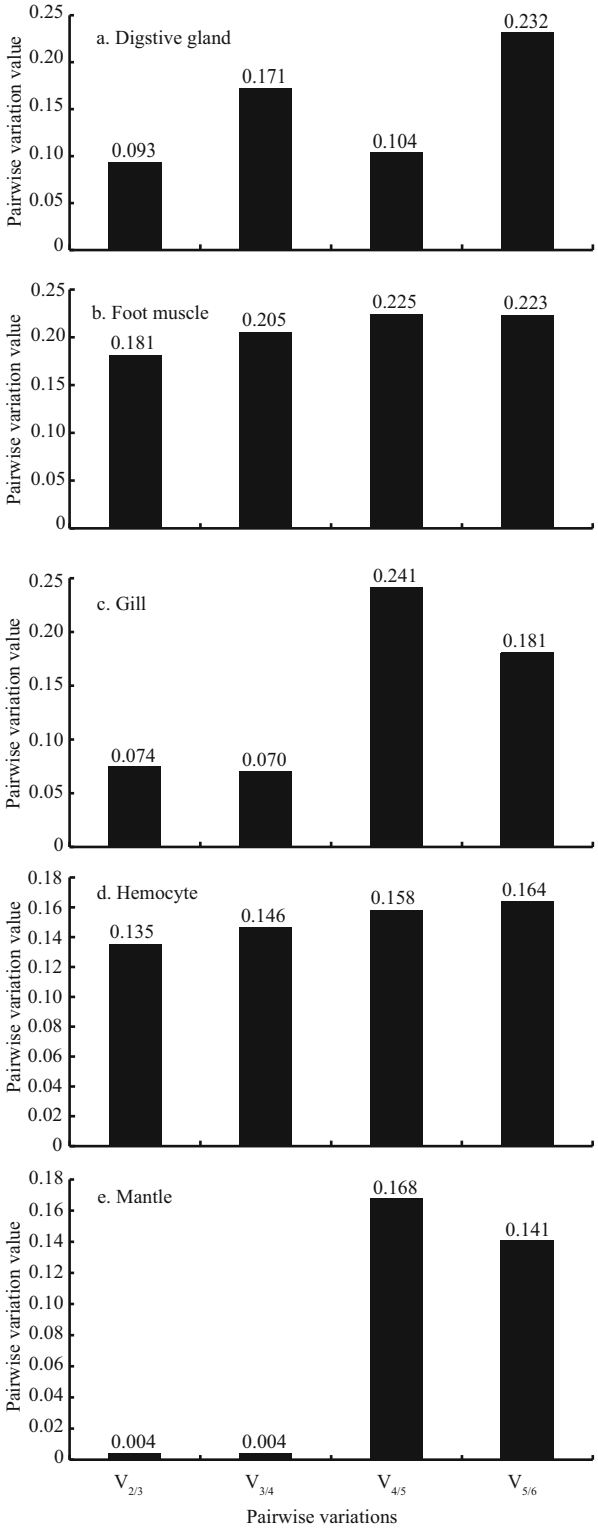


**Fig.3 Determination of the optimal number of reference genes for normalization at 12 h post-bacterial infection**

Abalone were infected with *Vibrio anguillarum* for 12 h, and the mRNA levels of the housekeeping genes in various tissues were determined by qRT-PCR. Pairwise variation between normalization factors was calculated by geNorm.

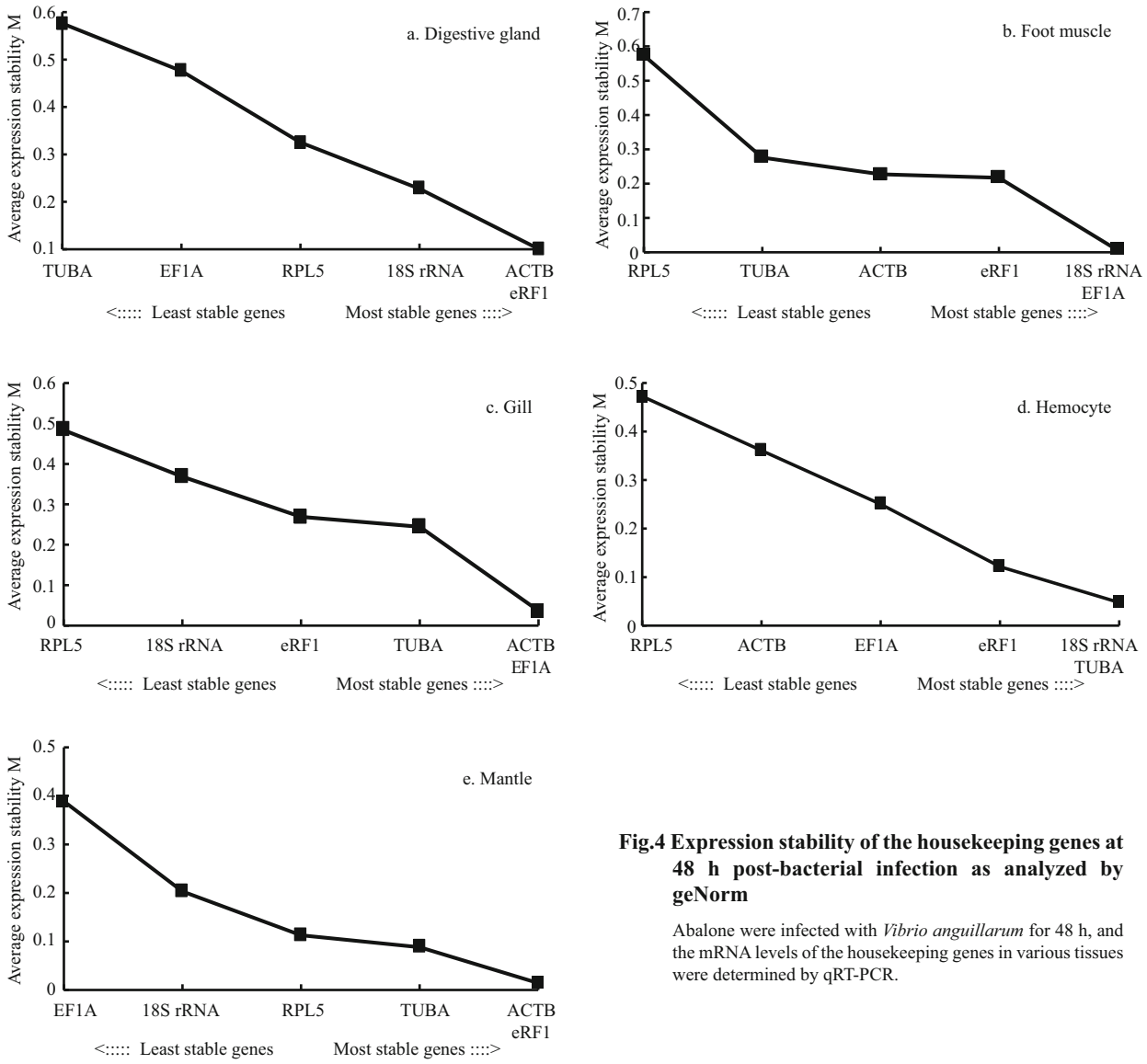


**Fig.4 Expression stability of the housekeeping genes at 48 h post-bacterial infection as analyzed by geNorm**

Abalone were infected with *Vibrio anguillarum* for 48 h, and the mRNA levels of the housekeeping genes in various tissues were determined by qRT-PCR.

NormFinder were eRF1/ACTB, eRF1/EF1A, ACTB/EF1A, 18S rRNA/EF1A, and 18S rRNA/eRF1, respectively (Table 6). Hence, for gill tissue, the optimal gene pairs predicted by geNorm and NormFinder were identical, while for the other four tissues, the optimal gene pairs ranked by geNorm and NormFinder overlapped by one gene. Pairwise variation analysis showed that the $V_{2/3}$ values for the digestive gland, foot muscle, gill, hemocyte, and mantle tissues were 0.097, 0.108, 0.116, 0.053, and 0.042, respectively (Fig.5), suggesting that in all these tissues, the two most stably expressed genes could serve as reliable references without the addition of the third gene. It should be noted that neither geNorm nor NormFinder identified ACTB as the most stable gene in the foot muscle or mantle tissue, suggesting that,

**Table 6 Ranking of candidate reference genes at 48 h post-infection by NormFinder**

| Ranking order | Digestive glands | Foot muscle | Gill | Hemocyte | Mantle |
|---|---|---|---|---|---|
| 1 | eRF1 (0.088) | eRF1 (0.088) | ACTB (0.0586) | 18S rRNA (0.096) | 18S rRNA (0.038) |
| 2 | ACTB (0.094) | EF1A (0.117) | EF1A (0.059) | EF1A (0.099) | eRF1 (0.042) |
| 3 | 18S rRNA (0.134) | ACTB (0.129) | TUBA (0.063) | TUBA (0.123) | ACTB (0.050) |
| 4 | EF1A (0.135) | 18S rRNA (0.142) | eRF1 (0.071) | eRF1 (0.148) | TUBA (0.054) |
| 5 | TUBA (0.137) | TUBA (0.167) | 18S rRNA (0.077) | ACTB (0.177) | RPL5 (0.080) |
| 6 | RPL5 (0.185) | RPL5 (0.270) | RPL5 (0.102) | RPL5 (0.237) | EF1A (0.099) |

The numbers in brackets indicate stability values.

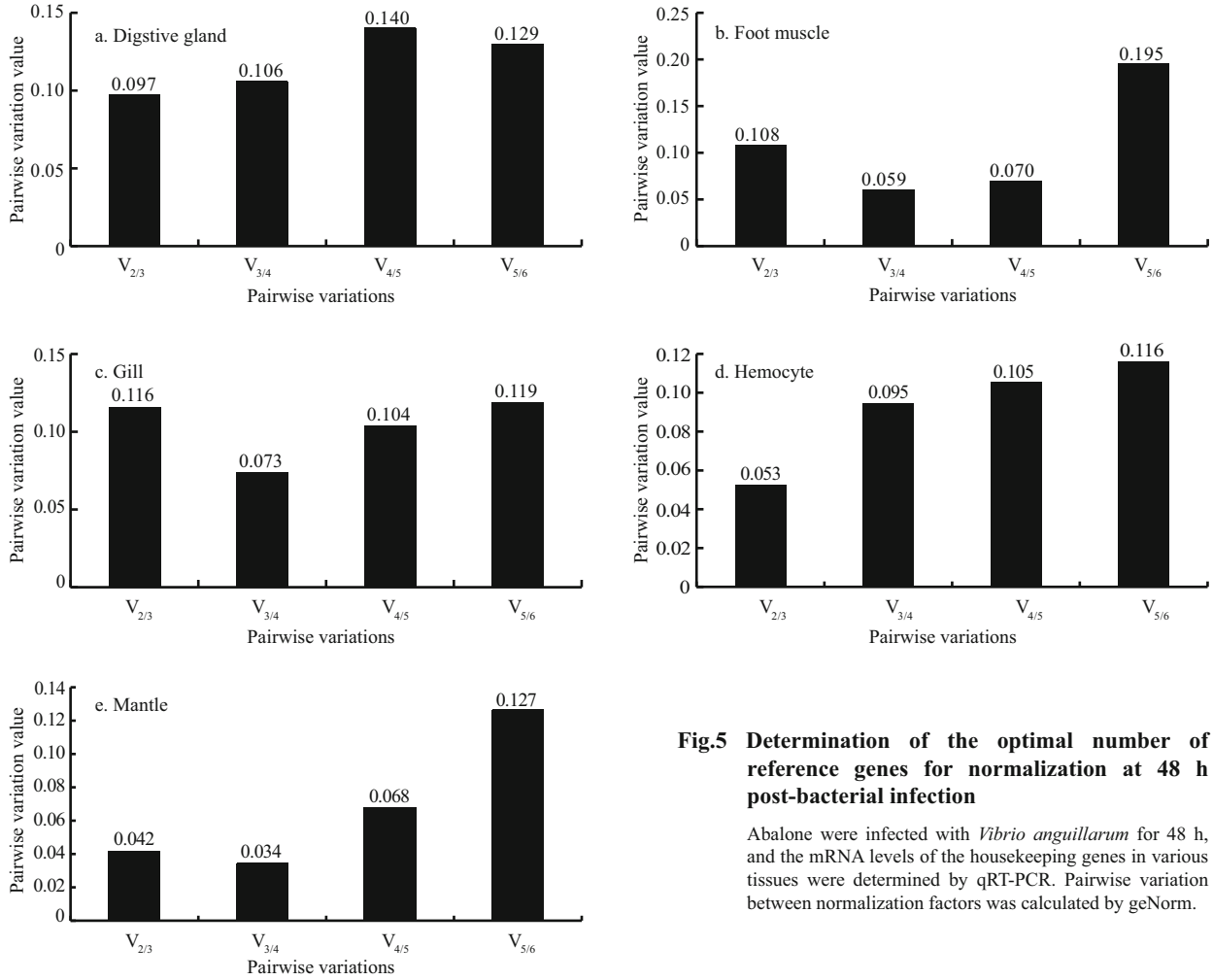


**Fig.5 Determination of the optimal number of reference genes for normalization at 48 h post-bacterial infection**

Abalone were infected with *Vibrio anguillarum* for 48 h, and the mRNA levels of the housekeeping genes in various tissues were determined by qRT-PCR. Pairwise variation between normalization factors was calculated by geNorm.

although ACTB has been most often used as an RT-PCR reference in abalone, it is not suitable for all tissues. When comparing the ranking results of 12 h post-infection to those of 48 h post-infection, with the exception of the gill tissue, which had the same most stable genes at these two points, all tissues exhibited optimal reference gene pairs that differed in one of the constituting genes. These results combined indicate that for bacterially infected abalone, expression of housekeeping genes varies with tissue type and duration of infection.

## 4 CONCLUSION

In conclusion, in this study we examined the expression stability of currently available housekeeping genes in abalone in the presence and absence of bacterial infection. We found that under normal physiological conditions, EF1A and TUBA were the most stably expressed genes across all tissue types examined, while following bacterial infection, the most stable genes varied with tissue type and infection time. Hence, when studying gene expression in bacterium-infected abalone via qRT-PCR, different internal references have to be used not only for different tissues but also for different infection time points. However, it has to be said that these conclusions were based on the specific conditions indicated in this report, and that if the conditions vary, e.g. if the samples were taken from a different season or a different geographical location, the results and conclusions may be different.

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