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RESEARCH ARTICLE

Selection of Reference Genes for RT-qPCR Analysis in the Monarch Butterfly, *Danaus plexippus* (L.), a Migrating Bio-Indicator

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Abstract

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) is a powerful technique to quantify gene expression. To facilitate gene expression study and obtain accurate results, normalization relative to stably expressed reference genes is crucial. The monarch butterfly, *Danaus plexippus* (L.), is one of the most recognized insect species for its spectacular annual migration across North America. Besides its great voyages, *D. plexippus* has drawn attention to its role as a bio-indicator, ranging from genetically modified organisms (GMOs) to natural ecosystems. In this study, nine reference genes from *D. plexippus* genome were selected as the candidate reference genes. The expression profiles of these candidates under various biotic and abiotic conditions were evaluated using the four readily available computational programs, *BestKeeper*, *Normfinder*, *geNorm*, and ΔC_t method, respectively. Moreover, *RefFinder*, a web-based computational platform integrating the four above mentioned algorithms, provided a comprehensive ranking of the stability of these reference genes. As a result, a suite of reference genes were recommended for each experimental condition. Specifically, *elongation factor 1 α* (*EF1A*) and *ribosomal protein 49* (*RP49*) were the most stable reference genes, respectively, under biotic (development, tissue, and sex) and abiotic (photoperiod, temperature, and dietary RNAi) conditions. With the recent release of a 273-million base pair draft genome, results from this study allow us to establish a standardized RT-qPCR analysis and lay a foundation for the subsequent genomic and functional genomic research in *D. plexippus*, a major bio-indicator and an emerging model for migratory animals.

Introduction

The advent of next-generation sequencing technologies has led to a significant increase in transcriptomic and genomic output for various organisms [1–3]. Validation of gene expression has become a standard for reporting and assessing the quality of these transcriptomic and genomic resources [4, 5]. Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) is a powerful technique to target and quantify gene expression [6], however, there remain limitations can significantly influence the normalization of gene expression, such as variations in RNA extraction, RNA quality and integrity, cDNA quality, and PCR efficiency [7–10]. In RT-qPCR, a commonly used technique to normalize the gene expression data is to introduce one or multiple reference genes, which are stably expressed across various experimental conditions and serve as the internal control [6, 9, 10]. Although these reference genes have been defined functionally as ‘constitutively expressed to maintain cellular function’, they do not necessarily meet prerequisites for a good reference gene that can be ‘expressed at constant levels across various biotic and abiotic conditions [4, 8, 10]. Many studies have proved that some commonly used reference genes express differentially across different experimental conditions; researchers have documented that multiple reference genes should be used for accurate normalization [4, 8, 9, 11, 12].

RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. In view of its high sequence specific reality, RNAi has drawn great attention from the crop protectors who recognized it as a novel and environmentally friendly ways for insect pest control [13, 14]. Recently, RNAi-based transgenic crops targeting insects have been developed [15–17]. One of the major ecological concerns with respect to the bio-safety of transgenic crops on the environment is their potential effects on non-target organisms [14]. The monarch butterfly is recognized as an important symbol of biodiversity and had been selected as a surrogate species for the risk assessment of *Bacillus thuringiensis* (*Bt*) and RNAi-based transgenic crops. Given the nature of RNAi mechanisms, non-target effects could occur via the modulation of gene expressions in these organisms [13]. Therefore, RT-qPCR will be a major research tool to evaluate potential non-target effects of this new biotechnology.

The monarch butterfly, *Danaus plexippus*(L.) (Lepidoptera: Danainae), is famous for its late summer/autumn southward migration from the United States and southern Canada to Mexico, and northward return in spring, which occurs over the lifespan of three to four generations of the butterfly [18]. In addition to this migration, *D. plexippus* has drawn attention as a surrogate species for the ecological risk assessment of genetically modified organisms (GMOs) [19–22] and as an eco-indicator for global climate change [23, 24]. The recent release of a 273-million base pair draft genome, including 16,866 protein-coding genes [25], provides an unprecedented opportunity to investigate the genetic basis governing monarch migration, including but not limited to genes involved in their migratory behavior, circadian rhythm, juvenile hormone modulation, and warning coloration [25–27]. Recent studies have identified the specific areas in the genome of the monarch that regulate migration. No genetic difference was observed between migrating and non-migrating monarchs but certain genes are expressed solely in migrating monarchs [25, 27].

The objective of this study was to determine suitable reference genes with stable expression in *D. plexippus* across various biotic and abiotic conditions. Here, nine reference genes from *D. plexippus* genome were selected as candidate reference genes [25], including *elongation factor 1 α* (*EF1A*), *glyceralde hyde-3-phosphate dehydro-genase* (*GAPDH*), *nicotinamide adenine dinucleotide* (*NADH*), *cyclophilins A* (*CypA*), *ribosomal protein S5* (*RPS5*), *ribosomal protein 49* (*RP49*), *vacuolar-type H⁺-ATPase* (*v-ATPase*), *28S ribosomal RNA* (*28S*), and *18S ribosomal*

RNA (18S). All of these candidates have been used previously as endogenous references for gene expression analyses [4, 8–12]. Stability of these genes was investigated under three biotic (developmental stage, tissue type, and sex) and three abiotic (temperature, photoperiod, and dietary RNAi) conditions. As a result, different sets of reference genes were recommended for each experimental condition. To validate the recommendations, effectiveness of these candidates were further examined by RT-qPCR analysis of a circadian clock gene *timeless (tim)* [28].

Materials and Methods

Ethics Statement

Eggs of *Danaus plexippus* were collected from common milkweed, *Asclepias syriaca*, near Ames, Iowa spring and summer 2013. No specific permit was required for the field collection. *Danaus plexippus* is a common American butterfly species in the United States. The permit to move live plant pests, noxious weeds, and soil were authorized by the United States Department of Agriculture Animal and Plant Health Inspection Service (Permit number: P526P-13-03521). Four to five generations of monarch adults were screened for presence of the protozoan parasite, *Ophryocystis elektroscirrha*, in order to eliminate it from the population. *D. plexippus* larvae from the maintenance colony were reared on either common milkweed or tropical milkweed, *Asclepias curassavica*. Larvae during the experiments were fed honeyvine milkweed, *Cynanchum laeve* (*syn. Ampelamus albidus*) at $25 \pm 1^\circ\text{C}$ (16L: 8D), and adults were fed 15% sugar solution.

Experimental conditions

Biotic factors. The different developmental stages including eggs, 1st, 2nd, 3rd, 4th instar larvae (collected at the first day of each instar), pupae, and adults. Tissues, including head, mid-gut, and carcass, were dissected from the 4th instar larvae. Male and female adults were collected separately to examine expression profiles of candidate genes between sexes.

Abiotic factors. To examine temperature influence, 2nd instar *D. plexippus* were exposed to 10°C, 22°C, and 37°C for 2 h. For photoperiod, 2nd instars were treated with exposure to 16:8 h, 12:12 h, and 8:16 h light: dark regime for 3 d. For the double-stranded RNA (dsRNA) feeding bioassay, there were three treatments including dsRNAs synthesized from *D. plexippus* *vATPase subunit A* (dsDP), *β -glucuronidase* (GUS) (dsGUS), and water control. Neonates (< 12 h) were given 1.6 μl of water solutions containing 5.0 $\mu\text{g}/\mu\text{l}$ dsRNA each day for two days. Larvae were collected on the third day of the experiment. Altogether, 16 μg of dsRNA was ingested by each *D. plexippus* larva.

For egg samples, six individuals were collected for each replicate; for pupa samples, one pupa was collected for each replicate. For the other biotic and abiotic conditions, approximately five individuals were collected for each treatment with three replicates. All samples were snap frozen with liquid nitrogen in 1.5 ml centrifuge tubes and then stored at -80°C .

Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to previously described methods [29, 30]. First-strand cDNA was synthesized from 700 ng of total RNA using the M-MLV reverse transcription kit (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. The cDNA was diluted 10-fold for the subsequent RT-qPCR studies.

Table 1. Primers used for RT-qPCR.

Gene	Accessionnumber	Primer sequences (5'-3')	Length(bp)	Efficiency(%)	R ²
<i>EF1A</i>	DQ157894	F: TGTCGCTTTTCGTACCCATT R: CCTTCAGCCTTACCCTCTTAC	114	97.1	0.989
<i>NADH</i>	U32457	F: GGTGTTTTAATTGGGGTTGC R:GCATCAGAAAAAGTTTGAATAATCC	122	102.3	0.9992
<i>GAPDH</i>	EU141486	F: CGTTCCCGTAGCTAATGTATCC R: GCTTCCTTGACCTTCTGTTAATG	93	106.1	0.9985
<i>CypA</i>	EHJ70135	F: CAACGGCTCTCAATTCTTCATC R: CCATGCCTTCAACCACATTAC	89	99.9	0.9898
<i>RPS5</i>	EU141382	F: GTCGTGTGAACCAGGCTATT R: GAGCTCATCAGCAACACATT	98	100.5	0.9991
<i>RP49</i>	AY971345	F: CCGGAAGGTGTTAGTCCACAAC R: CGGCGCAGTACTTCTATTCTG	71	98.6	0.9997
<i>18S</i>	AF394668	F: TGAGAAACGGCTACCACATC R: CGTAAGAGTCCCGTATCGTTATT	102	109.3	0.9997
<i>28S</i>	GQ229491	F: AACAAAGTACCGTGAGGGAAAG R: CATTTCGAGTTTCGCAGGTTTAC	94	102.7	0.9978
<i>v-ATPase</i>	EHJ63113	F: AGGACGACTTCTGCAACAGAACA R: TGTTCTTCAACATGCCACCCTCT	84	105.9	0.9998

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Reference gene primer design and RT-qPCR

Nine reference genes were selected (Table 1). The primers for the RT-qPCR were designed online (<https://www.idtdna.com/Primerquest/Home/Index>). The information of RT-qPCR amplification reaction and program were described in detail in our previous study [29, 30]. The standard curve and PCR efficiency of each candidate reference gene were constructed and calculated according to previously described methods [29, 30].

Data analysis

Stability of the nine candidate reference genes were evaluated by four computational programs, including *geNorm* [6], *NormFinder* [10], *BestKeeper* [31], and the ΔC_t method [32]. In addition, *RefFinder* (<http://www.leonxie.com/referencegene.php>), a web-based comprehensive platform, which integrates the four above mentioned algorithms, provided the overall ranking of the stability of these reference genes.

Validation of selected reference genes

The photoperiod-relevant *tim* gene of *D. plexippus* was used to evaluate the candidate reference genes. *tim* expression levels were determined in 2nd instars treated for 3 d under three photoperiod conditions (16L:8D, 12L:12D, and 8L:12D). Relative quantification of *tim* in different samples was performed using the $2^{-\Delta\Delta C_t}$ method [33].

Results

Performance and *Ct* values of candidate reference genes

Each of the tested genes displayed a single amplicon with the expected size on a 1.5% agarose gel (S1 Fig). Moreover, gene-specific of these candidates was affirmed by a single peak in

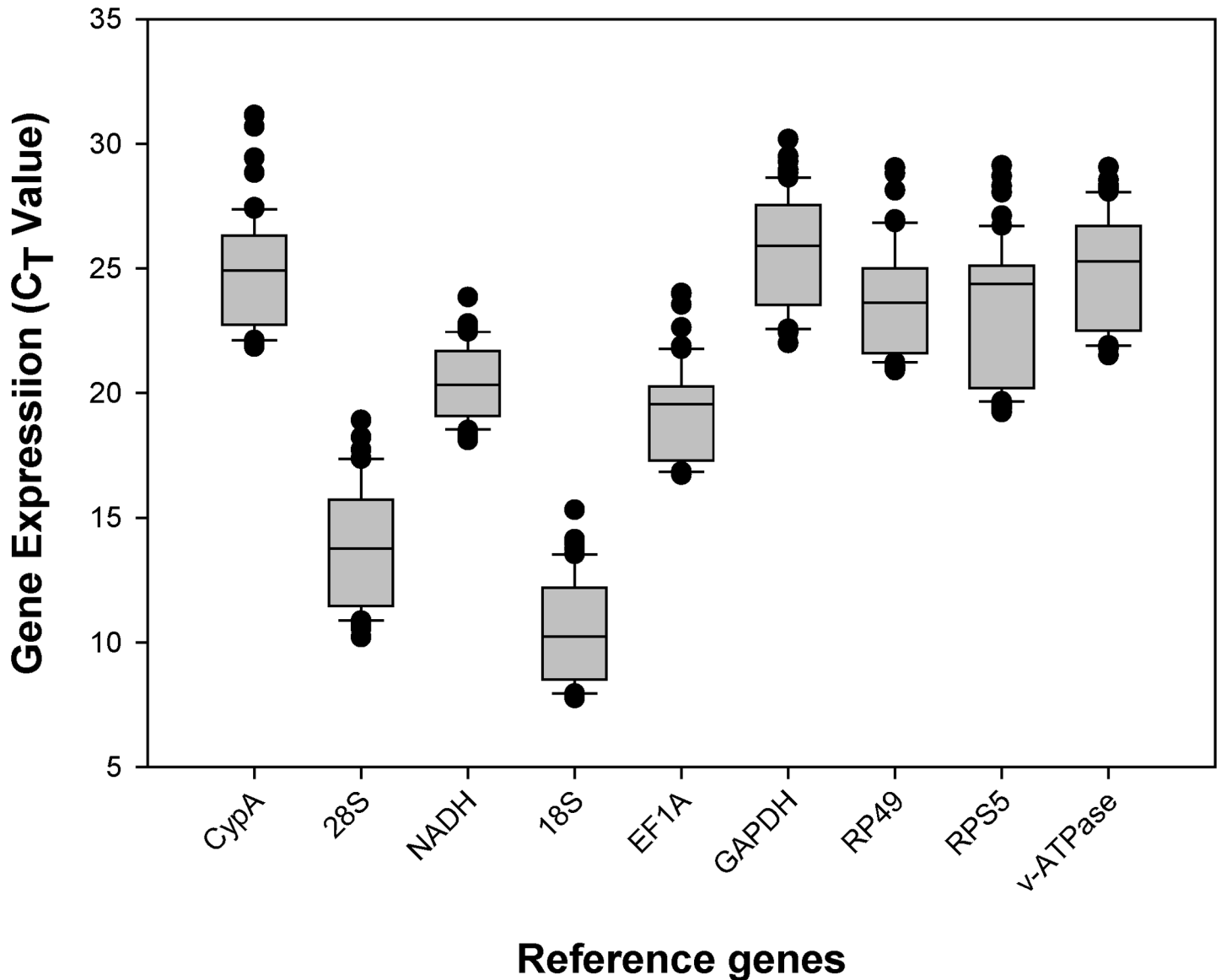


Fig 1. Expression profiles of candidate reference genes in *D. plexippus*. The expression level of candidate reference genes in 60 tested samples are documented in C_t -value. The dot indicates the maximum or minimum value of replicated samples, while whiskers indicate the standard error of the mean.

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melting curve analysis (S2 Fig). Standard curves were created for all the candidates, and the PCR efficiency and correlation coefficient for each standard curve were shown in Table 1.

Gene expression analyses of the nine reference genes exhibited a broad C_t range (Fig 1). C_t values ranged from 7 to 29, while most of the values were distributed between 11 and 22. The most abundant transcripts were 18S and 28S; and the least abundant transcripts were GAPDH and CypA.

Stability of candidate reference genes under biotic conditions

Developmental stage. All software programs, except *BestKeeper*, identified EF1A as the most stable gene (Table 2).

According to *RefFinder*, the overall order from the most stable to the least stable reference genes across different developmental stages was: EF1A, 28S, RPS5, RP49, v-ATPase, 18S,

Table 2. Stability of reference gene expression under biotic conditions.

Biotic conditions	Gene	<i>geNorm</i> Stability	Rank	<i>Normfinder</i> Stability	Rank	<i>BestKeeper</i> Stability	Rank	ΔCt Stability	Rank
Development	<i>EF1A</i>	0.53	1	0.61	1	1.35	6	1.07	1
	<i>NADH</i>	1.27	8	1.66	9	0.89	2	1.81	9
	<i>GAPDH</i>	1.12	7	0.93	7	0.98	4	1.33	8
	<i>RP49</i>	0.53	1	0.83	5	1.20	9	1.14	4
	<i>18S</i>	0.91	5	0.81	4	0.91	3	1.24	5
	<i>28S</i>	0.70	3	0.65	2	1.39	7	1.10	2
	<i>v-ATPase</i>	1.04	6	0.90	6	0.85	1	1.30	7
	<i>CypA</i>	0.80	4	0.99	8	1.40	8	1.30	6
	<i>RPS5</i>	0.66	2	0.75	3	1.20	5	1.14	3
Tissue	<i>EF1A</i>	0.45	1	0.51	2	0.80	7	0.87	2
	<i>NADH</i>	0.60	3	0.71	5	0.77	5	0.98	3
	<i>GAPDH</i>	1.01	8	1.22	9	0.73	4	1.37	9
	<i>RP49</i>	0.57	2	0.75	7	0.73	3	1.00	5
	<i>18S</i>	0.80	5	0.82	8	1.03	9	1.07	8
	<i>28S</i>	0.45	1	0.20	1	0.53	2	0.79	1
	<i>v-ATPase</i>	0.70	4	0.67	3	0.97	8	0.99	4
	<i>CypA</i>	0.91	7	0.68	4	0.41	1	1.00	6
	<i>RPS5</i>	0.87	6	0.75	6	0.77	6	1.05	7
Sex	<i>EF1A</i>	0.41	3	0.64	5	0.90	6	0.98	4
	<i>NADH</i>	1.23	8	2.21	9	1.45	9	2.27	9
	<i>GAPDH</i>	0.71	6	0.53	4	0.72	3	1.15	6
	<i>RP49</i>	0.35	2	0.10	1	0.91	4	0.89	1
	<i>18S</i>	0.29	1	0.15	2	0.68	2	0.89	2
	<i>28S</i>	0.29	1	0.35	3	0.82	5	0.93	3
	<i>v-ATPase</i>	0.93	7	1.24	7	0.64	1	1.55	8
	<i>CypA</i>	0.56	5	1.31	8	1.27	8	1.39	7
	<i>RPS5</i>	0.43	4	0.70	6	0.91	7	1.01	5

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NADH, *GAPDH*, *CypA* (Fig 2A). *geNorm* analysis revealed that the pair-wise variation value $V_{3/4}$ was 0.154, close to the proposed 0.15 cut-off (Fig 3), suggesting that three reference genes were required for reliable normalization throughout developmental stages.

Tissue. Majority of the software programs ranked *28S* as the most stable gene (Table 2). Based on *RefFinder*, the overall order from the most to the least stable reference genes in different tissue was: *28S*, *EF1A*, *CypA*, *NADH*, *RP49*, *v-ATPase*, *RPS5*, *GAPDH*, *18S* (Fig 2B). *geNorm* analysis revealed that the first V-value < 0.15 showed at $V_{3/4}$ (Fig 3), suggesting that three reference genes were sufficient for reliable normalization in different tissue types.

Sex. According to the results generated by *geNorm*, *18S* was ranked as the most stable gene whereas the other three programs identified it as the second stable gene (Table 2). According to *RefFinder*, the overall order from the most stable to the least stable reference genes in both female and male adults was: *18S*, *RP49*, *28S*, *v-ATPase*, *EF1A*, *GAPDH*, *RPS5*, *CypA*, *NADH* (Fig 2C). *geNorm* analysis revealed that the first V-value < 0.15 showed at $V_{2/3}$ (Fig 3), suggesting that two reference genes were enough for accurate normalization in female and male adults.

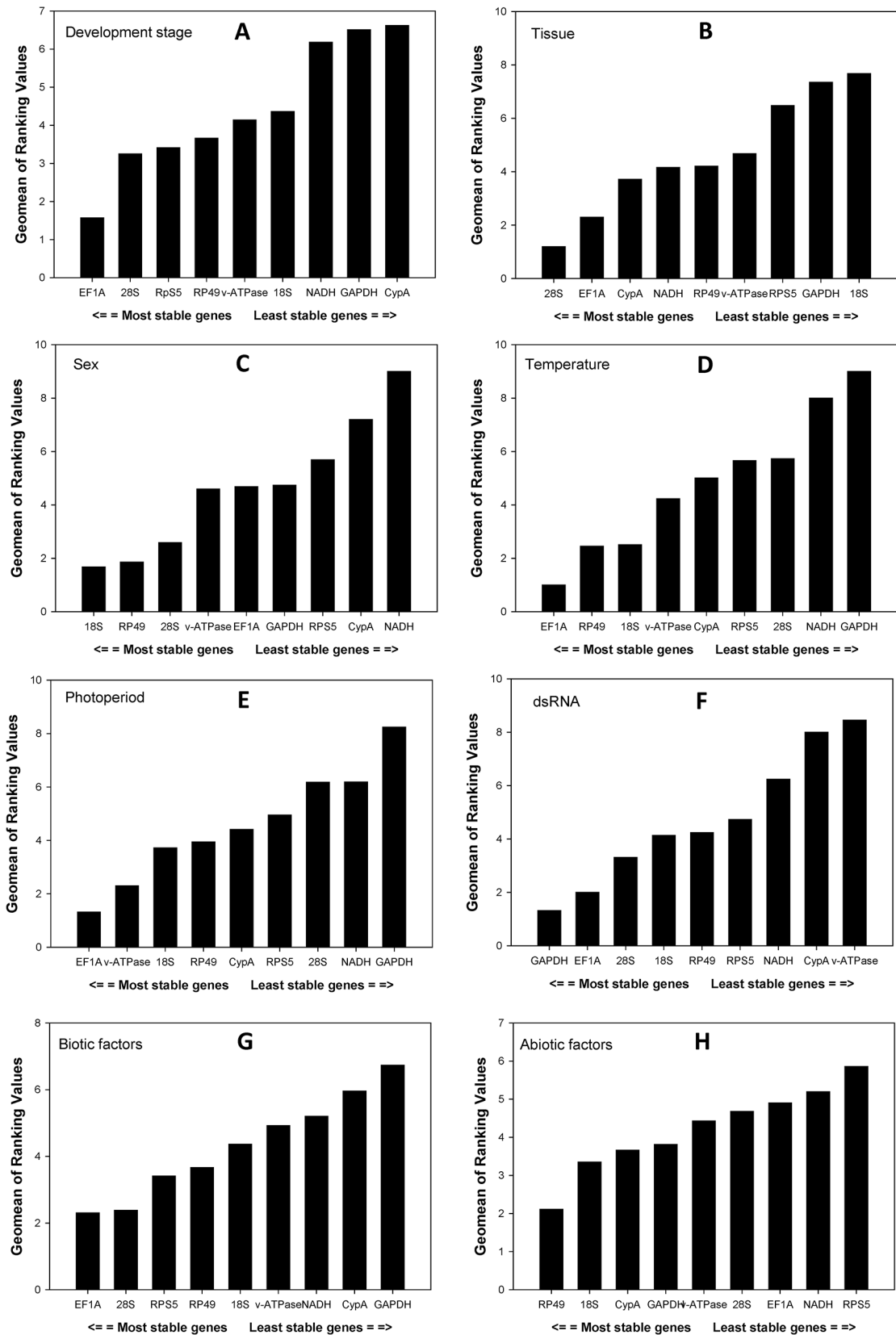


Fig 2. Stability of candidate reference genes expression under different treatment by RefFinder. A lower Geomean value indicates more stable expression.

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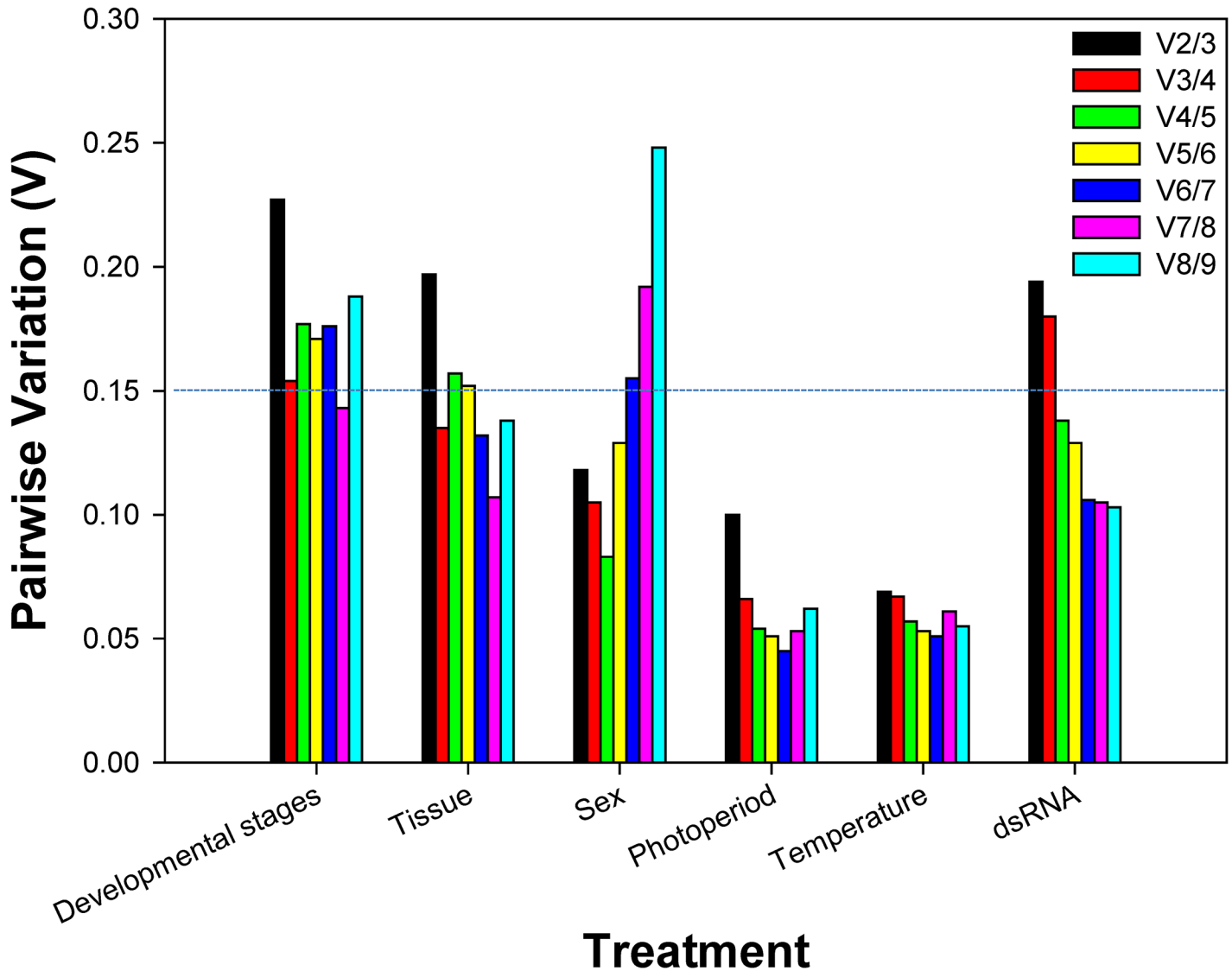


Fig 3. Determination of the optimal number of reference genes. Pairwise variation (V) is an index for determining the optimal number of reference genes for accurate RT-qPCR normalization. A cut-off value for pairwise variation of 0.15 was recommended [6].

doi:10.1371/journal.pone.0129482.g003

Stability of candidate reference genes under abiotic conditions

Temperature. All software programs identified *EF1A* as the most stable gene (Table 3). According to *RefFinder*, the overall order from the most stable to the least stable reference genes under the temperature stress was: *EF1A*, *RP49*, *18S*, *v-ATPase*, *CypA*, *RPS5*, *28S*, *NADH*, *GAPDH* (Fig 2D). *geNorm* analysis revealed that first V-value < 0.15 showed at V2/3 (Fig 3), suggesting that two reference genes were sufficient for accurate normalization across different temperatures.

Photoperiod. All software programs identified *EF1A* as the most or second to most stable gene (Table 3). According to *RefFinder*, the overall order from the most stable to the least stable reference genes under the photoperiod stress was: *EF1A*, *v-ATPase*, *18S*, *RP49*, *CypA*, *RPS5*, *28S*, *NADH*, *GAPDH* (Fig 2E). *geNorm* analysis revealed that first V-value < 0.15 showed at

Table 3. Stability of reference gene expression under abiotic conditions.

Abiotic conditions	Gene	<i>geNorm</i> Stability	Rank	<i>Normfinder</i> Stability	Rank	<i>BestKeeper</i> Stability	Rank	ΔCt Stability	Rank
Temperature	<i>EF1A</i>	0.18	1	0.05	1	0.15	1	0.31	1
	<i>NADH</i>	0.38	7	0.45	8	0.39	8	0.53	8
	<i>GAPDH</i>	0.42	8	0.49	9	0.45	9	0.55	9
	<i>RP49</i>	0.18	1	0.25	4	0.19	3	0.37	3
	<i>18S</i>	0.28	4	0.17	2	0.16	2	0.35	2
	<i>28S</i>	0.30	5	0.30	6	0.28	5	0.42	6
	<i>v-ATPase</i>	0.25	3	0.26	5	0.20	4	0.39	4
	<i>CypA</i>	0.33	6	0.24	3	0.29	6	0.40	5
	<i>RPS5</i>	0.21	2	0.37	7	0.29	7	0.44	7
	Photoperiod	<i>EF1A</i>	0.27	2	0.14	1	0.27	1	0.35
<i>NADH</i>		0.32	5	0.29	7	0.40	5	0.40	7
<i>GAPDH</i>		0.37	7	0.43	8	0.46	8	0.51	8
<i>RP49</i>		0.28	3	0.25	4	0.31	3	0.39	5
<i>18S</i>		0.17	1	0.27	6	0.46	8	0.39	4
<i>28S</i>		0.42	8	0.55	9	0.29	2	0.60	9
<i>v-ATPase</i>		0.17	1	0.20	2	0.46	7	0.35	2
<i>CypA</i>		0.33	6	0.23	3	0.41	6	0.39	3
<i>RPS5</i>		0.30	4	0.25	5	0.38	4	0.40	6
dietary RNAi		<i>EF1A</i>	0.37	1	0.47	2	0.54	4	0.79
	<i>NADH</i>	0.81	6	0.65	6	0.62	6	0.91	6
	<i>GAPDH</i>	0.37	1	0.46	1	0.51	3	0.79	1
	<i>RP49</i>	0.53	2	0.55	4	0.72	9	0.84	3
	<i>18S</i>	0.77	5	0.66	7	0.31	1	0.92	7
	<i>28S</i>	0.72	4	0.55	3	0.31	2	0.85	4
	<i>v-ATPase</i>	0.91	8	0.90	9	0.69	7	1.08	9
	<i>CypA</i>	0.86	7	0.87	8	0.69	8	1.05	8
	<i>RPS5</i>	0.66	3	0.63	5	0.58	5	0.90	5

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V2/3 (Fig 3), suggesting that two reference genes were adequate for accurate normalization under the photoperiod condition.

dietary RNAi. All software programs, except *BestKeeper*, identified *GAPDH* as the most stable gene (Table 3). According to *RefFinder*, the overall order from the most stable to the least stable reference genes under the dietary RNAi treatment was: *GAPDH*, *EF1A*, *28S*, *18S*, *RP49*, *RPS5*, *NADH*, *CypA*, *v-ATPase* (Fig 2F). *geNorm* analysis revealed that the pair-wise variation value V4/5 was below the proposed 0.15 cut-off (Fig 3). This result suggests that four reference genes were required for accurate normalization under the dietary RNAi condition.

According to *RefFinder*, the overall order from the most stable to the least stable reference genes under biotic conditions was: *EF1A*, *28S*, *RPS5*, *RP49*, *18S*, *v-ATPase*, *NADH*, *CypA*, *GAPDH* (Fig 2G); and the overall order from the most stable to the least stable reference genes under abiotic conditions was: *RP49*, *18S*, *CypA*, *GAPDH*, *v-ATPase*, *28S*, *EF1A*, *NADH*, *RPS5* (Fig 2H).

Validation of selected reference genes

Using two best reference gene combinations for normalization, two genes [*EF1A* and *v-ATPase*; NF (1–2)] or three genes [*EF1A*, *v-ATPase*, and *18S*; NF (1–3)], similar expression

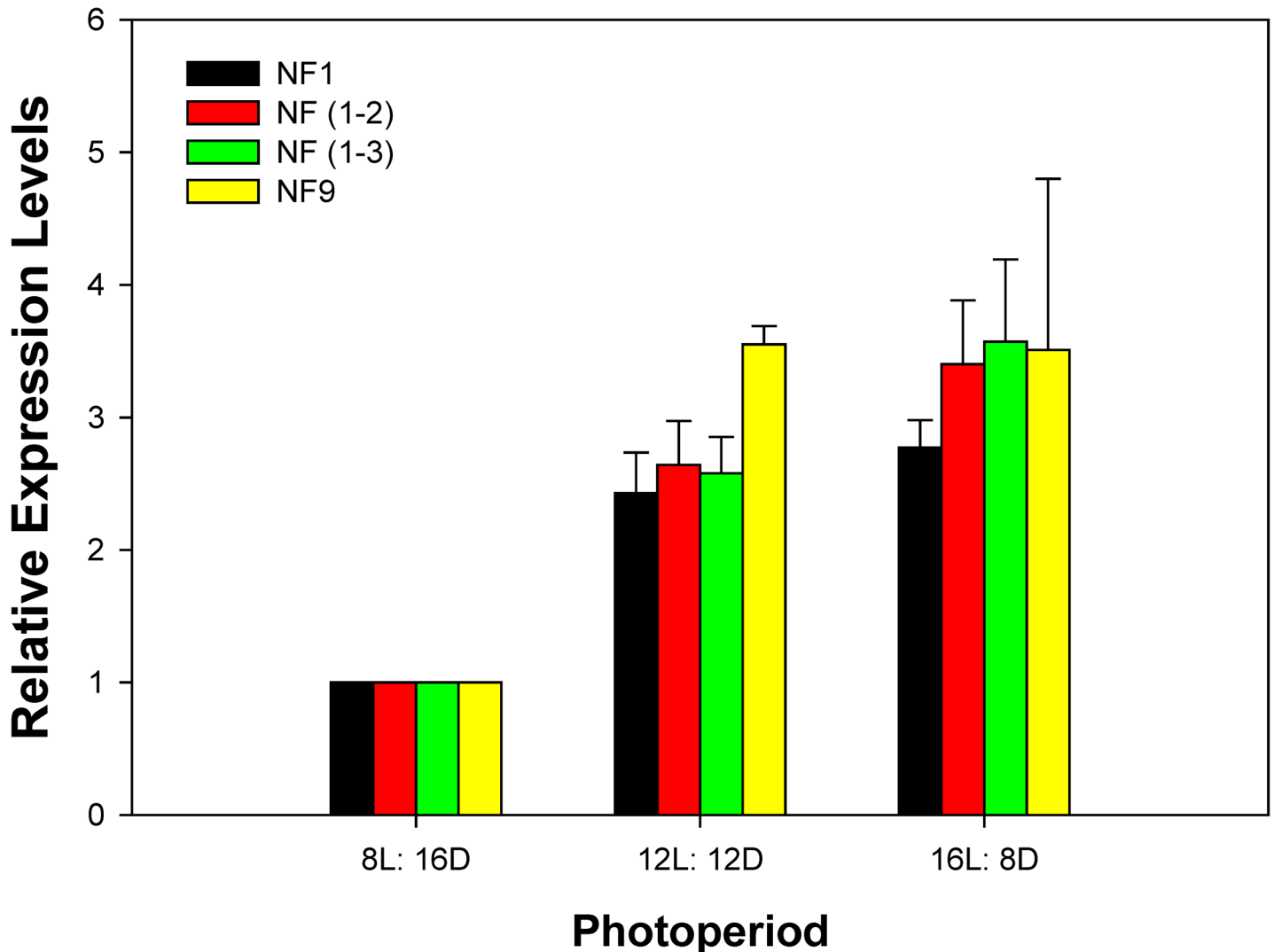


Fig 4. Validation of recommended reference genes. Expression profiles of *tim* gene under three photoperiod conditions were investigated using different combinations of reference genes. NF1, NF (1–2), NF (1–3), and NF9 indicate that the expression of *tim* was normalized using the best, the top two, the top three, or, the worst reference genes, respectively. Bar represents the mean and standard error of three biological replicates.

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levels of *tim* occurred within each photoperiod (Fig 4). Under the 12L: 12D photoperiod condition, the expression level of *tim* was the highest when the reference gene with the highest *Geo-mean* value (*GAPDH*; NF9) was used (Fig 4).

Discussion

The monarch butterfly is commonly used as a non-target insect for the ecological risk assessment of transgenic crops in the U.S.; plus interest in the migratory biology and genetics of this insect suggest future post-genome studies will occur. In this study, expression profiles of nine candidate reference genes from *D. plexippus* were evaluated under diverse experimental conditions. This research is the first step toward establishing a standardized RT-qPCR analysis for this insect.

Our results demonstrate that the best-suited reference genes can vary depending on biotic and abiotic factors (Tables 2 and 3). Thus, reference genes need to be selected based on the

experimental conditions. Unfortunately, a universal reference gene that covers all experimental conditions is unrealistic. For example, *EF1A*, which plays an important role in translation by catalyzing the GTP-dependent binding of aminoacyl-tRNA to the acceptor site of the ribosome, is the most stable reference gene for biotic factors (Fig 2G). This result is consistent with previous studies in other insects [34, 35]. However, *EF1A* ranked poorly (7th) as a reference gene for abiotic factors (Fig 2H).

To avoid biased normalization, many researchers have started to advocate the use of multiple reference genes to analyze gene expression [4, 5, 9, 11, 12, 29, 30, 36]. Our results demonstrate that two reference genes are required for reliable normalization under photoperiod, temperature, and sex conditions; three reference genes are required for reliable normalization in different tissue types and under different developmental stages; four reference genes are sufficient to normalize the expression and provide a more conservative estimation of target gene expression under dietary RNAi condition (Fig 3). As a result, we suggest that using different combinations of reference genes are necessary for studying gene expression in *D. plexippus*, especially with variable experimental conditions.

This work represents an important first step toward establishing a standardized gene analysis framework for *D. plexippus*. Analysis of our data indicate that *EF1A* and *RP49* are the most stable reference genes, respectively, under biotic (development, tissue, and sex) and abiotic (photoperiod, temperature, and dietary RNAi) conditions. With the recent release of *D. plexippus* genome, results from this study provide a critical piece for the subsequent genomics and functional genomics research in this emerging insect model, and shed light on the ecological risk assessment of RNAi-based biopesticides on this non-target bio-indicator agent.

Supporting Information

S1 Fig. The agrose gel profile of the nine candidate reference genes. M, EZ Load 100 bp Molecular Ruler; Templates in the PCR reactions were as follows: 1) *EF1A*, 2) *NADH*, 3) *GAPDH*, 4) *CypA*, 5) *RPS5*, 6) *RP49*, 7) *18S*, 8) *28S*, and 9) *v-ATPase*. (TIFF)

S2 Fig. Melting curve of the nine candidate reference genes. (TIFF)

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Author Contributions

Conceived and designed the experiments: XGZ HPP XWY. Performed the experiments: HPP XWY. Analyzed the data: HPP XWY. Contributed reagents/materials/analysis tools: KB XGZ. Wrote the paper: HPP XGZ RLH BDS.

References

1. Hudson ME (2008) Sequencing breakthroughs for genomic ecology and evolutionary biology. *Mol Ecol Resour* 8: 3–17. doi: [10.1111/j.1471-8286.2007.02019.x](https://doi.org/10.1111/j.1471-8286.2007.02019.x) PMID: [21585713](https://pubmed.ncbi.nlm.nih.gov/21585713/)

2. Xie W, Meng QS, Wu QJ, Wang SL, Yang X, Li RM, et al. (2012) Pyrosequencing the *Bemisia tabaci* transcriptome reveals a highly diverse bacterial community and a robust system for insecticide resistance. PLOS ONE 7: e35181. doi: [10.1371/journal.pone.0035181](https://doi.org/10.1371/journal.pone.0035181) PMID: [22558125](https://pubmed.ncbi.nlm.nih.gov/22558125/)
3. Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931. PMID: [17073008](https://pubmed.ncbi.nlm.nih.gov/17073008/)
4. Li RM, Xie W, Wang SL, Wu QJ, Yang NN, Yang X, et al. (2013) Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). PLOS ONE 8: e53006. doi: [10.1371/journal.pone.0053006](https://doi.org/10.1371/journal.pone.0053006) PMID: [23308130](https://pubmed.ncbi.nlm.nih.gov/23308130/)
5. Lourenço AP, Mackert A, dos Santos Cristino A, Simões ZLP (2008) Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. Apidologie 39: 372–385.
6. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: research0034.
7. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR—a perspective. J Mol Endocrinol 34: 597–601. PMID: [15956331](https://pubmed.ncbi.nlm.nih.gov/15956331/)
8. Strube C, Buschbaum S, Wolken S, Schnieder T (2008) Evaluation of reference genes for quantitative real-time PCR to investigate protein disulfide isomerase transcription pattern in the bovine lungworm *Dictyocaulus viviparus*. Gene 425: 36–43. doi: [10.1016/j.gene.2008.08.001](https://doi.org/10.1016/j.gene.2008.08.001) PMID: [18761062](https://pubmed.ncbi.nlm.nih.gov/18761062/)
9. Guo JL, Ling H, Wu QB, Xu LP, Que YX (2014). The choice of reference genes for assessing gene expression in sugarcane under salinity and drought stresses. Sci Rep 4: 7042. doi: [10.1038/srep07042](https://doi.org/10.1038/srep07042) PMID: [25391499](https://pubmed.ncbi.nlm.nih.gov/25391499/)
10. Andersen CL, Jensen JL, Ørntoft TF (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. PMID: [15289330](https://pubmed.ncbi.nlm.nih.gov/15289330/)
11. Zhu X, Yuan M, Shakeel M, Zhang YJ, Wang SL, Wang X, et al. (2014) Selection and evaluation of reference genes for expression analysis using qRT-PCR in the beet armyworm *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). PLOS ONE 9: e84730. doi: [10.1371/journal.pone.0084730](https://doi.org/10.1371/journal.pone.0084730) PMID: [24454743](https://pubmed.ncbi.nlm.nih.gov/24454743/)
12. Jacob F, Guertler R, Naim S, Nixdorf S, Fedier A, Hacker NF, et al. (2013). Careful selection of reference genes is required for reliable performance of RT-qPCR in human normal and cancer cell lines. PLOS ONE 8: e59180. doi: [10.1371/journal.pone.0059180](https://doi.org/10.1371/journal.pone.0059180) PMID: [23554992](https://pubmed.ncbi.nlm.nih.gov/23554992/)
13. Huvenne H, Smaghe G (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 56: 227–235. doi: [10.1016/j.jinsphys.2009.10.004](https://doi.org/10.1016/j.jinsphys.2009.10.004) PMID: [19837076](https://pubmed.ncbi.nlm.nih.gov/19837076/)
14. Burand JP, Hunter WB (2013) RNAi: Future in insect management. J Invertebr Pathol 112: S68–S74. doi: [10.1016/j.jip.2012.07.012](https://doi.org/10.1016/j.jip.2012.07.012) PMID: [22841639](https://pubmed.ncbi.nlm.nih.gov/22841639/)
15. Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, et al. (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25: 1322–1326. PMID: [17982443](https://pubmed.ncbi.nlm.nih.gov/17982443/)
16. Mao YB, Cai WJ, Wang JW, Hong GJ, Tao XY, Wang LJ, et al. (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotechnol 25: 1307–1313. PMID: [17982444](https://pubmed.ncbi.nlm.nih.gov/17982444/)
17. Zha W, Peng X, Chen R, Du B, Zhu L, He GC (2011) Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. PLOS ONE 6:e20504 doi: [10.1371/journal.pone.0020504](https://doi.org/10.1371/journal.pone.0020504) PMID: [21655219](https://pubmed.ncbi.nlm.nih.gov/21655219/)
18. Solensky MJ (2004) The effect of behavior and ecology on male mating success in overwintering monarch butterflies (*Danaus plexippus*). J Insect Behav 17: 723–743.
19. Jesse LCH, Obrycki JJ (2000) Field deposition of *Bt* transgenic corn pollen: lethal effects on the monarch butterfly. Oecologia 125: 241–248. doi: [10.1007/s004420000502](https://doi.org/10.1007/s004420000502) PMID: [24595835](https://pubmed.ncbi.nlm.nih.gov/24595835/)
20. Sears MK, Hellmich RL, Stanley-Horn DE, Oberhauser KS, Pleasants JM, Mattila HR, et al. (2001) Impact of *Bt* corn pollen on monarch butterfly populations: a risk assessment. Proc Natl Acad Sci U S A 98: 11937–11942. PMID: [11559842](https://pubmed.ncbi.nlm.nih.gov/11559842/)
21. Wraight CL, Zangerl AR, Carroll MJ, Berenbaum MR (2000) Absence of toxicity of *Bacillus thuringiensis* pollen to black swallowtails under field conditions. Proc Natl Acad Sci U S A 97: 7700–7703. PMID: [10840067](https://pubmed.ncbi.nlm.nih.gov/10840067/)
22. Zangerl AR, McKenna D, Wraight CL, Carroll M, Ficarello P, Warner R, et al. (2001) Effects of exposure to event 176 *Bacillus thuringiensis* corn pollen on monarch and black swallowtail caterpillars under field conditions. Proc Natl Acad Sci U S A 98: 11908–11912. PMID: [11559837](https://pubmed.ncbi.nlm.nih.gov/11559837/)
23. Dennis RL (1993) Butterflies and climate change. (Manchester University Press).

24. Woods JN, Wilson J, Runkle JR (2008) Influence of climate on butterfly community and population dynamics in western Ohio. *Environ Entomol* 37: 696–706. PMID: [18559175](#)
25. Zhan S, Merlin C, Boore JL, Reppert SM (2011) The monarch butterfly genome yields insights into long-distance migration. *Cell* 147: 1171–1185. doi: [10.1016/j.cell.2011.09.052](#) PMID: [22118469](#)
26. Stensmyr MC, Hansson BS (2011) A Genome Befitting a Monarch. *Cell* 147: 970–972. doi: [10.1016/j.cell.2011.11.009](#) PMID: [22118454](#)
27. Zhan S, Zhang W, Niitepöld K, Hsu J, Haeger JF, Zalucki MP, et al. (2014) The genetics of monarch butterfly migration and warning colouration. *Nature* 514: 317–321. doi: [10.1038/nature13812](#) PMID: [25274300](#)
28. Zhu HS, Sauman I, Yuan Q, Casselman A, Emery-Le M, Emery P, et al. (2008) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. *PLoS Biol* 6: e4. doi: [10.1371/journal.pbio.1000004](#) PMID: [19222305](#)
29. Yang CX, Pan HP, Liu Y, Zhou XG (2014) Selection of reference genes for expression analysis using quantitative real-time PCR in the pea aphid, *Acyrtosiphon pisum* (Harris) (Hemiptera, Aphididae). *PLOS ONE* 9: e110454. doi: [10.1371/journal.pone.0110454](#) PMID: [25423476](#)
30. Yang CX, Pan HP, Liu Y, Zhou XG (2015) Stably expressed housekeeping genes across developmental stages in the two-spotted spider mite, *Tetranychus urticae*. *PLOS ONE* 10: e0120833. doi: [10.1371/journal.pone.0120833](#) PMID: [25822495](#)
31. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pairwise correlations. *Biotechnol Lett* 26: 509–515. PMID: [15127793](#)
32. Silver N, Best S, Jiang J, Thein SL (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* 7: 33. PMID: [17026756](#)
33. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Methods* 25: 402–408. PMID: [11846609](#)
34. Hor áková D, Matoušková P, Kindl J, Valterová I, Pichová I (2010) Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. *Anal Biochem* 397: 118–120. doi: [10.1016/j.ab.2009.09.019](#) PMID: [19751695](#)
35. Ponton F, Chapuis MP, Pernice M, Sword GA, Simpson SJ (2011) Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *J Insect Physiol* 57: 840–850. doi: [10.1016/j.jinsphys.2011.03.014](#) PMID: [21435341](#)
36. Veazey KJ, Golding MC (2011) Golding selection of stable reference genes for quantitative RT-PCR comparisons of mouse embryonic and extra-embryonic stem cells. *PLOS ONE* 6: 27592.