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Supplementary material

Appendix 1

Table A1. GeneBank accession numbers of the sequences in other avian species used for the design of primers for qPCR for each gene in the study. Note that GAPDH and 18S were not used in the final analysis.

Gene	GeneBank accession numbers
SOD2	XM_010304404, XM_009696177, XM_009895538, XM_010120246, XM_010152840, XM_010193766
ALKBH3	XM_009895697, XM_010309137, XM_010125066, XM_010190876, XM_009702370, XM_009935354
HSPA8	XM_009890778, XM_009942237, XM_010192971, XM_010302520
NRLC5	XM_009894490, XM_010311015, XM_010117637, XM_010183872, XM_010154816, XM_009933622
TRIAP1	XM_009888591, XM_010305237, XM_010115739, XM_010180898, XM_009708736, XM_009936753
ACTB	XM_010121721, XM_010307594, XM_009889215, XM_010183222
GAPDH	XM_009892554, XM_009932317, XM_010309883, XM_010128473, XM_010190128
18S	AF173637, AF173638, AF173632

Table A2. Primer pairs of each gene used in the qPCR assays. Primer sequences, product sizes, final concentration in the qPCR reactions and efficiencies are reported. Note that GAPDH and 18S were not used in the final analysis.

Gene	Primer (5'-3')	Product size (bp)	Final concentration (mM)	Efficiency
SOD2	F: AAGGTGATGTTACAGCTCAGG R: CAGCTGTCAACTTCTCCTTG	188	300	1.91
ALKBH3	F: TTTGAGGAACCAAGGCTTAC R: GAGAAGGGAGTTGAAGGTATAGC	152	400	1.85
HSPA8	F: GAGAAGGAGGAGTTTGAGCAC R: TTTAGTCCACCTCCTCGATG	180	400	1.90
NLRC5	F: CCGTATGTCCATATCTGAGCTC R: CAACCTTCTCATGGTCAAACC	160	300	1.90
TRIAP1	F: AAGCGATCAAGGAGAAGGAC R: CCTAGAGAGCAGCTTAACAAAGG	192	400	1.91
ACTB	F: GAAATTGTGCGTGACATCAAG R: GGACTCCATACCCAAGAAAGATG	194	400	1.90
GAPDH	F: CAAGGCTGAGAATGGGAAAC R: GGCAGAGATGATAACACGCTTAG	189	400	1.94
18S	F: GGACAGGATTGACAGATTGAGAG R: ACGCCACTTGTCCCTCTAAG	179	400	1.87

Stability of reference genes

ACTB (*beta-actin*), GAPDH (*glyceraldehyde 3-phosphate dehydrogenase*) and ribosomal 18S genes were chosen as candidate reference genes, as used in previous studies of gene expression in birds (e.g. Jenko et al. 2012; Olias et al. 2014). Stability of reference genes was tested using a subset of 12 samples of the four experimental groups: first-hatched chicks from first-laid eggs; last-hatched chicks from last-laid eggs; first-hatched chicks from last-laid eggs and last-hatched chicks from first-laid eggs (N = 3 for each group). Samples were run in duplicate, and two negative controls were included for each gene. The amplification efficiency of reference genes was calculated using LinRegPCR (Ruijter et al. 2009) and their stability was analysed with BestKeeper (Pfaffl et al. 2004), NormFinder (Andersen et al. 2004) and GeNorm (Vandesompele et al. 2002). LinRegPCR analyses showed high mean efficiency values of all reference genes (efficiency average ± SD: ACTB = 1.88 ± 0.03; GAPDH = 1.94 ± 0.03; 18S = 1.87 ±0.02). ACTB was ranked as the most stable gene BestKeeper, NormFinder and GeNorm when samples were grouped by hatching order (Table A3). Moreover, when samples were grouped by laying order, NormFinder recommended the combination of GAPDH and 18S, but leaving ACTB as the most stable gene with similar stability value than the combination of the two genes (Table A3). Linear Models (LMs) showed no significant effect of laying order nor hatching order on the Cq mean of GAPDH, ACTB and 18S (Table A4). Thus, basing on the efficiency, stability values and LMs (see Material and methods), we selected ACTB as the best reference gene for normalisation of the expression of the target genes in our experiment.

Table A3. Stability ranking of the three reference genes tested in this study. Genes are ranked from the most to the least stable (1-4, respectively).

Rank	BestKeeper (SD)	BestKeeper (CV%)	NormFinder (HO)	NormFinder (LO)	Genorm
1	ACTB (1.35)	GAPDH (4.94)	ACTB (0.19)	GAPDH/18S (0.25)	ACTB (0.89)
2	GAPDH (1.41)	ACTB (5.62)	GAPDH (0.30)	ACTB (0.27)	GAPDH (0.99)
3	18S_2 (1.72)	18S_2 (20.88)	18S_2 (0.40)	GAPDH (0.43)	18S_2 (1.22)
4	-	-	-	18S_2 (0.60)	-

Stability values of each reference genes obtained by three different methods are shown in brackets. As NormFinder allows specifying the experimental group of the samples, results grouping for hatching order (HO) and laying order (LO) are shown. Standard Deviation (SD) and Coefficient of Variation (CV%) are shown for BestKeeper.

Table A4. Summary of the LMs of the quantification cycle (Cq) of the three candidate reference genes.

Variable	Source of variation	Estimate (SE)	d.f.	*F*	*p*
ACTB mean Cq	Intercept	23.85 (0.94)	1,9		
	Laying order	-0.13 (1.09)		0.01	0.911
	Hatching order	0.43 (1.09)		0.15	0.704
GAPDH mean Cq	Intercept	28.02 (1.00)	1,9		
	Laying order	0.23 (1.16)		0.04	0.849
	Hatching order	0.67 (1.16)		0.33	0.578
18S mean Cq	Intercept	9.32 (1.05)	1,9		
	Laying order	-1.65 (1.21)		1.85	0.207
	Hatching order	-0.49 (1.21)		0.16	0.695

Table A5. GenBank accession numbers of partial mRNA sequences of the studied genes and other genes sequenced during the tune-up of the study.

Abbreviation	Gene	GenBank accession number
SOD2	Superoxide dismutase 2, mitochondrial	KY905344
TRIAP1	TP53 regulated inhibitor of apoptosis 1	KY905345
NLRC5	NLR family CARD domain containing 5	KY905346
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	KY905347
FANCL	Fanconi anemia, complementation group L	KY905348
SOD1	Superoxide dismutase 1, soluble	KY905349
IL7	Interleukin 7	KY905350
SIRT1	Sirtuin 1	KY905351
CASP7	Caspase 7	KY905352
FTO	Fat mass and obesity associated gene	KY905353
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KY905354
ABL1	ABL proto-oncogene 1, non-receptor tyrosine kinase	KY905355
HMOX1	Heme oxygenase 1	KY905356

Table A6. Short nucleotide sequences (less than 200 nucleotide long) of partial mRNA or rRNA of the studied genes and other genes sequenced during the tune-up of the study (note that sequences less than 200 nucleotides long cannot be submitted to GenBank).

Abbreviation	Gene	Nucleotide sequence 5'-3'
ALKBH3	AlkB, alkylation repair homolog 3 (E. coli)	CAGGAAGTATCTTTTGAGGAACCAAGGCTTACCTCCTGGTATGGGGAACT TCCTTACACGTACTCCAGGATGACAATGCAGCCAAACCCAAACTGGCATC CTCTGCTGACCATGCTTAAGGAGCGCATTGAAGAGTTCAGTGGCTATACC TTCAACTCCCTTCTC
18S	18S RNA	GGACAGGATTGACAGATTGAGAGCTCTTTCTCGATTCCGTGGGTGGTGGT GCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAA CGAACGAGACTCTGGCATGCTAACTAGTTACGCGACCCCCGAGCGGTCGG CGTCCAACTTCTTAGAGGGACAAGTGGCGT
ACTB	Actin, beta	ATTGTGCGTGACATCAAGGAGAAGCTGGTCTATGTTGCCCTGGATTTTGA GCAGGAGATGGCCACAGCTGCCTCTAGCTCTTCTCTGGAGAAGAGCTATG AACTCCCTGATGGCCAGGTCATCACCATTGGCAACGAGAGGTTCAGGTGC CCTGAGGCCCTCTTCCAGCCATCTTTCTTGGGTATGGAGTCC
HSPA8	Heat shock 70kDa protein 8	TCAGACGGCCGAGAAGGAGGAGTTTGAGCACCAGCAGAAGGAGCTGGAGA AGGTGTGCAACCCCATAATCACCAAGCTGTACCAGAGTGCAGGAGGAATG CCTGGTGGGATGCCTGGTGGATTCCCTGGTGGTGGAGCTCCTCCATCTGG TGGAGCTTCATCTGGACCAACCATCGAGGAGGTGGACTAAAGGCAC

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