UNIVERSIDAD REGIONAL AMAZÓNICA IKIAM

FACULTAD DE CIENCIAS DE LA VIDA

CARRERA DE INGENIERÍA EN BIOTECNOLOGÍA

VALIDACIÓN DE GENES DE REFERENCIA PARA ANÁLISIS DE EXPRESIÓN GÉNICA EN *HELICONIUS ERATO LATIVITTA* (LEPIDÓPTERA: NYMPHALIDAE)

Proyecto de investigación previo a la obtención de título de:

INGENIERA EN BIOTECNOLOGÍA

AUTOR: PAOLA FERNANDA CALDERÓN OÑATE

TUTOR: PHD. CAROLINE NICOLE ORIANA BACQUET PÉREZ

Napo – Ecuador

2024

DE DERECHO DE AUTOR, AUTENTICIDAD Y RESPONSABILIDAD

De mi consideración:

Yo, Paola Fernanda Calderón Oñate con documento de identidad 0107559346, declaro que los resultados obtenidos en la investigación que presento en este documento final, previo a la obtención del título de Ingeniería en Biotecnología, son absolutamente inéditos, originales, auténticos y personales.

En virtud de lo cual, el contenido, criterios, opiniones, resultados, análisis, interpretaciones, conclusiones, recomendaciones y todos los demás aspectos vertidos en el presente documento son de mi autoría y de mi absoluta responsabilidad.

Tena, 15 de Febrero de 2024

Paola Fernanda Calderón Oñate
0107559346

AUTORIZACION DE PUBLICACION EN EL REPOSITORIO INSTITUCIONAL

Yo, Paola Fernanda Calderón Oñate con documento de identidad N°0107559346, en calidad de autor/a y titular de los derechos morales y patrimoniales del trabajo de titulación: “Validación de genes de referencia para análisis de expresión génica en *Heliconius erato lativitta* (Lepidoptera:Nymphalidae)” de conformidad con el Art. 114 del CÓDIGO ORGÁNICO DE LA ECONOMÍA SOCIAL DE LOS CONOCIMIENTOS, CREATIVIDAD E INNOVACIÓN, reconozco a favor de la Universidad Regional Amazónica Ikiam una licencia gratuita, intransferible y no exclusiva para el uso no comercial de la obra, con fines estrictamente académicos.

Así mismo autorizo a la Universidad Regional Amazónica Ikiam para que realice la publicación de este trabajo de titulación en el Repositorio Institucional de conformidad a lo dispuesto en el Art. 144 de la Ley Orgánica de Educación Superior.

Tena, 15 de Febrero de 2024

Paola Fernanda Calderón Oñate
0107559346

CERTIFICADO DE DIRECCIÓN DE TRABAJO DE TITULACIÓN

Certifico que el trabajo de titulación: “Validación de genes de referencia para análisis de expresión génica en *Heliconius erato lativitta* (Lepidoptera:Nymphalidae))” en la modalidad de: artículo, fue realizado por Paola Fernanda Calderón Oñate, bajo mi dirección.

El mismo ha sido revisado en su totalidad y analizado por la herramienta de verificación de similitud de contenido; por lo tanto, cumple con los requisitos teóricos, científicos, técnicos, metodológicos y legales establecidos por la Universidad Regional Amazónica Ikiam, para su entrega y defensa.

Tena, 15 de Febrero de 2024

Caroline Bacquet

Caroline Nicole Oriana Bacquet Pérez
C.I: 1756516041

AGRADECIMIENTOS

Deseo expresar mi agradecimiento a la Universidad Regional Ikiam por brindarme la oportunidad de formar parte de su programa de estudios. Esta institución me permitió establecer una conexión significativa con la naturaleza, así como intercambiar conocimientos y experiencias enriquecedoras con las comunidades indígenas locales, estudiantes provenientes de diversas regiones del país y el distinguido personal docente. Aprecio enormemente el conocimiento adquirido durante mi tiempo aquí, lo cual me ha motivado aún más para continuar mi trayectoria en la investigación.

Agradezco a la Dra. Caroline Bacquet por permitirme formar parte de su grupo de investigación y por gestionar los recursos necesarios para llevar a cabo este trabajo de investigación. A su vez, agradezco su continuo apoyo y motivación durante los momentos desafiantes, así como su mentoría, enseñanzas y en especial por su amistad.

Quiero agradecer al grupo de investigación de mariposas de la Universidad Ikiam por su apoyo y orientación durante el desarrollo de este proyecto de investigación. También quiero agradecer a mis amigos y colegas de laboratorio, destacando especialmente a Joshué, con quien compartí y validé información relevante de manera constante. Además, agradezco el valioso apoyo brindado por Ángel, quien me guió de manera efectiva en la realización de este trabajo.

Asimismo, quiero expresar mi sincero agradecimiento a mis amigos Majito, Jess, Majo, Leo y Alex, quienes han sido compañeros invaluables a lo largo de este recorrido universitario. De igual manera, quiero extender mi agradecimiento a Michael. Gracias por tu constante guía y apoyo. En conjunto, todos ustedes se han convertido en mi segunda familia.
Finalmente, agradezco a mis padres y hermanos, por su incansable esfuerzo, sabios consejos, constante apoyo y por motivarme siempre a ser la mejor versión de mí misma. Su apoyo ha sido invaluable y ha sido el pilar de mi crecimiento y desarrollo.

DEDICATORIA

A mis padres y hermanos,
por su apoyo y amor incondicional

TABLA DE CONTENIDO

INDICE DE TABLAS

INDICE DE FIGURAS

INDICE DE ANEXOS

RESUMEN

La mariposa neotropical *Heliconius erato lativitta* sirve como organismo modelo para el estudio de adaptaciones evolutivas, especiación y bases genéticas. Esta especie es reconocida principalmente por el mimetismo del patrón de color de las alas. Sin embargo, los mecanismos moleculares implicados en la adaptación a los cambios de temperatura siguen sin identificarse. En este sentido, la PCR cuantitativa en tiempo real (qPCR) ha resultado una de las herramientas más útiles para dilucidar el mecanismo molecular mediante la cuantificación del ARNm de genes implicados en la adaptación de estas especies. Sin embargo, la precisión y confiabilidad de los datos de qPCR dependen de la selección adecuada de genes de referencia, cuyos perfiles de expresión deben permanecer estables en diferentes condiciones experimentales. Por tanto, en este trabajo se seleccionaron seis genes (*ACT1, ACT2, ANX, AK, eEf1α* y *eEf1α2)* de *H. e. lativitta* que se utilizaron como genes candidatos de referencia y se evaluó su nivel de expresión a diferentes temperaturas en tres tipos de tejidos tanto para machos como para hembras. La estabilidad de los genes de referencia se evaluó utilizando tres algoritmos geNorm, NormFinder y RefFinder. Los resultados obtenidos con estos algoritmos indican que *AK* y *ACT2* son estables bajo estrés térmico. Sin embargo, a nivel de tejido, sólo *ACT1* y *AK* fueron los genes más estables, mientras que, a nivel de sexo, sólo *ACT1* y *ACT2* exhibieron una alta estabilidad. Por lo tanto, este estudio proporciona un conjunto estandarizado de genes de referencia para futuros análisis de expresión génica utilizando qPCR en *H. e. lativita.*

Palabras claves*:* *Heliconius, gen de referencia,* qPCR, expresión génica

ABSTRACT

The Neotropical butterfly *Heliconius erato lativitta* has been used as a model organism for the study of evolutionary adaptations, speciation, and genetic bases. This species is mainly recognized for its wing color pattern mimicry. However, the molecular mechanisms involved in adaptation to temperature changes remain unidentified. In this sense, quantitative real-time PCR (qPCR) has resulted one of the most useful tools to elucidate the molecular mechanism through the quantification of the mRNA of genes involved in the adaptation of this species. Nevertheless, the accuracy and reliability of qPCR data depends on the proper selection of reference genes, whose expression profiles must remain stable across different experimental conditions. Therefore, in this work six genes (*ACT1*, *ACT2*, *ANX*, *AK*, *eEf1α*, and *eEf1α2*) from *H. e. lativitta* were used as candidate reference genes and their expression level was assessed at different temperatures in three type of tissues both for males and females. The stability of reference genes was evaluated using three algorithms geNorm, NormFinder and RefFinder. The results obtained with these algorithms indicate that *AK* and *ACT2* are stables under temperature stress. However, at the tissue level, only *ACT1* and *AK* were the most stable genes, while at the sex level, only *ACT1* and *ACT2* exhibited a high stability. Hence, this study provides a standardized set of reference genes for future gene expression analysis using qPCR in *H. e. lativitta*

Keywords***:*** *Heliconius, reference gene,* qPCR, gene expression

1. INTRODUCTION

Climate change represents a significant threat to tropical and subtropical regions, mainly due to rising temperatures (Alves de Oliveira et al. 2021). The temperature change disrupts the adaptation and plasticity of tropical species, such as butterflies, which are unable to regulate their corporal temperature (Sheldon 2019). Extreme temperatures may affect the behavior, morphology, physiology, and genetic of butterflies, resulting in high mortality rates (Montejo-Kovacevich et al. 2019; Montejo-Kovacevich et al. 2020; Ashe-Jepson et al. 2023). However, along the years, butterflies have developed strategies to cope with temperature fluctuation, including the production of heat shock proteins (Harvey et al. 2020). Nonetheless, the molecular mechanisms underlying thermal stress adaptation in neotropical butterflies, particularly in the genus *Heliconius* remains largely unexplored.

The *Heliconius* genus includes 48 species and over 400 subspecies, such as the species *Heliconius erato lativitta* (*H. e. lativitta*), native of the Ecuadorian Amazon forest (Merrill et al. 2015; Montejo-Kovacevich et al. 2021). *H. e. lativitta* exhibits similar wing patterns than *Heliconius melpomene maletti*, despite they diverged approximately 8 million years ago, demonstrating a case of Müllerian mimicry (Ferguson and Jiggins 2009). On other hand, these butterflies maintain a close relationship with their *Passiflora* hostplant, from where they get cyanogenic compounds, making them unpleasant to their predators (Ehlers et al. 2021). Moreover, these butterflies can also scare away their predators due to their wings exhibit vibrant colors, which are a signal of their toxicity (Kronforst and Papa 2015). Owing to these facts, *H. e. lativitta* has been used as a model organism for the study of evolutionary adaptations, speciation process, and genetic bases through transcriptomic analysis and gene expression quantification (Montejo-Kovacevich et al. 2019; Montejo-Kovacevich et al. 2020; Montejo-Kovacevich et al. 2021).

Gene expression analysis investigates the function and genetic mechanism related to a particular phenotype or environmental response (Sellamuthu et al. 2022). This process involves the detection and quantification of the mRNA from target genes, employing molecular techniques, such as northern blot, microarray, and quantitative polymerase chain reaction (qPCR) (Romero et al. 2012). Among them, qPCR is widely favored in modern biology and biomedical sciences due to its high precision, efficiency, repeatability, speed, throughput, and sensitivity for the detection of low concentration of mRNA (Omondi et al. 2015; Khan et al. 2022). Nevertheless, the reliability and accuracy of qPCR depends on the quality of RNA extraction, cDNA synthesis, and primer efficiency and design (Bustin et al. 2009a). Therefore, it is crucial to address this issue to ensure precise gene expression analysis of *H. e. lativitta* through effective qPCR normalization, which includes the utilization of reliable reference genes.

Reference genes are characterized by their consistent expression levels across various physiological and experimental conditions (Khan et al. 2022). Common reference genes like *α-tubulin*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *actin* (*ACT*) or *elongation factor 1 alpha* (*eEf1α*) have been widely used as internal controls for qPCR normalization (Kozera and Rapacz 2013; Lü et al. 2018). For example, in several insect studies, *actin 1*(*ACT1*), *actin 2* (*ACT2*), *annexin IX-B* (*ANX*), *arginine kinase* (*AK*), *elongation factor 1 alpha* (*eEf1α*), and *elongation factor 1 alpha 2* (*eEf1α2*) genes were used as reference genes due to their high stability under different experimental treatments (Teng et al. 2012a; Arun et al. 2015; Piron Prunier et al. 2016; Shen et al. 2022; Tang et al. 2022). However, it has been reported that the expression level of these genes varies depending on cellular processes of each species in which they are participating (Panina et al. 2018). In this sense, although these genes have been identified as suitable for qPCR normalization, to date, their validity in *H. e. lativitta* remains unverified. Therefore, the selection and validation of appropriate reference genes from *H. e. lativita* is crucial to ensure the accuracy of the standardization of qPCR data and gene expression analysis in this species (Shen et al. 2022). In this study, the expression stability of various genes from different tissues of both sex of *H. e lativitta* were assessed both at normal conditions (25°C) and under thermal stress (4 and 40 ºC). Furthermore, the reference genes were validated using the target gene *Hsp90*. Thus, this research contributes valuable insights for future gene expression studies in *H. e. lativitta*.

2. MATERIALS AND METHODS

2.1 Insect rearing

This research was carried out under the authorization of the Ministerio del Ambiente, Agua y Transición Ecológica MAATE-DBI-CM-2021-0176. The Insect collection and rearing of *H. e. lativitta* butterflies were conducted in the surroundings of the Colonso Chalupas Biological Reserve located in the Ecuadorian Amazon Region. The collected wild specimens were housed in insectary cages (1 x 2.5 x 2 m) of the Universidad Regional Amazónica Ikiam in Tena, Ecuador, which were equipped with the host plant *Passiflora puntacta*. The butterflies were fed with *Lantana camara* and *Stachytarpheta jamaicensis* flowers and a solution of 15% sugar and 1% pollen (Ojeda 2023). Eggs laid in the host plant *Passiflora punctata* were collected and monitored every other day. Upon hatching, the larvae were individually reared in pots and fed with fresh leaves of the host plant until they reached pupation around 28 days. The newly emerged adult butterflies were maintained for a period of two weeks and each was identified with a unique code marked on their wings. All cages and pots were maintained at 25°C ±2, relative humidity 80% ±5, and 12:12 light: dark cycle.

2.2 Sample collection

A total of 18 butterflies, 9 females and 9 males, were used to investigate the effect of thermal stress on the expression level of candidate reference genes. These individuals were distributed in 3 groups, each consisting of 3 females and 3 males. The first one was placed inside a plastic container and exposed to 40 °C for 1 hour using a WB10 water bath (PoliScience®) to induce heat shock. In the same way, a second group was exposed to 4°C using an AlaskaH200B2 freezer (MABE) for 1 hour to induce cold shock (Luo et al. 2015; Montejo-Kovacevich et al. 2020). The third one was used as control group and was exposed to 25 ºC for 1 h using a WB10 water bath (PoliScience®). The temperature was monitored using a thermometer. After the treatment, the butterflies were sacrificed and dissected into head, thorax, and abdomen, and preserved in RNA Later™ (Invitrogen®) -80 °C until use.

2.3 Candidate reference genes and primer design

The candidate reference genes *ACT1*, *ACT2*, *ANX*, *AK*, *eEf1α*, *eEf1α2* were selected based on previous studies performed with *Bombyx mori* and *Heliconius numata* (Teng et al. 2012b; Piron Prunier et al. 2016). The preliminary sequences for each gene were obtained from *GenBank* database (Benson et al. 2017). After, the Basic Local Alignment Search Tool (BLAST) was employed to blast the obtained sequences against homologous sequences from version 3 of *H. e. lativitta genome* available in *The Lepidopteran Genome Database* (LepBase) (Challis et al. 2016 Jun 6; Lewis et al. 2016).Then, three primer pairs were designed for each gene using the selected sequences and the software Genius Prime (Dotmatics®) with the following parameters: amplicon length between 80 - 130 pb, melting temperature between 58 - 62 °C, and CG content of 40 – 60 %. Finally, Primer Blast was employed to find likely amplifications of unwanted genomic DNA (Ye et al. 2012). Additionally, the absence of additional bands of PCR-amplified products was verified on 2% agarose gel. Details of the reference genes are shown in **Table 1**.

2.4 Total RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from each sample by using the SV Total RNA Isolation System (Promega®) in accordance with the manufacturer's protocol. Concentration and purity of total RNA were analyzed using a NanoDrop One Spectrophotometer (Thermofisher™), and 4.5 µl of RNA samples with a concentration of 60 ng/µl and an A260/280 ratio between 1.8 and 2.2 were used for reverse transcription. Then, the first strand of cDNA was synthesized using retrotranscription GoScript® (Promega™) in accordance with the manufacturer's protocol. The process was carried out in a final volume of 20 µl and the resulting products were stored at -20 °C until use.

2.5 Quantitative real time PCR

Quantitative real-time PCR reactions were performed on the CFX-96 Touch System (Bio-Rad™). qPCR reactions were performed using BlastTaq 2X qPCR MasterMix (abm®) with a final volume of 10 µl and two technical replicates. The master mix contained 5 µl BlastTaq 2X qPCR MasterMix, 0.5 µl of each primer [10 µM], 1 µl of BSA [5 µg/µL], 1 µl of ultrapure water, and 2 µl diluted cDNA [10 ng/µL]. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles at 95°C for 30 s, annealing Tm for each gene (See **Table 1**) for 15 seg, and at 72°C for 1 min. Then, the specificity of amplification was verified by a melting curve with a temperature gradient from 65 to 95 °C with an increase of 0.5 °C/30 s. Finally, the amplification efficiency of the primers was obtained from the standard curve using a fivefold dilution series of gDNA (1:1, 1:3, 1:9, 1:27, and 1:81) with an initial concentration of 30 ng/µl and three technical replicates. The amplification efficiency (*E*) of each gene was calculated using the following equation (Radonić et al. 2004):

$$E = \left(10^{\left(-\frac{1}{m}\right)} - 1\right) * 100$$

where *m* is the slope value from the standard curve.

2.6 Expression stability of candidate reference genes

The expression levels of the candidate reference genes were evaluated using the threshold cycle (Ct value), and the variations were visualized using the geom_boxplot from the package ggplot2 3.4.3 in Rstudio 4.3.2 (Wickham 2016). It is important to mention, tissue and sex samples without any treatment and at 25°C were used for gene stability analyses in normal conditions. The expression stability of the candidate genes was then assessed using geNorm and NormFinder from the package NormqPCR 1.46.0 in Rstudio (Perkins et al. 2012). The geNorm algorithm determines the expression stability value (M) for each candidate gene, where genes with M values lower than 1.5 are considered the most stable. Moreover, geNorm also calculates the optimal number of reference genes for accurate qPCR normalization by pairwise variation (V). The Vn/ n+ 1 value is calculated between two consecutive normalization factors, where *n* is the number of genes used in the normalization. Thus, the inclusion of another stable gene may increase or decrease the Vn/ n+ 1 value, were values < 0.15 indicate that the addition of another gene is not necessary for normalization (Vandesompele et al. 2002).

On the other hand, the NormFinder model calculates the expression stability values as a function of the intra- intergroup variations of the candidate genes, where the genes with lower stability values were the most stable (Andersen et al. 2004). Then, the online RefFinder program was used to compare and rank the stability of candidate reference genes (Xie et al. 2023). This tool integrates four statistical algorithms (geNorm, NormFinder, BestKeeper, and comparative delta CT method) and assigns an appropriated value to an individual gene and calculates the geometric mean of these values for the overall final ranking. Subsequently, *Anova* function from the car package version 3.1-2 was utilized to assess the significance of the effects of sex and tissue on the number of copies of reference genes (Fox and Weisberg). Subsequently, Tukey's test was applied using the stats package version 4.3.2 to identify the specific pairs that show statistical differences in relation to the results previously obtained.

Finally, generalized linear models were employed to examine the relationship between temperature treatment and the absolute expression of candidate reference genes. These analyses were conducted in Rstudio after log-transformation to ensure data homogeneity. Model assumptions were validated using residual vs. fitted plots and normal Q-Q plots. The *Anova* function were using to estimate the significant effects of the temperature treatments.

2.7 Validation of the reference genes

Insects contain highly conserved heat shock proteins, such as *Hsp90* (Landais et al. 2001). These proteins participate in several biological processes such as the protection of the cell from damage caused by thermal stress (Landais et al. 2001). Then, the *Hsp90* gene was used as the target gen to demonstrate the usefulness of selected reference genes. The primer sequence of the target gene was: forward (5'- 3'): ATCAAAGCTCGACAGTGGCA and reverse (5'- 3'): AACCAAGTCGGCCTTTGTC. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and significant differences were analyzed using Rstudio after log transformation to obtain homogeneity in the data. Finally, generalized linear models and two-way ANOVA were used (Livak and Schmittgen 2001).

3. RESULTS

3.1 Evaluation of primer specificity and amplification efficiency

The candidate reference genes for *H. e. lativitta* were selected based on a study performed with other lepidopterans (Teng et al. 2012a; Piron Prunier et al. 2016). Three pairs of primers for each gene were evaluated to determine the optimal amplification efficiency (**Table 1**). The regression coefficient R^2 and amplification efficiency were determined by the slope of the standard curve of each gene. The efficiency values of the candidate reference genes ranged from 63.05% to 105.28%, being the minimum for *eEf1α2* and the maximum for *ACT2*. Therefore, *eEf1α2* was excluded from the study because its efficiency was lower than 80% (Piron Prunier et al. 2016). While the other candidate genes (*ACT1, ACT2, ANX, AK*, *eEf1α*) were considered robust and suitable for qPCR assays, since their efficiency was higher than 80% and their R^2 values were greater than 0.98 (**Table 1**) [15]. Moreover, gene specificity was confirmed by the presence of a single melt curve peak and a PCR amplification band observed on agarose gel electrophoresis (**Supplementary Figure1 and Supplementary Figure2**).

Table 1. Primer sequences, parameters, and efficiencies of amplification of reference genes used for qPCR normalization.

Gene	Lepbase ID	Primer Sequence	Length (bp)	Tm (°C)	Efficiency (%)	R^2
ACT1	HEL_011520-RA	F: CACACCGTGCCCATCTATGA R: GCGGTGGTGGTGAAAGAGTA	131	60	98.04	0.98
ACT2	HEL_008433-RA	F: CGAGCACGGTATCATCACCA R: TAGGGTTAAGTGGGGCCTCA	125	58	105.28	0.98
ANX	HEL_001985-RA	F: ACGTGGAATCGTACAACGCT R: TCATCAGAGCCACGATCACG	125	62	98.84	0.99
AK	HEL_012431-RA	F: GCCCCTGATGCTGAAGCTTA R: TTGCCGAGGGTCTCAACATC	131	60	92,58	0.99
eEf1α	HEL_002519-RA	F: CGTGAGCATGCCTTACTTGC R: AACATGTTGTCTCCGTGCCA	120	58	90.39	0.98
eEf1α 2	HEL_001340-RA	F: AGGACGTGTACAAGATCGGC R: TGCATCTCCACGGACTTCAC	133	58	63.05	0.99

3.2 Expression profiles of selected genes

The cycle of threshold (Ct) obtained for the candidate reference genes provides a general description of the variation in the genetic expression under different conditions. Ct values are crucial in the selection of reference genes, since the lower the Ct, the higher the expression level, and vice versa (Liu et al. 2023). As seen in **Figure 1**, *ANX* had the highest average Ct value (25.34), while *ACT2* had the lowest average Ct value (21.42). The other genes presented intermediate average Ct values (*ACT2* =22.20, *eEf1α* =22,60, and *AK* =22.93). Moreover, the highest dispersion of Ct values was identified for *ACT2* gene at 4.67 amplification cycles, followed by *AK* gene at 4.58 cycles, *ACT1* gene at 4.33 cycles, *ANX* gene at 3.96 cycles and eEf1α gene at 3.72 cycles (**Figure 1**).

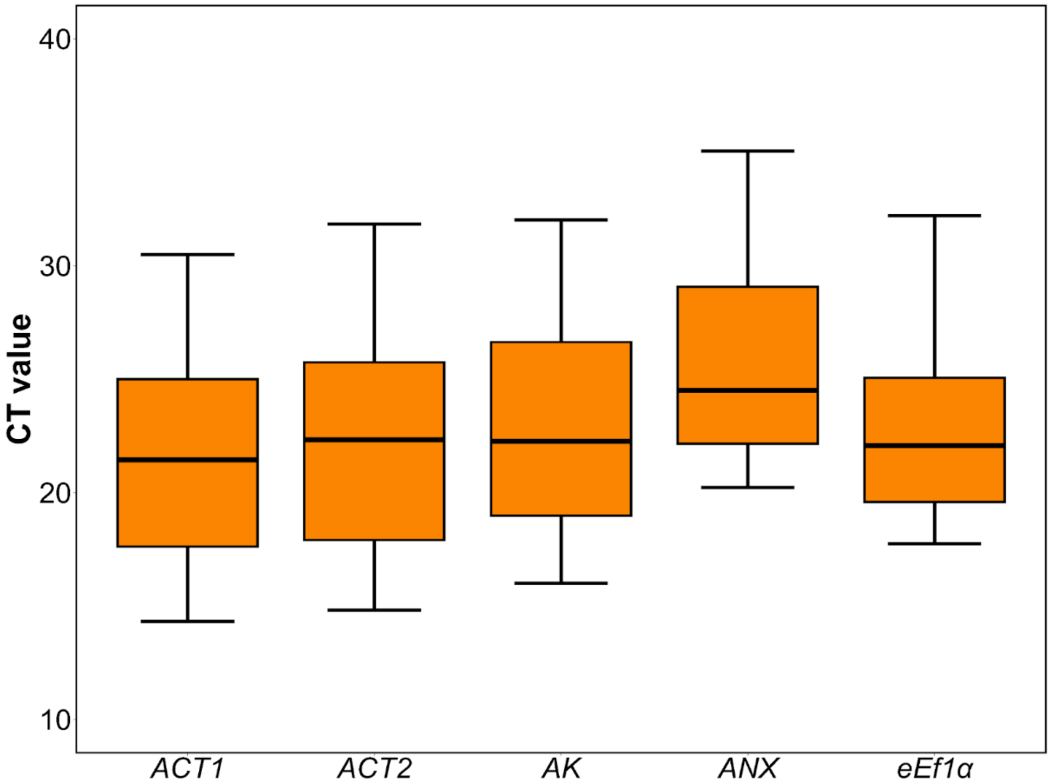


Figure 1. Expression *profiles of candidate genes reference under different conditions in* H. e. *lativitta*

On the other hand, absolute gene quantification shows the number of DNA copies of candidate reference genes (**Supplementary Figure 3**). *ACT* genes presented high variability in the thorax in both males and females. Also, *ANX* and *eEf1α* exhibited an increase in absolute expression in the head and thorax of both sexes. Finally, *AK* showed a high number of DNA copies in all treatments and tissues of both sexes (**Supplementary Figure 3**). Therefore, the variation in the CT values and the absolute quantification of the candidate genes indicates that a selection of suitable reference genes is required.

3.3 Expression stability of reference genes

3.3.1 Sex

The stability rankings generated by geNorm and NormFinder showed that *ACT2* and *ACT1* were the most stable genes and *ANX* was the least stable gene (**Table2**). This was according to the RefFinder results, in which the stability ranking of the genes was as follow: *ACT2* > *ACT1* > *AK* > ANX > *eEf1α* (**Figure2**). Furthermore, the *Anova test* revealed that there were not significant differences for the expression levels of *ACT1* and *ACT2* regarding sex ($p > 0.05$). Moreover, the pair-wise variation analysis revealed that V2/3 approached the 0.15 cut-off (**Figure 3**), suggesting that the most suitable gene combination for qPCR normalization is *ACT1* and *ACT2*.

Table 2. Ranking of the reference genes according to geNorm and NormFinder algorithms

Condition	Ranking	geNorm		NormFinder	
		Gene	Stability (M)	Gene	Stability (SV)
Sex	**1**	*ACT1/ACT2*	0.516	*ACT2*	0.167
	2	-	-	*ACT1*	0.500
	3	*AK*	0.682	*AK*	0.628
	4	*eEf1α*	0.742	*eEf1α*	0.695
	5	*ANX*	0.978	*ANX*	1.222
Tissue	**1**	*ACT2/AK*	0.826	*ACT1*	0.810
	2	-	-	*AK*	1.074
	3	*ACT1*	1.000	*ACT2*	1.097
	4	*eEf1α*	1.505	*eEf1α*	1.438
	5	*ANX*	1.915	*ANX*	2.284
Temperature	**1**	*ACT2/AK*	1.049	*AK*	0.937
	2	-	-	*ACT2*	1.375
	3	*ACT1*	1.567	*ACT1*	1.536
	4	*eEf1α*	1.896	*eEf1α*	1.569
	5	*ANX*	2.329	*ANX*	2.651

3.3.2 Tissue

The results obtained with the NormFinder and geNorm algorithms indicate that *eEf1α* and *ANX* were the least stable genes across different tissues in *H. e. lativitta*. As well as, NormFinder identified *ACT1* and *AK* as the most stable genes, while geNorm indicated that *ACT2* and *AK* were the most stable (**Table2**). These findings were agreed with the results of RefFinder that ranked *ACT1* and *AK* as the most stable genes. Additionally, the pairwise variation V2/3 determined by geNorm approached the 0.15 cut-off, suggesting the combination of *ACT1* and *AK* as reference genes to calculate the relative expression in different tissues (**Figure3**). However, *Anova* test revealed significant differences for the two candidate genes (*ACT1* F2/15=25, p= 1.67e-05*; AK* F2/15= 18.97, p= 7.81e-05) across the three tissues (thorax, abdomen, and head). Therefore, the Turkey test was performed to find out the tissue contributing to the significant difference. Thus, Tukey's results indicated that there are not significant differences in the interaction between head - abdomen (p > 0.05). While, the interaction between thorax – abdomen (*ACT1*, p = 1.8e-5; *AK*, p = 7.3e-5) and thorax – head (*ACT1*, p = 3.1e-4; *AK*, p = 1.6e-3) showed significant differences, indicating that the thorax is the significant factor.

Based on these results, suitable reference genes for thorax were identified. Thus, NormFinder and geNorm ranked *eEf1α* and *AK* as the most stable genes (**Supplementary Table 1**). These results are in agreement with the RefFinder outcomes, in which *eEf1α* and *AK* had the best stability in the thorax (**Supplementary Figure 4**). Hence, *ACT1* and *AK* is recommended for head and abdomen, while *eEf1α* and *AK* for thorax, and *AK* for the three tissues.

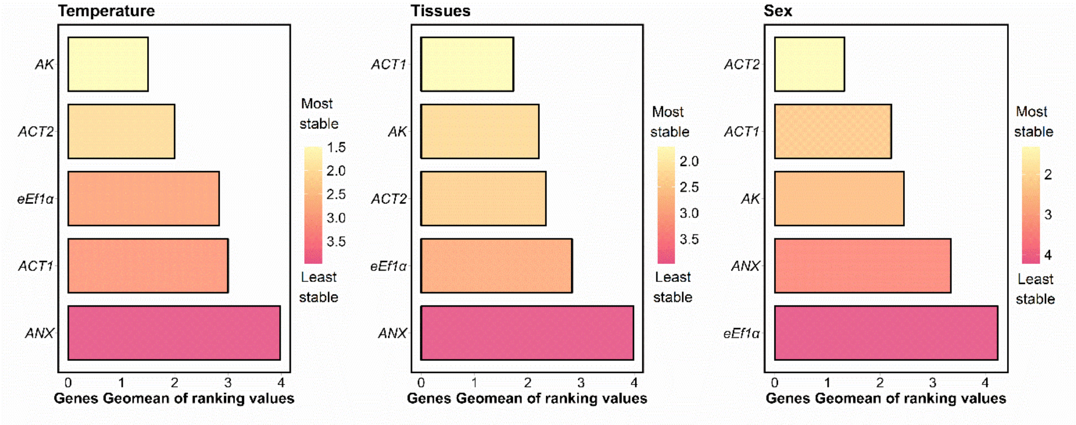


Figure 2. Expression stability of the reference genes under different conditions by RefFinder.

3.3.1 Temperature

The geNorm and NormFinder statistical algorithms provided similar results for the most stable genes. As is detailed in **Table 2**, *ACT2* and *AK* had the best stability under thermal stress conditions. These results were according to the RefFinder outcomes, in which *ACT2* and *AK* were the most stable genes, while *ANX* and *ACT1* were identified as the least stable genes (**Figure2**). On the other hand, the pairwise analysis performed with geNorm, revealed that the V2/3 value approached the proposed 0.15 cut-off, suggesting the use of *ACT2* and *AK* as reference genes for the evaluation of thermal response of butterflies under different temperature treatments **(Figure3**). Furthermore, it was confirmed with *Anova* test, that the recommended genes did not exhibit significant differences in response to temperature ($p > 0.05$).

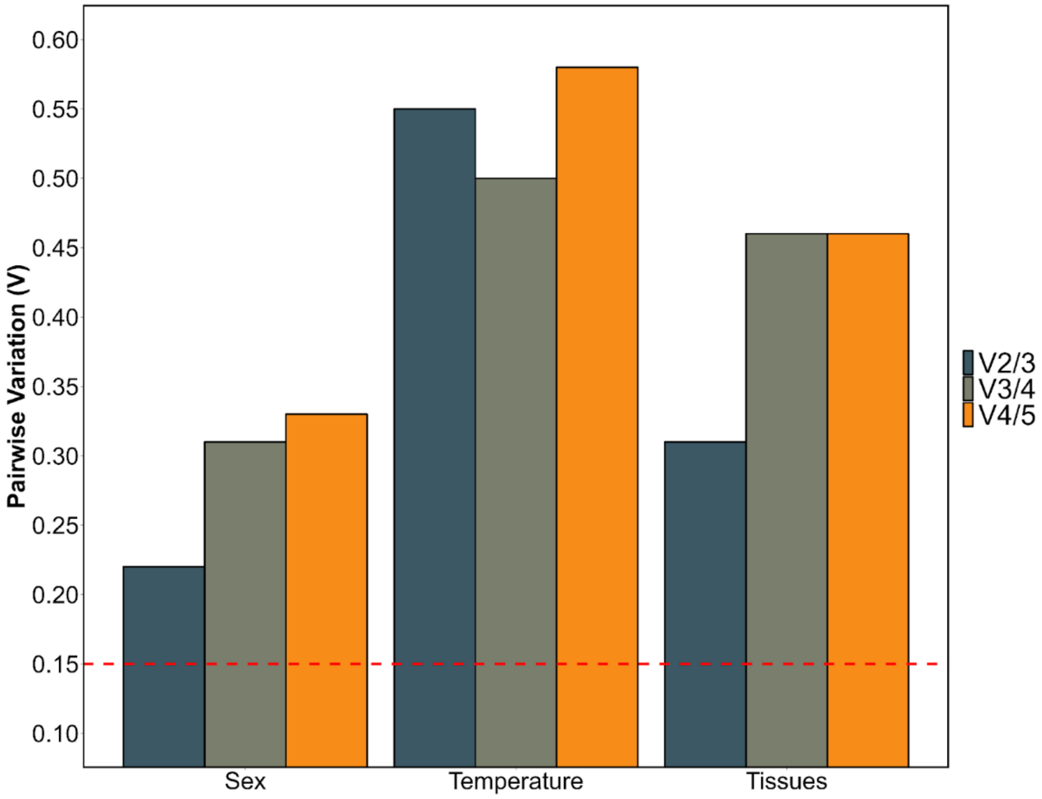


Figure 3. Determination of optimal number of reference genes under different conditions by geNorm.

3.4 Validation of reference genes

The relative expression of the target gene *Hsp90* under thermal stress was normalized to verify the stability of the selected reference genes. Two of the most stable genes (*AK* and *ACT2*) and two of the least stable genes (*ANX* and *ACT1*) of *H. e. lativitta* under thermal stress were selected and evaluated at different temperatures, as shown in **Figure 4**. The relative gene expression of *Hsp90* with the most stable combination in the heat treatment was 6.5 times higher in thorax (T) than in abdomen (A) and head (H) in both sexes (**Figure 4A)**. Furthermore, significant overexpression of *Hsp90* was observed under heat treatment in the abdomen of both sexes (**Figure 4A**). The cold treatment resulted in a significant overexpression of Hsp90, reaching up to 2 and 5 times in the thorax of females and males, respectively. Nonetheless, *Hsp90* exhibited an underexpression of up to 3-fold in the abdomen of both sexes. (**Figure 4A**).

Otherwise, it was observed an overexpression of *Hsp90* with the least stable genes of up to 5 and 10 time more under heat treatment in the thorax of females and males, respectively (**Figure 4B**). Likewise, there was an underexpression in the abdomen of the females. In contrast, under cold treatment, *Hsp90* was significantly overexpressed in all tissues of three male and significantly underexpressed in the head and thorax of females (**Figure 4B**).

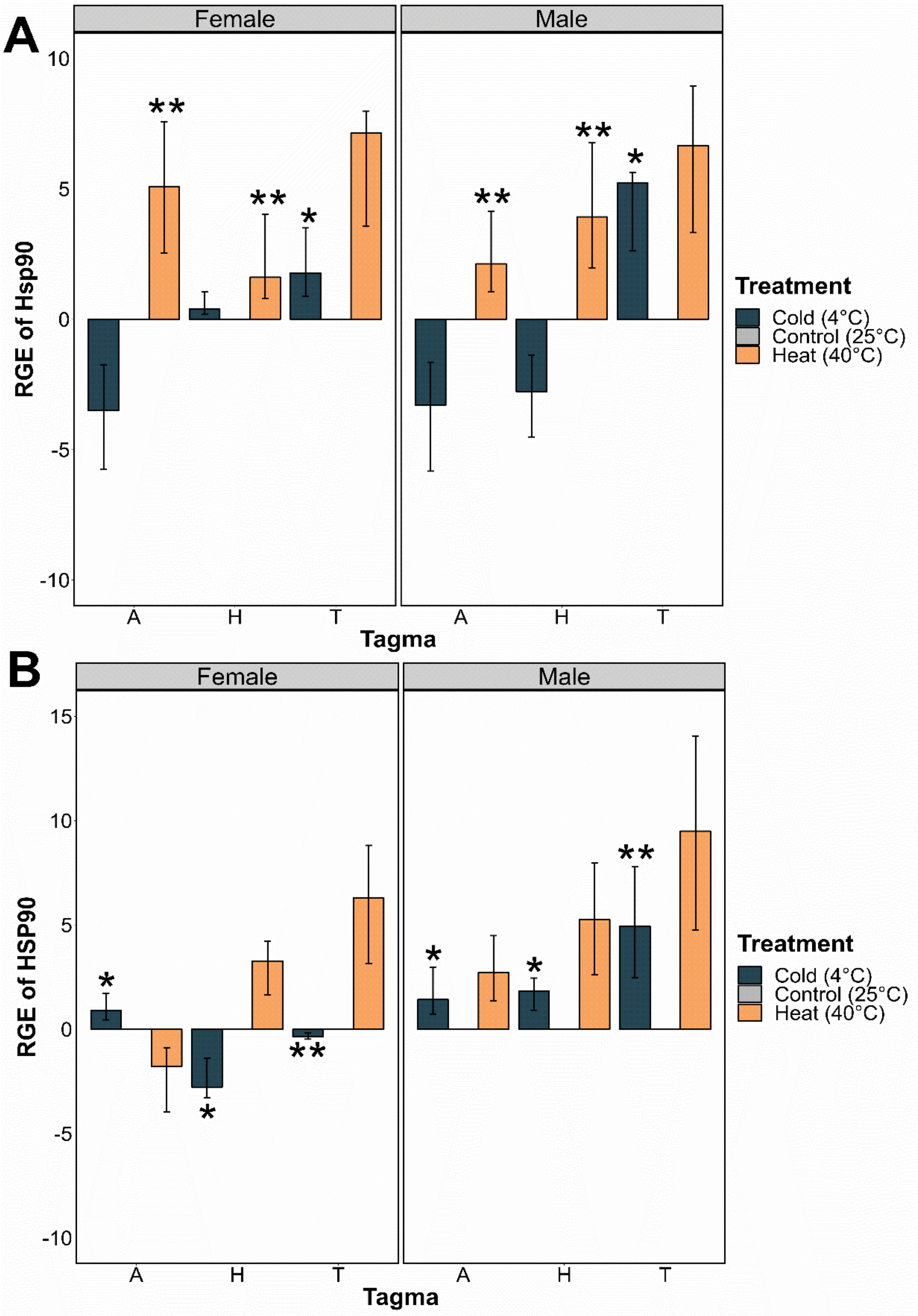


Figure 4. Relative gene expression (RGE) of *Hsp90* using the reference genes selected under different temperature treatments in H. e. lativitta.

A) Relative expression levels were normalized to the most stable genes (*AK* and *ACT2*). B) Relative expression levels using to the least stable genes (*ANX* and *ACT1*). Error bars show the SD of three biological replicates. The asterisk indicates significant difference ($P < 0.05$).

4. DISCUSSION

qPCR is widely employed to quantify target gene transcription levels in insects (Solà et al. 2017). However, the accuracy and reliability of qPCR data depend on the careful choice of reference genes. An ideal reference gene must present stable transcription levels under various experimental or environmental conditions (Tang et al. 2022). Unfortunately, several studies in insects have reported significant differences in the stability of commonly used reference genes under different experimental conditions and tissue where they are located. For example, the *eEf1α* gene has been shown to be stable under the presence of biotic factors but unstable under the influence of abiotic factors in Danaus plexippus (Pan et al. 2015). In another study performed with Spodoptera litura, it has been reported that *ACT* is stable under the effect of insecticides and starvation treatments, but it become unstable by changes in diet and depending on the tissue where is found (Lu et al. 2013). Likewise, it has been shown than *Actin A1* is stable during the developmental stages in Plutella xylostella, but unstable in Bombyx mori (Teng et al. 2012a). Hence, it is necessary to validate reference genes for each species and that they are stable under different experimental treatments and across different tissues.

In the present study, reference genes commonly employed in other lepidopterans were selected and validated for gene expression analysis in *H. e. lativitta*. The optimal amplification efficiency and regression coefficient determined by the slope of the standard curve play a crucial role in the selection process of reference genes (Bustin et al. 2009a).The ideal efficiency is considered 100%, assuming that PCR products duplicate in each cycle during the exponential phase and that data fit the standard curve (Piron Prunier et al. 2016). However, amplification efficiency may significantly vary due to decreased polymerase activity, quality of the sample, depletion of PCR components, and competition with PCR products (Wong and Medrano 2005; Zhang et al. 2015). Therefore, reference genes can be considered suitable if the efficiency is within the range of 80 - 120% and R^2 values are greater than 0.98 (Bustin et al. 2009a). In this sense, among the genes evaluated, only *eEf1α2* was not considered as candidate reference gene due to its lowest efficiency (63.05 %) (**Table1**), while than *ACT1, ACT2, AK, ANX* and *eEf1a* which showed a high efficiency were employed for further analysis.

ACT genes encode highly conserved proteins involved in fundamental processes, such as muscle contraction, cell motility, and cell division (Lu et al. 2013; Li et al. 2018). It has been stated that the stability of these genes could vary depending on the organism and specific experimental condition such as stage of development, tissues, sex, photoperiod, inanition, thermal stress, and diet (Lü et al. 2018). In the present study, *ACT* genes only showed the best stability at the sex and tissue level. However, when exposed to thermal stress their stability was slightly affected. These is according to the reported in other studies, in which these genes have exhibited high stability only under certain conditions such as temperature treatment, photoperiod, insecticide treatment, and diet in organisms like *Megalurothrips usitatus, Spodoptera litura*, *Drosophila melanogaster,* and *L. vinanasa* (Scharlaken et al. 2008; Ponton et al. 2011; Lu et al. 2013; Hou et al. 2023; Liu et al. 2023). On the other hand, *AK* gene is involved in cellular metabolism and catalyzes the reversible transfer of the phosphoryl group of ATP in invertebrates(Wang et al. 2009). Although *AK* is rarely used as a reference gene, its stability has been proven in tissues of *Bombus terrestris* and *Spodoptera litura* (Horňáková et al. 2010; Lu et al. 2013). Moreover, it has been reported that *AK* is stable in males and females of *L. japonica* and under insecticide treatments in *M. dirhodum* [41,42]. *However,* in this study, *AK* only exhibited high stability under thermal stress and in different tissues from *H. e. lativitta*, but it presented a high instability regarding sex.

On other hand, eEf1α participates in protein synthesis, specifically in the translation process by catalyzing GTP-dependent binding of aminoacyl-tRNA to the acceptor site of the ribosome (Lu et al. 2013). As a conserved nuclear gene, it has been used as a phylogenetic marker in insects and has been widely utilized as a reference gene (Martoni et al. 2017). For example, previous studies have reported the stability of *eEf1a* in different developmental stages of *Plagiodera versicolora* and *Oryctes rhinoceros,* in males and females of *H. theivora*, as well as, in individuals of *Mythimina separata* exposed to pesticides, and *M. usitatus* exposed to different temperatures (Li et al. 2018; Wang et al. 2019; Tu et al. 2022; Arvind et al. 2023; Hou et al. 2023). However, in this study, *eEf1a* only exhibited a high stability in the thorax, while in the other two tissues (head and abdomen), at the sex level, and under thermal stress, its expression level was not stable. On the contrary*, ANX* a gene than participate in Ca^{2+}-regulated phospholipid binding during the exocytosis and apoptosis, showed a high instability in the three assessed conditions (sex, tissue and temperature variation) (Moss and Morgan 2004). These results are different to the reported in other studies, in which the expression level of *ANX* gene was stable in the developmental stages of *Heliconius* butterflies (Reed et al. 2007; Piron Prunier et al. 2016). Therefore, these results highlight the importance of studying the expression levels of the candidate reference genes under different conditions in order to ensure the reliability of qPCR normalization.

Recent studies have indicated that a single reference gene used for the normalization of qPCR may influence the accuracy of expression levels (Bustin et al. 2009b; Teng et al. 2012a; Omondi et al. 2015; Liu et al. 2023). Hence, the use of two or more reference genes is recommended to reduce errors (Bustin et al. 2009b). In this work, the V values that indicate the optimal number of genes to be used were higher than the threshold value (0.15) for all conditions (Vandesompele et al. 2002). That is, these values may not be considered as the only criterion to determine the optimal number of reference genes to be used for qPCR normalization. In this sense, M values which also evaluate the stability of candidate reference genes were used to find the most stable genes. Then, according to the results shown in **Table 2**, at least two genes were considered as the most stable reference genes due to their M values were below 1.5 (Lu et al. 2013; Sun et al. 2019). Therefore, *AK* and *ACT2*, *ACT1* and *AK*, *eEf1α* and *AK*, and *ACT1* and *ACT2* were used to normalize gene expression in *H. e. lativitta* under thermal stress, for head-abdomen, thorax, and sex, respectively.

The validation of the two selected reference genes was evaluated in the relative expression analysis of *HSP90*, a key protein in the cellular response to thermal stress. The results suggested that relative expression of *HSP90* was high at 40°C in tagma of both sexes when was normalized by the recommended genes *AK* and *ACT2*. Nonetheless, when *Hsp90* was normalized using the least stable genes *ACT1* and *ANX*, it was observed a significant overexpression at high temperatures in the thorax of both sexes. Thus, these results emphasize that the choice of deficient reference genes may provide incorrect expression patterns in the target gene and misinterpretations (Pan et al. 2015). Therefore, the appropriate selection and use of reference genes for normalization is a fundamental prerequisite for accurate expression analysis of target genes.

5. CONCLUSIONS

The stability of six candidate reference genes from *H. e lativitta* was evaluated under different conditions and using three algorithms. It was found that the *ACT* genes are the most suitable to be used as reference genes for gene expression normalization due their high stability across different tissues and both in male and females. While the expression level of *ANX* gene was the most unstable becoming it the least appropriate gene for q-PCR data normalization. Additionally, the selected reference genes were validated by analyzing the expression of the *Hsp90* gene under heat stress, demonstrating that the choice of unstable genes may lead to incorrect interpretations of the expression patterns of the target genes. Therefore, careful selection and validation of reference genes are essential steps to ensure the accuracy of gene expression analyses, particularly in the study of heat stress responses in tropical butterflies. Moreover, the results of this work provide valuable information and a set of highly reliable reference genes for future research on gene expression in *Heliconius erato lativitt*a.

Acknowledgements

We are grateful to the Molecular Biology and Biochemistry Laboratory at Universidad Regional Amazónica Ikiam for access to it is facilities. We thank Leny Tacuri contributed to butterfly stock and Joshue Ruiz for assistance in the laboratory. We also thank Michael Zuarez for the thorough examination of the manuscript.

REFERENCES

Alves de Oliveira BF, Bottino MJ, Nobre P, Nobre CA. 2021. Deforestation and climate change are projected to increase heat stress risk in the Brazilian Amazon. Commun Earth Environ 2021 21. 2(1):1–8. doi:10.1038/s43247-021-00275-8. [accessed 2023 Jul 21]. https://www.nature.com/articles/s43247-021-00275-8.

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(15):5245–5250. doi:10.1158/0008-5472.CAN-04-0496. [accessed 2023 Dec 1]. https://pubmed.ncbi.nlm.nih.gov/15289330/.

Arun A, Baumlé V, Amelot G, Nieberding CM. 2015. Selection and validation of reference genes for qRT-PCR expression analysis of candidate genes involved in olfactory communication in the butterfly bicyclus anynana. PLoS One. 10(3):e0120401. doi:10.1371/journal.pone.0120401. [accessed 2023 Feb 1].
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0120401.

Arvind K, Antony G, Rajesh MK, Josephrajkumar A, Grace T. 2023. Reference genes for expression studies in different developmental stages of Oryctes rhinoceros, the coconut rhinoceros beetle. J Asia Pac Entomol. 26(2):102066. doi:10.1016/J.ASPEN.2023.102066.

Ashe-Jepson E, Arizala Cobo S, Basset Y, Bladon AJ, Kleckova I, Laird-Hopkins BC, Mcfarlane A, Sam K, Savage AF, Zamora AC, et al. 2023. Tropical butterflies use thermal buffering and thermal tolerance as alternative strategies to cope with temperature increase. J Anim Ecol. 92(9):1759–1770. doi:10.1111/1365-2656.13970. [accessed 2023 Dec 1]. https://onlinelibrary.wiley.com/doi/full/10.1111/1365-2656.13970.

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. 2017. GenBank. Nucleic Acids Res. 45(D1):D37–D42. doi:10.1093/NAR/GKW1070. [accessed 2024 Jan 17]. https://dx.doi.org/10.1093/nar/gkw1070.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. 2009a. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem. 55(4):611–622. doi:10.1373/CLINCHEM.2008.112797. [accessed 2023 Feb 1]. https://academic.oup.com/clinchem/article/55/4/611/5631762.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. 2009b. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem. 55(4):611–622. doi:10.1373/CLINCHEM.2008.112797. [accessed 2023 May 7]. https://academic.oup.com/clinchem/article/55/4/611/5631762.

Challis RJ, Kumar S, Dasmahapatra KKK, Jiggins CD, Blaxter M. 2016 Jun 6. Lepbase: the Lepidopteran genome database. bioRxiv.:056994. doi:10.1101/056994. [accessed 2023 May 8]. https://www.biorxiv.org/content/10.1101/056994v1.

Ehlers S, Szczerbowski D, Harig T, Stell M, Hötling S, Darragh K, Jiggins CD, Schulz S. 2021. Identification and Composition of Clasper Scent Gland Components of the Butterfly Heliconius erato and Its Relation to Mimicry. ChemBioChem. 22(23):3300–3313. doi:10.1002/CBIC.202100372. [accessed 2023 Feb 1]. https://onlinelibrary.wiley.com/doi/full/10.1002/cbic.202100372.

Ferguson LC, Jiggins CD. 2009. Shared and divergent expression domains on mimetic Heliconius wings. Evol Dev. 11(5):498–512. doi:10.1111/J.1525-142X.2009.00358.X. [accessed 2023 Feb 10]. https://onlinelibrary.wiley.com/doi/full/10.1111/j.1525-142X.2009.00358.x.

Fox J, Weisberg S. An R companion to applied regression.

Gao XK, Zhang S, Luo JY, Wang CY, Lü LM, Zhang LJ, Zhu XZ, Wang L, Lu H, Cui JJ. 2017. Comprehensive evaluation of candidate reference genes for gene expression studies in Lysiphlebia japonica (Hymenoptera: Aphidiidae) using RT-qPCR. Gene. 637:211–218. doi:10.1016/J.GENE.2017.09.057. [accessed 2023 Dec 1]. https://pubmed.ncbi.nlm.nih.gov/28964897/.

Harvey JA, Heinen R, Gols R, Thakur MP. 2020. Climate change-mediated temperature extremes and insects: From outbreaks to breakdowns. Glob Chang Biol. 26(12):6685. doi:10.1111/GCB.15377. [accessed 2023 Jul 21]. /pmc/articles/PMC7756417/.

Horňáková 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(1):118–120. doi:10.1016/J.AB.2009.09.019. [accessed 2023 Dec 1]. https://pubmed.ncbi.nlm.nih.gov/19751695/.

Hou Q, Yuan L, Jin H, Yan H, Li F, Wu S. 2023. Identification and validation of reference genes for normalization of gene expression analysis using qRT-PCR in Megalurothrips usitatus (thysanoptera: thripidae). Front Physiol. 14:1161680. doi:10.3389/FPHYS.2023.1161680/BIBTEX.

Khan MM, Guo CF, Peng J, Fan ZY, Hafeez M, Ali D, Wang K, Almarzoug MHA, Qiu BL. 2022. Screening and validation of reference genes using in RT-qPCR for gene expression studies in Paederus fuscipes, a medically and agriculturally important insect. J King Saud Univ - Sci. 34(1):101654. doi:10.1016/J.JKSUS.2021.101654.

Kozera B, Rapacz M. 2013. Reference genes in real-time PCR. J Appl Genet. 54(4):391–406. doi:10.1007/S13353-013-0173-X/METRICS. [accessed 2023 Jul 29]. https://link.springer.com/article/10.1007/s13353-013-0173-x.

Kronforst MR, Papa R. 2015. The Functional Basis of Wing Patterning in Heliconius Butterflies: The

Molecules Behind Mimicry. Genetics. 200(1):1. doi:10.1534/GENETICS.114.172387. [accessed 2024 Jan 16]. /pmc/articles/PMC4423356/.

Landais I, Pommet JM, Mita K, Nohata J, Gimenez S, Fournier P, Devauchelle G, Duonor-Cerutti M, Ogliastro M. 2001. Characterization of the cDNA encoding the 90 kDa heat-shock protein in the Lepidoptera Bombyx mori and Spodoptera frugiperda. Gene. 271(2):223–231. doi:10.1016/S0378-1119(01)00523-6.

Lewis JJ, van der Burg KRL, Mazo-Vargas A, Reed RD. 2016. ChIP-Seq-Annotated Heliconius erato Genome Highlights Patterns of cis-Regulatory Evolution in Lepidoptera. Cell Rep. 16(11):2855–2863. doi:10.1016/J.CELREP.2016.08.042.

Li HB, Dai CG, Zhang CR, He YF, Ran HY, Chen SH. 2018. Screening potential reference genes for quantitative real-time PCR analysis in the oriental armyworm, Mythimna separata. PLoS One. 13(4). doi:10.1371/JOURNAL.PONE.0195096. [accessed 2023 Dec 1]. https://pubmed.ncbi.nlm.nih.gov/29617430/.

Li Xinan, Gong P, Wang B, Wang C, Li M, Zhang Y, Li Xiangrui, Gao H, Ju J, Zhu X. 2020. Selection and validation of experimental condition-specific reference genes for qRT-PCR in Metopolophium dirhodum (Walker) (Hemiptera: Aphididae). Sci Rep. 10(1). doi:10.1038/S41598-020-78974-Z. [accessed 2023 Dec 1]. /pmc/articles/PMC7738536/.

Liu Y, Zhou J, Qiu Z, Hu P, Chen X, Yang Z. 2023. Identification and Validation of Reference Genes for Expression Analysis Using RT-qPCR in Leptocybe invasa Fisher and La Salle (Hymenoptera: Eulophidae). Insects 2023, Vol 14, Page 456. 14(5):456. doi:10.3390/INSECTS14050456. [accessed 2023 Dec 1]. https://www.mdpi.com/2075-4450/14/5/456/htm.

Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods. 25(4):402–408. doi:10.1006/METH.2001.1262.

Lü J, Yang C, Zhang Y, Pan H. 2018. Selection of reference genes for the normalization of RT-qPCR data in gene expression studies in insects: A systematic review. Front Physiol. 9(NOV):1560. doi:10.3389/FPHYS.2018.01560/BIBTEX.

Lu Y, Yuan M, Gao X, Kang T, Zhan S, Wan H, Li J. 2013. Identification and Validation of Reference Genes for Gene Expression Analysis Using Quantitative PCR in Spodoptera litura (Lepidoptera: Noctuidae). PLoS One. 8(7):e68059. doi:10.1371/JOURNAL.PONE.0068059. [accessed 2023 Dec 1].
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0068059.

Luo S, Ahola V, Shu C, Xu C, Wang R. 2015. Heat shock protein 70 gene family in the Glanville fritillary butterfly and their response to thermal stress. Gene. 556(2):132–141. doi:10.1016/J.GENE.2014.11.043. [accessed 2024 Jan 17]. https://pubmed.ncbi.nlm.nih.gov/25433328/.

Martoni F, Bulman SR, Pitman A, Armstrong KF. 2017. Elongation Factor-1α Accurately Reconstructs Relationships Amongst Psyllid Families (Hemiptera: Psylloidea), with Possible Diagnostic Implications. J Econ Entomol. 110(6):2618–2622. doi:10.1093/JEE/TOX261. [accessed 2023 Dec 1]. https://pubmed.ncbi.nlm.nih.gov/29040629/.

Merrill RM, Dasmahapatra KK, Davey JW, Dell'Aglio DD, Hanly JJ, Huber B, Jiggins CD, Joron M, Kozak KM, Llaurens V, et al. 2015. The diversification of Heliconius butterflies: what have we learned in 150 years? J Evol Biol. 28(8):1417–1438. doi:10.1111/JEB.12672. [accessed 2022 Jul 20]. https://onlinelibrary.wiley.com/doi/full/10.1111/jeb.12672.

Montejo-Kovacevich G, Martin SH, Meier JI, Bacquet CN, Monllor M, Jiggins CD, Nadeau NJ. 2020. Microclimate buffering and thermal tolerance across elevations in a tropical butterfly. J Exp Biol. 223(8). doi:10.1242/JEB.220426/VIDEO-1. [accessed 2023 May 7]. https://journals.biologists.com/jeb/article/223/8/jeb220426/223891/Microclimate-buffering-and-thermal-tolerance.

Montejo-Kovacevich G, Salazar PA, Smith SH, Gavilanes K, Bacquet CN, Chan YF, Jiggins CD, Meier JI, Nadeau NJ. 2021. Genomics of altitude-associated wing shape in two tropical butterflies. In: Molecular Ecology. Vol. 30. John Wiley and Sons Inc. p. 6387–6402.

Montejo-Kovacevich G, Smith JE, Meier JI, Bacquet CN, Whiltshire-Romero E, Nadeau NJ, Jiggins CD. 2019. Altitude and life-history shape the evolution of Heliconius wings. Evolution (N Y). 73(12):2436–2450. doi:10.1111/EVO.13865. [accessed 2023 May 7]. https://academic.oup.com/evolut/article/73/12/2436/6727012.

Moss SE, Morgan RO. 2004. The annexins. Genome Biol. 5(4):219. doi:10.1186/GB-2004-5-4-219. [accessed 2023 Dec 8]. /pmc/articles/PMC395778/.

Ojeda A. 2023. Expresión génica de hsp40 y hsp90 en respuesta al estrés témico en Heliconius erato lativitta. Univ Reg Amaz Ikiam.(October).

Omondi BA, Latorre-Estivalis JM, Rocha Oliveira IH, Ignell R, Lorenzo MG. 2015. Evaluation of reference genes for insect olfaction studies. Parasites and Vectors. 8(1):1–15. doi:10.1186/S13071-015-0862-X/FIGURES/3. [accessed 2023 Jan 31]. https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-015-0862-x.

Pan H, Yang X, Bidne K, Hellmich RL, Siegfried BD, Zhou X. 2015. Selection of Reference Genes for RT-qPCR Analysis in the Monarch Butterfly, Danaus plexippus (L.), a Migrating Bio-Indicator. PLoS One. 10(6):e0129482. doi:10.1371/JOURNAL.PONE.0129482. [accessed 2023 Jan 31]. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0129482.

Panina Y, Germond A, Masui S, Watanabe TM. 2018. Validation of Common Housekeeping Genes as Reference for qPCR Gene Expression Analysis During iPS Reprogramming
Process. Sci Reports 2018 81. 8(1):1–8. doi:10.1038/s41598-018-26707-8. [accessed 2023 Jul 29].

https://www.nature.com/articles/s41598-018-26707-8.

Perkins JR, Dawes JM, McMahon SB, Bennett DLH, Orengo C, Kohl M. 2012. ReadqPCR and NormqPCR: R packages for the reading, quality checking and normalisation of RT-qPCR quantification cycle (Cq) data. BMC Genomics. 13(1). doi:10.1186/1471-2164-13-296.

Piron Prunier F, Chouteau M, Whibley A, Joron M, Llaurens V. 2016. Selection of valid reference genes for reverse transcription quantitative PCR analysis in Heliconius numata (Lepidoptera: Nymphalidae). J Insect Sci. 16(1). doi:10.1093/jisesa/iew034. [accessed 2023 Feb 1]. https://academic.oup.com/jinsectscience/article/16/1/50/2726667.

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(6):840–850. doi:10.1016/J.JINSPHYS.2011.03.014. [accessed 2023 Dec 1]. https://pubmed.ncbi.nlm.nih.gov/21435341/.

Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun. 313(4):856–862. doi:10.1016/j.bbrc.2003.11.177. [accessed 2024 Jan 21]. https://pubmed.ncbi.nlm.nih.gov/14706621/.

Reed RD, McMillan WO, Nagy LM. 2007. Gene expression underlying adaptive variation in Heliconius wing patterns: non-modular regulation of overlapping cinnabar and vermilion prepatterns. Proc R Soc B Biol Sci. 275(1630):37–46. doi:10.1098/RSPB.2007.1115. [accessed 2023 Feb 10]. https://royalsocietypublishing.org/doi/10.1098/rspb.2007.1115.

Romero IG, Ruvinsky I, Gilad Y. 2012. Comparative studies of gene expression and the evolution of gene regulation. Nat Rev Genet. 13(7):505–516. doi:10.1038/nrg3229. [accessed 2023 Jan 31]. https://www.nature.com/articles/nrg3229.

Scharlaken B, De Graaf DC, Goossens K, Brunain M, Peelman LJ, Jacobs FJ. 2008. Reference Gene Selection for Insect Expression Studies Using Quantitative Real-Time PCR: The Head of the Honeybee, Apis mellifera, After a Bacterial Challenge. J Insect Sci. 8. doi:10.1673/031.008.3301. [accessed 2023 Dec 1]. /pmc/articles/PMC3061606/.

Sellamuthu G, Bílý J, Joga MR, Synek J, Roy A. 2022. Identifying optimal reference genes for gene expression studies in Eurasian spruce bark beetle, Ips typographus (Coleoptera: Curculionidae: Scolytinae). Sci Reports 2022 121. 12(1):1–17. doi:10.1038/s41598-022-08434-3. [accessed 2023 Jul 29]. https://www.nature.com/articles/s41598-022-08434-3.

Sheldon KS. 2019. Climate Change in the Tropics: Ecological and Evolutionary Responses at Low Latitudes. https://doi.org/101146/annurev-ecolsys-110218-025005. 50:303–333. doi:10.1146/ANNUREV-ECOLSYS-110218-025005. [accessed 2023 May 8]. https://www.annualreviews.org/doi/abs/10.1146/annurev-ecolsys-110218-025005.

Shen CH, Peng LJ, Zhang YX, Zeng HR, Yu HF, Jin L, Li GQ. 2022. Reference Genes for Expression Analyses by qRT-PCR in Phthorimaea operculella (Lepidoptera: Gelechiidae). Insects. 13(2):140. doi:10.3390/INSECTS13020140/S1. [accessed 2023 Apr 30]. https://www.mdpi.com/2075-4450/13/2/140/htm.

Solà M, Lundgren JG, Agustí N, Riudavets J. 2017. Detection and quantification of the insect pest Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae) in rice by qPCR. J Stored Prod Res. 71:106–111. doi:10.1016/J.JSPR.2017.02.002.

Sun H, Jiang X, Sun M, Cong H, Qiao F. 2019. Evaluation of reference genes for normalizing RT-qPCR in leaves and suspension cells of Cephalotaxus hainanensis under various stimuli. Plant Methods. 15(1):1–11. doi:10.1186/S13007-019-0415-Y/FIGURES/3. [accessed 2023 Dec 2]. https://plantmethods.biomedcentral.com/articles/10.1186/s13007-019-0415-y.

Tang J, Liang G, Dong S, Shan S, Zhao M, Guo X. 2022. Selection and Validation of Reference Genes for Quantitative Real-Time PCR Normalization in Athetis dissimilis (Lepidoptera: Noctuidae) Under Different Conditions. Front Physiol. 13:842195. doi:10.3389/FPHYS.2022.842195/FULL. [accessed 2023 Feb 1]. /pmc/articles/PMC8902415/.

Teng X, Zhang Z, He G, Yang L, Li F. 2012a. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in four lepidopteran insects. J Insect Sci. 12(1). doi:10.1673/031.012.6001/7999828/JIS12-0060.PDF. [accessed 2023 May 7]. https://academic.oup.com/jinsectscience/article/12/1/60/885157.

Teng X, Zhang Z, He G, Yang L, Li F. 2012b. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in four lepidopteran insects. J Insect Sci. 12(1). doi:10.1673/031.012.6001/7999828/JIS12-0060.PDF. [accessed 2023 Feb 1]. https://academic.oup.com/jinsectscience/article/12/1/60/885157.

Tu C, Xu P, Han R, Luo J, Xu L. 2022. Defining Suitable Reference Genes for qRT-PCR in Plagiodera versicolora (Coleoptera: Chrysomelidae) under Different Biotic or Abiotic Conditions. Agron 2022, Vol 12, Page 1192. 12(5):1192. doi:10.3390/AGRONOMY12051192. [accessed 2023 Jul 29]. https://www.mdpi.com/2073-4395/12/5/1192/htm.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3(7):1–12. doi:10.1186/GB-2002-3-7-RESEARCH0034/COMMENTS. [accessed 2023 Feb 1]. https://genomebiology.biomedcentral.com/articles/10.1186/gb-2002-3-7-research0034.

Wang H, Zhang Lan, Zhang Lee, Lin Q, Liu N. 2009. Arginine kinase: Differentiation of gene expression and protein activity in the red imported fire ant, Solenopsis invicta. Gene. 430(1–2):38–43. doi:10.1016/J.GENE.2008.10.021.

Wang Z, Meng Q, Zhu X, Sun S, Gao S, Gou Y, Liu A. 2019. Evaluation and Validation of Reference Genes for Quantitative Real-Time PCR in Helopeltis theivora Waterhouse (Hemiptera: Miridae). Sci Reports 2019 91. 9(1):1–10. doi:10.1038/s41598-019-49479-1. [accessed 2023 Jul 29]. https://www.nature.com/articles/s41598-019-49479-1.

Wickham H. 2016. Data Analysis. :189–201. doi:10.1007/978-3-319-24277-4_9. [accessed 2024 Jan 18]. https://link.springer.com/chapter/10.1007/978-3-319-24277-4_9.

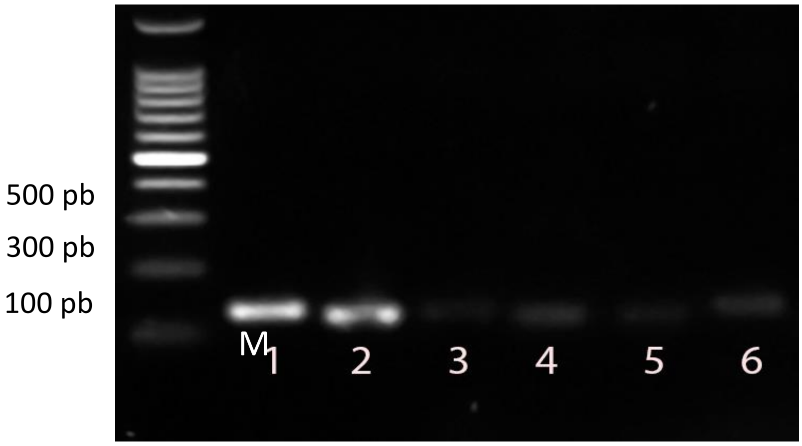
Wong ML, Medrano JF. 2005. Real-time PCR for mRNA quantitation. Biotechniques. 39(1):75–85. doi:10.2144/05391RV01/ASSET/IMAGES/LARGE/TABLE2.JPEG. [accessed 2023 Dec 1]. https://www.future-science.com/doi/10.2144/05391RV01.

Xie F, Wang J, Zhang B. 2023. RefFinder: a web-based tool for comprehensively analyzing and identifying reference genes. Funct Integr Genomics. 23(2):1–5. doi:10.1007/S10142-023-01055-7/METRICS. [accessed 2023 Dec 1]. https://link.springer.com/article/10.1007/s10142-023-01055-7.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 13(134).

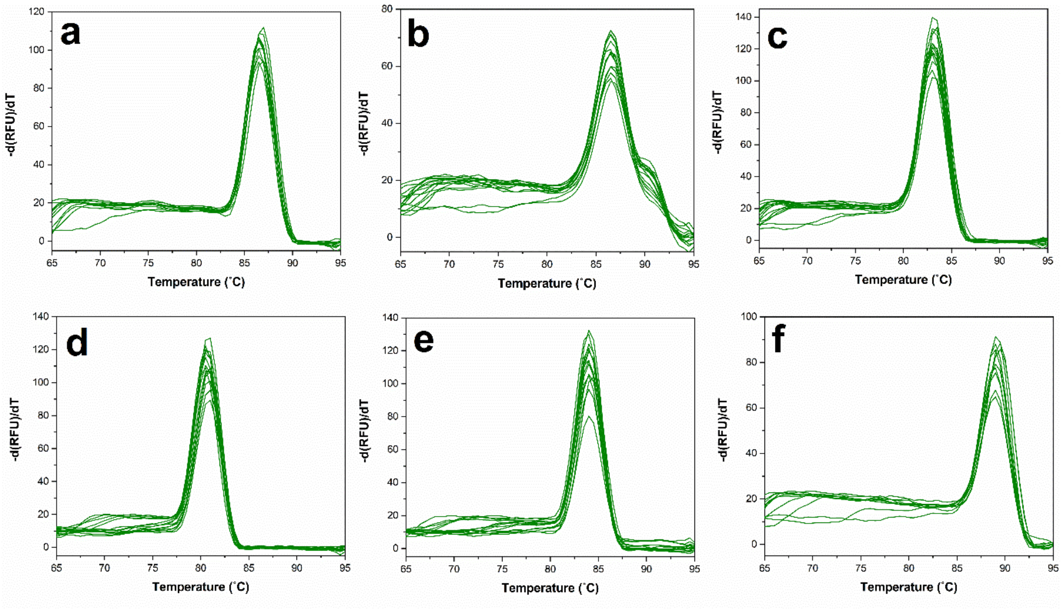
Zhang Q, Wang J, Deng F, Yan Z, Xia Y, Wang Z, Ye J, Deng Y, Zhang Z, Qiao M, et al. 2015. TqPCR: A Touchdown qPCR Assay with Significantly Improved Detection Sensitivity and Amplification Efficiency of SYBR Green qPCR. PLoS One. 10(7):e0132666. doi:10.1371/JOURNAL.PONE.0132666. [accessed 2023 Dec 1]. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0132666.

SUPPLEMENTARY INFORMATION

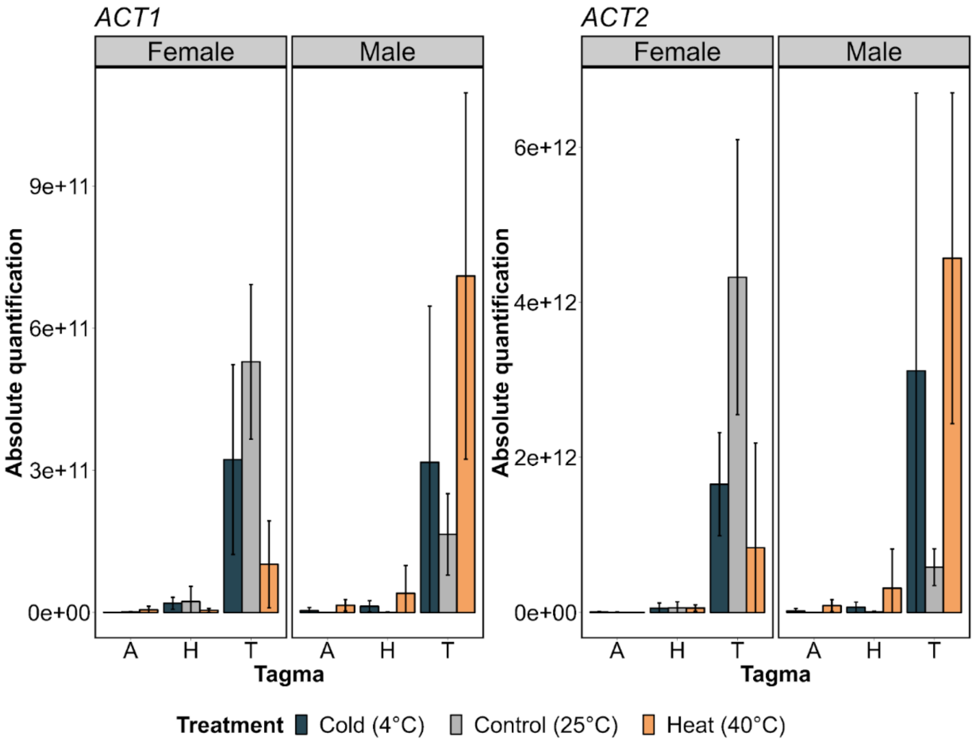


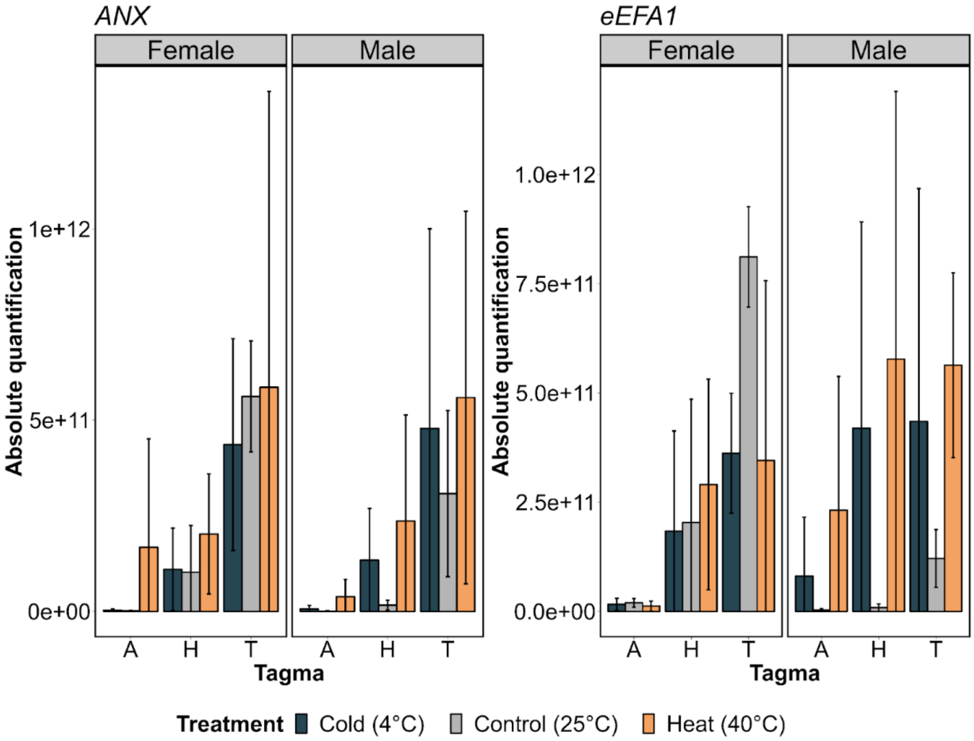
Supplementary Figure 1. Agarose gel electrophoresis of six candidate reference genes primers in PCR.

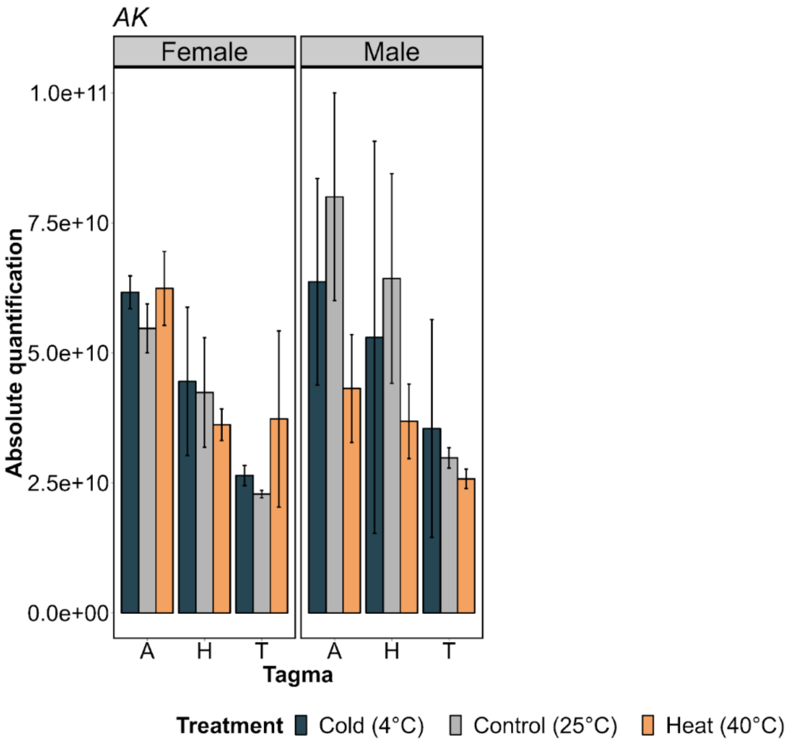
Lane M was DNA 5000pb Marker, PCR products were as follows: 1) ACT1; 2) ACT2; 3) AK; 4) eEf1a; 5) ANX; 6) eEf1a2.



Supplementary Figure 2. Melting curves analysis for each candidate gene in *H. e. lativitta*. Genes included: a) *ACT1*; b) *ACT2*; c) *AK*; d) *ANX*; e) *eEf1a; d) eEf1a2*


ACT1
Female
Male
Absolute quantification
9e+11
6e+11
3e+11
0e+00
A
H
T
A
H
T
Tagma
ACT2
Female
Male
Absolute quantification
6e+12
4e+12
2e+12
0e+00
A
H
T
A
H
T
Tagma
Treatment
Cold (4°C)
Control (25°C)
Heat (40°C)

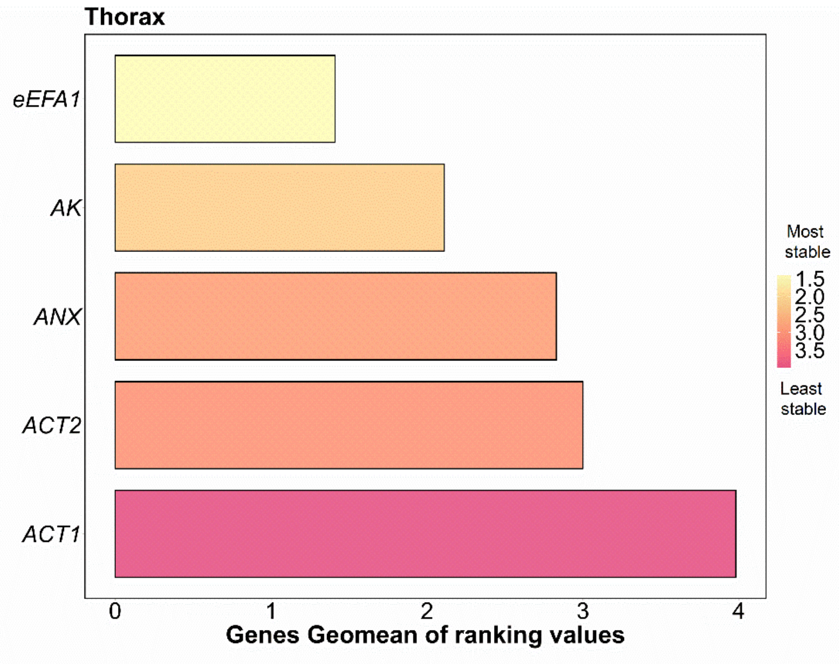

ANX
Female
Male
Absolute quantification
1e+12
5e+11
0e+00
A
H
T
A
H
T
Tagma
eEFA1
Female
Male
Absolute quantification
1.0e+12
7.5e+11
5.0e+11
2.5e+11
0.0e+00
A
H
T
A
H
T
Tagma
Treatment
Cold (4°C)
Control (25°C)
Heat (40°C)



Supplementary Figure 3. Absolute gene quantification of candidate reference genes

Supplementary Table 1. Ranking of the reference genes obtained geNorm and NormFinder of thorax

Condition	Ranking	geNorm		NormFinder	
		Gen	**Stability (M)**	**Gen**	**Stability (SV)**
Thorax	1	AK/ eEf1α	0	eEf1α	0.563
	2	-	-	AK	0.563
	3	ACT2	0.655	ACT2	0.679
	4	ANX	0.866	ANX	0.853
	5	ACT1	1.064	ACT1	1.195



Supplementary Figure 4. Expression stability of the reference genes of thorax by RefFinder