Transient growth-enhancing effects of elevated maternal thyroid hormones at no apparent oxidative cost during early postnatal period

Bin-Yan Hsu[1*], Blandine Doligez[2], Lars Gustafsson[3], Suvi Ruuskanen[1]

[1] Section of Ecology, Department of Biology, University of Turku, Finland

[2] Department of Biometry and Evolutionary Biology, CNRS UMR 5558, Université Lyon 1, Université de Lyon, France

[3] Department of Animal Ecology/Ecology and Evolution, Evolutionary Biology Centre, Uppsala University, Sweden.

* Correspondence author: Bin-Yan Hsu, biyahs@utu.fi

Running title: Transient effects of maternal THs

Abstract

Maternal thyroid hormones (THs) have been proven crucial for embryonic development in humans, but their influence within the natural variation on wild animals remains unknown. So far the only two studies that experimentally investigated the potential fitness consequences of maternal THs in birds found inconsistent results. More studies are thus required to assess the general effects of maternal THs and their influences on more behavioral and physiological parameters. In this study, we experimentally elevated yolk TH content in a wild migratory passerine species, the collared flycatcher (*Ficedula albicollis*), to investigate the effects on hatching success, nestling growth and oxidative stress. We found that TH-injected eggs had a higher hatching success, and the nestlings hatched from TH-injected eggs were heavier and larger than control nestlings, but only during the early postnatal period. These differences vanished by fledging. Nestlings from TH-injected eggs exhibited lower activity of the glutathione-s-transferase, a major antioxidant enzyme, than control nestlings at day 12, a few days before fledging, but they did not differ in oxidative damage and overall intracellular oxidative state. These results suggest that the early growth-enhancing effects incurred no observable oxidative stress. We hypothesize that such a transient growth-enhancing effect might be adaptive in advancing the development and maturation of the offspring so they are well-prepared in time for the upcoming migration. Further studies investigating whether such advancing effects can influence long-term fitness, will be more than valuable.

Keywords

Collared flycatchers, maternal effects, maternal hormones, nestling growth, oxidative stress, thyroid hormones

Introduction

Among all possible avenues for maternal effects, hormones of maternal origin have received a great deal of research interest in the past decades (Groothuis and Schwabl 2008, Podmokła et al. 2018). Such hormone-mediated maternal effects are widespread in all vertebrates. In placentotrophic species, including most mammals and some reptiles, maternal hormones can reach the embryos across the placenta while in lecithotrophic species, like birds, fish, amphibians, and many reptiles, maternal hormones are transferred into egg yolks. These maternal hormones represent a vital source of trans-generational phenotypic plasticity and a great potential for adaptive maternal effects (Groothuis and Schwabl 2008).

Among all taxa in which maternal hormones have been detected, birds are the most extensively studied models because their relatively big eggs largely facilitate experimental manipulation of these hormones. The studies on maternal hormones in avian eggs form two main research lines: the first concerns maternal androgens, in particular testosterone, which has been found to influence offspring phenotype and survival. Thus, it has been considered as a possible tool for mothers to adjust the outcome of hatching asynchrony (Groothuis et al. 2005). The second concerns maternal corticosterone, the major stress hormone in birds, because it represents trans-generational effects of maternal stress that could adaptively prepare offspring for future stress conditions (Love and Williams 2008, Haussmann et al. 2012). However, other steroid or non-steroid hormones are also indispensable for offspring development and can have profound effects on offspring phenotype and future fitness. One of such long-ignored group of hormones are the thyroid hormones.

The two most important forms of thyroid hormones (THs) are triiodothyronine (T3) and thyroxine (T4, McNabb and Darras 2015). Both THs are important for tissue development and differentiation, metabolism and growth, but play different roles: T3 binds to TH receptors and triggers the downstream cascade reactions, while T4 is the main product synthesized and secreted from the thyroid gland(s) and serve as a precursor, regulating the bioavailability of T3 via

deiodinases in local tissues (McNabb and Darras 2015). Medical studies on maternal thyroid dysfunction in humans (hyper- and hypo-thyroidism) have clearly established the crucial role of maternal THs for fetal development, as maternal hypothyroidism during pregnancy can impair fetal brain development and result in mental retardation and other neurological deficits (reviewed in Morreale de Escobar et al. 2004a, b). The physiological variation of maternal T4 was found to be linked to new-born weight in humans (Medici et al. 2013). The importance of maternal THs on the development of the embryonic central nervous system has also been demonstrated in poultry (Flamant and Samarut 1998, van Herck et al. 2012, 2013). The presence of TH receptors, transmembrane transporters and deiodinases in embryos during very early developmental stages, i.e. 35 hours to 4 days of incubation, has also been described (Flamant and Samarut 1998, van Herck et al. 2012), indicating that maternal THs can be functionally active in embryos before the start of their endogenous TH production. These results emphasize the crucial role of THs in the context of developmental biology. Nevertheless, the significance of maternal THs and the consequences of their intra- and inter-individual variation in an ecological and evolutionary context had been almost completely ignored until very recently (Ruuskanen and Hsu 2018).

Thus far, only two recent experimental studies in birds have investigated the potential fitness consequences of maternal THs in eggs, in which the researchers directly manipulated TH levels in egg yolks within the natural range of variation. Both studies found effects of elevated maternal THs that potentially linked to offspring future fitness, but the observed effects differed. In the first study in a wild population of great tits (*Parus major*), the elevation of yolk THs by 2SD enhanced growth in males but reduced it in females over the nestling period (Ruuskanen et al. 2016a). In the second study in a captive colony of wild-type rock pigeons (*Columba livia*), a similar yolk TH elevation enhanced hatching success but decreased nestling body mass during the late nestling stage independently of sex (Hsu et al. 2017). Moreover, in an earlier study in domestic Japanese quails (*Coturnix japonica*), the development of the embryonic pelvic cartilage was

74 enhanced in eggs of females orally dosed with T4 before laying, which likely caused
75 supraphysiological increase in yolk T3 and T4 (Wilson and McNabb 1997).
76 Besides growth, maternal THs may also influence nestling physiological functions, such as
77 metabolic rate and oxidative balance. As circulating TH levels are positively associated with basal and
78 resting metabolic rates (Elliott et al. 2013, Welcker et al. 2013), we may also expect an association
79 between metabolic rates and prenatal exposure to maternal THs. The potential effects of maternal
80 THs on metabolism and growth imply that the oxidative balance may also be affected by maternal
81 THs. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the
82 capacity of antioxidants to counteract the damaging effects of ROS (Monaghan et al. 2009). ROS are
83 inevitable by-products of cell respiration and metabolism and therefore viewed as potential
84 accompanying costs of accelerated growth (Alonso-Alvarez et al. 2007, Nussey et al. 2009).
85 Therefore, variation in maternal THs may affect offspring fitness via its impact on metabolic rate and
86 oxidative balance. Intriguingly, previous studies show inconsistent effects of maternal THs on resting
87 metabolic rates (RMR): in great tits, RMR of nestlings from TH-elevated eggs did not differ from
88 control nestlings (Ruuskanen et al. 2016a), while in rock pigeons, elevated yolk THs enhanced RMR in
89 females but reduced it in males (Hsu et al. 2017). The effects of maternal yolk THs on the oxidative
90 balance remain unexplored.
91 The discrepancies in the effects of maternal THs on offspring future fitness make it difficult
92 to predict in which situations females would benefit in increasing or decreasing yolk TH deposition.
93 To be able to do so, we need more studies in different species with various life histories or under
94 different environmental conditions to assess the generality. In this study, we experimentally studied
95 the consequences of variation in maternal yolk THs on nestling growth and oxidative balance by
96 manipulating yolk TH levels in a migratory passerine species, the collared flycatcher (*Ficedula*
97 *albicollis*). We injected a mixture of T3 and T4 to increase yolk TH levels by 2SD and monitored
98 subsequent nestling development by measuring body mass and tarsus length throughout the
99 nestling period. The contradicting results of previous studies make it difficult to *a priori* predict the

100 effect of the experimental treatment, but considering the well-known and essential function of THs
101 on metabolism and growth, we might expect enhanced growth for nestlings originating from TH-
102 injected eggs. We also measured three important biomarkers of oxidative balance: an antioxidant
103 enzyme (glutathione-s-transferase, GST), oxidative damage to lipids and general redox state (ratio of
104 reduced and oxidized glutathione). Because of the expected enhanced growth, we also expected
105 nestlings from TH-injected eggs to show elevated levels of oxidative stress, i.e. higher level of
106 oxidative damage and/or lower antioxidant capacity, compared to control nestlings (e.g. Alonso-
107 Alvarez et al. 2007, Nussey et al. 2009).

Materials and Methods

Experimental procedure

The study was conducted in the long-term monitored population of collared flycatchers on Gotland, Sweden, from late April to June, 2017. In total 801 nest-boxes in 18 plots were checked every 5 days before the start of egg-laying (around mid-May) and every 4 days after the first egg in the population was found (May 17). Clutch size was 5-7 eggs in most cases (Table S1). Egg injection started on the day when the 5th egg was laid and the same nest was visited in the following two days to inject the 6th and 7th egg, if present. We used a between-clutch design and randomly pre-assigned half of the nests to thyroid hormone elevation treatment (hereafter "TH nest") and the other half to the control treatment where the eggs were injected with vehicle only (hereafter "CO nest"). All the eggs in the same clutch were therefore assigned to the same treatment. The treatment assignment was balanced with respect to laying date. In total we injected 195 nests (97 TH and 98 CO nests). Among them, 10 nests were deserted during the incubation period and in another 3 nests all eggs were gone and presumed to have been preyed upon, yielding a final sample size of 182 nests (91 TH and 91 CO nests). Laying date did not differ between the two treatments (Mann-Whitney U test, U=4216.5, p=0.829), nor did clutch size (U=4493, p=0.273).

Twelve days after the last egg was laid for a given nest, we visited the nest after 16:00 to check for the presence of hatchlings. In case no hatchlings were found, the nest was inspected every day until at least one chick hatched or eggs were found cold, indicating nest abandonment (n=10). Hatching day (i.e. when the first hatchling was found in the nest) was considered day 0. At day 2, the nests were visited again to check the final number of hatchlings (calculated as the initial clutch size minus the number of remaining unhatched eggs).

Among the 182 experimental nests, we selected 60 nests in 8 plots over the whole study area for more detailed nestling biometric measurements. The 8 plots were selected due to practical reason but they represented the same habitat type as other plots in general. All nests in these 8 plots were included in our experiment and nests among them were selected for close growth

monitoring so as to balance yolk TH treatment and hatching date. In these selected nests, we weighed nestlings at day 2, 8 and 12 and measured tarsus length at day 8 and 12 post-hatching. Blood samples were taken at day 2 (<10 µl) and day 12 (< 50 µl) for sexing and measurement of markers of the oxidative balance. Blood samples were stored in coolers in the field and frozen at -80 °C on the same evening until analysis, ca. 5-6 months later. Nestlings were individually identified using nail-clipping codes at day 2, ringed at day 8 and fledging success was checked after day 18 (fledging usually occurs around day 16). In this paper, we analyzed and presented hatching success, fledging success, and pre-fledging survival from all the experimental nests, and the nestling growth data from the 60 selected nests.

The experimental procedure was approved by Jordbruksverkets (Swedish Board of Agriculture), permit number ID 872. www.jordbruksverket.se

In ovo hormone injection

Egg injection was conducted in the field. Eggs were first collected from each nest and temporarily replaced with dummy eggs during injection. A flashlight was used to back-illuminate the egg and show yolk position from beneath the egg. The prospective injection point was first sterilized with ethanol (>70%) and a hole was made on the eggshell using a sterile 25G needle. Five µl of TH or control solution was then injected into egg yolks with 25 µl Hamilton syringes with 26G needles. After injection, the hole was sealed with a very tiny drop of veterinary tissue adhesive (3M Vetbond™, No.1469SB) and eggs were returned to the nest. Hamilton syringes were cleaned with ethanol after the injection of each egg.

The injected hormone solution was prepared based on the yolk T3 and T4 levels measured from unincubated eggs collected in previous years from the same population (n=15, mean±SD: T3, 0.75±0.20 ng/yolk; T4, 2.74±0.42 ng/yolk, Ruuskanen et al. 2018). The injection dose was calculated so as to raise the average TH levels in the egg yolks by 2SD, i.e. 0.40 ng T3 and 0.84 ng T4 per 5 µl (i.e. the total volume injected). Crystalline T3 (3,3´,5-triiodo-L-thyronine, >95% HPLC, CAS number

6893-02-3, Sigma-Aldrich) and T4 (L-thyroxine, ≥98% HPLC, CAS number 51-48-9, Sigma-Aldrich) were first dissolved in 0.1 M NaOH separately and further diluted to twice the target concentration in 0.9% NaCl. T3 and T4 solutions were then mixed to produce the final solution to be injected. This TH solution was split into 300 µl plastic vials (Waters, USA) after being filtered through Sterile Syringe Filter (0.2 µm cellulose acetate, VWR) to achieve sterility. The hormone solution was stored in -20 °C until the day of injection. The control solution was prepared and stored in the same way, but contained no mixture of T3 and T4.

Molecular sexing

DNA extraction procedure followed Aljanabi and Martinez (1997). Primers P2 and P8 were used in PCR to amplify CHD-Z and CHD-W genes located on avian sex chromosomes (Griffiths et al. 1998). The PCR condition for each sample was as following: 5 µl 2X QIAGEN multiplex PCR kit + 0.2 µL of each primer (10 µM) + 1.6 µl H_2O + 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.

Analyses of the markers of the oxidative balance

To limit the number of samples to be analyzed, blood samples from day-12 nestlings of known sex were selected for the analysis of the markers of the oxidative balance. Samples were selected to balance sexes and yolk TH treatments, with 1 male and 1 female per experimental nest whenever possible. For those nests where there were only males (n=5 nests) or females (n=4 nests), 2 samples were randomly selected. A total of 102 samples were selected, from 50 female and 52 male nestlings, and from 50 CO and 52 TH nestlings.

We measured three biomarkers of the oxidative balance: (1) the activity of the antioxidant enzyme glutathione-s-transferase (GST) that catalyzes the glutathione cycle (Ercal et al. 2001,

Halliwell and Gutteridge 2007), (2) the ratio of reduced over oxidized glutathione (GSH/GSSG ratio), reflecting the overall cell oxidative state (e.g. Halliwell and Gutteridge 2007, Rainio et al. 2015), and (3) the lipid peroxidation (using malondialdehyde, MDA as a proxy), measured with the thiobarbituric acid test (TBARS, Halliwell and Gutteridge 2007).

The whole blood was first diluted in 0.9% NaCl to achieve protein concentration 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 is described in detail in Rainio et al. (2015). Briefly, GST-assay (Sigma CS0410) was adjusted from 96- to 384-well plate. We used 2 µl of each sample in triplicates and our own reagents: Dulbecco's phosphate buffered saline-buffer, 200 mM GSH (Sigma G4251), 100 mM CDNB (Sigma C6396) in EtOH. The ratio of GSH/GSSG was measured with the ThioStar® glutathione detection reagent kit (K006-F1D, Arbor Assays, USA) in triplicates following the kit instructions. MDA was analyzed using a 384-plate modification of TBARS-assay by Espín et al. (2017). Briefly, 50 µl of samples diluted in 0.9% NaCl were mixed with 100 µl of TBARS-BHT reagent (15% Trichloroacetic acid (TCA), 0.375% 2-Thiobarbituric acid (TBA), 0.02% Butylated hydroxytoluene (BHT) and 0.25 N HCl) and incubated in a water bath at 90° C for 30 min. Samples were then cooled in ice-water for 10 min to stop the reaction and centrifuged for 15 min at 6 °C in 2100 g. The standard was prepared from MDA (Sigma). The samples were analyzed in black 384-well plates, and fluorescence intensity (FI) was measured at an excitation/emission wavelength of 530/550 nm (EnSpire microplate spectrofluorometer). All biomarkers were measured in triplicate (intra-assay coefficient of variation <10% in all cases).

Because of technical issues, the final sample sizes varied for the three oxidative stress biomarkers: n=102, 86, 99 for GST, GSH/GSSG ratio, and lipid peroxidation, respectively.

Statistical analyses

All statistical analyses were performed in R 3.3.1 (R core team 2016). General or generalized linear mixed-effects models (LMMs and GLMMs) were used to analyze the effect of yolk injection treatment on egg hatching success, fledging success, body mass at day 2, body mass and tarsus length between day 8 and 12, and the three biomarkers of oxidative balance. In all models, yolk injection treatment (TH vs. CO) was included as a fixed factor and nest ID and plot ID were included as random intercepts to account for the non-independence of nestlings within a brood and broods within a plot. For hatching success (hatched vs. unhatched for each egg) and fledging success (fledged vs. dead), GLMMs were used and binomial residual distribution was specified, and no additional fixed factors were added as we did not measure any other egg-specific traits (e.g. egg mass). All other models were fitted with LMM, assuming Gaussian residual distribution. For body mass and tarsus length, nestling sex, hatching date, and brood size at day 2 were included as fixed factors. The interactions between yolk injection treatment and other fixed factors, and the interaction between sex and brood size were tested, but only retained in the model when significant. For nestling body mass and tarsus length between day 8 and 12, age was added as a fixed factor and the ID of each nestling was added as a random intercept. We chose to analyze body mass at day 2 separately from mass at day 8 and 12 for two reasons. (1) Body mass at such an early age reflects the effects of prenatal yolk TH exposure on prenatal development, while body mass at day 8 and 12 reflects the combination of the effects of prenatal yolk TH exposure and TH synthesized by the nestling on pre- and post-natal development. (2) Including three age points renders the growth curve non-linear, hampering the use of linear models. Because body mass and tarsus length were highly correlated ($r=0.81$ at day 8, $r=0.54$ at day 12), the effect of the treatment on body mass could simply reflect the effect on tarsus length (see *Results*). To test this, we included tarsus length as a covariate in the model on body mass to check whether the treatment has an additional effect on body mass independently from tarsus length. In this model, tarsus length was first centered and standardized by age class to avoid the collinearity between age and tarsus length.

All three biomarkers of oxidative status were first natural-log transformed to achieve normality and then fitted with separate models. Plot ID, nest ID, and the assay number were included in models as random intercepts; yolk injection treatment, nestling sex, hatching date, and brood size at day 2, and body mass at day 12 were included as fixed factors. Brood size at day 2 was included because brood size at early nestling stage can have profound effects on nestling growth and therefore oxidative balance (e.g. Losdat et al. 2011). All models were performed using the package *lme4* (Bates et al. 2015). The significance of fixed factors in LMMs was determined by model comparisons with Kenward-Roger approximation (package *pbkrtest*, Halekoh and Højsgaard 2014). When an interaction was significant, a post-hoc analysis was performed using the package *phia* and Holm-adjusted p-values were reported (de Rosario-Martínez 2015).

Pre-fledging survival in relation with yolk TH injection treatment was analyzed using Cox proportional hazards regression models (R package *survival*, Therneau 2015), clustered on nest ID and plot ID. Nestlings hatched in all nests (n=705) were included. Survival data were right-censored at fledging check (around day 18).

Results

Hatching success, fledging success, and pre-fledging survival

Among the 182 experimental nests (n=559 TH eggs and 575 CO eggs), the hatching success per nest was in average 65.1% and 59.3% for TH and CO nests, respectively, when including the nests (n=13) with complete hatching failure, i.e. where no egg hatched, likely due to an unknown cause other than the yolk injection treatment (e.g. infertile eggs or predation on the female). Excluding these nests yielded a hatching success of 70.4% and 63.1% for TH and CO nests, respectively (n=364 out of 517 TH eggs and 341 out of 540 CO eggs from in total 169 nests), and this difference was statistically significant (GLMM; effect of yolk TH injection treatment: z=2.339, p=0.019). Among these 169 nests, fledging success was monitored in 166 nests, with in total 267 out of 361 nestlings from TH nests (i.e. 74.0%, hereafter TH nestlings) and 264 out of 334 nestlings from CO nests (i.e. 79.0%, hereafter CO nestlings) fledging successfully. This difference was not statistically significant (GLMM, z=-0.142, p=0.887). Yolk TH treatment did not significantly influence pre-fledging survival (z=0.977, p=0.329, Fig. 1).

Body mass at day 2

In the subsample of 60 selected nests (see *Experimental procedure*), we measured and sexed a total of 272 day-2 hatchlings. Hatching date was highly concentrated between June 8 and 12 and did not differ between treatments (Mann-Whitney U test, W=489, p=0.552). TH nests had significantly larger brood sizes than CO nests (marginal mean±SE: TH, 4.92±0.26; CO, 4.22±0.26, LMM, $F_{1, 51.67}$=4.397, p=0.041) due to higher hatching success. Among the chicks that successfully hatched, there was no indication that the yolk TH treatment affected nestling sex ratio ($\chi 2$ =0.201, p=0.654, Table S2). Because of the relatively low hatching success of experimental nests, we can however not exclude an overall effect of THs via sex-specific hatching failure.

TH nestlings (n=142 from 29 nests) were significantly heavier at day 2 (marginal mean±SE = 3.51±0.11 g) than CO nestlings (n=130 from 31 nests, mean±SE = 3.09±0.12 g, $F_{1, 49.33}$=7.573, p=0.008,

Fig. 2). Female nestlings were heavier than male nestlings (marginal mean±SE = 3.37±0.10 g for females, and 3.22±0.09 g for males; $F_{1, 235.63}$=3.930, p=0.049, Fig. 2). The interaction between yolk TH injection and nestling sex was not significant ($F_{1, 234.88}$=0.741, p=0.390). Post-hoc comparisons confirmed that TH nestlings were significantly heavier than CO nestlings regardless of sex (females: χ^2=4.146, p=0.042; males: χ^2=8.305, p=0.008, Fig. 2). Hatching date and brood size did not affect body mass at day 2 ($F_{1, 54.84}$=1.142, p=0.290; $F_{1, 58.46}$=1.472, p=0.230, respectively).

Body mass and tarsus length at day 8 and 12

Out of the 272 nestlings measured at day 2, 247 (i.e. 90.8%) survived until day 8 and 229 (i.e. 84.2%) until day 12. Body mass differed between yolk TH injection and control treatments but the difference depended on nestling age, leading to a significant interaction between age and yolk TH treatment ($F_{1, 228.23}$=9.554, p=0.002, Fig. 3A). Post-hoc analyses showed that CO nestlings gained more weight from day 8 to day 12 than TH nestlings (adjusted slopes = 0.44±0.03 for CO nestlings and 0.31±0.03 for TH nestlings, χ^2=9.563, p=0.002, Fig. 3A), but there were no significant differences in body mass between TH and CO nestlings either at day 8 (χ^2=2.332, p=0.254) or day 12 (χ^2=0.085, p=0.771). Hatching date and brood size were both negatively correlated to nestling body mass (hatching date: estimate±SE = -0.154±0.072, $F_{1, 55.62}$=4.206, p=0.045; brood size: estimate±SE = -0.232±0.110, $F_{1, 55.42}$=4.086, p=0.048): nestlings that hatched later in the season and grew in larger broods were lighter. There was no sex difference ($F_{1, 208.71}$=0.187, p=0.666) or significant interaction between sex and yolk TH treatment ($F_{1, 208.99}$=0.018, p=0.892) in body mass.

TH nestlings had longer tarsi than CO nestlings, but the difference was larger at day 8 than day 12, leading to a significant interaction between yolk TH injection treatment and age ($F_{1, 226.45}$=7.459, p=0.007, Fig 3B). Post-hoc analyses showed the tarsi of CO nestlings grew more than those of TH nestlings (adjusted slope = 0.39±0.02 in CO nestlings, 0.32±0.02 in TH nestlings, χ^2=7.468, p=0.006); the difference in tarsus length between TH and CO nestlings was significant only at day 8 (χ^2=12.515, p<0.001) and not at day 12 (χ^2=2.627, p=0.105), suggesting that CO nestlings had

caught up with TH nestlings at day 12 (Fig. 3B). In addition, females had significantly longer tarsi (marginal mean±SE = 18.68±0.11 mm) than males (marginal mean±SE = 18.47±0.11 mm, $F_{1, 215.83}$=6.689, p=0.010). The interaction between nestling sex and yolk TH treatment in tarsus length was not significant ($F_{1, 216.36}$=0.072, p=0.789). Hatching date and brood size did not influence tarsus length (hatching date: $F_{1, 57.86}$=1.559, p=0.217; brood size: $F_{1, 57.04}$=0.109, p=0.743).

The model on body mass including tarsus length as a covariate yielded the same general result with two notable points: First, the sex difference on body mass became significant ($F_{1,204.39}$=4.108, p=0.044), suggesting that once controlled for tarsus length, males were on average 0.17 g (SE=0.08) heavier than females. Second, the interaction between age and yolk TH treatment was still significant ($F_{1,231.65}$=4.394, p=0.037), showing that the yolk TH treatment had a direct effect on body mass independently of its effect on tarsus length.

Oxidative stress

TH nestlings exhibited a significantly lower glutathione-s-transferase (GST) activity at day 12 than CO nestlings (back-transformed marginal mean±SE: TH nestlings, 0.004±0.0005 pmol/min/mg protein; CO nestlings, 0.005±0.0006, $F_{1, 41.58}$=4.827, p=0.034). Sex, hatching date, brood size, body mass, and their interactions with yolk TH injection treatment were all non-significant (F<1.95, p>0.17 in all cases). Yolk TH injection treatment had no effect on the ratio of reduced over oxidized glutathione (GSH/GSSG ratio, back-transformed marginal mean±SE: TH nestlings, 3.21±0.44; CO nestlings: 3.90±0.53, $F_{1, 38.25}$=1.022, p=0.319) or malondialdehyde levels (MDA, one end product of lipid peroxidation, back-transformed marginal mean±SE: TH nestlings, 0.039±0.005 nmol/mg; CO nestlings: 0.039±0.005 nmol/mg, $F_{1, 40.47}$=0.007, p=0.932)MDA levels were only influenced by sex with males exhibiting lower MDA levels than females (back-transformed marginal means±SE: males, 0.034±0.004; females: 0.044±0.006, $F_{1, 66.95}$=13.967, p<0.001). All other factors and interactions with yolk TH treatment on GST, GSH/GSSG ratio or MDA levels were non-significant (F<2.66, p>0.1 in all

cases). This suggests no difference in overall oxidative state and lipid peroxidation between the two treatment groups.

Discussion

In this study, we experimentally investigated the effects of maternal thyroid hormones (THs) on nestling growth and physiological processes, by elevating both yolk T3 and T4 simultaneously in the egg yolks of a wild passerine species, the collared flycatcher. Our treatment mimicked a higher maternal hormone deposition within the natural range. Interestingly, we found transient growth-enhancing effects of elevated yolk THs that disappeared by day 12 after hatching. At day 12, such growth-enhancing effects did not appear to lead to any apparent cost in terms of elevated oxidative stress. Taken together, these results suggest that maternal THs in egg yolks advance offspring early development and growth without translating into oxidative costs, but they do not lead to larger or heavier fledglings or affect fledging success. Consistent with our findings, previous studies also reported greater effects of maternal hormones at younger ages of the nestlings (e.g. Smiseth et al. 2011, Muriel et al. 2015a). However, our results are not in line with those of the two similar previous experiments in great tits (Ruuskanen et al. 2016a) and rock pigeons (Hsu et al. 2017), either in the direction or timing of the effect. This suggests between-species differences in the fitness consequences of maternal THs. Because the research of maternal transfer of THs to offspring in an eco-evolutionary context is still in its infancy, a detailed comparison of the results of these three studies will help to identify potential future research avenues for understanding the fitness consequences of the variation in maternal yolk THs.

Regarding hatching, we found higher hatching success for TH-injected eggs than control eggs after excluding the complete failures presumably unrelated to maternal THs. This finding is consistent with the higher hatching success for TH-injected eggs found in rock pigeons, emphasizing that elevated yolk THs may help the developing embryos overcome some unidentified developmental hurdles (Hsu et al. 2017). However, in great tits, yolk THs had no effect on hatching success (Ruuskanen et al. 2016a). This discrepancy may be due to the lower overall hatching success observed in the great tit study (only ~50%, Table S3). The lower observed hatching success in great tits suggests unknown factor(s) that may have dampened hatching success and blurred the

difference. Alternatively, the difference between great tits and the other two species may be related to the much lower yolk TH levels observed in great tits compared to the other two species (Table 1). Perhaps yolk THs played a permissive role in enhancing hatching success and in great tits the experimental manipulation did not meet the required threshold even after injection. Compared to uninjected eggs in the same population in the same year (ca. 92%, n=1646 chicks hatched from 1799 eggs out of 289 nests, Gustafsson et al. unpublished data), the hatching success of the injected eggs, no matter whether TH or CO, was much lower. Such a reduction in hatching success is, on the one hand, common in all egg-injection studies and is caused by the injection protocol itself (Groothuis and von Engelhardt 2005). On the other hand, such a reduction in hatching success appears to also differ between species: In rock pigeons, for instance, the hatching success of the TH-injected eggs is close to uninjected eggs (Hsu et al. 2017). The pigeon case implied that yolk THs might be able to compensate for the negative impact of the injection itself, but such compensation remained limited in collared flycatchers. Whether the reduction in hatching success of injected eggs may result in biases with respect to the treatment effects on nestlings is unclear but calls for caution when interpreting the results. The fledging success of our experimental nests (74% for TH nestlings and 79% for CO nestlings) was, however, similar to the fledging success of the non-experimental nests (77%, n=1274 fledglings, Gustafsson et al. unpublished data), suggesting that the injection protocol itself had no further effect after hatching.

The growth-enhancing effects of yolk THs in collared flycatchers were found in both body mass and tarsus length, with the latter being more pronounced. This result is consistent with our prediction, based on higher new-born mass with higher maternal THs in humans (Medici et al. 2013) and on enhanced pelvic cartilage development of the embryos from oral T4-dosed females in Japanese quails (Wilson and McNabb 1997). However, this result contradicts findings in rock pigeons and great tits. In great tits, elevated yolk THs had a sex-specific effect on nestling growth, with enhanced growth in males but reduced growth in females (Ruuskanen et al. 2016a), but here we observed no between-sex difference in collared flycatchers. Also, in rock pigeons, elevated yolk THs

reduced body mass by fledging independently of nestling sex (but had no effect on tarsus length; Hsu et al. 2017), but here we observed enhanced growth in collared flycatchers, on both body mass and tarsus length. Moreover, the discrepancy between studies is also observed regarding the developmental stages when the treatment effects were detected: in rock pigeons the treatment effect was only detected during the late nestling period while in collared flycatchers it was detected at an early stage and disappeared before fledging. In great tits, the treatment effects were detected using nestling measurements spanning over the whole nestling period (i.e. day 2, 7 and 14; Ruuskanen et al. 2016a).

These discrepancies between our results and previous studies probably result from multiple factors, including the intrinsic species differences formed over the evolutionary history and differences in the environmental contexts. For example, the different life histories of the three species may have co-evolved with differential prenatal sensitivity or responsiveness to maternal THs. Embryos of different species may also have evolved different ways to utilize or allocate maternal THs, for example by differentially expressing deiodinases at different developmental stages or in different tissues, because of their different life histories. Even though both collared flycatchers and great tits show a very similar breeding ecology and in particular use the same nesting sites in our study area, collared flycatchers are long-distance migrants, with a relatively shorter breeding window than great tits, which are sedentary. Elevated maternal THs may have been favored in collared flycatchers to advance the offspring's development and maturation so they are vulnerable to nest parasites and predators for a shorter time and will be ready for migration in time. If this hypothesis is true, in spite of no apparent differences in body mass and size at day 12, we might still expect earlier fledging or onset of migration, which remains to be tested. Moreover, clutches laid later in the season might contain higher levels of maternal THs in the yolks so the offspring can catch up with the schedule. So far, the variation of maternal yolk THs in relation with breeding phenology, maternal age or condition has not yet been characterized. By contrast, this selective pressure is probably weaker in resident great tits, let alone the highly fecund multi-brooded rock pigeons

(Johnston and Janiga 1995). Interestingly, among the three species, collared flycatchers showed the highest yolk concentrations of both T3 and T4 (about twice as high as the rock pigeon; Table 1), suggesting the importance of these maternal hormones in this species. More information on yolk THs of maternal origin in different species is now needed to assess whether the differences observed here are linked to different life histories. A comparative analysis would be valuable to shed light on how life history may shape the evolution of maternal THs. Another possible explanation to these discrepancies is that the effects of maternal THs depend on the environmental context. Such context-dependent effects have been reported for maternal androgens (e.g. Muriel et al. 2015b) and could be occurring for maternal THs as well. Experiments combining manipulations of yolk THs and a relevant environmental factor, such as food availability or ambient temperature, in a full factorial design would be the most effective way to reveal such context-dependent effects.

Along with morphological traits, we examined whether prenatal exposure to elevated yolk TH levels would lead to enhanced oxidative stress as a potential side effect of enhanced growth. We found no clear support for this hypothesis. At day 12, when the growth-enhancing effect in TH nestlings was not apparent anymore, we found lower blood GST activity in TH nestlings than in CO nestlings. At that age, CO nestlings had higher growth rate than TH nestlings, allowing them to catch up with the latter (Fig. 3), thus this result could suggest a short-term link between growth rate and oxidative stress (Alonso-Alvarez et al. 2007, Nussey et al. 2009). This also suggests that the early growth-enhancing effect of yolk THs did not seem to incur oxidative stress later on. However, we did not find a correlation between body mass gain from day 8 to day 12 and GST activity ($r=-0.03$, $p=0.75$). Therefore, analyzing whether TH nestlings showed higher GST activity at an earlier age (day 8), when they grew faster than CO nestlings, would still be needed to draw conclusions about the link between GST activity and growth here. Nevertheless, at day 12, oxidative damage to lipids and the overall oxidative state, indicated by the GSH/GSSG ratio, showed no difference between TH and CO nestlings, again suggesting no evidence for oxidative stress in relation to yolk TH injection treatment. Unfortunately, we did not measure here total circulating lipid levels, which might

confound the results of MDA levels (Pérez-Rodríguez et al. 2015). Nevertheless, to sum up, the transient growth enhancing effects found in this study does not appear to incur any oxidative costs to collared flycatcher nestlings based on our data. Whether or not any immunological costs were incurred, however, remains to be tested.

The present study applied a between-clutch design, assigning all eggs in a given nest with the same injection treatment. Part of the eco-evolutionary significance of maternal THs, however, might reside in the within-clutch variation, which was found to be substantial in great tits and rock pigeons (Hsu et al. 2016a, Ruuskanen et al. 2016b, c). The positive effects of maternal THs on growth during the early prenatal period might provide competitive advantage to some nestlings over their siblings and thus could influence the dynamics of sibling competition, similar to the long-proposed role for maternal androgens (Groothuis et al. 2005). To test this hypothesis about the function of maternal THs, a full factorial design combing the manipulations of both yolk THs and another relevant environmental variable or the context of sibling competition would be very enlightening.

The transient effects of maternal THs on nestling growth we found in this study call for the general question of their potential long-term fitness consequences. At this stage, it is not possible to make clear predictions since studies are still few and were conducted only during the nestling developmental stage. Nevertheless, the lack of difference in body mass and tarsus length at day 12, as found in this study, does not preclude the possibility of long-term effects induced by maternal THs. In this study, we focused on growth and oxidative stress during the pre-fledging period. Long-lasting effects might manifest in other traits that we did not measure. Previous studies of maternal THs in humans, rats, and chickens have shown that maternal THs are crucial for brain development (Morreale de Escobar et al. 2004a, Flamant and Samarut 1998, van Herck et al. 2012, 2013). This suggests potential organizational effects in the brain, which may in particular affect behavioral performance later in life, in line with results of many studies on maternal androgens (e.g. Hsu et al. 2016b). Moreover, THs are pleiotropic hormones that regulate not only metabolism, development, and tissue differentiation and maturation, but also bird molt and migration (e.g. Pérez et al. 2016,

2018). Effects of maternal THs on the regulation of the endocrine and immune system are also possible and may manifest on a longer term. The transient effects of maternal THs on growth might be part of the adjustment of the pace of life. In this case, we might expect advanced timing of migration, molt, first breeding, and even senescence for individuals that received higher maternal THs during development. Currently, studies investigating potential long-term effects of maternal TH exposure are still lacking but clearly needed to understand the fitness consequences of variation in yolk THs of maternal origin.

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Authors' Contributions:

BYH and SR designed and conducted the field experiment, and drafted the manuscript. BYH conducted sexing and statistical analyses. SR conducted lab analysis of oxidative stress. SR and BD coordinated the field work and logistics. All authors contributed to writing and approved the manuscript.

Conflict of Interest:

The authors declare no conflict of interest.

Table 1. Natural levels of maternal T3 and T4 levels (mean±SD) in the egg yolks of great tits, rock pigeons, and collared flycatchers.

	T3		T4	
Species	Concentration (pg/mg yolk)	Total content (ng/yolk)	Concentration (pg/mg yolk)	Total content (ng/yolk)
Great tit, ***Parus major***[a]	0.32±0.13	0.11±0.04	2.96±0.83	0.96±0.29
Rock pigeon, ***Columba livia***[b]	1.11±0.21	2.58±0.59	3.06±0.99	10.06±3.53
Collared flycatcher, ***Ficedula albicollis***[c]	1.98±0.49	0.75±0.20	7.21±1.00	2.74±0.42

[a] Ruuskanen et al. 2016a

[b] Hsu et al. 2016a

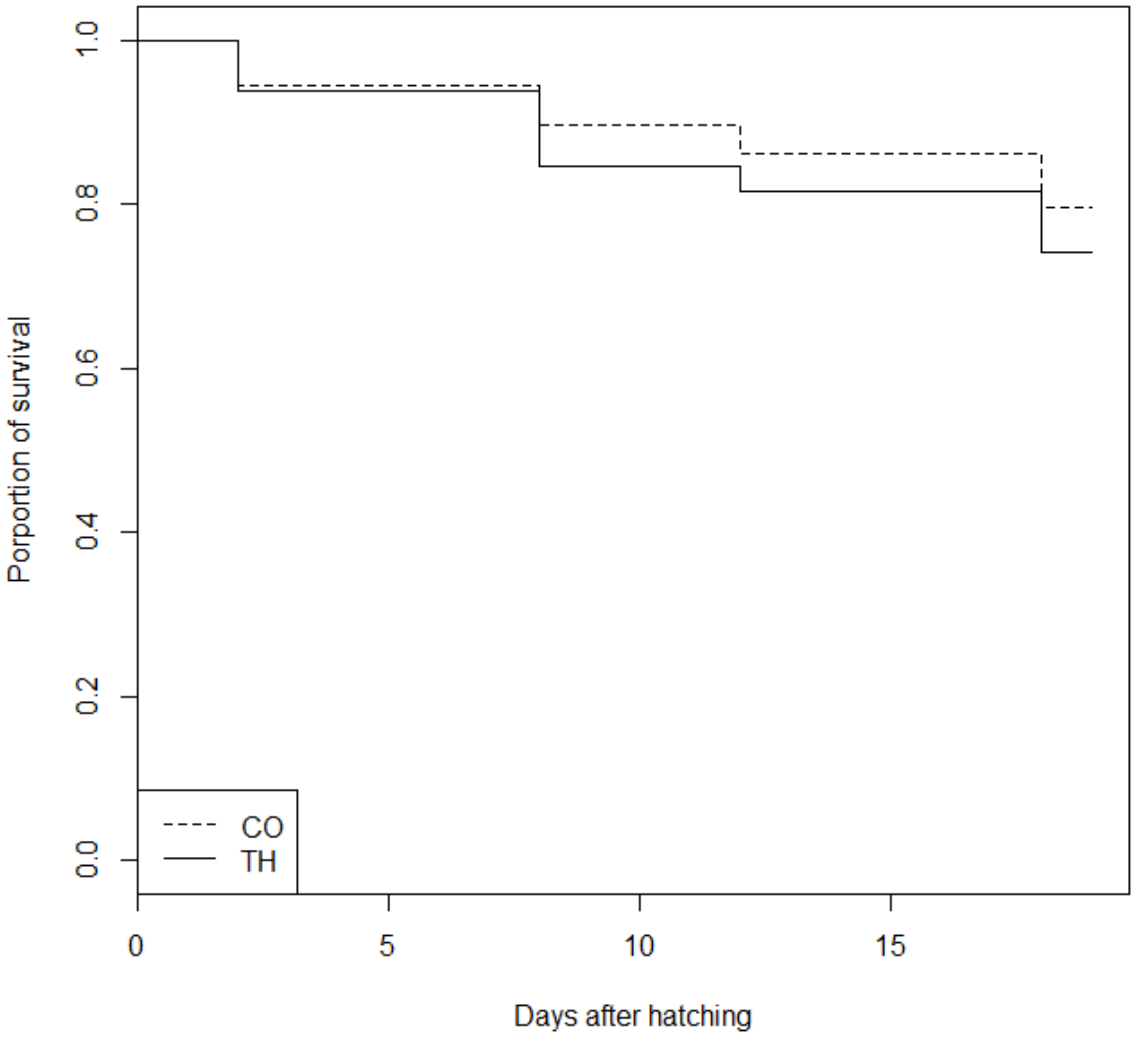
[c] Ruuskanen et al. 2018

Figure 1. Pre-fledging survival of collared flycatcher nestlings. Solid line: TH nestlings (n=364), dashed line: CO nestlings (n=341).

Figure 2. Body mass (mean±SE) of collared flycatcher nestlings at day 2 post-hatching in relation to yolk TH injection treatment and nestling sex. Filled dots: TH nestlings; open dots: CO nestlings. F: female, M: male. *: $p<0.05$, **: $p<0.01$.

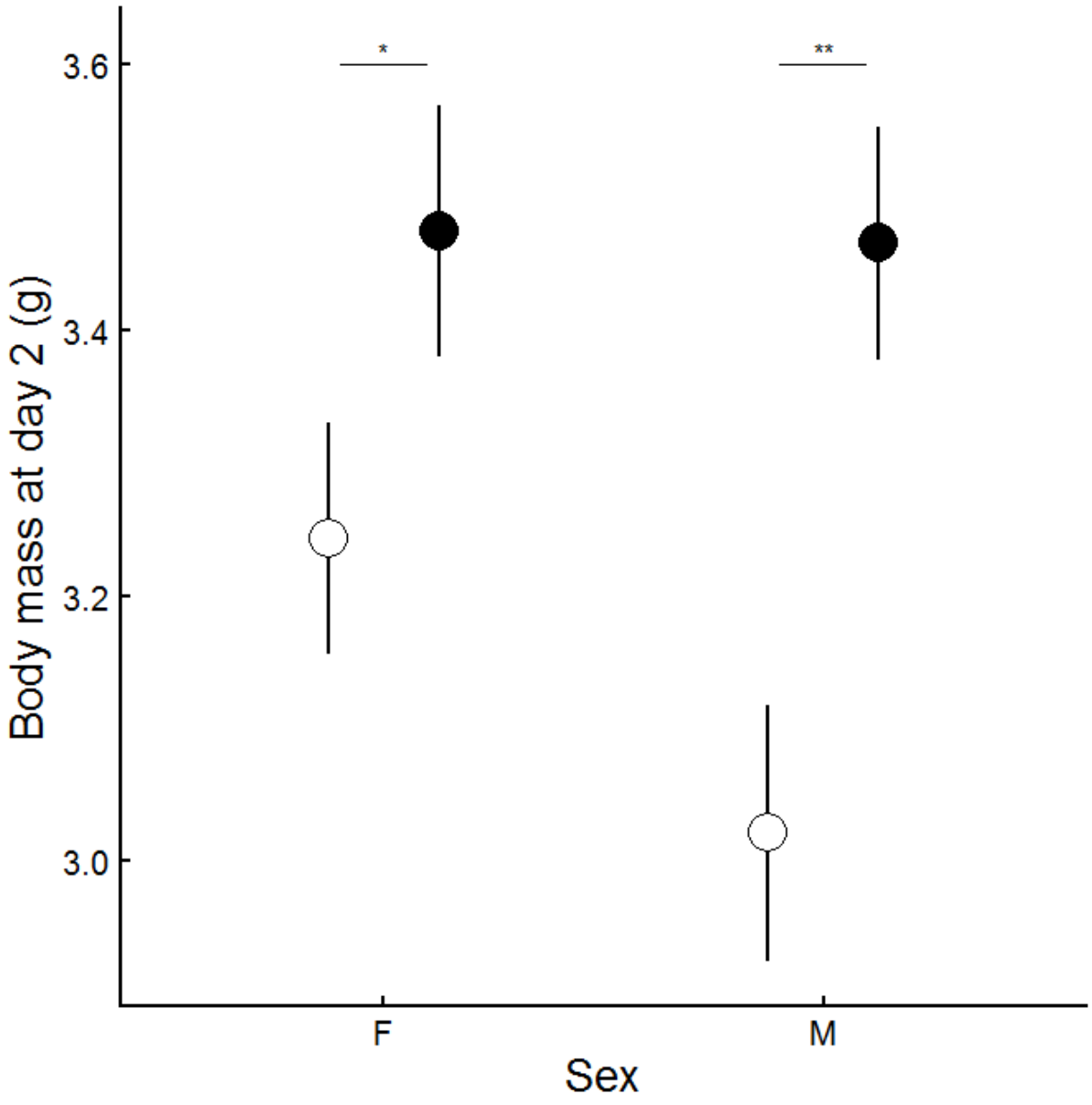
Figure 3. Body mass (A) and tarsus length (B) (mean±SE) of collared flycatcher nestlings at day 8 and 12 post-hatching for the two experimental treatments. Filled dots and solid line: TH nestlings; open dots and dashed line: CO nestlings.

497 **Figure 1.**



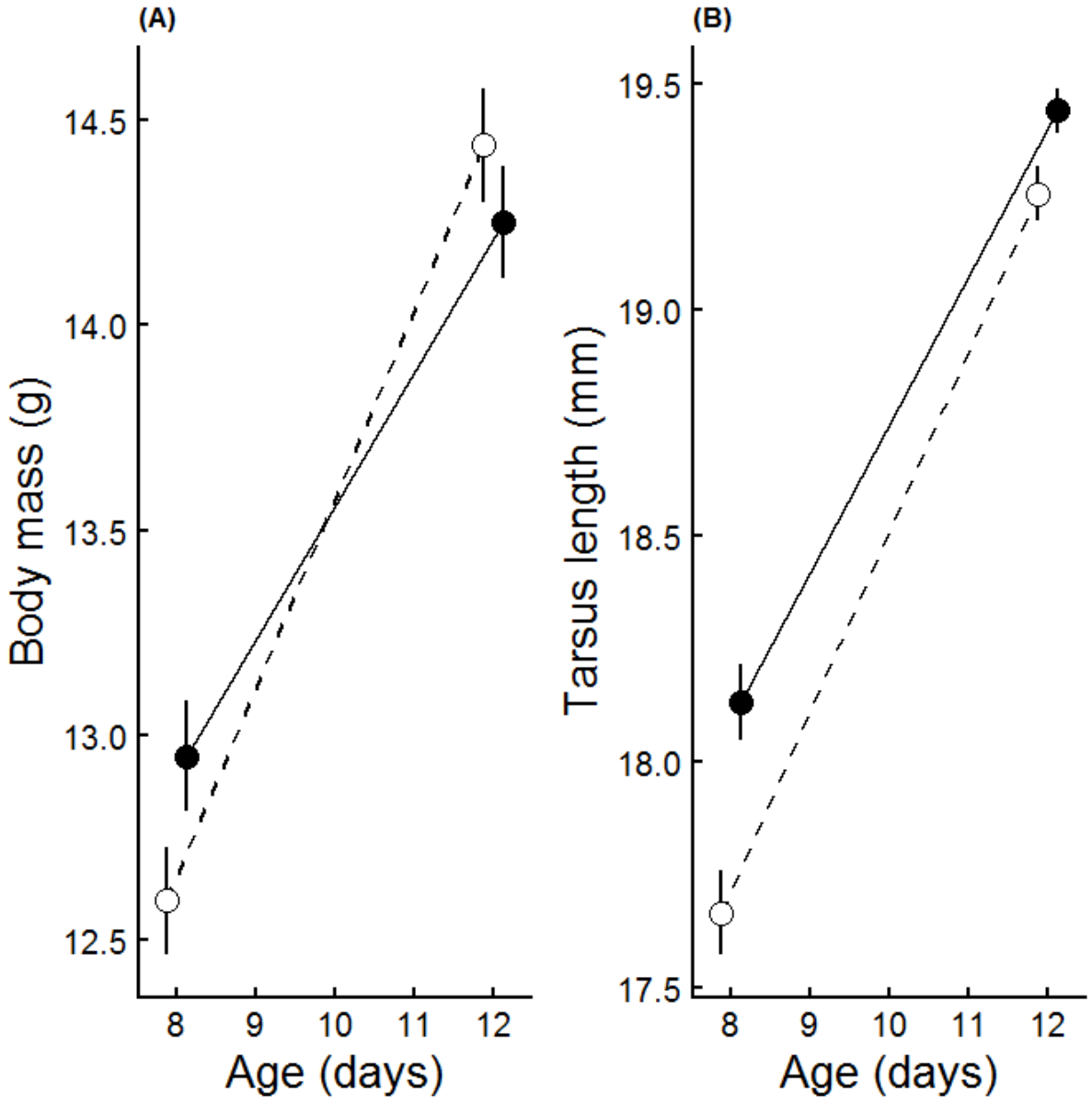
498

499 **Figure 2.**



500

501 **Figure 3.**



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