Heliyon 7 (2021) e06253

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Identification and evaluation of antioxidant and reference genes for quantitative real-time PCR in blood of *Caiman latirostris*

Lucia M. Odetti [a,b,*], Enrique V. Paravani [c], María F. Simoniello [a], Gisela L. Poletta [a,b,d]

[a] Cátedra de Toxicología, Farmacología y Bioquímica Legal, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje El Pozo S/N 3000, Santa Fe, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas Godoy Cruz 2290 C1425FQB Ciudad Autónoma de Buenos Aires, Argentina
[c] Lab. de Química Ambiental, Cátedra de Química General e Inorgánica. Facultad de Ingeniería–UNER, Oro Verde, Entre Ríos, Argentina
[d] Proyecto Yacaré-Lab. de Zoología Aplicada: Anexo Vertebrados, Facultad de Humanidades y Ciencias-Universidad Nacional del Litoral/Ministerio de Medio Ambiente, Av. Aristóbulo del Valle 8700 3000, Santa Fe, Argentina

ARTICLE INFO

Keywords:
Crocodiles
mRNA abundance pattern
Reference genes
Antioxidant genes

ABSTRACT

The quantitative real-time polymerase chain reaction (qPCR) has been one of the most promising approaches to perform rapid and accurate quantification of DNA in various biological systems. The aim of this study was to standardized the qPCR technique for the analysis of important genes involved in the main routes of antioxidant defense against reactive oxygen species (catalase: *cat* and superoxide dismutase: *sod*) and evaluate the stability of different reference genes in blood of *Caiman latirostris* hatchlings. The stability of the reference genes, *β-actin*, glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) and ribosomal protein L8 (*rpl8*) was determined using the comparative ΔCt, NormFinder, geNorm, BestKeeper and RefFinder. Then, *cat* and *sod* genes were normalized with each reference gene and their mRNA abundances were determined through the qPCR. Stability of genes was ranked through the different methods in the following order: *β-actin*, *rpl8* and *gapdh*, under normal physiological conditions. The results reveal that *cat* and *sod* genes present a similar relative mRNA abundance with *β-actin* and *rpl8*. This is the first report of the analysis of antioxidant mRNA as potential biomarkers of oxidative stress in blood for all crocodilians species. Besides, we determined the stability of different reference genes that can be used for normalization of mRNA abundance patterns in blood of *C. latirostris*, without the need to sacrifice the animals.

1. Introduction

In small-scale studies, the detection of RNA can be accomplished by common techniques including northern blot analysis, RNase protection assays, quantitative competitive reverse transcription–polymerase chain reaction (QC-RT-PCR), and quantitative real-time PCR (qPCR). Among these, qPCR has become increasingly popular compared to other methods because it has higher sensitivity, greater speed, and broader dynamic quantification range [1, 2]. It is a method that detects the accumulation of the amplification product as the reaction progresses, that is said in "real time" [3]. This is possible through the addition of fluorescent molecules to the reaction, including dyes that bind to DNA, and labeled primers or probes. Due to many technical advantages, as well as the low costs compared to other techniques, qPCR has been universally adopted as the choice for transcriptomic analysis [3].

Recently, a new family of biomarkers was developed for the analysis of gene expression related to cellular stress, which can be used as early warning sensors of xenobiotics exposure [4]. In this sense, it the analysis of relative mRNA abundances of target genes by qPCR must be standardized with a reference gene or housekeeping [5]. The reference gene, as internal control, is expressed in a stable way during experimental manipulations and is necessary for the regular physiological maintenance of the organism [2]. The target gene is normalized with the housekeeping gene to accurately estimate mRNA abundances. Various studies have shown that the stability of reference genes can vary between different species and experimental conditions [6, 7].

Caiman latirostris is a crocodilian species with great commercial and ecological value in South America, mainly due to sustainable use management. In Argentina, this activity began in Santa Fe province in the 1990's, through a program known as "*Proyecto yacaré*". This species is

* Corresponding author.
E-mail address: luodetti@gmail.com (L.M. Odetti).

https://doi.org/10.1016/j.heliyon.2021.e06253
Received 19 August 2020; Received in revised form 11 December 2020; Accepted 7 February 2021

considered a sentinel organism for evaluating the effect induced by different pesticides, as many populations living in the north-central region of Argentina are in the proximity of areas with high agricultural activity [8].

Most of the biomarkers used to date to identify contamination stress are based on alterations in biochemical parameters and/or cytological or histological abnormalities that can be correlated with the presence of toxins in the environment [9, 10]. Specifically, studies made to evaluate the impact of pesticides in *C. latirostris* include biomarkers of DNA damage, oxidative damage to DNA and lipids, antioxidant enzymes such as Catalase (CAT) and Superoxide dismutase (SOD) and immunological alterations [8, 11, 12, 13, 14, 15, 16, 17, 18]. These toxicological endpoints are useful and convenient tools for monitoring environmental quality, assessing chemical risks and safety, investigating threshold values, and evaluating the vulnerability of organisms to specific chemicals or toxins, such as pesticides [19]. Alterations in mRNA patterns offer new insights into the role of genes in the context of toxicity [20]. These changes are immediate and generally more sensitive than the endpoints traditionally used in toxicology, making them relevant markers in various stress conditions [21]. Several studies in fish and mammals have demonstrated their usefulness as gene expression markers, establishing them as a useful tool for biomonitoring and ecotoxicology tests [22, 23, 24, 25]. The possibility to apply molecular gene expression markers in *C. latirostris* would allow us to understand the specific meaning of many alterations produced by pesticides and observed through traditional biomarkers. In this sense, we believe it is necessary to study the stability of potential reference genes under normal conditions, since there is limited information in crocodilian species [26]. The aim of this study was to evaluate the catalase (*cat*) and superoxide dismutase (*sod*) genes transcript levels in *C. latirotris* blood, to propose them as biomarkers of oxidative stress induced by environmental stressors. Besides, we evaluated through different methods (comparative ΔCt, NormFinder, geNorm, BestKeeper and RefFinder) the stability of *β-actin*, glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) and ribosomal protein L8 (*rpl8*) genes, to be used as housekeeping.

2. Materials and methods

2.1. Animals

The study was evaluated and approved by the Research Ethics and Safety Advisory Committee of the Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral (Santa Fe, Argentina) (Form N° 01–15). We used eight *C. latirostris* hatchlings (five days old), coming from two different clutches harvested by the Proyecto Yacaré sustainable used program. Five days after hatching, blood samples (0.5 mL) were taken from the spinal vein with heparinized syringes and 25G x 5/8" needles [27].

2.2. RNA extraction, determination of quality and cDNA synthesis

Total-RNA was isolated from whole blood (1:5) using the reagent TRIzol™ LS (Invitrogen™) through a protocol adapted for *C. latirostris* [28]. The ratio of absorbance at 260 nm/280 nm was used to verify the quality of the RNA in each sample. RNA integrity was evaluated trough the traditional electrophoresis agarose gel (2%). Subsequently, the treatment with DNase (Invitrogen) was carried out to eliminate any remaining genomic DNA, following the procedures detailed by the manufacturers and previous studies [29]. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific®) was used for cDNA synthesis (RT-PCR). The cDNA amplification was performed in a volume of 25 μl. The reaction was done as follows: 2.5 μl of 10X *EasyTaq* buffer® (with Mg^{2+}), 0.5 μl of *EasyTaq* DNA polymerase®, 2 μl of dNTPS (2.5 mM), 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 2 μl of cDNA and 16 μl of H_2O.

2.3. Selection of gene and primer design

Three reference genes (*β-actin*, *gapdh* and *rpl8*) and two genes involved in the antioxidant system (*cat* and *sod*) were evaluated in this work. The primers for qPCR were designed through the Integrated DNA Technologies page (https://www.idtdna.com/pages) from the following sequences published for *Alligator mississippiensis*: *β-actin* mRNA (GenBank: DQ421415.1), *gapdh* mRNA (GenBank: XM_006258364.3), *rpl8* mRNA (GenBank: XM_006266675.3) and *cat* mRNA (GenBank: XM_006259972.3). For the *sod* gene, we used primers reported by Sujiwattanarat et al. [30], for *Crocodylus siamensis*.

2.4. qPCR analysis

qPCR was performed on a StepOne™- 48 Real-Time PCR System (Applied Biosystems™). Each sample was run in triplicate under following conditions: 4 uL 5X HOT FIREPol® EvaGreen® qPCR Mix Plus, 14 μL PCR H_2O,0.5 μL each forward and reverse primers (10 μM), and 1 μL cDNA template. qPCR program was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s, 57 °C for 20s, 72 °C for 20s, 77 °C for 10s, and finally, 95 °C for 15s, 60 °C for 1 min and 95 °C for 15s. To verify the effectiveness of the designed primers before being used in the qPCR technique, a conventional PCR was performed.

In the present work, we observed the melting curves, amplification efficiency, linear standard curve and slope for each gene by qPCR. In these sense, standard curve of 1/10 dilution series (run in triplicate) from pooled cDNA (mix of cDNA from eight samples) were generated for each primer pair.

In turn, we determine the impact of normalization with reference genes in the expression level of the *cat* and *sod* genes, according to the $2^{-\Delta\Delta ct}$ method [31].

2.5. Determination of the stability of reference genes

The comparative ΔCt, NormFinder, geNorm, BestKeeper and RefFinder were the methods selected to determine the stability of reference genes. The comparative ΔCt method analyzes relative mRNA abundances of "pairs of genes'' within each tissue sample [32, 33]. The NormFinder, geNorm and BestKeeper are computational programs based on different algorithms [34, 35, 36, 37]. The RefFinder integrates and compare the geNorm, Normfinder, BestKeeper, and the comparative ΔCt method and rank the candidate reference genes through comprehensive ranking [38]. In all cases, the lower the stability value, the higher mRNA abundance stability of the reference genes.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 15.0 software for Windows [39]. Clutches were compared for mRNA abundances of all genes using the T test. As there were no differences between clutches, the analysis of mRNA abundances were performed considering data of all the hatchlings and expressed as mean values ±standard deviation (SD).

3. Result

The mean blood RNA concentration after DNAse treatment was 87.23 ng/ul ± 6.02 ng/ul (mean ± SD). The absorbance at 260 nm/280 nm optical density ratio measured with Nanodrop spectrophotometer was 1.89 ± 0.06 (mean ± SD). The integrity of the RNA was evaluated by means of an agarose gel under denaturing conditions, which indicates two well-defined bands corresponding to rRNA (28s and 18s), in addition, the band corresponding to 28s was observed to have an intensity approximately twice than that of the 18s band (Figure 1).

Table 1 shows the characteristics of the specific primers, the length of the amplicons, and PCR reactions efficiencies for this work. Amplification efficiencies for the candidate reference and antioxidant genes ranged

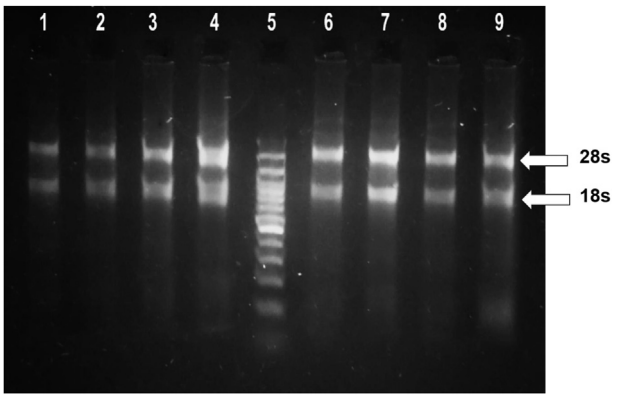

Figure 1. Agarose gel electrophoresis (2%) showing RNA extraction of all samples through the protocol adapted for *C. latirostris*. Lane 1–4 and 6–9: Blood RNA extractions without genomic DNA contamination; Lane 5: ladder 100 bp (Plus DNA - TransGen Biotech).

from 96% to 110%, the linear correlation coefficient (R^2) ranged from 0.992 to 0.999 and the slope between -3.114 and -3.399.

Results from the conventional PCR verified the effectiveness of the designed primers. In turn, the melting curve analysis revealed that all primer pairs amplified a single qPCR product (Figure 2).

The three reference genes and those of the antioxidant enzymes were classified according to their abundant profiles: 1) *β-actin* (average Ct: 21.8), *rpl8* (average Ct: 25.02), *sod* (Ct average: 23.83) and *cat* (average Ct: 22.91) in medium level and 2) *gapdh* (Ct average 27.1) in low level of expression.

Table 2 shows the stability value of the three reference genes tested in five-days-old *C. latirostris* blood samples. The result obtained by ΔCt method, NormFinder and BestKeeper revealed that *β-actin* is the most stable gene and that *rpl8* and *gapdh* were least stable. The geNorm determined that the two most stable genes were *β-actin* and *rpl8*.

The RefFinder analysis ranked the gene stability according to the comprehensive ranking as follows: *β-actin* > *rpl8* > *gapdh*.

To evaluate the impact of the reference genes on the qPCR data, the expression of *sod* and *cat* genes was normalized with each one of them. Then, the ΔΔCt method was performed to visualize the mRNA abundances of antioxidant genes in normal conditions (Figure 3).

4. Discussion

The determination of the mRNA abundances of genes related to cellular stress belongs to a new family of biomarkers that can be used to detect exposure to xenobiotics, acting as early warning sensors. In this sense, in a previous work, our group standardized the technique for preservation and isolation of high-quality RNA from *C. latirostris* blood [28], which is the first necessary step to perform good transcriptomic studies. In this work, we identify and validate reference genes to obtain reliable and accurate data in qPCR from blood of *C. latirostris* hatchling. The evaluation and validation of reference genes in crocodilians is limited [26] and most gene studies have used a single reference gene without evaluating the stability or the expression of other housekeepings [40, 41, 42, 43, 44].

As there are no reports in mRNA abundances in blood of *C. latirostris*, we first verified that RNA extraction, designed primers, and efficiencies of PCR reactions were consistent with those recommended by the background literature. In this sense, we observed an approximate 260 nm/230 nm ratio of 1.90 for all samples, indicating RNA of good quality, without protein or phenolic contamination. The quantity and quality of RNA extracted from *C. latirotris* blood complied with the established standards to perform qPCR [46]. The quantification of mRNA abundances through qPCR can be affected by several factors, so it is extremely important to have good quantity and quality of RNA, an efficient cDNA synthesis, a good performance of the primers and the proper statistical analysis [47]. Several authors confirm that the primers are essential to guarantee a specific and efficient amplification of the products [48]. Taylor et al. [45], also indicate that the target sequences should be unique, 75–150 bp, have a GC content of 50–60%, not contain secondary structures and have a melting temperature of 55–65 °C. Besides, the dynamics of the reaction itself, the use of non-optimal reagent concentrations, the quality of the enzyme (which can generate efficiencies below 90%), and the presence of PCR inhibitors in one or more of the reagents can influence the efficiency of the reaction. In the present work, the primers designed meet the required standards and the curves were in the range between 96% and 110%, the slope between -3.114 and -3.399 and the linear correlation coefficient (R^2) between 0.992 and 0.999 (Table 1).

The normalization of mRNA abundances through the housekeeping gene is necessary to decrease the variation in RNA quantification and other experimental errors [46]. In this sense, different methods have been validated, such as the use of the comparative ΔCt, NormFinder, geNorm, BestKeeper and RefFinder. These methods permit the determination of the most stable gene or combination of genes in different experimental conditions, or life stages, in the species under study [30, 31]. *β-actin*, *rpl8* and *gapdh* are the most common reference genes used to normalize mRNA abundance data [49, 50]. Specifically, in crocodilians, there are no reports about validation of reference genes in blood using these methods. In the present study, we found that *β-actin* is the most stable reference gene under normal conditions. Similarly, studies in other species demonstrated that *β-actin* is the most stable gene in different tissues (liver, gill, muscle, spleen, kidney, heart, brain, intestine, skin) and conditions (under high-temperature stress, under normal physiological state, under exposure to metals, among others) [35, 51, 52]. *β-actin* is a gene universally expressed in most animal tissues and has long been used as an internal standard for gene expression analysis studies

Table 1. Gene identification, characterization of the designed primers and PCR reactions efficiencies.

Gene abbr.	Forward (F) and Reverse (R) primer sequence (5′–3′)	Amplicon Length (bp)	GC%	Amplification efficiency curve (%).	R^2 (linear standard)	Slope
Housekeeping						
β-actin	*F*: TCACGAGACCACTTTCAACTC *R*: AGGGCTGTGATTTCCTTCTG	138	F: 47.62 R: 50	102.83	0.999	-3.256
gapdh	*F*: GGCTGAGAATGGAAAACTTGTG *R*: TCCCCACTTGATGTTGCTG	82	F: 45.5 R: 46.2	109.48	0.996	-3.114
rpl8	*F*: CCAGAAGGCACCATTGTTTG *R*: ATAGTTTCCAGAAGCACGGG	78	F: 50 R: 50.2	103.68	0.992	-3.237
Genes involved in the antioxidant system pathways						
sod	*F*: GATGAGAGGCATGTTGGAG *R*: CCACCATGGTACGTCCA	124	F: 52.6 R: 58.8	96.86	0.999	-3.999
cat	*F*: TGAGCCTAGCCCTGATAAAATG *R*: CTCTGATAGTTAGCGACACGAG	135	F: 45.45 R: 50	101.53	0.996	- 3.28

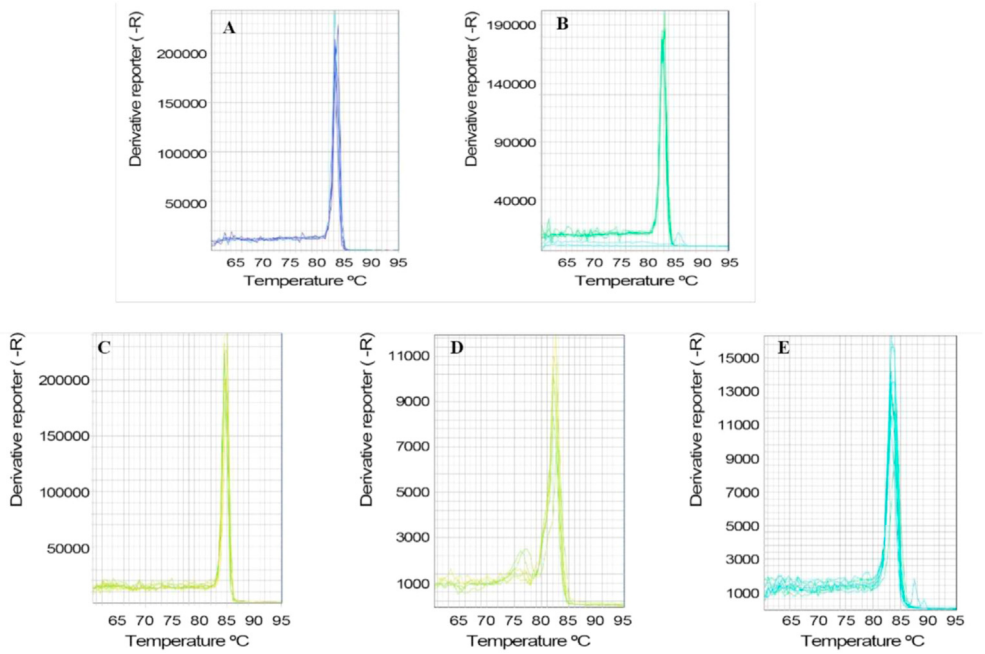

Figure 2. Melting curves generated for all genes. A: *cat*; B: *sod*; C: *β-actin*; D: *gapdh*; E: *rpl8*.

Table 2. Evaluation of stability of the reference genes in blood of *C. latirostris* hatchlings.

Reference genes	ΔCt		NormFinder		BestKeeper		geNorm	
	Mean SD[1]	Rank	Stability index	Rank	SD	Rank	M value	Rank
β-actin	0.69	1	0.201	1	0.204	1	0.58	1
rpl8	0.91	2	0.544	2	0.661	3	0.58	1
gapdh	1.021	3	0.666	3	0.442	2	0.874	2

[1] Mean standard deviation (SD).

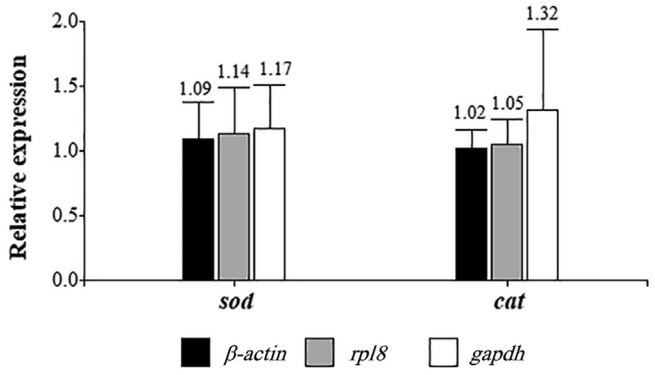

Figure 3. *sod* and *cat* mRNA abundances (mean ± SD) in blood of *C. latirostris* hatchlings, using different reference genes for normalization: *β-actin* (black bar), *rpl8* (grey bar) and *gapdh* (white bar).

[53, 54]. However, some studies reported that it shows less stability in certain conditions [55, 56].

rpl8 gene is a structural constituent of the 60S ribosomal subunit. This work demonstrated that it could be used as a reference gene because its moderate instability. Other studies showed that it can be useful as a normalizing gene depending on the species, the stage and the experimental conditions [4, 57].

gapdh gene is an important enzyme in the carbohydrate metabolism [58]. *gapdh* was the least stable gene in blood of *C. latirostris* hatchling. Similarly, several studies demonstrated low *gapdh* expression stability in certain life stages and tissues [56, 59], while other works showed that it can be suitable as a housekeeping [32, 60].

In general, these reports demonstrate that the mRNA abundances of reference genes can be affected by experimental treatments, stage of development and cell type [61, 62]. Therefore, there is no universal reference gene, so it is necessary to verify the expression levels of the candidate reference genes and prevent incorrect expression profiling [63].

In relation to target genes, in the present work we identified two genes related to the antioxidant defense systems (*cat* and *sod*) in order to propose them as potential biomarkers of oxidative stress induced by environmental stressors. This is the first report of the analysis of these genes in blood for all crocodilian species. The relative abundances of these two mRNA was evaluated through the three proposed housekeeping. We consider that both *β-actin* and *rpl8* could be used as normalizing genes for *sod* and *cat* in blood samples, while *gapdh* is less recommended because the relative expression level of *cat* increases considerably (1.32 with *gapdh* vs. 1.02 and 1.05 with *β-actin* and *rpl8*, respectively). For *sod* mRNA, the abundances are quite similar with the three normalizing genes. Moreover, when we observe the abundance of *gapdh* it is low (Ct average 27.1) compared to the other genes.

It is important to note that this work was done in blood of *C. latirostris* hatchlings under normal conditions. It is necessary to deepen the analysis of expression levels of reference and target genes in future studies, under different conditions and life stages of this species. Moreover, future research could compare these genes in different crocodilian species, in order to assess the usefulness of these techniques for other crocodilian species, maybe with little modifications.

The mRNA abundances of *sod* and *cat,* together with the other biomarkers of oxidative stress routinely applied by our group in the broad snouted caiman, would allow us to analyze, in an integrated way, the response induced in these animals to different xenobiotics. Besides, this study provides compelling methodologies in crocodilians for the selection, stabilization, and normalization of reference genes to obtain reliable and accurate data in the analysis of target mRNA abundance with real-time quantitative RT-PCR.

Declarations

Author contribution statement

Lucia M. Odetti: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Enrique V. Paravani, María F. Simoniello: Analyzed and interpreted the data; Wrote the paper.

Gisela L. Poletta: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2016-2020 to GLP), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 11220130100478CO to GLP) and Proyecto Yacaré - Yacarés Santafesinos (MMA/MUPCN).

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] D.G. Ginzinger, Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream, Exp. Hematol. 30 (2002) 503–512.
[2] S.A. Bustin, Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, J. Mol. Endocrinol. 29 (2002) 3–39.
[3] S.A. Bustin, et al., Quantitative real-time RT-PCR – a perspective, J. Mol. Endocrinol. 34 (2005) 597–601.
[4] H.K. Barman, et al., Identification and characterization of differentially expressed transcripts in the gills of freshwater prawn (*Macrobrachium rosenbergii*) under salt stress, Sci. World J. 2012 (2012) 1–11.
[5] A. Radonić, et al., Guideline to reference gene selection for quantitative real-time PCR, Biochem. Biophys. Res. Commun. 313 (2004) 856–862.
[6] S. Hibbeler, et al., Housekeeping genes for quantitative expression studies in the three spined stickleback *Gasterosteus aculeatus*, BMC Mol. Biol. 9 (2008) 18.
[7] X. Wu, et al., Variation of expression levels of seven housekeeping genes at different life-history stages in *Porphyra yezoensis*, PloS One 8 (2013) 60740.
[8] G.L. Poletta, et al., Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and the Micronucleus test, Mutat. Res. 672 (2009) 95–102.
[9] R. Van der Oost, et al., Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ Toxicol Pharmacol. 13 (2003) 57–149.
[10] M.N. Moore, et al., An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management, Mutat. Res. 552 (2004) 247–268.
[11] G.L. Poletta, et al., Genetic, enzymatic and developmental alterations observed in Caiman latirostris exposed *in ovo* to pesticide formulations and mixtures in an experiment simulating environmental exposure, Ecotoxicol. Environ. Saf. 74 (2011) 852–859.
[12] G.L. Poletta et al, Biomarkers of oxidative damage and antioxidant defense capacity in Caiman latirostris blood. Comp. Biochem. Physiol., C. 179 (2016) 29–36. https://doi.org/10.1016/j.cbpc.2015.08.003.
[13] P.A. Siroski, et al., Immunotoxicity of commercial-mixed glyphosate in broad snoutedcaiman (*Caiman latirostris*) Chemico-Biol, Interac 244 (2016) 64–70.
[14] E.C. Lopez Gonzalez, et al., Induction of micronuclei in broad snouted caiman (*Caiman latirostris*) hatchlings exposed *in vivo* to Roundup® (glyphosate) concentrations used in agriculture, Pestic. Biochem. Physiol. 105 (2013) 131–134.
[15] E.C. Lopez Gonzalez, et al., Micronuclei and other nuclear abnormalities on *Caiman latirostris* (Broad-snouted caiman) hatchlings after embryonic exposure to different pesticide formulations, Ecotoxicol. Environ. Saf. 136 (2017) 84–91.
[16] E.C. Lopez Gonzalez, et al., Genotoxicity induced by widely used pesticide binary mixtures on *Caiman latirostris* (Broad-snouted caiman), Chemosphere 232 (2019) 337–344.
[17] P.M. Burella, et al., Oxidative damage and antioxidant defense in *Caiman latirostris* (Broad snouted caiman) exposed *in ovo* to pesticide formulations, Ecotoxicol. Environ. Saf. 161 (2018) 437–443.
[18] L.M. Odetti, et al., Genotoxicity and oxidative stress in Caiman latirostris hatchlings exposed to pesticide formulations and their mixtures during incubation period, Ecotoxicol. Environ. Saf. 193 (2020) 110312.
[19] S. Ullah, M.J. Zorrieh-Zahra, Ecotoxicology: a review of pesticides induced toxicity in fish, Adv. Anim. Vet. Sci. 3 (2015) 40–57.
[20] M.J. Aardema, J.T MacGregor, Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies, Mutat. Res. 499 (2003) 13–25.
[21] M. Ghelichpoura, et al., Expression of immune, antioxidant and stress related genes indifferent organs of common carp exposed to indoxacarb, Aquat. Toxicol. 208 (2019) 208–216.
[22] P.M. Craig, et al., Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*), Am. J. Physiol. Regul. 293 (2007) 1882–1892.
[23] H. Xing, et al., Effects of atrazine and chlorpyrifos on cytochrome P450 in common carp liver, Chemosphere 104 (2014) 244–250.
[24] Z. Zhang, et al., Chlorpyrifos exposure in common carp (*Cyprinus carpio L.*) leads to oxidative stress and immune responses, Fish Shellfish Immunol. 67 (2017) 604–611.
[25] M. Velki, et al., Pesticides diazinon and diuron increase glutathione levels and affect multixenobiotic resistance activity and biomarker responses in zebrafsh (*Danio rerio*) embryos and larvae, Environ. Sci. Eur. 31 (2019) 1–18.
[26] S. Kohno, et al., Stress responses in the chemistry and mRNA abundance of the peripheral blood in the American alligator, J. Exp. Zool. 333 (2019) 151–163.
[27] J.G. Myburgh, et al., The post-occipital spinal venous sinus of the Nile crocodile (*Crocodylus niloticus*): its anatomy and use for blood sample collection and intravenous infusions, J. S. Afr. Vet. Assoc. 85 (2014) 19.
[28] E.C. Lopez-Gónzalez, et al., Optimizing protocols for high-quality RNA extraction from blood and liver tissues of the Broad-snouted caiman, Russ. J. Herpetol. (1802) in press.
[29] E. Angeli, et al., Protein and gene expression of relevant enzymes and nuclear receptor of hepatic lipid metabolism in grazing dairy cattle during the transition period, Res. Vet. Sci. 123 (2019) 223–231.
[30] P. Sujiwattanarat, et al., Molecular cloning and characterization of Siamese crocodile (*Crocodylus siamensis*) copper, zinc superoxide dismutase (CSI-Cu,Zn-SOD) gene. Comparative Biochemistry and Physiology, Part. A. 191 (2016) 187–195.
[31] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\triangle\triangle}$ Ct Method, Methods 25 (2001) 402–408.
[32] N. Silver, et al., Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR, BMC Mol. Biol. 7 (2016) 33.
[33] D. Chen, et al., Evaluation and identification of reliable reference genes for pharmacogenomics, toxicogenomics, and small RNA expression analysis, J. Cell. Physiol. 226 (2011) 2469–2477.
[34] C.L. Andersen, et al., 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 (2004) 5245–5250.
[35] K. Shekh, et al., Expression stability and selection of optimal reference genes for gene expression normalization in early life stage rainbow trout exposed to cadmium and copper, Aquat. Toxicol. 190 (2017) 217–227.
[36] J. Vandesompele, et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) 1–12.
[37] M.W. Pfaffl, et al., Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper — excel-based tool using pair-wise correlations, Biotechnol. Lett. 26 (2004) 509–515.
[38] F. Xie, et al., miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs, Plant Mol. Biol. 80 (2012) 75–84.
[39] SPSS for Windows, SPSS Inc., Chicago, USA, 2008. Version 14.0.
[40] S. Kohno, et al., Gene expression patterns in juvenile American alligators (*Alligator mississippiensis*) exposed to environmental contaminants, Aquat. Toxicol. 88 (2008) 95–101.
[41] B.C. Moore, et al., Gonadal mRNA expression levels of TGF superfamily signaling factors correspond with post-hatching morphological development in American alligators, Sex Dev. 4 (2010) 62–72.
[42] K. Jin-He, et al., Apoptosis mechanism of human cholangiocarcinoma cells induced by bile extract from crocodile, Appl. Biochem. Biotechnol. 166 (2012) 942–951.
[43] M. Hale, et al., AHR and CYP1A expression link historical contamination events to modern day developmental effects in the American alligator, Environ. Pollut. 230 (2017) 1050–1061.
[44] A. Martínez-Juárez, et al., Expression of the Sox9, Foxl2, Vasa, and TRPV4 genes in the ovaries and testes of the Morelet's crocodile, Crocodylus moreletii, J. Exp. Zool. 330 (2018) 148–164.

[45] S. Taylor, et al., A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines, Methods 50 (2010) S1–S5.
[46] S.A. Bustin, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiment, Clin. Chem. 55 (2009) 611–622.
[47] A, Maroufi, et al., Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR, BMC Mol. Biol. (2010) 11–15.
[48] S. Bustin, J. Hugget, qPCR primer design revisited, Biomol. Detection Quantification 14 (2017) 19–28.
[49] R. Barber, et al., GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues, Physiol. Genom. 21 (2005) 389–395.
[50] J. Huggett, et al., Real-time RT-PCR normalization; strategies and considerations, Gene Immun. 6 (2005) 279–284.
[51] G. Purohit, et al., Evaluation of housekeeping genes as references for quantitative real-time PCR analysis of gene expression in the murrel *Channa striatus* under high-temperature stress, Fish Physiol. Biochem. 42 (2016) 125–135.
[52] W. Zheng, L. Sun, Evaluation of housekeeping genes as references for quantitative real time RT-PCR analysis of gene expression in Japanese flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 30 (2011) 638–645.
[53] M. Sopezki, et al., Validation of housekeeping genes as internal controls for the study of the effects of microcystin-LR in zebrafish by real-time PCR, Zebrafish (2018).
[54] Y. Li, et al., Transcriptome-based evaluation and validation of suitable housekeeping gene for quantification real-time PCR under specific experiment condition in teleost fishes, Fish Shellfish Immunol. 98 (2020) 218–223.
[55] E.M. Glare, et al., b-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalizing mRNA levels, Thorax. 57 (2002) 765–770.
[56] M. Jaramillo, et al., Identification and evaluation of reference genes for expression studies by RT-qPCR during embryonic development of the emerging model organism, *Macrobrachium olfersii*, Gene 598 (2017) 97–106.
[57] Q. Xu, Y. Liu, Gene expression profiles of the swimming crab *Portunus trituberculatus* exposed to salinity stress, Mar. Biol. 158 (2011) 2161–2172.
[58] M.E. Campanella, et al., Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane[J], Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 2402–2407.
[59] W. Sun, et al., Suitable reference gene selection for different strains and developmental stages of the carmine spider mite, *Tetranychus cinnabarinus*, using quantitative real-time PCR, J. Insect Sci. 10 (2010) 208.
[60] J. Shim, Keeping house: evaluation of housekeeping genes for real-time PCR in the red alga, *Bostrychia moritziana* (Florideophyceae), ALGAE 31 (2016) 167–174.
[61] D.W. Liu, Choice of endogenous control for gene expression in no small cell lung cancer[J], Eur. Respir. J. 26 (2005) l002–l008.
[62] A.T. McCurley, G.V. Callard, Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment, BMC Mol. Biol. 9 (2008) 102.
[63] A.L. Filby, C.R. Tyler, Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish, BMC Mol. Biol. 8 (2007) 10.