ESM Supplementary methods and results of:

'Pace and stability of embryonic development affect telomere dynamics: an experimental study in a precocial bird model'

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ESM1: Incubation temperature treatment groups

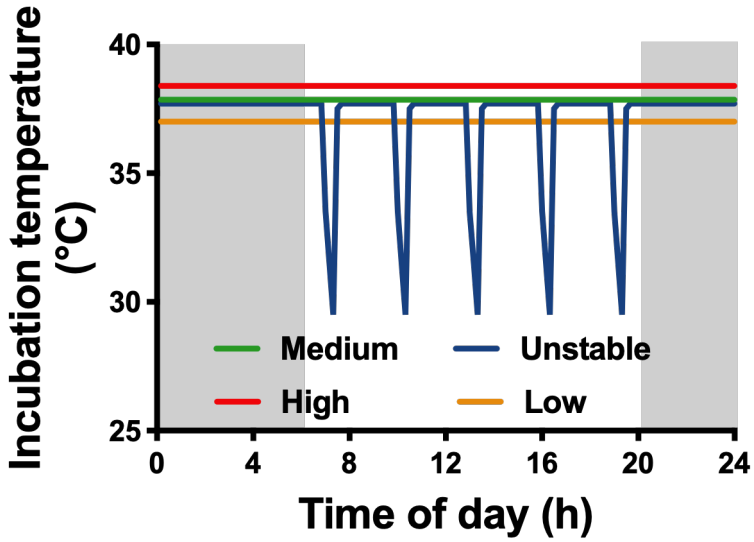

Fig S1: Incubation temperature treatments. Grey and white areas represent dark and light periods respectively. The Unstable group was incubated at the same temperature as the Medium group, but experienced 5 incubation recesses of 30min per day so that its average daily temperature was the same as the Low group.

ESM2: Methods for measurement of embryo heart rate and plasma corticosterone

Embryo heart rate

We measured embryo heart rate, as an indicator of embryo metabolism, using a non-invasive infrared methodology (Buddy, Avitronics, Truro, UK; [1]) on the 60 eggs being sacrificed at stED13 to control for known effects of developmental stage on embryo heart rate [1]. Heart rate was however measured one day earlier (*i.e.* stED12) to avoid interference with plasma sampling for corticosterone measurement at stED13 (see below). Measurements were always conducted within 90s of taking the egg out of the incubator (*i.e.* to limit cooling down) and never conducted during incubation recess or the following 30min for the Unstable group.

To ascertain that unstable incubation treatment induced developmental instability (*i.e.* metabolism/growth deceleration during incubation recess periods), we measured heart rate at ED6 on eggs from M and U group that were sacrificed at ED13 both before (*i.e.* 37.7°C) and halfway through the incubation recess (*i.e.* 15 min after incubator switch-off; *ca.* 32°C) for the unstable group. While heart rate was stable in the medium temperature group (*i.e.* not experiencing incubation recess), it significantly decreased by *ca.* 15% in the unstable temperature group (Fig S1, Time*Treatment interaction, GEE: Wald-$\chi^2_{1,1}$ = 57.3, p < 0.001).

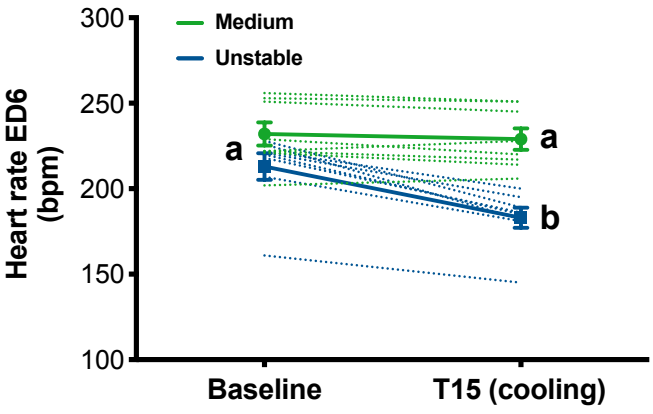

Fig S2: Effect of incubation recess on embryo (ED6) heart rate. The unstable temperature group experienced a 15 min incubation recess, while the medium temperature group was incubated at the same temperature (37.7°C) but did not experience incubation recess. Data plotted as individual responses (dotted lines) and means ± SE (joined by solid lines). Details of statistical tests are given in the text, letters indicate significant differences according to post-hoc tests (N = 8 / group).

Plasma corticosterone measurement in embryos

Corticosterone (CORT) is the main glucocorticoid hormone in birds. Plasma total CORT levels of embryos at stED13 were determined by immunoassay according to guidelines provided by the manufacturer (DetectX® Corticosterone Enzyme Immunoassay Kit, Arbor Assays, USA). To ensure sampled plasma CORT levels were at baseline levels, the time interval from taking the egg out of the incubator to euthanasia and blood sampling was always kept < 3min (mean interval = 1'40'' ± 21''), and embryos from the Unstable group were not sampled during incubation recess or the following 30min. The intra-individual coefficient of variation based on duplicates was 8.00 ± 2.36% and inter-plate coefficient of variation based on one repeated sample over plates was 10.37%.

ESM3: Methods for measurement of DNA damage and telomere length

DNA damage in RBCs

8-OHdG is one of the predominant forms of free radical-induced oxidative lesions in DNA, and widely used as a marker of oxidative damage [2]. Levels of 8-OHdG in RBC DNA was quantified using a competitive immunoassay (300ng DNA, EpiQuick 8-OHdG DNA Damage Quantification Direct Kit Colorimetric, Epigentek, USA) following the manufacturer recommendations. RBC DNA damage is expressed as pg of 8-OHdG/ng of DNA. The intra-individual coefficient of variation based on duplicates was 7.91 ± 1.10% and inter-plate coefficient of variation based on one sample repeated over all plates was 8.38%. A few samples (n = 6) did not contain sufficient DNA for the assay.

Telomere length determination using Terminal Restriction Fragment (TRF)

Different methods have been used to measure telomere length, the two most common being qPCR and TRF (Terminal Restriction Fragment), the latter being considered as the gold-standard methodology in the field [3]. While trying to validate the qPCR approach for use in Japanese quail by comparing results of qPCR to *in-gel* and denatured TRF (see [4] for details), we noticed the presence of large amounts of interstitial telomeric sequences (ITS), and a pronounced variability in ITS amount between samples. Thereby, methods that could not distinguish between true telomeres and ITS (*i.e.* qPCR and denatured TRF) were found to be poorly indicative of true telomere length in this species, and so we opted for using *in-gel* TRF. We followed a published protocol that has been used successfully in numerous avian species [5]. In brief, DNA was extracted from 5 µL of RBCs using the Gentra Puregene Tissue Kit (Qiagen) and kept frozen at -80°C until analysis. Digestion with restriction enzymes was then conducted on 10µg of DNA using *Hae III* (75U), *Hinf I* (15U), and *Rsa I* (40U) in 1X CutSmart enzyme-buffer

and incubated overnight at 37°C. DNA samples and ^{32}P labelled size ladder (2-40kb, 1 kb DNA Extension Ladder Invitrogen) were then loaded into a 0.8% non-denaturing agarose gel. DNA was separated using pulse-field gel electrophoresis (14°C at 3 V cm^{-1}, initial switch time 0.5 seconds, final switch time 7.0 seconds) for 19 hours, followed by in-gel hybridization overnight with the ^{32}P γ-ATP probe $(5'\text{-CCCTAA-}3')_4$. Hybridized gels were placed on a phosphor screen for 48 hours, and subsequently scanned with a Typhoon Variable Mode Imager (Amersham Biosciences). Finally, average telomere length was quantified by densitometry in the program ImageJ (version 2.0) within the limits of our molecular size markers (2-40kb). Samples were run over 13 different gels with one standard sample being present in duplicate in each gel to ensure between-gel consistency. Different RBC samples from the same individual (*i.e.* D1, D20 and D60 from a given bird) were always placed on the same gel to ensure the quality of longitudinal data, especially considering the moderate within-individual changes compared to between-individual differences (see ESM3 for details). Experimental groups were balanced within each gel. The intra-gel CV based on duplicates was 2.54 ± 0.40%, and inter-gel CV based on the repeated standard sample was 3.16%.

ESM4: Postnatal growth

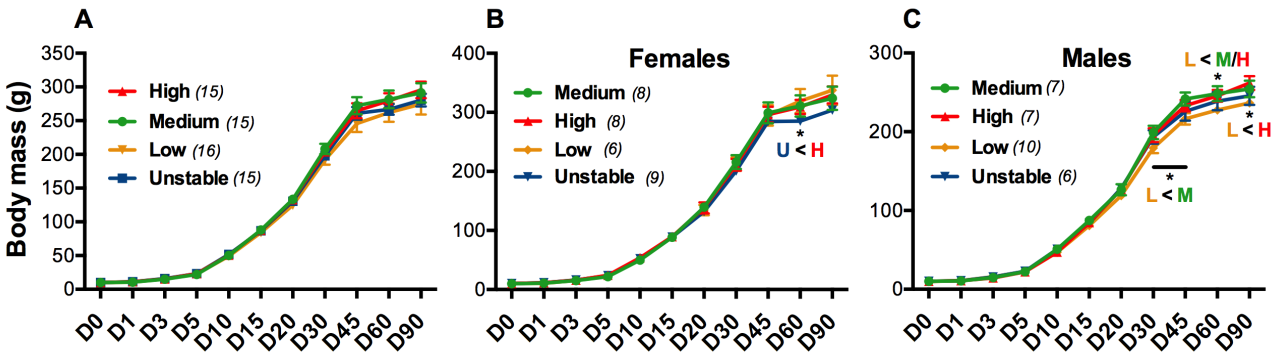

Fig S3: Postnatal body mass growth curves of (A) all individuals, (B) female individuals, (C) male individuals. Data plotted as means ± SE. Details of statistical tests are given in Table S2. * in B and C indicate significant differences according to GEE post-hoc tests. Numbers in brackets represent sample size.

Table S1: Summary of the most parsimonious GEE models explaining the variability in postnatal body mass growth rate. Estimates for fixed factors are given for the following levels: Age = D0, Treatment = medium and Sex = female. Significant factors are presented in bold.

Body mass growth	Estimate	SE	df	Wald-χ^2	p-value
Intercept	245,7	10.6	1	4848.0	**< 0.001**
Age	-235.5	10.4	10	6132.3	**< 0.001**
Treatment	8.6	14.4	3	2.0	0.58
Sex	58.1	12.3	1	39.2	**< 0.001**
Age x Treatment	-8.6	14.1	30	115.3	**< 0.001**
Age x Sex	-58.3	12.3	10	204.7	**< 0.001**
Treatment x Sex					ns
Age x Treatment x Sex	-0.1	0.7	33	63.4	**< 0.001**

ESM5: Telomere length and dynamics

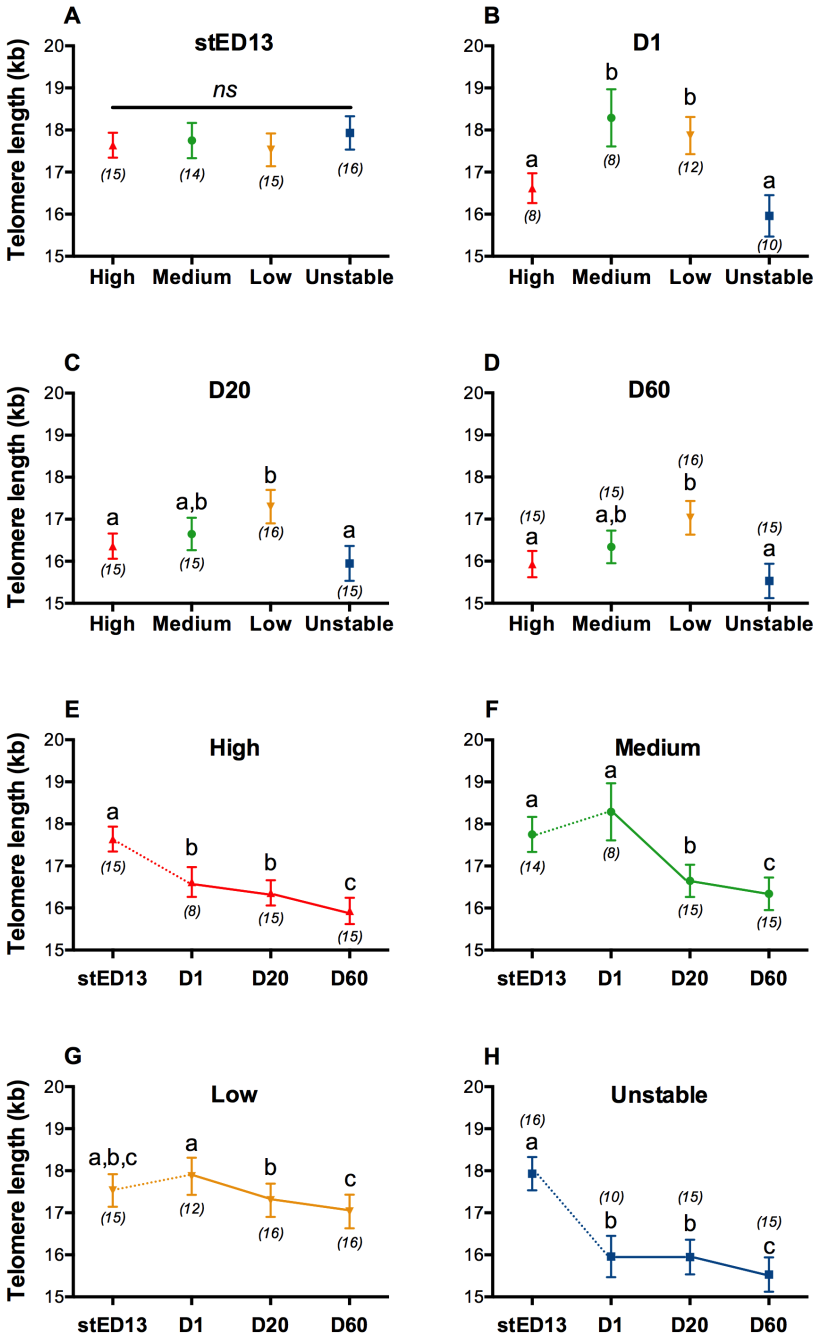

Fig S4: Telomere length in red blood cells according to prenatal experimental conditions and age. stED13 represents a standardized developmental stage between experimental groups as explained in the methods**. (A-D) Telomere length between experimental groups at each stage. (E-H) Age-related changes in telomere length for each experimental group**. Data plotted as means ± SE. Solid lines indicate longitudinal data and dotted lines cross-sectional data, letters indicate significant differences according to post-hoc tests, and numbers in brackets represent sample sizes.

ESM6: DNA damage

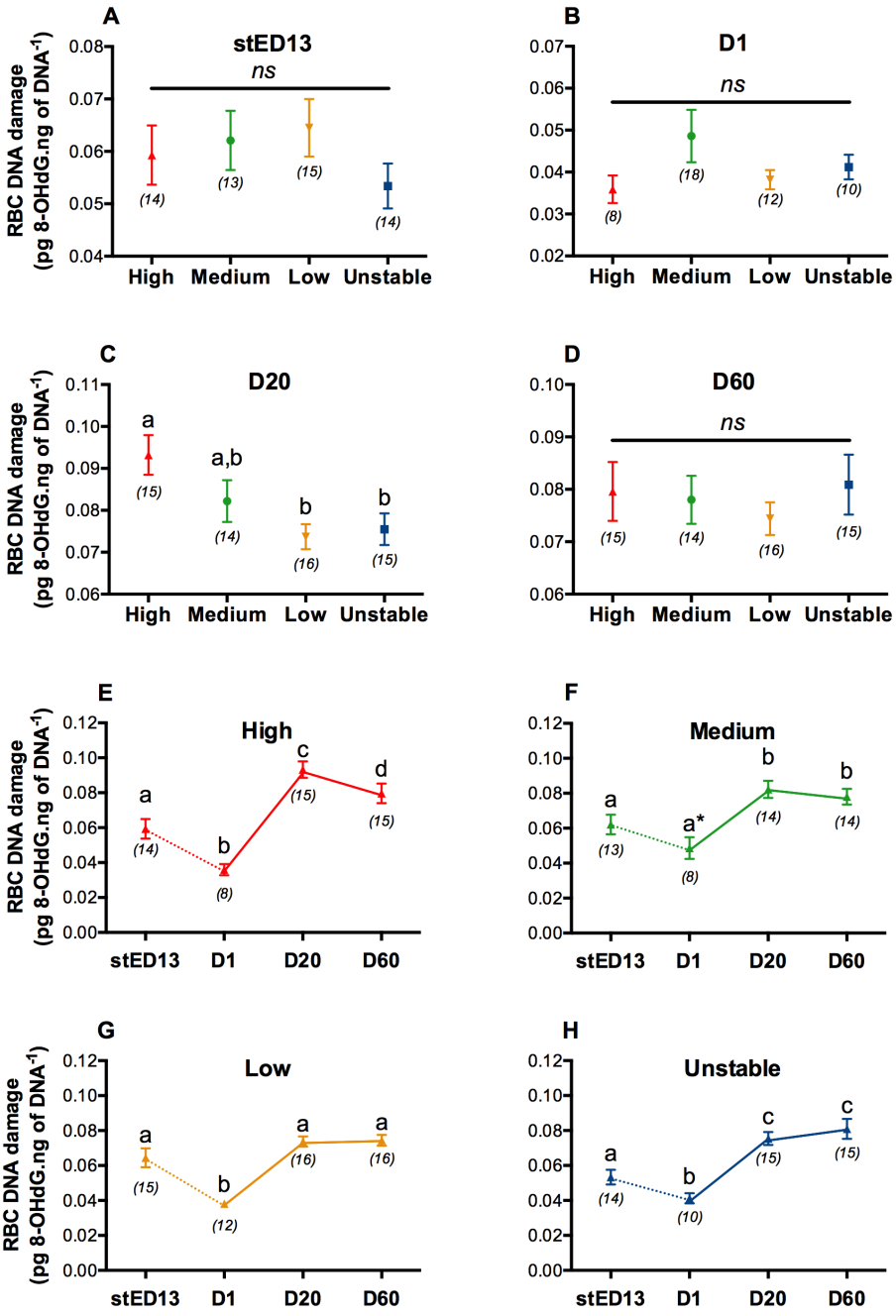

Fig S5: DNA damage levels in red blood cells according to prenatal experimental conditions and age. stED13 represents a standardized developmental stage between experimental groups as explained in the methods**. (A-D) DNA damage levels between experimental groups at each stage. (E-H) Age-related changes in DNA damage for each experimental group**. Data plotted as means ± SE. Solid lines indicate longitudinal data and dotted lines cross-sectional data, letters indicate significant differences according to post-hoc tests, and numbers in brackets represent sample sizes.

ESM7: Stage-dependent correlations between phenotypic and physiological traits

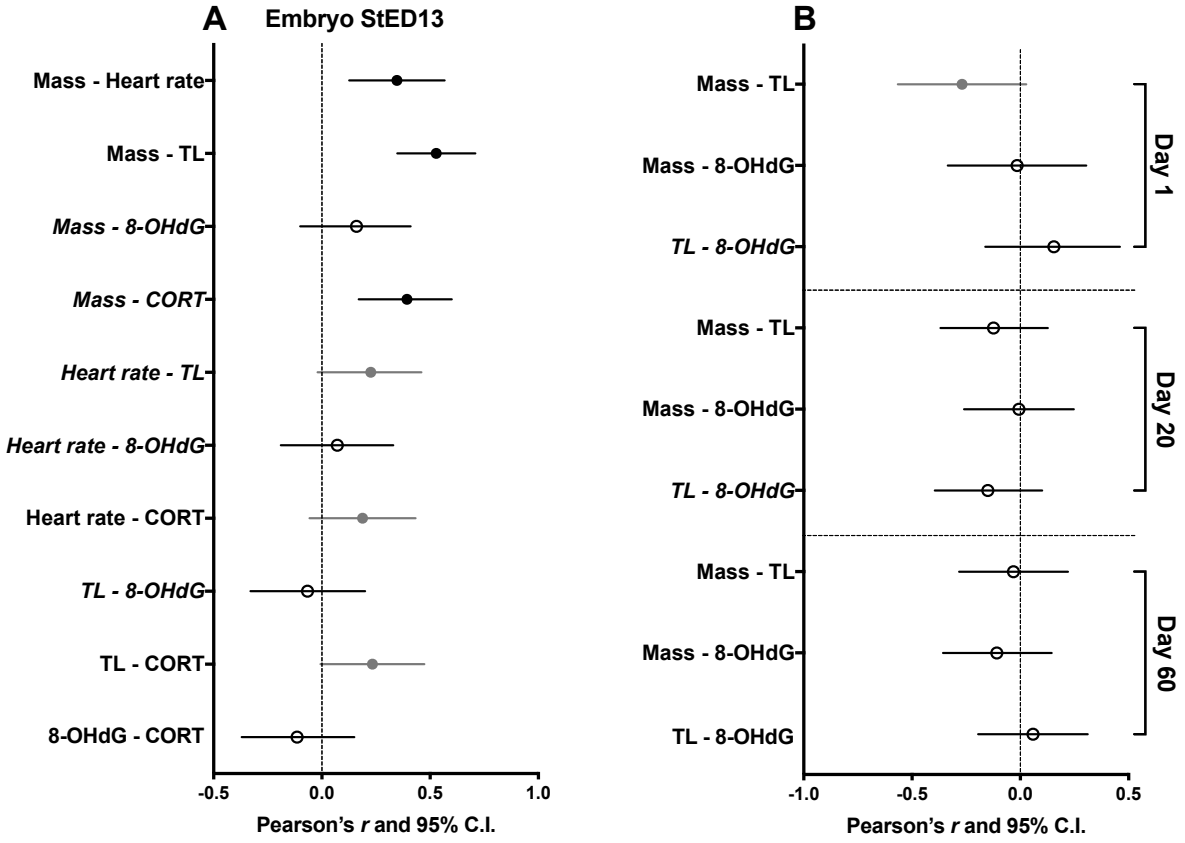

Fig S6: Stage-dependent correlations between phenotypic and physiological traits, (A) in embryos at the standardized embryonic day 13 (StED13), and (B) at the postnatal stages: day 1, day 20 and day 60. Mass = embryo or body mass, CORT = corticosterone, 8-OHdG = 8-hydroxydeoxyguanosine, TL = telomere length. Pearson correlation coefficients *r* and associated 95% confidence interval are reported. Significant ($p < 0.05$) correlations are plotted in black, non-significant trends ($p < 0.10$) are plotted in grey, and non-significant correlations ($p > 0.10$) are plotted with empty symbols.

ESM references

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