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Manipulation of prenatal thyroid hormones does not affect growth or physiology in nestling pied flycatchers

Running page head: Prenatal thyroid hormones in pied flycatchers

Keywords: maternal effects; maternal hormones; thyroid hormones; bird; growth; oxidative stress;

What is already known

Maternal hormones are a means for mothers to adapt their offspring to expected environmental conditions. Thyroid hormones are key metabolic hormones in vertebrates, involved in embryonic development, growth, thermoregulation and reproduction but hardly studied as maternal effects. Studies on maternal THs have so far given contrasting results that could be attributed to species or context differences.

What this study adds

We found that elevated yolk thyroid hormones did not affect growth, contradicting results from a previous study in a closely related species. This suggests that maternal THs may have context-dependent effects.

Abstract

Hormones transferred from mothers to their offspring are thought to be a tool for mothers to prepare their progeny for expected environmental conditions, thus increasing fitness. Thyroid hormones (THs) are crucial across vertebrates for embryonic and postnatal development and metabolism. Yet, yolk THs have mostly been ignored in the context of hormone-mediated maternal effects. In addition, the few studies on maternal THs have yielded contrasting results that could either be attributed to species or to environmental differences. In this study, we experimentally elevated yolk THs (within the natural range) in a wild population of a migratory passerine, the European pied flycatcher *Ficedula hypoleuca*, and assessed their effects on hatching success, nestling survival, growth and oxidative status (lipid peroxidation, antioxidant enzyme activity and oxidative balance). We also sought to compare our results with those on a closely related species, the collared flycatcher *Ficedula albicolis* that has strong ecological and life-history similarities with our species. We found no effects of yolk THs on any of the responses measured. We could only detect a weak trend on growth: elevated yolk THs tended to increase growth during the second week post hatching. Our results contradict the findings of previous studies including those in the collared flycatcher. However, differences in fledging success and nestling growth between both species in the same year suggest a context-dependent influence of the treatment. This study should stimulate more research on maternal effects mediated by thyroid hormones, and their potential context-dependent effects.

Introduction

Maternal effects are all the non-genetic influences of a mother on her offspring and receive increasing attention in evolutionary and behavioral ecology (Moore et al. 2019; Yin et al. 2019). Via maternal effects, mothers may influence the fitness of their progeny by adapting their phenotype to expected environmental conditions (Mousseau and Fox 1998; "adaptive maternal effects" in Marshall and Uller 2007), and a recent meta-analysis found strong support for adaptive effects (Yin et al. 2019). Maternal effects are observed in plants, invertebrates and vertebrates, and can have many possible mediators (Danchin et al. 2011; Kuijper and Johnstone 2018). One intriguing pathway is via the hormones transmitted from the mother to her progeny. These hormone-mediated maternal effects have been found to profoundly influence offspring phenotype in many different taxa (e.g. in mammals, Dantzer et al. 2013; birds, von Engelhardt and Groothuis 2011; reptiles, Uller et al. 2007 and invertebrates, Schwander et al. 2008). Most studies in the field of hormone-mediated maternal effects have focused on steroid hormones, such as glucocorticoids and androgens (Groothuis and Schwabl 2008; von Engelhardt and Groothuis 2011). However, mothers transfer other hormones to their embryo (Williams and Groothuis 2015), including thyroid hormones (Ruuskanen and Hsu 2018).

Thyroid hormones (THs) are metabolic hormones produced by the thyroid gland and are present in two main forms: thyroxine (T_4) and triiodothyronine (T_3). T_3 has a greater affinity with thyroid hormone receptors and is therefore responsible for most of the receptor-mediated effects. T_4, on the other hand, is mostly a precursor of T_3, although it may carry non-genomic effects (i.e. independent of TH receptors) (Davis et al. 2016). Thyroid hormones have pleiotropic effects that serve several biologically important functions across vertebrates (Ruuskanen and Hsu 2018), and have been studied previously to some extent in various taxa (e.g. in birds, Wilson and McNabb 1997; fish, Brown et al. 1988 and amphibians, Duarte-

72 Guterman et al. 2010). In early-life, they participate in the maturation of multiple tissues (e.g.
73 birds, McNabb and Darras 2015; mammals, Pascual and Aranda 2013), and interact with
74 growth hormones to increase growth (e.g. structural growth: Wilson and McNabb 1997;
75 McNabb and Darras 2015). THs also regulate metabolism, and, during adult life, are necessary
76 for normal reproductive functions (e.g. birds, McNabb and Darras 2015; mammals, Norris
77 and Carr 2013). In wild bird species, plasma THs correlate positively with metabolic rate
78 (Elliott et al. 2013; Welcker et al. 2013), and studies on mammalian model species found
79 mechanistic evidence on the influence of THs on metabolism (Mullur et al. 2014). THs can
80 alter the concentration of sodium and potassium in the cells (Haber and Loeb 1986; Ismail-
81 Beigi et al. 1986), hence requiring ATP consumption to restore a normal gradient, which in
82 turn stimulate metabolism (Mullur et al. 2014).
83 THs could further influence cell oxidative status, a biomarker that may underlie life-
84 history trade-offs and ageing (Metcalfe and Alonso-Alvarez 2010) via multiple pathways.
85 Oxidative stress occurs when the reactive oxygen species (ROS) production exceeds the
86 capacity of antioxidant defenses (Monaghan et al. 2009). It results in oxidative damage on, for
87 example, DNA, lipids and proteins (Monaghan et al. 2009). As previous studies have shown
88 that accelerated growth could increase oxidative stress (e.g. Alonso-Alvarez et al. 2007; Stier
89 et al. 2014), the stimulating effects of THs on growth and metabolism likely contribute to the
90 production of ROS, hence increase oxidative stress (Asayama et al. 1987; Villanueva et al.
91 2013).
92 Studies on the effect of maternal thyroid hormones on offspring development in wild
93 animals are scarce. In humans and rats, hypothyroid condition of the mother impairs brain
94 development and cognition in her children (Moog et al. 2017). A potential problem here is that
95 in mammalian species, maternal thyroid variation or manipulation inevitably influences other
96 aspects of maternal physiology, which confounds the direct effects on the offspring.

97 Oviparous species, such as birds, are therefore suitable models for studying the role of
98 maternal hormones on the progeny because embryos develop in eggs outside the mother's
99 body and maternally-derived hormones are deposited in egg yolks (Prati et al. 1992; Schwabl
100 1993). This allows the measurement and experimental manipulation of maternal hormone
101 transfer to be independent of maternal physiology. Together with their relatively well-known
102 ecology and evolution, birds have become the most extensively studied taxa in research on the
103 function of maternal hormones (Groothuis et al. 2019).
104 Maternal thyroid hormones have long been detected in egg yolks of chicken (Hilfer
105 and Searls 1980; Prati et al. 1992) and Japanese quail (Wilson and McNabb 1997). To date,
106 only three studies have investigated the effects of physiological variation in yolk THs on
107 offspring development (great tits, *Parus major*, Ruuskanen et al. 2016; rock pigeons,
108 *Columba livia*, Hsu et al. 2017; collared flycatchers, *Ficedula albicollis,* Hsu et al. 2019).
109 These studies revealed potential biological relevance and fitness consequences but also some
110 discrepancies on the role of yolk THs. For example, yolk THs improved hatching success in
111 rock pigeons (Hsu et al. 2017) and in collared flycatchers (Hsu et al. 2019) but had no effect
112 in great tits (Ruuskanen et al. 2016). Moreover, TH injection in great tit eggs increased
113 offspring growth in males but decreased it in females (Ruuskanen et al. 2016). Conversely,
114 yolk THs decreased growth during the second half of the nestling phase in rock pigeons (Hsu
115 et al. 2017), whereas they increased early growth, but decreased later postnatal growth in
116 collared flycatchers (Hsu et al. 2019). Finally, great tits showed no response to elevated yolk
117 THs in resting metabolic rate (RMR) (Ruuskanen et al. 2016), whereas RMR was increased in
118 females but decreased in males rock pigeon hatchlings (Hsu et al. 2017). These studies
119 suggest that yolk THs may exert costs and benefits on the offspring in a species-specific
120 manner. Another non-mutually exclusive hypothesis is that yolk THs may have context-
121 dependent effects if the costs and benefits of THs differ across environments. For example, if

prenatal THs increase RMR (as suggested by Hsu et al. 2017), the elevated RMR may lead to increased growth in benign conditions, but decreased growth when resource availability is poor (Auer et al. 2015). Therefore, further studies on other species and contexts are needed to understand these contradicting findings.

Moreover, the study on collared flycatchers is the only one so far that investigated the association between yolk THs and oxidative stress in offspring (Hsu et al. 2019). This study surprisingly showed no adverse effect of yolk THs on whole blood oxidative damage or oxidative balance, despite the early growth-enhancing effects in the same study (Hsu et al. 2019). This absence of influence on oxidative stress contradicts the general knowledge of THs, with hyperthyroid tissues exhibiting higher oxidative damage in mammals (liver and heart, Venditti et al. 1997; brain, Adamo et al. 1989), calling for additional studies to confirm or contradict these findings.

To explore the origin of the discrepancies between previous studies (i.e. species- or context-dependency), we conducted a similar experiment as Hsu et al. (2019) in a closely related species with a similar ecological niche, the pied flycatchers (*Ficedula hypoleuca*). Pied and collared flycatchers are sister species that have very similar life-histories, reproductive ecology and morphology, and can also hybridize (Lundberg and Alatalo 1992). Importantly, the similarity between the two species offers us an opportunity to explore the potential role of the environment in modulating the effect of maternal hormones, which may contribute to explain the discrepancies of TH-related effects in the previous studies. To this end, we manipulated the concentrations of yolk THs in a wild population of pied flycatchers by injecting a combination of T_4 and T_3 in their eggs. We ensured that the treatment was within the physiological range. As proxies of environmental quality, we also collected data on temperature, precipitation, and fledging success of pied flycatchers. These data were then compared with those collected previously for collared flycatchers (Hsu et al 2019). If the

environmental contexts were similar between the two studies, we would expect to observe similar effects of elevated yolk THs, namely enhanced embryo development, hatching success, body mass and structural growth. By contrast, if the environmental context and the effects of elevated yolk THs differed between the studies, it would lend some support for the potential of context-dependent modulation. Finally, elevated yolk THs may result in higher oxidative stress (a general trend from the literature, e.g. Villanueva et al. 2013) either directly via the stimulating effects of THs on metabolism or indirectly via increased growth, or show no association with oxidative stress at all (as suggested by Hsu et al. 2019).

Material and Methods

Study site and study species

The experiment was conducted during the spring 2017 in Turku, South-West of Finland (60°26'N, 22°10'E). The study species is the pied flycatcher, a small (ca. 15 g) migratory passerine that breeds in Finland from May to July. Pied flycatchers are secondary cavity nesters that also breed in artificial nest boxes. At this latitude, females generally lay a single clutch of 5 to 8 eggs.

Nest monitoring and experimental design

Yolk thyroid hormone concentrations were elevated via injections into unincubated eggs using a between-clutch design (i.e. all eggs of the same clutch received the same injection). In total, 29 clutches (170 eggs) received a thyroid hormone injection (hereafter TH-treatment), and 28 clutches (169 eggs) received a control injection (hereafter CO-treatment). In two nests, one in each treatment, none of the eggs hatched due to desertion before incubation. These two clutches were therefore removed from the analysis. The final sample size is 28 TH-nests (166 eggs) and 27 CO-nests (164 eggs).

Nest boxes were monitored twice a week during nest construction until egg laying. On the morning when the fifth or sixth egg was laid, all eggs were temporarily removed from the nest for injection, replaced with dummy eggs and returned after injection. Nests were then visited every following morning to inject freshly laid eggs until clutch completion, marked by the absence of freshly laid eggs and females incubating their eggs. Females generally start incubating their eggs after the last egg has been laid.

The clutches were randomly assigned to one of the treatments. In addition, treatments were alternated across clutches to balance the order of treatments within a day. Similarly, we also balanced the treatments across the laying period. There was no difference in the average (± SD) laying date (TH = 27.00 ± 2.64 vs. CO = 27.19 ± 2.65, 1 = 1st of May, Wilcoxon unpaired test, W = 402.5, p = 0.68), nor in the average (± SD) clutch size (TH = 5.93 ± 0.81 eggs vs. CO = 6.07 ± 0.78 eggs, Wilcoxon unpaired test, W = 439.5, p = 0.26).

Preparation of the solution and injection procedure

The thyroid hormone solution (TH solution) was composed of a mix of T_4 (L-thyroxine, ≥ 98% HPCL, CAS number 51-48-9, Sigma-Aldrich) and T_3 (3,3',5-triiodo-L-thyronine, >95% HPCL, CAS number 6893-02-3, Sigma-Aldrich), first dissolved in 0.1M NaOH and then diluted in 0.9% NaCl. The concentration of each hormone was based on hormone measurements in 15 pied flycatcher eggs, from 15 clutches, collected during the spring 2016 in Turku. The average hormone contents of these eggs were the following: T_4 = 2.307 ng/yolk (SD = 0.654) and T_3 = 0.740 ng/yolk (SD = 0.238). We injected twice the standard deviation of each hormone (1.308 ng/yolk of T_4 and 0.477 ng/yolk of T_3), a standard and recommended procedure for hormone manipulation within the natural range (Ruuskanen et al. 2016; Hsu et al. 2017; Podmokła et al. 2018). The control solution (CO) was a saline solution (0.9% NaCl).

Before the injection, the shell was disinfected with a cotton pad dipped in 70%

195 alcohol. The injection procedure consisted of four steps. First, a disposable and sterile 25G
196 needle (BD Microlance ™) was used to pierce the shell. To locate the yolk, the egg was lit by
197 a small torch from underneath. Second, the injection of 5µl was performed with a Hamilton®
198 syringe (25 µl, Hamilton Company) directly into the yolk. Third, the hole in the shell was
199 sealed with a veterinary tissue adhesive (3M Vetbond ™) and the eggs were marked with a
200 permanent marker (Stabilo OHPen universal). Finally, all eggs of a clutch were returned to the
201 nest at the same time, and the dummy eggs removed.

202 *Nestling growth monitoring and blood sampling*

203 Nests were checked daily for hatching two days before the expected hatching date.
204 The date of hatching for a particular nest was recorded as the day the first hatchlings were
205 observed (day 0). Two days after hatching, nestlings were coded by clipping down feathers to
206 identify them individually. Nestlings were ringed at day 7 after hatching. Body mass (0.01 g)
207 was recorded at day 2, 7 and 12 after hatching. Tarsus (0.1 mm) and wing length (1 mm) were
208 recorded at day 7 and 12. At day 12, blood samples from all nestlings were also collected (ca.
209 40 µl) from the brachial vein in heparinized capillaries and directly frozen in liquid nitrogen
210 for analyses of oxidative stress biomarker and molecular sexing. All nestlings from the same
211 nest were sampled within 20 min. Samples were stored at -80°C until analyses. Finally,
212 fledging was monitored from day 14 after hatching. Fledging date was recorded when all the
213 nestlings had fledged from the nest, and fledging success (fledged/not) was scored for each
214 hatchling.
215 Finally, we collected data on temperature (hourly averages) and precipitation from the
216 European Climate Assessment & Dataset (ECA&D, Klein Tank et al. 2002), and calculated
217 the daily averages and length of periods of continuous rain, a key factor affecting mortality in
218 flycatchers (Siikamäki 1996; Eeva et al. 2002). Temperature data (hourly averages) were
219 extracted from a station located approximately 3 km away from our field site. To compare

environmental conditions between the collared flycatcher study by Hsu et al. (2019) and our study, we also collected similar data for the study period from a field station close to the collared flycatcher population (See Figs. A1 and A2 and Table A3, available online). In addition, we used overall fledging success as a proxy for environmental quality. In both populations, nest predation and adult mortality rates are low and are not main determinants of fledging success (Doligez and Clobert 2003; B. Doligez pers. comm.; S. Ruuskanen pers. comm). Thus, fledging success may be a good indicator of environmental conditions during the nestling phase. The data in Hsu et al. (2019) and in our experiment were collected on the same year (2017), and both nest-box populations were located in mixed forest habitats.

Sexing method

DNA extraction procedure from the blood cells followed Aljanabi and Martinez (1997), using approximately 5 µl of whole-blood samples. The method of sexing followed that described by Ruuskanen and Laaksonen (2010) with minor changes on the PCR condition: 5 µl QIAGEN multiplex PCR kit + 0.1 µl of each primer (20 µM) + 1.8 µl H2O + 3 µl DNA, yielding 10µl for the final PCR volume. The initial denaturation was at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 90 s, and 72 °C for 60 s. The samples were then held at 72 °C for 10 min and 20 °C for 5 mins. PCR products were analyzed with 3% agarose gel under 100 V for 90 min.

Oxidative stress analysis methods

Samples from two individuals per clutch were analyzed. Whenever possible, one male and one female were chosen of approximately the same body mass since body mass is known to covary with oxidative status (Rainio et al. 2015). The average difference in mean body mass between the chicks selected for oxidative stress analysis within each clutch is -0.01 g (SD = 0.43; range = -1.80–0.77 g). If samples from both sexes were not available for a clutch,

then two individuals of the same sex were selected. In total, 103 nestlings were included in the analysis (TH, N = 27 nests and 50 nestlings; CO, N = 27 nests and 53 nestlings).

Three biomarkers of oxidative status were measured: the activity of the antioxidant enzyme glutathione S-transferases (GSTs), the ratio of reduced and oxidized glutathione (GSH:GSSG ratio) and lipid peroxidation (using malonaldehyde, MDA, as a proxy) (Sheenan et al. 2001; Halliwell and Gutteridge 2015). GST enzymes catalyze the conjugation of toxic metabolites to glutathione (Sheenan et al. 2001; Halliwell and Gutteridge 2015). In normal cells, GST activity is expected to be lower than in damaged cells (Rainio et al., 2013). The GSH:GSSG ratio represents the overall oxidative state of cells, and a low ratio reveals oxidative stress (e.g. Rainio et al. 2013; Halliwell and Gutteridge 2015; Rainio et al. 2015). Lipid peroxidation is commonly measured with the thiobarbituric acid test (TBARS, Alonso-Alvarez et al. 2008; Halliwell and Gutteridge 2015). This test relies on the ability of polyunsaturated fatty acids contained in cell membranes to readily react with oxygen radicals by donating a hydrogen atom. The fatty acid radical is unstable, and a chain of reactions occurs. Malonaldehyde is an end product of this reaction (Marnett 1999) and thus used as a measure of lipid peroxidation.

Whole blood was first thawed and then diluted in 0.9% NaCl to achieve protein concentrations ranging 4–13 mg/ml. Overall protein concentration (mg/ml) was measured using BCA protein assay (Thermo Scientific) with a BSA standard (bovine serum albumin, Sigma). The methodology for measuring GST and GSH:GSSG ratio followed Rainio et al. (2015). The marker of lipid peroxidation, MDA, was analyzed using a 384-plate modification of TBARS-assay described by Espín et al. (2017). All biomarkers enzyme activities were measured in triplicate (intra-assay coefficient of variability [CV] <10% in all cases).

Statistical analysis

Data were analyzed with the software R version 3.5.3 (R Core Team 2019). General

and generalized linear mixed models (LMMs and GLMMs, respectively) were performed using the R package *lme4* (Bates et al. 2015). All mixed models included nest as a random intercept. P-values in LMMs were obtained by model comparison using Kenward-Roger approximation from the package *pbkrtest* (Halekoh and Højsgaard 2014). The significance of the predictors in GLMMs was determined by parametric bootstrapping with 1,000 simulations using the package *pbkrtest*. Model residuals were checked for normality and homogeneity by visual inspection. Significant interactions were further analyzed by post-hoc comparison with the package *phia* (de Rosario-Martinez 2015). Estimated marginal means and standard errors (EMMs ± SE) were derived from models using the package *emmeans* (Lenth 2019).

To analyze hatching success, a dummy code was given to each egg: 0 for unhatched egg and 1 for hatched egg. A GLMM was performed with a binomial error distribution (logit link). Treatment was included as the predictor and two covariates were included: the average temperature over the egg laying period and clutch size. Fledging success was coded similarly: 0 for dead and 1 for fledged nestling. A similar GLMM was fitted, with treatment as a predictor and the average temperature over the nestling period and brood size at day 2 as covariates.

Duration of the embryonic period and duration of the nestling phase were fitted in separate linear models with treatment as the fixed effect, and the average ambient temperature over these two phases as covariates to control for potential temperature-related effects (Olson et al. 2006; Salaberria et al. 2014). Laying date and brood size were added as additional covariates for nestling phase duration as they both may influence nestling growth and thereby nestling phase duration (Williams 2012).

Early body mass (i.e. at day 2 after hatching) was analyzed separately from growth during the second week post-hatching (i.e. from day 7 to day 12) for two reasons. First, variation in the former may represent better the influence of maternal THs on prenatal

development, while the variation in the latter also reflects the influence during the postnatal stage when the yolk that contain the hormones is totally consumed. Second, including the three time points in a single model created a non-linear growth curve, hampering proper statistical analyses. The model to analyze early body mass included laying date and mean temperature between hatching and day 2 as covariates. To analyze growth between day 7 and 12, we used the scaled mass index by Peig and Green (2009), a recommended method to estimate changes in body condition. The SMI was calculated as follows:

$$\mathrm{SMI}_i = M_i \times (L_0/L_i)^b$$

where M_i and L_i are body mass and tarsus length of the individual i, respectively. L_0 is the mean value of tarsus length for the whole population (L_0 = 17.0 mm, N = 228), and b is the slope estimate of a regression of ln-transformed body mass on ln-transformed tarsus length (b = 1.83). Furthermore, we analyzed growth in wing and tarsus length separately, given that THs may also influence structural size (e.g. Wilson and McNabb 1997), independently of mass. Models to analyze the morphological variables included sex as a fixed factor to test for potential sex-dependent effects of THs, as found by Hsu et al. (2017) and Ruuskanen et al. (2016). Treatment and age were added as fixed factors together with their 2- and 3- way interactions with sex. Brood size at day 2, laying date and average temperature were included as covariates. Individual identity was added as a random intercept to account for repeated measures.

The models to analyze growth (SMI and structural size) included age and treatment as fixed factors. Brood size at day two, laying date and average daily temperature (between day 3 and 7 for measurements at day 7, and between day 8 and 12 for measurements at day 12) were added as covariates, and nestling identity as an additional random intercept to account for repeated measures.

The models of oxidative stress biomarkers (i.e. GST activity, MDA concentration and

GSH:GSSG ratio) included treatment and sex as the predictors, and brood size at day 2, laying date and mean daily temperature as covariates. Body mass at day 12, which was the day of blood sampling, as an additional covariate, because body mass is known to be associated with oxidative status (e.g. Rainio et al., 2015). In a separate model, body mass at day 12 was replaced with growth rate (in grams/day) between day 7 and 12, to test the association of growth rate on oxidative stress. Assay number was also added as a random intercept to account for inter-assay variation. Response variables were log-transformed to achieve normal distribution of the residuals.

Ethical note

The study complied with Finnish regulation and was approved by the Finnish Animal Experiment Board (ESAVI/2389/04.10.07/2017) and by the Finnish Ministry of Environment (VARELY580/2017).

Results

Hatching and fledging success, duration of embryonic and nestling periods

Hatching success (TH = 75.3% vs. CO = 76.8%) and fledging success (TH = 92.2% vs. CO = 92.3%) were similar between the two treatments (GLMMs, $p > 0.71$). Hatching success was not affected by clutch size or by ambient temperature during incubation (GLMMs, both $p > 0.09$). Likewise, fledging success was not correlated to brood size at day 2 or ambient temperature (GLMMs, both $p > 0.10$). Duration of the embryonic period did not differ between the groups ($t = -0.59$, $p = 0.56$). Injection of yolk THs did not affect the duration of the nestling period either ($t = -1.01$, $p = 0.32$). Likewise, there was no association between laying date or brood size at day 2 and the duration of the nestling period (all $t < 1.87$, $p > 0.07$). Finally, there was no association between temperature and duration of embryonic or

342 nestling periods (all $p > 0.09$).

343 *Growth*

344 Experimental elevation of yolk thyroid hormones did not affect early postnatal body
345 mass (day 2 Estimated marginal means [EMMs] ± SE: CO = 3.63 ± 0.13 g vs TH = 3.50 ±
346 0.13 g), and neither did sex (Table 1). We detected a tendency of an interaction between
347 treatment and age on nestling scale mass index (SMI) between day 7 and 12 that just did not
348 reach statistical significance ($p=0.07$, Table 1). Although the interaction was not significant,
349 we performed post-hoc analyses to explore the trend further. We found that TH-treated
350 nestlings tended to grow faster than control nestlings during the second week post-hatching
351 (adjusted slopes ± SE = 1.32 ± 0.11 for CO nestlings, 1.61 ± 0.12 for TH nestlings, $\chi^2 = 3.41$,
352 Holm-adjusted $p = 0.06$, Fig. 1). Though, there was no significant difference in mass index
353 between the treatments at day 7 ($\chi^2 = 0.06$, Holm-adjusted $p = 0.81$) or day 12 ($\chi^2 = 1.04$,
354 Holm-adjusted $p = 0.62$), indicating that the interaction likely originates from small
355 differences in the opposite directions at day 7 and day 12 between TH and control groups. On
356 average, males had a slightly higher mass index than females between day 7 and day 12
357 (EMMs ± SE: males = 80.78 ± 0.59, females = 79.86 ± 0.61 g, Table 1). For structural size
358 measurements, tarsus and wing lengths, however, no effects of yolk TH treatment were
359 detected (Table 1). Ambient temperature was negatively correlated with SMI, and positively
360 associated with wing length (Table 1).

361 *Oxidative stress and oxidative damage*

362 Experimental elevation of yolk THs did not affect antioxidant enzyme activity (mean ±
363 SE: CO and TH nestlings = 0.006 ± 0.0003 pmol GST/min/mg protein), oxidative damage on
364 lipids (mean ± SE: CO = 0.051 ± 0.003 and TH = 0.053 ± 0.004 nmol MDA/mg protein) or
365 oxidative status (mean ± SE: CO = 3.86 ± 0.47 and TH = 4.51 ± 0.73 GSH:GSSG ratio)

(Table 2). None of the other predictors or covariates (i.e. sex, body mass, growth rate, temperature and brood size) were associated with these oxidative stress biomarkers, except laying date, which was negatively correlated with MDA concentration (Table 2).

Environmental context

Patterns of temperature and precipitation during the different stages of breeding are shown in Figures A1 and A2 (available online) for pied flycatchers and collared flycatchers. There were only minor differences in mean temperature across the stages and species: For pied flycatchers, the average temperatures over the laying, incubation and nestling periods were 12.14 °C, 13.43 °C and 15.02 °C respectively, and for collared flycatchers 12.48 °C, 13.67 °C and 14.56 °C. Likewise, the number of days with rain was rather similar (Table A3, available online) and we could not reliably associate peaks in precipitation with peaks in nestling mortality (Fig. A4, available online). Importantly, however, collared flycatchers experienced lower fledging success (ca. 75%, Hsu et al. 2019) compared to pied flycatchers (ca. 90%) during the study year, whereas both species have similar fledging success of about 90% when the environmental conditions are good (Qvarnström et al. 2009), suggesting that the collared flycatchers experienced harsher environmental conditions than the pied flycatchers.

Discussion

We replicated an experimental study on the effect of egg thyroid hormones on offspring development in collared flycatchers in a closely related and ecologically similar species, the pied flycatcher while at the same time monitoring environmental factors. This would allow us to study the generality of results found earlier but also potential environmentally dependent hormone effects.

Overall, our results on pied flycatchers differ substantially from those on collared

390 flycatchers (Hsu et al. 2019). We found no effect of prenatal THs on hatching success or
391 growth (in body mass, body condition or structural growth), where Hsu et al. (2019) found an
392 increase in hatching success and in early growth, but lower growth during the second week of
393 the nestling period. Because these two species are closely related and display ecological
394 similarities (Lundberg and Alatalo 1992), we predicted that such discrepancies in the results
395 could arise if THs influence growth differently in different environmental conditions. We
396 observed that fledging success, a proxy for environmental harshness, was lower in collared
397 than the pied flycatcher experiment. Yet, temperatures and rainfall did not generally seem to
398 differ across the studies, suggesting that other environmental factors may interact with yolk
399 THs. Furthermore, collared flycatchers generally have a slightly higher early body mass
400 (Qvarnström et al. 2009) and a higher fledging mass (Myhrvold et al. 2015) than pied
401 flycatchers. Yet, when comparing the present study with Hsu et al. (2019), collared flycatchers
402 had a lower early body mass than pied flycatchers and a similar body mass close to fledging,
403 suggesting poorer growth of collared flycatchers during the study year. Prenatal
404 environmental conditions (i.e. during egg laying and incubation) were rather similar between
405 the two species, and thus cannot explain why yolk THs enhanced hatching success and early
406 body mass in collared flycatchers (Hsu et al. 2019) but not in pied flycatchers (this study).
407 More experimental studies on the context-dependent effects of yolk THs are thus needed.
408 Despite no clear differences in temperature and precipitation, the lower growth and
409 survival of nestling collared flycatchers suggest that the environmental conditions may have
410 been harsher in this population than in the pied flycatcher population. Such environmental
411 conditions may have contributed to the contrasting results on the effects of yolk THs on
412 postnatal growth. We can speculate that a potential underlying mechanism is linked to
413 metabolic rates. Hsu and colleagues suggested that prenatal THs increase basal metabolic
414 rates (Hsu et al. 2017). Increased basal metabolic rates may lead to decreased postnatal

415 growth in harsh conditions, such as for the collared flycatcher, but have no effect or even
416 increase growth when resource availability is good, as the case in pied flycatchers.
417 Nevertheless, despite the high degree of ecological similarity between the two species, the
418 possibility that species difference actually explained the contrasting results remains to be
419 examined.

420 We observed no effect on antioxidant enzyme activity (GST) or in the oxidative
421 balance (GSH:GSSG) and no increase in oxidative damage in lipids (MDA) in response to
422 elevated yolk THs. The earlier study on collared flycatchers reported similar levels of
423 oxidative stress biomarkers and found no increase in oxidative stress in response to elevated
424 prenatal THs (Hsu et al. 2019). These results may suggest that egg THs do not affect the
425 oxidative status of nestlings as it would be expected from the literature. However, the absence
426 of detrimental consequences on oxidative stress may be due to the experimental design of
427 both studies, with an increase in yolk THs within the natural range of the species. Thus,
428 individuals may have been able to raise their antioxidant capacities (other than those
429 measured in this study) to avoid oxidative damage. That said, physiological elevation (i.e.
430 within the natural range) of yolk THs was necessary to get ecologically relevant results.
431 Furthermore, due to fieldwork constraints, there are some limits to our approach. We
432 measured a limited number of markers of oxidative status at a single time point in one tissue,
433 and therefore lack an overview of the variation that may have happened over the course of the
434 whole nestling phase, also in other tissues and for other biomarkers. Further studies with more
435 comprehensive measures of oxidative stress would help understanding the relationship
436 between yolk THs and oxidative stress.

437 In conclusion, this study shows no convincing effect of yolk THs on nestling
438 development. We found that yolk THs did not increase growth and incurred no extra oxidative
439 damage, nor affected nestling survival. Our results differ from a study on a closely related

species, suggesting that the role of prenatal THs may differ according to the environment experienced by the progeny. The study adds to the small body of literature on so far largely neglected thyroid hormone-mediated maternal effects. Research on maternal THs would greatly benefit from further studies with the same species in different, experimentally manipulated, contexts. It would also profit from comparative studies on species with different life-histories that are likely to influence the effects induced by exposure to maternal THs.

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627 **Figure legend**

628 Figure 1: Scale mass index raw data (mean ± SE, left) and marginal means (right) at
629 day 7 and day 12 after hatching in TH-treated nestlings (N = 125) and controls (N = 126).
630 The interaction between the treatment and age of the nestlings approached significance (p =
631 0.07). Empty circle: CO = control injection; solid circle: TH = yolk thyroid hormone
632 elevation.