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 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,

(Hsu et al., 2020, 2019a, 2017; Medici et al., 2013; Ruuskanen et al., 2016a; Sarraude et al., 2020a; Sarraude et al., 2020b; Vrijkotte et al., 2017; Zhang et al., 2019), which can directly contribute to telomere attrition through increasing cellular division (Monaghan and Ozanne, 2018; Stier et al., 2020), or indirectly accelerate telomere shortening through increasing oxidative stress (Monaghan and Ozanne, 2018; Reichert and Stier, 2017; Smith et al., 2016). (ii) Elevated TH levels are often associated with higher metabolic rates (Liu et al., 2006; Mullur et al., 2014; Welcker et al., 2013) and stimulate mitochondrial aerobic metabolism (Cioffi et al., 2013), both of which can potentially increase reactive oxygen species (ROS) and oxidative damage (Stier, Massemin, and Criscuolo 2014), accelerating telomere shortening (Reichert and Stier, 2017). It was recently shown that exposure to elevated prenatal TH levels can lead to a sex-specific increase in metabolic rate and circulating thyroid hormone levels shortly after hatching (rock pigeons *Columba livia*, Hsu et al. 2017, but see Ruuskanen et al. 2016), which suggests that prenatal hormones may program postnatal metabolism and TH-axis function. (iii) The 'metabolic telomere attrition hypothesis' (Casagrande and Hau, 2019) postulates that telomere shortening might be adaptive and have signaling functions related to metabolic demand (*i.e.* accentuated shortening when catabolism is favored over anabolism via mTOR inhibition). Since THs can have both anabolic and catabolic actions (Mullur et al., 2014), predictions can be made in both directions between prenatal THs and telomere length. From an evolutionary perspective, increased offspring metabolism or growth, which may be beneficial for the mother/offspring, diverts resources from somatic maintenance if resources are limited. This can accelerate damage to biomolecules and/or decrease repair/maintenance processes, and therefore accentuate telomere shortening. Therefore, prenatal TH levels would be expected to vary in relation to predicted environmental conditions (as observed in

terms of temperature and laying order, e.g. Ruuskanen, Groothuis, et al. 2016; Hsu, Verhagen, et al. 2019) to optimize this trade-off.

In contrast to most predictions relating prenatal THs to telomere length (see above), we recently reported that prenatal exposure to experimentally elevated THs increased telomere length in nestlings of a wild passerine, the collared flycatcher (*Ficedula albicollis,* Stier et al. 2020). To better understand the potential generality of this surprising finding as well as assess underlying mechanisms and potential carry-over effects of variation in prenatal THs on later life-stages and survival, we conducted a more detailed study in another passerine species, the great tit *(Parus major*). The aim of this study was to comprehensively investigate the influence of prenatal THs on growth, oxidative stress, plasma THs, mitochondrial density and telomere dynamics as well as survival via an experimental manipulation of prenatal THs in a wild population. We monitored offspring multiple times during development and as juveniles a few months after fledging. Based on the majority of prior literature, we would predict that elevated prenatal THs could lead to faster growth, increased plasma THs, oxidative stress and mitochondrial density, ultimately accelerating telomere shortening. Alternatively, if our previous finding in the collared flycatcher reflected a general pattern, we would predict that despite accelerating growth, elevated prenatal THs could increase telomere length. We also predict that elevated prenatal TH could increase post-fledging survival of the juveniles (*e.g.* both due to accelerated postnatal growth and potential beneficial effects for thermoregulation under low autumn-winter temperatures). However, longer-term survival, that we were not able to evaluate here, could be decreased for example due to shorter telomeres. As a previous study in the same species reported sex-dependent effects of prenatal THs on growth (Ruuskanen et al., 2016), we may also expect the effects on physiology and survival to differ between sexes.

Methods

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

Field experiment

The nest boxes were monitored with five-day intervals to track egg laying. Yolk T3 and T4 (i.e. a combined injection of the two hormones) levels were elevated in half of the nests (n = 21 TH nests and n = 21 control nests) by injection into the egg, following methods in Ruuskanen, Darras, et al. (2016). Control nests were injected with the vehicle (0.9% NaCl) only. The TH concentration in great tit eggs is in the population is 0.11 ± 0.04 ng/yolk for T3 and 0.96 ± 0.29 ng/yolk for T4 (Ruuskanen et al., 2019). We aimed to raise the amount of yolk TH by 2SD via injection into the egg yolk, a dose that has been recommended in relation to the natural hormone range of the study species (Podmokla et al., 2018). This corresponded to target doses of 0.041 ng/yolk for T3 and 0.325 ng/yolk for T4. To make sure injections would be performed on unincubated eggs mimicking maternal hormone levels (great tits can start incubation before the clutch is complete), injections were conducted on the day the 5^{th} egg was laid to all eggs in the clutch. Thereafter, injections were conducted each day for the newly laid egg.

Hatching date and success was monitored by visiting nests daily starting before the estimated hatching day. Nestling body mass (~0.01g) and tarsus (~0.5 mm) were measured on day 2 (mass only), 7 and 14 post-hatching. All nestling measurements were conducted between 8 am and 2 pm. After measurements on day 2, nestlings were nail-coded and about half of the brood were cross-fostered with same-age nestlings in another nest (preferentially between control and TH treatments) to increase statistical power by having chicks from TH and control-injected eggs experiencing the same postnatal environment. On day 7 a blood sample (ca 40µl) was taken, kept on ice, centrifuged within 8 hours, and RBCs frozen at -80ºC for molecular analyses (telomere length, mitochondrial density and molecular sexing). On day 14 a small blood sample (10-15µl) was snap-frozen in liquid nitrogen for oxidative stress biomarker analyses and stored at -80ºC, while the rest of the sample was kept on ice, and centrifuged. Plasma was used for TH analyses and RBCs for mitochondria and telomere measurements as above. After day 7 measurements half of the nest were subject to a temperature manipulation (nest temperature increased for *ca.* 2^{o}C during day 7 – day 14) for the purposes of another study. To avoid potential confounding effects, we here only analyze data from the nests which were not temperature-manipulated for day 14 and in juveniles (N = 19 nests, 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 = 41 nests, 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, 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.

Thyroid hormones and oxidative stress assays

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

Total glutathione (tGSH), the most abundant intra-cellular antioxidant, and the ratio between reduced and oxidized gluthatione (GSH:GSSG, an indicator of oxidative challenge) were measured from whole-blood samples with the ThioStar® Glutathione Fluorescent Detection Kit (K005-FI, Arbor Assays, USA) (Sarraude et al., 2020a). As a measure of oxidative damage, we assessed blood lipid peroxidation (malonaldehyde, MDA) using the TBARS-assay following Espin et al. (2017). Both measurements had CV% < 10 and are expressed per mg of protein (measured via BCA protein assay, ThermoFisher Scientific). Biomarkers of oxidative

stress were measured from 2 randomly selected nestlings per nest (n = 33 TH, n = 26 control nestlings).

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

We extracted DNA from blood cells using a standard salt extraction alcohol precipitation method (Aljanabi and Martinez, 1997). Extracted DNA was diluted in elution buffer BE for DNA preservation. DNA concentration and quality (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. 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. Here, we used RAG1 as a SCG (verified as single copy using a BLAST analysis on the great tit genome) and cytochrome oxidase subunit 2 (COI2) as a mitochondrial gene (verified as non-duplicated in the nuclear genome using a BLAST analysis). Forward and reverse telomere primers were 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' (Tel-1b) and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Tel-2b) respectively. Forward and reverse RAG1 primers were 5'-TCGGCTAAACAGAGGTGTAAA-3' and 5'-CAGCTTGGTGCTGAGATGTAT-3', respectively. Forward and reverse COI2 primers were 5'-CAAAGATATCGGCACCCTCTAC-3'; 5'- GCCTAGTTCTGCACGGATAAG-3', respectively. For the

qPCR assays, the 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 mean reaction efficiencies were 109.1 ± 1.8% for telomere, 102.2 ± 1.6% for RAG1, 96.3 ± 1.1% for COI2. The relative telomere length and mtDNAcn of each sample were calculated as $(1+Ef_{\text{Tel or COI2}})^{\Delta\text{Cq Tel or COI2}}/(1+Ef_{\text{RAG1}})^{\Delta\text{CqRAG1}}$; *Ef* being the amplicon efficiency, and ΔCq the difference in Cq-values between the reference sample and the focal sample. Intra-plate technical repeatabilities of telomere and *mtDNAcn* based on triplicates were 0.87 (95% C.I. [0.85-0.89]) and 0.96 (95% C.I. [0.95-0.97]) respectively. Inter-plate technical repeatabilities of telomere and *mtDNAcn* based on one repeated plate were 0.98 (95% C.I. [0.97-0.99]) and 0.77 (95% C.I. [0.59-0.88]) respectively.

The use of *mtDNAcn* as an index of mitochondrial density has been questioned in human (Larsen et al., 2012), but we have previously shown good correlations between mtDNAcn and mitochondrial respiration rates in pied flycatcher (Stier et al. 2019). Great tits have quite peculiar telomeres, characterized notably by some ultra-long telomeres that do not seem to shorten with age in adults(Atema et al., 2019). Since qPCR only provides an estimate of overall telomere length, it has been suggested 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 (Stier et al., 2016, 2015), to respond to environmental factors (*e.g.* hatching asynchrony: (Salmón

et al., 2016; Stier et al., 2016, 2015) and to predict adult survival (Salmón et al., 2017). 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 (Kärkkäinen et al., n.d.), and the biological repeatability in our dataset was R = 0.66 [0.55-0.79], which is above the average reported by qPCR studies (*i.e.* R = 0.47), and within the upper range of what has been reported for great tits (Kärkkäinen et al. 2021)

Birds were molecularly sexed using a qPCR approach adapted from(Chang et al., 2008; Ellegren and Fridolfsson, 1997). 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).

Statistical analyses

We ran several linear or generalized linear mixed models (LMMs/GLMMs) for both nestling and juvenile data. In order to account for genetic effects and the effects of growing environment other than prenatal hormones, we included the IDs of both the nest of origin (i.e. where a nestling hatched) and the nest of rearing (i.e. where a nestling grew up) as

257 random intercepts in all suitable models. In models with repeated measurements of the same
258 individuals, the ID of each individual was included as a random intercept and age as a random
259 slope to interact with both the original and rearing nests, accounting for the variation in
260 growth rate between nests. For physiological measurements, assay batch ID was included
261 either as random intercept or fixed factors (depending on the number of levels, see Tables for
262 details).

263 To analyze nestling growth, we separated the data into the early (i.e. from day 2 to day
264 7) and late (i.e. from day 7 to day 14) phases. Because of the start of heating treatment at day
265 7, this separation allows us to make use of the larger sample size during the early phase and
266 focus on the nestlings from non-heated nests during the late phase. The fixed factors of these
267 models included the prenatal hormone treatment (TH versus CO), age, and cross-fostering
268 (yes or no). For tarsus length, we additionally controlled for the measurer ID as a fixed factor.
269 The potential effect of brood size (at day 2) was initially explored but subsequently removed
270 from the models for simplicity because no significant effect was found. Because molecular
271 sexing was only conducted on nestlings having DNA extracted for mtDNAcn and rTL, not all
272 nestlings included in the growth analyses were sexed. We therefore did not include sex in
273 order to make use of the full data set. Repeating our models with only the sexed nestlings still
274 gave qualitatively the same results and did not show sex-specific effects of prenatal THs on
275 growth. For the juveniles captured during autumn/winter we ran LMMs with hormone
276 treatment, date of capture, and sex (because of clearer sexual size dimorphism at this age) as
277 fixed factors.

278 For all models of physiological measurements fixed factors included prenatal hormone
279 treatment, nestling sex and body mass. Because both mtDNAcn and rTL are ratios to a single

280 copy gene, we also z-transformed the data to allow across-study comparison (Verhulst, 2020).
281 Age was included as a three-level categorical variable (day 7, day 14, and autumn) in order to
282 estimate the changes of mtDNAcn and rTL between each time point.

283 We used GLMMs to estimate the influence of prenatal thyroid hormones on hatching
284 success, fledging success (i.e. pre-fledging survival), and post-fledging survival. We modelled
285 the outcome of each egg (i.e. hatched or not) or nestling (i.e. survived or not) using a logit link
286 function and specifying a binomial residual distribution. For hatching success, two nests in
287 which eggs never hatched (i.e. cause of failure likely unrelated to our treatment) were
288 excluded, giving a final sample size of 354 eggs from 42 nests. The nest ID was treated as a
289 random intercept, and prenatal hormone treatment and laying date of each nest as fixed
290 factors. For fledging success, all 131 nestlings (reared in 22 nests after cross-fostering) that
291 successfully hatched were included in the analysis. The ID of rearing nest was treated as a
292 random intercept and prenatal hormone treatment, laying date, brood size, and cross-
293 fostering as fixed factors. For post-fledging survival, the 99 nestlings that successfully fledged
294 were included in the analysis. The individuals that were never recaptured in the autumn were
295 presumed dead. In this model, including nest ID as random intercepts caused convergence
296 problem. We therefore fitted a binomial GLM instead, with prenatal hormone treatment,
297 cross-fostering, and body mass at day 14 (i.e. the last measurement before fledging) as
298 predictors.

299 In all statistical models described above, hormone-related two-way interactions were
300 of interest and therefore included. Following the suggestion by (Schielzeth, 2010), input
301 variables (except for the technical variables) were mean-centered before model fitting.

Significant interactions were further examined by post-hoc interaction analysis using the R package *emmeans* (Lenth 2021).

All LMMs and GLMMs were conducted in the R environment 3.6.1, using the package *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017). P values were determined using the Kenward-Roger method to approximate the denominator degrees of freedom (R package *pbkrtest*, (Halekoh and Hojsgaard, 2014), implemented within *lmerTest*) for LMMs and by Laplace approximation for GLMMs. Model assumptions were diagnosed by using the R package *DHARMa* (Hartig 2021) and no clear violations were observed except for the models for plasma T3. Nevertheless, given the fact that the model of T3 did not detect any significant effect and the general robustness of LMMs (Schielzeth et al., 2020), this did not influence our conclusion.

Results

Early body mass growth (between day 2 and day 7 post-hatching) did not differ between TH-supplemented and control groups (Table 1a, Fig 1a), but individuals from TH-supplemented eggs grew faster at the later nestling stage, i.e. between day 7 and day 14 post-hatching (Hormone×age interaction, $F_{1,20.18} = 5.423$, $p = 0.030$, Table 1b, Fig 1b; body mass gain (mean±SE) in TH-nestlings 0.898 ± 0.042 g/day, CO-nestlings 0.785±0.043 g/day). Similarly, individuals from TH-supplemented eggs expressed faster tarsus growth rate between day 7 and day 14 post-hatching ($F_{1,32.40} = 4.134$, $p=0.050$, Table 1c, TH: 0.567±0.032 mm/day, CO: 0.475±0.033 mm/day, Fig 1c). 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

325 at any age (Fig 1, Table S1). In juveniles, there was no difference in body mass or wing length

326 between the treatment groups (Body mass: $F_{1,7.24} = 0.012$, $p = 0.916$; wing length: $F_{1,11.48} =$

327 0.005, $p = 0.948$).

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

329 supplementation.

(a) Early nestling growth (day 2 – day 7)						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=224)	Intercept	0.843	0.918			
Nest of origin (n=42)	Intercept	0.628	0.793			
	Age	0.005	0.070			
Nest of rearing (n=42)	Intercept	0.057	0.239			
	Age	0.007	0.083			
Residual		0.203	0.450			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	8.229	0.146	56.516			
Hormone (TH)	-0.181	0.142	-1.277	1, 38.27	1.632	0.209
Age	1.730	0.019	89.617	1, 45.65	8031.211	<0.001
Cross-foster (yes)	-0.139	0.069	-2.006	1, 187.94	4.025	**0.046**
Hormone × Age	0.009	0.016	0.573	1. 28.88	0.328	0.571
Hormone × Cross-foster	0.037	0.076	0.485	1, 34.61	0.235	0.631
(b) Late nestling body mass growth (day 7 – day 14)						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=111)	Intercept	0.567	0.753			
Nest of origin (n=36)	Intercept	0.381	0.617			
	Age	0.007	0.084			
Nest of rearing (n=20)	Intercept	0.003	0.058			
	Age	0.015	0.120			
Residual		0.534	0.731			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	15.496	0.152	101.728			
Hormone (TH)	-0.240	0.310	-0.774	1, 29.58	0.598	0.445
Age	0.841	0.035	24.228	1, 19.41	586.992	<0.001
Cross-foster (yes)	-0.100	0.269	-0.686	1, 32.46	0.138	0.713
Hormone × Age	0.113	0.049	1.853	1, 20.18	5.423	**0.030**
Hormone × Cross-foster	0.321	0.643	0.500	1, 20.30	0.250	0.623
(c) Late nestling tarsus growth (day 7 – day 14)						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=111)	Intercept	0.239	0.489			
Nest of origin (n=36)	Intercept	0.221	0.470			
	Age	0.012	0.111			
Residual		0.376	0.613			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	20.607	0.168	122.819			
Hormone (TH)	-0.126	0.216	-0.583	1, 34.02	0.340	0.563
Age	0.520	0.023	22.379	1, 36.42	500.810	<0.001
Cross-foster (yes)	-0.141	0.171	-0.824	1, 51.71	0.679	0.414
Measurer 2	0.235	0.180	1.306	2, 122.11	7.626	<0.001
Measurer 3	0.601	0.169	3.551			
Hormone × Age	0.092	0.045	2.033	1, 32.40	4.134	**0.050**
Hormone × Cross-foster	0.436	0.342	1.276	1, 52.56	1.628	0.208

Sample sizes:

Early growth, n = 99 control and 119 TH-supplemented at day 2, 104 control and 117 TH-supplemented at day 7
Late growth, n = 57 control and 54 TH-supplemented at day 7, 50 control and 49 TH-supplemented at day 14

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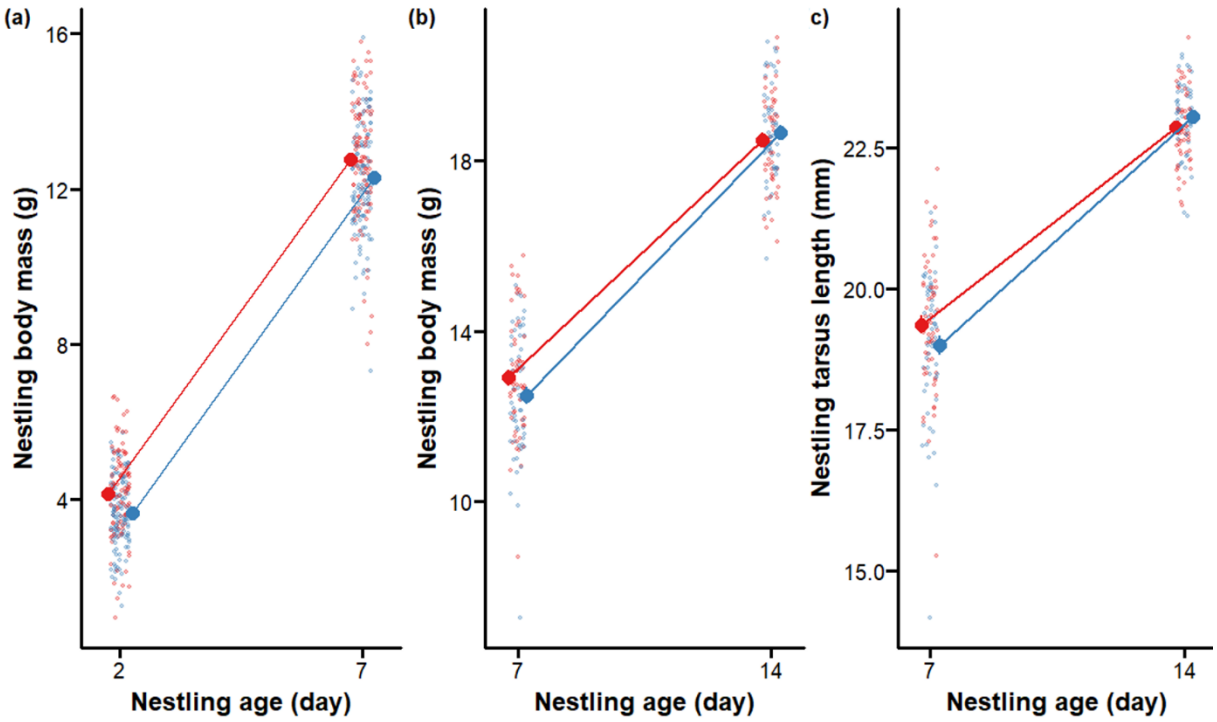

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333 Fig. 1. Nestling growth in prenatally TH-supplemented (blue dots) and control (red dots)
334 groups. (a) body mass growth during early nestling stage (day 2 to day 7 post-hatching) (b)
335 body mass growth late nestling stage (day 7 to day 14 post-hatching),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

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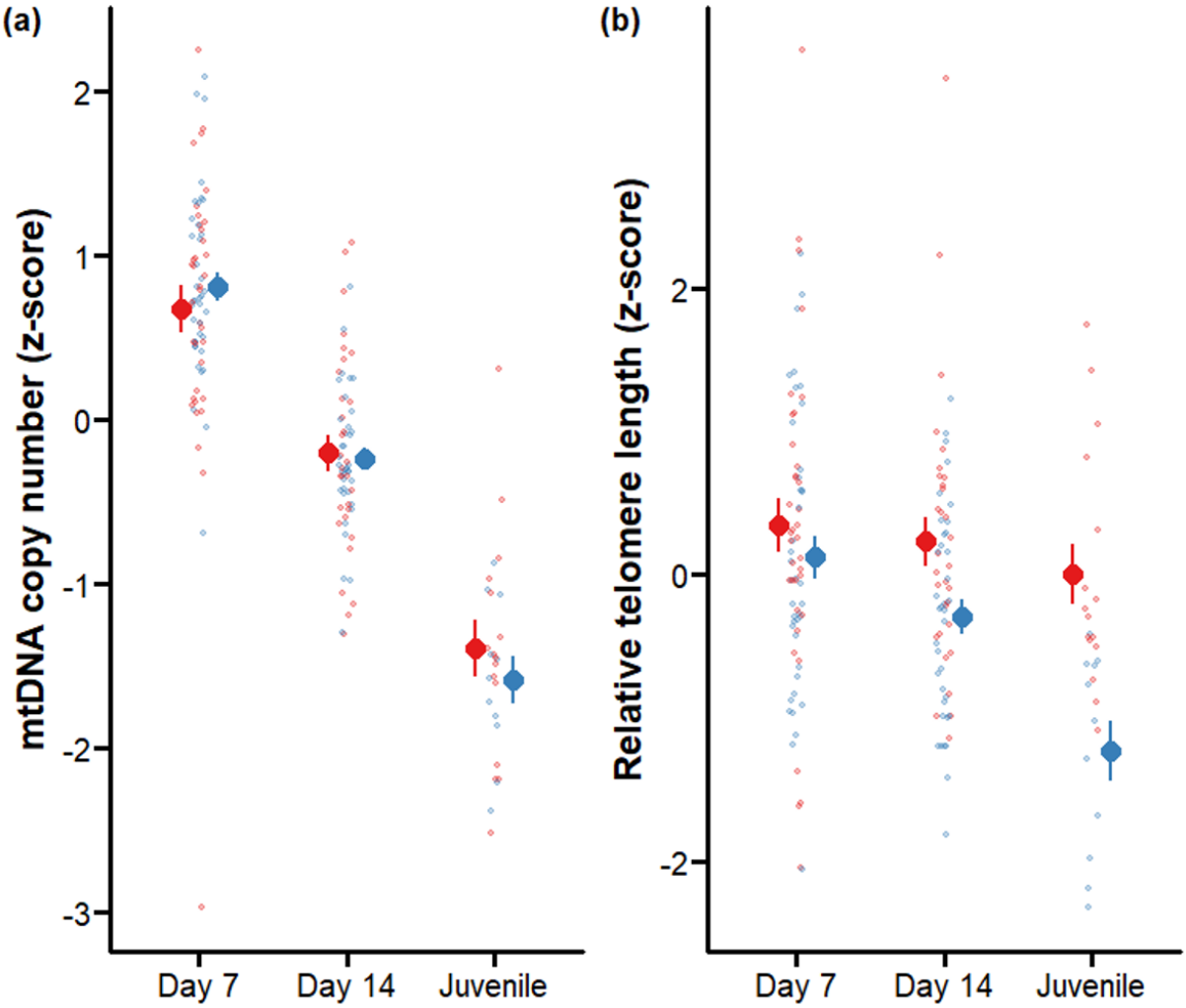

Fig 2. (a) Relative mitochondrial density (z-scale) and (b) relative telomere length in prenatally TH-supplemented (blue dots) and control groups (red dots) at different developmental stages (day 7 and 14 post-hatching and as juveniles, ca. 3 months of age). Means±SE (large dots) and scatter of the raw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

Biomarkers of intracellular oxidative status (total GSH, GSH:GSSG ratio) and damage to lipids 14 days post-hatching did not clearly differ among the treatment groups (marginal means±SE: total GSH µmol/mg protein: TH-supplemented, 0.256±0.024, control, 0.244±0.024; GSH:GSSG ratio: TH-supplemented, 0.051±0.008, control, 0.065±0.011; MDA nmol/mg protein: TH-

supplemented, 0.038±0.005, control, 0.037±0.005; Table S2). Plasma T3 and T4 also did not differ among prenatally TH-supplemented and control groups 14 days post hatching (marginal means±SE pmol/ml T3: TH-supplemented, 1.24±0.21, control, 1.22±0.23; T4: TH-supplemented, 7.38±1.05, control, 6.60±1.18; Table S3). Biomarkers of oxidative stress or THs were not associated with body mass or sex ($p > 0.06$, Table S3)

Mitochondrial density decreased with age, but there were no clear differences between prenatally TH-supplemented and control groups at any age (d7, d14 or juveniles, Fig 2a, Table 2). Post-hoc analyses indicated significant decrease between each age category (all Tukey-adjusted $p < 0.001$), regardless of the hormone treatment. Mitochondrial density was not associated with sex or body mass (Table 2).

Telomere length decreased with age (Table 2, Fig 2b), but the pattern was different between TH-supplemented and control group (Table 2, Fig 2b): There was no difference among the prenatally TH-supplemented and control group early in the nestling phase (d7, Tukey post-hoc test: TH vs control: $t_{40.6} = 1.357$, $p = 0.182$). Telomeres were significantly shorter (13%) in the TH group on day 14 post-hatching (Tukey post-hoc test, TH vs Control: $t_{44.1} = 2.548$, $p = 0.014$), and this difference was accentuated in juveniles, offspring from TH supplemented eggs having telomeres being ca. 33% shorter than offspring from control group (Tukey post-hoc test, TH vs Control: $t_{106.0} = 3.806$, $p = 0.0002$).

371 Hatching success did not clearly differ among prenatally TH supplemented and control
372 individuals (TH 67.6%, control 69.1%; z =0.314, p = 0.754). Nestling survival to fledging was
373 marginally, but not statistically significantly higher in differ prenatally TH supplemented than
374 and control nestlings (TH: 83.05% vs CO 69.44%, z = 0.56, p = 0.575). Fledging success was not
375 associated with laying date or brood size (p> 0.6). Juvenile recapture rate was not significantly
376 different across the groups (TH: 28.57% vs CO 34.00%, z=-0.46, p = 0.645).

377 **Table 2.** Linear mixed models of mitochondrial density and telomere length in blood cells
378 growth in response to prenatal TH-supplementation.

(a) Mitochondria density						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=78)	Intercept	0.088	0.297			
Nest of origin (n=35)	Intercept	0.044	0.211			
Nest of rearing (n=19)	Intercept	<0.001	<0.001			
DNA batch (n=8)	Intercept	0.022	0.148			
Residual		0.238	0.488			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	-0.017	0.086	-0.198			
Hormone (TH)	-0.014	0.140	-0.098	1, 23.49	0.010	0.923
Sex	0.225	0.128	1.756	1, 67.12	3.083	0.084
Age day 7	2.196	0.121	18.216	1, 107.26	331.826	**<0.001**
Age day 14	1.227	0.122	10.042	1, 107.07	100.849	**<0.001**
Day-7 body mass	-0.069	0.068	-1.015	1, 64.04	1.030	0.314
Hormone × Sex	-0.035	0.261	-0.134	1, 67.03	0.018	0.894
Hormone × Age day 7	0.363	0.240	1.518	1, 108.03	2.305	0.132
Hormone × Age day 14	0.196	0.242	0.808	1, 107.99	0.652	0.421
Hormone × Body mass	0.003	0.136	0.026	1, 66.85	0.001	0.980
Sex × Age day 7	0.370	0.241	1.532	1, 106.83	2.347	0.129
Sex × Age day 14	0.076	0.245	0.311	1, 106.76	0.097	0.757
(b) relative telomere length						
Random effects:		**Variance**	**Std. Dev.**			
Individual ID (n=77)	Intercept	0.090	0.301			
Nest of origin (n=35)	Intercept	0.028	0.167			
Nest of rearing (n=19)	Intercept	0.057	0.238			
DNA batch (n=8)	Intercept	0.249	0.499			
Residual		0.518	0.720			
Fixed factors	**Estimate**	**SE**	**t**	**df**	**F**	**p**
Intercept	-0.086	0.202	-0.427			
Hormone (TH)	-0.550	0.174	-3.168	1, 18.03	10.035	**0.005**
Sex	-0.318	0.168	-1.890	1, 60.80	3.571	0.064
Age day 7	0.909	0.176	5.175	1, 113.09	26.778	**<0.001**

Age day 14	0.341	0.092	3.726	1, 112.25	13.886	**<0.001**
Day-7 body mass	0.100	0.090	1.116	1, 63.08	1.246	0.268
Hormone × Sex	0.087	0.334	0.262	1, 58.01	0.069	0.794
Hormone × Age day 7	0.992	0.347	2.859	1, 116.76	8.172	**0.005**
Hormone × Age day 14	0.708	0.351	2.018	1, 114.95	4.074	**0.046**
Hormone × Body mass	-0.149	0.175	-0.847	1, 62.53	0.718	0.400
Sex × Age day 7	-0.231	0.351	-0.657	1, 113.82	0.432	0.513
Sex × Age day 14	-0.495	0.355	-1.396	1, 111.55	1.950	0.165

Sample sizes: n = 37 control and 41 TH-supplemented

379

Discussion

By manipulating prenatal exposure to THs in a wild passerine species, we demonstrate that an increase in prenatal THs can accelerate both postnatal growth and telomere shortening. Yet, we did not detect significant effects of elevated prenatal THs on postnatal oxidative stress levels, cellular energetics measured as mtDNA copy number, circulating TH levels, or short to medium-term survival (*i.e.* hatching success, fledging success, and apparent survival to the next autumn/winter). While previous findings suggested potential sex-dependent effects of prenatal THs in the same species (Ruuskanen et al., 2016a), our results do not support such a hypothesis.

Shorter telomeres in the TH-supplemented group were only detected from 14 days after hatching onwards, which seems to exclude direct effects or prenatal TH on telomere dynamics during embryonic development. Yet, this coincides with the accelerated postnatal body mass and tarsus growth observed in the TH-group (between day 7 and 14) compared to controls, and faster growth can accelerate telomere shortening either through enhanced cell division or through inducing oxidative stress (Monaghan and Ozanne, 2018). Considering the lack of impact of prenatal THs on oxidative stress reported here and in previous work on birds (Hsu et al., 2020, 2019a; Sarraude et al., 2020a; Sarraude et al., 2020b), it seems unlikely that the accelerated telomere shortening observed in this study would be linked to increased oxidative damage on DNA. Yet, we cannot fully rule out this explanation since DNA damage was not directly assessed and oxidative stress sensitivity might vary between different biomolecules (Reichert and Stier, 2017). The effect of prenatal THs on telomere length seemed to increase with age (*i.e.* stronger effect observed in juveniles than at day 14), which could be explained by a delayed effect of fast postnatal growth (day 7 to day 14) because telomeres

403 only shorten at the subsequent cellular division, and therefore delayed effects are likely to be
404 observed (Monaghan and Ozanne, 2018). Our hypothesis that prenatal THs could program
405 postnatal metabolic and endocrine function (and thus affect telomere shortening indirectly)
406 was not supported as we found no evidence for differences in mitochondrial density or plasma
407 TH levels (key coordinators of metabolism) across the treatment groups. The latter results are
408 not fully surprising considering the limited evidence supporting a prenatal programming of
409 plasma THs (sex-specific effect on T4 only, Hsu et al. 2017; no effect, Hsu et al. 2020) or
410 mitochondrial density (no effect, Stier et al. 2020; Hsu et al. 2020). According to the *metabolic*
411 *telomere attrition hypothesis*, telomere shortening is likely to be increased during energy-
412 demanding periods, and accelerated growth under limited resources is likely carrying a
413 metabolic cost (Casagrande and Hau, 2019).

414 The effects observed here on telomeres in response to increased prenatal TH levels
415 are in sharp contrast with our previous findings in another passerine species, the collared
416 flycatcher (Stier et al. 2020). Indeed, increasing prenatal THs in the collared flycatcher
417 increased telomere length measured very shortly after hatching (day 2), while not affecting
418 telomere shortening during postnatal growth (Stier et al. 2020). There are several alternative
419 explanations for these contrasted findings: (1) we cannot exclude that prenatal THs increased
420 telomere length during embryonic development in great tits since our first telomere length
421 measurement (day 7, *ca.* 70% of fledging body mass) was done considerably later than in
422 collared flycatchers (*i.e.* day 2, *ca.* 20% of fledging body mass). (2) THs differently influenced
423 post-natal growth in the two species: great tits from the TH-supplemented eggs were initially
424 slightly smaller, but grew faster in the late nestling period, whereas collared flycatchers from
425 the TH-supplemented eggs were bigger soon after hatching, but grew slightly slower during
426 the nestling stage (Hsu et al., 2019a). These contrasting growth patterns may explain, at least

partly, the contrasted findings in these two species: an increased metabolic demand for fast postnatal growth in great tit in response to prenatal TH could accelerate telomere shortening, while a reduced metabolic demand in collared flycatcher from TH-injected eggs could enable telomere maintenance (Casagrande and Hau, 2019). Measurements of the mTOR signaling pathway could shed light on the validity of this hypothesis (Casagrande and Hau, 2019). (3) The effects of maternal signals on offspring are expected to differ based on environmental conditions, so called context-dependent effects: For example, prenatal androgens have been found to differentially influence offspring growth depending on season or food availability (Groothuis et al., 2020; Muriel et al., 2015). We recently found no evidence for prenatal THs differentially influencing growth and early-life survival depending on rearing temperature (Hsu et al. 2020), yet the influence of resource availability was not tested. In two sister species, collared and pied flycatchers, differential effects of prenatal THs on growth may have been explained by differences in food resources (Sarraude et al. 2020b). (4) These two species exhibit different life-histories, as collared flycatchers are migratory and great tits (relatively) sedentary. Migratory species have generally higher metabolism (Jetz et al., 2008) as well as an overall faster pace of life (Soriano-Redondo et al. 2021), and may thus present differences in TH physiology. We may speculate that the telomere maintenance, and role of THs in the regulation of telomere maintenance may differ across species with different life-histories. Telomere maintenance is known to be influenced by e.g. species lifespan (Haussmann et al., 2004) and migratory populations within a species have shorter telomeres (Bauer et al., 2016). Yet, to our knowledge, studies have not considered migratory vs. non-migratory species in telomere maintenance. Species-specific effects of the prenatal hormonal environment on telomere dynamics have already been described: elevated prenatal glucocorticoid levels led to shorter telomeres in domestic chicken and female zebra finch (Haussmann et al., 2012;

Tissier et al., 2014), but to longer telomeres in yellow-legged gull (Noguera, da Silva, and Velando 2020). To understand if any of these hypotheses is likely to explain the contrasted effects found here and in Stier et al. (2020), more studies on the impact of prenatal THs on telomere biology across taxa are needed.

To understand the selective pressures on maternal allocation and signaling, it is important to characterize true fitness effects from development to survival and lifetime reproductive success. In contrast to our predictions, we found no effects of elevated prenatal TH-supplementation on apparent survival or individual condition during their first autumn/ winter. Unfortunately, longer-term effects on survival and reproductive success are very challenging to measure in such an experimental setting in the wild, as the recruitment to first breeding is usually very low (e.g. < 10% Radersma et al., 2015), which requires a very high number of nests to be manipulated, something often not feasible for both logistical and ethical reasons. Yet, long-term effects of accelerated early-life telomere shortening may lead to a decrease in longer-term survival and lifespan (Eastwood et al., 2019; Heidinger et al., 2012). The negative effects on later stages suggest that there need to be benefits of high prenatal THs for the offspring or mother, potentially during early-life stages, where selection can be strong. In this study, the elevated growth rates could increase offspring competitive abilities early in life, yet, the effects of prenatal THs on growth seem to be highly inconsistent across studies (Hsu et al., 2020, 2019a, 2017; Ruuskanen et al., 2016a; Sarraude et al., 2020a; Sarraude et al., 2020b). While circulating THs are known to be associated with health and ageing in humans and mammalian models (Bano et al. 2017, 2019; Møllehave et al. 2020), our study is the first to show that exposure to higher prenatal thyroid hormones can lead to accelerated cellular ageing measured through telomere length. This should stimulate further

474 research using both epidemiological and experimental approaches across taxa to uncover the
475 potential regulation of telomere biology by thyroid hormones both pre- and postnatally.

476

477 **Author contribution**

478 BYH, AS & SR designed the study and collected field data. NCS, AS & SR conducted laboratory
479 work. BYH analyzed the data. BYH, AS & SR wrote the manuscript, with input from NCS.
480

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489

490 **Data accessibility**

491 All data will be archived and available in Figshare upon acceptance.

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