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Effects of experimentally manipulated yolk thyroid hormone levels on offspring development in a wild bird species

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ABSTRACT

Maternal effects are a crucial mechanism in a wide array of taxa to generate phenotypic variation, thereby affecting offspring development and fitness. Maternally derived thyroid hormones (THs) are known to be essential for offspring development in mammalian and fish models, but have been largely neglected in avian studies, especially in respect to natural variation and an ecological context. We studied, for the first time in a wild species and population, the effects of maternally derived THs on offspring development, behavior, physiology and fitness-related traits by experimental elevation of thyroxine and triiodothyronine in ovo within the physiological range in great tits (*Parus major*). We found that elevated yolk TH levels had a sex-specific effect on growth, increasing male and decreasing female growth, relative to controls, and this effect was similar throughout the nestling period. Hatching or fledging success, motor coordination behavior, stress reactivity and resting metabolic rate were not affected by the TH treatment. We conclude that natural variation in maternally derived THs may affect some offspring traits in a wild species. As this is the first study on yolk thyroid hormones in a wild species and population, more such studies are needed to investigate its effects on pre-hatching development, and juvenile and adult fitness before generalizations on the importance of maternally derived yolk thyroid hormones can be made. However, this opens a new, interesting avenue for further research in the field of hormone mediated maternal effects.

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1. Introduction

Maternal effects are a crucial mechanism in a wide array of vertebrate taxa to generate phenotypic variation, potentially adapting offspring to their expected environment (Mousseau and Fox, 1998). Especially prenatal maternal effects, such as maternal effects mediated via prenatal exposure to hormones, are important as there can be organizing effects on the developing embryo (see McCormick, 1999; Uller and Olsson, 2006; von Engelhardt and Groothuis, 2011; Helle et al., 2013). Oviparous species, such as birds, are good model species for studying prenatal maternal effects, as eggs facilitate manipulation of maternal resources and signals. So far, the literature on maternal hormones in bird eggs, especially in non-domesticated bird species, has almost solely focused on steroid hormones: There is extensive data on their effects (within physiological range) on development, phenotype, behavior and even fitness, as well as variation among and within clutches due to environmental factors (reviewed by Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). However, from poultry

(reviewed by McNabb, 2007), and recently also from few wild species (great tit, Ruuskanen et al., 2015), it is known that bird eggs also contain the thyroid hormones (THs), L-thyroxine (T4) and 3,5,3'-triiodothyronine (T3, the biologically more active form). Interestingly, our recent results show that in wild populations of great tits (*Parus major*), there is variation in yolk TH levels among females, in relation key environmental conditions, temperature and timing of breeding (Ruuskanen et al. unpubl), and yolk TH levels increase over the egg laying sequence within clutches (Ruuskanen et al., 2015). If this variation in yolk THs within physiological range is associated with offspring fitness-related traits, it opens up the possibility for an adaptive maternal effect mediated via maternal deposition of thyroid hormones in wild populations. Nevertheless, knowledge of the importance and function of maternally derived THs in birds is limited, and data on the significance of natural variation of yolk TH levels in ecological or evolutionary contexts is lacking in wild bird species.

There are several reasons to expect maternal THs to play an important role in offspring development in avian species: Exposure to THs is indispensable for normal development and metabolism in vertebrates (Norris and Carr, 2013), including birds (McNabb and Wilson, 1997; Decuyper et al., 2005; McNabb, 2007; McNabb and Darras, 2014), as they are needed for differentiation and maturation of the central nervous system, muscle, skeletal, skin/feather, gut and lung tissues, and

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are important in the hatching process (McNabb and Wilson, 1997; McNabb, 2007). In adult birds THs play multiple important roles in the initiation of gonadal maturation, egg-laying, molt, and multiple aspects of thermogenesis and metabolism, including metabolic rates (McNabb, 2007; McNabb and Darras, 2014). Furthermore, (bio)medical research in humans and rodent models suggests that exposure to *maternal* THs in early foetal stages is crucial for normal growth and development, especially neural development, as hypothyroid conditions lead to severe pathological consequences on brain and behavior (e.g. Zoeller et al., 2007; Patel et al., 2011). Also in fish eggs, maternal THs play an important role in early development (hatching, growth, pigmentation) and even survival (Power et al., 2001; Campinho et al., 2014). However, data on the effects of THs within the natural variation is scarce. Nevertheless, extensive data from other egg hormones, such as steroids, suggests that variation within physiological range can affect multiple offspring traits (von Engelhardt and Groothuis, 2011). Importantly, there is good evidence that also the early avian embryo is sensitive to maternal THs: essential components for TH action (transporters, deiodinase enzymes and receptors) are present in the avian (chicken) embryo from a very early age, before the development of its own TH production, and expression of these proteins changes in response to TH administration already during the first days of embryonic development (Flamant and Samarut, 1998; Darras et al., 2009; Geysens et al., 2012; Van Herck et al., 2013, 2012). This opens the intriguing possibility that maternally derived yolk THs could substantially affect embryonic development (and have potential long-lasting effects) also in birds. To our knowledge, in the only experimental study where yolk THs were artificially elevated prior to incubation (via dosing females with T4 and T3), supraphysiological doses led to increased cartilage growth and differentiation in quails (Wilson and McNabb, 1997). However, to understand the ecological and evolutionary significance of maternal THs in the egg and their adaptive potential, the potential effects of natural variation within the physiological range in yolk THs on fitness-related traits in wild populations need to be addressed.

We studied the effects of yolk THs on offspring development, physiology, behavior and fitness-related traits by experimentally manipulating yolk TH (T4 and T3) levels within the physiological range in a field population of great tits, and cross-fostered chicks to create a within-clutch experimental design. To our knowledge, this is the first such study in a wild species in a field population – where the fitness-related effects can be investigated. We measured various traits, all of which have been previously found to be affected by THs (either maternal or produced by the embryo/nestling itself) in birds or other taxa. The measured traits are important in a wild population, as they are likely to be related to nestling pre- or post-fledging survival. We measured: 1) Hatching success: embryonic thyroid hormones stimulate a variety of metabolic and developmental processes necessary for successful hatching process, and exogenous T4 close to hatching increases hatching success (Christensen, 1985; McNabb, 2007), thus likely maternal THs can also play a role; 2) Growth: embryo circulating THs are known to be positively associated with growth pre- and post-hatching (but severe hypo- and hyperthyroidism may decrease growth) (King and May, 1984; McNabb and Wilson, 1997; McNabb, 2007). We measured both skeletal size and body mass as THs are found to be important for both skeletal and muscle growth in many taxa. Furthermore, in precocial quails, also maternal THs were associated with increased embryonic skeletal growth (Wilson and McNabb, 1997). Importantly, offspring size is related to post-fledging survival, thus offspring growth is directly related to fitness; 3) Metabolic rates: In adult birds, higher circulating TH levels are associated with higher metabolic rates (Bishop et al., 1995; Welcker et al., 2013), thus we hypothesize that maternal THs could also affect offspring metabolic rates. Increased nestling metabolic rates may be related to increased energy consumption, and, especially in poor rearing conditions, this may cause a fitness cost in wild populations; 4) Stress reactivity: Maternal hyperthyroidism has been found to impair stress coping in e.g. rat offspring (Zhang et al., 2008).

Individuals in wild populations are likely to encounter many types of stressors (abiotic, resource-based, inter/intraspecific), and coping with these challenges is likely related to their fitness; 5) Motor coordination: Establishment of brain architecture during central nervous system development in vertebrates (including birds) is critically affected by THs and several studies in mammals point to the effect of maternal THs on behavior (Rainwater et al., 2008; Patel et al., 2011). Maternal THs have been found to play a role in the early stages of nervous system differentiation in chicken embryos prior to the onset of their own thyroid function (see Flamant and Samarut, 1998). We hypothesized that adverse effects of altered maternal THs could also be manifested in traits such as motor coordination or cognition/learning in birds. In wild populations, chick motor coordination such as coordination of the begging behavior is important for food solicitation from the parents; 6) Fledging success: This is a crucial fitness-related trait in wild populations. We hypothesize that the effect of maternal THs on fledging success is likely to be indirect, mediated via increased growth or other effects of THs on development. In short, we predicted that nestlings from eggs with higher yolk TH levels would show 1) increased hatching success 2) increased growth 3) higher metabolic rates 4) higher stress reactivity 5) poorer motor coordination and 6) higher fledging success. We also determined nestling sex and studied the potential sex-dependent effects, as growth is sex-dependent in great tits (Räberg et al., 2005; Nicolaus et al., 2009), and there is some indication for variation in plasma THs across the sexes in birds (Newcombe et al., 1992).

2. Methods

The experiment was conducted in a nest box population (155 nest-boxes) in Bennekom in the Netherlands (51° 59' 53 N, 5° 40' 35 E). The study species, the great tit, is an abundant, non-migratory, insectivorous hole-breeding passerine, and a common model species in ecological research. Both parents take part in nestling feeding, and nestlings usually fledge at age of 18–21 days.

2.1. Yolk thyroid hormone treatment

The nest boxes were monitored with three day intervals for initiation of nest building, and as soon as nest-building was halfway, monitored every day. From 44 nests, we collected the 4th egg and from 14 nests the 1st and the 7th egg (in total 34 eggs from CO nests and 38 eggs from TH-treatment nests, see below) for the purposes of another study. The rest of the eggs of these clutches were used for the manipulation experiment, see Table 1 for sample sizes. Yolk T3 and T4 levels were elevated by injection into the egg yolk before incubation had started (i.e. a combined injection of the two hormones). All eggs of the clutch received the same treatment (hereafter control or yolk TH treatment). We conducted a between-clutch design instead of a within-clutch design as the latter was practically not feasible: it would have required tracking which chick hatches from which egg. In practice, monitoring the hatching of each egg in great tit nests in a wild population is not possible, as this would require many visits within a day, which is too much disturbance for the female and will lead to desertion of the nest.

The injection dose was calculated based on a sample of 113 eggs (58 clutches) from the same population from the previous year (in 2013, see Ruuskanen et al., 2015). Average concentrations were: T4 2.97 pg/mg (SD 0.84, range 1.4–6.6) and T3 0.33 pg/mg, (SD 0.13, range 0.12–0.6).

Table 1

Sample sizes; numbers of nests, injected eggs and chicks in each treatment. CO = control, TH = thyroid hormone elevation.

Sample sizes	CO	TH	Total
Nests	30	32	62
Eggs injected	198	222	420
Chicks hatched	101	111	212

The average content of T4 was 0.964 ng/yolk (SD 0.29, range 0.28–1.98, i.e. 7-fold variation) and T3 0.110 ng/yolk (SD 0.04, range 0.04–0.38, i.e. 10-fold variation). The injection dose was ± 2 SD of the yolk hormone content, which corresponds to 0.58 ng T4/yolk and 0.08 ng T3/yolk. Sterile stock solution of T3 (3,3',5-triiodo-L-thyronine, $>95\%$ HPLC, CAS number 6893–02–3, Sigma-Aldrich) and T4 (L-thyroxine, $\geq 98\%$ HPLC, CAS number 51–48–9, Sigma-Aldrich) was prepared in 0.1 M NaOH and further diluted 1:10,000 in sterile 0.9% NaCl in a laminar flow cabinet. Control clutches were injected with 0.9% NaCl only. The solution was stored in aliquots in -20°C and thawed to room temperature on the day of injection.

To make sure injections would be performed on unincubated eggs (as great tits can start incubation before the clutch is complete), injections were conducted on the day the 6th egg was laid to all eggs in the clutch. Thereafter, injections were done each day for the newly laid egg. None of the clutches had been incubated before the injections started. Eggs were carefully removed from the nest, replaced by dummy eggs (which great tits readily accept) and transported to a closelyby mobile lab. The injections were conducted following a method that has been successfully used in tens of bird species, including great tits (Tsichirren et al., 2005; Ruuskanen et al., 2012): Briefly, the position of the yolk was visualized using a light source positioned beneath the egg. 4 μl of the hormone – saline solution (TH treatment) or saline solution only (control) was injected into the yolk using a Hamilton syringe, and the hole in the egg shell was sealed with a drop of tissue adhesive (VetBond, 3 M). This procedure only took a few minutes; hereafter the eggs were immediately returned to the nest and dummy eggs removed.

2.2. Hatching and cross-fostering procedure

Hatching date and success was monitored by visiting nests daily (always in the morning), starting before the estimated hatching day. Hatching success (excluding collected eggs or abandoned nests) was low, on average 50% (see Results and Supplementary file S1). The average hatching success of non-manipulated nests in local great tit populations in the study year was 87% (Visser, M.E., Ruuskanen, S., unpubl). Unfortunately, the reasons for this low hatching success are not clear: for example, it may be related to infections, internal damage in the process of transporting eggs or potentially due to the use of cyanoacrylate tissue-glue (VetBond). However, similar hatching success has previously been reported in great tits in other injection studies (Podlas et al., 2011; Podlas and Richner, 2013). The authors have used similar injection protocol, including tissue glue, previously in pied and collared flycatchers (*Ficedula hypoleuca*, *Ficedula albicollis*), where the hatching success was ca 70% (Ruuskanen and Laaksonen, 2010; Ruuskanen et al., 2012). Unhatched eggs were collected and frozen in -20°C on day 2 after the other eggs had hatched. Later they were dissected and embryonic development (number of days) was estimated following Murray et al. (2013). In total 156 eggs (68 control eggs and 88 TH treatment eggs) were opened. Of these, nine eggs were completely dried/destroyed and the stage of development could not be determined. We discuss the effects of low hatching success in detail in the discussion.

Due to the low hatching success and thus (unnaturally) small numbers of nestlings per nest, we combined nestlings from two or more nests, with opposite treatments, into one nest (originally either control or TH treatment nest) to 1) avoid any effects of treatment masked by very small broods (or parents even deserting the brood); 2) increase statistical power using a within-clutch experimental design, given that post-hatching conditions vary widely across territories (broods). On day 2 after hatching (hatching date = d0) we measured mass (~ 0.01 g), wing length (~ 0.5 mm) and marked nestlings individually by cutting a part of the down feathers. We then transferred nestlings to foster nests with nestlings of the same hatch date and similar mass (matched as closely as possible) but opposite treatment, thus creating mixed broods with chicks from both TH-elevated and control eggs. The relative difference between average body mass of 2d chicks

between the two types of nestlings within a brood, taking into account the direction of the difference, was on average -1.2% (SD 17.7%). We aimed to create foster nests with equal number of treatment and control chicks (proportion of TH-chicks per nest on average 0.52 ± 0.18 SD, controlled for in the statistical analyses), but this was not always possible due to lack of similar aged and sized nestlings. Except for two nests, which contained only control chicks, each foster nest contained at minimum two nestlings from each treatment. The new brood size in the foster nests was determined as the original clutch size of that pair minus one nestling (accounting for normal hatching failure of one egg; mean 7.1, SD 1.2 range 4–9 nestlings). We considered that it was very important to keep the original and natural brood sizes, as we did not want to cause additional variation because of apparent brood size manipulation. In total we created 30 foster broods, with 101 nestlings from control eggs and 111 nestlings from yolk TH injected eggs.

2.3. Nestling morphological measurements and fledging success.

Nestlings were measured on day 7 and 14 after hatching (mass ~ 0.01 g, wing ~ 0.5 mm). All nestling measurements were conducted between 8 am and 2 pm, and by the same observer (SR). On day 14 a drop of blood from the brachial vein was collected for sex determination analysis (following Griffiths et al., 1998, using FavorGen kit). Sex determination produced data of 66 females and 70 males. Around fledging (from d17 onwards) nests were visited daily to record fledging date to calculate the length of the nestling period (hatch date to fledge date) and to record individual fledging success.

2.4. Motor coordination behavioral test

Multiple neurological effects, behavioral alterations and motor skills problems have been found in relation to altered TH exposure in humans and mammals (e.g. Patel et al., 2011, Vasudevan et al., 2013), and there is also some evidence for such effects in birds (Kagami and Nishigori, 2010, Rainwater et al., 2008). On day 7, we conducted a simple motor coordination behavioral test by gently placing the bird on its back on a X shaped platform, and counted the time (seconds) it took to return to standing again (with a maximum of 60 s, after which the test was terminated). All birds started moving immediately after placing them on their back.

2.5. Handling stress test

The stress axis has been found to be affected by maternal thyroid hormone levels in mammals, offspring exposed to high maternal THs showing higher stress reactivity and stress-induced corticosterone levels (e.g. Zhang et al., 2008). We conducted a handling stress test using breath rate as a stress response, before and after social isolation, following Fucikova et al. (2009) who showed these stress responses are repeatable in the species studied. This test was conducted on 14-day old nestlings, immediately after taking the nestlings out from the nest to a cotton bag. Briefly, one by one, the bird was held in hand and its breathing rate (number of breast movements) was counted for 1 min in 15-second intervals: the increase of stress (breath rate) over the 1-min period was our response. After handling, we put each individual into a wooden box (consisting of 12 compartments of $6 \times 6 \times 6$ cm size) for 15 min where nestlings were spatially and visually separated from their siblings (social isolation) but could hear each other. This was repeated until we tested the whole brood. After the first nestling had been in the wooden box for 15 min, we measured the breath rate in the same way for 1 min again. Thereby, periods 1 and 2 refer to the periods of 1 min before and after social isolation subsequently. Other measurements (see above) were taken after the handling test.

2.6. Metabolic measurements

For determining resting metabolic rate (RMR), we used protocol following [Amo et al. \(2011\)](#), using the exact same equipment at the laboratory of the Netherlands Institute of Ecology in Wageningen. Briefly, at the age of 14 days, a minimum of two nestlings per nest were taken from the nest at sunset (ca 21.00) and brought to the laboratory (ca 10 min drive) for overnight metabolic measurements in the respirometer, and returned to their nest at sunrise (ca 6.00). To increase sample size, we also measured additional birds on day 15 (see below). Birds were weighed before and after measurements, and the two nestlings from each nest (one control and one TH treatment) were matched for body mass as much as possible (average difference between nestlings from different treatments from the same foster nest; 5.7%, SD 3%). Birds spent the night in individual respirometer chambers (ø 7 cm, length 20 cm) at 27 °C (temperatures recorded by loggers inside the measuring chambers during 00.00–04.00: mean $\pm$ SE 27.7 $\pm$ 0.02 °C, controlled for in the analyses). In the equipment, air flows with a precise flow rate through chambers and from the air that comes out, CO₂ and O₂ are measured. The CO₂ and O₂ from a control (empty) chamber are used as a reference. The energy consumption during rest (measurements of the lowest consumption during the night, i.e. resting metabolic rate, as kJ/24 h) was calculated from the amount of oxygen consumed, corrected for temperature and body mass. The RMR was measured from 69 nestlings in total (day 14: 25 CO, 28 TH, day 15: 7 CO, 9 TH). All nestlings that were measured in the respirometer fledged in the following days, suggesting that the measurement did not notably affect their condition. All experimental procedures were approved by the The Animal Experimentation Committee (DEC) of the Royal Netherlands Academy of Arts and Sciences (KNAW) (license number: NIOO1402).

2.7. Statistical analyses

All statistics were conducted using SAS 9.3 statistical software. Hatching success was analysed with a generalized linear model (proc GENMOD) with binomial distribution (hatched/eggs, event/trials syntax, logit link) and treatment as a predictor. Individual fledging success (fledged/not) was analysed with a generalized linear mixed model (proc GLIMMIX) with binomial distribution (logit link), treatment as the predictor, with covariates hatching date (i.e. timing of breeding), brood size (at day 2) and brood composition (percentage of birds from the TH treatment), and foster brood ID as the random effect (N = 212). Interactions with sex could not be tested as only nine out of 136 sexed nestlings did not fledge. The length of the nestling period was analysed with mixed models with predictors: treatment, sex and their interaction. Covariates were hatching date, brood size and brood composition. We included both original and foster brood ID as random effects (to control for the genetic origin and rearing environment).

A principal component analysis of the growth measurements (mass and wing) including all measuring periods (day 2, 7 and 14) was conducted. PC1 explained 96% of the variation. We then conducted a repeated measures mixed model with PC1 as the response and with predictors treatment, sex, age (2, 7 or 14 days) and their interactions. Individual (nested in foster brood ID) was included as a repeated subject effect and current and original brood IDs as the random effect.

Resting metabolic rate (kJ/24 h) was analysed with a similar mixed model as the length of nestling period. Furthermore, we always included temperature within the measurement chamber, body mass after the measurements and nestling age (14 or 15 days) as covariates, and additionally the measurement chamber ID as a random effect.

Data from the motor skills behavioral test was bimodally distributed with many very short times (<10 s; 52% of the cases), and many very long times (>60 s; 15% of the cases). Thus we divided the data to fast or slow birds (below and above median, 7.5 s) and used generalized linear mixed models (proc GLIMMIX, binomial error distribution), with

similar predictors and random factors as above, but also including body mass and time of day as covariates.

In the handling stress test, our main interest was in the increase in breath rate over the 1-min period. Thus we first calculated individual estimates of slopes over the 1-min period (corrected for environmental variation) using a following mixed model (see [Fucikova et al., 2009](#)): The response was the number of breaths per 15-s bouts and the fixed factors were bout number (continuous, nested within period), period, interaction between individual ID and bout number (nested in period) (i.e. slopes for each individual, separately for the 2 periods). Covariates were mass, tarsus, time of day, temperature, date and individual as a random effect. The obtained estimates are therefore a measurement of the individual deviations from the mean handling stress (i.e. individual deviations from the slopes). We then used these estimates to test the differences among treatment groups with a mixed model where the response was the estimate and fixed factors treatment, sex, period and their interactions, and similar covariates and random effects as above.

Importantly, as sample sizes in d2 and d7 measurements were smaller when sex was included (N = 136 with sex, N = 212 without sex), we also analysed all above-described traits excluding the sex effect. There were no significant treatment effects, thus we report the results of the analysis always including sex. All models were reduced by removing interactions, main effects and covariates, starting from the highest p-values ($\alpha > 0.05$) with the exception of treatment, sex and their interaction which were always retained in the model. Removed factors were reintroduced into the reduced model one by one to confirm their non-significance, and these statistics are reported in the tables. Kenward-Roger method was used for calculating the degrees of freedom. Finally, we also calculated the effect sizes for each predictor (partial R², following [Edwards et al., 2008](#) and Cohen's d, see Table S2).

3. Results

3.1. Hatching and fledging success and developmental time

Hatching success was low, 50.3% (SD 19.1) for yolk TH treatment nests, and 49.8% (SD 22.8) for control nests, and did not differ between control and TH treated eggs ($\chi^2 = 0.12$, $p = 0.38$). The timing of the mortality of the embryos of the unhatched eggs did not differ in relation to the treatment ($\chi^2 = 0.15$, $p = 0.69$). The average day (days of development) of mortality day was 5.1 (SD 3.5, range 0–12) in unhatched TH treatment eggs and 4.9 (SD 3.4, range 0–12) in unhatched control eggs, respectively. Mortality occurred throughout the incubation period (range 0–12 days) in both treatments (see Supplementary file S1).

In control nestlings, 70 out of 101 fledged, and in TH treatment 71 nestlings out of 111 fledged: There was thus no difference between the treatments in fledging success (treatment: $F_{1,32.6} = 1.28$ $p = 0.27$; $R^2 = 0.05$). Fledging success was not correlated with timing of breeding, brood size or composition ($p > 0.52$). The length of the nestling period was also not affected by treatment ($F_{1,11.4} = 3.16$ $p = 0.10$, $R^2 = 0.20$: length of the nestling period, nb of days $\pm$ SE: Female CO: 19.5 $\pm$ 0.15; Female TH 19.5 $\pm$ 0.21; Male CO 19.8 $\pm$ 0.18; Male TH 19.4 $\pm$ 0.23). The length of the nestling period was not correlated with timing of breeding, brood size or composition ($p > 0.10$).

3.2. Growth

The original data on mass and wing length for each treatment and sex is provided in Table S1. There was a sex-specific effect of yolk TH treatment on nestling size (PC1 of mass and wing length) ($F_{1, 373} = 9.07$, $p = 0.0028$, $R^2 = 0.11$; Table 2, Fig 1): TH treatment increased the growth of male nestlings, and decreased the growth of females. This effect was similar at all nestling ages (2, 7 and 14 days; i.e. interactions age * treatment or age * treatment * sex, $p > 0.05$, Table 2). Tukey post-hoc tests did not reveal differences among treatments within sexes (p -values > 0.11), but sex differences across treatments (TH treatment:

Table 2

Nestling growth (PC1 of mass and wing) in relation to experimental yolk thyroid hormone elevation in great tits (*Parus major*). Repeated measured mixed models using data from day 2, 7 and 14 after hatching (hatching date = d0) with individual (nested in brood ID) as the repeated subject. For further details see text. Predictors in the final, reduced model are shown in bold and statistically significant ($p < 0.05$) with underlining. Partial R2s are calculated following Edwards et al., 2008.

Predictors	F, ddf	p	Effect size (partial R2)
Treat	0.03 _{1,388}	0.85	0.0004
Sex	1.7 _{1,370}	0.19	0.02
Age	<u>4598₂₃₇₅</u>	<u><0.001</u>	0.98
Treat * sex	<u>9.07_{1,373}</u>	<u>0.0028</u>	0.11
Treat * age	2.4 _{2,367}	0.10	0.03
Sex * age	0.69 _{2,359}	0.50	0.01
Treat * sex * age	0.26 _{2,354}	0.77	0.005
Date	4.37 _{1,473}	0.04	0.31
Brood size	2.49 _{1,150}	0.12	0.09
Sample size	N = 396 (135 individuals)		

females vs males: $t = -3.1$, $p = 0.01$, CO: females vs males: $d = 1.2$, $p = 0.61$), suggesting opposite effects on the sexes. Nestling size decreased across the season (hatching date: $\beta \pm SE$: -0.014 ± 0.0067). The effect sizes were moderate, and also showed the opposite effects of the TH treatment on growth of males and females (Table S2).

3.3. Physiology and behavior

Resting metabolic rate (RMR) was not affected by the yolk TH treatment or its interaction with sex (Table 3, means $\pm SE$ (kJ/24 h): Female CO: 31.10 ± 4.17 ; Female TH 31.62 ± 6.85 ; Male CO 30.18 ± 4.76 ; Male TH: 32.75 ± 5.70). Resting metabolic rate decreased with increasing proportion of TH treated chicks in the nest (Table 3, $\beta \pm SE$: -10.45 ± 3.80). Breathing rate increased with measurement order in the 1-min handling stress (4 successive 15-s bouts, Fig 3) and the increase was more rapid post-isolation (Table 3, Fig 2). However, this handling stress response (slopes over the 1-min period) was not affected by TH treatment or sex either pre- or post-isolation (Table 3, Fig 2). Motor coordination behavior, i.e. the time it took for a nestling to return to standing, was not affected by treatment, sex or their interaction (Table 3, Fig 3.). The probability of being fast in the motor coordination task increased across the season (Table 2, hatching date: $\beta \pm SE$ 0.15 ± 0.08). Effect sizes for RMR, stress reactivity and motor coordination were moderate between CO vs TH males, but small for females (Table S2).

Fig. 1. PC1 of size (mass and wing) (marginal means $\pm SE$) in relation to yolk thyroid hormone treatment, separately for each sex, in a wild population of great tits. Number of individuals/group are indicated above the bars. See text for statistics.

4. Discussion

We predicted that embryonic exposure to elevated maternal TH levels would increase hatching success, growth and metabolic rates, impair motor coordination, and increase sensitivity to stress. In addition, we predicted that elevated maternal TH levels would increase fledging success, a direct fitness-measure, likely via the positive effects of elevated yolk THs on growth. We found that there was a sex-specific effect of elevated yolk THs on nestling size. This effect was similar during the whole nestling period. In contrast to our predictions, we did not find any effect on the measured physiological or behavioral traits. Also hatching and fledging success were not affected, but sex-specific analyses could not be conducted on these data.

The sex-specific effect on growth, elevated yolk THs increasing male growth and decreasing female growth in an altricial wild bird is a novel result, but in contrast to our predictions and previous research. The effect was similar in both early and late nestling phase. The mechanisms behind this sex-specific effect of yolk THs are not clear. One hypothesis is that yolk THs may advance male and decrease female development, potentially via increasing male embryonic growth, or increasing hatching speed, with opposite effects on females (see McNabb, 2007), thus males getting fed earlier by the parents. Consequently males from yolk TH-elevated eggs could reach larger size earlier and this initial size difference could persist into late nestling phase. However, hatching speed or timing could not be measured accurately, as this is practically impossible in the wild population: frequent visits would have caused the disturbance-intolerant great tit females to leave the nest and subsequently risk getting the chicks cold, even deserted. Also, an organizing effect of yolk THs on nestling metabolic or endocrine function (circulating thyroid hormone levels) could explain such an effect on growth that lasts to late nestling period. In tits, male nestlings are commonly slightly larger during the nestling period (Räberg et al., 2005; Nicolaus et al., 2009); however we have no data on potential sex-differences in yolk or circulating THs (or their cross-talks with other hormones responsive for growth), in thyroid development or receptor sensitivity in the species, which could explain the sex-specific effects. In previous experimental studies in precocial quails, elevated (supraphysiological doses in female diet) yolk T4 increased embryonic alkaline phosphatase activity (a marker of differentiation) and pelvic cartilage wet and dry weights (Wilson and McNabb, 1997) and in chickens, elevated yolk THs affected embryonic neuronal development (Flamant and Samarut, 1998). However, the effects on post-hatching growth or sex-dependent effects were not tested.

We did not find any long-lasting effects of the maternal yolk THs on metabolic rates, stress reactivity or motor coordination, which is in contrast to our predictions and previous research. Yolk TH treatment also did not affect our fitness measures, hatching and fledging success. However, long-term effects for example on neurological function are commonly found in humans and mammals (e.g. Haddow et al., 1999; de Escobar et al., 2004; Zhang et al., 2008), suggesting organisational effects of maternal TH exposure. Long-term effects have also been found for avian yolk steroids, on behavioral, physiological and plumage traits, potentially affecting fitness (e.g. Groothuis et al., 2005; Ruuskanen and Laaksonen, 2010; Ruuskanen et al., 2012). Before making any conclusions about the lack of effect of elevated maternal THs on physiological or behavioral traits, it needs to be noted that in this study 1) the hatching success was low, which may have clouded some effects, if there was selective mortality in the treatment groups. However, hatching success was similar in both treatment groups, mortality was similarly distributed across the embryonic period in both treatments, and the average timing of mortality did not differ among treatment. Nevertheless, high mortality decreased our sample size and thus we may have limited statistical power; 2) This was the first field study in altricial birds, and we could only measure a limited number of traits, thus more studies on multiple TH-related traits are needed; 3) In this experiment we used a physiologically relevant dose (+ 2SD). The (bio)medical research on

Table 3

Results of nestling physiological and behavioral measurements relation to experimental yolk thyroid hormone elevation in great tits (*Parus major*). General or generalized linear mixed models with original and foster nest ID as random factors (and in the models for breathe rate, also individual ID). For further details see text. Predictors in the final, reduced model are shown in bold and statistically significant ($p < 0.05$) with underlining. RMR = resting metabolic rate. Motor coordination = the time to return from back to standing (slow/fast). Handling stress = slope over the 1-min period (in breathe rate). Period = pre- or post-social isolation in the handling stress test. Partial R2s are calculated following Edwards et al., 2008.

Predictors	RMR (kJ/24 h)			Motor coordination			Handling stress		
	F, ddf	p	Partial R2	F, ddf	p	Partial R2	F, ddf	p	Partial R2
Treat	0.38 _{17,5}	0.54	0.08	3.2 ₂₈	0.08	0.30	0.72 _{36,2}	0.40	0.07
Sex	0.48 _{55,2}	0.49	0.03	2.8 ₁₂₈	0.09	0.08	2.45 ₄₈	0.12	0.16
Treat * sex	0.05 ₄₇	0.82	0.004	1.60 ₁₂₈	0.21	0.04	1.23 ₂₄₈	0.21	0.019
Date	0.09 _{15,6}	0.76	0.02	4.00 _{21,6}	0.06	0.42	2.1 _{20,5}	0.15	0.33
Brood size	0.96 _{19,4}	0.33	0.19	0.13 _{22,2}	0.74	0.03	2.62 ₂₆	0.12	0.33
Brood composition	7.53 ₂₄	0.01	0.55	0.69 ₂₃	0.41	0.13	0.77 _{24,2}	0.38	0.13
						Period	10.8 ₂₁₀	0.001	0.2
						Period * treat	0.04 ₂₀₉	0.83	0.0009
						Period * sex	0.59 ₂₀₉	0.44	0.013
						Period * treat * sex	1.27 ₂₀₇	0.26	0.03
Sample size	N = 65			N = 135			N = 257 (134 individuals)		

mammalian models suggests that abnormal TH levels cause for example alternations in (neural) development (see introduction). In birds, the studies with experimental manipulations of maternal THs, or exogenous THs during later embryonic stages, often also used supraphysiological doses (>20 fold; Wilson and McNabb, 1997; Bishop et al., 1995; but see Christensen, 1985) or used anti-thyroid chemicals, which seriously altered thyroid status (e.g. Rainwater et al., 2008). However in this study, inspired by the observed variation in yolk TH levels in this species in relation to environmental conditions, our aim was specifically to investigate the potential effects of variation in yolk TH levels within this physiological range, to understand potential fitness effects of such variation in wild populations. This has been rarely studied (but see extensive literature on the effects of such variation in steroid hormones on multiple offspring fitness-related traits, Von Engelhardt and Groothuis, 2011). It is thus possible that larger doses or especially the reduction of yolk TH levels may cause more prominent and potentially long-lasting effects on physiology and behavior. Studies on endocrine disruptors, for example PCBs, show reduced yolk TH levels with potential effects on offspring traits (McNabb, 2007). Indeed, seriously reduced yolk TH levels may be more critical for development than hyperthyroidism, given that, as suggested by van Herck et al. (2013) individuals may even be able to compensate for the lack of

decreased yolk T4 content in the egg yolk by a more efficient T4 uptake. Also in human and rodent models, hypothyroidism during pregnancy causes severe developmental problems with long-lasting consequences (Burrow et al., 1994; de Escobar et al., 2004; Porterfield and Hendrich, 1993).

Finally, the effects of yolk THs on growth may have consequences for sibling competition within a brood (and thus fitness) if there is within-clutch variation in yolk TH levels. Interestingly, we recently found in great tits that yolk T4 levels increase with laying order within the clutch (Ruuskanen et al., 2015). Certainly more studies in various other species are needed to understand if and how yolk TH levels (within normal physiological range) affect offspring development and whether there are patterns within clutches, which may be related to sibling competition. Ultimately, the fitness consequences of variation in the physiological levels of maternally derived yolk THs for the offspring and the mother need to be further studied to understand their potential adaptive value and role in ecological and evolutionary context. In conclusion, this study experimentally demonstrates for the first time in a wild bird population the potential biological relevance of variation in maternal thyroid hormones in egg yolk on offspring growth.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yhbeh.2016.03.006>.

Fig. 2. Breath rate (number of breast movements per 15-s bout $\pm$ SE) in the handling stress test, separately for each treatment and sex. B1–B4 = number of breaths at the four 15-s measurement bouts. Period 1 is 1 min before a 15-min of social isolation (black thick bar) and Period 2 is 1 min after isolation.

Data accessibility

All data will be archived and available in Dryad.

Fig. 3. The numbers of fast (black) and slow (grey) individuals in the motor coordination test (return to standing), separately for each treatment (CO = control, TH = elevated yolk thyroid hormone levels) and sex.

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