Can increased prenatal exposure to thyroid hormones alter physiology and behaviour in the long-term? Insights from an experimental study in Japanese quails.

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Abstract

Maternal thyroid hormones (triiodothyronine T3 and thyroxine T4) are important regulators of embryonic development and gene expression. While maternal thyroid disorders are known to cause developmental issues in humans, variation in maternal thyroid hormones in healthy mothers have also been found to correlate with infant and child phenotypes. This suggests a potential impact of maternal thyroid hormones on offspring phenotype in an eco-evolutionary context. In chickens, prenatal thyroid hormone treatment has been shown to influence embryonic gene expression and postnatal treatment to influence imprinting and learning. However, the potential long-term effects of maternal thyroid hormones on physiology and behaviour are unclear. This study aims to investigate the long-term effects of maternal thyroid hormones on behaviour, plasma thyroid hormone levels and brain gene expression using the Japanese quail (*Coturnix japonica*) as a model. Egg hormone levels were elevated by injecting unincubated eggs with either saline (control), T3, T4 or a mixture of T3 and T4. Social motivation, boldness and fearfulness to predators were tested shortly after hatching and as adults. Plasma thyroid hormone levels and pallial expression of thyroid hormone receptor A, type 2 deiodinase, and nuclear receptor coactivator 1 were measured in adulthood. We found no evidence that elevated thyroid hormone levels in eggs affected behaviour, plasma hormone levels, or gene expression. This is the first study examining the potential long-term effects of elevated maternal thyroid hormones within the natural range. Although we found no evidence of long-term effects, other traits may still be affected and remain to be studied.

Keywords: maternal hormones, maternal effects, endocrinology, phenotypic plasticity, bird

Introduction

Maternal effects refer to the effect of the maternal phenotype or environment on the phenotype of the offspring. The effect can be mediated by hormones passed from the mother to the offspring, the maternal hormones. As the levels of maternal hormones are influenced by the maternal environment, they can transmit information about the maternal environment to the offspring. This allows the development of the offspring phenotype to be controlled by environmental factors (Marshall and Uller, 2007; Mousseau and Fox, 1998). Such hormone-mediated maternal effects can be observed in a wide range of traits, including physiology and behaviour (Groothuis et al., 2019, 2005; Ruuskanen and Hsu, 2018), and can have long-lasting effects into adulthood with potential fitness consequences (Groothuis et al., 2005; Schoech et al., 2011; von Engelhardt and Groothuis, 2011).

Compared to the more extensively studied androgens and glucocorticoids (Podmokła et al., 2018), the effects of maternally transferred thyroid hormones (THs) have been less explored (Darras, 2019; Ruuskanen and Hsu, 2018). THs (triiodothyronine, T3, and thyroxine, T4) play important roles in metabolism and development, where they regulate the expression of numerous genes (Bernal, 2017; Darras, 2019; Moog et al., 2017). TH action is regulated by several components of the thyroid system. TH receptors (THRA and THRB) and coactivators control the transcription of TH-responsive genes by forming a complex with the active form, T3 (Brent, 2012). The intracellular availability of T3 is regulated by several transmembrane TH transporters (e.g. MCT8, OATP1c1) that differentially transport either T4 or T3 (Groeneweg et al., 2020). Inside the cell, T3 availability can be further regulated by deiodinase enzymes that either convert T4 to T3 (deiodinase 2, D2) or convert both to inactive forms (deiodinase 3, D3) (Gereben et al., 2008). The expression of these components (TH receptors, transporters and deiodinases) begins very early in embryonic

development, allowing the embryo to regulate TH levels in an organ and cell-specific manner (Darras, 2019; Darras et al., 2011; Flamant and Samarut, 1998; Ruuskanen et al., 2022; Van Herck et al., 2012). However, the development of the thyroid gland occurs much later in development (Forhead and Fowden, 2014; McNichols and McNabb, 1988; Porazzi et al., 2009). Thus, in almost all vertebrates studied so far, proper embryonic development depends on maternally transferred THs (Brown et al., 2014; Darras, 2019; Moog et al., 2017; Patel et al., 2011).

In mammals, maternal THs are transferred across the placenta, whereas in egg-laying animals such as birds, maternal THs are deposited in the egg yolk. In birds, high natural variation in egg TH levels has been observed, up to 5-fold in magnitude (Ruuskanen and Hsu, 2018). This variation may be due to environmental factors such as food availability or temperature (Hsu et al., 2016; Ruuskanen et al., 2016c), and has been reported at both the inter- and intra-individual levels (Hsu et al., 2016, 2019b; Ruuskanen et al., 2016a, 2016c). As THs are important in development (Darras et al., 2009; Decuypere et al., 2005; McNabb, 2007; Wilson and McNabb, 1997), and as increasing yolk TH levels has been found to increase embryonic TH levels (Van Herck et al., 2012), high variation in egg TH levels could therefore be a source of offspring phenotypic variation.

To date, most of what is known about phenotypic differences due to maternal THs comes mainly from biomedical studies (Moog et al., 2017). However, studies using pathological TH concentrations do not provide relevant information on the ecology or evolution of maternal THs. Therefore, the effects of maternal THs within the natural range need to be studied to understand whether variation has effects on traits affecting offspring fitness. In addition, experiments manipulating maternal THs in mammals would inevitably interfere with maternal physiology, making it difficult to attribute results solely to maternal

THs. Egg-laying animals, such as birds, have therefore become common models for studying the functions of maternal hormones since maternal THs deposited in eggs can be manipulated directly and independently. So far, elevated TH levels in the egg within the natural range have been found to affect hatching success, offspring growth, plasma T3 level and telomere length (Hsu et al., 2023a, 2019a, 2017; Ruuskanen et al., 2016b; Sarraude et al., 2020; Stier et al., 2020). Nevertheless, some of the reported effects appear to be transient during the early post-hatching period (Hsu et al., 2019a) Whether the effects of maternal THs can be long-lasting and potentially influence offspring fitness remains unclear.

To investigate the potential long-term effects of variation in maternal THs, we experimentally elevated egg TH levels within the natural range in Japanese quail (*Coturnix japonica*) and measured changes in behaviour, plasma TH levels and brain gene expression. While maternal hyperthyroidism has been shown to affect behaviour, for example anxiety (Laureano-Melo et al., 2019; Zhang et al., 2010, 2008), it is not known whether increasing maternal THs within the natural range affects behaviour. Also, as postnatal THs have been shown to influence filial imprinting and learning behaviour (Yamaguchi et al., 2012), it is interesting to study whether maternal THs, acting earlier in development, may also have behavioural effects. Potential behavioural effects were investigated in both chicks and adults, allowing for a broader timescale, by examining boldness, social motivation and fearfulness to predators. Due to the lack of studies with natural levels of elevated maternal THs and inconsistent results from studies on maternal hyperthyroidism and offspring anxiety (Laureano-Melo et al., 2019; Zhang et al., 2010, 2008) , the direction of potential behavioural changes is difficult to predict. In addition, plasma TH levels were measured in adults, and changes in these would indicate long lasting effects on TH function. While plasma TH levels have been reported to be affected by elevated egg TH levels in a sex-

dependent manner in the rock pigeons (Hsu et al., 2017), some more recent studies in wild passerines found no clear effects of egg THs on nestling plasma THs (Hsu et al., 2023b, 2020). In terms of gene expression, previous studies have examined the effect of the natural variation in maternal THs on brain gene expression only in bird embryos or young chicks (Van Herck et al., 2015, 2012; Yamaguchi et al., 2012), while the potential long-term effects of prenatal TH exposure remain unknown. Following our previous experiment manipulating egg TH levels in Japanese quail (Sarraude et al., 2020), here we studied several genes involved in TH function: *THRA* (thyroid hormone receptor A), *NCOA1* (nuclear receptor coactivator 1) and *DIO2* (type 2 deiodinase) in the pallium of adults. Expression of these genes has been shown to be influenced by THs in other species (Misiti et al., 1998; Walpita et al., 2007).

Materials and methods

1.1 Materials

This study was conducted in accordance with the Finnish legislation and approved by the Finnish Animal Ethics Committee (Eläinkoelautakunta; ESAVI / 1018 / 04.10.07 / 2016). Japanese quail (*Coturnix japonica*), a commonly used laboratory model with a reference genome, was used. Twenty-four breeding pairs were housed in cages with food and water available ad libitum. The photoperiod was long (16L:8D) and the temperature stable at 20° C. Eggs were collected from the cages in the morning and stored (15° C, 50% relative humidity) for seven days before treatment.

1.2 Treatment of eggs, rearing of chicks and collection of samples

We manipulated egg TH levels by injecting hormones directly into the eggs prior to embryonic development at a dose that mimicked increased maternal transfer (see details in Sarraude et al., 2020). Eggs from each breeding pair were randomly assigned to four treatment groups: T3, T4, T3 + T4 and control (0.9% saline). Eggs laid by the same pair were always evenly distributed among all treatment groups. The injected hormone solutions were prepared from crystallised powder (T3: CAS number 6893-02-3, Sigma-Aldrich, St. Louis, MO, USA and T4: CAS number 51-48-9, Sigma-Aldrich, St. Louis, MO, USA). The amount of hormone injected was based on previous measurements in the parental generation (mean ± SD: T4, 15.3 ± 4.43 ng / egg; T3, 7.58 ± 2.32 ng / egg) and the aim was to increase hormone contents in the yolk by two standard deviations (as recommended by Podmokla et al., 2018). The injection protocol has been described in detail in Sarraude et al. (2020). Briefly, each egg was injected with 50 µl of either the respective hormone solution (T3, T4 or T3+T4, in 0.9% saline) or 0.9% saline (control, CO), delivered by using 27G insulin syringes (BD Microlance™). After sealing the hole with sterile plaster (OPSITE Flexigrid, Smith & Nephew), the eggs were incubated artificially (37.8 °C and 55% relative humidity). A total of 158 eggs were treated (39 T3, 39 T4, 40 T3+T4 and 40 CO), of which 66 chicks hatched (15 T3, 20 T4, 21 T3 + T4 and 10 CO), with sex distributions of chicks surviving to adulthood (females / males): 7/4 for T3, 10/8 for T4, 9/12 for T3 + T4 and 3/4 for CO. Sex was determined at maturity by examining the presence of the cloacal gland. The overall hatching rate (~40%) was consistent with previous studies in domestic quail (Okuliarová et al., 2007).

The rearing conditions of the chicks have been described in detail in a previous publication (Sarraude et al., 2020). In short, chicks were reared in groups (4-16 individuals, depending on age) with treatments and sexes mixed, until sexual maturity (ca. 6–8 weeks

after hatching in Japanese quails), after which the birds were separated by sex to smaller groups (4-5 individuals). Photoperiod was 16L:8D for the first 7 months and 8L:16 thereafter. At approximately 4.5 months of age, we collected blood samples from the brachial vein, separated plasma, and stored it at -80° C. Birds were euthanised by decapitation at 10 months of age. After decapitation, 40 mg of tissue was collected from the pallium in PBS buffer, snap frozen in liquid nitrogen and stored at -80° C.

1.3 Behavioural experiments

To assess the potential effects of maternal THs on behaviour, we conducted a series of behavioural tests (open arena, emergence and tonic immobility tests) at different life stages. We followed previously published protocols that have been used extensively with many different model organisms. The tests were conducted in separate rooms where the birds had no visual or auditory contact with other individuals. The exception was the open arena test, where the subject was able to hear the vocalisations of another bird being tested at the same time in another room. All experiments were recorded, and the videos were analysed blindly of the treatment. All videos of the same test at both ages (chick/adult) were analysed by one person, except for the open arena test, where one person analysed chick videos and another person analysed adult videos.

The open arena test measured social motivation and boldness and it followed a setup developed by Forkman et al. (2007). The test was performed on both sexes at three days and approximately 4.5 months of age. In the test, the bird was placed in a circular illuminated arena and the movement duration and the number of escape attempts (attempt to jump/fly over the wall) were measured from videos for 10 minutes (from the time the researcher left the room). Only 10 of the 55 adults attempted to escape, so adult escape

attempts were not analysed. Subjects who moved less and made fewer escape attempts were interpreted as less bold and less socially motivated.

The emergence test measured social motivation and boldness. The test was performed on both sexes, twice on chicks (6-9d old) and once on adults (4.5 months old). The test followed a setup developed by Forkman et al. (2007), in which a bird was placed in a dark chamber connected to another, bright chamber. One minute after the subject was placed in the dark chamber, a door connecting the chambers was removed. After removing the door, the researcher left the observation room and the time it took for the subject to completely exit the dark chamber was measured. Subjects who left the dark chamber more quickly were interpreted as bolder and more socially motivated. The test lasted five minutes. Whether the subject remained in the dark chamber (0) or left it (1) during the test was also recorded.

The tonic immobility test (Mills and Faure, 1991) measured fearfulness to predators. The test was performed on both sexes at 12 days and approximately 4.5 months of age. To induce immobility, the bird was placed on its back in the centre of a V-shaped test apparatus and restrained by hand for 15 seconds, after which the hand was removed, and the time taken for the bird to resume a standing posture was measured. If the subject resumed the standing posture in less than 10 seconds, this was recorded as a failed induction and the induction procedure was repeated. Four adult subjects who failed five times were excluded. If the subject remained stationary for more than 10 seconds, then the time to recovery was measured. The longer a subject took to recover, the more fearful it was interpreted. The test lasted 10 minutes. Five subjects (1 chick and 4 adults) did not recover for 10 minutes and were excluded from the analysis (approximately 4% of all tests).

224 ### *1.4 Plasma thyroid hormone levels*

225 To test whether elevated maternal THs affect plasma TH levels later in life (4.5 months
226 of age), we extracted THs from plasma samples and measured them using a previously
227 validated LC-MS / MS protocol (Ruuskanen et al., 2018). The sample size was 48 individuals
228 (T3: 10, T4: 16, T3T4: 15, and 7 in the control). Hormone concentrations are expressed in
229 pmol/ml plasma.

230

231 ### *1.5 Gene expression*

232 To test for potential long-term changes in the expression of TH related genes (*DIO2*,
233 *THRA*, and *NCOA1*), we measured gene expression from pallium samples (10 months old).
234 RNA was extracted from the homogenised pallium samples using the NucleoSpin RNA Plus
235 kit (MACHEREY-NAGEL). The sample size was 31 (7 in control and 8 per treatment group).
236 Extraction was done from 50 µl of the homogenised brain sample according to the
237 manufacturer's instructions, except that the gDNA removal column was centrifuged longer
238 (1 min, 11000 g) and the RNA was eluted once with 40 µl of RNase-free water. RNA
239 concentration and purity were quantified by optical density, and all samples not meeting
240 our quality criteria (i.e. RNA concentration > 30 ng/µl and 260/280 > 1.80) were excluded
241 from further analysis. RNA integrity was checked using an E-Gel 2% electrophoresis system
242 (Invitrogen), and the ribosomal RNA 18S vs. 28S band intensity and was deemed
243 satisfactory. Samples were stored at -80° C for 2 weeks prior to cDNA synthesis. cDNA
244 synthesis was performed using the SensiFAST ™ cDNA Synthesis Kit (Bioline) according to
245 the manufacturer's instructions. The reaction was performed using 500 ng of RNA as the

starting material. A control (noRT) was prepared without reverse transcriptase enzyme. The cDNA was diluted with water to a concentration of 1.2 ng/µl and stored at -20° C.

GAPDH and *PGK* were used as control genes, using primers previously designed and validated in Japanese quail (Vitorino Carvalho et al., 2019). Stability of expression between experimental groups was further verified using geNorm software (geNorm M < 0.5, geNorm V < 0.15), and thus the geometric mean of these two genes was used as our reference gene. Primers for the target genes were designed using the Primer-BLAST tool (NCBI). The primer pairs (Table 1) were designed to have at least one intron between them. The suitability of the primers in qPCR was tested prior to the actual analysis using different primer concentrations and different amounts of cDNA. The tests confirmed the sufficient amplification efficiency of the primers (between 90 and 110% based on the standard curve) and the formation of only one product of the expected size based on both the presence of a single narrow peak in the melting curve, and the presence of a single band of the expected size on the agarose gel.

Table 1: qPCR primer set information and performance for gene expression assays. Cq refers to qPCR quantification cycle, efficiency was evaluated using the LinReg method based on individual well efficiency. Technical precision estimates are given for Cq as coefficient of variation CV (%) and for expression (ratio of target to reference genes) as technical repeatability (*R*).

Gene	Primers	Cq ± SD	*Efficiency*	CV Cq	*Repeatability expression*
GAPDH	F: TCTCTGTTGTTGACCTGACCTG R: ATGGCTGTCACCATTGAAGTC	15.0±0.50	98.0%	0.59%	
PGK1	F: CAAGCTCACCCTGGACAAGT R: GGACGGCTGCCTTGATTCTT	21.1±0.86	85.5%	0.48%	
DIO2	F: CTGGAACAGCTTCCTCCTGG R: ATCCAGAAGACGGCACTTGG	23.6±0.83	92.9%	0.40%	0.91
NCOA1	F: CGACTCACGGAAGAGGAAGG R: GTGTCGATGTCCCCGATGTT	20.4±2.82	91.9%	0.38%	0.84
THRA	F: TGGTGAAGAGCAGCATGTCA R: AGCACCCATCGTACTTGCAG	21.4±0.82	96.0%	0.46%	0.82

qPCR assays were performed on a Mic qPCR (Bio Molecular Systems) in a final volume of 12 µL containing 6 ng of cDNA, 800 nM of forward and reverse primers and SensiFAST SYBR Lo-ROX (Bioline). We ran gene-specific qPCR plates and each sample was analysed in duplicate. Two plates were run for each gene and experimental groups and sex were always balanced within each plate. We used a cDNA reference sample (*i.e.* ratio = 1), which was a pool of 10 different individuals on each plate. We used a two-step cycling with the following conditions: 2 min at 95°C; then 45 cycles of 5s at 95°C followed by 20s at 60°C (fluorescence reading) for all reactions. The expression of each gene was calculated as $(1+Ef_{Target})^{\Delta Cq(Target)}$/ geometric mean $[(1+Ef_{GAPDH})^{\Delta Cq^{GAPDH}}; (1+Ef_{PGK1})^{\Delta Cq^{PGK1}}]$, where Ef is the amplification's efficiency and ΔCq is the difference between the Cq-values of the reference sample and the sample of interest. Amplification in the no-RT controls never occurred until at least 8 cycles after the lower Cq sample, and thus contamination by genomic DNA could not interfere with

our results. Technical precision and repeatability were generally good (Table 1), but a few samples with a CV% of duplicates (based on the final ratio) greater than 15% were excluded from the analysis (final sample size: N_{DIO2} = 27, N_{NCOA1} = 28, N_{THRa} = 26)

1.6 Statistical analysis

All statistical analyses were performed with R software (version 3.6.2), using the packages lme4 version 1.1-23 (Bates et al., 2015) and emmeans version 1.6.0 (Lenth, 2021). Normality and homoscedasticity of the residual distributions were checked visually. For the behavioural analysis, recovery time (tonic immobility) and exit time (emergence test), were transformed (natural logarithm) to ensure that the residuals were normally distributed. Linear mixed models (LMM) were used to analyse time to exit in the emergence test, movement duration and number of escape attempts in the open arena test, recovery time in the tonic immobility test, plasma TH concentrations and qPCR results. Generalised linear mixed model (GLMM) with a binomial error distribution was used to analyse the proportion of individuals leaving the dark chamber in the emergence test. Fixed effects were treatment, sex, and test number/age (in repeated tests). Interactions between sex and treatment, and between treatment and age (in repeated tests), were also analysed. Random effects were individual ID (in repeated tests) and parental ID (to account for relatedness between siblings in all experiments). F- and p-values for LMMs were obtained using the Kenward-Roger approximation on the denominator degree of freedom, implemented in the package *lmerTest* (Kuznetsova et al., 2017). For binomial regression models, main effects and interactions were tested by comparing the model with and without the factor. Non-

significant ($p > 0.05$) interactions were excluded from the final models. Post-hoc tests were performed using Tukey tests where appropriate.

Results

Effects of prenatal thyroid hormone supplementation on behaviour

In general, no statistically significant differences in chick or adult behavioural traits were observed between the egg TH manipulation groups (T3, T4, T3 + T4, control). Elevated THs had no significant effect on social motivation or boldness (Table 2, Fig. 1-2), nor on fearfulness to predators (Table 2, Fig. 3). A statistically significant interaction between treatment and age was found in the emergence test ($F = 3.58$, ddf = 67.2, $p = 0.018$, Fig. 2): In all treatments except the control group (Tukey $t=0.59$, $p=0.56$), chick exit times were significantly longer than those of adults (all $t > 3.21$, $p < 0.002$) while no differences between treatments were found within each age-category (all $t < 2.48$, all $p > 0.07$). There was also a statistically significant interaction between treatment and sex in the tonic immobility test ($F = 2.92$, ddf = 37.5, $p = 0.047$, Fig. 3): In males, the recovery time was longer in the T4-group (marginal mean ± SE: 177.1 ± 47.8 s) than in the T3-group (marginal mean ± SE: 56.1 ± 20.8 s, Tukey $t = 2.71$, $p = 0.047$), whereas no differences between treatments were observed in females (all $t < 2.29$, all $p > 0.12$). In the T4-group, the recovery time of males (marginal mean ± SE: 177.1 ± 47.8 s) was longer than that of females (marginal mean ± SE: 85.2 ± 19.9 s) ($t=2.16$, $p= 0.037$), while no sex differences were observed within the other treatments (all $t<1.80$, all $p>0.079$).

Table 2. Marginal means and standard errors of behavioural tests. Results for chicks (age: 3d, 6-9d, 12d) and adults (age: 4.5 months) are shown separately for each variable. F- and p-values for treatment from linear models (ANOVA, Kenward-Roger approximation). Test statistics (χ^2) and p-values for binomial regressions (model with treatment compared to model without treatment) were calculated with ANOVA.

Test: measurement	*Age*	*Control*	*T3*	*T3T4*	*T4*	F_{ddf}/χ^2 (df)	*P*
Tonic immobility:	12 d	92.0±25.5	84.6±21.1	68.4±13.1	123±24.7	$2.52_{36.2}$	0.073
Recovery time (s)	4.5 m	90.6±25.3	83.3±21.4	67.3±13.6	121±25.5		
Open arena:	3 d	77.2±31.1	100±27.1	73.3±19.1	63.3±20.8	$0.44_{46.6}$	0.72
Movement duration (s)	4.5 m	181±31.6	204±28.1	177±19.3	167±21.3		
Open arena:	3 d	5.85±3.87	4.14±3.53	8.25±2.14	7.21±2.43	$0.35_{41.7}$	0.79
Escape attempts (number)							
Emergence:	6–9 d	34.8±14.7	42.7±16.4	74.7±20.9	121±36.1	$1.92_{39.6}$	0.14
Exit time (s)	4.5 m	25.6±12.5	6.59±2.75	23.0±7.64	12.6±4.30		
Emergence:	6–9 d	0.80±0.11	0.70±0.12	0.62±0.10	0.58±0.10	2.71 (3)	0.44
Likelihood of emergence (0-1)	4.5 m	0.91±0.06	0.86±0.08	0.81±0.08	0.78±0.09		

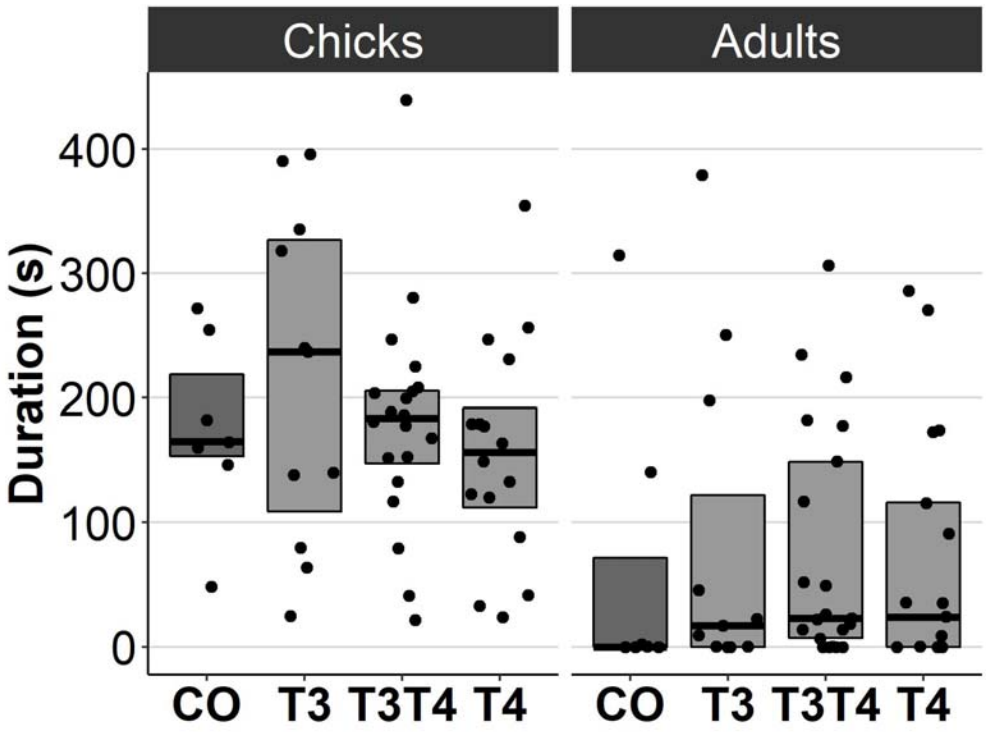

Figure 1. Movement duration for chicks (3 d) and adults (4.5 months) in the open arena test for different prenatal thyroid hormone treatment groups. The lines represent the medians of the treatments, and the bars represent the interquartile range of the original observations. Sample size (chicks/adults): CO 7/7, T3 11/11, T3T4 20/21 and T4 16/17.

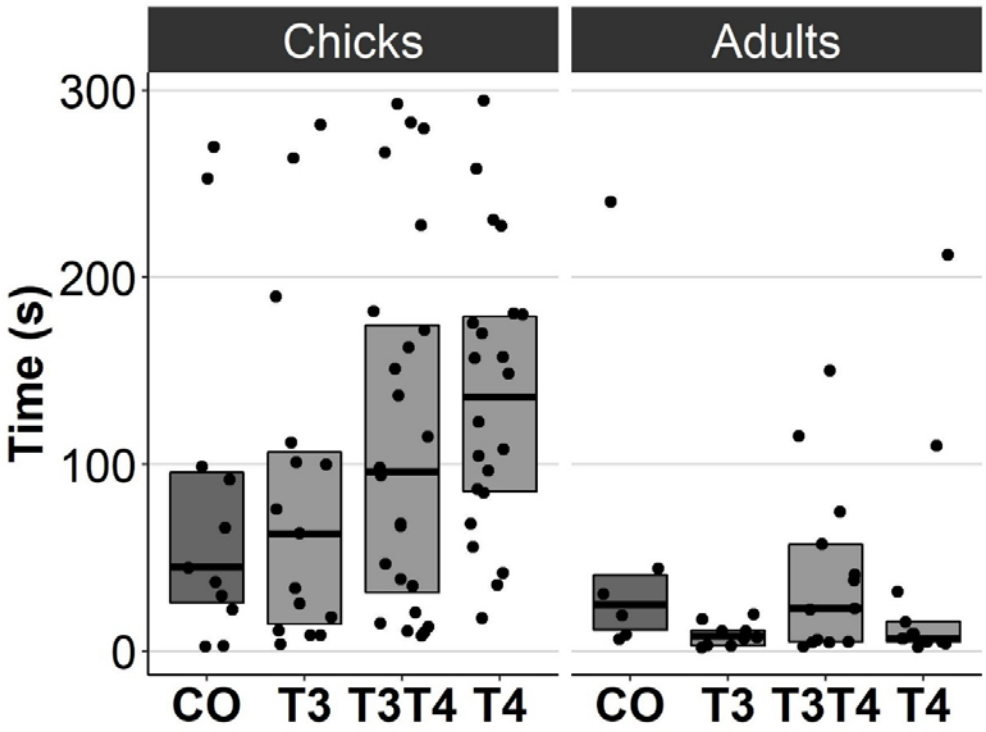

Figure 2. Time to exit the dark chamber for chicks (6-9 d) and adults (4.5 months) in the emergence test for different prenatal thyroid hormone treatment groups. The test was performed twice for chicks and once for adults. The lines represent the medians of the treatments, and the bars represent the interquartile range of the original observations. Sample size (chicks/adults): CO 11/6, T3 15/9, T3T4 24/13 and T4 22/13.

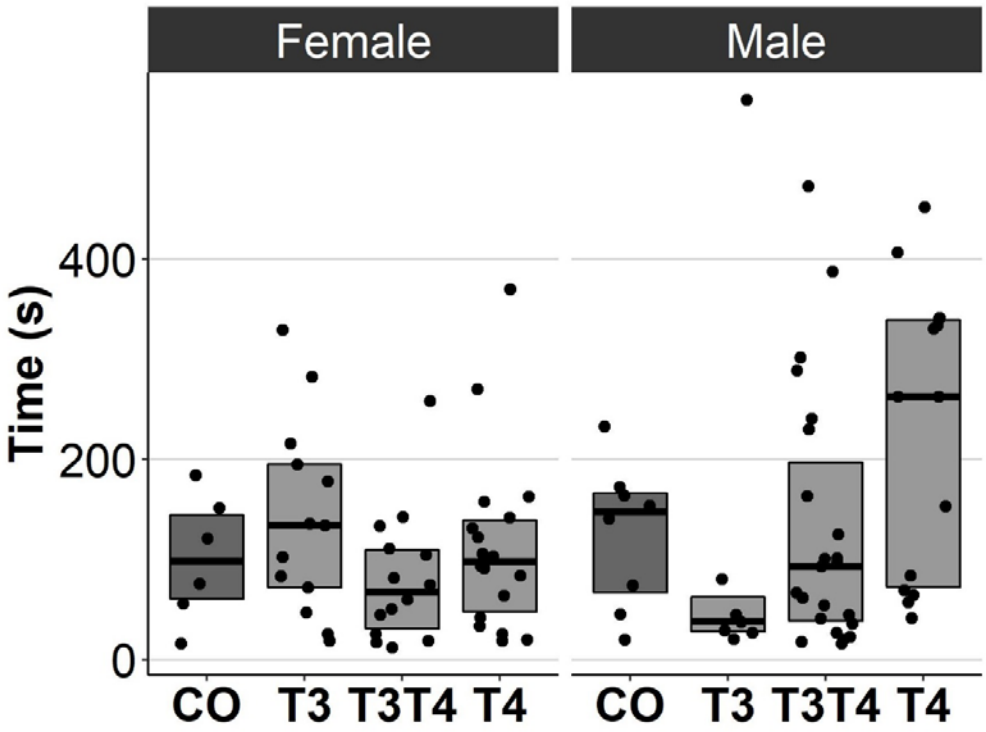

Figure 3. Time to resume standing position for females and males, chicks (12 d) and adults (4.5 months) combined, in the tonic immobility test for different prenatal thyroid hormone treatment groups. The lines represent the medians of the treatments, and the bars represent the interquartile range of the original observations. Sample size (female/male: CO 6/8, T3 13/7, T3T4 14/23 and T4 18/14.

Effects of prenatal thyroid hormone supplementation on plasma thyroid hormone levels

Increasing TH levels in eggs did not significantly affect plasma TH levels in 4.5-month-old birds (T3: $F = 0.13$, ddf = 37.7, $p = 0.94$, T4: $F = 2.1$, ddf = 41.5, $p = 0.12$, Fig. 4). Treatment also had no sex-dependent effect on plasma T3 or T4 (treatment-sex interactions, $p > 0.36$).

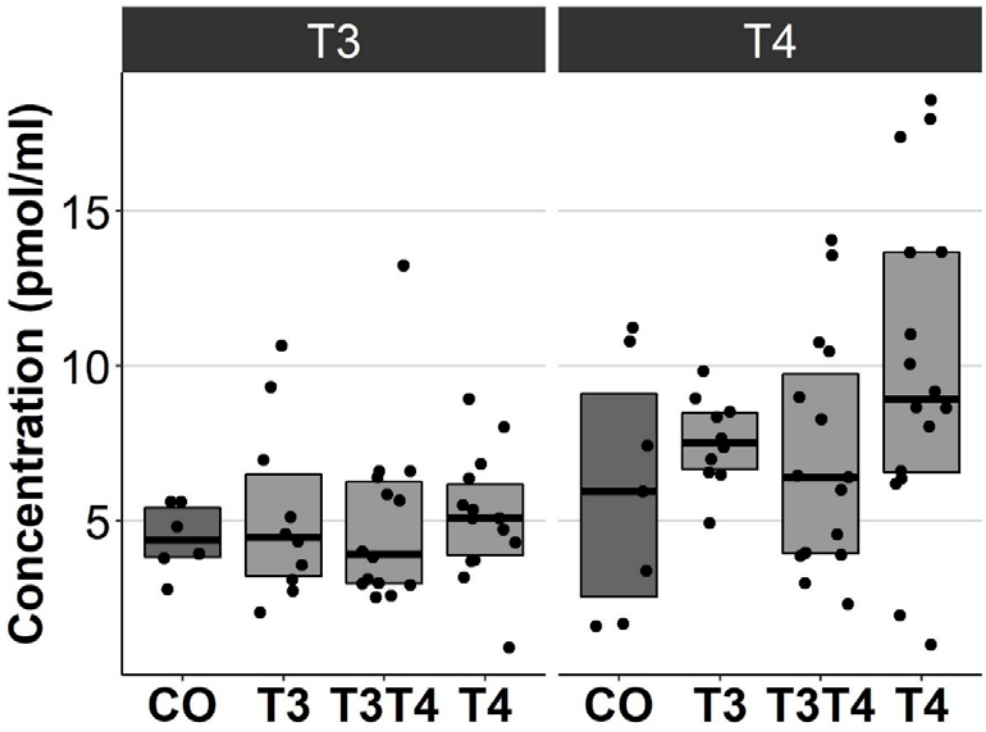

Figure 4. Plasma T3 and T4 concentrations in 4.5-month-old birds between different prenatal thyroid hormone treatment groups. The lines represent the medians of the treatments, and the bars represent the interquartile range of the original observations. Sample size (T3/T4 concentrations): CO 6/7, T3 10/10, T3T4 14/15 and T4 14/16.

Effects of prenatal thyroid hormone supplementation on pallial gene expression

Increasing TH levels in eggs did not affect pallial gene expression of TH-related genes in 10-month-old birds (*DIO2*: F = 2.49, ddf = 17.3, p = 0.09, *NCOA1*: F = 0.092, ddf = 21.1, p = 0.96, *THRA*: F = 0.47, ddf = 18.9, p = 0.71, Fig. 5). Treatment also did not have sex-dependent effect on gene expression (treatment-sex interactions, p > 0.26). Females had higher expression of *NCOA1* (F=7.68, p=0.012) and *THRA* (F=5.17, p=0.034) than males.

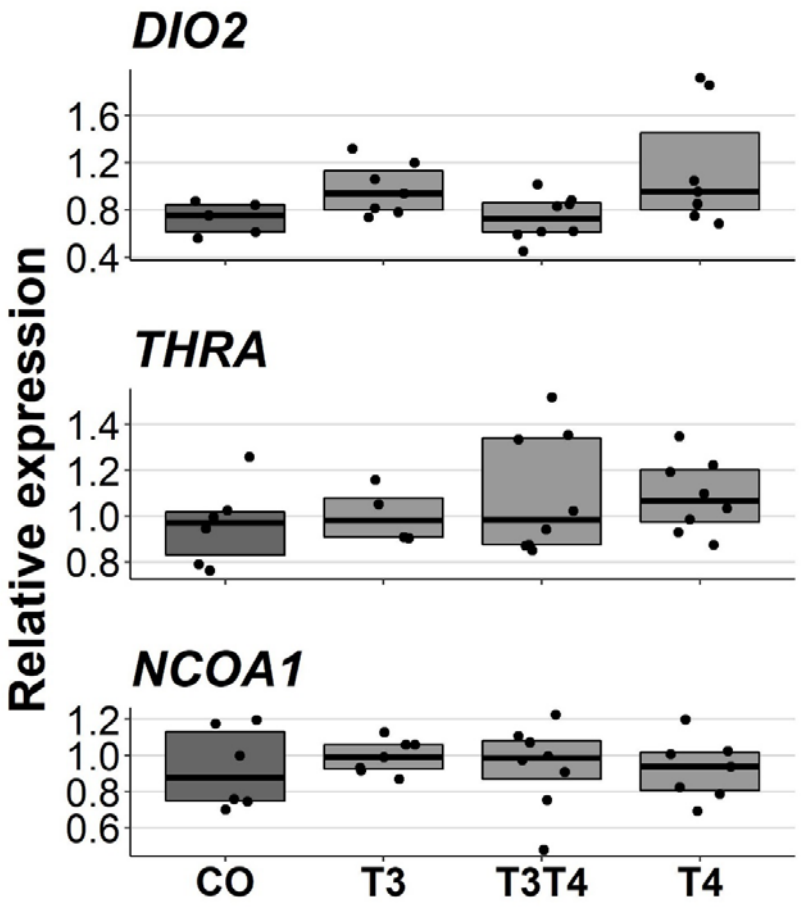

Figure 5. Relative expressions of *DIO2*, *THRA* and *NCOA1* in the pallium of 10-month-old birds between different prenatal thyroid hormone treatments. The lines represent the medians of the treatments, and the bars represent the interquartile range of the original observations. Sample sizes (CO/T3/T3T4/T4): DIO2 5/7/8/7, THRA 6/4/8/8 and NCOA1 6/7/8/7.

Discussion

In this study, we tested for long-term changes in behaviour, plasma TH levels and pallial gene expression following increased exposure to maternal THs. In terms of behaviour, we observed no significant changes in boldness, social motivation or fearfulness to predators in chicks (3-12 d) or adults (4.5 months) when the egg TH level was increased. To our knowledge, there are no similar previous studies, and it is

391 therefore difficult to assess whether there may have been changes in other
392 behavioural traits. The detection of changes was also partly limited by the small
393 sample size, which meant that we may not have had enough statistical power to detect
394 subtle changes in behaviour. Nevertheless, we found some age-dependent and sex-
395 dependent effects of TH treatment on behaviour. The age-dependent effect in the
396 emergence test suggests that elevated maternal TH leads to a decrease in boldness
397 and social motivation with age (from chick to adult), whereas such a decrease is not
398 observed in controls. However, the lack of differences between control and treatment
399 within a given age suggests that such an effect (if not associated with type I statistical
400 error) is rather mild. The sex-dependent effect in the tonic immobility test also seems
401 relatively unconvincing because no sex differences were observed within treatments,
402 nor did T3T4 females or T4 males differ from the same-sex controls. Given the
403 important role of THs in brain development, we expected that elevated maternal THs
404 would have led to at least some behavioural changes. Yet, our data suggest that an
405 experimental increase in maternal THs within the natural range has no clear effect on
406 the behavioural traits measured. However, in contrast to maternal THs, post-hatch TH
407 treatment has been shown to affect imprinting and learning (Yamaguchi et al., 2012).
408 The discrepancy between our results and Yamaguchi et al. (2012) could suggest that
409 the sensitive period during which THs can influence behaviour might occur at a later
410 timing of development, but this requires further investigation.

411 We found that elevated maternal THs did not significantly affect plasma TH
412 levels in adulthood (4.5 months of age). This was the first study to test for long-term
413 changes, whereas previously changes in response to elevated prenatal THs have been
414 observed at younger ages: Experimental elevation of egg THs was found to increase
415 male and decrease female plasma TH levels in rock pigeons at 14 days of age (Hsu et

al., 2017), but such effects were not observed in two other wild passerine species (Hsu et al., 2023b, 2020). In contrast to these studies in altricial species, not only was this study conducted on a precocial species, but our measurements were also made at a different life stage (adulthood). Overall, this suggests that an increase in maternal THs may only lead to transient changes in offspring plasma TH levels and that the effects may not be universal across species.

We also found no significant effect of elevated maternal THs on the expression of *THRA*, *DIO2* or *NCOA1* in the pallium of adult (10 months old) individuals. However, changes in gene expression have been observed in other TH-related genes, but again, at much shorter time spans: In chickens, injection of THs into the yolk decreased the expression of the TH-transporter *OATP1C1* and increased the expression of the TH-transporter *MCT8* in 4-day-old embryos (Van Herck et al., 2012). The expressions of our target genes, *THRA*, *DIO2* and *NCOA1,* have also been shown to be at least transiently responsive to THs in other species: *THRA* and *DIO2* in zebrafish embryos (Walpita et al., 2007), and *NCOA1* in adult rats (Misiti et al., 1998). It is therefore possible that the gene regulation by maternal THs is restricted to early development when the embryo is not yet able to produce THs. As the embryonic TH production begins, the influence of maternal THs might be expected to decrease, but studies on the relative contributions of maternal vs embryonic THs are lacking. Following embryonic TH production, the initially higher manipulated TH concentration may begin to level off and the differences in gene expression may disappear. Differences could still exist in adulthood, if higher maternal THs could lead to epigenetic changes, but this has not been studied with prenatal THs in their natural range. There is also no information on possible long-term changes in other brain regions or genes. Therefore,

it remains unknown whether higher maternal THs can lead to long-term changes in gene expression.

In general, the lack of changes in the measured traits following the elevation of egg THs could be explained if the higher egg TH levels did not lead to a higher TH concentration in the embryo. This could be due to the precise regulation of the transport of THs from the egg to the embryo. However, we have previously shown in the same experimental birds, that an increase in prenatal THs influences hatching success (Sarraude et al., 2020), suggesting that THs do influence prenatal development. Effects of prenatal THs on offspring growth and physiology have also been reported in other species (Hsu et al., 2023b, 2019a, 2017; Ruuskanen et al., 2016b; Stier et al., 2020). In terms of brain gene expression, it could be that tightly regulated TH transport to the developing brain prevents elevated maternal THs from affecting gene expression, but this would contradict the previously observed changes in brain gene expression following TH injection in chickens (Van Herck et al., 2012). It is also possible that embryos could buffer the effect of maternal THs by altering the expression of deiodinases (in the brain or other tissues). It has recently been shown in several species, including Japanese quail, that embryos express *DIO2* and *DIO3* at very early stages (Ruuskanen et al., 2022; Too et al., 2017). Metabolisation of THs to inactive forms in key tissues could reduce phenotypic variation caused by differences in egg TH levels. However, it is unlikely that buffering alone could explain the complete lack of changes in this study, given the effects on development previously observed in the same experimental birds (Sarraude et al., 2020). As it is, the lack of changes in TH-related gene expression in this study is likely to be explained by the stage of ontogeny, prenatal THs not leading to long-lasting effects. However, the lack of prenatal

measurements means that this cannot be fully confirmed, and it remains possible that elevated maternal THs have no effect on the measured traits in Japanese quails.

In conclusion, we found no evidence that elevated maternal THs cause long-term changes in behaviour, plasma TH levels or pallial gene expression. This is consistent with our previous findings in the same experimental birds, where elevation of prenatal THs (T3, T4, or in combination) did not lead to long-term effects on oxidative stress, moult or reproduction (Sarraude et al., 2020). While behavioural changes have not been previously studied, plasma TH concentrations and gene expression have been shown to respond to elevated maternal THs in the short-term (Hsu et al., 2017; Van Herck et al., 2012). However, based on this study, these changes would appear to be limited to early development or shortly after hatching with the offspring's own TH production later replacing the effects of elevated maternal THs. Maternal THs could theoretically influence the offspring's own TH production at a later age by altering the function of the HPT-axis, but no evidence has yet been found to support this hypothesis. The long-term effects of variation in maternal THs remain unclear, but this study has provided new information on some previously untested traits. Understanding long-term effects in addition to short-term effects will help to better understand the evolutionary and ecological importance of maternal THs.

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Data availability statement

Data will be available on Figshare (DOI: 10.6084/m9.figshare.22266211) at publication.

Author contributions

S.R., T.S., B-Y.H, A.S and K.A. designed the study. S.R. obtained funding. T.S., B-Y.H and S.R conducted the experiment. K.A. and T.S. collected the behavioural data from videos. K.A., A.S and S.R. collected the physiological data and conducted laboratory work. K.A. analysed all data with support from all co-authors, and wrote the first draft of the manuscript. A.S, B-Y.H, T.S. and S.R commented on the manuscript.

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