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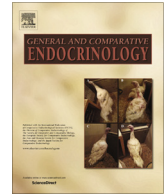
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Research paper

Maternal thyroid hormones enhance hatching success but decrease nestling body mass in the rock pigeon (*Columba livia*)Bin-Yan Hsu^{a,*}, Cor Dijkstra^a, Veerle M. Darras^b, Bonnie de Vries^a, Ton G.G. Groothuis^a^aGroningen Institute for Evolutionary Life Sciences, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands^bLaboratory of Comparative Endocrinology, Section of Animal Physiology and Neurobiology, KU Leuven, Naamsestraat 61, B-3000 Leuven, Belgium

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ABSTRACT

Thyroid hormones (THs) – triiodothyronine (T3) and thyroxine (T4) – are essential for embryonic development in vertebrates. All vertebrate embryos are exposed to THs from maternal origin. As maternal TH levels are known to be essential to embryonic development, the natural variation of maternal THs probably represents a pathway of maternal effects that can modify offspring phenotype. However, potential fitness consequences of variation of maternal TH exposure within the normal physiological range and without confounding effects of the mother have never been experimentally investigated. We experimentally manipulated the levels of yolk T3 and T4 within the physiological range in a species in which the embryo develops outside the mother's body, the Rock Pigeon (*Columba livia*) eggs. Making use of the natural difference of yolk testosterone between the two eggs of pigeon clutches, we were also able to investigate the potential interaction between THs and testosterone. Elevated yolk TH levels enhanced embryonic development and hatching success, and reduced body mass but not tarsus length between day 14 and fledging. The yolk hormones increased plasma T4 concentrations in females but reduced it in males, in line with the effect on metabolic rate at hatching. Plasma concentrations of T3 and testosterone were not significantly affected. The effects of treatment did not differ between eggs with high or low testosterone levels. Our data indicate that natural variation in maternal yolk TH levels affects offspring phenotype and embryonic survival, potentially influencing maternal and chick fitness.

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1. Introduction

In recent decades, maternal effects have been widely recognised as one of the major inducers of adaptive phenotypic plasticity (Mousseau and Fox, 1998). Prenatal maternal effects, although often overlooked, are especially important, since they act on the embryo when it is especially sensitive to the early organizing effects of environmental cues. Maternal hormones are excellent tools for the mother to translate environmental information to her embryo (anticipatory maternal effects, Marshall and Uller, 2007), as her own hormone production is affected by the environment and maternal hormones can reach the embryo in many taxa, from insects to humans. (e.g. mammals: Dloniak et al., 2006; Helle et al., 2013; birds: Gil, 2008; Groothuis et al., 2005; von Engelhardt and Groothuis, 2011; reptiles: Uller and Olsson, 2006; Warner et al., 2009; fish: Brown et al., 2014; McCormick, 1998, 1999; insects, Mousseau and Dingle, 1991; Sonobe and Yamada, 2004).

Birds have been the most widely used model for the study of maternal hormones because they have relatively large eggs and their embryos develop outside their mothers' body, facilitating experimental research. Almost all these studies have focused on androgens. However, thyroid hormones (THs) of maternal origin are known to be present in avian egg yolks already around 1990 (Sechman and Bobek, 1988; Prati et al., 1992). There are at least four reasons for the importance of yolk thyroid hormones as maternal signals. First, THs are indispensable for normal embryonic development, growth and metabolism (McNabb et al., 1998; van Herck et al., 2013). Second, essential proteins involved in cellular function of THs, like TH-receptors, TH-transporters, and deiodinating enzymes, are already expressed long before the embryonic thyroid gland becomes functional (Darras et al., 2011; Flamant and Samarut, 1998; van Herck et al., 2012), suggesting responsiveness to maternal THs in early development. Third, accumulating evidence indicates that the hypothalamus-pituitary-thyroid (HPT) axis can regulate the function of the hypothalamus-pituitary-gonad (HPG) axis (Duarte-Guterman et al., 2014; Flood et al., 2013; Sechman, 2013), suggesting that androgen and TH mediated maternal effects should not be studied in isolation from

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each other. Fourth, unlike steroids, which are made of an abundant precursor, cholesterol, the production of THs requires an essential component, iodine, that cannot be synthesized *de novo* but has to be taken up from the environment. Therefore, unlike androgens, THs may be a costly investment resulting in an evolutionary trade-off for the mother between allocating the hormones to self or to her offspring. Indeed we recently found evidence for regulation of yolk TH deposition independent from the TH levels in maternal circulation depending on food conditions (Hsu et al., 2016). The variation in yolk TH deposition thus probably represents an important mediator of maternal effects that is still unexplored.

The hormone L-thyroxine (T4) is produced by the thyroid gland, transported to target tissue by the circulation where it is converted to its biologically active metabolite 3,5,3'-triiodothyronine (T3). This hormone affects several metabolic processes, growth, differentiation, brain development and behaviour. Currently, the best knowledge about the potential effects of maternal THs on offspring comes from (bio)medical studies on human mothers affected by hypothyroidism and experiments on rodent models, and aquacultural studies on the development of fish. The human studies indicate that not only maternal hypo- or hyper-thyroxinemia can lead to detrimental effects on foetal development (e.g. Andersen et al., 2013; Morreale de Escobar et al., 2004a,b), but also that normal variation in TH levels in the mother correlates with foetal birth weight (Medici et al., 2013; Shields et al., 2011). In fish, TH manipulation by immersion of eggs generally enhances organ differentiation, embryonic development and larval growth and survival, but high doses sometimes lead to morphological abnormalities (reviewed in Brown et al., 2014). All these studies have suggested essential roles of maternal THs in embryonic development. The actual ecological significance and fitness consequences, however, still require experimental studies manipulating maternal THs within the natural range to verify.

In terrestrial animals, experimental data on the effects of maternal THs on offspring development are very scarce and, although interesting, were based on supra-physiological dosages. In birds, the only available study that manipulated maternal thyroid hormone levels in the egg mimicking elevated maternal deposition was conducted in Japanese quails (*Coturnix japonica*) by oral dosing of the hens with T4, resulting in increased T3 and T4 levels in both maternal blood plasma and egg yolks (Wilson and McNabb, 1997). Embryos from females treated with the highest dose had significantly larger pelvic cartilage weight although embryo plasma THs levels were not significantly higher (Wilson and McNabb, 1997). These authors did not find an effect on hatching success, but in an early study in chickens (*Gallus domesticus*), in ovo injection of T4 or thiourea (an anti-thyroid agent) at the end of embryonic development showed that T4 accelerated hatching while thiourea blocked hatching (Balaban and Hill, 1971).

Recently we found that eggs of the rock pigeon, *Columba livia*, the wild ancestor of domesticated pigeons, contained both T3 and T4 and that these levels varied with food conditions (Hsu et al., 2016), suggesting that yolk TH levels could be a mediator of prenatal maternal effects. In this present study, we tested the effects of elevated prenatal TH exposure within the physiological range by injection in freshly laid eggs, mimicking elevated maternal hormone deposition. In the rock pigeons, the modal clutch size is two. In the previous experiment where we measured TH levels in un-incubated eggs, we documented that androgen levels (testosterone and androstenedione) are much higher in the second laid egg than in the first laid egg (also see Goerlich et al., 2009), whereas the within-clutch variation for T3 or T4 does not show a systematic pattern across the laying order (Hsu et al., 2016, see Supplementary methods for more details). This species therefore provides an excellent model to study yolk THs not only for their

general effects on offspring development, but also under different concentrations of yolk androgens. Based on the available literature on TH effects we analysed the effect of the treatment on timing and success of hatching, metabolic rate at hatching, and growth until fledging. In order to study a possible pathway for the effects, we assessed whether plasma THs and testosterone levels in chicks were modulated by prenatal THs exposure.

2. Materials and methods

2.1. Species and housing

This study was conducted from June to August 2014, in a large pigeon aviary (45 m long * 9.6 m wide * 3.75 m high) at the outdoor animal facility of University of Groningen under *ad lib.* food and water conditions. All pigeons were outbred wild type rock pigeons, descendants of wild caught birds. At the start of the experiment, 146 pigeons (70 females and 76 males) were present. In order to induce breeding, 78 nest-boxes with a nest-bowl and nesting materials were provided. All experimental procedures were approved by the Animal Welfare Committee and the Animal Welfare Body of University of Groningen (DEC No. 5635G).

2.2. Egg collection and experimental procedure

Nest-boxes were checked every morning between 9:00 and 11:00. Any newly-laid egg was marked with a permanent marker, collected and replaced with a dummy egg. Collected eggs were then stored in a climate cell (a chamber where temperature and humidity can be controlled) between 12 and 16 °C and relative humidity ~50% for no longer than 4 days. Once a sufficient number of eggs was collected, we injected half of the eggs with 50 µl TH solution (TH-eggs, the dose was to elevate yolk TH levels by ~2 SD according to our previous data, Hsu et al., 2016, see also Supplementary methods and Fig. S1) and the other half with 50 µl saline (0.9% NaCl; C-eggs) as control. After injections, a pair of TH- and C-eggs matched for laying order, weight and date of laying was returned to the colony but cross-fostered to a genetically-unrelated nest for incubation. As incubation and rearing condition is known to profoundly influence embryo development, cross-fostering allows a more robust design and randomization of uncontrolled correlations between egg and parental qualities.

In total we injected 160 eggs. TH-eggs and C-eggs were not significantly different in egg mass (mean ± SD: TH-eggs, 16.60 ± 1.33 g; C-eggs, 16.61 ± 1.24 g; *t*-test, $t_{157.337} = 0.037$, $P = 0.971$) or storage duration (Mann-Whitney *U* test, $U = 3161$, $P = 0.8925$).

2.3. Measurements

The researchers were kept blind from knowing the egg injection treatment of each egg while taking all measurements.

2.3.1. Hatching time and hatching success

Pigeon eggs take 17–19 days to hatch (Johnston and Janiga, 1995). On day 16 after egg injection, we collected the eggs again (nests were again provided with dummy eggs). Eggs were illuminated with a flashlight to evaluate embryo development. Eggs that were clearly well-developed were put in an incubator at 37.5 °C and >70% relative humidity until they hatched.

The incubator was checked every 4 h from 9:00 to 21:00. The hatching time of a new hatchling was estimated as accurately as possible based on checking time and the dryness of their down feather. After hatching, the chick body mass was measured with a digital scale to the nearest 0.1 g and tarsus length was measured

with a digital calliper to the nearest 0.01 mm, after which the chick was put back in the incubator until the indirect calorimetry (see Section 2.4). After the indirect calorimetry, a drop of blood was taken from the medial metatarsal vein of each chick for molecular sexing. Chicks were then paired by body mass and opposite hormone treatment and then allocated to genetically-unrelated foster nests. Only those chicks that were successfully paired in this manner were included in the growth analysis.

2.3.2. Chick growth

Among the 99 chicks that successfully hatched, we generated 33 experimental pairs that hatched on the same day as another egg of the same laying order, but with opposite injection treatment. These pairs of chicks were included for the analysis of body growth and plasma hormone levels.

Chick growth was regularly followed by measuring their body mass and tarsus length every second day until post-hatching day 14, and every third day from post-hatching day 14 to day 23 or day 26. In our colony, pigeon fledglings are usually able to fly on ~day 25 after hatching. If chicks already flew out of the nest before day 26, we abandoned taking the biometry on day 26. On day 14 we also took a blood sample of ~500 μ l with 1 ml syringes (BD Plastipak™) and 25G needles (100 Sterican®, B.Braun). All syringes were pre-heparinized on the day of blood-sampling and the blood samples were immediately spun down to separate plasma. Plasma samples were then stored in -20°C until hormone assay.

2.4. Indirect calorimetry

To measure the oxygen consumption rate of pigeon chicks within 24 h after hatching, we used a four-channel open-flow system with a modified incubator where chicks were kept at 37.5°C . To induce resting metabolic rate (RMR) the incubator was covered with a thick black cloth to induce darkness in order to reduce chick activity and any potential disturbance. All measurements lasted for 1.5–3 h, depending on how quickly we could obtain a relatively stable period of oxygen consumption rate for at least one hour. For details see [Supplementary methods](#).

2.5. Molecular sexing

Our protocol of molecular sexing was the same as described previously ([Goerlich et al., 2009, 2010](#)).

2.6. Hormone extraction and assay

We used radioimmunoassay to quantify the concentrations of testosterone, and two THs: triiodothyronine (T3) and thyroxine (T4), in the blood plasma of day 14 pigeon chicks.

2.6.1. Testosterone

For testosterone extraction and radioimmunoassay, we followed the same protocol as [Goerlich et al. \(2009\)](#) with slight adjustments according to the amount of plasma and the expected testosterone levels (for details see [Supplementary methods](#)). Testosterone was measured using a commercial RIA kit (TESTO-CT2, Cisbio Bioassays, Codolet, France). Standards were prepared using dilution series from a pre-prepared stock and ranged from 0.08 to 20 ng/ml testosterone. Recoveries were calculated by comparison to non-extracted ^3H -labelled testosterone and averaged 83% (SD = 4.8%). Because we did not have prior knowledge of pigeon plasma testosterone levels at this age but we already have abundant and reliable data on testosterone levels in egg yolks of pigeons, we used ‘pools’ of extracted yolk with known concentrations instead of pools of plasma as external quality controls. The intra-assay CV for testosterone was 2.99%.

2.6.2. Thyroid hormones (T3 and T4)

Total T3 and T4 levels in pigeon chick plasma were measured by standard radioimmunoassay. This includes using antibodies (20-TR40 and 20TR-45) from Fitzgerald Industries International (US-Ireland) and a standard in hormone-free human serum (Byk-Sangtec Diagnostica, Germany) ([van der Geyten et al., 2001](#)). [3^{\prime} - ^{125}I]T3 and [3^{\prime} ,5'- ^{125}I]T4 were prepared using the chloramine-T method according to [Visser et al. \(1977\)](#), using ^{125}I from PerkinElmer (Zaventem, Belgium) and unlabelled 3,5-T2, 3,3',5-T3 from Henning Berlin GmbH (Berlin, Germany). The T3 RIA had a detection limit of 2 fmol and an intra-assay variability of 2.2%. The T4 RIA had a detection limit of 5 fmol and an intra-assay variability of 2.8%. For the T3 RIA cross-reactivity with T4 was 0.1–0.5%, whereas for the T4 RIA cross-reactivity with T3 was 3.5%. All samples were measured within a single assay.

2.7. Statistical analysis

All data were analysed with general and generalized linear mixed model by the package *lme4* ([Bates et al., 2014](#)) in R 3.0.2 ([R Core Team, 2013](#)). Alpha was set at 0.05 and *P* values of linear mixed models were derived from log-likelihood ratio tests (LRT). Model residuals and VIFs (Variance Inflation Factor, using the function *vif.mer*, [HLP/jaeger lab blog, 2011](#)) were all checked for assessing homogeneity, normality and model collinearity. All VIFs were less than 2 and no substantial multi-collinearity occurred. For all models, TH-injection and laying order (representing different testosterone levels) were included as fixed factors as they were the main interests of this study. Sex was also included as a fixed factor in most models, except for the models of embryo development and hatching success because we did not sex unhatched eggs. In all models, two interaction terms (TH-injection by sex and TH-injection by laying order) were tested, and only presented when $P < 0.05$ or for the sake of comparison with other models. When significant interaction effects were found, post hoc interaction contrasts were tested by R package *phia* (Holm-adjusted *P* values were presented, [de Rosario-Martinez, 2015](#)). Egg mass is strongly associated with many traits of chicks ([Krist, 2011](#)) and therefore was included as a covariate. Furthermore, the nest where eggs were laid and the nest where eggs were incubated, were considered as random factors in the models for the parameters before and around hatching (embryo development, hatching success, time to hatch, body mass and tarsus length at hatching). For the parameters measured at or after day 14, only the nest where chicks were raised was included as a random factor. In the model of RMR, we took natural log on both RMR data and chick body mass as metabolic rate has a well-established association with body mass ([Glazier, 2008; Hudson et al., 2013; Nagy et al., 1999](#)). In this model, body mass was of course included as a covariate, and the nests where the eggs were laid and incubated were considered as random factors. Details on the statistical models that were not presented can be found in the [Supplementary materials](#).

In some models strong correlations between model residuals and fitted values were detected, likely resulting from the shrinkage effects of mixed models. In these cases, parametric bootstrapping model comparisons with 1000 times simulation (package *pbkrtest*, [Halekoh and Højsgaard, 2014](#)) were run to confirm whether the LRT *P* values were biased. In all such cases, LRT and parametric bootstrapping model comparisons gave very similar results, and LRT *P* values were therefore presented.

In most variables, there was at least one outlier (see [Supplementary methods and Fig. S2](#)). These outliers did not always belong to the same individuals and the real cause was very difficult to identify. To avoid influential points that may have unbalanced effects, we therefore ran the statistical models twice: including

and excluding the outliers, and report the results of both in the text.

3. Results

3.1. Hatching success and hatching time

At incubation day 16, TH-eggs had a significantly higher proportion of developing embryos (C-eggs 63.75%, TH-eggs 77.5%, $P = 0.041$). TH-eggs also had a significantly higher hatching success (C-eggs 52.5% and TH-eggs 71.25%, $P = 0.019$, for model details see Table S1). As for hatching time, in the model where the outliers were removed, TH-injection did not have a significant overall effect, nor did egg laying order or the interaction between TH-injection and egg laying order ($P > 0.14$, for details see Table S2). When including the outliers, there was a significant interaction of treatment by laying order ($P = 0.046$, Table S2, Fig. S3). Post-hoc interaction analysis indicated that TH-injection accelerated hatching only on second laid eggs (Holm-adjusted $P = 0.020$), but not on first laid eggs (Holm-adjusted $P = 0.952$). Irrespective of whether the outliers were excluded or not, second laid eggs required on average about 0.8 days less to hatch than first laid eggs ($P < 0.001$, Table S2, Fig. S3).

3.2. Chick body mass, tarsus length, and metabolic rate at hatching

On the day of hatching, none of the predictors in the model (TH-injection, laying order, sex and their interactions) yielded significant effects on chick body mass or tarsus length, when the outliers were excluded (all $P > 0.1$, Table S3). Including the outliers led to a significant but unexpected sex difference such that females had longer tarsi than males on the hatching day ($P = 0.017$, Table S3). For RMR, in the model excluding the outliers, we found a significant interaction between TH-injection and chick sex (estimate \pm SE = -0.078 ± 0.030 , $t = -2.587$, $P = 0.009$), suggesting that TH-injection raised RMR in female chicks but reduced it in male chicks (Fig. S4). This interaction was only approaching significance if the outliers were included (Table S3). The main effects of TH-injection, egg laying order and chick sex, were all non-significant, irrespective of whether the outliers were excluded or included.

3.3. Plasma hormone levels at day 14

Regardless of the inclusion or exclusion of the outliers, neither the in ovo TH-injection nor the egg laying order had significant effects on plasma T3 or T4 levels ($P > 0.2$). However, in both cases there was a significant interaction between TH-injection and sex for plasma T4 levels ($P < 0.01$, Table S4), but not for plasma T3 levels (Fig. 1A). This indicates that TH-females had higher plasma T4 than C-females ($\chi^2_1 = 8.021$, Holm-adjusted $P = 0.009$, Fig. 1B), while TH-males tended to have lower plasma T4 than C-males ($\chi^2_1 = 3.331$, Holm-adjusted $P = 0.068$, Fig. 1B), similar to the above-mentioned effect on metabolic rates. The interaction between TH-injection and egg laying order only showed a significant effect on plasma T3 levels when the outliers were removed ($P = 0.018$, Table S4), suggesting that TH-injection reduced plasma T3 levels in chicks from first laid eggs but increased it in chicks from second laid eggs (Fig. S5).

For plasma testosterone, regardless of the inclusion or exclusion of the three outliers, the treatment of TH-injection did not show significant effects, nor its interaction by sex or by egg laying order ($P > 0.08$, Table S4). However, only in the model where the outliers were excluded, the effect of laying order was significant, indicating chicks from second laid eggs had significantly lower plasma testosterone levels (estimate \pm SE = -0.024 ± 0.01 , $t = -2.315$, $P = 0.020$).

3.4. Chick body mass and tarsus length at day 14 and day 23

At day 14 post-hatching, TH-injection significantly reduced chick body mass ($P = 0.028$, Table 1, Fig. 2A), but not tarsus length ($P = 0.524$, Table 1, Fig. 2B), when the outliers were removed from the model. In the same model, males also showed significant larger body mass ($P = 0.003$) and longer tarsi ($P = 0.004$), reflecting the sexual dimorphism in this species. In contrast, when the outliers were included, not only the effect of TH-injection became non-significant ($P = 0.366$), but also the sex effect on body mass ($P = 0.117$). The effect of egg laying order was not significant ($P > 0.2$) no matter the outliers were included or not.

In order to test whether any of the three plasma hormones sampled at this day predicted chick body mass and tarsus length, we added these data into the model. When all outliers were excluded, none of the three hormones significantly associated with chick body mass ($P > 0.27$), but plasma T3 showed a negative association (estimate \pm SE = -0.717 ± 0.194 , $t = -3.689$, $P = 0.001$, Fig. S6) and

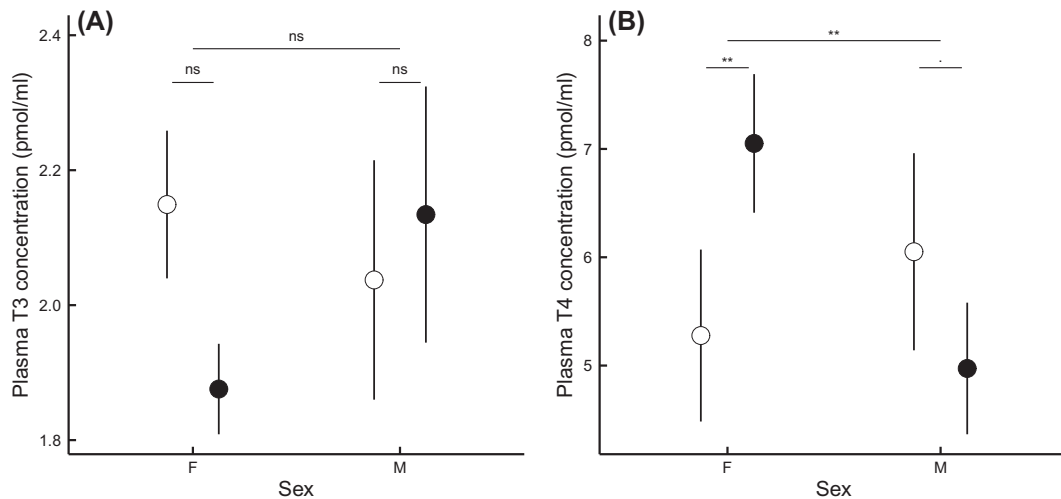


Fig. 1. Mean \pm SE of plasma T3 (A) and T4 (B) levels across sexes between in ovo TH-injection treatments at post-hatching day 14 (outliers excluded). In ovo TH-injection did not significantly influence chick plasma T3 levels (A) while it showed sex-specific effects plasma T4 (B). Filled circle: TH-chicks; open circle: C-chicks. ($^*P < 0.01$; $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.1$; ns, $P > 0.1$).

Table 1
Linear mixed models of chick body mass and tarsus length at day 14.

	Estimates	SE	<i>t</i>	<i>P</i> ^a	Estimates	SE	<i>t</i>	<i>P</i> ^a
Body mass at day 14		(outliers included, <i>N</i> = 66)				(outliers included, <i>N</i> = 62)		
TH injection	−4.925	5.566	−0.885	0.366	−10.078	4.592	−2.195	0.028
Laying order (2nd laid eggs)	2.993	7.085	0.422	0.658	−4.087	5.149	−0.794	0.401
Sex (male)	9.348	6.162	1.517	0.117	14.294	4.829	2.960	0.003
Egg mass	−0.426	3.083	−0.138	0.884	0.616	2.306	0.267	0.821
Tarsus length at day 14		(outliers included, <i>N</i> = 66)				(outliers included, <i>N</i> = 63)		
TH injection	<0.000	0.263	0.000	>0.999	−0.149	0.249	−0.598	0.524
Laying order (2nd laid eggs)	−0.101	0.430	−0.235	0.805	−0.357	0.305	−1.169	0.224
Sex (male)	0.790	0.318	2.489	0.012	0.787	0.275	2.863	0.004
Egg mass	−0.030	0.172	−1.175	0.848	0.108	0.137	0.791	0.414

The nest where chicks were raised was considered as the random factor.

^a *P* values were derived from log-likelihood ratio test.

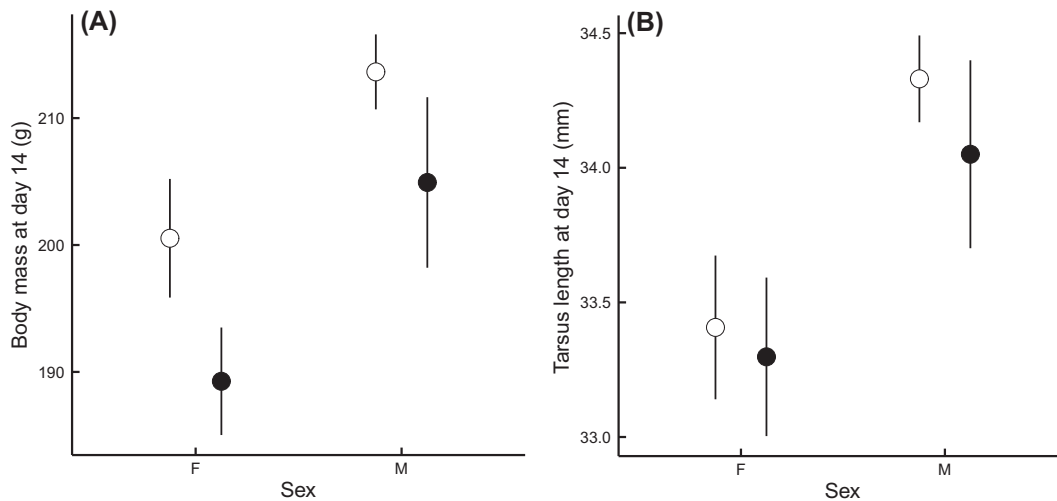


Fig. 2. Mean \pm SE of body mass (A) and tarsus length (B) at post-hatching day 14 (outliers excluded). In ovo TH-injection significantly decreased chick body mass, irrespective of sex ($P = 0.028$, $N = 62$) but did not significantly influence tarsus length ($P = 0.524$, $N = 63$). Filled circle: TH-chicks; open circle: C-chicks.

testosterone a positive association (estimate \pm SE 14.821 ± 2.699 , $t = 5.492$, $P < 0.001$, Fig. S6) with chick tarsus length. When the outliers were included, plasma T3 and testosterone did not significantly predict chick tarsus length, nor did plasma T4 ($P > 0.1$). Unexpectedly, in this model plasma T3 showed a positive associa-

tion with chicks' body mass (estimate \pm SE = 11.572 ± 4.991 , $t = 2.319$, $P = 0.02$).

At day 23 post-hatching, a couple of days before leaving the nest, TH-chicks were still significantly lighter than C-chicks ($P = 0.001$, Fig. 3A, Table 2), but the tarsus length of TH-chicks

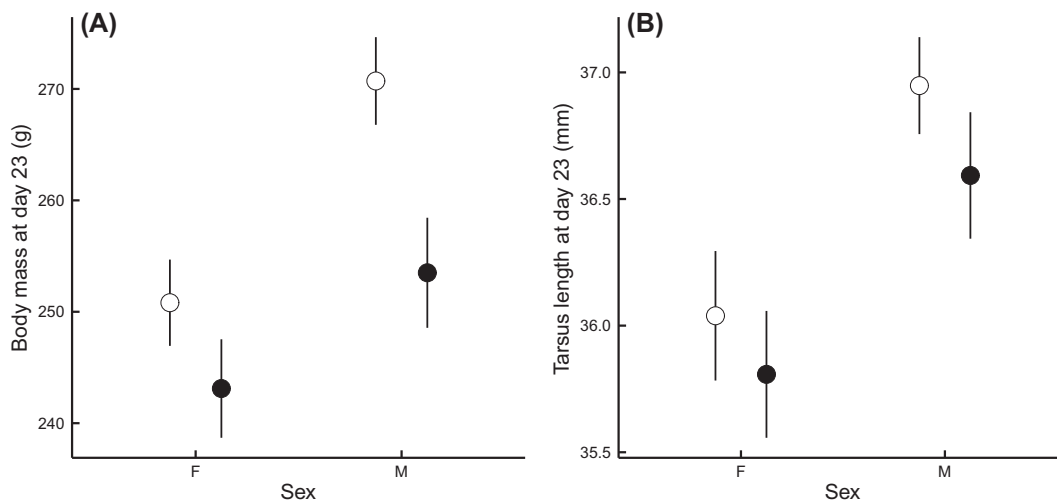


Fig. 3. Mean \pm SE of body mass (A) and tarsus length (B) at post-hatching day 23 (outliers excluded). In ovo TH-injection significantly decreased chick body mass, irrespective of sex ($P = 0.001$, $N = 62$) but did not significantly influence tarsus length ($P = 0.340$, $N = 61$). Filled circle: TH-chicks; open circle: C-chicks.

Table 2

Linear mixed models of chick body mass and tarsus length at day 23.

	Estimates	SE	t	P ^a	Estimates	SE	t	P ^a
Body mass at day 23								
		(outliers included, N = 65 ^b)				(outliers included, N = 62)		
TH injection	-10.157	4.152	-2.446	0.016	-11.270	3.333	-3.381	0.001
Laying order (2nd laid eggs)	-4.929	6.027	-0.818	0.393	-8.557	5.011	-1.708	0.080
Sex (male)	15.811	4.865	3.250	0.001	13.083	4.043	3.236	0.001
Egg mass	-0.492	2.519	-0.196	0.841	2.352	2.100	1.120	0.244
Tarsus length at day 23								
		(outliers included, N = 65 ^b)				(outliers included, N = 61)		
TH injection	-0.088	0.231	-0.381	0.692	-0.157	0.171	-0.917	0.340
Laying order (2nd laid eggs)	-0.459	0.354	-1.297	0.180	-0.400	0.310	-1.289	0.188
Sex (male)	1.011	0.276	3.666	<0.001	0.649	0.225	2.889	0.004
Egg mass	-0.046	0.145	-0.321	0.732	-0.041	0.122	-0.338	0.719

The nest where chicks were raised was considered as the random factor.

^a P values were derived from log-likelihood ratio test.^b N = 65 because one chick died at day 20 and was thus removed.

was not significantly different from that of C-chicks ($P = 0.340$, Fig. 3B, Table 2), when excluding the outliers. This result remained the same even when the outliers were included ($P = 0.016$ and 0.692 , respectively, Table 2). Interactions between TH-injection and laying order and between TH-injection and sex were not significant ($P > 0.34$). At this age, no matter the outliers were included or excluded, males were significantly larger than females, both in body mass ($P = 0.001$) and tarsus length ($P = 0.004$, Table 2).

At this age, when all outliers were excluded, the levels of the three plasma hormones sampled 10 days earlier still did not correlate with body mass, similar to what we found at day 14. Tarsus length at day 23, which showed a significant correlation with plasma T3 and testosterone at day 14, still showed an almost significant correlation with plasma T3 concentrations ($P = 0.054$) while the concentrations of plasma T4 and testosterone did not correlate with tarsus length at this age ($P = 0.356$ and 0.644 , respectively). When all outliers were included, none of these plasma hormones showed any significant correlations with body mass and tarsus length (all $P > 0.12$, data not shown).

3.5. Survival

Out of the 66 chicks, only 1 chick died before fledging. After fledging, 5 chicks died before the winter time. Among these 6 chicks, 4 were from TH-eggs and 2 from a C-egg. These mortality rate were not significantly different (Chi-square test with Yates' continuity correction, $\chi^2_1 = 0.183$, $P = 0.669$). In our housing condition, there was no recorded death during the winter time.

4. Discussion

This study, to our knowledge, is the first study (together with a complementary study on great tits: Ruuskanen et al., 2016) experimentally investigating the effects of prenatal exposure to maternal thyroid hormones within the physiological range on offspring development. We adopted in ovo hormone injection so that the manipulation would not interfere with the mother and we could avoid potentially confounding effects from changed maternal physiology. We found that TH-injection in ovo affected fitness relevant traits: enhancing hatching success, reducing chick body mass from the second week after hatching till around the time of fledging, and affecting metabolic rate and plasma TH concentration in a sex-specific way. Most of the key results did not change by the inclusion or exclusion of the few outliers in our results and thus are considered robust. In most other cases, the models including the few outliers resulted in inconsistent results, for example, the significant longer tarsi of females at hatching irrespective of treatment (see Section 3 and Table S3), which contradicts the well-known sexual dimorphism in this species with males being larger

(Johnston and Janiga, 1995). Including the outliers also yielded in the inconsistency between the results on hormone concentrations and metabolic rate. Although all results are explicitly presented in this paper, for sake of conciseness and consistency our discussion is based on the results from the models without the few outliers as these seem to have in some cases a disturbing and unbalanced effect on the analyses.

The increase in hatching success by the physiologically-relevant elevation of yolk TH levels is consistent with the essential role of THs in embryonic development (birds: McNabb et al., 1998; fish: Brown et al., 2014). Our finding that TH treatment increased the proportion of eggs with well-developed embryos two days before the expected hatching day suggests that higher prenatal TH exposure may help counteract some hurdles in embryonic development. Beneficial effects on the embryonic metabolism, efficiency of glucose utilization, and tissue differentiation are possible but not exclusive explanations. For example, studies in commercial strains of turkeys showed that supplementation of iodine, a critical and perhaps limiting component of THs (Hsu et al., 2016), in the maternal diet increased egg hatchability (Christensen and Davis, 2001; Christensen and Donaldson, 1994), and increased embryonic gluconeogenesis that helped maintain blood glucose concentrations (Christensen and Donaldson, 1994). However, in Japanese quails, orally T4-dosed females laid eggs containing high yolk T3 and T4 levels but those eggs did not have higher hatching success than controls (Wilson and McNabb, 1997). But in that study, the hens were made hyperthyroid (Wilson and McNabb, 1997). This might have deviated yolk TH levels from the normal physiological range and altered the physiological state in females, which may have affected other egg substances, too. However, in the complementary study in the great tits (*Parus major*), enhanced hatching success was not observed either (Ruuskanen et al., 2016). Clearly, similar yolk THs manipulating studies should be conducted in more species to investigate general principles and potential species difference on hatching success induced by elevated maternal yolk THs.

Although TH-injection enhanced the proportion of well-developed embryos and hatching success, we did not find effects on body mass or tarsus length in the hatchlings. One possible explanation could be that elevated yolk THs have helped low-quality chicks, that should have failed to hatch otherwise, successfully emerge out of the eggs, which may have masked differences between the experimental and control groups. This could also explain the lower body mass of TH-chicks since day 14 after hatching. However, if this was the case, one would also expect lower tarsus length or lower chick survival, neither of which was observed. Alternatively, prenatal THs within the physiological range may function in a subtle way that only manifests itself in a later stage of development due to an organizational influence with lasting

effects on metabolic or endocrine function. As our data showed that in ovo TH-injection resulted in a lower body mass at day 14 as well as around the time of fledging and no difference in survival between TH- and C-chicks was observed, the latter seems more possible, and further studies examining metabolic rates and endocrine function in a later stage should be able to shed more light on the possible mechanisms.

According to our results, modified T4 production by the chicks is probably not the direct cause of this lower body mass, because the effect of treatment on plasma T4 levels was sex-specific (increased in females but decreased in males, Fig. 1), in contrast to the effect on body mass (Figs. 2 and 3). Due to the limited amount of blood plasma, we could not measure corticosterone and growth hormone levels. These hormones might mediate the effects of prenatal treatment given their demonstrated relationship with THs (Brown et al., 2014; Cogburn et al., 2000; McNabb et al., 1998). Our finding that only body mass was affected by the treatment and tarsus length was not (Figs. 2 and 3) is consistent with the synergistic effect between growth hormone and T3 on the reduction of the deposition of body fat by altering hepatic gene expression (Cogburn et al., 2000; Wang et al., 2007). Also, given the role of THs on metabolism, the treatment could have elevated metabolic rate, resulting in higher energy expenditure at the cost of lower body mass. Intriguingly, when we removed the outliers, the metabolic rates of the nestlings were affected by TH-injection in a sex-specific manner, increased in females while decreased in males, consistent with the effect on plasma concentration of T4 at day 14. Albeit not significant, plasma T3 also showed an opposite pattern from plasma T4, suggesting that early exposure to THs probably affect the sex-specific conversion by deiodinase from T4 to T3 (e.g. Chan et al., 2005), although the actual mechanism is still unclear. These results seem to provide a link between metabolism and endogenous T4 production or T3 to T4 conversion, but neither of them could support the sex-independent effects of TH-injection on chick body mass. In order not to disturb parental care, we did not measure metabolic rate at later ages when an effect on body mass became apparent. Therefore, the possible mechanisms underlying the sex-independent effects on body mass and the sex-specific effects on plasma T4 require further study.

In conclusion, our data indicate that natural variation of concentrations of maternally-derived THs in bird eggs can have clear and in some aspects sex-specific effects that are biologically relevant. The effect on embryo survival and hatching is clearly fitness enhancing whereas the negative effect on nestling body mass might be detrimental. This may pose a trade-off for the mother in how much thyroid hormone to allocate. Indeed, in an earlier study we found that T3 concentrations in the egg were higher in a poor food condition (Hsu et al., 2016). Poor food can lead to lower-quality eggs with less nutrients (e.g. Williams, 1996; for our species: Hsu et al., 2016) and thus lower hatching success (Krist, 2011; Williams, 2012), so that especially in that case boosting hatching success by elevated allocation of T3 at the cost of lower fledging weight would be evolutionarily favourable since by that time the juvenile offspring is less vulnerable. Although our study was conducted on *ad lib.* food conditions, perhaps masking potential effects on survival and hampering the translation of the results to the field situation, the fact that chick body mass was still affected by the treatment around fledging does show that *ad lib.* food is not necessarily overruling the effects of maternal THs on offspring phenotype. Nevertheless, whether the adjustment of yolk THs deposition is an adaptive maternal effect preparing offspring for the upcoming environmental context can be tested by applying parental food restriction after the hatching of chicks and following up this study with manipulations in the field. Given the potential effects of THs on energy utilization, underlying

mechanisms and the ultimate fitness consequences all need further studies.

Authors' contributions

B.Y.H., C.D., and T.G.G.G. designed the experiment. B.Y.H. conducted the research and collected data. B.Y.H., V.M.D., and B.D.V. carried out the lab work. B.Y.H. and T.G.G.G. carried out the statistical analysis. B.Y.H. and T.G.G.G. mostly drafted the manuscript. T. G.G.G. coordinated and supervised the study. All authors approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2016.10.011>.

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