From climate warming to accelerated cellular ageing: an experimental heating study in a wild passerine bird

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Abstract

Climate change is increasing both the average ambient temperature and the frequency and severity of heat waves. While direct mortality induced by heat waves is increasingly reported, sub-lethal effects are also likely to impact wild populations. We hypothesized that accelerated ageing could be a cost of being exposed to higher ambient temperature, especially in early-life when thermoregulatory capacities are not fully developed. We tested this hypothesis in wild great tits (*Parus major*) by experimentally increasing nest box temperature by *ca.* 2°C during postnatal growth and measuring telomere length, a biomarker of cellular ageing predictive of survival prospects in many bird species. While increasing early-life temperature had no detectable effect on growth or survival to fledging, it accelerated telomere shortening, and although non-significantly, reduced medium-term survival from 34% to 19%. Heat-induced telomere shortening was not explained by oxidative stress, but more likely by an increase in energy demand (*i.e.* higher thyroid hormones levels, increased expression of glucocorticoid receptor, increased mitochondrial density) leading to a reduction in telomere maintenance mechanisms (*i.e.* non-significant decrease in the gene expression of telomerase and protective shelterin). Our results thus suggest that climate warming can affect rate of ageing in wild birds, with potential impact on population dynamics and persistence.

Introduction

Climate change is increasing both the average ambient temperature and the frequency and severity of heat waves (IPCC 2014). While direct mortality induced by heat waves is increasingly reported (*e.g.* [1]), sub-lethal effects are also likely to impact population dynamics and persistence [2]. Despite usually regulating their body temperature within a narrow range, endotherms can also be sensitive to (even small) changes in temperature, especially during early-life stages when their thermoregulation is not yet fully developed [3]. For instance, songbird nestlings have recently been shown to have a narrower thermoneutral zone than adults, as well as really poor cooling capacities [4] . Given that early-life experiences are known to have long-lasting effects on health, reproduction and even longevity (*e.g.* [5,6]), changes in early-life thermal environment associated with climate change are predicted to impact offspring phenotype and survival. Accordingly, some studies demonstrated associations between pre- or postnatal temperatures and survival in wild endotherm populations (*e.g.* [7–11]), and even longevity in humans [12]. Yet, the underlying physiological mechanisms remain poorly understood.

The physiological mechanisms of heat stress have been rather well characterized in laboratory endotherms: (i) Acute heat stress is known to increase the levels of key thermoregulatory and metabolic hormones, namely thyroid hormones (THs) [13,14]. (ii) Heat stress influences mitochondria, the cell powerhouse, as it reduces its efficiency to convert nutrients to ATP and increases its number [15] and (iii) increases the production of reactive oxygen species [16] contributing to oxidative stress and cellular ageing [17]. Yet, extrapolating findings from laboratory studies to wild populations in the context of climate change is challenging at best, partly because the range of temperature manipulations often exceeds temperature changes experienced in natural populations. A few studies in wild populations have experimentally increased early-life postnatal temperatures in endotherms [11,18,19], and report alterations of growth and body temperature. However, the potential mid to long-term consequences of such effects have not been characterized.

A key challenge, especially in relatively long-lived animals and wild populations, is to quantify the potential long-term deleterious effects of early-life conditions. Using the length of telomeres (*i.e.* the protective structure located at the end of chromosomes that progressively shorten with age), a key hallmark of ageing, as a molecular biomarker may help to overcome this challenge. Indeed, telomere length has been shown to predict survival (*i.e.*

meta-analysis in [20]), and even lifetime reproductive success [21], and most of the telomere shortening is known to occur early in life (*e.g.* [22]). Various early-life environmental stressors, including high incubation temperature in birds (*e.g.* [22]), have been shown to accelerate telomere shortening [23]. Telomere shortening is accelerated by oxidative stress [24], mitochondrial dysfunction [25] and changes in metabolic demand [26], which are predicted to increase in response to thermal stress (see above). While thermal environment has been shown to affect telomere shortening in ectotherms (*e.g.* [27–29]), we critically lack data from endotherm species (but see [30,31]).

In this study, we comprehensively assessed the effects of an experimental increase in temperature during early postnatal development on growth, short and medium-term survival (*i.e.* to fledging and to the next autumn-winter), as well as on key physiological and ageing markers (thyroid hormones, mitochondrial density, oxidative stress, telomere length and gene expression) in a wild great tit (*Parus major*) population. The temperature elevation mimicked a scenario of *ca.* 2°C temperature increase, as forecasted by 2060 under the IPCC dangerous scenario (SSP3-7.0). It was applied during a vulnerable period of postnatal growth, *i.e.* when offspring are not anymore brooded by their mother, but still not fully capable of thermoregulation. We predicted that increasing nest temperature would lead to (i) reduced growth due to possible energetic costs of heat dissipation and/or lowered mitochondrial efficiency and/or reduced parental provisioning [32], (ii) reduced survival prospects, (iii) increased THs, (iv) increased oxidative stress, (v) increased mitochondrial density and (vi) shorter telomeres due to oxidative stress-induced shortening and/or a decrease in telomere maintenance mechanisms.

Material & methods

Experimental design

The experiment was conducted in 2018 in a nest box population of great tits on the island of Ruissalo ('60°26.055 N,'22°10.391 E) in Finland. Nest microclimate differs between natural nests and artificial nestboxes, and it is important to note here that we used custom-made wooden nestboxes. Great tits are mostly ectothermic until 9 days post-hatching, and then mostly endothermic from 12 days post-hatching, with a gradual transition from ectothermy to endothermy [33] . The upper critical temperature of great tit nestling increases with age (*i.e.* 6 days: 24°C; 9 days: 28°C; 12-15 days: 30°C), and the risk of lethality at high ambient temperature has been stressed previously [33]. At our field site, average temperature during the study period was mean ± SD: 15.85 ± 2.31°C and maximum daily temperature was 21.22 ± 4.43°C and was above 24.0°C for 22% of the days within the study period (data provided by the Finnish Meteorological Institute and collected by Artukainen weather station in Turku, 2 to 4 km from the study sites). Additionally, nestbox temperature is always warmer than ambient temperature, by approximately 2.5°C based on our own data (mean ± SD: 18.43 ± 2.41°C). Few breeding pairs initiate a second breeding attempt in our population, so only the first breeding attempt was used in this experimental study.

Half of the nestlings of each nest (total N =32 nests) were swapped between nests two days after hatching to account for the effects of genetic background and rearing environment. To increase nest box temperature during growth of *ca.* 2°C, one heating pad (Uniheat Shipping warmer®, USA) was installed under the ceiling in half of the nest boxes (N = 17) between d7 and d14 (hereafter, “heated” nests), *i.e.* second half of postnatal development. For the other half (N= 15), a control pad (identical to the heating pads but not producing heat) was installed (hereafter “control” nests). Heating pads were checked and replaced every second day, and control nests were also visited to standardize human disturbance between the experimental groups. The actual nest box temperature was recorded with a thermo-logger (iButton thermochron, measuring at 3min intervals, 0.0625°C accuracy) placed *ca.* 10cm above the nest cup, and daily average, minimum and maximum temperatures over the course of the heating treatment were calculated for each nest box.

Nestling body mass and tarsus length were measured prior to the treatment on d7 and post-treatment on d14 after hatching. Blood samples were collected from the brachial vein at

d14 (*ca.* 70µl) using heparinized capillaries. Whole-blood samples for oxidative stress and DNA/RNA extraction were immediately snap-frozen in liquid nitrogen, and an aliquot of whole-blood was stored on ice pack until centrifugation in the laboratory at the end of the day to assess plasma thyroid hormone levels.

To study potential long-term and delayed effects of early-life heating treatment, we recaptured juvenile great tits during the following autumn-winter using mist-nets at seven feeding stations located across the field site (total of 126 hours of mist-netting). Mass and wing length of the juveniles were recorded and a blood sample (*ca.* 80µl) was collected and stored as explained above. Our method of recapture provides an estimate of post-fledging survival (*i.e.* apparent survival), but could be slightly biased by dispersal.

Plasma thyroid hormones and oxidative stress

Plasma thyroid hormones (T3 and T4, expressed as pg/µL) were measured from d14 nestlings with nano-LC-MS/MS following [34]. Total glutathione (tGSH), the most abundant intra-cellular antioxidant, was measured from whole-blood samples with the ThioStar® Glutathione Fluorescent Detection Kit (K005-FI, Arbor Assays, USA; technical repeatability: R = 0.97 (95% C.I. [0.96-0.98]). As a measure of oxidative damage, we assessed blood lipid peroxidation (malonaldehyde, MDA) using the TBARS-assay following [35] (technical repeatability: R = 0.92 (95% C.I. [0.88-0.94])).

Mitochondrial density, telomere length and molecular sexing

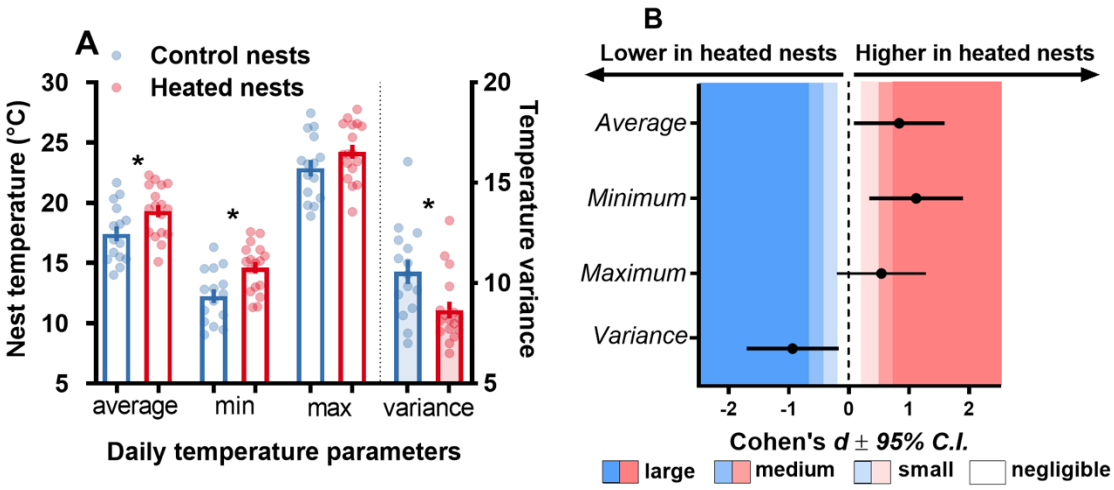
Relative telomere length (*rTL*) and mitochondrial DNA copy number (*mtDNAcn*, an index of mitochondrial density) were quantified on DNA extracted from blood cells using real-time quantitative PCR (qPCR) assays, following [36] (see details in ESM). This technique estimates relative telomere length by determining the ratio (T/S) of telomere repeat copy number (T) to a single copy gene (SCG), and the relative mtDNAcn as the ratio between one mitochondrial gene and the same single copy gene. Birds were molecularly sexed using a qPCR approach adapted from [37,38] (see details, including on technical repeatability, in ESM).

Gene expression analysis

We used RT-qPCR to quantify the relative expression levels of 6 genes of interest from RNA extracted from blood cells (see ESM for details on methods). We quantified the expression of

167 genes related to (i) cellular stress response: the glucocorticoid receptor (GCR) nr3c1, two heat
168 shock proteins of the HSP70 (*i.e.* HSPA2) and HSP90 (*i.e.* HSP90B1) families, as well as the nuclear
169 factor erythroid 2-related factor 2 NRF2 (an oxidative-stress-induced regulator of several
170 antioxidants and cellular protective genes); and to (ii) telomere maintenance processes: the
171 telomeric repeat binding factor 2 TERF2 (a shelterin protein helping in protecting telomeres) and
172 the telomerase reverse transcriptase TERT (catalytic subunit of the enzyme responsible for
173 telomere elongation).
174
175 **Statistical analysis**
176 For each trait of interest (see Fig. 2), we fitted a generalized linear mixed models
177 (GLMMs, R package *lme4*) to assess the effects of heating treatment. Maximum sample size was
178 N = 32 nests, n = 98 nestlings d14 and n = 26 juveniles, but the final sample size is often smaller
179 and varies between physiological parameters according to sample availability and success of
180 laboratory analyses (see specific sample size in Fig. 2). In all models, the identity of nest of origin
181 (*i.e.* where a nestling was born) and the nest of rearing (i.e. where a nestling grew up after cross-
182 fostering at day 2) were treated as random factors. Other random factors and fixed-effect
183 covariates are included when relevant for the trait in question (see details in ESM tables S2-
184 S21). Two-way interactions were initially tested, and removed from final models if non-
185 significant (*e.g.* heating treatment * sex); yet p-value before removal are indicated in ESM tables
186 for completeness. Main statistical outputs from type III analysis of variance with Satterthwaite's
187 method are reported in main text, while full model outputs with model estimates are reported
188 in ESM tables. We report standardized effect size for each trait as Cohen's *d* and 95% confidence
189 interval using the *emmeans* package in *R*. For survival analyses, ln Odds Ratios were transformed
190 to Cohen's *d* using the formula: $d = \ln\text{OddsRatio} \times (\sqrt{3}/\pi)$ following [39]
191

192 ## Results



193

194 **Fig. 1: Effects of the heating treatment on nest temperature: daily average, minimum temperature,**
195 **maximum and variance. (A) raw data points and mean ± SE**, **(B) standardized effect size and 95%**
196 **confidence interval**. The standard Cohen's *d* scale for negligible <0.2, small <0.5, medium <0.8 or
197 large >0.8 effect size is presented with a colour scale and statistical significance is indicated by an *
198 when p < 0.05. Sample size: 15 control nests *vs.* 17 heated nests.

199

200 The heating treatment was effective in raising average nest temperature by 1.9°C
201 (large effect size; $F_{1,30}$ = 5.58, p = 0.025; Fig. 1) and minimum temperature by 2.3°C (large
202 effect size; $F_{1,30}$ = 10.03, p = 0.003; Fig. 1) over the heating period, while maximum
203 temperature was only increased by 1.3°C, which was not significant (medium effect size; $F_{1,30}$
204 = 2.31, p = 0.14; Fig. 1). The variance in nest temperature was significantly lower in heated
205 nests (large effect size; $F_{1,30}$ = 7.02, p = 0.013; Fig. 1).

206 Nestlings from heated nests were not significantly lighter or smaller than control ones
207 at day 14 (negligible and small effect size respectively; p = 0.748 and 0.969; Fig. 2, Tables S2-
208 S3). Juveniles from heated nests were non-significantly heavier (large effect size; p = 0.078)
209 but not larger (small effect size; p = 0.723) than control ones when recaptured the following
210 autumn/winter (Fig. 2, Tables S4-S5).

211 Survival to fledging was very high overall and not significantly influenced by the heating
212 treatment (control: 94% *vs.* heated: 98%; p = 0.82; Fig. 2, Table S6). Birds from heated nests
213 were less likely (large effect size, control: 34% *vs.* heated: 19%;) to be recaptured as juveniles,
214 although not significantly so: p = 0.22 (Fig. 2, Table S7).

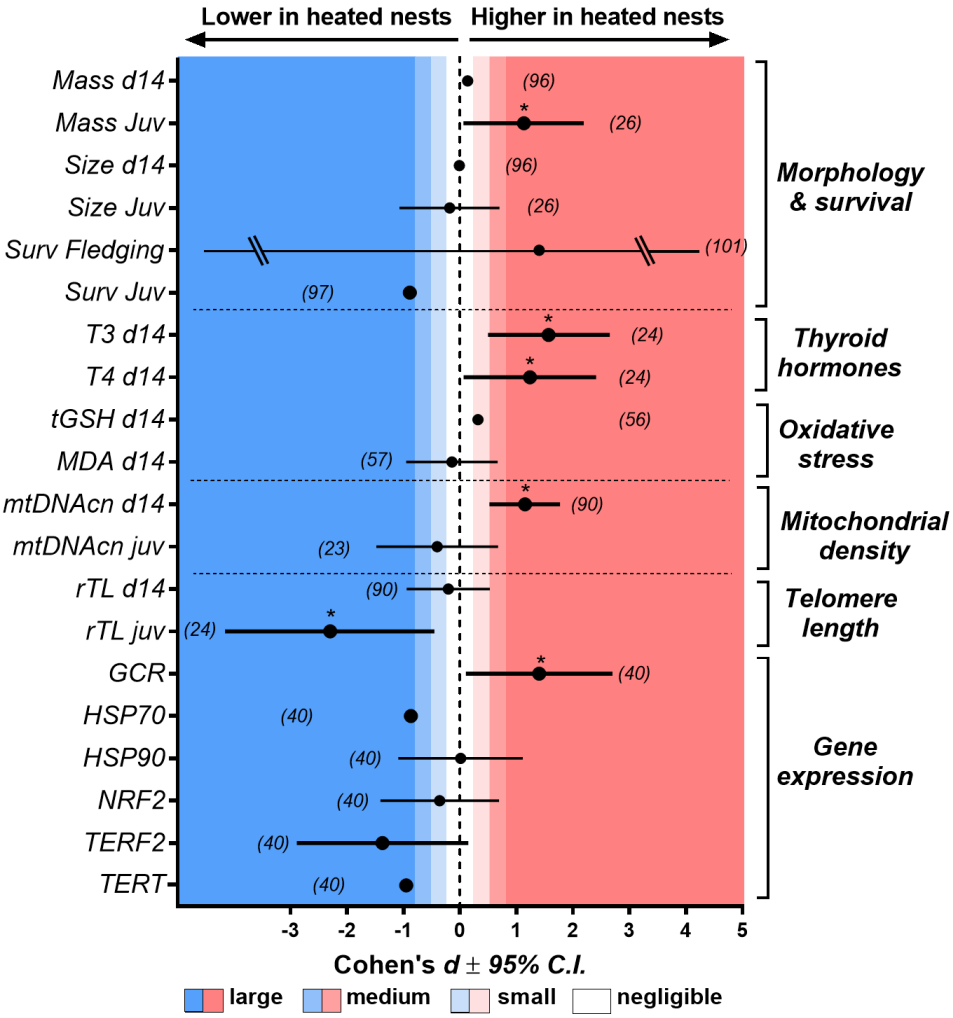


Fig. 2: Effects of nest heating treatment on key life-history and physiological traits in wild great tits. Effects are presented as standardized effect sizes (Cohen's *d*, or transformed to Cohen's *d*) and 95% confidence intervals. d14: nestlings being 14 days old, Juv: juveniles (*i.e.* captured during the autumn/winter following their fledging); surv: survival; T3 and T4: plasma thyroid hormones; *MDA*: biomarker of oxidative damage to lipids measured in blood, *tGSH*: total glutathione (*i.e.* intra-cellular antioxidant molecule) content in blood cells; *mtDNAcn*: mtDNA copy number, a proxy for mitochondrial density measured in blood cells; *rTL*: relative telomere length (*i.e.* a biomarker of ageing) of blood cells; GCR: gene expression of the glucocorticoid receptor, HSP70 and HSP90: gene expression of heat shock proteins, NRF2: gene expression of an oxidative-stress-induced regulator of several antioxidants and cellular protective genes, TERF2: gene expression of a shelterin protein helping in protecting telomeres, TERT: gene expression of the catalytic subunit of the enzyme responsible for telomere elongation. Detailed information on statistics is available in ESM Tables S2-S21. Significant effects according to full statistical models are presented with * symbols, and large effects presented in bold. The standard Cohen's *d* scale for negligible <0.2, small <0.5, medium <0.8 or large >0.8 effect size is presented with a colour scale. Sample size for each trait is presented between brackets.

Nestlings from heated nests were not significantly lighter or smaller than control ones at day 14 (negligible and small effect size respectively; $p = 0.748$ and 0.969; Fig. 2, Tables S2-S3). Juveniles from heated nests were non-significantly heavier (large effect size; $p = 0.078$)

but not larger (small effect size; $p = 0.723$) than control ones when recaptured the following autumn/winter (Fig. 2, Tables S4-S5).

Survival to fledging was very high overall and not significantly influenced by the heating treatment (control: 94% *vs.* heated: 98%; $p = 0.82$; Fig. 2, Table S6). Birds from heated nests were less likely (large effect size, control: 34% *vs.* heated: 19%;) to be recaptured as juveniles, although not significantly so: $p = 0.22$ (Fig. 2, Table S7).

Nestlings from heated nests had higher circulating thyroid hormones levels than controls, significantly so for T3 (large effect size; $p = 0.032$) but not for T4 (large effect size; $p = 0.090$; Fig. 2, Tables S8-S9). Heating treatment did not significantly alter oxidative stress levels (glutathione: small effect size, $p = 0.347$; oxidative damage to lipids: negligible effect size, $p = 0.682$; Fig. 2, Tables S10-11).

Mitochondrial density in blood cells decreased sharply during postnatal development (Fig. 3A), and nestlings from heated nests had a higher mitochondrial density at the end of the heating treatment (large effect size; $p = 0.002$; Fig. 2 & 3A, Table S10), but not when recaptured as juveniles (negligible effect size; $p = 0.554$; Fig. 2 & 3A, Table S11). Heating treatment did not significantly influence nestlings' telomere length (negligible effect size; $p = 0.580$; Fig. 2 & 3B, Table S12), but juveniles from heated nests had markedly shorter telomeres than controls (large effect size; $p = 0.033$; Fig. 2 & 3B, Table S13).

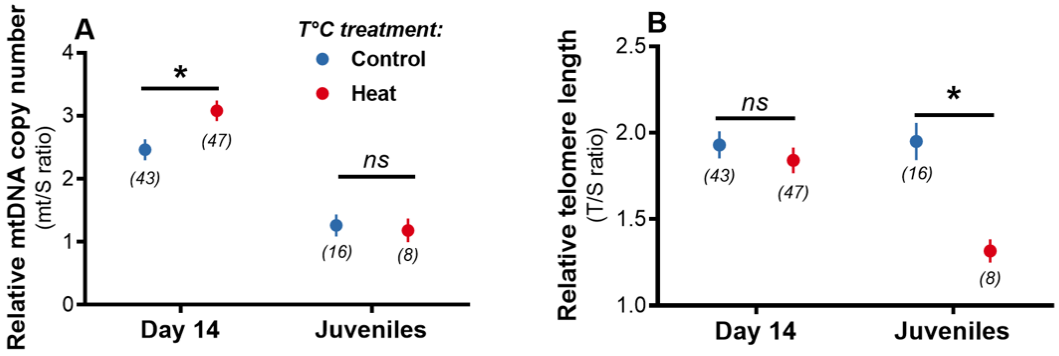


Fig. 3: Effects of age and nest heating treatment on: (A) mtDNA copy number as a proxy of mitochondrial density and cellular energetics, and (B) relative telomere length as a biomarker of ageing. Means are plotted ± SE and full statistical models are presented in Tables S12-S15.

Gene expression was partly altered by nest heating treatment, with nestlings from heated nest showing significantly higher expression levels of the glucocorticoid receptor (GCR: large effect size; $p = 0.045$; Fig. 2, Table S16), and although non-significantly, lower expression

284 levels of the telomere-related genes TERF2 (large effect size; p = 0.088; Fig. 1, Table S17) and
285 TERT (large effect size; p = 0.085; Fig. 1, Table S18). There was however no clear effect on the
286 expression of heat shock proteins (HSP70: medium effect size, p = 0.298; HSP90: negligible
287 effect size, p = 0.985; Fig. 2, Tables S19-20) or the regulator of antioxidant protection NRF2
288 (small effect size, p = 0.504; Fig. 2, Table S21).
289

290 ## Discussion

291 By experimentally manipulating early postnatal temperature of *ca.* 2°C (in line with
292 predictions of climate change), we demonstrated that exposure to higher ambient
293 temperature during early-life could affect several physiological pathways (*i.e.* thyroid
294 hormones, mitochondrial biogenesis, glucocorticoid signalling) and ultimately lead to
295 accelerated cellular ageing (*i.e.* shorter telomeres) through a potential deregulation of
296 telomere maintenance processes (*i.e.* shelterin protein and telomerase). While immediate
297 survival was not impacted by the experimental increase in nest temperature, birds from
298 heated nests were less likely to be seen alive the following autumn-winter, although non-
299 significantly so.

300 Growth rate is known to be influenced in a complex manner by weather conditions
301 [40], and previous experimental studies increasing nest temperature have reported
302 contrasted results (positive effect: [18]; negative effects: [11,19]). Our experimental
303 manipulation did not affect body size or mass during postnatal growth. Apparent survival to
304 autumn-winter was lower for birds originating from heated nests (19% *vs.* 34%, large effect
305 size), but not significantly so. Obtaining large enough sample size to detect significant effects
306 on survival in experimental studies on wild animals is unfortunately challenging. Yet, our
307 results are in accordance with observational data from a 12-year monitoring study of great tit
308 survival in Spain, showing a negative association between ambient temperature during
309 postnatal growth and post-fledging survival [7].

310 While there is some evidence from laboratory acute heat stress studies that thyroid
311 hormones could be up-regulated when facing increased ambient temperatures [13,14], we
312 show here that even a small increase in early-life temperature can increase thyroid hormones
313 levels. Importantly, high thyroid hormone levels have been linked to increased mortality risks
314 in adult humans [41] and increased susceptibility to free radicals in birds [42]. Yet, we found

no significant effect of nest heating on two oxidative stress biomarkers (glutathione and oxidative damage to lipids), nor on NRF2 gene expression (an oxidative-stress-induced regulator of several antioxidants). Similarly, the gene expression of two heat shock proteins (HSP70 and HSP90 families) was not clearly impacted by nest heating. This suggests that a *ca.* 2°C increase in temperature might be too low to induce oxidative stress or a heat shock response (compared to heat stress experiments in the lab that often use > +10°C; *e.g.* [43]). Yet, the gene expression of the glucocorticoid receptor nr3c1 was increased in heated nests, suggesting either a response to a stressful stimulus (*e.g.* [44]) or potentially an increase in metabolic demand [45].

Both the rise in thyroid hormones and the observed increase in mitochondrial density at the end of the heating period would support the hypothesis of an increase in metabolic demand. In line with our results on mitochondrial density, mild heat stress has been shown to increase mitochondrial biogenesis *in vitro* [15] and *in vivo* [46]. This could be a way to compensate the typical decrease in mitochondrial coupling efficiency observed at higher body temperature (*e.g.* [47]), but measuring both body temperature (*i.e.* [11] showed that nest heating induced hyperthermia) and mitochondrial coupling efficiency [48] would be needed here to test this hypothesis.

While telomeres were not immediately impacted by the heating treatment (*i.e.* no effect at the end of the heating period on day 14), we discovered some important delayed effect since juveniles from heated nests had markedly shorter telomeres than control individuals. Sample size of juveniles was relatively limited, and the lower apparent post-fledging survival of birds from heated nests could lead to bias associated with selective disappearance. Yet, this is unlikely to bias our results since we would expect individuals with shorter telomeres to disappear earlier from the population (and not the opposite), as this has previously been shown in our model species [49]. Additionally, the trend towards a decreased expression of genes related to telomere maintenance (TERF2 and TERT) observed at day 14 in nestlings from heated nests support the results observed on telomere length in juveniles. Our results are in accordance with recent findings in humans showing: 1. a negative effect of warm temperature and a positive effect of cold temperature during gestation on newborn telomere length [50]; 2. shorter telomeres in adults exposed to acute (i.e. 1-13 days) warm environmental conditions [51]. Additionally, it has recently been shown at the correlative level that warm and dry conditions were associated with shorter telomere in an Australian

passerine species [30] . Here, we provide experimental evidence suggesting a causal link between environmental temperature and telomere shortening, as well as some hints on the underlying molecular mechanisms. While oxidative stress-induced telomere shortening [24] is unlikely to explain our results (see above), the 'metabolic telomere attrition hypothesis' [26] could be a good candidate. Indeed, this theory stipulates that increased metabolic demand mediated by increased glucocorticoid signalling (*i.e.* increased nr3c1 expression observed here) can decrease investment in telomere maintenance processes (*i.e.* decreased expression of TERF2 and TERT observed here) and be associated with mitochondrial dysfunction [52].

To conclude, our study provides the first experimental evidence that a moderate increase in ambient temperature during early-life can accelerate cellular ageing in a wild endotherm species. While the exact consequences of such increase in early-life temperature on individual fitness and population dynamics remain to be determined, previous evidence at the inter-population level has shown that shorter telomeres might precede population extinction and be used as an early warning sign [53].

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Data accessibility

All data are publicly available on FigShare: 10.6084/m9.figshare.28838297

Ethical permits

All procedures were approved by the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESAVI/2902/2018) and by the Environmental Center of Southwestern Finland (license number VARELY549/2018) granted to SR.

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ESM of '**From climate warming to accelerated cellular ageing: an experimental heating study in a wild passerine bird**'

qPCR assays for telomere length, mtDNA copy number and molecular sexing

We extracted DNA from blood cells using a standard salt extraction alcohol precipitation method ([1]). Extracted DNA was diluted in elution buffer BE for DNA preservation. DNA concentration and purity (260/280 > 1.80 and 260/230 > 2.00) were checked with a ND-1000-Spectrophotometer (NanoDrop Technologies, Wilmington, USA). DNA integrity was verified in 24 samples chosen randomly using gel electrophoresis (50 ng of DNA, 0.8 % agarose gel at 100 mV for 60 min) and DNA staining with Midori Green. Each sample was then diluted to a concentration of 1.2 $ng.\mu L^{-1}$ for subsequent qPCR analysis.

Relative telomere length (*rTL*) and mitochondrial DNA copy number (*mtDNAcn*, an index of mitochondrial density) were quantified using qPCR. Here, we used recombination activating gene 1 RAG1 as a single copy gene (verified as single copy using a BLAST analysis on the great tit genome) and cytochrome oxidase subunit 1 (COI1) as a mitochondrial gene (verified as non-duplicated in the nuclear genome using a BLAST analysis). The qPCR reactions were performed on a 384-QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher), in a total volume of 12µL including 6ng of DNA, primers at a final concentration of 300nM and 6µL of SensiFAST^{TM} SYBR lo-ROX (Bioline). Telomere, RAG1 and COI2 reactions were performed in triplicates on the same plates (10 plates in total); the qPCR conditions were: 3min at 95°C, followed by 35 cycles of 10 s at 95°C, 15 s at 58°C and 10s at 72°C. A DNA sample being a pool of DNA from 10 adult individuals was used as a reference sample and was included in triplicate on every plate. The efficiency of each amplicon was estimated from a standard curve of the reference sample ranging from 1.5 to 24ng. The primer sequences, as well as qPCR efficiencies and technical precision estimates (coefficient of variation and technical repeatability) are provided in Table S1. The relative telomere length and mtDNAcn of each sample were calculated as $(1+Ef_{\text{Tel or COI2}})^{\Delta Cq\ \text{Tel or COI2}}/(1+Ef_{\text{RAG1}})^{\Delta CqRAG1}$; *Ef* being the amplicon efficiency, and ΔCq the difference in Cq-values between

the reference sample and the focal sample.

The use of mtDNAcn as an index of mitochondrial density has been questioned in human [2], but we have previously shown good correlations between mtDNAcn and mitochondrial respiration rates in pied flycatcher [3] and great tit (Cossin-Sevrin et al. *in revision*). Great tits have quite peculiar telomeres, characterized notably by some ultra-long telomeres that do not seem to shorten with age in adults [4]. Since qPCR only provides an estimate of overall telomere length, it could be suboptimal for this study species. Yet, relative telomere length (*i.e.* measured using qPCR) in this species has been shown to shorten during the nestling stage [5,6], to respond to environmental factors (e.g. hatching asynchrony: [5]; altitude: [6]; urbanization: [7]) and to predict adult survival [8]. Within-individual repeatability of telomere length has recently been suggested to be an important factor to evaluate the pertinence of telomere length data in a given study/species [9], and the biological repeatability in our dataset was $R = 0.44$ [0.25-0.60], which is close to the average reported by qPCR studies (*i.e.* $R = 0.47$), and well within the range of what has been reported for great tits [9].

Birds were molecularly sexed using a qPCR approach adapted from [10,11]. Forward and reverse sexing primers were 5'- CACTACAGGGAAAACTGTAC-3' (2987F) and 5'- CCCCTTCAGGTTCTTTAAAA -3' (3112R), respectively. qPCR reactions were performed in a total volume of 12µL including 6ng of DNA, primers at a final concentration of 800nM and 6µL of SensiFAST™ SYBR® Lo-ROX Kit (Bioline). qPCR conditions were: 3 min at 95°C, followed by 40 cycles of 45 s at 95°C, 60 s at 52°C and 60s at 72°C, then followed by a melting curve analysis (95°C 60s, 45°C 50s, increase to 95°C at 0.1°C/s, 95°C 30s). Samples were run in duplicates in a single plate and 6 adults of known sex were included as positive controls. Sex was determined by looking at the dissociation curve, with two peaks indicating the presence of a Z and W chromosome (female), and one peak indicating the presence of only the Z chromosomes (male).

RT-qPCR assays for evaluating gene expression

We used RT-qPCR to quantify the expression levels of 6 genes of interest in d14 nestlings (Table S1). First, RNA was extracted from 10 µL of blood (immediately after the first thawing following -80°C storage) using Nucleospin RNA Plus extraction kit (Macherey-Nagel) following manufacturer instructions. Second, RNA concentration and purity were quantified using optical density. Samples not meeting quality criteria (i.e. RNA concentration > 25 ng/µl, 260/280 and 260/230 > 1.80) were excluded for further analysis. RNA integrity was checked using E-Gel 2% electrophoresis system (Invitrogen), and the ribosomal RNA 18S *vs.* 28S bands intensity, and deemed satisfactory. Samples were stored at -80°C for 2 weeks before cDNA synthesis. 600ng of RNA were used for cDNA synthesis using the SensiFAST™ cDNA Synthesis kit (Bioline) following manufacturer instructions. cDNA was diluted at a final concentration of 1.2 ng/µL for qPCR analysis. No-RT control samples were prepared following the same protocol, but without reverse transcriptase enzyme.

We assessed the expression genes related to 1) cellular stress response: the glucocorticoid receptor (GCR) nr3c1, two heat shock proteins of the HSP70 (*i.e.* HSPA2) and HSP90 (*i.e.* HSP90B1) families, as well as the nuclear factor erythroid 2-related factor 2 NRF2 (an oxidative-stress-induced regulator of several antioxidants and cellular protective genes); and 2) telomere maintenance processes: the telomeric repeat binding factor 2 TERF2 (a shelterin protein helping in protecting telomeres) and the telomerase reverse transcriptase TERT (catalytic subunit of the enzyme responsible for telomere elongation). qPCR was performed in a total volume of 12µl containing 5µl of each diluted cDNA sample (i.e. 1.2ng/µl) and 7µl of reaction mix containing primers (forward and reverse) at a final concentration of 300nM and Sensifast SYBR®No-ROX Mix (Bioline). The succinate dehydrogenase complex subunit A (SDHA; [12]) and the ribosomal protein L13 (RPL13, [13]) were used as reference genes. SDHA and RPL13 were identified as the most stable reference genes using geNorm software (geNorm M < 0.7, geNorm V < 0.15) and the geometric mean of these two genes was thus used as our reference gene. Primers (Table S1) have been designed whenever possible on exon-exon junction using NCBI primer designing tool using the *Parus major* reference genome. Specificity has

been checked using BLAST analysis and confirmed by a single narrow peak in melting curve analyses and the presence of a single PCR product of the expected size on agarose gel. Amplification in no-RT controls never occurred before at least 5 cycles after the lower Cq sample (> 8 Cq for all genes but TERT), and thus contamination by genomic DNA could not interfere with our results.

We ran gene-specific qPCR plates and each sample was analyzed in duplicate. Experimental groups were always balanced within each plate. We used a cDNA reference samples (*i.e.* ratio = 1) being a pool of 5 different individuals on every plate. One inter-plate standard sample was also run on every plate. qPCR assays were performed on a Mic qPCR instrument (Bio Molecular Systems) and included a two-step cycling with the following conditions: 2 minutes at 95°C; then 40 cycles of 5s at 95°C followed by 20s at 60°C (fluorescence reading) for all reactions. The expression of each gene was calculated as $(1+Ef_{Target})^{\Delta Cq(Target)}$/ geometric mean $[(1+Ef_{SDHA})^{\Delta CqSHDA}$; $(1+Ef_{RPL13})^{\Delta CqRPL13}]$, Ef being the amplification's efficiency and ΔCq being the difference between the Cq-values of the reference sample and the sample of interest.

Table S1: Information on qPCR primer sets and performance for relative mtDNA copy number, telomere length and gene expression assays. Cq refers to qPCR quantification cycle, efficiency has been evaluated using a standard curve for genomic DNA qPCR and using the LinReg method based on individual well efficiency for RT-qPCR for gene expression. Cq of non-template control (NTC) for genomic DNA qPCR and of non-reverse transcription control (NRT) for RT-qPCR are provided. Technical precision estimates (coefficient of variation CV and technical repeatability *R*) are provided for final ratios both at the intra-plate (based on duplicates) and the inter-plate levels.

Target	Primer F	Primer R	Ref	Product length	Cq ± SE	Efficiency ± SE	NTC or NRT Cq	CV-intra ± SE	CV-inter	*R*-intra	*R*-inter
RAG1	TCGGCTAAACAGAGGTGTAAAG	CAGCTTGGTGCTGAGATGTAT	This study	100	25.39 ± 0.02	109.80 ± 1.8	NA	-	-	-	-
COI1	CAAAGATATCGGCACCCTCTAC	GCCTAGTTCTGCACGGATAAG	This study	91	20.38 ± 0.04	102.21 ± 1.62	NA	9.65 ± 0.32	8.11	0.96	0.77
Tel	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	[14]	-	8.09 ± 0.02	96.27 ± 1.09	37.44 ± 1.18	10.74 ± 0.42	11.29	0.87	0.98
SDHA	GGGCAATAACTCCACGGCAT	TTGTATGGCAGGTCTCTACGA	[13]	99	22.02 ± 0.16	97.57 ± 0.12	35.51 ± 0.25	-	-	-	-
RPL13	TACTCCTTCAGCCTCTGCAC	ACAAGAAGTTTGCCCGGACT	[13]	99	20.37 ± 0.15	89.92 ± 0.17	34.08 ± 0.28	-	-	-	-
HSP70 (HSPA2)	GGGGCACTTTCGATGTCTCA	CAAAGTGGTTCACCATGCGG	This study	118	19.93 ± 0.15	95.60 ± 0.26	30.02 ± 0.43	4.28 ± 0.47	3,63	0.99	-
HSP90 (HSP90B1)	TCACATCTGTGGATTCTTCTGT	GTGGATGAGGAACCTGAAGAG	This study	114	20.40 ± 0.11	92.46 ± 0.28	37.98 ± 0.21	5.03 ± 0.40	7,1	0.99	-
GCR (nr3c1)	GGAATAGGTGCCAGGGATCG	TTCCAGGGCTTGAATAGCCA	[12]	102	26.99 ± 0.16	95.35 ± 0.25	37.81 ± 0.28	11.82 ± 1.08	10,15	0.85	-
NRF2 (NFE2EL2)	CAGAAAAGAATCCTGAACTGACTGC	TGTCGATCAAGTTCATGTCCAAGT	This study	155	24.02 ± 0.15	89.63 ± 0.29	36.24 ± 0.53	5.28 ± 0.49	5,2	0.99	-
TERF2	CCCTACAAAAGTATGGATGCCG	AACAACCACAGCAGCTTCCT	This study	175	24.89 ± 0.17	90.20 ± 0.17	36.01 ± 0.51	6.57 ± 0.56	4,87	0.97	-
TERT	GAAAACATTAAATGCAGGGATTGCC	CTCTGGGACATTCCTGATTAGCC	This study	187	30.03 ± 0.16	90.04 ± 0.17	37.72 ± 0.31	17.48 ± 1.44	5,67	0.79	-

Table S2: Results of linear mixed models for nestling body mass

Day-14 body mass (n=96)

Random effects:		**Variance**				
Nest of rearing (n=32)	Intercept	0.3767				
Nest of origin (n=21)	Intercept	0.2964				
Residual		0.5562				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	16.538	1.269	13.032			
Heating (No-heat)	-0.100	0.302	-0.332	1, 11.93	0.110	0.746
Brood size	**-0.272**	**0.098**	**-2.772**	**1, 26.26**	**7.684**	**0.010**
Day-7 body mass	**0.394**	**0.067**	**5.870**	**1, 77.44**	**32.458**	**<0.001**
Date	**-0.073**	**0.029**	**-2.518**	**1, 38.84**	**6.342**	**0.016**
Sex (male)	**0.436**	**0.190**	**2.299**	**1, 75.83**	**5.284**	**0.024**
Heating × sex	-0.495	0.374	-1.321	1, 75.49	1.744	0.191

Table S3: Results of linear mixed models for nestling body size

Day-14 body size (tarsus length, n=96)

Random effects:		**Variance**				
Nest of rearing (n=32)	Intercept	<0.0001				
Nest of origin (n=21)	Intercept	0.1960				
Residual		0.2155				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	18.502	1.043	17.745			
Heating (No-heat)	0.005	0.123	0.041	1, 83.32	0.002	0.967
Brood size	<0.001	0.044	0.012	1, 81.96	<0.001	0.990
Day-7 tarsus length	0.216	0.053	4.067	1, 87.29	15.540	<0.001
Date	-0.012	0.017	-0.699	1, 32.79	0.488	0.490
Sex (male)	0.623	0.112	5.585	1, 74.60	31.188	<0.001
Measurer B	0.149	0.179	0.832	2, 85.48	1.092	0.340
Measurer C	0.272	0.185	1.474			
Heating × sex	-0.431	0.225	-1.920	1, 78.80	3.685	0.058

Table S4: Results of linear mixed models for juvenile body mass

Juvenile body mass (n=25)						
Random effects:		**Variance**				
Nest of rearing (n=16)	Intercept	0.0125				
Nest of origin (n=13)	Intercept	<0.0001				
Residual		0.8796				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	13.911	3.985	3.491			
Heating (No-heat)	**-1.057**	**0.418**	**-2.532**	**1, 13.97**	**6.413**	**0.024**
Brood size	0.141	0.134	1.051	1, 14.39	1.105	0.311
Sex (male)	0.408	0.419	0.973	1, 19.02	0.946	0.343
Day-14 body mass	0.225	0.204	1.104	1, 19.96	1.219	0.283
Heating × sex	0.051	0.956	0.053	1, 19.00	0.003	0.958

Table S5: Results of linear mixed models for juvenile body size

Juvenile body size (wing length, n=26)

Random effects:		**Variance**				
Nest of rearing (n=17)	Intercept	<0.0001				
Nest of origin (n=13)	Intercept	<0.0001				
Residual		2.9310				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	80.931	7.178	11.275			
Heating (No-heat)	0.315	0.726	0.433	1, 21.00	0.188	0.669
Brood size	0.314	0.241	1.307	1, 21.00	1.709	0.205
Sex (male)	1.664	0.735	2.264	1, 21.00	5.126	0.034
Day-14 body mass	-0.365	0.366	-0.999	1, 21.00	0.999	0.329
Heating × sex	0.169	1.625	0.104	1, 20.00	0.011	0.918

Table S6: Results of generalized linear mixed models for nestling survival

For survival, binomial GLMMs were fitted with maximum likelihood by Laplace approximation. As there were only 7 nestlings that failed to fledge, other covariates (sex and day-7 body mass) were excluded in these models to enable model convergence.

Nestling survival from day 7 to fledging (n=101)				
Model I. Heating treatment				
Random effects:		**Variance**	**Std. Dev.**	
Nest of rearing (n=34)	Intercept	397.8000	19.9500	
Nest of origin (n=21)	Intercept	<0.0001	<0.0001	
Fixed effects:	**Estimate**	**Std. Error**	**z**	**p**
Intercept	12.574	6.237	2.016	
Heating (No-heat)	-1.399	6.175	-0.227	0.821

Table S7: Results of generalized linear mixed models for juvenile survival

Post-fledging survival (n=97)				
Random effects:		**Variance**	**Std. Dev.**	
Nest of rearing (n=32)	Intercept	1.0590	1.0293	
Nest of origin (n=21)	Intercept	<0.0001	0.0014	
Fixed effects:	**Estimate**	**Std. Error**	**z**	**p**
Intercept	-9.411	4.840	-1.944	
Heating (No-heat)	0.837	0.687	1.218	0.223
Day-14 body mass	0.399	0.257	1.553	0.120
Sex (male)	0.812	0.629	1.291	0.197
Heating × sex	-0.174	1.157	-0.151	0.880

Table S8: Results of linear mixed models for nestling plasma T3 levels

Day-14 nestling blood T3 level (n=24)

Random effects:		**Variance**				
Nest of rearing (n=19)	Intercept	<0.0001				
Nest of origin (n=13)	Intercept	0.0942				
Residual		0.0952				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	1.458	1.294	1.127			
Heating (No-heat)	**-0.484**	**0.151**	**-3.204**	**1, 14.15**	**10.265**	**0.006**
Sex (male)	-0.078	0.157	-0.497	1, 14.22	0.247	0.627
Day-14 body mass	-0.070	0.071	-0.987	1, 13.78	0.974	0.341
Heating × sex	0.631	0.336	1.880	1, 18.79	3.533	0.076

T3 was natural-log transformed

Table S9: Results of linear mixed models for nestling plasma T4 levels

Day-14 nestling blood T4 level (n=24)

Random effects:		**Variance**				
Nest of rearing (n=19)	Intercept	<0.0001				
Nest of origin (n=13)	Intercept	0.0934				
Residual		0.0525				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	2.423	1.004	2.413			
Heating (No-heat)	**-0.283**	**0.118**	**-2.403**	**1, 14.69**	**5.776**	**0.030**
Sex (male)	0.233	0.122	1.905	1, 14.68	3.627	0.077
Day-14 body mass	-0.033	0.055	-0.599	1, 14.10	0.358	0.559
Heating × sex	0.293	0.288	1.019	1, 17.80	1.038	0.322

T4 was natural-log transformed.

Table S10: Results of linear mixed models for nestling blood oxidative damage to lipids (MDA) levels

Day-14 nestling blood MDA level (n=57)						
Random effects:		**Variance**				
Nest of rearing (n=26)	Intercept	0.0005				
Nest of origin (n=18)	Intercept	<0.0001				
Batch of TBARS (n=6)	Intercept	0.0994				
Residual		0.0519				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-2.991	0.530	-5.642			
Heating (No-heat)	0.033	0.074	0.447	1, 22.68	0.200	0.659
Sex (male)	0.017	0.071	0.240	1, 47.99	0.058	0.811
Day-14 body mass	-0.018	0.028	-0.629	1, 37.33	0.396	0.533
Heating × sex	0.027	0.146	0.183	1, 47.53	0.034	0.855

Table S11: Results of linear mixed models for nestling blood total glutathione (tGSH) levels

Day-14 nestling blood tGSH level (n=56)						
Random effects:		**Variance**				
Nest of rearing (n=27)	Intercept	<0.0001				
Nest of origin (n=18)	Intercept	0.0237				
Batch of GSH (n=2)	Intercept	0.0208				
Residual		0.0936				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-0.226	0.773	-0.292			
Heating (No-heat)	-0.098	0.095	-1.031	1, 49.86	1.062	0.308
Sex (male)	-0.007	0.097	-0.067	1, 50.20	0.005	0.947
Day-14 body mass	-0.056	0.042	-1.351	1, 49.76	1.824	0.183
Heating × sex	0.247	0.194	1.278	1, 47.89	1.634	0.208

Table S12: Results of linear mixed models for nestling blood cell mtDNA copy number

Day-14 nestling mtDNA copy number (n=90)

Random effects:		**Variance**				
Nest of rearing (n=32)	Intercept	0.1105				
Nest of origin (n=21)	Intercept	0.3423				
Residual		0.5128				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	0.028	1.431	0.019			
Heating (No-heat)	**-0.821**	**0.220**	**-3.735**	**1, 15.120**	**13.948**	**0.002**
Sex (male)	0.127	0.193	0.655	1. 77.96	0.429	0.514
Day-14 body mass	0.018	0.078	0.224	1, 84.21	0.050	0.823
Heating × sex	-0.174	0.374	-0.466	1, 78.66	0.217	0.642

Table S13: Results of linear mixed models for juvenile blood cell mtDNA copy number

Juvenile mtDNA copy number (n=23)

Random effects:		**Variance**				
Nest of rearing (n=14)	Intercept	<0.0001				
Nest of origin (n=13)	Intercept	<0.0001				
Residual		0.9976				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-7.514	4.240	-1.772			
Heating (No-heat)	0.404	0.515	0.784	1, 19.00	0.615	0.443
Sex (male)	-0.237	0.441	-0.538	1, 19.00	0.289	0.597
Juvenile body mass	0.404	0.224	1.803	1, 19.00	3.250	0.087
Heating × sex	0.833	0.936	0.891	1, 17.45	0.794	0.385

Table S14: Results of linear mixed models for nestling blood cell relative telomere length

Day-14 nestling telomere length (n=90)

Random effects:		**Variance**				
Nest of rearing (n=32)	Intercept	0.3542				
Nest of origin (n=21)	Intercept	0.0614				
Residual		0.6041				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-1.977	1.534	-1.288			
Heating (No-heat)	0.161	0.280	0.574	1, 15.83	0.330	0.574
Sex (male)	-0.212	0.210	-1.011	1, 81.66	1.022	0.315
Day-14 body mass	0.110	0.084	1.317	1, 84.37	1.735	0.191
Heating × sex	-0.264	0.399	-0.663	1, 76.04	0.439	0.510

Table S15: Results of linear mixed models for juvenile blood cell relative telomere length

Juvenile telomere length (n=23)

Random effects:		**Variance**				
Nest of rearing (n=14)	Intercept	<0.0001				
Nest of origin (n=13)	Intercept	0.1109				
Residual		0.4093				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	1.648	2.887	0.571			
Heating (No-heat)	**1.475**	**0.373**	**3.952**	**1, 16.48**	**15.619**	**0.001**
Sex (male)	0.260	0.313	0.833	1, 17.97	0.694	0.416
Juvenile body mass	-0.153	0.153	-0.999	1, 9.79	0.999	0.342
Heating × sex	0.088	0.683	0.129	1, 17.84	0.017	0.899

Table S16: Results of linear mixed models for nr3c1 expression levels

Day-14 nestling nr3c1 expression (n=40)

Random effects:		**Variance**				
Nest of rearing (n=31)	Intercept	0.6435				
Nest of origin (n=20)	Intercept	<0.0001				
Residual		0.2912				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	2.460	2.414	1.019			
Heating (No-heat)	**-0.756**	**0.340**	**-2.226**	**1, 26.00**	**4.957**	**0.035**
Sex (male)	0.032	0.274	0.117	1, 25.26	0.014	0.908
Day-14 body mass	-0.103	0.129	-0.799	1, 34.95	0.639	0.429
PlateID	-0.444	0.260	-1.705	1, 22.53	2.907	0.102
Heating × sex	0.651	0.563	1.157	1, 30.59	1.339	0.256

Table S17: Results of linear mixed models for TERF2 expression levels

Day-14 nestling TERF2 expression (n=40)

Random effects:		**Variance**				
Nest of rearing (n=31)	Intercept	0.6949				
Nest of origin (n=20)	Intercept	<0.0001				
Residual		0.2067				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-2.937	2.292	-1.281			
Heating (No-heat)	0.625	0.336	1.860	1, 26.52	3.460	0.074
Sex (male)	-0.149	0.250	-0.595	1, 21.33	0.354	0.558
Day-14 body mass	0.138	0.122	1.126	1, 33.76	1.267	0.268
PlateID	0.184	0.236	0.781	1, 19.06	0.609	0.445
Heating × sex	0.340	0.517	0.657	1, 23.57	0.432	0.518

Table S18: Results of linear mixed models for TERT expression levels

Day-14 nestling TERT expression (n=40)

Random effects:		**Variance**				
Nest of rearing (n=31)	Intercept	<0.0001				
Nest of origin (n=20)	Intercept	0.4313				
Residual		0.5662				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-1.171	2.426	-0.483			
Heating (No-heat)	0.537	0.274	1.959	1, 27.07	3.837	0.060
Sex (male)	-0.116	0.293	-0.397	1, 30.38	0.158	0.694
Day-14 body mass	0.051	0.131	0.389	1, 34.85	0.151	0.700
PlateID	0.039	0.278	0.141	1, 27.25	0.020	0.889
Heating × sex	0.070	0.621	0.113	1, 32.79	0.013	0.320

Table S19: Results of linear mixed models for HSP70 expression levels

Day-14 nestling HSP70 expression (n=40)

Random effects:		**Variance**				
Nest of rearing (n=31)	Intercept	0.7109				
Nest of origin (n=20)	Intercept	<0.0001				
Residual		0.2268				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-2.537	2.353	-1.078			
Heating (No-heat)	0.378	0.342	1.104	1, 27.26	1.219	0.279
Sex (male)	-0.008	0.259	-0.033	1, 23.05	0.001	0.974
Day-14 body mass	0.119	0.125	0.950	1, 34.19	0.902	0.349
PlateID	0.183	0.244	0.749	1, 20.76	0.561	0.462
Heating × sex	0.022	0.539	0.040	1, 26.08	0.002	0.968

Table S20: Results of linear mixed models for HSP90 expression levels

Day-14 nestling HSP90 expression (n=40)

Random effects:		**Variance**				
Nest of rearing (n=31)	Intercept	0.4735				
Nest of origin (n=20)	Intercept	<0.0001				
Residual		0.3562				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-7.956	2.331	-3.414			
Heating (No-heat)	-0.006	0.317	-0.019	1, 25.35	<0.001	0.985
Sex (male)	-0.153	0.275	-0.558	1, 29.55	0.311	0.581
Day-14 body mass	**0.419**	**0.125**	**3.360**	**1, 34.56**	**11.290**	**0.002**
Plate	0.308	0.263	1.170	1, 26.56	1.370	0.252
Heating × sex	0.1885	0.557	0.338	1, 31.31	0.115	0.734

Table S21: Results of linear mixed models for NRF2 expression levels

Day-14 nestling NRF2 expression (n=40)

Random effects:		**Variance**				
Nest of rearing (n=31)	Intercept	0.4850				
Nest of origin (n=20)	Intercept	<0.0001				
Residual		0.4215				
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**F**	**p**
Intercept	-6.242	2.446	-2.552			
Heating (No-heat)	0.233	0.330	0.705	1, 26.04	0.497	0.487
Sex (male)	0.294	0.291	1.011	1, 31.03	1.022	0.320
Day-14 body mass	**0.308**	**0.131**	**2.354**	**1, 34.33**	**5.541**	**0.024**
Plate	0.316	0.279	1.131	1, 28.30	1.278	0.268
Heating × sex	-0.047	0.591	-0.080	1, 32.64	0.006	0.937

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