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

4

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32 **Abstract**

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

54

55 **Keywords:** maternal hormones, maternal effects, endocrinology, phenotypic plasticity,
56 bird

57 **Introduction**

58 Maternal effects refer to the effect of the maternal phenotype or environment on
59 the phenotype of the offspring. The effect can be mediated by hormones passed from the
60 mother to the offspring, the maternal hormones. As the levels of maternal hormones are
61 influenced by the maternal environment, they can transmit information about the maternal
62 environment to the offspring. This allows the development of the offspring phenotype to be
63 controlled by environmental factors (Marshall and Uller, 2007; Mousseau and Fox, 1998).
64 Such hormone-mediated maternal effects can be observed in a wide range of traits,
65 including physiology and behaviour (Groothuis et al., 2019, 2005; Ruuskanen and Hsu,
66 2018), and can have long-lasting effects into adulthood with potential fitness consequences
67 (Groothuis et al., 2005; Schoech et al., 2011; von Engelhardt and Groothuis, 2011).

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

81 development, allowing the embryo to regulate TH levels in an organ and cell-specific
82 manner (Darras, 2019; Darras et al., 2011; Flamant and Samarut, 1998; Ruuskanen et al.,
83 2022; Van Herck et al., 2012). However, the development of the thyroid gland occurs much
84 later in development (Forhead and Fowden, 2014; McNichols and McNabb, 1988; Porazzi et
85 al., 2009). Thus, in almost all vertebrates studied so far, proper embryonic development
86 depends on maternally transferred THs (Brown et al., 2014; Darras, 2019; Moog et al., 2017;
87 Patel et al., 2011).

88 In mammals, maternal THs are transferred across the placenta, whereas in egg-laying
89 animals such as birds, maternal THs are deposited in the egg yolk. In birds, high natural
90 variation in egg TH levels has been observed, up to 5-fold in magnitude (Ruuskanen and Hsu,
91 2018). This variation may be due to environmental factors such as food availability or
92 temperature (Hsu et al., 2016; Ruuskanen et al., 2016c), and has been reported at both the
93 inter- and intra-individual levels (Hsu et al., 2016, 2019b; Ruuskanen et al., 2016a, 2016c).
94 As THs are important in development (Darras et al., 2009; Decuypere et al., 2005; McNabb,
95 2007; Wilson and McNabb, 1997), and as increasing yolk TH levels has been found to
96 increase embryonic TH levels (Van Herck et al., 2012), high variation in egg TH levels could
97 therefore be a source of offspring phenotypic variation.

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

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

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

129 dependent manner in the rock pigeons (Hsu et al., 2017), some more recent studies in wild
130 passerines found no clear effects of egg THs on nestling plasma THs (Hsu et al., 2023b,
131 2020). In terms of gene expression, previous studies have examined the effect of the natural
132 variation in maternal THs on brain gene expression only in bird embryos or young chicks
133 (Van Herck et al., 2015, 2012; Yamaguchi et al., 2012), while the potential long-term effects
134 of prenatal TH exposure remain unknown. Following our previous experiment manipulating
135 egg TH levels in Japanese quail (Sarraude et al., 2020), here we studied several genes
136 involved in TH function: *THRA* (thyroid hormone receptor A), *NCOA1* (nuclear receptor
137 coactivator 1) and *DIO2* (type 2 deiodinase) in the pallium of adults. Expression of these
138 genes has been shown to be influenced by THs in other species (Misiti et al., 1998; Walpita
139 et al., 2007).

140

141 **Materials and methods**

142

143 *1.1 Materials*

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

151

152 *1.2 Treatment of eggs, rearing of chicks and collection of samples*

153 We manipulated egg TH levels by injecting hormones directly into the eggs prior to
154 embryonic development at a dose that mimicked increased maternal transfer (see details in
155 Sarraude et al., 2020). Eggs from each breeding pair were randomly assigned to four
156 treatment groups: T3, T4, T3 + T4 and control (0.9% saline). Eggs laid by the same pair were
157 always evenly distributed among all treatment groups. The injected hormone solutions were
158 prepared from crystallised powder (T3: CAS number 6893-02-3, Sigma-Aldrich, St. Louis,
159 MO, USA and T4: CAS number 51-48-9, Sigma-Aldrich, St. Louis, MO, USA). The amount of
160 hormone injected was based on previous measurements in the parental generation (mean \pm
161 SD: T4, 15.3 ± 4.43 ng / egg; T3, 7.58 ± 2.32 ng / egg) and the aim was to increase hormone
162 contents in the yolk by two standard deviations (as recommended by Podmokla et al.,
163 2018). The injection protocol has been described in detail in Sarraude et al. (2020). Briefly,
164 each egg was injected with 50 μ l of either the respective hormone solution (T3, T4 or T3+T4,
165 in 0.9% saline) or 0.9% saline (control, CO), delivered by using 27G insulin syringes (BD
166 Microlance™). After sealing the hole with sterile plaster (OPSITE Flexigrid, Smith &
167 Nephew), the eggs were incubated artificially (37.8 °C and 55% relative humidity). A total of
168 158 eggs were treated (39 T3, 39 T4, 40 T3+T4 and 40 CO), of which 66 chicks hatched (15
169 T3, 20 T4, 21 T3 + T4 and 10 CO), with sex distributions of chicks surviving to adulthood
170 (females / males): 7/4 for T3, 10/8 for T4, 9/12 for T3 + T4 and 3/4 for CO. Sex was
171 determined at maturity by examining the presence of the cloacal gland. The overall hatching
172 rate (~40%) was consistent with previous studies in domestic quail (Okuliarová et al., 2007).

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

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

182

183 *1.3 Behavioural experiments*

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

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

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

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

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

223

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 μ l 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 μ l 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/ μ l 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

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

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

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270 **Table 1: qPCR primer set information and performance for gene expression assays.** Cq refers to
 271 qPCR quantification cycle, efficiency was evaluated using the LinReg method based on individual well
 272 efficiency. Technical precision estimates are given for Cq as coefficient of variation CV (%) and for
 273 expression (ratio of target to reference genes) as technical repeatability (*R*).
 274

Gene	Primers	Cq ± SD	Efficiency	CV Cq	Repeatability expression
GAPDH	F: TCTCTGTTGTTGACCTGACCTG R: ATGGCTGTACCATTGAAGTC	15.0±0.50	98.0%	0.59%	
PGK1	F: CAAGCTCACCTGGACAAGT 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: GTGTCGATGTCCCGATGTT	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

275

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

288 our results. Technical precision and repeatability were generally good (Table 1), but a few
289 samples with a CV% of duplicates (based on the final ratio) greater than 15% were excluded
290 from the analysis (final sample size: $N_{DIO_2} = 27$, $N_{NCOA1} = 28$, $N_{THR_a} = 26$)

291

292 *1.6 Statistical analysis*

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

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

312

313 **Results**

314 *Effects of prenatal thyroid hormone supplementation on behaviour*

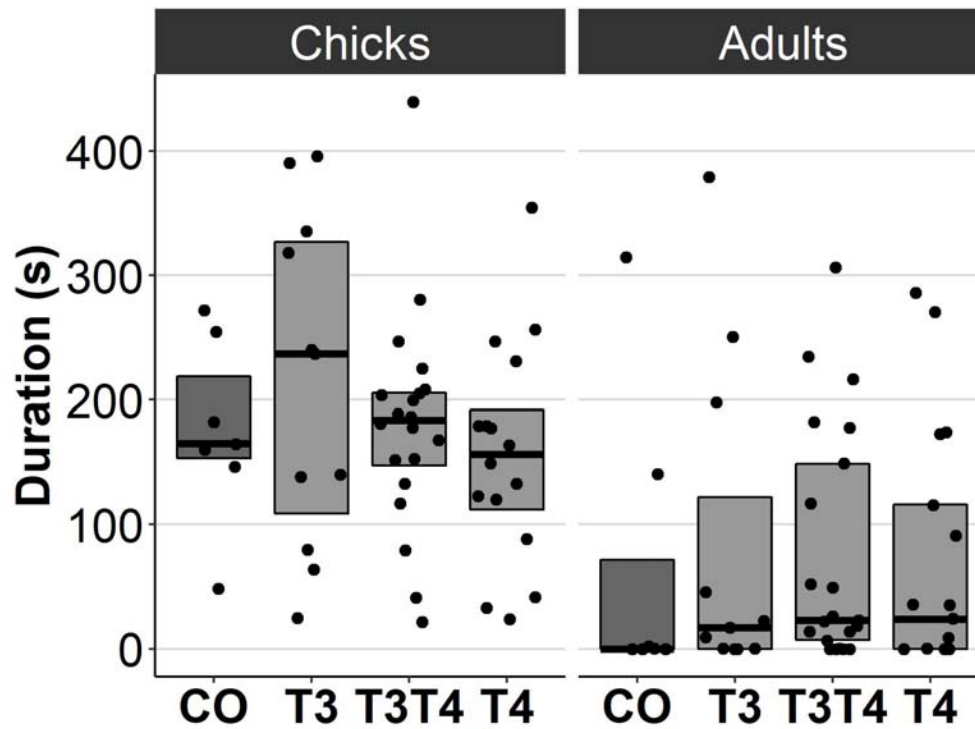
315 In general, no statistically significant differences in chick or adult behavioural traits
316 were observed between the egg TH manipulation groups (T3, T4, T3 + T4, control). Elevated
317 THs had no significant effect on social motivation or boldness (Table 2, Fig. 1-2), nor on
318 fearfulness to predators (Table 2, Fig. 3). A statistically significant interaction between
319 treatment and age was found in the emergence test ($F = 3.58$, $ddf = 67.2$, $p = 0.018$, Fig. 2):
320 In all treatments except the control group (Tukey $t=0.59$, $p=0.56$), chick exit times were
321 significantly longer than those of adults (all $t > 3.21$, $p < 0.002$) while no differences between
322 treatments were found within each age-category (all $t < 2.48$, all $p > 0.07$). There was also a
323 statistically significant interaction between treatment and sex in the tonic immobility test (F
324 $= 2.92$, $ddf = 37.5$, $p = 0.047$, Fig. 3): In males, the recovery time was longer in the T4-group
325 (marginal mean \pm SE: 177.1 ± 47.8 s) than in the T3-group (marginal mean \pm SE: 56.1 ± 20.8
326 s, Tukey $t = 2.71$, $p = 0.047$), whereas no differences between treatments were observed in
327 females (all $t < 2.29$, all $p > 0.12$). In the T4-group, the recovery time of males (marginal
328 mean \pm SE: 177.1 ± 47.8 s) was longer than that of females (marginal mean \pm SE: 85.2 ± 19.9
329 s) ($t=2.16$, $p= 0.037$), while no sex differences were observed within the other treatments
330 (all $t < 1.80$, all $p > 0.079$).

331

332 **Table 2.** Marginal means and standard errors of behavioural tests. Results for chicks (age: 3d,
 333 6-9d, 12d) and adults (age: 4.5 months) are shown separately for each variable. F- and p-values
 334 for treatment from linear models (ANOVA, Kenward-Roger approximation). Test statistics (χ^2)
 335 and p-values for binomial regressions (model with treatment compared to model without
 336 treatment) were calculated with ANOVA.
 337

<i>Test: measurement</i>	<i>Age</i>	<i>Control</i>	<i>T3</i>	<i>T3T4</i>	<i>T4</i>	<i>F_{ddf}/χ² (df)</i>	<i>P</i>
<i>Tonic immobility:</i>	12 d	92.0±25.5	84.6±21.1	68.4±13.1	123±24.7	2.52 _{36,2}	0.073
<i>Recovery time (s)</i>	4.5 m	90.6±25.3	83.3±21.4	67.3±13.6	121±25.5		
<i>Open arena:</i>	3 d	77.2±31.1	100±27.1	73.3±19.1	63.3±20.8	0.44 _{46,6}	0.72
<i>Movement duration (s)</i>	4.5 m	181±31.6	204±28.1	177±19.3	167±21.3		
<i>Open arena:</i>	3 d	5.85±3.87	4.14±3.53	8.25±2.14	7.21±2.43	0.35 _{41,7}	0.79
<i>Escape attempts (number)</i>							
<i>Emergence:</i>	6-9 d	34.8±14.7	42.7±16.4	74.7±20.9	121±36.1	1.92 _{39,6}	0.14
<i>Exit time (s)</i>	4.5 m	25.6±12.5	6.59±2.75	23.0±7.64	12.6±4.30		
<i>Emergence:</i>	6-9 d	0.80±0.11	0.70±0.12	0.62±0.10	0.58±0.10	2.71 (3)	0.44
<i>Likelihood of emergence (0-1)</i>	4.5 m	0.91±0.06	0.86±0.08	0.81±0.08	0.78±0.09		

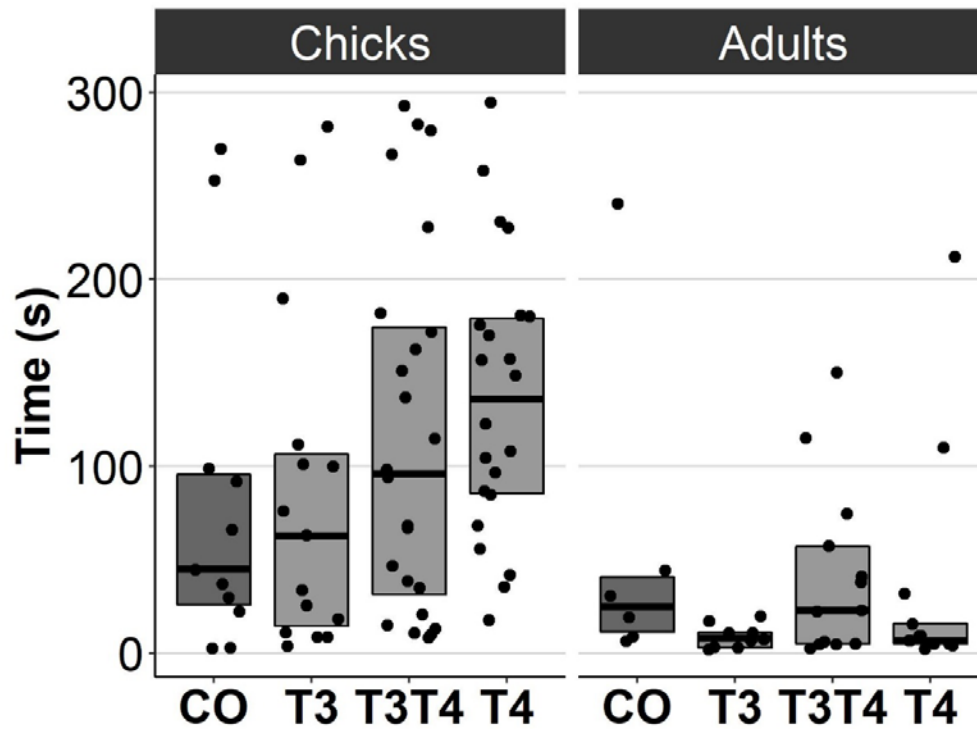
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339

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

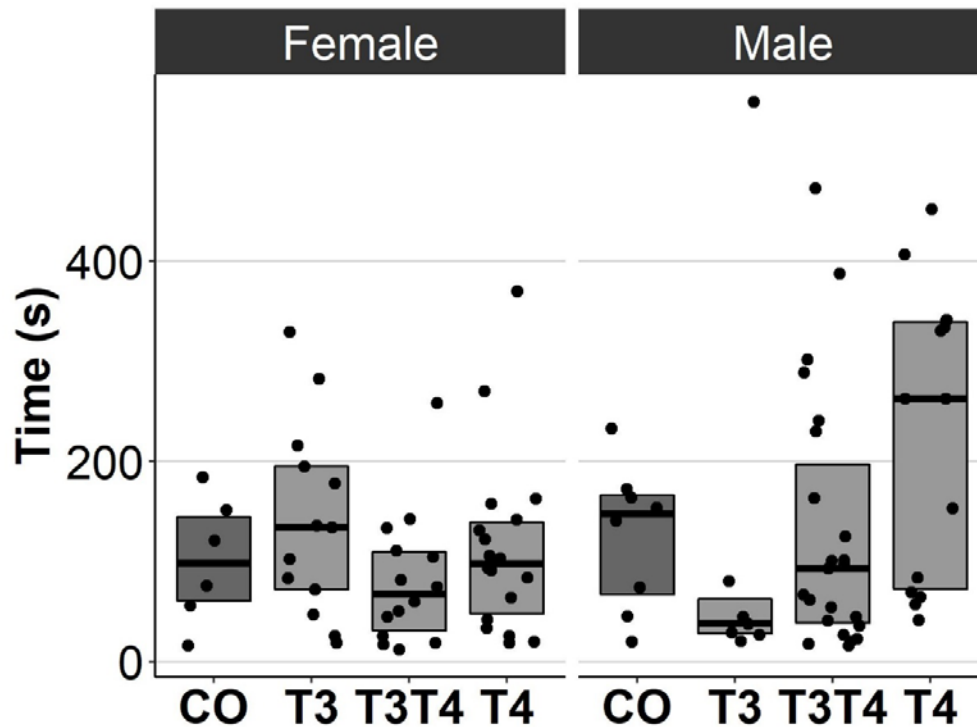
344



345

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

351



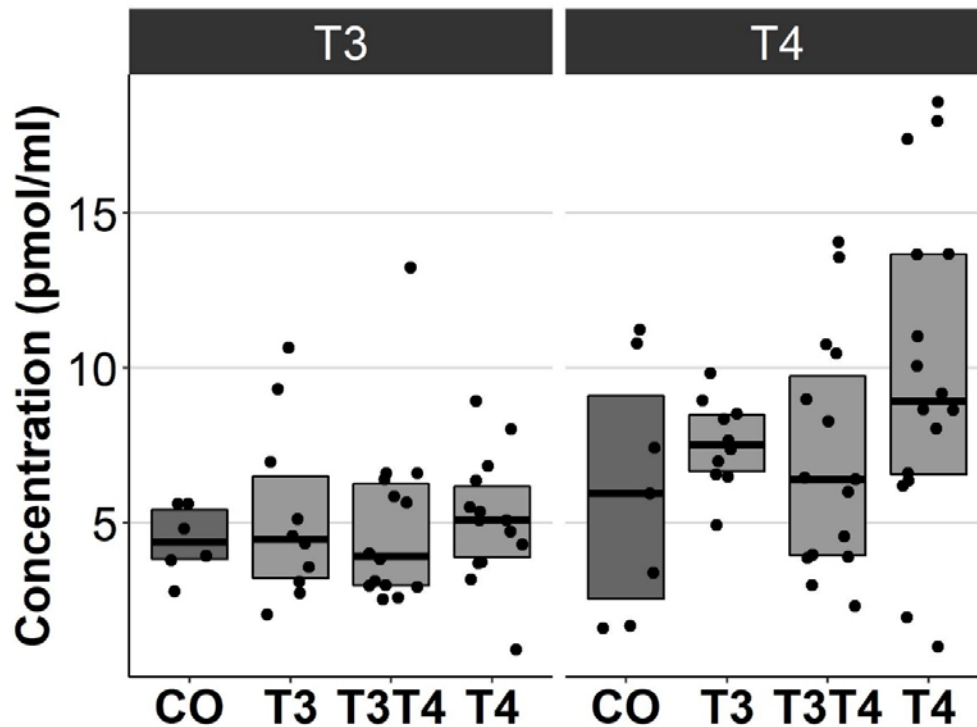
352

353 **Figure 3.** Time to resume standing position for females and males, chicks (12 d)
354 months) combined, in the tonic immobility test for different prenatal thyroid hormone
355 treatment groups. The lines represent the medians of the treatments, and the bars represent
356 the interquartile range of the original observations. Sample size (female/male: CO 6/8, T3
357 13/7, T3T4 14/23 and T4 18/14.

358

359 *Effects of prenatal thyroid hormone supplementation on plasma thyroid*
360 *hormone levels*

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



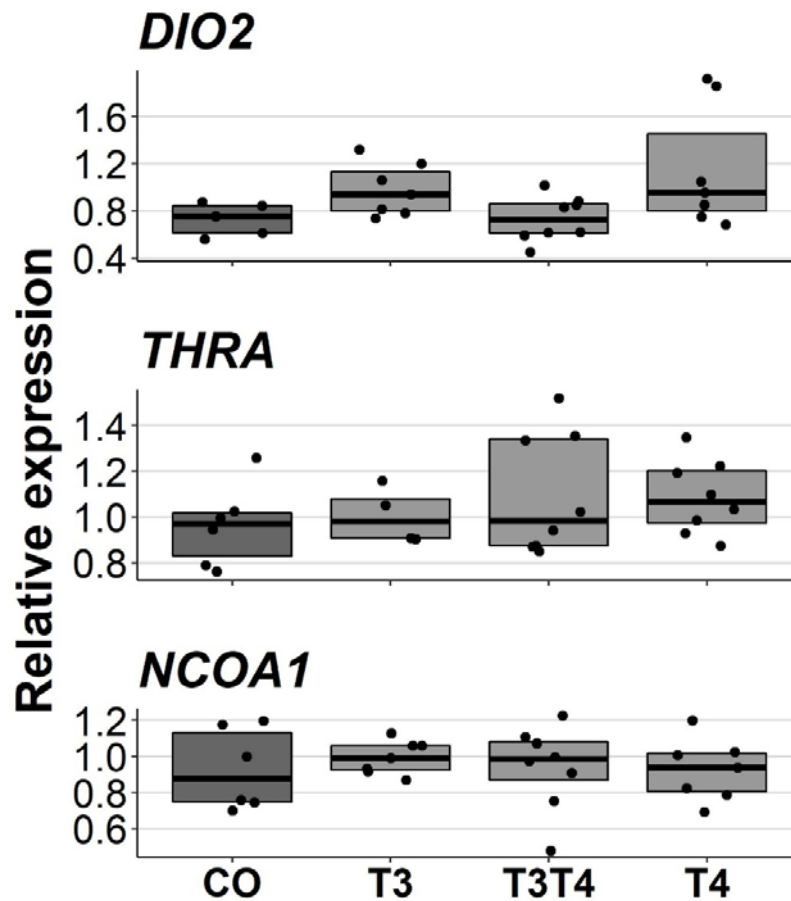
365

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

370

371 *Effects of prenatal thyroid hormone supplementation on pallial gene*
372 *expression*

373 Increasing TH levels in eggs did not affect pallial gene expression of TH-related
374 genes in 10-month-old birds (*DIO2*: $F = 2.49$, $ddf = 17.3$, $p = 0.09$, *NCOA1*: $F = 0.092$, ddf
375 $= 21.1$, $p = 0.96$, *THRA*: $F = 0.47$, $ddf = 18.9$, $p = 0.71$, Fig. 5). Treatment also did not
376 have sex-dependent effect on gene expression (treatment-sex interactions, $p > 0.26$).
377 Females had higher expression of *NCOA1* ($F=7.68$, $p=0.012$) and *THRA* ($F=5.17$,
378 $p=0.034$) than males.



379

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

384

385 **Discussion**

386 In this study, we tested for long-term changes in behaviour, plasma TH levels
387 and pallial gene expression following increased exposure to maternal THs. In terms of
388 behaviour, we observed no significant changes in boldness, social motivation or
389 fearfulness to predators in chicks (3-12 d) or adults (4.5 months) when the egg TH level
390 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

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

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

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

442 In general, the lack of changes in the measured traits following the elevation of
443 egg THs could be explained if the higher egg TH levels did not lead to a higher TH
444 concentration in the embryo. This could be due to the precise regulation of the
445 transport of THs from the egg to the embryo. However, we have previously shown in
446 the same experimental birds, that an increase in prenatal THs influences hatching
447 success (Sarraude et al., 2020), suggesting that THs do influence prenatal
448 development. Effects of prenatal THs on offspring growth and physiology have also
449 been reported in other species (Hsu et al., 2023b, 2019a, 2017; Ruuskanen et al.,
450 2016b; Stier et al., 2020). In terms of brain gene expression, it could be that tightly
451 regulated TH transport to the developing brain prevents elevated maternal THs from
452 affecting gene expression, but this would contradict the previously observed changes
453 in brain gene expression following TH injection in chickens (Van Herck et al., 2012). It is
454 also possible that embryos could buffer the effect of maternal THs by altering the
455 expression of deiodinases (in the brain or other tissues). It has recently been shown in
456 several species, including Japanese quail, that embryos express *DIO2* and *DIO3* at very
457 early stages (Ruuskanen et al., 2022; Too et al., 2017). Metabolisation of THs to
458 inactive forms in key tissues could reduce phenotypic variation caused by differences
459 in egg TH levels. However, it is unlikely that buffering alone could explain the complete
460 lack of changes in this study, given the effects on development previously observed in
461 the same experimental birds (Sarraude et al., 2020). As it is, the lack of changes in TH-
462 related gene expression in this study is likely to be explained by the stage of ontogeny,
463 prenatal THs not leading to long-lasting effects. However, the lack of prenatal

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

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

482

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486

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492

493 Data availability statement

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

495

496 Author contributions

497 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

498 S.R conducted the experiment. K.A. and T.S. collected the behavioural data from

499 videos. K.A., A.S and S.R. collected the physiological data and conducted laboratory

500 work. K.A. analysed all data with support from all co-authors, and wrote the first draft

501 of the manuscript. A.S, B-Y.H, T.S. and S.R commented on the manuscript.

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