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Selection of Reference Gene for Expression Studies in the Ovary and Pituitary of Spotted Scat (*Scatophagus argus*) at Different Ovarian Stages

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Abstract: The spotted scat (*Scatophagus argus*) is the most widely commercially cultured fish in eastern and southern Asia that possess potential economic value. However, up to date, there are no studies reported on the selection of suitable reference genes in this fish, which is crucial for guaranteeing accurate and reliable results in RT-qPCR analyses. This study aimed at screening the most stable reference genes in the ovary and pituitary at different ovarian stages, which is of great significance for further research on the reproductive regulation mechanism of female *S. argus*. In this study, we selected twenty-one genes as the candidate reference genes, including four genes in the ovary (*EEF1A*, *ACTB*, *B2M*, and *HPRT1*), and seventeen genes in the pituitary (*B2M*, *APOA1*, *CGBA*, *RPLP0*, *GNRHR*, *GUSB*, *TBP*, *SLC25A5*, *RPL4*, *PLA1A*, *GAPDH*, *GNB2L1*, *CTFS*, *HPRT1*, *RPS2*, *TFRC*, and *TUBB4B*). To analyze the stability of the potential reference genes, we first used four commonly used software, BestKeeper, GeNorm, NormFinder, and ΔCt, and then we established comprehensive rankings with ReFinder. The results indicate that *RPL4* and *GNB2L1*, and *EEF1A* and *HPRT1* were the most appropriate reference genes for the pituitary and ovary of *S. argus*, respectively, in the three developmental stages of the ovary. Additionally, GeNorm analysis suggested that for the accurate normalization of gene expression, two reference genes were reliable in the ovary and pituitary of *S. argus*. To the best of our knowledge and understanding, this is the first study to identify appropriate reference genes for analyzing gene expression in *S. argus*. Consequently, this study can be used as a foundation for future research on the reproduction of *S. argus*.

Keywords: *Scatophagus argus*; reference gene; RT-qPCR; pituitary; ovary

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1. Introduction

The reverse transcription–quantitative polymerase chain reaction (RT-qPCR) is recognized as one of the most precise and popular technologies for examining and assessing gene expression [1]. This approach provides various advantages, including easy operation, offering high sensitivity, excellent repeatability, and accurate quantification [2]. However, the accuracy of quantitative analysis is affected by several factors, such as primer amplification efficiency among different samples, reverse transcription efficiency, and RNA concentration and quantity [3]. To minimize these variations, a suitable normalization method is pivotal to achieving accurate results [1]. Endogenous reference genes are considered to be the most common and popular method for normalizing RT-qPCR data [4,5]. Ideally, a reference gene can indicate constant expression in a specific tissue type, regardless of the ovary developmental stages or experimental treatment states [1,6].

Generally, reference genes for RT-qPCR studies are usually used as housekeeping genes, such as 18S ribosomal RNA (*18S*), beta-actin (*ACTB*), and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* [7,8]. However, it became clear that the expression of these genes

may not be the same in all tissues, experimental states, or developmental stages [1,2,6]. For example, *GAPDH* and *ACTB* were found as unstable genes in the gonads of adult female and male zebrafish (*Danio rerio*), where significant expression differences were found [9]. A study on rice field eels (*Monopterus albus*) indicated *GAPDH*, *18S*, and *ACTB* are not appropriate as reference genes during gonad development due to their high levels of expression variations [7]. Many similar studies were reported [4,6,7,10,11]. Consequently, it is crucial to identify relevant reference genes in expression research, particularly under certain experimental treatment states. Currently, a great number of software and algorithms, such as NormFinder [12], GeNom [13], ΔCt [14], and BestKeeper [15] were discovered to help identify stable internal reference genes. However, because these programs were created using various mathematical models [16,17], rankings produced using the same set of expression data are often unalike upon the usage of different software [11,18]. RefFounder is shown to be a user-friendly web-based tool that integrates the four methods mentioned above, which provides an extensive rating for experimental reference genes. The higher the expression stability, the lower the rank value [19–21].

Spotted scat (*Scatophagus argus*) is the most widely commercially cultured fish in eastern and southern Asia that possesses potential economic value. Females usually grow faster than males under identical cultural conditions [22,23], making monosexual female populations' culture very valuable economically. In addition, *S. argus* can eat algae, sick shrimp, parasites on fish bodies, and shellfish attached to the pond wall and net cage, making it a good "garbage fish". It is thus suitable for mixed cultivation with other marine shrimp and fish. However, artificial propagation is still not effective in the actual breeding process. Elucidating the reproductive regulation mechanism of *S. argus* will help its artificial reproduction. Although in recent years, studies were conducted based on the reproductive biology of *S. argus* [23–27], there is no research conducted on the reference genes of *S. argus,* making the study of its reproductive regulation mechanism difficult. Considering the important roles of the pituitary and ovary in the reproductive regulation of female *S. argus*, appropriate reference genes for these two tissues at different ovarian developmental stages are of great significance for future research.

This study aims to identify a suitable reference gene that can be expressed for RT-qPCR investigation in the ovary and pituitary of *S. argus* at different ovarian stages (stage II, stage III, and stage IV). In previous studies on reference genes in other species and on the female pituitary transcriptome of *S. argus* (unpublished data), for analysis of candidate reference genes, four ovarian genes and seventeen pituitary genes were selected. These genes include elongation factor 1-alpha (*EEF1A*), Beta-2-microglobulin-like (*B2M*), hypoxanthine-guanine phosphoribosyl transferase (*HPRT1*), and beta-actin (*ACTB*) in the ovary, and apolipoprotein A-IV (*APOA1*), Fsh beta subunit (*CGBA*), 60S acidic ribosomal protein *PO* (*RPLPO*), gonadotropin-releasing hormone II receptor (GNRHR), TATA box binding protein (*TBP*), beta-glucuronidase (*GUSB*), tricarboxylate transport protein (*SLC25A5*), 60S ribosomal protein L4 (*RPL4*), phospholipase A1 member A isoform XI (*PLA1A*), receptor of activated protein C kinase 1 (*GNB2L1/RACK1*), cathepsin L-like (*CTFS*), 40S ribosomal protein S2 (*RPS2*), Transferrin receptor protein 1 (*TFRC*), tubulin beta-2b chain (*TUBB4B*), *B2M*, *HPRT1*, and *GAPDH* in the pituitary. The RT-qPCR results were analyzed using the four-software mentioned above. Ultimately, RefFinder was used to provide a comprehensive ranking of the stability of these candidate reference genes. This study provided a reliable number of reference genes that are appropriate for RT-qPCR analysis in the pituitary and ovary of *S. argus* at different ovarian stages.

2. Materials and Methods

2.1. Ethical Statement

The Experimental Animal Care Committee of Guangdong Ocean University gave its approval for the experimental animals used in this study.

2.2. Experimental Fish and Sample Collection

A total number of 20 two-year-old female *S. argus* (weight: 242.83 ± 50.90 g, length: 19.48 ± 1.13 cm) were collected from Donghai Island in Zhanjiang, Guangdong Province, China. The fish fasted for 24 h and then were anaesthetized with MS-222 (Sigma, St. Louis, MO, USA) before sampling. Then the fish were dissected for ovarian and pituitary tissues. Then liquid nitrogen was used to snap freeze the pituitary and ovary that were then stored at −80 °C, ready for RNA extraction. Part of the ovaries was fixed in Bouin's solution overnight. The fixed ovaries were then used for preparing paraffin sections and stained with hematoxylin-eosin according to the manipulation of Jiang et al. [23]. The stained fragments were observed under a Nikon Eclipse Ti-E microscope (Tokyo, Japan). The development stages of the ovary were determined according to previous findings [23,25].

2.3. RNA Extraction and cDNA Synthesis

The Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from ovarian and pituitary tissue following the manufacturer's instructions. An Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to assess the RNA integrity value (RIN). The RNA quality was also checked using agarose gel electrophoresis. A total of 1 μg RNA of each sample was used to synthesize the first strand of cDNA using Prime ScriptTM RT Reagent Kit with gDNA Eraser (RR047A; Takara Bio, Dalian, China).

2.4. Primer Design

We selected seventeen candidate reference genes for the pituitary. The internal reference genes *B2M, HPRT1, GAPDH, TUBB4B, GUSB, TBP,* and *GNB2L1* were selected, as they were used in previous reports [16,28–31]. According to our transcriptome data (unpublished data), these genes *APOA1, CGBA, RPLP0, GNRHR, SLC25A1, PLA1A, CTFS, RPL4, RPS2,* and *TFRC* were selected because they are highly expressed in the pituitary of *S. argus*. We selected these genes, *EEF1A*, *B2M*, *GAPDH*, and *HPRT1* for analysis since they were established as good reference genes for the ovary in *Magang geese* [6], *Mozambique tilapia* [8], mouse [32], and sows [33]. Based on the candidate reference gene sequences obtained from the reference genome of *S. argus* [34], the oligo primer analysis software Version 7.0 was used to design the primers [35] (Table 1). The primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Table 1. Primer sequences used in the present study.

Gene Name	Primer Sequence (5′-3′) Forward	Primer Sequence (5′-3′) Reverse	Amplification Efficiency (%)	Product Length (bp)	Gene ID
APOA1 #	GCCCATCATCACAGAGTACCAAA	AGTCTTGGTCTCTTCCACGTTG	110.27	124	XM_046389465
B2M #,*	GAACTTCCTGGCGCTAAGCA	TATGATGTCCCCATGAGTGACC	93.91	132	KJ868824
CGBA #	ACCACGAGGATCCGGTCTACAT	GCCCGCAGAACGTGTTTCCTGTGTTA	95.80	174	KY129603.1
CTFS #	GCTGCCGTCAAATGCT	GTCCATTCTCAGCCAACCAA	109.42	188	XM_046405387.1
GAPDH #,*	ATGGCCTTCCGTGTCCC	ACTGTCGCCATTGAAATCTGT	98.73	180	XM_046405387.1
GNB2L1 #	CCCAAATCGCCACTACGC	GTCCCATCCCAAGCACCA	106.85	202	MT774146.1
GNRHR #	TACTGGTTCTTCCCCGACGA	TCGCTGCACAATATGAACCTC	92.71	284	XM_046405904.1
GUSB #	GTCGTTCTGTCGCCTCC	GCCGCCATTAATCGAACCTC	93.99	130	XM_046410012.1
HPRT1 #	CGCTCCATCCCAATGACAGT	ACGTTCTTGCCTGTCAGTGT	108.42	119	XM_046405836.1
PLA1A #	TCTACAACAGCCACGTCT	GCCACCAAACAAGTCCAG	95.56	182	XM_046403220.1
RPL4 #	GCTGGCACCGCAGGATCAACA	CCTTGGTCTTCTTGTAGCCCTCA	101.09	168	XM_046394990.1
RPLP0 #	GCCCTTCTCCTACGGTCTCA	CTAGCGATGTTCCTCACACCC	96.99	129	XM_046384377.1
RPS2 #	TCTGCCCATCAAGGAGTCTG	GCTTGCCGATCTTGTTCCC	101.14	275	XM_046414974.1
SLC25A1 #	CGCATCCCGGAAAAGCCATC	ACCTGTGGCATCTCGCATT	98.23	284	XM_046411134.1
TBP #	GGTTAGCTGCGAGAAAATATGC	GATCATTCGGTAAATCAGTCCT	97.83	197	XM_046384317.1
TFRC #	CCGCACTCCTATACACGTT	CAGGTAGCCAATCAAGAACCC	96.03	246	XM_046408402.1
TUBB4B #	GGCTTCCACCTTCATTGGCAAC	GTACTCGGACACCAGGTCGTTC	100.40	178	XM_046386004.1
ACTB *	GAGAGGTTCCGTTGCCCAGAG	CAGACAGCACAGTGTTGGCGT	100.72	145	KF649214.1
EEF1A *	CACATCAACATCGTGGTCATT	ACTTGCTGGTCTCAAACTTCC	95.83	235	XM_046418492.1

Note: "#" and "*" indicates the candidate reference gene for the pituitary and ovary, respectively.

2.5. Real-Time Quantitative PCR (RT-qPCR) Analysis and Determination of the Amplification Efficiencies

For real-time quantitative PCR, the SYBR Green PCR Mix (TransGen Biotech, Beijing, China) was used. The RT-qPCR reaction system (total volume 20 μL) consisted of the 10 μL of SYBR Green Mix, 1 μ template, 0.5 μL forward and reverse primer (100 μmol /mL), and 8 μL of water. Reaction conditions included one initial cycle of denaturation at 95 °C for 300 s, then followed by 40 cycles (95 °C for 30 s, annealing temperature for 20 s, and conducting elongation at 72 °C for 30 s). Melting curve analysis was used for all reactions to determine the specificity of the PCR-amplified products. For each set of primers, the amplification efficiencies (AE) were determined using standard curves with the ten-fold dilution of the cDNA template (1, 1/10, 1/100, and 1/1000). The equation AE = (10[−1/slope] −1) × 100 [36] is used to calculate the AE value. RT-qPCR was performed in triplicate using a Light Cycler 96 (Roche Diagnostics, Shanghai, China).

2.6. Stability Analysis of Candidate Reference Genes

The stability of candidate reference genes was tested using the ovary and pituitary cDNA isolated from all samples, respectively. The ovarian and pituitary cDNA for RT-qPCR reactions were performed in triplicate as well. After that, NormFinder, GeNorm, ΔCt, and BestKeeper were used to analyze the stability of the genes using the average Ct values of each sample. GeNorm calculates a M value for each gene, and the gene with the lowest M value has the highest stability [10]. Each candidate gene's stability was ranked by inter-group variation by NormFinder (SV) [14]. To determine each gene's stability, the ΔCt method was used by obtaining the standard deviation of Cq differences within each sample for each pairwise comparison with the over genes and averaging them [13,17]. The best-stabilized gene with the lowest coefficient of variance and standard deviation (SD) was selected by BestKeeper. Reference genes with the SD value of <1 are considered to be stable [15]. Finally, RefFinder integrates the four techniques mentioned above and provides a thorough ranking for the tested candidate reference genes [19]. Additionally, the pairwise variation ($V_{n/n+1}$), which was carried out using the GeNorm tool, was used to select the total number of reference genes for normalizing gene expression. Universally, ($V_{n/n+1}$) less than the threshold value of 0.15 denotes that n is the most appropriate number and that the n + 1 reference gene is not required for normalization.

3. Results

3.1. Characteristics of Ovarian Stages

The HE staining revealed that a significant amount of primary growth oocyte (PG) occupied the ovary stage at stage II (*N* = 6), while much previtellogenic oocyte (PV) and early vitellogenic oocyte (EV) appeared in the ovary at stage III (*N* = 3), and plenty of late vitellogenic oocytes (LV) were found in the ovary as stage IV (*N* = 11) (Figure 1 and Supplementary Table S1).

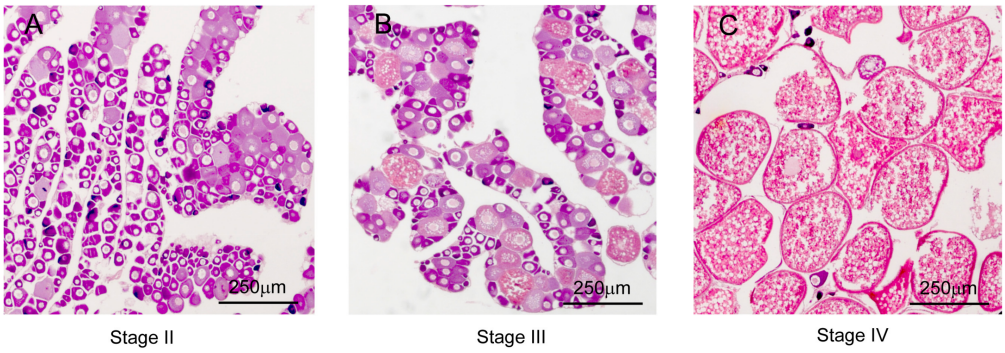

Figure 1. Gonadal histology of the *S. argus*.

3.2. RNA Extraction and Quality

All the 28S:18S ratios of the total RNA samples were greater than 1, and the OD260/280 ratios were 1.8~2.0. Except for one ovary sample in stage IV, the RIN of all other sample RNAs was above 7, indicating good quality (Figure 2A,B). RT-PCR revealed that *ACTB* primers could amplify a single band with both ovary and pituitary cDNA, indicating that the cDNA is good for further analysis (Figure 2C,D).

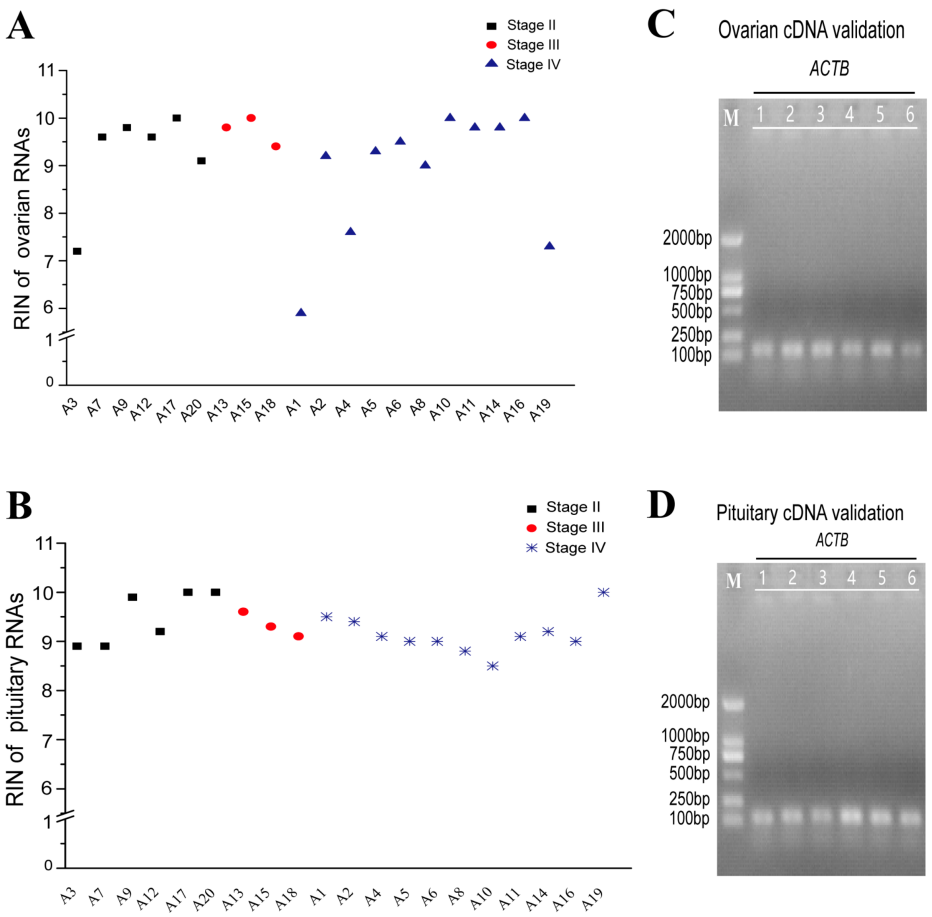

Figure 2. Quality detection of total RNA and cDNA. RIN of ovarian (**A**) and pituitary (**B**) RNAs (RIN above 7 indicates high RNA integrity). Amplification of ovarian (**C**) and pituitary (**D**) cDNA by *ACTB*.

3.3. Specificity and Efficiency of the Candidate Primers

The specificity of the primers was identified by PCR amplification and testing the PCR product length using agarose to get electrophoresis. The results indicate that all the primers could amplify a single band in the ovary or pituitary samples (data are not shown). The analysis of the melting curve showed that all the primers present a single peak at the expected primer annealing temperature (Supplementary Figures S1 and S2), confirming the primers pairs' specificity. Additionally, the AE of all primer pairs ranged from 92.71 to 110.27%, meeting the basic requirement of the RT-qPCR (Table 1).

3.4. Expression Profiles of Candidate Reference Genes

RT-qPCR revealed that the candidate reference genes' mean Ct values varied from 9.52 to 21.95 cycles in the ovary and 13.42 to 28.03 cycles in the pituitary (Figure 3A,B). In both the ovary and pituitary tissues, the candidate reference genes expression varied with the ovary development.

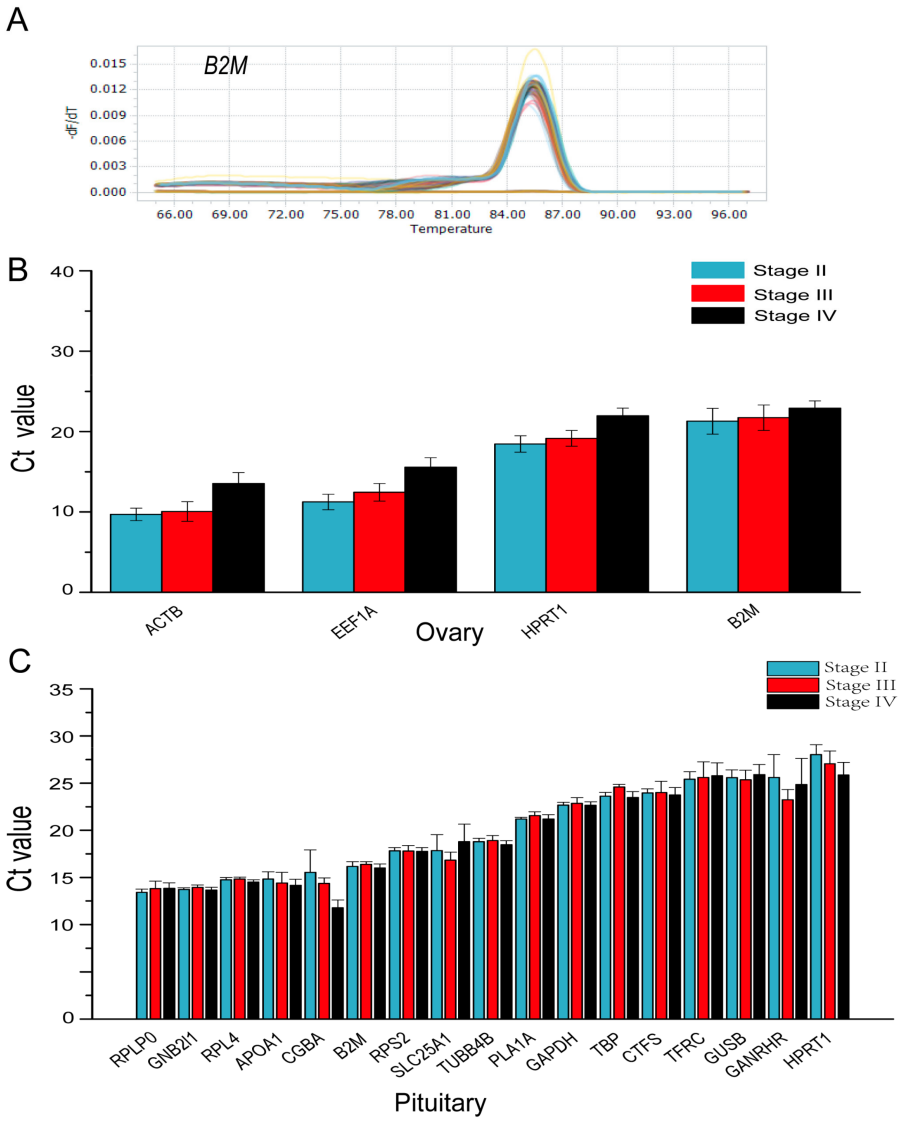

Figure 3. Primer-specific detection and expression levels of candidate reference genes in the ovary and pituitary tissues of *S. argus*. (**A**) Expression levels of four candidate reference genes in the ovary at different stages. (**B**) Expression levels of four candidate reference genes in the ovary at different stages. (**C**) Expression levels of seventeen candidate reference genes in the pituitary at different stages.

3.5. Stability of Candidate Reference Genes in Ovaries

The GeNorm analysis indicated that in the different stages of ovaries, stage II, stage IV, and all stages, the ranking for stability was *EEF1A* = *HPRT1* > *ACTB* > *B2M* (Figure 4A,C,D), while in the ovaries at stage III, the ranking for stability was *ACTB* = *HPRT1* > *EEF1A* > *B2M* (Figure 4C). Interestingly, the NormFinder analysis and the ΔCt analysis share the same results: the ranking for stability was *EEF1A* > *HPRT1* > *ACTB* > *B2M* in the ovaries at stages II and IV (Figure 4E,G,I,K). Meanwhile, in the ovaries at stage III, the ranking for ranking was *ACTB* > *HPRT1* > *EEF1A* > *B2M* (Figure 4F,J). In ovarian samples of all stages, the ranking for stability was *HPRT1* > *EEF1A* > *ACTB* > *B2M* (Figure 4H,L). The findings of the BestKeeper analysis revealed that the *B2M* gene in stage II and stage III ovaries, the *ACTB* gene in stage IV ovaries, and the *EEF1A*, *HPRT1*, and *ACTB* genes in ovarian samples of all stages were not stable for their SD > 1 (Figure 4M–P). Thus, according to the BestKeeper analysis, the stability ranking was *ACTB* > *EEF1A* > *HPRT1* in ovaries at stage II, *HPRT1* > *EEF1A* > *ACTB* in ovaries at stage III, and *B2M* > *HPRT1* > *EEF1A* > *ACTB* in ovaries at stage IV.

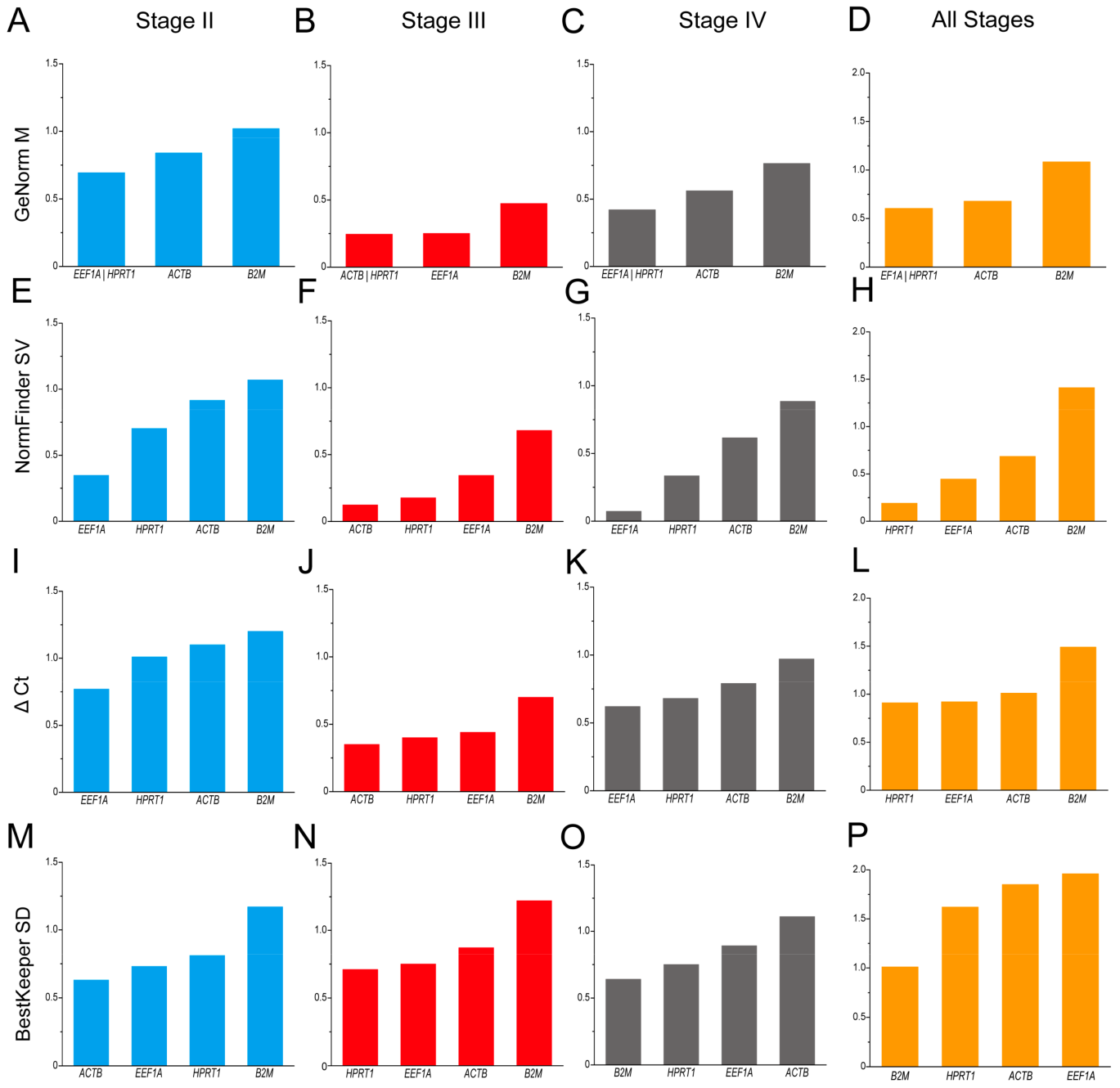

Figure 4. Analysis of the candidate reference gene stability in ovaries at different stages based on GeNorm (**A**–**D**), NormFinder (**E**–**H**), ΔCt (**I**–**L**), and BestKeeper (**M**–**P**) programs.

Based on the four methods above, the potential gene stability was comprehensively ranked throughout all stages using RefFinder. The results indicate that the ranking of stability was *EEF1A* > *HPRT1* > *ACTB* > *B2M* in ovaries at stage II (Figure 5A), *ACTB* > *HPRT1* > *EEF1A* > *B2M* in ovaries at stage III (Figure 5B), *EEF1A* > *HPRT1* > *B2M* > *ACTB* in ovaries at stage IV (Figure 5C), and finally, *HPRT1* > *EEF1A* > *B2M* > *ACTB* in ovaries at all stages (Figure 5D).

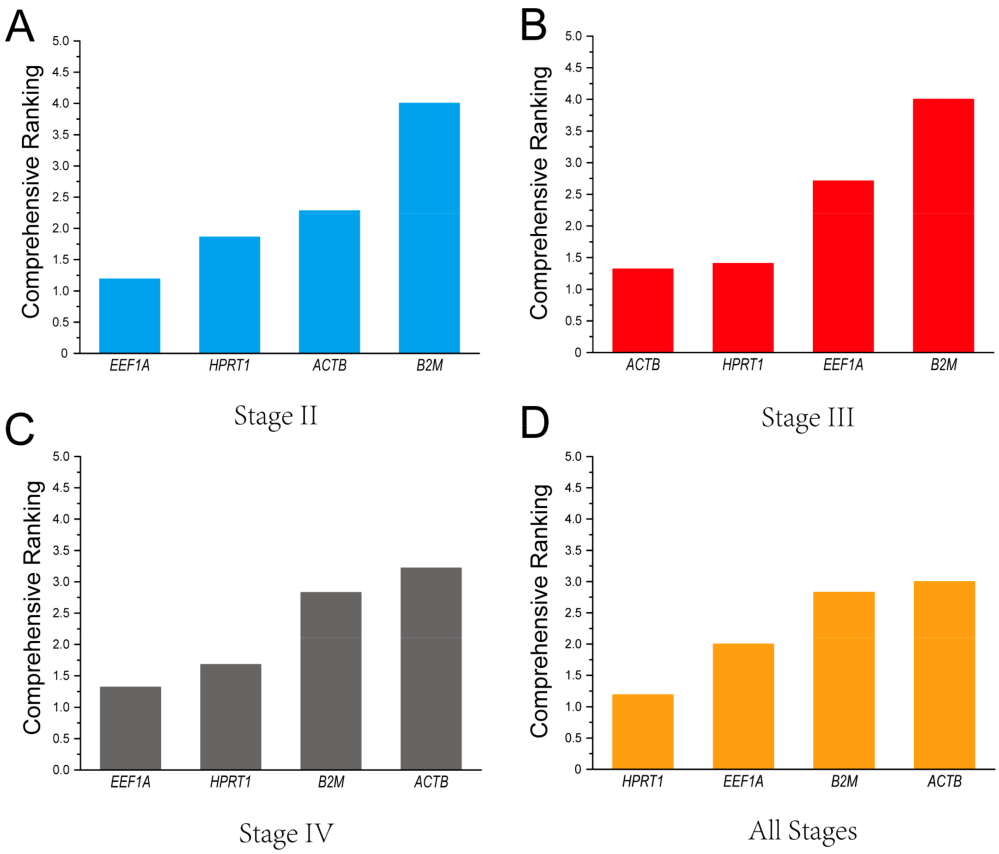

Figure 5. Ranking of the candidate reference genes' stability in ovaries at different stages using RefFinder. (**A**) Stage II; (**B**) Stage III; (**C**) Stage IV; (**D**) All stages.

3.6. Stability of Candidate Reference Genes in the Pituitary

In the pituitary, NormFinder, ΔCt, BestKeeper, and GeNorm analyses revealed that *RPL4*, *GNB2L1*, *PLA1A*, *TUBB4B*, *GAPDH*, *RPS2*, *TBP*, and *B2M* were more stable than other candidate reference genes because their stability ranking was always in the forefront, although their stability ranking order was different in samples of different stages (Figure 6A–D). In the GeNorm analysis, *GAPDH* and *TUBB4B* were more stable reference genes in the pituitary at stage II, *GNB2L1* and *PLA1A* in the pituitary at stage III, *RPL4* and *TUBB4B* in the pituitary at stage IV, and *RPL4* and *GNB2L1* in the pituitary at all stages (Figure 6A). In the BestKeeper analysis, the results show that *SLC25A1*, *GNRHR*, and *CGBA* in the pituitary at stage II; *HPRT1* and *TFRC* in the pituitary at stage III; *HPRT1*, *SLC25A1*, and *GNRHR* in the pituitary at stage IV; and *SLC25A1*, *HPRT1*, *GNRHR*, and *CGBA* in the pituitary at all stages were not stable for SD > 1 (Figure 6C).

According to the RefFinder analysis, the comprehensive rankings in the pituitary at stage II were as follows: *GNB2L1* > *RPL4* > *PLA1A* > *GAPDH* > *TUBB4B* > *TBP* > *CTFS* > *RPS2* > *RPLP0* > *B2M* > *APOA1* > *TFRC* > *GUSB* > *HPRT1* > *SLC25A1* > *GNRHR* > *CGBA* (Figure 7A). In the pituitary at stage III, the stability ranking was *RPL4* > *TUBB4B* > *GNB2L1* > *GAPDH* > *PLA1A* > *RPS2* > *TBP* > *B2M* > *SLC25A1* > *GUSB* > *CGBA* > *CTFS* > *GNRHR* > *APOA1* > *RPLP0* > *HPRT1* > *TFRC* (Figure 7B). In the pituitary at stage IV, the stability ranking was *RPL4* > *GNB2L1* > *RPS2* > *TUBB4B* > *PLA1A* > *GAPDH* > *TBP* > *B2M* > *RPLP0* > *CGBA* > *APOA1* > *CTFS* > *GUSB* > *TFRC* > *HPRT1* > *SLC25A1* > *GNRHR* (Figure 7C). In the pituitary at all stages, the stability ranking was *RPL4* > *GNB2L1* > *RPS2* > *PLA1A* > *TUBB4B* > *GAPDH* > *B2M* > *TBP* > *CTFS* > *RPLP0* > *APOA1* > *GUSB* > *TFRC* > *HPRT1* > *SLC25A1* > *CGBA* > *GNRHR* (Figure 7D).

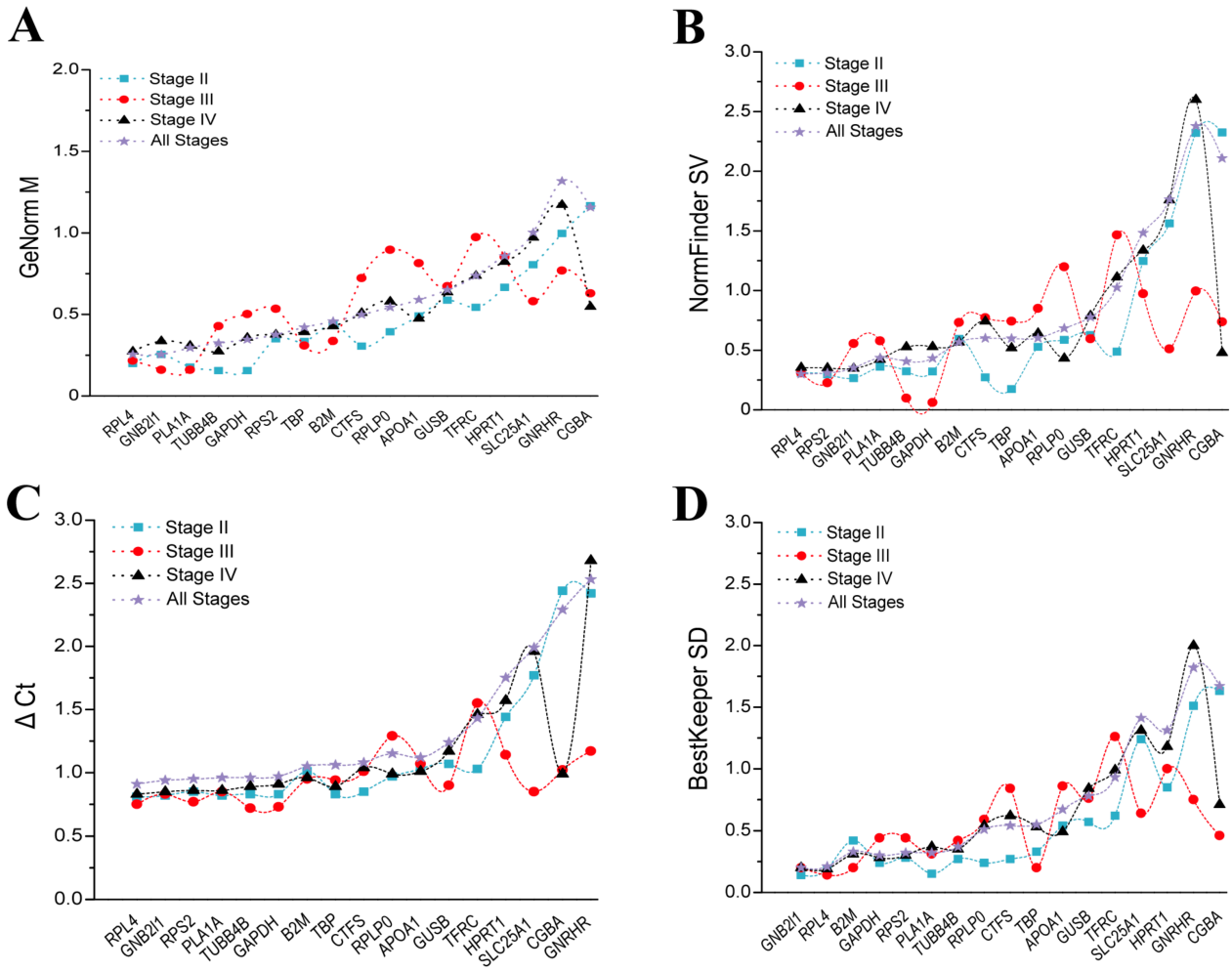

Figure 6. Analysis of the candidate reference gene stability in the ovaries at different stages based on GeNorm (**A**), NormFinder (**B**), ΔCt (**C**), and BestKeeper (**D**) programs.

3.7. Determination of the Optimal Number of Reference Genes

The GeNorm analysis showed that the V2/3 was below 0.15 across the different developmental stages in the ovary and pituitary tissues, respectively. The two reference genes were reliable for the accurate normalization of gene expression; therefore, there is no need to introduce the next internal reference gene for correction (Figure 8). Combined with the analysis of the reference gene stability, EEF1A and HPRT1 and RPL4 and GNB2L1 can be used to combine optimal reference genes in the ovary and pituitary, respectively, at stage II, stage IV, and for all stages. In stage III, the combination of optimal reference genes was *ACTB* and *HPRT1*, and *RPL4* and *TUBB4B* in the ovary and pituitary, respectively.

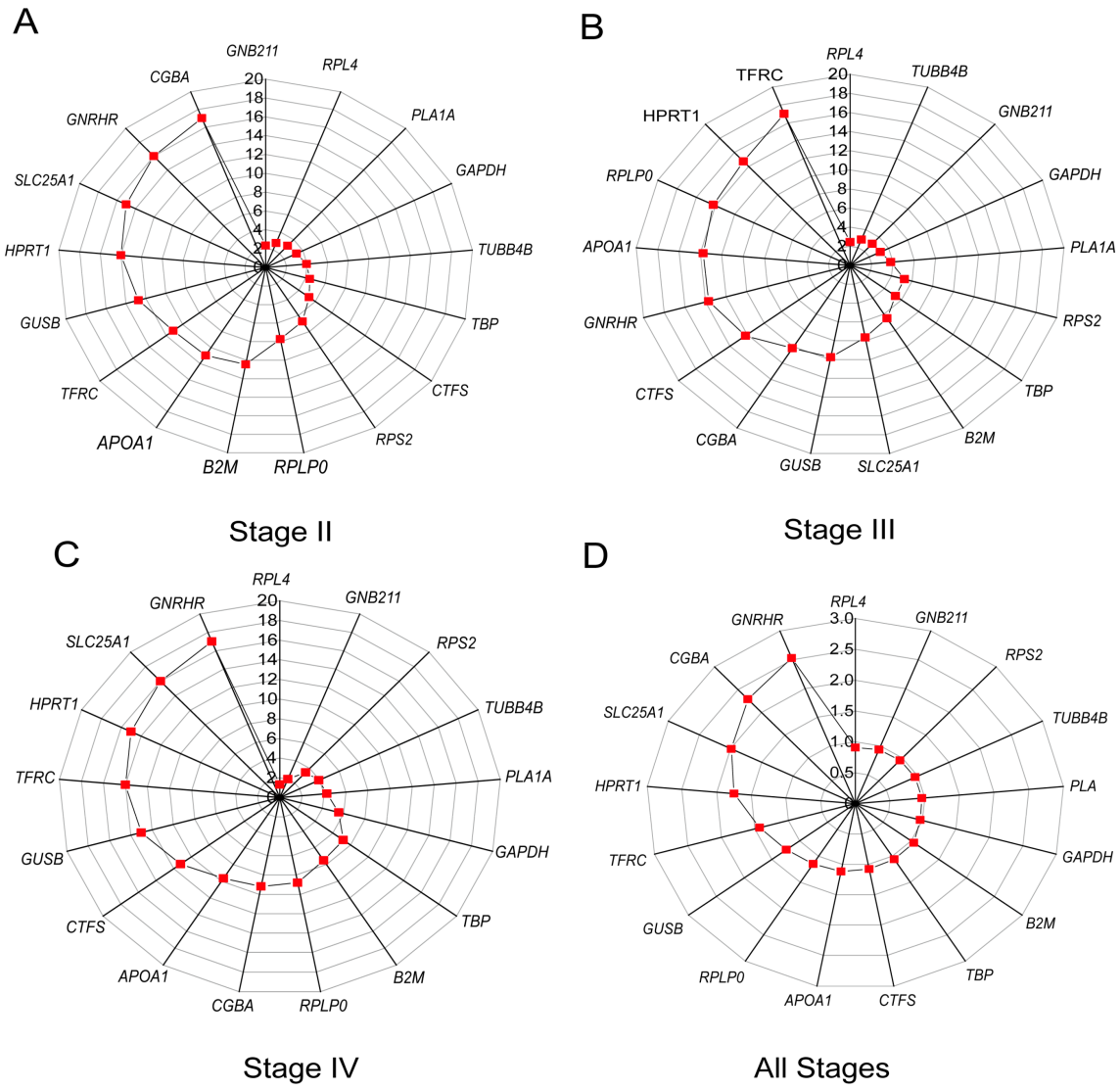

Figure 7. Ranking of the candidate reference gene stability in the pituitary at different stages by RefFinder. (**A**) Stage II; (**B**) Stage III; (**C**) Stage IV; (**D**) All stages.

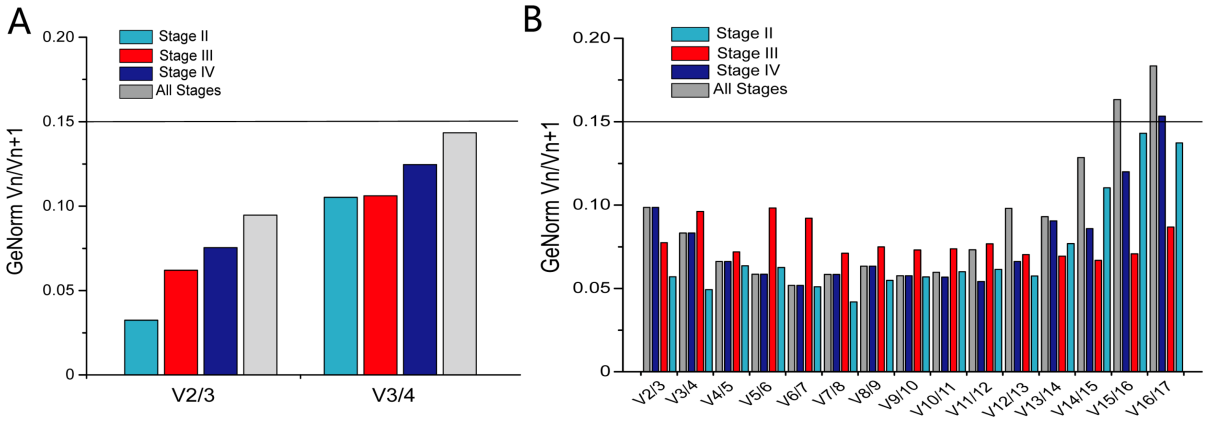

Figure 8. The optimal number of reference genes for normalization of gene expression in the ovary (**A**) and pituitary (**B**). GeNorm was used to calculate the pairwise variation (V_n/V_{n+1}, and "n" represents the number of reference genes).

4. Discussion

It is widely recognized that the selection of the appropriate endogenous reference genes is important for the study of gene expression with RT-qPCR since various studies indicated that reference genes are development-dependent, conditional, and tissue-specific [1,28,37]. The reference genes for RT-qPCR underwent validation for various tissues in different organisms [6–8,17,19,20,28,38–40]. However, there is no evidence of suitable reference genes in tissues of *S. argus*. In this research, RT-qPCR analysis was used to evaluate the expression levels of 21 candidate reference genes, including 17 genes in the pituitary and 4 genes in the ovary of *S. argus*, and we identified which genes are suitable for the ovary and pituitary at different ovarian stages.

In choosing a candidate reference gene, a key characteristic that should be considered is expression stability, regardless of the tissue's development stages or physiological conditions that they are expressed in. In the present study, five types of software were used to evaluate the candidate reference gene stability, including GeNorm [13], NormFinder [12], the comparative ΔCt method [14], BestKeeper [15], and RefFinder [19]. Interestingly, the rankings of GeNorm, NormFinder, comparative ΔCt, and RefFinder are always consistent with each other and different from those of BestKeeper in samples of the same stage, whether in the ovary or pituitary. This variation is probably because NormFinder and GeNorm evaluate the reference genes' stability according to the variation in Ct values, while BestKeeper calculates stability values based on the Ct values' correlation coefficient [17,28], as reported in previous studies [21,28,37,41,42]. According to Robbledo et al. [17], the ranking generated by the NormFinder method is the most reliable ranking for choosing reference genes for RT-qPCR analysis when the results of gene stability measurement methods differ. Therefore, several methods need to be applied to evaluate the reference genes' stability to make a final decision.

The ovary is the hypothalamus–pituitary–gonadal (HPG) axis target organ that exhibits functional and morphological differences during developmental stages [43]. The stability of reference gene expression in the ovaries of vertebrates was validated in a number of studies. Ji et al. [44] found that in the liver and ovaries of Zi geese (*Anser cygnoides*), there were the most stable *GAPDH* expressions. Gao et al. [28] found that *ACTB* and *CTSD* were best-suited gene combinations for normalization in the ovary of turbot (*Scophthalmus maximus*). Cai et al. [3] found that *TBP* is the appropriate reference gene for examining clock gene expression in vitro in the rat (*Rattus norvegicus*) ovarian granulosa cells. *EEF1* was reported by Mahanty et al. [45], to be a more appropriate reference gene in the minnows' (*Puntius sophore*) ovaries. According to Hu et al. [7], the gonads of *M. albus* may have reference genes, such as *EF1* and *RPL17*, in the various stages of development. Meanwhile, due to the level of expression variability, the reference genes 18S, *GAPDH*, and *ACTB* are not appropriate. In this study, we found that the optimal internal reference gene combination was *EEF1A* and *HPRT1* in stage II, stage IV, and all-stages ovaries, and *ACTB* and *HPRT1* in the ovaries of stage III after combining the results of four software analyses. All cells expressed *EEF1*, which performs a number of functions during cell growth and proliferation [45–47]. In the gonads of zebrafish exposed to endocrine disruptors, *EEF1* was also found to be an appropriate reference gene [7], and *M. albus* in different stages of development [17]. *HPRT1* is an important enzyme in gout and uric acid metabolism [48]. In fathead minnows (*Pimephales promelas*), *HPRT1* was recommended as the appropriate internal control in real-time PCR studies of the effects of estrogen in fish [49]. *HPRT1* was identified as the top candidate housekeeping gene in the zebrafish xenograft model [50]. In the ovaries of zebrafish exposed to Roundup, *HPRT1* was reported as the best stable ranked gene [37]. Therefore, *EEF1A* and *HPRT1* might be widely used in fish as ovary reference genes.

In teleosts, the pituitary is an important organ that regulates the growth and maturation of the ovary. Selecting appropriate reference genes to use for normalization in this tissue is rarely investigated. According to Zhang et al. [6], *HPRT1* and *RPL4* were found to be the best reference genes for the pituitary of the Magang goose at different stages of reproduction. Gao et al. [28] found that *ACTB, 18S*, and *CTSD* were the most suited gene

combination for normalization in the pituitary of *S. maximus*. GeNorm rated *18S* as the most stable gene in the pituitary of sparrows [51]. In this study, except for *RPL4* and *TUB4B* in fish with ovaries in stage III, *GNB2L1* and *RPL4* were the most stable reference gene combinations in the pituitary of *S. argus*. In any case, *GNB2L1, RPL4*, and *TUB4B* were among the top five most stable reference genes in the pituitary. *GNB2L1*, also known as *RACK1*, plays a part in cellular signaling pathways [52,53]. *GNB2L1* was suggested to be a suitable reference gene for studying gene expression in neutrophils [54] and the ovary of sows [33]. To the best of our knowledge, *GNB2L1* was identified for the first time in fish as a reference gene. *RPL4* encodes a protein that is a component of the 60S ribosomal subunit [55] and was previously suggested as a suitable reference gene in many studies, such as in exfoliated cervical cells [56], in ovarian tumors [30], in the pituitary of Magang geese [6], in *Ophraella communa* [2], and in Atlantic cod (*Gadus morhua*) [57]. In the pituitary of *S. argus*, *GNB2L1, RPL4*, and *TUB4B* may serve as appropriate reference genes.

Following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) [1], the results of RT-qPCR data analysis may differ when a single reference gene is used. Therefore, it is recommended to use more than one reference gene in the experiment. In this study, the two reference genes that are most stably expressed in the ovary and pituitary both had GeNorm V values of <0.15, which means two reference genes are enough to normalize the data expression in the ovary and pituitary of each developmental stage, respectively. Consistently, numerous studies reported that the two reference genes were the optimal number required in the experiment [2,6,11,28]. Taken together, this study recommends using at least two reference genes for RT-qPCR to avoid variation in reference genes.

5. Conclusions

For the first time, the ovaries and pituitary of *S. argus* at different ovarian stages were used to authenticate a group of candidate reference genes in this study. In conclusion, analysis of reference gene stability using five types of software revealed that the combination of two reference genes in the ovary (*HPRT1, EEF1A*) and pituitary (*GNB2L1, RPL4*) is highly recommended to be used as reference genes in *S. argus* at different ovarian stages. We expect these findings will contribute to the development and advancement of molecular research on the reproduction of *S. argus*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fishes8020120/s1, Figure S1: The melting curves of the cDNA samples amplified with candidate genes in ovary; Figure S2: The melting curves of the cDNA samples amplified with candidate genes in pituitary; Table S1: Ovarian stages of the 20 female spotted scat. Table S2: Expression stability analysis of each candidate reference gene in the ovary based on five softwares; Table S3: Expression stability analysis of each candidate reference gene in the pituitary based on five softwares.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

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