Prenatal thyroid hormones accelerate postnatal growth and telomere shortening in wild great tits

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Running headline: Prenatal thyroid hormones shorten telomeres

Abstract

Early-life environment is known to affect later-life health and disease, which could be mediated by the early-life programming of telomere length, a key hallmark of ageing. According to the *fetal programming of telomere biology hypothesis*, variation in prenatal exposure to hormones is likely to influence telomere length. Yet the contribution of key metabolic hormones, *i.e.* thyroid hormones (THs), has been largely ignored. We recently showed that in contrast to predictions, exposure to elevated prenatal THs increased postnatal telomere length in wild collared flycatchers, but the generality of such effect, its underlying proximate mechanisms and consequences on survival have not been investigated. We therefore conducted a comprehensive study evaluating the impact of THs on potential drivers of telomere dynamics (growth, post-natal THs, mitochondria and oxidative stress), telomere length and medium-term survival using wild great tits as a model system. While prenatal THs did not significantly affect telomere length a week after hatching (*i.e.* day 7), they influenced postnatal telomere shortening (*i.e.* shorter telomeres at day 14 and the following winter) but not apparent survival. Circulating THs, mitochondrial density or oxidative stress biomarkers were not significantly influenced, whereas TH-supplemented group showed accelerated growth, which may explain the observed delayed effect on telomeres. We discuss several alternative hypotheses that may explain the contrast with our previous findings in flycatchers. Given that shorter telomeres in early life tend to be carried until adulthood and are often associated with decreased survival prospects, the effects of prenatal THs on telomeres may have long-lasting effects on senescence.

Keywords: developmental programming, thyroid hormone, telomere, ageing, mitochondria, metabolism, oxidative stress, maternal effects

Introduction

Early-life environment has been repeatedly observed to affect adult health and survival prospects in human and non-human vertebrates (Barnes and Ozanne, 2011; Godfrey and Barker, 2001; Metcalfe and Monaghan, 2001). While the mechanisms underlying such delayed effects remained somewhat elusive (Barnes and Ozanne, 2011), the early-life programming of telomere length (i.e. the protective end caps of chromosomes) has emerged as a key candidate (Entringer et al., 2018). Telomere length is considered as a hallmark of ageing (López-Otín et al., 2013) since telomeres shorten with age, and shorter telomeres are often predictive of lower survival or lifespan in both epidemiological and experimental studies (Arbeev et al., 2020; Heidinger et al., 2012; Muñoz-Lorente et al., 2019; Wilbourn et al., 2018). The prenatal hormonal environment, such as exposure to elevated glucocorticoid levels, has been coined as an important factor influencing early-life telomere length and its associated long-term outcomes (Criscuolo et al., 2020; Haussmann et al., 2012; Marchetto et al., 2016; Parolini et al., 2019). While there has been a considerable interest in prenatal glucocorticoids (Haussmann et al. 2012; Tissier, Williams, and Criscuolo 2014; Noguera, da Silva, and Velando 2020) and to a lesser extent androgens (Parolini et al., 2019; Tissier et al., 2014) in the context of the '*fetal programming of telomere biology*' hypothesis (Entringer et al., 2018), the potential impact of prenatal thyroid hormones has been mostly ignored so far (Stier et al. 2020).

Thyroid hormones (THs) are key coordinators of development and metabolism (McNabb, 2007), which are transferred from mothers to offspring (Ruuskanen and Hsu, 2018). Variation in exposure to prenatal thyroid hormones (T3, triiodothyronine, and T4, thyroxine) could influence telomere length via several mutually non-exclusive proximate pathways: (i) Prenatal THs can stimulate growth (yet results are inconsistent across studies and species,

70 (Hsu et al., 2020, 2019a, 2017; Medici et al., 2013; Ruuskanen et al., 2016a; Sarraude et al.,
71 2020a; Sarraude et al., 2020b; Vrijkotte et al., 2017; Zhang et al., 2019), which can directly
72 contribute to telomere attrition through increasing cellular division (Monaghan and Ozanne,
73 2018; Stier et al., 2020), or indirectly accelerate telomere shortening through increasing
74 oxidative stress (Monaghan and Ozanne, 2018; Reichert and Stier, 2017; Smith et al., 2016).
75 (ii) Elevated TH levels are often associated with higher metabolic rates (Liu et al., 2006; Mullur
76 et al., 2014; Welcker et al., 2013) and stimulate mitochondrial aerobic metabolism (Cioffi et
77 al., 2013), both of which can potentially increase reactive oxygen species (ROS) and oxidative
78 damage (Stier, Massemin, and Criscuolo 2014), accelerating telomere shortening (Reichert
79 and Stier, 2017). It was recently shown that exposure to elevated prenatal TH levels can lead
80 to a sex-specific increase in metabolic rate and circulating TH levels shortly after hatching (rock
81 pigeons *Columba livia*, Hsu et al. 2017, but see Ruuskanen et al. 2016), which suggests that
82 prenatal hormones may program postnatal metabolism and TH-axis function. (iii) The
83 'metabolic telomere attrition hypothesis' (Casagrande and Hau, 2019) postulates that
84 telomere shortening might be adaptive by amplifying the cellular signaling of energy debt to
85 re-direct critical resources to immediately important processes. In this scenario, we would
86 expect accentuated shortening when catabolism is favored over anabolism via mTOR
87 inhibition (i.e. mechanistic target of rapamycin, a key regulator of cellular homeostasis)
88 leading to a decrease in telomere maintenance processes and an active shortening through
89 exonuclease action (Casagrande and Hau, 2019). Since THs can have both anabolic and
90 catabolic actions (Mullur et al., 2014), predictions can be made in both directions between
91 prenatal THs and telomere length.

92 From an evolutionary perspective, increased offspring metabolism or growth, diverts
93 resources from somatic maintenance if resources are limited. This can accelerate damage to

94 biomolecules and/or decrease repair/maintenance processes, and therefore accentuate
95 telomere shortening. Therefore, prenatal TH levels would be expected to vary in relation to
96 predicted environmental conditions (as observed in terms of temperature and laying order,
97 e.g. Ruuskanen, Groothuis, et al. 2016; Hsu, Verhagen, et al. 2019) to optimize this trade-off.

98 In contrast to most predictions relating prenatal THs to telomere length (see above),
99 we recently reported that prenatal exposure to experimentally elevated THs increased
100 telomere length in nestlings of a wild passerine, the collared flycatcher (*Ficedula albicollis,*
101 Stier et al. 2020). To better understand the potential generality of this surprising finding as
102 well as assess underlying mechanisms and potential carry-over effects of variation in prenatal
103 THs on later life-stages and survival, we conducted a more detailed study in another passerine
104 species, the great tit *(Parus major*). The aim of this study was to comprehensively investigate
105 the influence of prenatal THs on growth, oxidative stress, plasma THs, mitochondrial density
106 (i.e. mitochondrial DNA copy number) and telomere dynamics as well as survival via an
107 experimental manipulation of prenatal THs in a wild population. We monitored offspring
108 multiple times during development and as juveniles a few months after fledging. Based on the
109 majority of prior literature, we would predict that elevated prenatal THs could lead to faster
110 growth, increased plasma THs, oxidative stress and mitochondrial density (higher density can
111 lead to higher ROS production), ultimately accelerating telomere shortening. Alternatively, if
112 our previous finding in the collared flycatcher reflected a general pattern, we would predict
113 that despite accelerating growth, elevated prenatal THs could increase telomere length. We
114 also predict that elevated prenatal TH could increase post-fledging survival of the juveniles
115 (*e.g.* both due to accelerated postnatal growth and potential beneficial effects for
116 thermoregulation under low autumn-winter temperatures). However, longer-term survival,

117 that we were not able to evaluate here, could be decreased for example due to shorter
118 telomeres.

119 **Methods**

120 The experiment and all methods we used were in accordance with all relevant guidelines and
121 regulations and have been approved by the Animal Experiment Board of the Administrative
122 Agency of South Finland (ESAVI/2902/2018) and the Environmental Center of Southwestern
123 Finland (license number VARELY549/2018). The experiment was conducted in 2018 in a nest
124 box population (314 nest-boxes distributed over seven forest plots) on the island of Ruissalo
125 in southwestern Finland (60° 25′ N, 22° 10′ E).

126

127 *Field experiment*

128 The nest boxes were monitored with five-day intervals to track egg laying. Yolk T3 and T4 (i.e.
129 a combined injection of the two hormones) levels were elevated in half of the nests (n = 21
130 TH nests and n = 21 control nests) by injection into the egg, following methods in Ruuskanen,
131 Darras, et al. (2016). Control nests were injected with the vehicle (0.9% NaCl) only. The TH
132 content (±SD) in great tit eggs in the population is 0.053 (±0.02) ng/yolk for T3 and 0.46 (± 0.16
133 ng/yolk) for T4 (Ruuskanen et al. unpubl data). We aimed to raise the amount of yolk TH by
134 2SD via injection into the egg yolk, a dose that has been recommended in relation to the
135 natural hormone range of the study species (Podmokla et al., 2018). This corresponded to
136 target doses of 0.041 ng/yolk for T3 and 0.325 ng/yolk for T4. To make sure injections would
137 be performed on unincubated eggs mimicking maternal hormone levels (great tits can start
138 incubation before the clutch is complete), injections were conducted on the day the 5th egg

139 was laid to all eggs in the clutch. Thereafter, injections were conducted each day for the newly
140 laid egg.

141 Hatching date and success was monitored by visiting nests daily starting before the
142 estimated hatching day. Nestling body mass (~0.01g) and tarsus (~0.5 mm) were measured on
143 day 2 (mass only), 7 and 14 post-hatching. All nestling measurements were conducted
144 between 8 am and 2 pm. After measurements on day 2, nestlings were nail-coded, the brood
145 was split and nestlings were cross-fostered with same-age nestlings in another nest of the
146 opposite hormone treatment. This "split-brood design" allows chicks from TH and control-
147 injected eggs to be raised under the same postnatal environment and is in theory more
148 sensitive to detect effects that may otherwise require a much larger number of nests in an
149 experiment of "between-brood" design (e.g. Tschirren et al. 2005). Preferentially, half of the
150 brood was swapped between the nests with opposite treatments whenever possible, but we
151 also split a brood for cross-fostering when we did not have an equal number of nests with
152 opposite treatments on a given day. The proportion of TH-nestlings (i.e. number of TH-
153 nestlings divided by the brood size) in each nest after cross-fostering was therefore recorded.
154 All nestlings, cross-fostered or not, were included in the statistical analyses. There was no clear
155 bias on the number of nestlings that were cross-fostered or stayed in the original nests with
156 respect to the hormone treatment (CO-nestlings: 58 stayed and 41 moved, TH-nestlings: 68
157 stayed and 51 moved, $\chi^2 = 0.006$, $p = 0.939$). On day 7 a blood sample (ca 40µl) was taken and
158 snap-frozen in liquid nitrogen for molecular analyses (telomere length, mitochondrial density
159 and molecular sexing). On day 14 a small blood sample (10-15µl) was snap-frozen in liquid
160 nitrogen for oxidative stress biomarker analyses and stored at -80ºC, while the rest of the
161 sample (ca 40 µl) was kept on ice, and centrifuged. RBCs (15 µl) were used for mitochondria
162 and telomere measurements as above and plasma (15 µl) for TH analyses. After day 7

measurements half of the nest were subject to a temperature manipulation (nest temperature increased for *ca.* 2°C during day 7 – day 14) for the purposes of another study. To avoid potential confounding effects, for data after day 7 and in juveniles, we only included the individuals from the nests which were not temperature-manipulated (N = 20 nests, 111 nestlings on day 7 and 99 nestlings on day 14). Yet, for day 2 and day 7 measurements (prior to the temperature manipulation), we prefer to include all data (N = 42 nests, in total 224 nestlings, 218 nestlings on d2 and 221 nestlings on d7) to make use of the larger sample size.

To study long-term effects of prenatal thyroid hormones and offspring apparent post-fledging survival, we recaptured great tits during the following autumn-winter using mist-netting. Seven feeding stations were mounted in the study plots in August (generally any given nest box had a station within 200m), and nets were positioned close to the feeding stations. The distances between adjacent feeding stations were ca. 500 m, and most birds were captured at a different feeding station than their natal forest plot. In addition, 5 birds were captured at a constant effort site > 3 km from the study plots, suggesting that all stations were potentially accessible to all birds. Circa 20 kg of peeled sunflower seeds and 2 kg of fat were provided at each station, checked and filled bi-weekly, and consumption noted. Only in a very few cases the feeders were completely empty. Mist-netting was conducted at each feeding station for 3 hours on three different days in September-October and similarly again in February, summing up to a total 126 hours of mist-netting. The time of day (morning/day/afternoon) was rotated for each site. Nets were checked every 30 minutes and mass (~0.01g), wing (~0.5 mm) were recorded for each bird. A small blood sample (40-60µl) was taken, kept on ice, centrifuged within 8 hours, and RBCs frozen at -80ºC for telomere and mitochondria density analyses.

187 *Thyroid hormones and oxidative stress assays*

188 Plasma thyroid hormones, the biologically active form triiodothyronine (T3), and a
189 precursor thyroxine (T4) (expressed as ng/mL) were measured from 14-day-old nestlings with
190 nano-LC-MS/MS following Ruuskanen et al. (2019; 2018). Due to practical constraints (time,
191 effort and finances), we randomly selected one to four nestling per nest, n = 13 TH and n = 11
192 control nestlings (from 18 nests of origin and 11 nests of rearing) for analysis.

193 Total glutathione (tGSH), the most abundant intra-cellular antioxidant, and the ratio
194 between reduced and oxidized gluthatione (GSH:GSSG, an indicator of oxidative challenge)
195 were measured from whole-blood samples (12µl of 0.9% NaCl-diluted whole blood) with the
196 ThioStar® Glutathione Fluorescent Detection Kit (K005-FI, Arbor Assays, USA) (Sarraude et al.,
197 2020a). As a measure of oxidative damage, we assessed blood lipid peroxidation
198 (malonaldehyde, MDA, 50µl of 0.9% NaCl-diluted whole blood) using the TBARS-assay
199 following Espin et al. (2017). Both measurements had CV% < 10 and are expressed per mg of
200 protein (measured via BCA protein assay, ThermoFisher Scientific), following Espin et al. 2017.
201 We aimed to measure biomarkers of oxidative stress from 2 control or 2 TH-nestlings per nest,
202 yet sometimes fewer nestlings were available and some samples failed in the laboratory
203 analysis, yielding the final sample size as n = 33 TH and 26 control nestlings from 18 rearing
204 nests.

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

206 We extracted DNA from blood cells using a standard salt extraction alcohol
207 precipitation method (Aljanabi and Martinez, 1997). Extracted DNA was diluted in elution
208 buffer BE for DNA preservation. DNA concentration and quality (260/280 > 1.80 and 260/230
209 > 2.00) were checked with a ND-1000-Spectrophotometer (NanoDrop Technologies,
210 Wilmington, USA). DNA integrity was verified in 24 samples chosen randomly using gel

211 electrophoresis (50 ng of DNA, 0.8 % agarose gel at 100 mV for 60 min) and DNA staining with
212 Midori Green. Each sample was then diluted to a concentration of 1.2 ng.μl^{-1} for subsequent
213 qPCR analysis.

214 Relative telomere length (*rTL*) and mitochondrial DNA copy number (*mtDNAcn*, an
215 index of mitochondrial density) were quantified using qPCR. This technique estimates relative
216 telomere length by determining the ratio (T/S) of telomere repeat copy number (T) to a single
217 copy gene (SCG), and the relative mtDNAcn as the ratio between one mitochondrial gene and the
218 same single copy gene. Here, we used RAG1 as a SCG (verified as single copy using a BLAST
219 analysis on the great tit genome) and cytochrome oxidase subunit 2 (COI2) as a mitochondrial
220 gene (verified as non-duplicated in the nuclear genome using a BLAST analysis). Forward and
221 reverse telomere primers were 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
222 (Tel-1b) and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Tel-2b) respectively.
223 Forward and reverse RAG1 primers were 5'-TCGGCTAAACAGAGGTGTAAA-3' and 5'-
224 CAGCTTGGTGCTGAGATGTAT-3', respectively. Forward and reverse COI2 primers were 5'-
225 CAAAGATATCGGCACCCTCTAC-3'; 5'- GCCTAGTTCTGCACGGATAAG-3', respectively. For the
226 qPCR assays, the reactions were performed on a 384-QuantStudio™ 12K Flex Real-Time PCR
227 System (Thermo Fisher), in a total volume of 12µL including 6ng of DNA, primers at a final
228 concentration of 300nM and 6µL of SensiFAST™ SYBR lo-ROX (Bioline). Telomere, RAG1 and
229 COI2 reactions were performed in triplicates on the same plates (10 plates in total); the qPCR
230 conditions were: 3min at 95°C, followed by 35 cycles of 10 s at 95°C, 15 s at 58°C and 10s at
231 72°C. A DNA sample being a pool of DNA from 10 adult individuals was used as a reference
232 sample and was included in triplicate on every plate. The efficiency of each amplicon was
233 estimated from a standard curve of the reference sample ranging from 1.5 to 24ng. The mean
234 reaction efficiencies were 109.1 ± 1.8% for telomere, 102.2 ± 1.6% for RAG1, 96.3 ± 1.1% for

235 COI2. The relative telomere length and mtDNAcn of each sample were calculated as $(1+Ef_{\text{Tel or COI2}})^{\Delta Cq\ \text{Tel or COI2}}/(1+Ef_{RAG1})^{\Delta CqRAG1}$; *Ef* being the amplicon efficiency, and ΔCq the difference in
236
237 Cq-values between the reference sample and the focal sample. Intra-plate technical
238 repeatabilities of telomere and *mtDNAcn* based on triplicates were 0.87 (95% C.I. [0.85-0.89])
239 and 0.96 (95% C.I. [0.95-0.97]) respectively. Inter-plate technical repeatabilities of telomere
240 and *mtDNAcn* based on one repeated plate were 0.98 (95% C.I. [0.97-0.99]) and 0.77 (95% C.I.
241 [0.59-0.88]) respectively.

242 The use of *mtDNAcn* as an index of mitochondrial density has been questioned in human
243 (Larsen et al., 2012), but we have previously shown good correlations between mtDNAcn and
244 mitochondrial respiration rates in pied flycatcher (Stier et al. 2019). Great tits have quite peculiar
245 telomeres, characterized notably by some ultra-long telomeres that do not seem to shorten with
246 age in adults (Atema et al., 2019). Since qPCR only provides an estimate of overall telomere
247 length, it has been suggested it could be suboptimal for this study species. Yet, relative telomere
248 length (*i.e.* measured using qPCR) in this species has been shown to shorten during the nestling
249 stage (Stier et al., 2016, 2015), to respond to environmental factors (*e.g.* hatching asynchrony:
250 (Salmón et al., 2016; Stier et al., 2016, 2015) and to predict adult survival (Salmón et al., 2017).
251 Within-individual repeatability of telomere length has recently been suggested to be an important
252 factor to evaluate the pertinence of telomere length data in a given study/species (Kärkkäinen et
253 al., n.d.), and the biological repeatability in our dataset was R = 0.66 [0.55-0.79], which is above
254 the average reported by qPCR studies (*i.e.* R = 0.47), and within the upper range of what has been
255 reported for great tits (Kärkkäinen et al. 2021)

256 Birds were molecularly sexed using a qPCR approach adapted from(Chang et al., 2008;
257 Ellegren and Fridolfsson, 1997). Forward and reverse sexing primers were 5'-

258 CACTACAGGGAAAACTGTAC-3′ (2987F) and 5′- CCCCTTCAGGTTCTTTAAAA -3′ (3112R),
259 respectively. qPCR reactions were performed in a total volume of 12µL including 6ng of DNA,
260 primers at a final concentration of 800nM and 6µL of SensiFAST™ SYBR® Lo-ROX Kit (Bioline).
261 qPCR conditions were: 3 min at 95°C, followed by 40 cycles of 45 s at 95°C, 60 s at 52°C and
262 60s at 72°C, then followed by a melting curve analysis (95°C 60s, 45°C 50s, increase to 95°C at
263 0.1°C/s, 95°C 30s). Samples were run in duplicates in a single plate and 6 adults of known sex
264 were included as positive controls. Sex was determined by looking at the dissociation curve,
265 with two peaks indicating the presence of a Z and W chromosome (female), and one peak
266 indicating the presence of only the Z chromosomes (male).
267
268 *Statistical analyses*

269 We ran several linear or generalized linear mixed models (LMMs/GLMMs) for both
270 nestling and juvenile data. In order to account for genetic effects and the effects of growing
271 environment other than prenatal hormones, we included the IDs of both the nest of origin
272 (i.e. where a nestling hatched) and the nest of rearing (i.e. where a nestling grew up) as
273 random intercepts in all suitable models. For physiological measurements, assay batch ID was
274 included either as random intercept or fixed factors (depending on the number of levels, see
275 Tables for details). All initial models included the prenatal hormone treatment (TH vs. CO),
276 date (continuous variable), cross-fostering (yes/no), brood size (continuous variable),
277 proportion of TH-nestlings (continuous variable) in a nest, and their interactions with hormone
278 treatment as fixed factors. Except for hormone treatment, other variables were initially
279 included because of their potential confounding effects on nestling growth and development
280 but only retained in the final models when $p < 0.15$ in order not to miss potentially significant
281 factors. Nevertheless, in the final models, significance level was still set at 0.05. In models of

282 nestling growth (i.e. body mass and tarsus length), the ID of each individual was included as a
283 random intercept.

284 To analyze nestling growth, we separated the data into the early (i.e. from day 2 to day
285 7) and late (i.e. from day 7 to day 14) phases. Because of the start of heating treatment at day
286 7, this separation allows us to make use of the larger sample size during the early phase and
287 focus on the nestlings from non-heated nests during the late phase. In these models, age (as
288 a continuous variable) and hormone × interaction were included as additional fixed factors,
289 and individual ID as a random intercept. In order to account for the variation in growth rate
290 between nests, age (continuous variable) was also treated as a random slope to interact with
291 both the original and rearing nests. For tarsus length, we additionally controlled for the
292 measurer ID as a fixed factor. Because molecular sexing was only conducted on nestlings
293 having DNA extracted for mtDNAcn and rTL, not all nestlings included in the growth analyses
294 were sexed. We therefore did not include sex in order to make use of the full data set.
295 Repeating our models with only the sexed nestlings still gave qualitatively the same results
296 and did not show sex-specific effects of prenatal THs on growth. For the juveniles captured
297 during autumn/winter we ran LMMs with hormone treatment, date of capture, and sex
298 (because of clear sexual size dimorphism at this age) as fixed factors.

299 For all models of physiological measurements fixed factors included prenatal hormone
300 treatment, nestling sex and body mass. If not showing any trends (i.e. $p<0.15$), sex and body
301 mass were then removed from the final models, given the smaller sample sizes for the
302 physiological measurements. Natural log transformation was conducted on highly-skewed
303 data: mtDNAcn and three oxidative stress biomarkers to ensure normal residual distributions.
304 rTL and plasma THs were untransformed as residuals qualified the assumption of normality.

305 Because both mtDNAcn and rTL are ratios to a single copy gene, we also z-transformed the
306 data to allow across-study comparison (Verhulst, 2020). The ages of all juveniles were pooled
307 as a single age category "autumn", and age was thus included as a three-level categorical
308 variable (day 7, day 14, and autumn) in order to estimate the changes of mtDNAcn and rTL
309 between each time point.

310 We used GLMMs to estimate the influence of prenatal thyroid hormones on hatching
311 success, fledging success (i.e. pre-fledging survival), and post-fledging survival. We modelled
312 the outcome of each egg (i.e. hatched or not) or nestling (i.e. survived or not) using a logit link
313 function and specifying a binomial residual distribution. For hatching success, two nests in
314 which eggs never hatched (i.e. cause of failure likely unrelated to our treatment) were
315 excluded, giving a final sample size of 354 eggs from 42 nests. The nest ID was treated as a
316 random intercept, and prenatal hormone treatment and laying date of each nest as fixed
317 factors. For fledging success, we only included the 131 nestlings (reared in 27 nests after cross-
318 fostering) that successfully hatched and were subsequently reared in the nests that were not
319 subject to temperature manipulation in the analysis. Among the 131 nestlings, 99 successfully
320 fledged and were included in the analysis of post-fledging survival. According to the nestling
321 ringing records since 1999, only 8 out of >6200 birds ringed on the Ruissalo island were
322 recaptured outside the island, suggesting a very low dispersal rate. The individuals that were
323 never recaptured in the autumn were therefore presumed dead. The ID of rearing and original
324 nests was treated as a random intercept and prenatal hormone treatment as the fixed factor.
325 Laying date, brood size, and cross-fostering, as well as their interactions with the hormone
326 treatment, were initially included but only retained in the final model when $p < 0.15$.

327 In all statistical models described above, hormone-related two-way interactions were
328 of interest and therefore included. Following the suggestion by (Schielzeth, 2010), input
329 variables (except for the technical variables) were mean-centered before model fitting.
330 Significant interactions were further examined by post-hoc interaction analysis using the R
331 package *emmeans* (Lenth 2021).

332 All LMMs and GLMMs were conducted in the R environment 3.6.1, using the package
333 *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017). P values were determined
334 using the Kenward-Roger method to approximate the denominator degrees of freedom (R
335 package *pbkrtest*, (Halekoh and Hojsgaard, 2014), implemented within *lmerTest*) for LMMs
336 and by Laplace approximation for GLMMs. Model assumptions of normality and
337 homoscedasticity were diagnosed by visual inspection on simulated residuals using the R
338 package *DHARMa* (Hartig 2021). Clear violation was only observed in the models for plasma
339 T3 in which residuals were heteroscedastic. Nevertheless, given the fact that the model of T3
340 did not detect any significant effect and the general robustness of LMMs (Schielzeth et al.,
341 2020), this did not influence our conclusion.

342

343 **Results**

344 Early body mass growth (between day 2 and day 7 post-hatching) did not differ
345 between TH-supplemented and control groups (Table 1a, Fig 1a), but individuals from TH-
346 supplemented eggs grew faster at the later nestling stage, i.e. between day 7 and day 14 post-
347 hatching (Hormone × age interaction, $F_{1,20.18} = 5.449$, $p = 0.030$, Table 1b, Fig 1b; body mass
348 gain (mean±SE) in TH-nestlings 0.899 ± 0.042 g/day, CO-nestlings 0.786±0.043 g/day).
349 Similarly, individuals from TH-supplemented eggs expressed marginally faster tarsus growth

rate between day 7 and day 14 post-hatching ($F_{1,31.06}$ = 3.737, p=0.062, Table 1c, TH: 0.567±0.033 mm/day, CO: 0.476±0.034 mm/day, Fig 1c). Cross-fostering was retained in the final model of early body mass growth and suggested that fostered chicks had a lower growth rate (-0.139±0.069 g/day) than non-fostered chicks (p = 0.046, Table 1a). When analyzing the body mass and tarsus separately for each age, body mass was slightly smaller on day 2 and day 7 in the TH-supplemented group compared to control, yet there was no statistically significant difference between the groups at any age (Fig 1, Table S1). At day 7, fostered nestlings tended to have a lower body mass by 0.347 g (SE = 0.179, p=0.053) on average, and had significantly shorter tarsi by 0.33 mm (SE = 0.146, p=0.025), yet such differences disappeared by day 14 (Table S1). In juveniles, there was no difference in body mass or wing length between the treatment groups (Body mass: $F_{1,9.79}$ = 0.003, p = 0.959; wing length: $F_{1,10.34}$ = 0.011, p = 0.919).

362 **Table 1.** Linear mixed models of early and late nestling growth in response to prenatal TH-

363 supplementation.

(a) Early nestling body mass growth (day 2 – day 7)						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=224)	Intercept	0.839	0.916			
Nest of origin (n=42)	Intercept	0.627	0.792			
	Age	0.005	0.070			
Nest of rearing (n=42)	Intercept	0.061	0.248			
	Age	0.007	0.083			
Residual		0.202	0.450			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	8.232	0.146	56.478			
Hormone (TH)	-0.182	0.142	-1.284	1, 38.29	1.650	0.207
Age	1.730	0.019	89.716	1, 45.64	8045.995	**<0.001**
Cross-foster (yes)	-0.139	0.069	-2.008	1, 188.30	4.031	**0.046**
Hormone × Age	0.009	0.016	0.560	1. 28.84	0.314	0.580
(b) Late nestling body mass growth (day 7 – day 14)						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=111)	Intercept	0.554	0.744			
Nest of origin (n=36)	Intercept	0.374	0.611			
	Age	0.007	0.083			
Nest of rearing (n=20)	Intercept	0.001	0.037			
	Age	0.015	0.122			
Residual		0.534	0.731			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	15.525	0.141	101.416			
Hormone (TH)	-0.272	0.287	-0.947	1, 29.68	0.896	0.351
Age	0.841	0.035	24.076	1, 19.49	579.635	<0.001
Hormone × Age	0.113	0.049	2.334	1, 20.18	5.449	**0.030**
(c) Late nestling tarsus growth (day 7 – day 14)						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=111)	Intercept	0.235	0.485			
Nest of origin (n=36)	Intercept	0.213	0.461			
	Age	0.012	0.111			
Nest of rearing (n=20)	Intercept	0.000[§]	0.000[§]			
	Age	<0.001	<0.001			
Residual		0.376	0.613			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	20.660	0.164	126.251			
Hormone (TH)	-0.172	0.21	-0.818	1, 30.22	0.669	0.420
Age	0.521	0.024	22.090	1, 27.16	487.968	<0.001
Measurer 2	0.2220	0.184	1.197	2, 95.70	6.832	0.002
Measurer 3	0.586	0.173	3.381			
Hormone × Age	0.091	0.047	1.933	1, 31.06	3.737	0.062

Sample sizes:
Early growth, n = 99 control and 119 TH-supplemented individuals at day 2, 104 control and 117 TH-supplemented individuals at day 7. Please noticed that in total n = 42 nests during the early growth, but n = 41 nests at day 2 and n = 41 nests at day 7, because we missed out the

measurements for one nest at day 2 (n = 7 nestlings), while by day 7, nestlings from another nest died.
Late growth, n = 57 control and 54 TH-supplemented at day 7, 50 control and 49 TH-supplemented at day 14
[§] Singular fit (i.e. insufficient data to reliably estimate the variance)

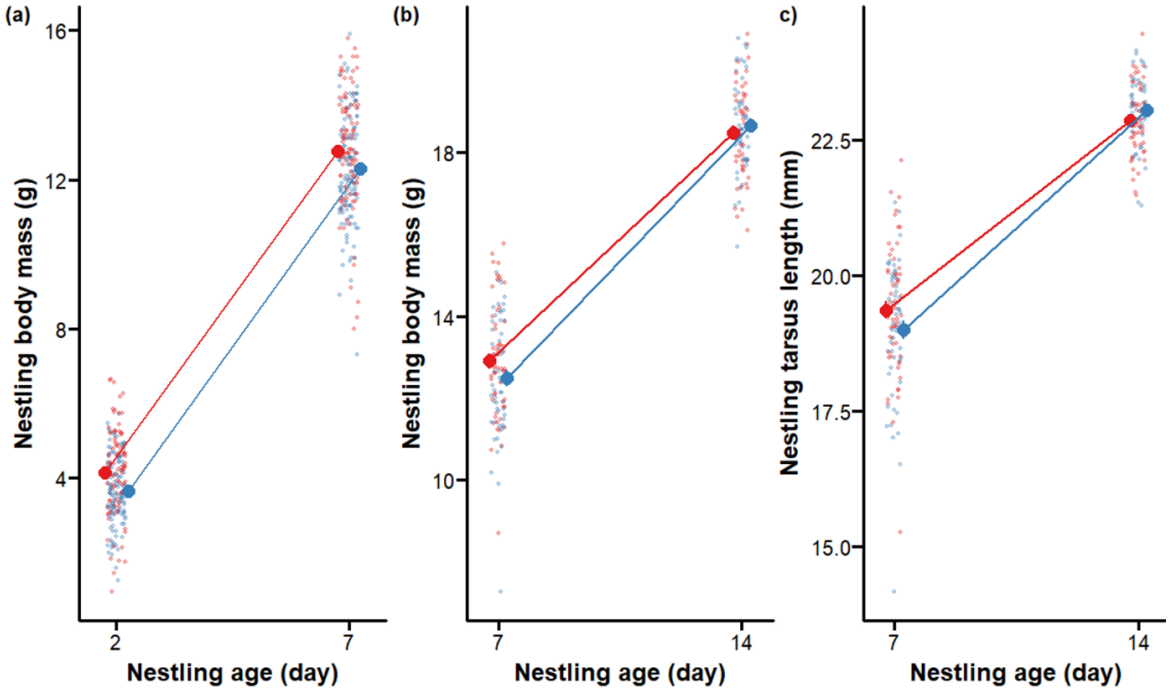
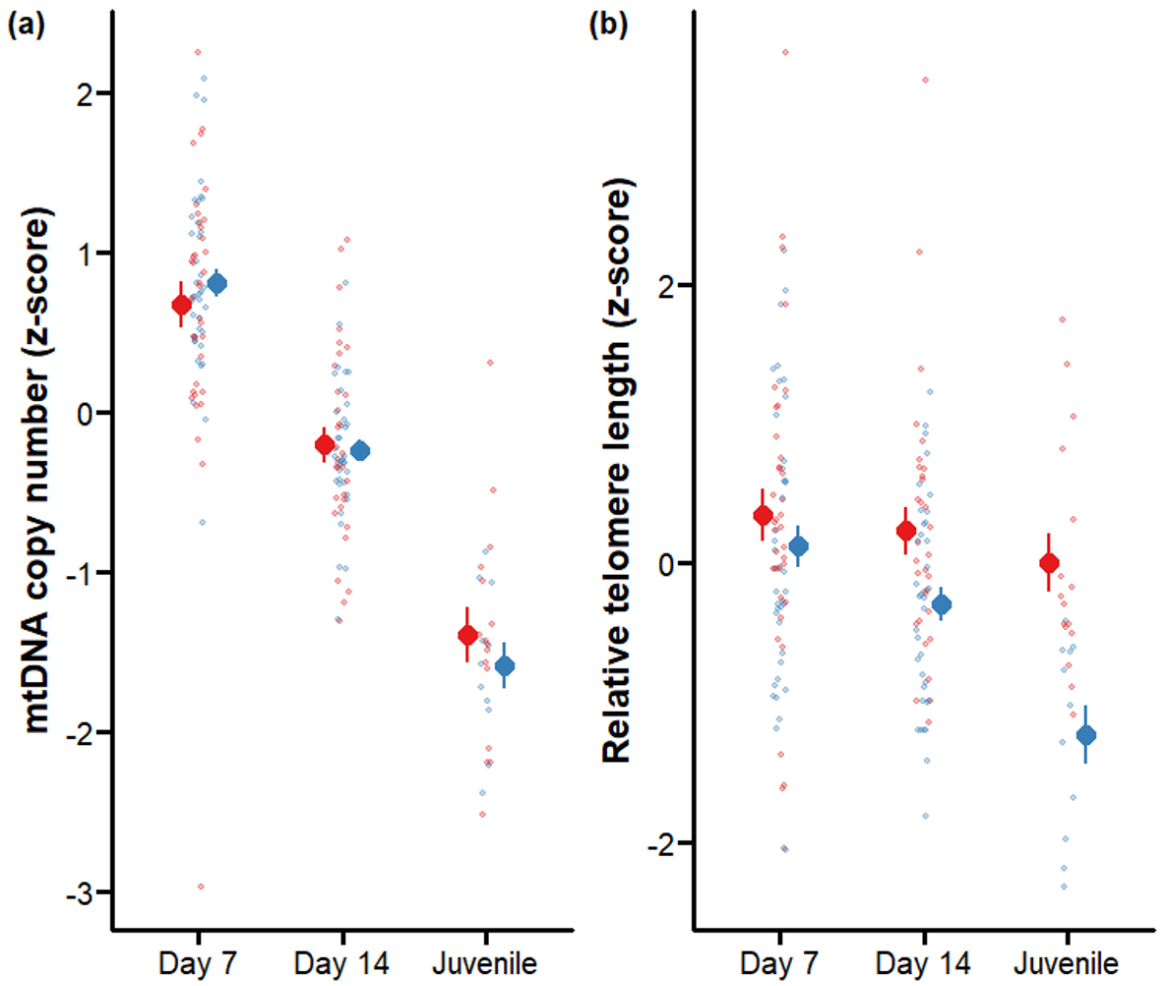


Fig. 1. Nestling growth in prenatally TH-supplemented (blue dots) and control (red dots) groups. (a) body mass growth during early nestling stage (day 2 to day 7 post-hatching) (b) body mass growth late nestling stage (day 7 to day 14 post-hatching), (c) structural size (tarsus) growth during late nestling stages. Means±SE (large dots) and scatter of the raw data (small, semi-transparent dots) are shown. See sample sizes in the text.



373

374 Fig 2. (a) Relative mitochondrial density (z-scale) and (b) relative telomere length in prenatally
375 TH-supplemented (blue dots) and control groups (red dots) at different developmental stages
376 (day 7 and 14 post-hatching and as juveniles, ca. 3 months of age). Means±SE (large dots) and
377 scatter of the raw data (small, semi-transparent dots) are shown. See sample sizes in the text.

378

379 Biomarkers of intracellular oxidative status (total GSH, GSH:GSSG ratio) and damage to lipids
380 14 days post-hatching did not clearly differ among the treatment groups (marginal means±SE:
381 total GSH µmol/mg protein: TH-supplemented, 0.253±0.022, control, 0.249±0.023; GSH:GSSG
382 ratio: TH-supplemented, 0.045±0.008, control, 0.053±0.010; MDA nmol/mg protein: TH-

383 supplemented, 0.038±0.005, control, 0.037±0.005; Table S2). Plasma T3 and T4 also did not
384 differ among prenatally TH-supplemented and control groups 14 days post hatching (marginal
385 means±SE pmol/ml T3: TH-supplemented, 1.21±0.17, control, 1.23±0.19; T4: TH-
386 supplemented, 7.35±0.946, control, 7.39±1.02; Table S3).

387 Mitochondrial density decreased with age, but there were no clear differences between
388 prenatally TH-supplemented and control groups at any age (d7, d14 or juveniles, Fig 2a, Table
389 2). Post-hoc analyses indicated significant decrease between each age category (all Tukey-
390 adjusted $p < 0.001$), regardless of the hormone treatment.

391 Telomere length decreased with age (Table 2, Fig 2b), but the pattern was different
392 between TH-supplemented and control group (Table 2, Fig 2b): Early in the nestling phase the
393 difference between the prenatally TH-supplemented and control group approached
394 significance (d7, Tukey post-hoc test: TH vs control: $t_{46.2} = 1.804$, $p = 0.078$). On day 14 post-
395 hatching, telomeres were significantly shorter (13%) in the TH group (Tukey post-hoc test, TH
396 vs Control: $t_{46.8} = 3.166$, $p = 0.003$), and this difference was accentuated in juveniles, offspring
397 from TH supplemented eggs having telomeres being ca. 33% shorter than offspring from
398 control group (Tukey post-hoc test, TH vs Control: $t_{109.8} = 4.311$, $p < 0.0001$). Further, we added
399 the percentage of body mass gain from day 7 to day 14 in the model to examine whether the
400 effect of prenatal TH supplementation was via its effect on nestling growth, but this did not
401 qualitatively change the results. The percentage of body mass gain did not show a clear effect
402 on telomere length (estimate±SE = -0.575±0.580, $F_{1,64.54}=0.981$, $p=0.326$). Similarly, the three
403 oxidative stress biomarkers also did not explain the difference in telomere shortening (all p's
404 > 0.31).

Hatching success did not clearly differ among prenatally TH supplemented and control individuals (TH 67.6%, control 69.1%; z =0.306, p = 0.760, Table 3a). While nestling survival to fledging was higher in prenatally TH supplemented than control nestlings (TH: 83.05% vs CO 69.44%), our model did not provide clear support for such a difference (z = 0.136, p = 0.892, Table 3b) and an analysis from egg to fledgling stage yielded qualitatively similar results (treatment p = 0.86). Fledging success was lower in the nests with larger brood sizes (z = -2.333, p = 0.020, Table 3b). Juvenile recapture rate was not significantly different across the groups (TH: 28.57% vs CO 34.00%, z=-0.993, p = 0.321, Table 3c).

413 **Table 2.** Linear mixed models of mitochondrial density and telomere length in blood cells
414 growth in response to prenatal TH-supplementation. In these models, age was as a categorical
415 factor with three levels. For the purpose of mean-centering, two dummy variables ("Age day
416 7" and "Age day 14") were created to represent the respective contrast against the juvenile
417 age.

(a) Mitochondria density						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=78)	Intercept	0.084	0.290			
Nest of origin (n=35)	Intercept	0.041	0.203			
Nest of rearing (n=19)	Intercept	0.000§	0.000§			
DNA batch (n=8)	Intercept	0.017	0.129			
Residual		0.244	0.494			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	-0.023	0.079	-0.295			
Hormone (TH)	0.021	0.135	0.155	1, 24.00	0.024	0.878
Sex	0.200	0.121	1.651	1, 73.16	2.723	0.103
Age day 7	2.202	0.121	18.134	1, 111.27	328.825	**<0.001**
Age day 14	1.225	0.123	9.968	1, 111.12	99.357	**<0.001**
Hormone × Age day 7	0.352	0.240	1.464	1, 111.78	2.142	0.146
Hormone × Age day 14	0.197	0.243	0.807	1, 111.70	0.652	0.421
(b) relative telomere length						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=77)	Intercept	0.093	0.305			
Nest of origin (n=35)	Intercept	0.000§	0.000§			
Nest of rearing (n=19)	Intercept	0.026	0.162			
DNA batch (n=8)	Intercept	0.394	0.628			
Residual		0.513	0.716			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	-0.085	0.236	-0.361			
Hormone (TH)	-0.635	0.153	-4.150	1, 16.06	17.223	**<0.001**
Sex	-0.268	0.154	-1.742	1, 65.94	3.035	0.086
Age day 7	0.904	0.174	5.197	1, 117.27	27.008	**<0.001**
Age day 14	0.654	0.175	3.728	1, 115.66	13.895	**<0.001**
Brood size at day 2	0.213	0.094	2.271	1, 10.18	5.157	**0.046**
Hormone × Age day 7	1.039	0.343	3.027	1, 120.32	9.165	**0.003**
Hormone × Age day 14	0.741	0.347	2.137	1, 119.21	4.567	**0.035**
Hormone × Brood size	-0.255	0.151	-1.681	1, 32.83	2.827	0.102

Sample sizes: n = 37 control and 41 TH-supplemented individuals at day 7; 33 control and 38 TH-supplemented individuals at day 14; 16 control and 13 TH-treatment recaptured juveniles
§ Singular fit (i.e. insufficient data to reliably estimate the variance)

418 **Table 3.** Generalized linear mixed models of hatching success, fledging success, and post-
419 fledging survival. For fledging success and post-fledging survival, only the nestlings that were
420 reared in the nests without temperature manipulation were included. The models were fit
421 by maximum likelihood approach using Laplace approximation with binomial distribution
422 and logit link function. All input variables were mean-centered before model-fitting.

(a) Hatching success (n = 354 eggs)				
Random effects:		**Variance**	**Std. Dev.**	
Nest of origin (n=42)	Intercept	0.052	0.228	
Fixed factors	**Estimate**	**SE**	**z**	**p**
Intercept	0.781	0.123	6.339	
Hormone (TH)	0.074	0.241	0.306	0.760
(b) Fledging success (n = 131 nestlings)				
Random effects:		**Variance**	**Std. Dev.**	
Nest of origin (n=39)	Intercept	0.00[§]	0.00[§]	
Nest of rearing (n=27)	Intercept	582.60	24.14	
Fixed factors	**Estimate**	**SE**	**z**	**p**
Intercept	-0.263	3.036	-0.087	
Hormone (TH)	0.195	1.436	0.136	0.892
Brood size	-10.640	4.562	-2.333	**0.020**
(c) Post-fledging survival (n = 99 fledlings)				
Random effects:		**Variance**	**Std. Dev.**	
Nest of origin (n=34)	Intercept	0.000[§]	0.000[§]	
Nest of rearing (n=19)	Intercept	1.584	1.258	
Fixed factors	**Estimate**	**SE**	**z**	**p**
Intercept	-0.925	0.395	-2.339	
Hormone (TH)	-0.569	0.573	-0.993	0.321

[§] Singular fit (i.e. insufficient data to reliably estimate the variance)
Sample size:
Hatching success: n = 176 control eggs and 178 TH-eggs
Fledging success: n = 72 control nestlings and 59 TH-nestlings

423

424 **Discussion**

425 By manipulating prenatal exposure to THs in a wild passerine species, we demonstrate that an
426 increase in prenatal THs can accelerate both postnatal growth and telomere shortening. Yet,
427 we did not detect significant effects of elevated prenatal THs on postnatal oxidative stress
428 levels, cellular energetics measured as mtDNA copy number, circulating TH levels, or short to
429 medium-term survival (*i.e.* hatching success, fledging success, and apparent survival to the
430 next autumn/winter).

431 Shorter telomeres in the TH-supplemented group were only detected from 14 days
432 after hatching onwards, which seems to exclude direct effects or prenatal TH on telomere
433 dynamics during embryonic development. Yet, this coincides with the accelerated postnatal
434 body mass and tarsus growth observed in the TH-group (between day 7 and 14) compared to
435 controls, and faster growth can accelerate telomere shortening either through enhanced cell
436 division or through inducing oxidative stress (Monaghan and Ozanne, 2018). Considering the
437 lack of impact of prenatal THs on oxidative stress reported here and in previous work on birds
438 (Hsu et al., 2020, 2019a; Sarraude et al., 2020a; Sarraude et al., 2020b), it is not surprising the
439 accelerated telomere shortening observed in this study could not be explained by the
440 measured oxidative stress biomarkers. Yet, we cannot fully rule out this explanation since DNA
441 damage was not directly assessed and oxidative stress sensitivity might vary between different
442 biomolecules (Reichert and Stier, 2017). The effect of prenatal THs on telomere length seemed
443 to increase with age (*i.e.* stronger effect observed in juveniles than at day 14), which could be
444 explained by a delayed effect of fast postnatal growth (day 7 to day 14) because telomeres
445 only shorten at the subsequent cellular division, and therefore delayed effects are likely to be
446 observed (Monaghan and Ozanne, 2018). Nevertheless, including body mass growth between

447 day 7 and day 14 in the model did not explain the difference in telomere shortening. Our
448 hypothesis that prenatal THs could program postnatal metabolic and endocrine function (and
449 thus affect telomere shortening indirectly) was not supported as we found no evidence for
450 differences in mitochondrial density or plasma TH levels (key coordinators of metabolism)
451 across the treatment groups. The latter results are not fully surprising considering the limited
452 evidence supporting a prenatal programming of plasma THs (sex-specific effect on T4 only,
453 Hsu et al. 2017; no effect, Hsu et al. 2020) or mitochondrial density (no effect, Stier et al. 2020;
454 Hsu et al. 2020). Yet, our measurements are limited to blood and tissue-specific effects of TH
455 on mitochondrial density or TH-related cellular machinery (receptors and converting enzymes;
456 Ruuskanen et al. 2022) may occur as this has been shown for instance for glucocorticoids
457 (Weber et al. 2002). According to the *metabolic telomere attrition hypothesis*, telomere
458 shortening is likely to be increased during energy-demanding periods, and accelerated growth
459 under limited resources is likely carrying a metabolic cost (Casagrande and Hau, 2019).

460 The effects observed here on telomeres in response to increased prenatal TH levels
461 are in sharp contrast with our previous findings in another passerine species, the collared
462 flycatcher (Stier et al. 2020). Indeed, increasing prenatal THs in the collared flycatcher
463 increased telomere length measured very shortly after hatching (day 2), while not affecting
464 telomere shortening during postnatal growth (Stier et al. 2020). There are several alternative
465 explanations for these contrasted findings: (1) we cannot exclude that prenatal THs increased
466 telomere length during embryonic development in great tits since our first telomere length
467 measurement (day 7, *ca.* 70% of fledging body mass) was done considerably later than in
468 collared flycatchers (*i.e.* day 2, *ca.* 20% of fledging body mass). (2) THs differently influenced
469 post-natal growth in the two species: great tits from the TH-supplemented eggs were initially
470 slightly smaller, but grew faster in the late nestling period, whereas collared flycatchers from

471 the TH-supplemented eggs were bigger soon after hatching, but grew slightly slower during
472 the nestling stage (Hsu et al., 2019a). These contrasting growth patterns may explain, at least
473 partly, the contrasted findings in these two species: an increased metabolic demand for fast
474 postnatal growth in great tit in response to prenatal TH could accelerate telomere shortening,
475 while a reduced metabolic demand in collared flycatcher from TH-injected eggs could enable
476 telomere maintenance (Casagrande and Hau, 2019). Measurements of the mTOR signaling
477 pathway could shed light on the validity of this hypothesis (Casagrande and Hau, 2019). (3)
478 The effects of maternal signals on offspring are expected to differ based on environmental
479 conditions, so called context-dependent effects: For example, prenatal androgens have been
480 found to differentially influence offspring growth depending on season or food availability
481 (Groothuis et al., 2020; Muriel et al., 2015). We recently found no evidence for prenatal THs
482 differentially influencing growth and early-life survival depending on rearing temperature
483 (Hsu et al. 2020), yet the influence of resource availability was not tested. In two sister species,
484 collared and pied flycatchers, differential effects of prenatal THs on growth may have been
485 explained by differences in food resources (Sarraude et al. 2020b). (4) These two species
486 exhibit different life-histories, as collared flycatchers are migratory and great tits (relatively)
487 sedentary. Migratory species have generally higher metabolism (Jetz et al., 2008) as well as an
488 overall faster pace of life (Soriano-Redondo et al. 2021), and may thus present differences in
489 TH physiology. We may speculate that the telomere maintenance, and role of THs in the
490 regulation of telomere maintenance may differ across species with different life-histories.
491 Telomere maintenance is known to be influenced by e.g. species lifespan (Haussmann et al.,
492 2004) and migratory populations within a species have shorter telomeres (Bauer et al., 2016).
493 Yet, to our knowledge, studies have not considered migratory vs. non-migratory species in
494 telomere maintenance. Species-specific effects of the prenatal hormonal environment on

495 telomere dynamics have already been described: elevated prenatal glucocorticoid levels led
496 to shorter telomeres in domestic chicken and female zebra finch (Haussmann et al., 2012;
497 Tissier et al., 2014), but to longer telomeres in yellow-legged gull (Noguera, da Silva, and
498 Velando 2020). To understand if any of the hypotheses presented above is likely to explain the
499 contrasted effects found here and in Stier et al. (2020), more studies on the impact of prenatal
500 THs on telomere biology across taxa are needed.

501 As mentioned above, in the collared flycatcher, elevated yolk THs increased nestlings
502 body mass at day 2 (Hsu et al. 2019), whereas in this great tit study, elevated yolk THs only
503 non-significantly reduced nestling body mass at day 2 (marginal mean ± SE = -0.402 ± 0.245,
504 p=0.109). In both studies, the lighter group of nestlings caught up with the other group before
505 fledging. It therefore appears that the main difference lies on the differential effects of THs
506 during the peri-hatching period. Interestingly, another experiment in the same population of
507 great tits found that elevated yolk THs significantly shortened the time needed to hatch by 0.6
508 days on average (Cossin-Sevrin et al. 2022). Despite the shorter developmental time, the
509 nestling body mass at day 2 was also found non-significant (Cossin-Sevrin et al. 2022). Thus,
510 the exact peri-hatching effects of prenatal THs in altricial birds still need more information to
511 clarify.

512 To understand the selective pressures on maternal allocation and signaling, it is
513 important to characterize true fitness effects from development to survival and lifetime
514 reproductive success. In contrast to our predictions, we found no effects of elevated prenatal
515 TH-supplementation on apparent survival or individual condition during their first autumn/
516 winter. Unfortunately, longer-term effects on survival and reproductive success are very
517 challenging to measure in such an experimental setting in the wild, as the recruitment to first
518 breeding is usually very low (e.g. < 10% Radersma et al., 2015), which requires a very high

519 number of nests to be manipulated, something often not feasible for both logistical and ethical
520 reasons. Yet, long-term effects of accelerated early-life telomere shortening may lead to a
521 decrease in longer-term survival and lifespan (Eastwood et al., 2019; Heidinger et al., 2012).
522 The negative effects on later stages suggest that there need to be benefits of high prenatal
523 THs for the offspring or mother, potentially during early-life stages, where selection can be
524 strong. In this study, the elevated growth rates could increase offspring competitive abilities
525 early in life, yet, the effects of prenatal THs on growth seem to be highly inconsistent across
526 studies (Hsu et al., 2020, 2019a, 2017; Ruuskanen et al., 2016a; Sarraude et al., 2020a;
527 Sarraude et al., 2020b). While circulating THs are known to be associated with health and
528 ageing in humans and mammalian models (Bano et al. 2017, 2019; Møllehave et al. 2020), our
529 study is the first to show that exposure to higher prenatal thyroid hormones can lead to
530 accelerated cellular ageing measured through telomere length. This should stimulate further
531 research using both epidemiological and experimental approaches across taxa to uncover the
532 potential regulation of telomere biology by thyroid hormones both pre- and postnatally.

533

534 **Author contribution**

535 BYH, AS & SR designed the study and collected field data. NCS, AS & SR conducted laboratory
536 work. BYH analyzed the data. BYH, AS & SR wrote the manuscript, with input from NCS.
537

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546

547 **Data accessibility**

548 All data is archived and available in Figshare (DOI: 10.6084/m9.figshare.20110409).

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Supplementary material to

Prenatal thyroid hormones accelerate postnatal growth and telomere shortening in wild great tits

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Table S1. Linear mixed models (GLMM) on the effects of prenatal thyroid hormone elevation on nestling body mass and tarsus length at day 2, day 7, and day 14 post-hatching. The denominator degree of freedom (ddf) were approximated by the Kenward-Roger method to calculate p values.

(a) Body mass at day 2 (n = 218 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=41)	Intercept	0.481	0.694			
Residual		0.625	0.791			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	3.849	0.123	31.426			
Hormone (TH)	-0.402	0.245	-1.639	1, 38.69	2.686	0.109
Sample size: n = 99 control and 119 TH-supplemented						
The total sample size at day 2 (n =218 nestlings from 41 nests) was smaller than that at day 7 because we missed the day 2 for one nest (n = 7 nestlings)						
(b) Body mass at day 7 (n = 221 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=41)	Intercept	0.944	0.972			
Nest of rearing (n=41)	Intercept	<0.001	<0.001			
Residual		1.482	1.217			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	12.495	0.175	71.459			
Hormone (TH)	-0.367	0.353	-1.040	1, 38.93	1.081	0.305
Cross-foster (yes)	-0.347	0.179	-1.945	1, 179.22	3.782	0.053
Brood size	-0.141	0.072	-1.956	1, 25.71	3.828	0.061
Hormone × Cross-foster	0.668	0.368	1.813	1, 28.49	3.286	0.080
(c) Tarsus length at day 7 (n = 221 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=41)	Intercept	0.4916	0.700			
Nest of rearing (n=41)	Intercept	0.000[§]	0.000[§]			
Residual		0.982	0.991			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	18.940	0.205	92.592			
Hormone (TH)	-0.275	0.322	-0.855	1, 42.78	0.731	0.397
Cross-foster (yes)	-0.330	0.146	-2.268	1, 178.84	5.145	0.025
Proportion of TH nestlings	-0.297	0.482	-0.617	1, 54.90	0.381	0.540
Measurer 2	0.211	0.199	1.059	2, 204.00	3.357	0.037
Measurer 3	0.523	0.204	2.561			
Hormone × proportion of TH nestlings	-1.711	0.972	-1.762	1, 58.65	3.103	0.083
Sample size: n = 104 control and 117 TH-supplemented						
[§] Singular fit (i.e. insufficient data to reliably estimate the variance)						

Table S1 (cont.)

(d) Body mass at day 14 (n = 99 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=34)	Intercept	0.310	0.557			
Nest of rearing (n=19)	Intercept	0.228	0.477			
Residual		0.801	0.895			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	18.603	0.176	105.693			
Hormone (TH)	0.099	0.303	0.747	1, 22.45	0.130	0.722
(e) Tarsus length at day 14 (n = 99 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=34)	Intercept	0.132	0.363			
Nest of rearing (n=19)	Intercept	0.000[§]	0.000[§]			
Residual		0.283	0.532			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	23.199	0.238	97.313			
Hormone (TH)	0.157	0.175	0.899	1, 27.77	0.808	0.376
Measurer 2	-0.488	0.265	-1.845	2, 61.90	4.014	0.023
Measurer 3	-0.066	0.256	-0.258			

Sample sizes: n = 50 control and 49 TH-supplemented

[§] Singular fit (i.e. insufficient data to reliably estimate the variance)

Table S2. Linear mixed models of blood oxidative stress biomarkers in response to prenatal TH-supplementation.

(a) total GSH µmol/mg protein (n = 58 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=29)	Intercept	0.031	0.177			
Nest of rearing (n=18)	Intercept	0.050	0.223			
Residual		0.044	0.210			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	-1.492	0.114	-13.148			
Hormone (TH)	0.014	0.105	0.128	1, 15.46	0.016	0.900
GSH assay batch	0.221	0.145	1.526	1, 16.57	2.328	0.146
(b) GSH:GSSG ratio (n = 58 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=29)	Intercept	0.210	0.458			
Nest of rearing (n=18)	Intercept	0.031	0.175			
Residual		0.134	0.367			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	-3.106	0.181	-17.207			
Hormone (TH)	-0.171	0.243	-0.703	1, 16.75	0.456	0.509
Body mass	0.106	0.059	1.794	1, 41.88	2.986	0.091
Proportion of TH-nestlings	-0.173	0.462	-0.375	1, 21.30	0.124	0.728
GSH assay batch	0.133	0.237	0.561	1, 16.87	0.307	0.587
Hormone × Proportion of TH-nestlings	1.787	0.926	1.930	1, 19.81	3.553	0.074
(c) MDA nmol/µg protein (n = 59 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=30)	Intercept	0.004	0.059			
Nest of rearing (n=18)	Intercept	0.004	0.060			
TBARS batch (n = 6)	Intercept	0.100	0.317			
Residual		0.039	0.198			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	-3.286	0.135	-24.340			
Hormone (TH)	0.036	0.067	0.536	1, 16.86	0.287	0.599

Sample sizes:
total GSH and GSH:GSSG ratio, n = 26 control and 32 TH-supplemented
MDA, n = 26 control and 33 TH-supplemented

Table S3. Linear mixed models of blood plasma thyroid hormones growth in response to prenatal TH-supplementation.

(a) T3 pmol/ml (n = 24 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=18)	Intercept	0.054	0.232			
Nest of rearing (n=11)	Intercept	0.127	0.357			
Residual		0.132	0.363			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	1.218	0.147	8.273			
Hormone (TH)	-0.026	0.202	-0.128	1, 7.37	0.016	0.902
(b) T4 pmol/ml (n = 24 nestlings)						
Random effects:		**Variance**	**Std. Dev.**			
Nest of origin (n=18)	Intercept	0.738	0.859			
Nest of rearing (n=11)	Intercept	2.934	1.713			
Residual		5.721	2.392			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	7.452	0.758	9.837			
Hormone (TH)	-0.041	1.113	-0.037	1, 6.57	0.001	0.973
Sex	2.101	1.119	1.877	1, 14.99	2.532	0.132

Sample sizes: n = 11 control and 13 TH-supplemented