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**Born to be young? Prenatal thyroid hormones increase early-life telomere length in wild collared flycatchers**

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Relevant information will appear here if provided.

**Ethics**

*Does your article include research that required ethical approval or permits?:*

Yes

*Statement (if applicable):*

The study was conducted in the long-term monitored population of collared flycatchers on Gotland, Sweden (Jordbruksverkets permit no. ID 872)

**Data**

*It is a condition of publication that data, code and materials supporting your paper are made publicly available. Does your paper present new data?:*

Yes

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Data [25] used in this article is publicly available at:  
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**Conflict of interest**

I/We declare we have no competing interests

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**Authors' contributions**

This paper has multiple authors and our individual contributions were as below

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All authors agree to be held accountable for the content therein and approve the final version of the manuscript. AS, BYH & SR designed the study. BYH and SR conducted the fieldwork, AS and CM conducted laboratory work. BD, LG & PB contributed to data collection. AS analyzed the data and wrote the manuscript with input from all authors.

1 **Born to be young? Prenatal thyroid hormones increase early-life telomere length in**  
2 **wild collared flycatchers**

3  
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25 **Keywords**

26 Ageing, mitochondria, telomere length, bird, fetal programming.

28 **Abstract**

29           The underlying mechanisms of the lifelong consequences of prenatal environmental condition  
30 on health and ageing remain little understood. Thyroid hormones (THs) are important regulators of  
31 embryogenesis, transferred from the mother to the embryo. Since prenatal THs can accelerate early-  
32 life development, we hypothesized that this might occur at the expense of resource allocation in  
33 somatic maintenance processes leading to premature ageing. Therefore, we investigated the  
34 consequences of prenatal THs supplementation on potential hallmarks of ageing in a free-living avian  
35 model in which we previously demonstrated that experimentally elevated prenatal THs exposure  
36 accelerates early-life growth. Using a cross-sectional sampling, we first report that mitochondrial DNA  
37 (mtDNA) copy number and telomere length significantly decrease from early-life to late adulthood,  
38 thus suggesting that these two molecular markers could be hallmarks of ageing in our wild bird model.  
39 Elevated prenatal THs had no effect on mtDNA copy number but counter-intuitively increased  
40 telomere length both soon after birth and at the end of the growth period (equivalent to offsetting *ca.*  
41 4 years of post-growth telomere shortening). These findings suggest that prenatal THs might have a  
42 role in setting the 'biological' age at birth, but raise questions about the nature of the evolutionary  
43 costs of prenatal exposure to high THs levels.

## 44 Introduction

45 Prenatal environmental conditions can have lifelong consequences on health and ageing, but  
46 much remains to be done to uncover the mechanisms linking the pre- and postnatal stages [1]. Thyroid  
47 hormones (THs) are master regulators of development, health and ageing [2-4]. They are transferred  
48 from the mother to the embryo [4] and thyroid disorders during pregnancy can induce developmental  
49 pathologies in humans [5]. There is natural variation in the amount of TH being transferred from the  
50 mother to the embryo, and some of this variation is linked to the environmental conditions  
51 experienced by the mother (*e.g.* food availability or temperature [4]). This suggests that maternal THs  
52 could be a potential signal transferred from the mother to the embryo to adjust offspring phenotype  
53 to current environmental cues in order to maximize fitness [4]. While the potential for prenatal THs to  
54 mediate adaptive maternal effects remains mostly untested, some of our recent data in wild collared  
55 flycatcher (*Ficedula albicollis*) suggest that increasing prenatal THs exposure increases both hatching  
56 success and early-life growth [6]. From an evolutionary perspective, such apparent beneficial effects  
57 are likely to come with associated costs if there is no increase in resource acquisition. This could occur  
58 for instance through a reduction in resource allocation towards somatic maintenance, which could  
59 impair subsequent health and accelerate ageing [7]. Yet, we currently lack experimental data on the  
60 effects of prenatal THs on postnatal health and ageing.

61 Avian models offer a great potential to study this question since prenatal conditions can be  
62 directly manipulated through hormonal injection in the egg [4]. Yet, one important challenge remains  
63 to be able to assess long-term consequences on ageing under an ecologically-realistic scenario. When  
64 direct effects on survival and lifespan cannot be estimated, the use of biomarkers that are mirroring  
65 age-related health impairments could be useful. Two promising biomarkers are telomere length and  
66 mitochondrial DNA (mtDNA) copy number since both markers are frequently reported to decrease  
67 with age and to be associated with increased mortality risks [8-10]. There is evidence that telomere  
68 length could reflect phenotypic quality since early-life telomere length has been shown for instance to  
69 predict lifespan [11] and fitness [12] in avian species. Interestingly, a large part of the inter-individual

70 variation in telomere length could already be set at birth, and thus may be partially caused by different  
71 exposure to maternal hormones [13,14].

72 In this study, we aim to evaluate whether mtDNA copy number and telomere length decline  
73 with age in a free-living bird population of collared flycatchers, and to test the effects of prenatal THs  
74 on those potential hallmarks of ageing. To this aim, we first investigated age-related changes in  
75 telomere length and mtDNA copy number using cross-sectional data covering most of the lifespan  
76 spectrum for this population. While it is established that telomeres shorten with age in most bird  
77 species, there is no information to date regarding age-related variation of mtDNA copy number in  
78 avian models [15]. Then, using egg-injection of THs, we investigated the effect of prenatal THs on  
79 postnatal mtDNA copy number and telomere length. Based on the known stimulation of mitochondrial  
80 biogenesis by THs (among other through the up-regulation of PGC1 $\alpha/\beta$  [16]), we predicted that  
81 increasing prenatal THs might increase early-life mtDNA copy number, which could be a cellular  
82 pathway supporting the transient growth-enhancing effect we previously demonstrated in this species  
83 [6]. Conversely, since THs have been reported in some studies to increase oxidative stress and in our  
84 model species to enhance growth (two pathways accelerating telomere shortening [7,17]), we  
85 predicted that increasing prenatal THs levels should shorten telomere length at birth, and/or increase  
86 early postnatal telomere shortening.

87

## 88 **Materials & Methods**

89 This study was conducted in the long-term monitored population of collared flycatchers on  
90 Gotland, Sweden. In 2016, 44 randomly selected adult birds (30 females and 14 males) of known-age  
91 (1 to 7 years old, *i.e.* cross-sectional data; maximum lifespan = 9.8 years) were sampled across different  
92 woodlands. Data and samples from 30 nests of a previous study [6] were used to investigate the effects  
93 of a prenatal THs manipulation on telomeres and mtDNA copy number. We used 14 *Control* (vehicle-  
94 injected) and 16 *TH* nests in which all eggs were injected with *ca.* a 2SD increase of THs egg content  
95 based on natural range, following the procedure described in detail in [6] and ESM1. The original study

96 [6] demonstrated that THs-injected eggs had a higher hatching success, that early-life survival was not  
97 significantly affected by prenatal THs manipulation, and that chicks from THs-eggs were heavier at day  
98 2 and larger at day 8 but did not significantly differ from controls in mass or size at fledging [6]. Two  
99 chicks per nest were randomly selected among those surviving to day 12. These chicks were blood  
100 sampled twice: soon after hatching (day 2, < 10 $\mu$ L of blood) and at the end of growth (*i.e.* day 12; <  
101 50 $\mu$ L of blood). Relative mtDNA copy number of blood cells has been measured as described in [18]  
102 and ESM2. Both relative telomere length (*rTL* measured using qPCR) and absolute telomere length (*TL*,  
103 measured using *in-gel* TRF from day 12 samples only) have been measured as described in [19] and  
104 ESM2. Two day 2 samples failed to meet DNA quality control criteria, three samples failed to amplify  
105 for the mtDNA copy number analysis, and one day 12 DNA sample was excluded from TRF analysis due  
106 to insufficient DNA quantity, explaining the observed differences in sample sizes among analyses. Age-  
107 related variation in mtDNA copy number and telomere length were tested using Pearson correlation  
108 tests, and age-related regression equations have been calculated to infer the amount of telomere  
109 length or mtDNA copy number lost per year post-growth (Fig. 1). The effects of prenatal TH elevation  
110 and age on mtDNA copy number and telomere length (*rTL* and *TL*) were tested using linear mixed  
111 models, with nest identity and bird identity as random effects (to control for multiple birds per nest  
112 and multiple samples per bird), as well as age (2 vs. 12 days), treatment (TH vs. Control) and their  
113 interaction as fixed effects. mtDNA copy number and *rTL* were z-transformed prior to statistical  
114 analysis [20]. Standardized effect sizes (*ES*) for mixed models have been calculated using the emmeans  
115 package in R [21]. Non-significant interactions were removed from final models. The effect of sex was  
116 tested but excluded from final analyses since it was never significant.

117

## 118 **Results and Discussion**

119 We found strong evidence that both mtDNA copy number ( $r = -0.57$ ,  $p < 0.001$ , Fig. 1A) and  
120 telomere length measured either using a relative qPCR method (*rTL*,  $r = -0.30$ ,  $p = 0.022$ , Fig. 1B) or an  
121 absolute *in-gel* quantification (*TL*,  $r = -0.48$ ,  $p < 0.001$ , Fig. 1C) significantly decreased from growth



122 completion (*i.e.* day 12) to late-adulthood using a cross-sectional approach. Restricting our analyses to  
123 adult individuals only (*i.e.* age  $\geq 1$ ) reveals that mtDNA copy number ( $r = -0.42$ ,  $p = 0.004$ ) and absolute  
124 telomere length ( $r = -0.43$ ,  $p = 0.003$ ) still significantly decrease with age, while this relationship is not  
125 significant anymore for relative telomere length ( $r = -0.23$ ,  $p = 0.14$ ). These findings in our avian model  
126 are in accordance with reports from the human literature [9,10]. Since individuals exhibiting short  
127 telomeres (both in human and various avian species [8,10]) or low mtDNA copy number (only tested  
128 in human to the best of our knowledge [9]) have been shown to disappear earlier from the population,  
129 the age-related slopes from our cross-sectional sampling presented in Fig. 1 could underestimate the  
130 real age-related decline in both telomere length and mitochondrial density.

131 We found no significant impact of prenatal THs on mtDNA copy number (TH:  $ES = -0.26$  [-1.03;  
132 0.51],  $p = 0.46$ , ESM3 Table S1A, Fig. 2A), despite a considerable early-life reduction in mtDNA copy  
133 number during the growth period (Age:  $p < 0.001$ , equivalent to the reduction occurring over 3.5 years  
134 post-growth based on the age-related regression equation in Fig. 1A). Contrary to our predictions,  
135 increasing prenatal THs led to longer telomeres (measured as  $rTL$ ) soon after hatching (day 2), and this  
136 effect was maintained at the end of the growth (day 12) period (TH:  $ES = 1.11$  [0.03;2.19],  $p = 0.036$ ,  
137 ESM3 Table S1B, Fig. 2B). This is confirmed by the analysis of absolute telomere length ( $TL$ ) at day 12,  
138 showing longer telomeres in birds hatched from THs-injected eggs (TH:  $ES = 1.91$  [0.01;3.81],  $p = 0.044$ ,  
139 ESM3 Table S1C, Fig. 2C) compared to control eggs. The effect of increasing prenatal THs on telomere  
140 length was substantial (*i.e.* large 'biological' effect size), being equivalent to offsetting *ca.* 4.3 years  
141 ( $rTL$ ) and 3.6 years ( $TL$ ) of post-growth telomere shortening (based on age-related regression equations  
142 in Fig. 1B and 1C). It was unfortunately not possible to evaluate any long-lasting impact of prenatal THs  
143 supplementation on adult telomere length. Yet, considering that most telomere dynamics occurs  
144 during the growth period and that telomere length is a repeatable trait [13,14], it is likely that the  
145 effects of prenatal THs observed here on early-life telomere length would be carried over the adult  
146 stage.

147           The positive effect of prenatal THs on telomere length is unlikely related to oxidative stress  
148 prevention, since we previously found no differences in oxidative stress markers in these experimental  
149 birds [6], or in a similar experiment in a closely-related species [22]. The selective disappearance of  
150 THs-embryos with short telomeres could potentially explain why chicks hatched from THs-eggs have  
151 longer telomeres than controls. Yet, this seems unlikely to explain our results since we found a higher  
152 hatching success of THs-eggs [6]. One previous study in humans reported that the promoter of *hTERT*  
153 (the catalytic subunit of the enzyme telomerase, responsible for elongating telomeres) contains a  
154 binding site for THs [23]. Consequently, one hypothesis would be that prenatal THs could elongate  
155 telomeres early in life through the activation of the telomerase enzyme. Yet, we are currently lacking  
156 both *in vitro* and *in vivo* studies testing such an hypothesis.

157           While an evolutionary trade-off is generally expected to occur between fast growth and  
158 telomere shortening [7], prenatal THs supplementation both enhances early-life growth [6] and leads  
159 to longer early-life telomeres in our study system. Egg THs could be a potential signal transferred from  
160 the mother to the embryo to adjust offspring phenotype to the expected environmental conditions  
161 [4]. Yet, if raising egg THs levels only had benefits, natural selection would have favoured higher  
162 maternal transfer of THs into eggs, and/or offspring maximizing growth and telomere length without  
163 such a maternal signal. Consequently, it is likely that high egg THs levels have costs that we have not  
164 been able to detect here (*e.g.* reduced immunity) or that could only become visible later in life (*e.g.*  
165 altered reproductive potential), which clearly deserves further investigation. Importantly, we can  
166 expect the potential cost-benefit balance of low or high egg THs levels to depend on environmental  
167 conditions [4]. Such potential context-dependent effects will require experimental manipulations of  
168 both egg THs and environmental conditions to be revealed (*e.g.* [22]).

169           While the mechanisms remain to be identified, our study demonstrates that prenatal THs  
170 levels have the potential to modulate telomere length in early-life, and thus to influence the 'biological'  
171 age at birth. It has previously been shown that prenatal exposure to glucocorticoids could shorten  
172 telomeres [24], but our study is the first to show that telomere length at birth could be increased by

173 modulating the prenatal hormonal environment. Thyroid function is known to influence cardiovascular  
174 disease risk and life expectancy in adult humans [3], but no information is currently available regarding  
175 the impact of prenatal THs exposure on adult health and lifespan. Epidemiological and long-term  
176 experimental studies investigating the impact of prenatal THs on adult phenotype, health and lifespan  
177 are now required to establish if the effect observed here on early-life telomere length could be  
178 translated into a longevity gain, but also to identify potential costs of elevated prenatal exposure to  
179 THs.

180  
181

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191

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261

263 **Figure captions**

264

265 **Fig. 1: Age-related variation in potential hallmarks of ageing in wild collared flycatchers: (A) decrease in relative**  
266 **mtDNA copy number, (B) decrease in relative telomere length** measured with qPCR, and **(C) decrease in absolute**  
267 **telomere length** measured with *in-gel* TRF. Data is cross-sectional, adult birds were of known-age and chicks (*i.e.*  
268 age 0) were 12 days old (from control group only, 1 chick per nest). *rTL* and *mtDNA copy number* have been z-  
269 transformed, Pearson's coefficients of correlation *r* and associated p-values are presented along with the age-  
270 related regression equations. Regression lines are plotted  $\pm$  95% C.I., N = 58 (44 adults, 14 nestlings). See also the  
271 main text for statistical analyses restricted to adult individuals.

272

273 **Fig. 2: Effects of experimental prenatal thyroid hormone elevation on: (A) early-life dynamics of mtDNA copy**  
274 **number, (B) early-life dynamics of relative telomere length, and (C) absolute telomere length** at the end of  
275 growth period (day 12). *rTL* and *mtDNA copy number* have been z-transformed. Means are plotted as symbols  $\pm$   
276 SE, individual responses are plotted as solid lines, and *p*-values are indicated within each panel. See ESM3 Table  
277 S1 for details on statistics.

278

279

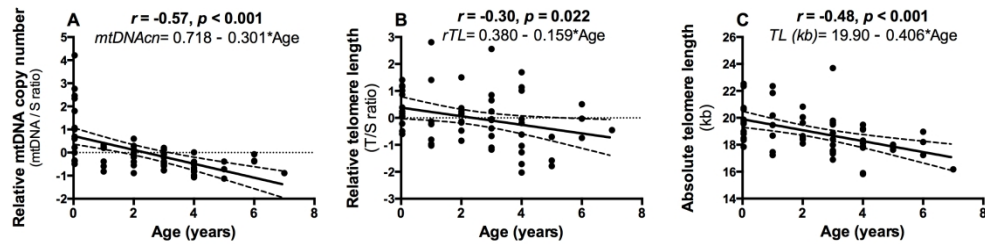


Fig. 1: Age-related variation in potential hallmarks of ageing in wild collared flycatchers: (A) decrease in relative mtDNA copy number, (B) decrease in relative telomere length measured with qPCR, and (C) decrease in absolute telomere length measured with in-gel TRF. Data is cross-sectional, adult birds were of known-age and chicks (i.e. age 0) were 12 days old (from control group only, 1 chick per nest). rTL and mtDNA copy number have been z-transformed, Pearson's coefficients of correlation  $r$  and associated  $p$ -values are presented along with the age-related regression equations. Regression lines are plotted  $\pm$  95% C.I.,  $N = 58$  (44 adults, 14 nestlings). See also the main text for statistical analyses restricted to adult individuals.

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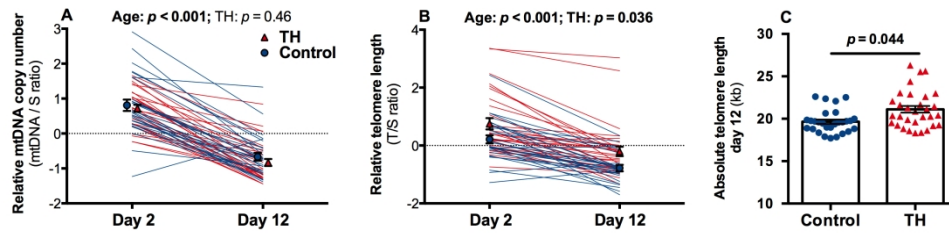


Fig. 2: Effects of experimental prenatal thyroid hormone elevation on: (A) early-life dynamics of mtDNA copy number, (B) early-life dynamics of relative telomere length, and (C) absolute telomere length at the end of growth period (day 12). rTL and mtDNA copy number have been z-transformed. Means are plotted as symbols  $\pm$  SE, individual responses are plotted as solid lines, and p-values are indicated within each panel. See ESM3 Table S1 for details on statistics.

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