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Abstract

The level of mRNA expression varies greatly under different physiological and experimental conditions. Therefore, when conducting gene expression analysis through qPCR, it is essential to employ reference genes for the normalization of RNA quantities.

Two reference genes were selected to determine their validity as reference genes in Molly Fish. The evaluation of genes was performed in three cases; the first was an adult untreated fish, the second was a treated fish by calcitonin drug, and the third was by using different developmental stages of fish. The fish specimens were categorized into two sets. The first group received an injection of 4 microliters of calcitonin; At the experiment end, pregnant fish were anatomized to obtain embryos of successive stages in addition to organs of the adult. The samples were fixed following special methods of RNA extraction. The primers were designed for the studied genes. The RNA was extracted, tested and purified, and cDNA building, amplification, and RT-PCR interactions were performed.

After analyzing the data, it was found that the expression of GAPDH gene was twice the EF1a expression. Also found that GAPDH was more stable and consistent than EF1a under the calcitonin influence. It turns out that GAPDH was the most consistent in its expression during the developmental stages. Unlike EF1a, which showed significant differences among some of these stages.

Thus, the study deduces that GAPDH may be valid as a reference gene, and excludes EF1a for this purpose in this species.

Keywords: GAPDH, EF1a, Reference Genes, Fish, *Poecilia latipinna*

Introduction

In gene expression analysis, reference genes serve the purpose of normalizing total RNA quantities, addressing mechanical errors, compensating for variations in reverse transcription efficiency, Adjusting for variations in RNA integrity across samples, and mitigating the impact of PCR inhibitors. (Bustin *et al.*, 2005). Real-time RT-PCR relies on endogenous controls such as housekeeping genes (HKGs), rRNA, and total RNA as references for estimating the expression levels of target genes. The primary aim of employing these controls is to minimize or alleviate variations introduced during sampling. Specifically, these differences pertain to both the quantity and quality of RNA (Olsvik *et al.*, 2005). HKGs are expected to demonstrate uniform expression across cells, organs, individual organisms, diverse developmental stages, and various experimental conditions. Numerous HKGs are utilized as reference genes in qPCR. However, even HKGs, in the absence of chemical treatment, have a tendency to undergo changes influenced by the tissue type and developmental stage (Kumari *et al.*, 2015). Undetected and surprising alterations in the expression of housekeeping genes (HKGs) may lead to incorrect conclusions regarding actual biological effects (Dheda *et al.*, 2005). Several investigations have demonstrated that no single gene universally maintains a consistent expression level across all developmental or experimental

conditions. The selection of the optimal reference gene as an endogenous control depends on the specific tissues under examination. Consequently, a diverse set of genes was chosen to normalize mRNA expression data (Dheda *et al*., 2004; Radonić *et al*., 2004). Thus, the selected HKGs should be validated for each new experimental setup (Øvergård *et al*., 2010). For expression studies using qPCR, at least two reference genes, must be used (Mitter *et al*., 2009). Fish have many characteristics that distinguish species among themselves, and distinguish them from other vertebrates, which has led to interest in them in studies and research. Its importance has also recently been proven in various scientific fields (Ribas and Piferrer, 2014; Bootorabi *et al*., 2017). Real-time RT-PCR has evolved into a crucial instrument for examining gene expression in fish. (Øvergård *et al*., 2010).

The current study investigates two genes within HKGs to test their suitability as reference genes in fish *Poecilia latipinna*. The first is the GAPDH gene, that holds significance in various cellular processes. (Sirover, 1999; Olsvik *et al*., 2005). The second one is EF1a, the elongation factor, which plays a crucial role in the translation process and participates in a variety of cellular functions (Thornton *et al*., 2003; Olsvik *et al*., 2005).

The specificity of the calcitonin hormone in fish has been demonstrated in previous studies (Srivastav *et al*., 2009; Al-Saray, 2020; Alsaray and Alali, 2020), This hormone has been chosen as a crucial factor to elucidate the molecular-level mechanisms of its action. Leveraging the sensitivity, speed, simplicity, and specificity of qPCR technologies enables the detection of minute amounts of nucleic acid across a variety of samples. To our knowledge, there is no previous study investigated the validity of reference genes in the studied species, possibly in the genus Poecilia in general. This is crucial because reference genes, frequently borrowed from the literature and applied without adequate validation across diverse experimental conditions, are assumed to maintain a consistent expression level (Faheem *et al*., 2018).

Materials and methods

The Laboratory animals

Fully developed *P. latipinna* fish were acquired from ornamental fish shops and transported to the laboratory. Fish used were average 5 cm lengths and 5 g weight, divided into two groups at a rate of one male for every five females. Each group contained approximately 50 fish, and they were under the same laboratory conditions. Fish in group 1 received injections of 4 microliters of calcitonin per 5 grams of fish weight, corresponding to 1 IU per 100 grams of fish weight (Srivastav *et al*., 2009). Group 2 fish were injected with a 0.6% concentration of normal saline and served as the control group. Injections were administered daily for the initial ten days, after which the injected was every two days until the experiment end that took about a month and a half. Pregnant females from both groups, were anatomized at the end of the experiment and embryos and tissue samples were collected in successive stages. The embryos were measured using a dissection microscope. The stages were determined by the lengths because of their small size, starting from 2 mm to 7 mm.

Sample preparation

The samples were prepared according to the Morel and Raccurt (2002) method known as Formalin Fixed Paraffin Embedded (FFPE) for RNA extraction to study gene expression through quantitative real-time polymerase chain reaction (Q-RT-PCR).

Primers design

The primers of the two studied genes (EF1a and GAPDH) were designed using data in the Data Banks based on Parameters appropriate for Real-Time PCR processes (Table 1).

Table (1) Primers designed for the studied genes EF1a and GAPDH.

Genes	Primers
EF1a-F	GTTAAGTCCGTTGAGATGCAC
EF1a-rt-R	GATGATGACCTGAGCATTGAAG
GAPDH-F	CACTGTCAAGGCTGAGAACGG
GAPDAH-R	GAGATGATAACACGCTTAGCACCA

RNA extraction

RNA was extracted from embedded samples in paraffin wax moldings using a special kit from MACHEREY-NAGEL (MN, Germany). The NanoDrop device was used to assess both the quantity and quality of the extracted RNA. The RNA purification and removal of DNA by DNase was performed using a special Deoxyribonuclease I Kit (Invitrogen, Cat # 18068-015).

CDNA construction, amplification, and RT-PCR reactions

The cDNA was synthesized using BIO-RAD's iScript kit, and subsequent real-time PCR reactions were carried out on the Light Cycler 96 (Roche Co.) utilizing SYBR Green Master Mix (5x).

Data analysis

[2 ^ - Ct] equation was adopted in calculating gene expression (Al-Asadi, 2018), and the SPSS statistical analysis program was used to analyze the data in the current study. using both T-Test and ANOVA.

Results

The average Ct values for the EF1a gene ranged from 38.63 - 38.5 for the control group and from 14.14 - 38.19 for the treatment group (Fig. 1). Ct values for the GAPDH gene ranged from 19.31 - 20.84 for control and from 19.06 - 21.09 for the treatment group (Fig. 2). The results showed that the rates variance, among the Ct values of the EF1a gene of the control group, were greater than the rates variance among the Ct values of the GAPDH gene, as the highest rate of the EF1a gene at 3 mm stage (38.5), and the lowest rate at 4 mm stage (34.63) (Fig. 1). Whereas the highest rate of GAPDH gene was at 6 mm stage (20.84), and the lowest rate was at adult (19.31) (Fig. 2). The rate variance among the stages of the EF1a gene was (3.87). which is greater than the rate variance among the stages of the GAPDH gene, whose value was (1.53).

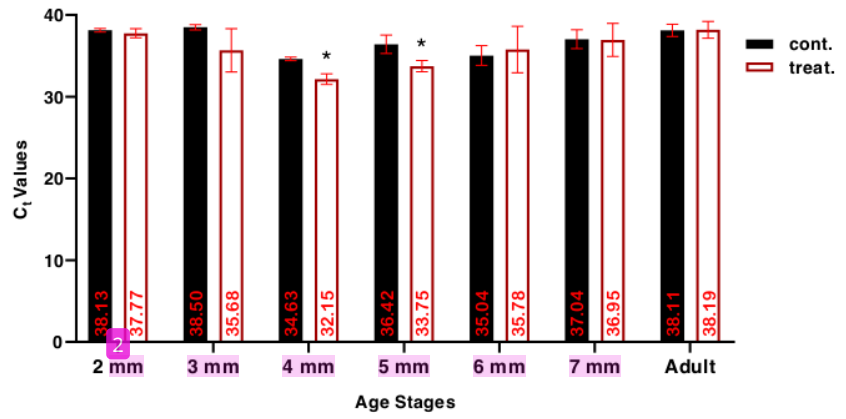

Fig. (1) Rates of Ct values of the EF1a gene for the control and treatment groups at the studied stages.

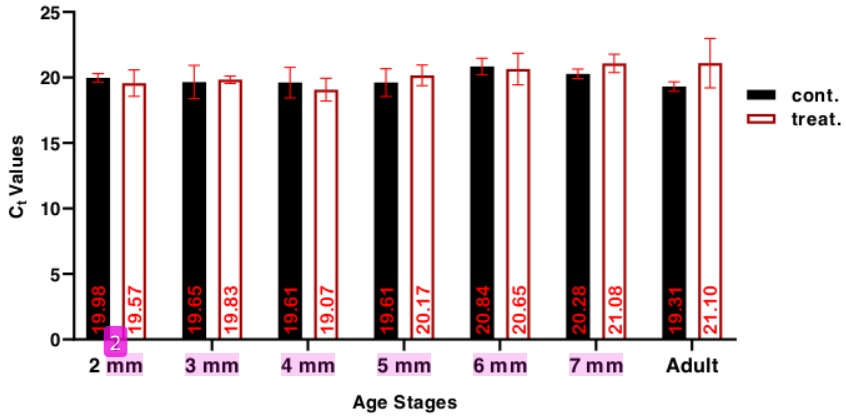

Fig. (2) Rates of Ct values of the GAPDH gene for the control and treatment groups at the studied stages.

The effect of calcitonin hormone on gene expression

One: The effect of calcitonin on EF1a

The results of the statistical analysis of the gene expression values for the two genes studied for each stage revealed notable distinctions between the control and treatment groups at the 4 mm and 5 mm stages for the EF1a gene (Fig. 1).

Two: The effect of calcitonin on GAPDH

The results of the statistical analysis did not record significant differences between the groups with any of the GAPDH gene stages (Fig. 2).

3.2 Gene expression among stages

One: The gene expression of EF1a stages

The results showed that there are clear differences in the Ct values between stages of the EF1a gene and reached the level of significance in some stages. The results of the statistical analysis recorded significant differences between stages (2 & 4 mm), (2 & 6 mm), (3 & 4) Mm), (3 & 6 mm), (4 mm & an adult) and (6 mm & an adult), (Table 2, Fig. 3).

Two: The gene expression of GAPDH stages

Statistical analysis of the Ct values for the GAPDH gene did not show significant differences between stages (Table 3, Fig. 3).

Table (2) Statistical analysis of gene expression values between the EF1a gene stages according to the ANOVA test, at the probability level P ≤ 0.05

Ages	P-value	Ages	P-value	Ages	P-value
2 & 3 mm	1.00	3 & 5 mm	0.89	4mm & Adult	0.02*
2 & 4 mm	0.02*	3 & 6 mm	0.03*	5 & 6 mm	0.26
2 & 5 mm	0.92	3 & 7 mm	0.99	5 & 7 mm	0.99
2 & 6 mm	0.04*	2mm & Adult	1.00	5mm & Adult	0.93
2 & 7 mm	0.99	4 & 5 mm	0.16	6 & 7 mm	0.12
2mm & Adult	1.00	4 & 6 mm	1.00	6mm & Adult	0.04*
3 & 4 mm	0.02*	4 & 7 mm	0.07	7mm & Adult	0.99
Note: The asterisk marker signifies statistically significant differences.					

Table (3) Statistical analysis of gene expression values between the GAPDH gene stages according to the ANOVA test, at the probability level P ≤ 0.05

Ages	P-value	Ages	P-value	Ages	P-value
2 & 3 mm	0.97	3 & 5 mm	1.00	4mm & Adult	1.00
2 & 4 mm	0.96	3 & 6 mm	0.71	5 & 6 mm	0.72
2 & 5 mm	0.97	3 & 7 mm	0.90	5 & 7 mm	0.90
2 & 6 mm	0.99	2mm & Adult	1.00	5mm & Adult	1.00
2 & 7 mm	1.00	4 & 5 mm	1.00	6 & 7 mm	1.00
2mm & Adult	0.95	4 & 6 mm	0.69	6mm & Adult	0.66
3 & 4 mm	1.00	4 & 7 mm	0.88	7mm & Adult	0.86

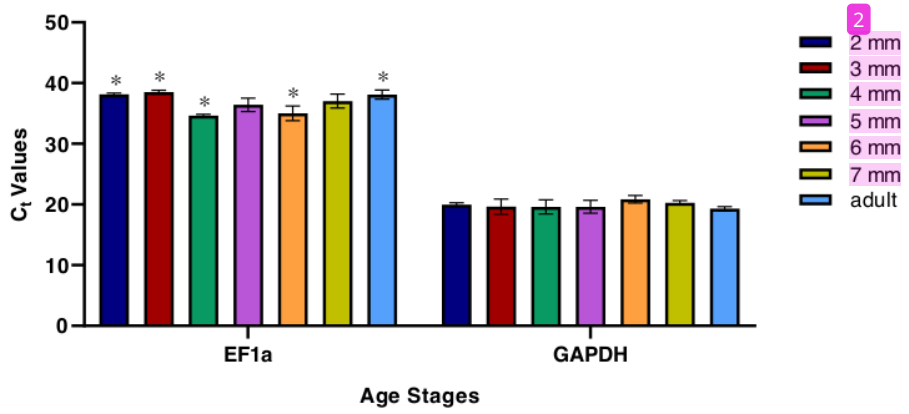

Fig. (3) Rates of Ct values among the stages for the studied genes.

Discussion

Gene expression analysis during qPCR requires proper normalization since the activity of the majority of genes tends to fluctuate based on the physiological state and varying conditions. Hence, it is crucial to identify and validate reference genes that remain stable for the normalization and analysis of data. (Faheem *et al*., 2018). Reference genes are suitable for adoption when they exhibit consistent expression levels across diverse tissues and throughout all developmental stages of the organism. They remain unaffected by experimental treatments and should ideally be expressed at a level similar to the RNA being studied. Nevertheless, the challenge of data normalization in qPCR persists, especially in the context of absolute quantification. (Bustin and Nolan, 2004). This study was distinguished for testing the validity of two from HKGs, EF1a and GAPDH, as reference genes in *P. latipinna*. The high Ct values for the EF1a gene compared to the Ct values for the GAPDH gene indicate the abundance of the GAPDH gene expression compared to the EF1a in this species. Also, the rates variance of Ct values among the studied stages showed that the GAPDH gene was less different than the EF1a in its expression during those stages. Through the results of the statistical analysis of the gene expression values between the control and treatment groups for the stages studied for the two genes which showed significant differences between the two groups at some stages of the EF1a gene. With no significant differences for the GAPDH gene, it is clarified that the GAPDH gene was the most constant under the influence of the calcitonin hormone. The results of the statistical analysis of the Ct values among the stages resulted in significant differences among some stages of the EF1a gene, whereas no significant differences were recorded among the stages of the GAPDH gene, which indicates the stability of the GAPDH gene expression, and the fluctuation of the EF1a gene expression During the various developmental stages, from the above, it appears that the EF1a gene is not an ideal candidate for the normalization of the target genes in *P. latipinna*. This conflicts with many studies on various species of fish. EF1a was always at the forefront of the preferred genes being tested as *Dicentrarchus labrax* (Mitter *et al*., 2009), *Hippoglossus hippoglossus* (Øvergård *et al*., 2010). As for the GAPDH gene, it appears to be a good candidate to be a reference gene in the studied species. Although, it did not achieve the results obtained by its counterpart except in limited studies and in specific tissues and specific experimental conditions (Spinsanti *et al*., 2006; *Pei et al*., 2007). Most studies that dealt with the

two genes together, EF1a gene was the most stable and the GAPDH gene was the least stable among the genes studied in various fish species (Olsvik *et al*., 2005: 2008; McCurley and Callard, 2008; Zhao *et al*., 2014; Wang *et al*., 2015; Xia *et al*., 2017). It is interesting to find here that GAPDH is characterized by constancy and stability at the various stages and under the influence of the hormone. Unlike the EF1a gene, which lost its preference with this species, the results varied in other studies. It was found that both the EF1a and GAPDH genes were suitable as a reference gene in most tissues of *Paralichthys olivaceus* (Zheng and Sun, 2011), both of which are not suitable in *Ictalurus punctatus* and *Oreochromis niloticus*, respectively (Small *et al*., 2008; Yang *et al*., 2013).

Conclusion

The GAPDH gene expression was stronger, more constant and stable than the EF1a gene at all stages studied, and also under the influence of the hormone. Therefore, it is possible that GAPDH may be a suitable reference gene with *P. latipinna*. Exclude the EF1a gene. For lack of basic conditions that qualify it as a suitable reference gene in this species of fish.

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