Antioxidants safeguard telomeres in bold chicks (by S.-Y. Kim & A. Velando)

SUPPLEMENTARY MATERIAL

Telomere length assay

Telomere length was measured in DNA from red-blood cells using quantitative PCR (qPCR) on a StepOnePlus (Applied Biosystems). DNA was extracted by using DNeasy Blood and tissue kit and following the manufacturer's instructions (Qiagen). The quantity and purity of the genomic DNA were measured using Take3 on a Synergy microplate reader spectrophotometer (BioTek). We estimated telomere length as the ratio (T/S) of telomere repeat copy number (T) to single gene copy number (S), relative to a reference (golden) sample [1]. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control, single copy gene, with primer sequences designed on PRIMER EXPRESS (Applied Biosystems) from *Larus michahellis* GADPH gene intron (GenBank: FM209949.1; Forward GGAPDH1: 5'-GGAGGGTGTATGGAATTCTTCCT-3'; Reverse GGAPDH2: 5'-CACAACCGAACCACTCAGCT-3'). The telomeres were amplified using the following primers forward tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and reverse tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). Melt curve analysis indicated that a single amplicon was generated by qPCR with the designed GAPDH primers as also confirmed in an electrophoresis analyses (figure S1).

For the quantitative PCR assay, we used 10 ng of DNA per reaction and at a concentration of 150 µM. Primers were mixed with 10 µl Luminaris Color HiGreen High ROX qPCR Master Mix (ThermoScientific) for a total volume of 20 µl. Telomere and GAPDH reactions were performed on separate plates, and we added 1 µl of betaïne in the telomere reaction. The qPCR conditions for GAPDH were: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C and 30 s at 72°C; conditions for telomeres were: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 34 s at 60°C and 30 s at 72°C.

A pool of three individual samples was used as reference 'golden' sample, which was serially diluted to produce a standard curve (40 ng, 20 ng, 10 ng, 5 ng of

DNA per well) in triplicate on every plate. Our samples always fell within the limits of the standard curves. We ran 20 plates (10 plates for each primer set) and the mean reaction efficiencies were 104.5 (range 95.45–111.0) for telomeres and 103.12 (range 94.5–110.5) for GAPDH. The golden sample was also used to calculate both the within- and among-plate variations. Mean intra-assay variations (CV) of the C_q values (quantification cycles, [2]) for telomere and GAPDH reactions were 0.7 and 0.3, respectively, and inter-assay variations (CV) were 2.8 and 1.1. Samples were ran in duplicate and we used the mean C_q values of the duplicates in all calculations (C_q values, mean ± SD; telomere: 17.76 ± 0.65; GAPDH: 21.11 ± 0.52). Three wells (one in one telomere plate and two in two GAPDH plates) failed to produce positive amplicons. Duplicates were highly repeatable (average intra-class correlation coefficient [95% interval]; telomere: 0.93 [0.90-94], $p < 0.001$; GAPDH: 0.97 [0.96-98], $p < 0.001$, $n = 220$). T/S ratio of each sample was calculated using the mean value as: $(1+Ef_{\text{tel}})^{\Delta C_q\ \text{tel}}/(1+Ef_{\text{GAPDH}})^{\Delta C_t\ \text{GAPDH}}$. Here, Ef represents the plate efficiency, and ΔC_q the difference in C_q-values between the golden sample and the focal sample.

Since the quantitative PCR assay measures the total number of telomeric repeats [3], our measure of the telomere length (T/S ratio) includes interstitial repeats that do not change with time. A previous study has shown that interstitial repeats were not a significant problem in the estimation of telomere length in a similar gull species (*Larus fuscus*) to our study species [4]. Nevertheless, if there is a large variation in interstitial telomere signal among- and within-individual levels, this may add noise to our telomere length data. However, probably it is unlikely that this variation, if any, confound our results unless interstitial repeats varied between randomly allocated samples to the two experimental treatments.

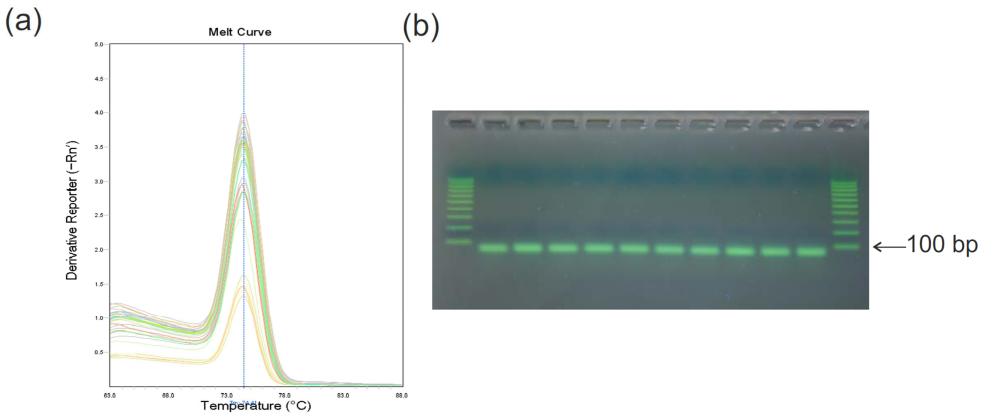

Figure S1. Validation of reaction products obtained from the designed gull GAPDH primers. (a) Melt-curve analysis showing the first derivative of the change in fluorescence intensity as a function of temperature. (b) Electrophoresis analyses of the reaction products, which were separated in 2% agarose gel, and visualized with Red Safe Nucleic Acid Staining Solution (Intron Biotechnology). First and last lanes, molecular weight markers 100-1,000 bp in 100 bp increments; the rest (lanes 2-11), single PCR products using the designed gull GAPDH primers corresponding to ten different chicks. This single product was around 69 bp, as confirmed in an additional electrophoresis analysis with a 50bp increment rule. This was the predicted amplicon size based on the alignment of primers of the yellow-legged gull GAPDH gene sequence.

Table S1. Summary of the full and final mixed models of tonic immobility, growth rate and telomere length (*n* = 212 chicks).

dependent variable	source of variation	full model				final model			
		estimate (SE)	*F*	*d.f.*	*p*-value	estimate (SE)	*F*	*d.f.*	*p*-value
tonic immobility	intercept	-1.70 (5.55)				0.013 (0.17)			
	treatment (control)	-6.11 (9.21)	0.36	1,48.96	0.552				
	sex (male)	-0.66 (0.44)	0.26	1,200	0.614				
	hatching date	0.19 (0.13)	0.12	1,44.34	0.726				
	hatching order (A)	0.23 (0.60)	1.60	2,200	0.205				
	hatching order (B)	0.56 (0.57)							
	egg volume	0.00003 (0.00004)	1.62	1,156.6	0.205				
	treatment x sex	0.95 (0.63)	2.22	1,200	0.132				
	treatment x hatching date	0.12 (0.28)	0.17	1,44.34	0.679				
	treatment x hatching order (A)	0.32 (0.91)	0.09	2,200	0.911				
	treatment x hatching order (B)	0.37 (0.86)							
	treatment x egg volume	0.00002 (0.00006)	0.12	1,156.6	0.731				
	random factors[1]								
	original nest (cross-foster group)					0.13 (0.31)			0.655
	foster nest (cross-foster group)					0			1.0
	cross-foster group					0.25 (0.23)			0.195
growth rate	Intercept	0.26 (0.09)				0.33 (0.052)			
	treatment (control)	-0.0016 (0.16)	0.02	1,130	0.900				
	sex (male)	-0.0087 (0.0094)	0.13	1,155	0.723				
	hatching date	-0.0073 (0.0026)	8.49	1,124	0.004	-0.008 (0.002)	20.91	1,202	<0.001
	hatching order (A)	-0.0087 (0.0094)	1.81	2,115	0.169				
	hatching order (B)	-0.0005 (0.0087)							
	egg volume	$5.84 \cdot 10^{-7}$ (0)	0.64	1,111	0.424				
	treatment x sex	0.010 (0.0097)	1.10	1,155	0.297				
	treatment x hatching date	0.00073 (0.0047)	0.88	1,124	0.605				
	treatment x hatching order (A)	0.025 (0.014)	1.83	2,115	0.164				

	treatment x hatching order (B)	0.022 (0.13)							
	treatment x egg volume	$-4.59 \cdot 10^{-7}$ (0)	0.25	1,111	0.606				
	random factors[1]								
	original nest (cross-foster group)					$1.56 \cdot 10^{-21}$ (0)			1.0
	foster nest (cross-foster group)					0.007 (0.0002)			<0.001
	cross-foster group					0			1.0
telomere length	intercept	0.87 (1.57)				1.41 (0.14)			
	treatment (control)	1.63 (2.42)	0.50	1,196	0.480	-0.23 (0.14)	0.09	1,206	0.758
	tonic immobility (bold)	-0.0099 (0.26)	0.44	1,196	0.510	-0.0099 (0.13)	4.01	1,206	0.047
	growth	2.75 (1.81)	5.20	1,196	0.024	2.66 (1.15)	5.33	1,206	0.022
	sex (male)	0.086 (0.14)	4.42	1,196	0.037	0.190 (0.095)	3.94	1,206	0.048
	hatching date	0.016 (0.041)	0.45	1,196	0.502				
	hatching order (A)	0.14 (0.19)	1.12	2,196	0.327				
	hatching order (B)	0.085 (0.18)		1,196					
	egg volume	$2.91 \cdot 10^{-7}$ (0.00001)	0.29	1,196	0.590				
	treatment x tonic immobility	0.35 (0.20)	3.17	1,196	0.076	0.40 (0.19)	4.41	1,206	0.037
	treatment x growth	0.18 (2.39)	0.01	1,196	0.941				
	treatment x sex	0.24 (0.20)	1.53	1,196	0.217				
	treatment x hatching date	-0.084 (0.073)	1.31	1,196	0.253				
	treatment x hatching order (A)	-0.28 (0.28)	1.57	2,196	0.211				
	treatment x hatching order (B)	-0.48 (0.27)		1,196					
	treatment x egg volume	$8.47 \cdot 10^{-6}$ (0.00001)	0.25	1,196	0.617				
	random factors[1]								
	original nest (cross-foster group)					0			1.0
	foster nest (cross-foster group)					0			1.0
	cross-foster group					$4.92 \cdot 10^{-20}$			1.0

[1] *p*-values of random effects were estimated in the final models by the likelihood ratio test

References

1. Cawthon RM. 2002 Telomere measurement by quantitative PCR. *Nucleic Acids Res.* **30**, e47-e47.
2. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009 The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611-622.
3. Nakagawa S, Gemmell NJ, Burke T. 2004 Measuring telomeres: applications and limitations. *Mol. Ecol*. **13**, 2523–2533.
4. Foote CG, Gault EA, Nasir L. Monaghan P. 2011 Telomere dynamics in relation to early growth conditions in the wild in the lesser black-backed gull. *J. Zool.* **283**, 203–209.