Causes and consequences of natural variation in maternal thyroid hormones in avian eggs

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Chapter 1

General introduction, aims and methods, general discussion and synthesis

Tom Sarraude

Phenotypic plasticity and parental effects

Evolution by natural selection predicts that the variety of phenotypes among individuals results in fitness differences according to the encountered environmental conditions. Such phenotypic and fitness differences have a heritable component that is transmitted to the following generations, and evolution selects the variants that have the highest fitness.

In the Modern Synthesis (MS), the current paradigm in evolutionary biology, inheritance is necessarily genetic; that is, parents transmit genes to their offspring that are responsible for the offspring performance and fitness. This traditional view is now challenged by the growing and undisputable evidence that inheritance is not only genetic, but also non-genetic. Biological information transmitted from one generation to the next include epigenetic modifications of gene expression (epigenetic inheritance), transmission of environmental changes induced by individuals (ecological inheritance), and transmission of non-genetic components affecting offspring phenotype (parental non-genetic effects), all reviewed by Danchin et al. (2011). Because parental influence on offspring phenotype and fitness can no longer be seen as genetic inheritance only (as assumed by the MS), several authors are calling for a revision of the current paradigm. The Extended Evolutionary Synthesis (EES) aims at incorporating all the non-genetic inheritance (inclusive inheritance) (Pigliucci, 2007; Laland et al., 2014). The need for such an EES has been under debate for about a decade now (advocates, Laland et al., 2015; Müller, 2017; Tanghe et al., 2018; opponents, Welch, 2017; Stoltzfus, 2017).

Phenotypic, or developmental, plasticity is defined as the ability of a genotype to produce different phenotypes under different environments. This plasticity allows an individual to rapidly cope with changing conditions by producing a phenotype matching the new environment (Levis & Pfennig, 2019). According to Uller (2008), parental effects are a form of phenotypic plasticity, with parental phenotype affecting offspring phenotype. Although both parents can exert so-called parental effects, most of the research has been conducted on maternal effects. Once regarded as background noise in genetic studies, maternal effects are now viewed as a potential tool for mothers to prepare their offspring for expected environmental conditions (i.e. “adaptive maternal effect”, (Mousseau & Fox, 1998a; Marshall & Uller, 2007). There is now strong evidence that maternal effects can be adaptive and are widespread across many taxa (Moore, Whiteman & Martin, 2019; Yin et al., 2019). Maternal effects are an important source of plasticity as they can prepare the progeny for expected environmental conditions. Embryos only have limited possibilities to assess their own environment and must therefore rely on maternal signalling. By responding to maternal signalling, the progeny can develop the appropriate phenotype to anticipate its future environment (Kuijper & Johnstone, 2018).

Maternal hormones

Maternal hormones when transmitted from the mother to the offspring are one potential pathway for mothers to influence their offspring's phenotype, and have been identified as such across many taxa (insects, Mousseau & Dingle, 1991; fish, Campinho et al., 2014; reptiles, Uller, Astheimer & Olsson, 2007; birds, Groothuis et al., 2005; mammals, Dufty, 2002). Hormonal signalling is highly dynamic, with a short reaction time to environmental cues and a rapid turnover. Therefore, mothers may use this signalling pathway to communicate reliable information about its environment to the offspring. In vertebrates, most of the research on maternal hormones has so far focused on steroid hormones, i.e., androgens and glucocorticoids (Ruuskanen & Hsu, 2018; Groothuis et al., 2019). These hormones have been found to be associated with a broad suite of offspring traits related to their fitness (e.g., begging behaviour, growth, immune function, stress response; reviewed by Groothuis et al., 2019). This long list of traits reflects the pleiotropy of hormones, a key aspect of hormonal signalling. This pleiotropy implies that variations in hormone levels may bring costs as well as benefits on offspring fitness. Because of these costs, individuals may face trade-offs between traits (e.g., elevated testosterone enhances aggressiveness and competitiveness but lowers immune functions). Therefore, the balance between the benefits and costs associated with maternal hormones may depend on the environmental context. For example, in an environment with high parasitism, mothers may transfer less androgens into their eggs to favour nestlings' immune system over growth (e.g., Tschirren, Richner & Schwabl, 2004).

As mentioned above, maternal THs have been much less studied than steroid hormones, despite clear effects of these hormones on human foetus development for example (Medici et al., 2013; Korevaar et al., 2016), but also in wild species such as fish, amphibians and birds (Ruuskanen & Hsu, 2018). Besides, thyroid hormone production requires iodine, a trace element that cannot be synthesised *de novo* by an organism. Therefore, THs may be costly to produce contrary to steroid hormones that are produced from cholesterol, abundantly present in an organism. Such potential cost of TH production may be a source of conflict between parents and offspring (discussed in the synthesis).

Thyroid hormones

What are THs?

Thyroid hormones are produced by the thyroid gland, located in the lower neck and found in all vertebrates (Angell, Huang & Alexander, 2018). The thyroid is under the control of the hypothalamic–pituitary axis and thus form the hypothalamic-pituitary-thyroid (HPT) axis. The hypothalamus produces two hormones that have stimulatory effects on the pituitary: the thyrotropin-releasing hormone (TRH) and the corticotropin-releasing hormone (CRH). TRH is the main regulatory hormone of the pituitary in mammals, while CRH exerts most of the hypothalamic control on the pituitary in non-mammalian vertebrates (McNabb & Darras, 2015). Both hormones stimulate the production of the thyroid-stimulating hormone (TSH) by the pituitary, which is the main regulatory hormone of thyroid hormone synthesis (Angell, Huang & Alexander, 2018). THs in turn exert a negative feedback on the HPT axis.

Thyroid hormones are metabolic hormones that are present in two main forms: thyroxine (T_4) and triiodothyronine (T_3). The thyroid gland production consists mostly of T_4, with lesser amounts of T_3. TH action mainly depends on TH receptors that have a greater affinity for T_3 than for T_4 (10 to 50 times greater: Zoeller, Tan & Tyl, 2007; Angell, Huang & Alexander, 2018). This is why T_4 is mostly seen as a precursor of T_3, the biological active form. The main pathway of TH metabolism consists in sequential removal of an iodine atom from the outer ring (outer ring deiodination, ORD) or from the inner ring (inner ring deiodination, IRD). ORD and IRD are catalysed by deiodinase enzymes and occur in the tissues (i.e., peripheral conversion) (Figure 1). There are 3 types of deiodinases: D1, D2 and D3. D1 can catalyse mostly ORD with some IRD activity, while D2 only catalyses ORD and D3 only IRD. Therefore, D1 and D2 are the main activators of T_4 by converting it into T_3, while D3 mostly deactivates T_4 and T_3 by conversion into reverse-T_3 and diiodothyronine (T_2), respectively. As shown in Figure 1, iodine is a key component of THs. Iodine cannot be synthesised by an organism and has therefore to be found in the environment. This need in iodine may bring a cost to TH production, especially when iodine availability is limited.

THs exert most of their actions by binding to nuclear receptors, the thyroid hormone receptors (TRs). TRs bind to DNA next to transcription control region of target genes. In addition to genomic effects (by binding to TRs), THs can also exert non-genomic effects (reviewed by Davis, Goglia & Leonard, 2016).

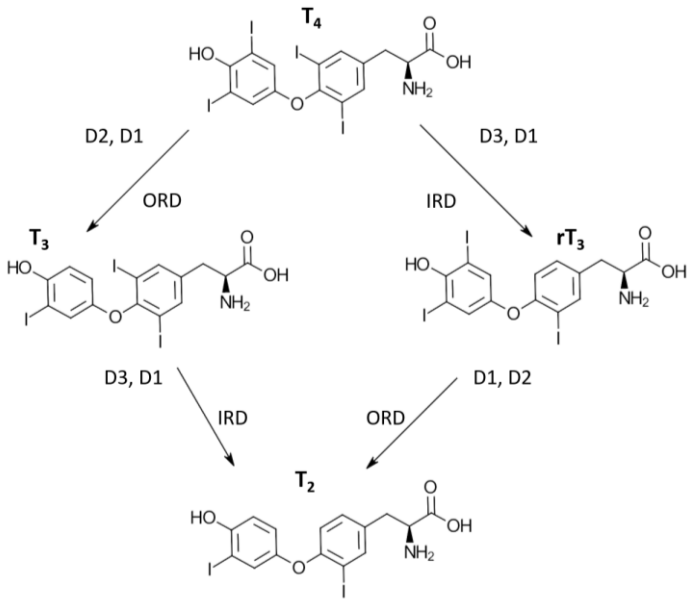

Figure 1: TH metabolism. D1 = deiodinase type 1; D2 = deiodinase type 2; D3 = deiodinase type 3; ORD = outer ring deiodination; IRD = inner ring deiodination. Inner ring refers to the ring closest to the carbon chain, while outer ring refers to the ring farthest from the carbon chain.

Role and importance of THs

THs are pleiotropic hormones that play an essential role in development, metabolism and reproduction in all vertebrates. THs are important for normal embryonic and juvenile growth (mammals, Pascual & Aranda, 2013; birds, McNabb & Darras, 2015; fish, Deal & Volkoff, 2020). They influence metabolism, nutrient efficiency and energy expenditure (Kim, 2008; McNabb & Darras, 2015; Deal & Volkoff, 2020). In homeotherms, THs are responsible for heat production, necessary for thermoregulation (McNabb & Darras, 2015). THs are also involved in reproductive processes, such as gonadal development and, in oviparous species, egg-laying (McNabb & Darras, 2015; Deal & Volkoff, 2020). THs also participate in life stage transitions. In oviparous species, THs facilitate hatching as seen by the surge in THs in the perihatch period (De Groef, Grommen & Darras, 2013). Likewise, THs are necessary for metamorphosis in fish and amphibians (e.g., Laudet, 2011; De Groef, Grommen & Darras, 2018).

THs and TH-derivative are also found in lower vertebrates and invertebrates, with similar structure and mode of action (genomic and non-genomic), and TH signalling plays a role on development and metamorphosis (Holzer, Roux & Laudet, 2017; Taylor & Heyland, 2017).

Environmental influences on THs

Environmental factors such as ambient temperature and food intake are known to influence the HPT axis (reviewed by McNabb & Darras, 2015). Cold temperatures stimulate TH availability (mostly T_3) to increase metabolism and thermogenesis, primarily via increased conversion from T_4 to T_3 (McNabb & Darras, 2015). On the other hand, warm temperatures have a suppressive effect on thyroid function. Food availability also affects circulating THs, with food restriction generally decreasing plasma T_3, while the effect on plasma T_4 may differ between species (Darras et al., 1995).

Environmental contamination to endocrine-disruptive chemicals (EDCs) also alters thyroid function, with PCBs being among the first EDCs identified in birds (McNabb & Fox, 2003). Prenatal exposure to PCBs can have long-lasting consequences in wild populations such as reduced reproductive success in herring gulls (McNabb & Fox, 2003) or reduced growth in alligators (Boggs et al., 2013). Other EDCs have been identified more recently as thyroid disruptors, such as metals (e.g., mercury, copper), perchlorate, products of oil combustion (e.g., PAHs) (reviewed by Matthiessen, Wheeler & Weltje, 2018), and polyfluorinated chemicals (PFCs). PFCs and their derivatives are synthetic substances that are widely used for their lipophobic and hydrophobic properties in various industries, such as textile, clothing, cosmetics or food packaging (Jensen & Leffers, 2008). PFCs are global contaminants that are found in all environmental compartments (i.e., air, water and soil). Because PFCs are stable compounds, they travel long distances with oceanic or atmospheric currents, are even found in polar areas, and accumulate and magnify along the food chain (Jensen & Leffers, 2008). PFCs and their derivatives are also considered EDCs as they affect sex hormone production in rat (reviewed by Jensen & Leffers, 2008). Recently, perfluoroalkyl and polyfluoroalkyl substances (PFASs), which are PFC derivatives, have been studied as potential disruptors of the HPT axis and metabolic activity. PFASs were positively correlated with circulating THs in wild peregrine falcon nestlings (*Falco peregrinus*, Sun et al., 2021), and positively associated with BMR in breeding female black-legged kittiwakes (*Rissa tridactyla*, Blévin et al., 2017), although no correlations were found in that study between PFASs and circulating THs (Blévin et al., 2017). Another study found a negative association between PFASs and breeding parameters in great tits, though the overall effects on breeding success were mild (Groffen et al., 2019). A recent experimental study investigated the effects of a mixture of PFASs on chicken embryos and found a decrease in TH production (Mattsson et al., 2019). Clearly, more experimental studies are needed to assess the consequences of PFASs, but current evidence points towards a disruptive effect of PFCs on the HPT axis, with potential effects on metabolic rate and reproduction.

Thyroid hormones in avian eggs

Maternal thyroid hormones have long been detected in egg yolks of chicken (Hilfer & Searls, 1980; Prati et al., 1992) and Japanese quail (*Coturnix japonica*, Wilson & McNabb, 1997). THs are mostly present in the egg yolk (75–95%, McNabb & Wilson, 1997), with fewer amount in the albumen. Recent studies in altricial species have demonstrated the effect of physiological variation of prenatal THs on early life (great tits, *Parus major*, Ruuskanen et al., 2016; rock pigeons, *Columba livia*, Hsu et al., 2017; collared flycatchers, *Ficedula albicollis*, Hsu et al., 2019; pied flycatchers, *Ficedula hypoleuca*, Stier et al., 2020). However, the studies have also shown some discrepancies in their results (Table 1). For example, yolk THs improved hatching success in rock pigeons and in collared flycatchers but had no effect in great tits. Moreover, TH injection in great tit eggs increased offspring growth in males but decreased it in females. Conversely, yolk THs decreased growth during the second half of the nestling phase in rock pigeons, whereas they increased early growth, but decreased later postnatal growth in collared flycatchers. Finally, great tits showed no response to elevated yolk THs in resting metabolic rate (RMR), whereas RMR was increased in females but decreased in males rock pigeon hatchlings.

Environmental conditions also influence yolk THs. Recent studies showed ambient temperature correlated with yolk T_4 but not yolk T_3 (Ruuskanen et al., 2016b), and that food restriction affected yolk THs (Hsu et al., 2016). Besides, yolk T_3 was found to be heritable but not yolk T_4 (Ruuskanen et al., 2016). However, yolk or nestling THs were not correlated with environmental pollution to metals and metalloids in a recent study (Ruuskanen et al., 2019).

Birds as a study model

Oviparous species, such as birds, are suitable models for studying the role of maternal hormones on the progeny because embryos develop in eggs outside the mother's body and maternally derived hormones are deposited in egg yolks (Prati et al. 1992; Schwabl 1993). This allows the measurement and experimental manipulation of maternal hormone transfer to be independent of maternal physiology. Birds, with their relatively well-known ecology and evolution, have become the most extensively studied taxa in research on the function of maternal hormones (Groothuis et al. 2019).

Table 1: Summary of the results obtained so far on the effects of increased prenatal THs in avian species.

	Hatching success	**Metabolism**	**Telomeres**	**Growth**	**Reference**
Japanese quails	0	-	-	↑ embryonic pelvic cartilage	Wilson & McNabb, 1997
Great tits	0	0	-	↑ in males ↓ in females	Ruuskanen et al., 2016
Rock pigeons	↑	↓ RMR in males ↑ RMR in females	-	↓	Hsu et al., 2017
Collared flycatchers	↑	0	-	↑ early growth ↓ later growth	Hsu et al., 2019
	-	-	↑ length	-	Stier et al., 2020

General aims and methods

In my thesis I aimed at addressing several unanswered questions on the causes and consequences of variation in maternal THs in avian eggs:

1) Is iodine limiting TH production and transfer to the yolk? As mentioned above, iodine is a key element of THs. If iodine availability is limited, TH production and transfer to the yolk may become costly for mothers. If so, they may face trade-offs between allocating iodine and THs to themselves or to their progeny.
2) Are mothers able to regulate yolk TH transfer independently from their own circulating THs? If mothers are able to regulate yolk TH transfer, this would free them from potential trade-offs between optimising circulating or yolk THs.
3) Are there any organisation effects of prenatal THs? Previous studies have shown some short-term effects of prenatal THs in avian species. Organisational effects (i.e., early phenotypic modifications that only have effects during adulthood) have not been studied yet. Yet, such long-term effects are important to assess whether prenatal THs can be adaptive.
4) Are the effects of prenatal THs context-dependent? Previous studies have shown discrepancies in the effects of prenatal THs. These apparent contradictions may be due to species differences and/or to differences in the environmental conditions (i.e., context), such as ambient temperature or food availability.

In my thesis, I have used several study models to address my different questions (Figure 2). I have used a wild free-living species (Pied flycatcher) to answer eco-evolutionary questions. I could also compare the effects of prenatal THs in this species

and in a closely related species, the Collared flycatcher, to discuss the potential context-dependent effects of prenatal THs. The use of captive Japanese quails made it easier to study the independent and additive effects of prenatal T_3 and T_4, the long-term effects of prenatal THs, and to control the environment (food availability, photoperiod). Besides, all the recent studies on prenatal THs used altricial species as study models. Therefore, the role of variation in prenatal THs in precocial species is as yet unclear. Embryonic development differs drastically between these two developmental modes, with precocial species having a more advanced development than altricial ones. In addition, the thyroid gland becomes functional around mid-incubation in precocial species, whereas it only becomes functional after hatching in altricial species. Thus, precocial embryos may react differently to maternal THs than altricial embryos. Finally, rock pigeons have an extended breeding period compared to other altricial species, which makes it an interesting study model to investigate the potential trade-offs arising between self-maintenance and reproductive costs.

First and foremost, I wanted to know whether environmental conditions can influence the transfer of maternal THs. Iodine is a key component of THs and cannot be synthesised by an organism, which has to find it in its environment. I therefore tested whether iodine availability may constrain deposition of THs in the eggs **(Chapter 2)**, thus addressing the questions of whether THs are costly to produce and transfer, and whether mothers have developed regulatory mechanisms to cope with these potential costs. To answer these questions, I manipulated the iodine content in captive rock pigeons feed to induce a trade-off in laying females between allocation iodine and THs to themselves or to their eggs. Breeding pairs were fed either with an iodine-restricted or an iodine-supplemented diet for ca. 10 weeks. I collected two clutches and two blood samples per females distant by ca. 3 weeks. With this experimental design, I could also test whether a long exposure to low iodine availability increases the chance of inducing a trade-off in breeding mothers. I expected that, if iodine resources are limiting TH production, females under the restricted diet would not be able to maintain normal levels of plasma and yolk iodine and THs. Thus, either one or both compartments would have reduced iodine and/or TH concentrations. This would be even accentuated after a longer exposure to limited iodine availability. Second, if mothers are able to regulate yolk TH deposition, they may preferentially allocate resources (i.e., iodine and THs) to either their own circulation or to the eggs, thus resulting in a different balance between both compartments compared to control females. In addition, females may also regulate egg production to reduce the total amount of iodine or THs allocated to reproduction.

Next, I wanted to tackle the question of whether females are able to regulate TH transfer to their eggs **(Chapter 3)**. Independent regulation of maternal hormone transfer is a key aspect for mothers to avoid potential physiological trade-offs and to adjust

offspring phenotype to the future conditions when these conditions are different from those experienced by mothers. To do so, I implanted free-living pied flycatcher females with T_4 implants. I collected two eggs per females: the first egg of the clutch (pre-implant) serving as a within-clutch control, and the last egg of the clutch (post-implant) to assess whether the T_4 implant affected the transfer of maternal THs to the yolk. I also took one blood sample per female early in the incubation to evaluate the effect of the implant on female circulating THs. In parallel, I monitored the short-term effects (up to 3 days after implantation) of exogenous T_4 on circulating THs in captive females to make sure that the implants released T_4 fast enough to influence yolk THs of the last egg. If the implant successfully increased circulating T_4, we would expect an increase in the conversion to T_3 in the tissue and thus an increase in circulating T_3. On the other hand, females may be able to regulate the conversion from T_4 to T_3 in order to maintain normal circulating T_3. Second, if mothers can regulate TH transfer independently from their circulating THs, one would expect plasma and yolk THs to respond differently to exogenous T_4. For example, mothers may favour their own circulating TH levels, and thus get rid of excess THs in the eggs, potentially at the expense of embryo's metabolism. Alternatively, if mothers cannot regulate TH transfer independently, one would expect both plasma and yolk THs to respond similarly to exogenous T_4.

Then, I wanted to know what are the short- and long-term consequences of maternal THs in a captive precocial species **(Chapter 4)** and in a free-living altricial species **(Chapter 5)**. In **Chapter 4**, I manipulated THs in unincubated eggs from Japanese quails. I increased egg THs by yolk injection within the physiological range of the species. For the first time in prenatal TH studies, I not only injected a combination of both hormones, but I also injected T_3 and T_4 separately. Injection of either T_3 or T_4 alone allowed me to tell apart the effects of each hormone and their additive effects. I then measured a wide array of traits, both during development and adulthood, potentially affected by prenatal THs: embryonic development and survival, chick growth, chick and adult oxidative stress, and adult responsiveness to change in photoperiod. I expected hatching success to be positively affected by prenatal THs (Hsu et al., 2017a, 2019b), while growth to be positively (Hsu et al., 2019b), negatively (Hsu et al., 2017a) or sex-specifically affected (Ruuskanen et al., 2016). I also predicted that yolk THs would have organisational effects on life stage transitions, with advanced timing of puberty and moult.

In **Chapter 5**, I tested the short-term effects of prenatal THs, but this time in a free-living passerine species, the Pied flycatcher. Similar to Chapter 4, I injected, within the physiological range of the species, a combination of T_3 and T_4 in unincubated eggs. I then assessed the effects of prenatal THs on various responses: hatching success, nestling growth, physiology and survival. As in chapter 4, I expected elevated yolk THs to increase hatching success and affect growth. Elevated yolk THs may also increase

nestling oxidative stress through increased metabolism (Asayama et al., 1987; Villanueva, Alva-Sanchez & Pacheco-Rosado, 2013). Finally, by comparing my results with these of a similar study on Collared flycatchers, a closely related species, I proposed an explication for the apparent discrepancy in the literature about the effects of prenatal THs in birds.

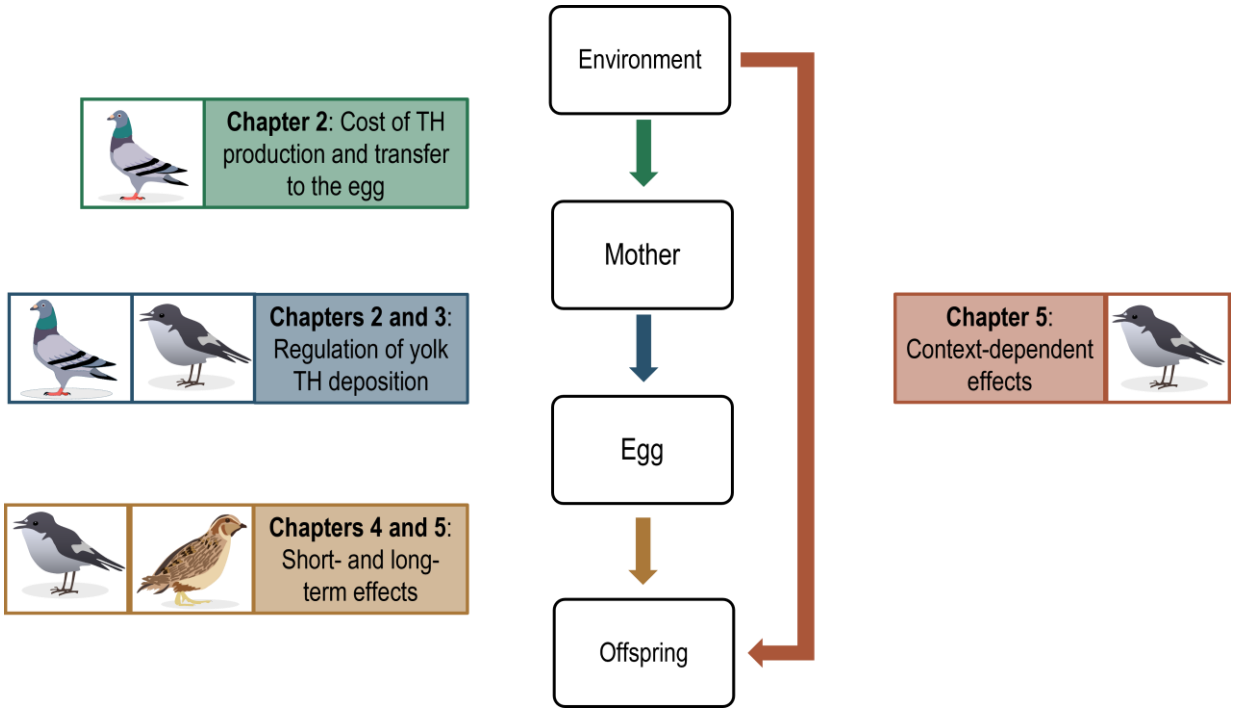

Figure 2: Schematic view of the main questions in the thesis. The effects of the environment on circulating and yolk THs were studied in Chap 2 and 5, respectively. The potential regulation of yolk TH transfer was tested in Chap 3, and the short- and long-term effects of yolk THs were studied in Chap 4 and 5.

General discussion

Is iodine limiting TH production and transfer to the yolk? (chapter 2)

In the first chapter of my thesis, I aimed to answer the question whether THs are costly to produce and to transfer to the eggs, thus inducing a trade-off in mothers between self-maintenance and reproduction when iodine resources become limited. I also wanted to know whether mothers may regulate yolk TH deposition to cope with these potential trade-offs. I thus provided breeding pairs of Rock pigeons with either an iodine-restricted or an iodine-supplemented diet. I collected two clutches and two blood samples (after clutch completion) per female.

I found a decrease in circulating and yolk iodine with restricted iodine. However, mothers that laid eggs managed to maintain normal circulating and yolk TH concentrations. These females likely did not face any constraints or trade-offs due to restricted iodine. Exposure duration to restricted iodine also had no effect on circulating and yolk iodine and THs. However, I did find that fewer females laid eggs in the iodine-restricted group. Therefore, restricted availability of iodine may induce a cost for egg production. Previous studies have demonstrated that hypothyroid females reduce (Van Herck et al., 2013) or even stop (Wilson & McNabb, 1997) egg production. We may therefore speculate that our restricted diet rendered some females hypothyroid to the extent they could not lay eggs. This result may also suggest that mothers may not be able to regulate yolk TH deposition independently from their own circulating TH levels (in line with results in **chapter 3**). However, these females could not be captured as they would not use nest boxes. If we had been able to sample those females, we may have observed decreased circulating THs, contrary to the females that laid eggs. Overall, our results indicate that, when facing limiting iodine availability, mothers may prioritise their circulating THs, to maintain their own physiology, and offspring quality over offspring quantity by reducing the number of eggs produced.

Are mothers able to regulate TH transfer to their eggs? (chapter 3)

In this chapter, I aimed at assessing whether mothers are able to regulate maternal TH transfer to the eggs independently from their circulating THs. To do so, I inserted subcutaneous T_4 implants in egg-laying female Pied flycatchers.

The results of this study showed that exogenous T_4 successfully increased plasma and yolk T_4, but not plasma or yolk T_3. In addition, although the yolk T_3/T_4 ratio decreased due to T_4 implants, this ratio was not different from the ratio in females' plasma. These results indicate that, within the range of the manipulation, females are not able to regulate the transfer of T_4 into their eggs independently from their circulation, and that yolk T_4 simply mirror plasma T_4 at the time of yolk formation (Groothuis & Schwabl, 2008). This absence of regulatory mechanisms may impose trade-offs on

mothers between optimising T_4 for herself or for her eggs. Second, because our T_4 implants failed to affect plasma T_3, we cannot test the potential regulation of the transfer of T_3. These results are in contradiction with previous studies that found evidence for independent regulation of TH transfer. The first one orally administrated either a low dose (1x the daily T_4 production) or a high dose (3x the daily production) of T_4 in Japanese quails for several weeks. The authors found that low dose of T_4 increased yolk T_3 but not plasma T_3 (Wilson & McNabb, 1997). In the second study, breeding hens treated with methimazole, an anti-TH drug, showed a decrease in yolk T_3 but not in plasma T_3 while plasma and yolk T_4 remained unaffected (Van Herck et al., 2013). In both studies, mothers favoured maintenance of their circulating levels of THs over yolk TH concentrations. The apparent contradictions with my results may be explained by the fact that these two studies administrated supraphysiological doses and/or by the fact that the studies manipulated circulating THs for a long period of time (up to 16 weeks). Conversely, I manipulated circulating THs within the physiological range, apart from a short surge in plasma T_4 (less than 3 days), and for a shorter period of time (ca. 3 weeks).

The unaffected plasma T_3, despite increased plasma T_4, suggests that females were able to regulate the peripheral conversion from T_4 to T_3 to maintain normal concentrations of plasma T_3. Previous research has shown that THs can mediate deiodinases expression and activity (Bianco et al., 2002). This mechanism could be a tool for females to regulate TH transfer to their eggs if independent regulation of TH transfer were not possible. However this mechanism is not very flexible and does not free females from potential trade-offs.

Do prenatal THs exert organisational effects? Do prenatal T_3 and T_4 have independent effects as well as additive effects? (chapter 4)

In this chapter I injected unincubated eggs from Japanese quails maintained in captivity with either T_4 or T_3 alone, a combination of both hormones, or a saline (control) solution. This design allowed us to explore the effects of T_4 and T_3 separately, which has not been done in previous studies. I measured traits known to be influenced by circulating and yolk THs: embryo development and survival, chick growth, transition between life-history stages (i.e., reproductive state and moult) and chick and adult oxidative stress.

I found that elevated yolk THs (T_4 alone or in combination with T_3) increased hatching success, in line with previous studies (Hsu et al., 2017; Hsu et al., 2019, but see Ruuskanen et al., 2016 and Sarraude et al., 2020). I found no other short- or long-term effects on any of the measured traits. Previous studies have reported positive (Wilson & McNabb, 1997; Hsu et al., 2019; **chapter 5**), negative (Hsu et al., 2017a), or sex-specific effects (Ruuskanen et al., 2016) of prenatal THs on growth. Yet, our study failed to detect different growth trajectories between the treatments. I also found no effect on the timing or speed of postnuptial moult, or on oxidative stress. Finally, I found no evidence for

differential effects of maternal T_4 and T_3. Yet, I should emphasise that due to an overall low hatching success (ca. 50%), our control and T_3 groups had few individuals, thus limiting statistical power to detect differences between groups.

What are the short-term effects of prenatal THs in a free-living altricial species? Are prenatal TH effects context-dependent? (chapter 5)

In this last chapter, I tested the short-term effects of elevated prenatal THs in a precocial species, the Pied flycatcher. I compared my results to a similar study on a closely related species, the Collared flycatcher (Hsu et al., 2019b). In this chapter, I elevated, within the physiological range, both T_3 and T_4 in unincubated eggs. I then measured traits potentially affected by prenatal THs: hatching success, growth, oxidative stress and survival.

Our results showed no effect of elevated yolk THs on hatching success, oxidative stress or survival. We found a non-statistically significant trend on growth, with TH nestlings growing slightly faster than control nestlings in the second week post hatching. Our results differ substantially from those of Hsu and collaborators (2019b) who found positive effects on hatching success and growth in a sister species. Because these species are phylogenetically close, and capable of hybridising, species differences alone may not explain the contradicting results. In an attempt to explain this discrepancy, we compared environmental conditions (ambient temperature and precipitation) during the breeding season of these two species. We found no clear differences the environmental variables measured. Yet, because nestling survival notably differed between the two studies (90% in this study vs 75% in Hsu et al., 2019), we may still expect some components of the environment, such as food availability, to play a role in the potential context-dependent effects of prenatal THs. As mentioned in the general introduction, ambient temperature correlates with circulating and yolk THs, and is thus a potential factor interacting with prenatal THs. This is why we further tested in a fully factorial experiment whether prenatal THs interact with post-hatching nest temperature and found no clear context-dependent effects (Hsu et al., 2020).

Synthesis

In my thesis, I aimed at answering several questions on the causes and consequences of natural variation in maternal THs in birds. In **chapter 2**, I demonstrated that iodine availability can limit egg production and thus potentially limit TH transfer to the eggs. However, some females appeared to maintain normal circulating and yolk THs while also maintaining normal egg production. Therefore, better quality individuals may be able to cope with the potential costs of TH production under restricted iodine. In addition, I found no short- or long-term negative consequences of elevated yolk THs on offspring (**chapters 4 and 5**, but see negative effects on growth in Ruuskanen et al., 2016; Hsu et al., 2017). Therefore, one could ask why females do not pack their eggs with THs to boost offspring growth (as found in Hsu et al., 2019 and suggested in **chapter 5**) and hatching success (as found in **chapter 4** and in Hsu et al., 2017 and Hsu et al., 2019). The results in **chapter 3** may bring an answer to that question. In **chapter 3**, I found that females may not be able to regulate TH transfer to the eggs independently from their own circulating levels. Such an impossibility to regulate yolk TH transfer may impose on mothers a trade-off between optimising circulating THs and yolk THs. Thus, mothers cannot increase yolk THs without increasing their own circulating THs, potentially exposing themselves to high THs.

In my thesis, I could not find evidence for the ability of mothers to regulate yolk TH deposition independently from their own circulating TH levels (**chapters 2 and 3**), in contradiction with previous studies (Wilson & McNabb, 1997; Van Herck et al., 2013; Hsu et al., 2016). This contradiction may partly be explained by differences in the experimental designs (as discussed above). The potential absence of regulatory mechanism may impose certain trade-offs on mothers between optimising circulating and yolk THs.

In the case of thyroid hormones, iodine may be a limiting factor in yolk TH deposition and egg production (**chapter 2**). Thus, if mothers cannot independently adjust yolk THs, both mothers and embryos may suffer from reduced THs when iodine is limited. On the other hand, independent regulation of yolk THs may allow mothers to prioritise either themselves or their progeny. Since such regulatory mechanism may not be available to mothers, they may be left with one extreme solution to cope with restricted iodine: stop egg production, as found in **chapter 2**. This solution may allow females to maintain iodine stores and circulating THs and wait for more favourable conditions to reproduce. Rock pigeons lay a maximum of two eggs per clutch; thus, females faced with restricted dietary iodine may lay either 100%, 50% or 0% of their maximum clutch size. Repeating the experiment with a species that lays multiple eggs in a clutch would allow to quantify more precisely the effects of limited dietary iodine

on clutch size. Nevertheless, rock pigeons are able to lay multiple clutches per breeding season, which makes it a relevant study model to test for trade-offs between current vs future breeding attempts. In **chapter 2**, despite restricted iodine, some females maintained normal egg production despite reduced circulating and yolk iodine. Nevertheless, these females managed to maintain normal circulating and yolk THs. Previous studies showed that reduced yolk iodine can impair embryonic and hatchling TH function, with reduced iodine and T_4 thyroid stores (McNabb, Dicken & Cherry, 1985; Stallard & McNabb, 1990). These studies did not report yolk THs, but our results show that reduced yolk iodine does not necessarily imply reduced yolk THs. Thus, the negative effects of low yolk iodine may not be directly due to reduced yolk THs, and the exact consequences of low yolk iodine should deserve further investigation.

Independent regulation of yolk THs may allow mothers to flexibly adjust the signals transferred to their progeny to match the future environmental conditions, which may be different from those experienced by the mothers at the time of yolk formation. However, if mothers are not able to regulate yolk THs independently from their own circulating levels, the information they transmit to their progeny may not be completely accurate regarding the future environment. This may hamper the potential for maternal hormones to be adaptive, and may even lead to non-adaptive effects. Thus, embryos may have developed mechanisms to ignore, at least partly, maternal signalling.

When the field of maternal effects developed embryos were first considered as mere passive recipient of maternal information and signalling. This view has then been challenged (Müller et al., 2007), and new evidence show that embryos can metabolise maternal hormones early in the development (Kumar et al., 2018) and can adjust the number of hormone receptors to the presence of maternal hormones (Kumar et al., 2019). Embryos can also respond differently to maternal signalling according to their rank in the clutch (Kumar et al., 2018). This recent thesis (Neeraj 2019, PhD thesis) has given a new role for embryos that clearly deserve more attention. In the context of thyroid hormones, embryos may have several levers available to adjust their response to maternal signalling. They may increase or decrease their sensitivity to maternal THs by up- or down-regulating nuclear TH receptors, TH transporters and/or deiodinases. Previous studies in precocial species have demonstrated that embryos express such receptors, transporters and enzymes very early in the development, before their own thyroid gland are functional (reviewed by Darras, 2019). To date, such information is not available in altricial species. Recent studies aimed at filling this knowledge gap and suggest similar results in wild Pied flycatchers and Blue tits (*Cyanistes caeruleus*): early embryos (ca. 3 days into incubation) express mRNA of TH transporters, TH receptors and all deiodinases (Ruuskanen et al. unpublished results). It will then be important to assess whether embryos can adjust their response to maternal THs according to the pre- and

post-natal environment they are experiencing, such as yolk composition or rank in the clutch. The ability of embryos to respond to maternal THs deserves further examination, especially if mothers show little potential for independent regulation of yolk TH deposition.

The ability of embryos to modulate their response to maternal signalling is also interesting in the context of parent-offspring conflicts. Such conflicts arise when the evolutionary interests of the parents and of the progeny do not align. In iteroparous species, which can undergo several reproductive cycles in a lifetime, parents tend to maximise their overall reproductive output, and will thus not invest all of their energy in one breeding attempt. On the other hand, offspring from one breeding attempt will seek to maximise the investment of their parents in the current breeding attempt, hence creating a conflict. In the context of maternal hormones, mothers may use hormones to "manipulate" offspring development and phenotype to their own advantage (Müller et al., 2007). However, the ability to "manipulate" offspring while maintaining optimal circulating hormones requires that mothers can regulate yolk TH deposition independently from their circulating THs. Since the results in **chapter 2 and 3** suggest no independent regulation of yolk TH deposition, embryos may have the upper hand in family conflicts involving THs. **Chapter 3** also shows between-females differences in yolk T_4 deposition in response to exogenous T_4, with an up to 6-fold difference in yolk T_4 concentration (Figure 1 of **chapter 3**). This difference may be due to implants releasing hormones at different rates, but it may also be due to mothers using different strategies to clear excess T_4. Some females may simply deposit more of the excess hormone, while others may metabolise such hormone into other inactive forms, such as T_2 or rT_3. Measuring such metabolites may yield important information on the strategies employed by breeding females during yolk formation and hormone deposition.

In **chapters 4 and 5**, where I looked at the short- and long-term effects of prenatal THs, I could not replicate most of the previous results from similar studies. To my knowledge, **chapter 4** was the first study to look at the potential organisational effects of yolk THs into adulthood. The absence of such effects on the traits measured (growth, moult, oxidative stress) suggest that embryos can buffer the initial increase in yolk THs and are not simply passive to maternal signalling. In addition, unpublished results from the same experiment showed no differences in behaviour (in chicks and adults) caused by prenatal THs (Ruuskanen et al., unpublished results). This is coherent with the analysis of the expression of four genes involved in TH metabolism (2 genes coding for TH receptors, 1 gene for DIO2, and 1 gene for a coactivator) in adult brains (Ruuskanen et al., unpublished results from the same experiment). In **chapter 5**, I could

not find the same short-term effects shown by previous similar studies on hatching, growth or metabolism (Ruuskanen et al., 2016; Hsu et al., 2017, 2019).

The discrepancies between the different studies cannot be solely due to species differences. As discussed in **chapter 5,** prenatal THs may have context-dependent effects that can be difficult to capture in field studies. Context-dependence means that the same amount of yolk THs will result in a different outcome related to the environment experienced by the progeny. For example, the stimulating effects of THs on metabolism may increase growth when there are enough resources but may decrease growth when resources are limited. Such context-dependent effects have been studied recently by manipulating, in a full factorial design, yolk THs and nest temperature in wild pied flycatchers. The results showed no clear interaction between yolk THs and nest temperature on nestling growth, survival and physiology (Hsu et al., 2020). However, other environmental conditions may interact with yolk THs, such as food availability or other yolk hormones.

Evaluating the potential context-dependent effects is important to assess under which conditions variation in prenatal THs can be adaptive. Maternal hormones can be considered adaptive when they increase mothers' fitness. Mothers can increase their own fitness by anticipating the future conditions and adjust the offspring phenotype accordingly. These are referred to as anticipatory maternal effects (AME, Marshall & Uller, 2007), and are considered to be widespread. Such effects require that mothers can accurately predict the future environmental conditions. If the future conditions match the current conditions, mothers should adjust the offspring phenotype to resemble the mothers' phenotype. This is a simple case scenario, in which mothers only need to produce one phenotype. Conversely, if the future conditions do not match the current ones, mothers need to produce another phenotype that would be adequate for the expected conditions. This more complex scenario requires mothers to have evolved mechanisms to predict future conditions, and regulatory mechanisms to flexibly adjust yolk THs. **Chapters 2 and 3** in my thesis do not show evidence that such regulatory mechanisms exist, thus leaving little scope for AE mediated by maternal THs.

Alternatively, Bonduriansky and Crean argue that some non-anticipatory effects, such as condition-transfer effects (CTE) can also be adaptive, and may even be more widespread than AME (Bonduriansky & Crean, 2018). CTE suppose a positive correlation between the condition, or quality, of the parents and the condition of the offspring, regardless of the environment (Bonduriansky & Crean, 2018). Therefore, evolution of CTE does not need environmental conditions to be predictable or specific regulatory mechanisms (Bonduriansky & Crean, 2018). Following Bonduriansky and Crean, transfer of yolk THs can still be adaptive even if mothers cannot regulate yolk

TH deposition independently from their circulating THs. Thus, research on maternal hormones would benefit from investigating the potential adaptive value of maternal THs.

Other aspects of maternal THs deserve further investigation in the future

This thesis was faced with some methodological limitations. First, increasing yolk traits necessitates injections that are rather invasive methods thought to reduce hatching success. Thus, there is a need for developing less invasive methods such as egg-dipping or albumen injection. However, such methods are for now less reliable than yolk injections, with uncertainties about how much of the hormones will actually be taken up by the embryo. Using a full-factorial design, one would be able to tell apart the effects of manipulation (injection or dipping) and maternal THs on hatching success. A recent study confirms that yolk injections induce the highest mortality rate compared with the other two methods (Birker et al., unpublished results). Albumen injection may be a good method to inject accurate amount of hormones, especially for lipophilic hormones, which may migrate towards the yolk (Birker et al., unpublished results). Finally, egg-dipping is as safe albumen injections, but should only be used under specific conditions such as small sample sizes, harsh field conditions, or inexperienced users (Birker et al., unpublished results).

Second, in my thesis I only manipulated prenatal THs, though interactions between THs and other hormonal axes are well documented across vertebrates (Flood, Fernandino & Langlois, 2013; Brown et al., 2014; McNabb & Darras, 2015; Tovo-Neto, 2018). Manipulating yolk THs together with another yolk component (e.g., steroid hormones, antioxidants) would yield addition information on the importance of interactions between prenatal THs and other yolk components. In fish, combined manipulations of yolk cortisol and THs have demonstrated synergistic effects of these two hormones on larval development and survival (reviewed by Brown et al., 2014). In birds, simultaneous manipulation of yolk THs and corticosterone in great tit eggs have shown little interaction effects of both hormones (Ruuskanen et al., unpublished results). Nevertheless, such ecological studies in wild species should be encouraged.

Third, it is easier to increase a yolk component than to decrease it. In the case of thyroid hormones, one could administrate (by injection or some other method) a thyroid antagonist. However, this involves other problems regarding the potential undesired effects (independent of the HPT axis) of such antagonists on embryonic development. According to Darras (2019): “Decreasing maternal TH availability throughout development can only be achieved by rendering laying hens hypothyroid, which is typically done by addition of goitrogens [TH antagonists] to their food or drinking water”. However, these molecules are also transferred to the eggs and found in embryonic tissue (Van Herck et al., 2013), potentially causing other adverse effects. Besides, injecting goitrogens directly in eggs can block the embryonic thyroid gland and

decrease endogenous TH production, although only when the gland has become functional, ca. around mid-incubation in precocial species (Darras, 2019). Using goitrogens to reduce the conversion of T_4 into T_3 earlier in the development is impossible without administrating toxic doses (Darras, 2019).

Increasing or decreasing a yolk component should lead to the same conclusions regarding simple relationships between yolk THs and fitness-related traits, such as positive or negative correlation, or no correlation at all (Figure 3, A - C). Experiments manipulating yolk THs in both directions would enable detecting more complex relationships between yolk THs and fitness-related traits such as ceiling or threshold effects (Figure 3, D - G), or quadratic effects (Figure 3, H and I). A recent meta-analysis on hormone manipulation found support for the Optimal Endocrine Phenotype Hypothesis, which poses that individuals display the optimal, or near-optimal, endocrine phenotype for a certain environment (Bonier & Cox, 2020). Therefore, any manipulation of their hormonal status will deviate them from the optimal phenotype and decrease their fitness (Bonier & Cox, 2020), and is illustrated in Figure 3H. The authors also acknowledge that they lacked data to properly test the alternative hypothesis, the Ongoing Selection Hypothesis. This hypothesis assumes that individuals mostly fail at expressing the optimal phenotype. Thus, endocrine manipulation should bring an individual closer to its optimal phenotype and increase its fitness (Bonier & Cox, 2020), which is depicted in Figure 3I.

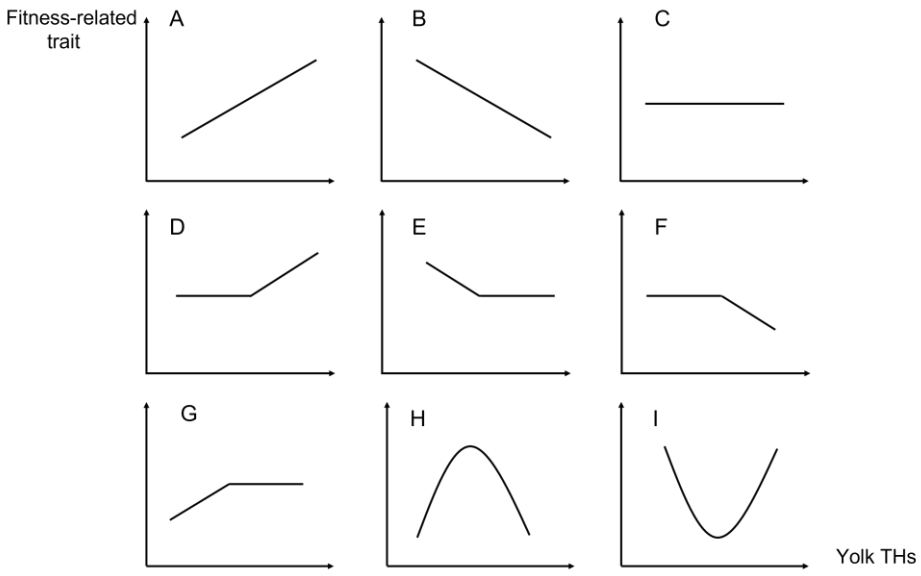

Figure 3: Possible theoretical relationships between yolk THs and fitness-related traits, such as growth, metabolism or survival.

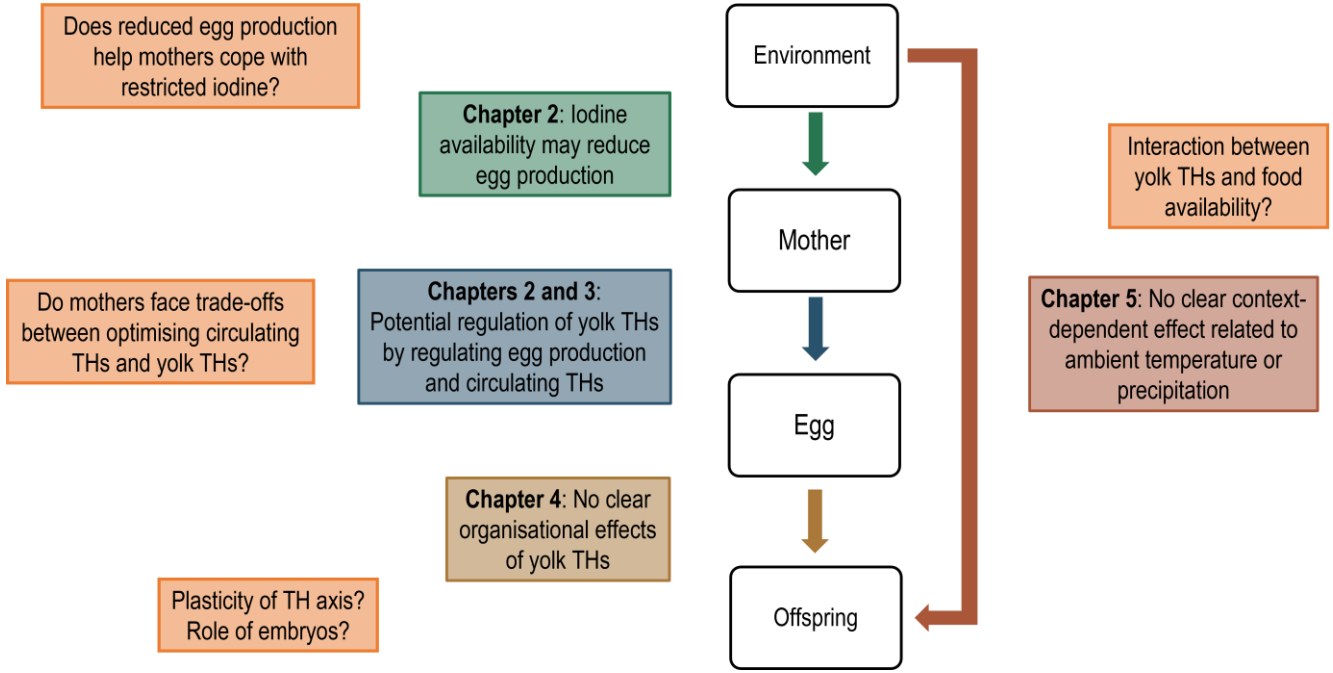

Figure 4: Schematic view of the main results of the thesis. Future questions that could be further investigated are in bright orange.

Conclusion

In conclusion, thyroid hormones have long been known for playing important roles in all vertebrates on a wide variety of traits, such as embryonic development, juvenile growth, reproduction and metabolism. However, these hormones have been overshadowed by steroid hormones in the context of hormone-mediated maternal effects. Only recently natural variation of prenatal THs have gained attention in an eco-evolutionary context. My thesis aimed at answering some important questions on the potential cost of TH production and transfer to the eggs, whether mothers are able to regulate yolk TH deposition, and on the potential long-term and context-dependent effects of prenatal THs (Figure 4). Research on maternal THs should learn from the existing literature on maternal androgens and corticosterone and aim at answering questions such as the adaptive value of maternal THs or how embryos respond to prenatal THs.

Chapter 2

Is maternal thyroid hormone deposition subject to a trade-off between self and egg because of iodine? An experimental study in rock pigeon

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Abstract

Maternal hormones constitute a key signalling pathway for mothers to shape offspring phenotype and fitness. Thyroid hormones (THs; triiodothyronine, T_3 and thyroxine, T_4) are metabolic hormones known to play crucial roles in embryonic development and survival in all vertebrates. During early developmental stages, embryos exclusively rely on the exposure to maternal THs, and maternal hypothyroidism can cause severe embryonic maldevelopment. The TH molecule includes iodine, an element that cannot be synthesised by the organism. Therefore, TH production may become costly when environmental iodine availability is low. This may yield a trade-off for breeding females between allocating the hormones to self or to their eggs, potentially to the extent that it even influences the number of laid eggs. In this study, we investigated whether low dietary iodine may limit TH production and transfer to the eggs in a captive population of Rock pigeons (*Columba livia*). We provided breeding females with an iodine-restricted (I- diet) or iodine-supplemented diet (I+ diet) and measured the resulting circulating and yolk iodine and TH concentrations and the number of eggs laid. Our iodine-restricted diet successfully decreased both circulating and yolk iodine concentrations compared to the supplemented diet, but not circulating or yolk THs. This indicates that mothers may not be able to independently regulate hormone exposure for self and their embryos. However, egg production was clearly reduced in the I- group, with fewer females laying eggs. This result shows that restricted availability of iodine does induce a cost in terms of egg production. Whether females reduced egg production to preserve THs for themselves or to prevent embryos from exposure to low iodine and/or THs is as yet unclear.

Introduction

Non-genetic inheritance is defined as the transmission of information between generations beyond coding genes (Danchin et al., 2011). Parental effects are included in this non-genetic inheritance and may be considered adaptive (Mousseau & Fox, 1998a; Moore, Whiteman & Martin, 2019), although the strength and ubiquity of adaptive parental effects is still under debate (Yin et al., 2019; Sánchez-Tójar et al., 2020; Zhang et al., 2020). Parental effect of maternal origin, i.e. maternal effects, have received increasing attention since the 1990's (Bernardo, 1996; Mousseau & Fox, 1998a). Hormones of maternal origin can be transferred to the offspring and constitute a potential pathway for mothers to influence their offspring's phenotype (Groothuis et al., 2019). Hormone allocation to offspring could be costly for mothers as it could induce a trade-off between allocating hormones to their own metabolism or to their offspring's. Steroid hormones, the most studied hormones in the context of hormone-mediated maternal effects, may not be that costly to produce as they are derived from cholesterol, which is abundant in the organism (Groothuis & von Engelhardt, 2005). On the other hand, thyroid hormones (THs) may be considered costly, as their molecular structure includes iodine, a trace element that cannot be synthesised by organisms and must therefore be found in the environment.

Thyroid hormones are metabolic hormones that are present in two main forms: thyroxine (T_4) that contains four atoms of iodine, and triiodothyronine (T_3) that contains three atoms of iodine. Iodine is concentrated into the thyroid gland and incorporated into tyrosines that will be combined to form T_4 and T_3 (McNabb & Darras, 2015). The thyroid gland produces mostly T_4 and lesser amounts of T_3. In the peripheral tissues (e.g. liver, kidney, muscle), T_3 is mostly obtained from T_4 via removal of an iodine atom by deiodinase enzymes (McNabb & Darras, 2015). TH action mainly depends on TH receptors that have a greater affinity for T_3 than for T_4 (Zoeller, Tan & Tyl, 2007). This is why T_4 is mostly seen as a precursor of T_3, the biologically active form. THs play important roles in growth, reproduction, metamorphosis and thermoregulation, and the TH signalling pathway is well conserved throughout the animal kingdom, from invertebrates (Holzer, Roux & Laudet, 2017; Taylor & Heyland, 2017) to vertebrates (reviewed by Ruuskanen & Hsu, 2018).

The main sources of iodine for terrestrial animals is via uptake of food, where plants absorb it from the soil, and via drinking water (Anke, 2004). Because iodine availability differs across environments and food sources (Anke, 2004; Boggs et al., 2011), THs may be costly to produce when iodine availability is limited. Low iodine diet in rodents under laboratory conditions generally decrease circulating THs (e.g. Santisteban et al., 1982; Zhimei et al., 2014; but see Bocco et al., 2020). Furthermore, in wild alligators (*Alligator mississippiensis*) overall higher plasma THs were reported in

the populations exposed to higher iodine concentrations compared to low iodine habitat (Boggs et al., 2011, 2013). In breeding animals, this potential cost may induce trade-offs between allocating resources (i.e., iodine and THs) to themselves or to their progeny because embryos rely exclusively on THs of maternal origin during early development (humans, Stepien & Huttner, 2019; fish, Castillo et al., 2015; birds, Darras, 2019). In humans, TH deficiency arising from iodine deficiency can severely impair brain development and increase foetus mortality (reviewed by Zimmermann, 2009). Although the importance of THs has long been acknowledged, such a potential trade-off has never been studied.

Birds provide an excellent model organism to study hormone-mediated maternal effects and the associated trade-offs (Groothuis et al., 2019). Unlike mammals, avian embryos develop in eggs, outside of mothers' body, which facilitates the measurement of not only maternal physiological status but also the actual embryonic exposure to maternal hormones deposited in egg yolks. It has been demonstrated that early avian embryos have all the necessary machinery (i.e., transporters, receptors, deiodinases) to make use of the THs deposited by mothers (reviewed by Darras, 2019). Maternally transferred thyroid hormones play crucial roles in the development of the central nervous system (Darras, 2019) and several other tissues and organs (e.g., muscles, heart, eye, bones; McNabb and Darras, 2015). In addition, recent studies in birds have shown that physiological variation in prenatal THs can affect hatching success, growth and metabolism (Ruuskanen et al., 2016; Hsu et al., 2017, 2019; Sarraude et al., 2020b).

Three older studies investigated the effect of different dietary iodine concentrations on the thyroid function in different species and life stages. In egg laying Japanese quails (*Coturnix japonica*), McNabb, Blackman and Cherry (1985) found that circulating and yolk iodine were proportional to dietary iodine. In addition, thyroid T_4 was decreased by restricted dietary iodine but plasma THs were not affected in quails (McNabb, Blackman & Cherry, 1985), suggesting the presence of regulatory mechanisms maintaining normal circulating TH concentrations. In contrast, McNichols and McNabb (1987) found that both circulating iodine and T_4 concentrations decreased in response to low dietary iodine in the ring doves (*Streptopelia risoria*). This discrepancy indicates that species may respond differently to limited iodine availability. In quails, embryos and hatchlings from the eggs with low iodine concentrations suffered from thyroid gland hypertrophy, but their circulating TH levels were not different from those from the eggs with moderate or high yolk iodine concentrations (McNabb, Dicken & Cherry, 1985). Thus, developing embryos may already be able to cope with limited iodine resources and maintain TH production. However, none of these studies measured yolk THs, which is necessary information to investigate the potential trade-offs between mothers' circulation and embryonic exposure to THs.

The aim of our study was two-fold. First, to investigate whether limited iodine may constrain TH production, and second, whether mothers in that case can prioritise TH allocation to either self or their eggs. To this end we provided breeding pairs of rock pigeons (*Columba livia*) with a diet restricted in iodine (hereafter I- group), to create a potential trade-off between circulating and yolk THs, and compared this group with a reference group provided with ample iodine (hereafter I+ group), removing such a trade-off in the female. Based on the literature we did not expect our supplemented diet to be toxic (see "Iodine restricted and supplemented diet" in the methods section). If restricted dietary iodine limits TH production, we may expect a decrease in circulating THs in the mother, as found in ring doves, and/or a decrease in yolk THs. To cope with limited TH production due to iodine limitations, mothers may have developed regulatory mechanisms allowing them to trade off THs for self or for the egg. This would be reflected in a different distribution of TH concentration between mothers' circulation and in the egg in iodine-restricted compared to iodine-supplemented group. Alternatively, mothers may prioritise circulating and yolk TH concentrations by limiting egg production and thereby the total amount of TH allocated to yolks, thus prioritising quality over quantity of offspring. If this is true, we may expect iodine-restricted females to lay fewer eggs (i.e., smaller clutch sizes) than iodine-supplemented females. This would be consistent with two studies that showed that hypothyroidism ceased egg laying in Japanese quails (Wilson & McNabb, 1997), and reduced egg production in chickens (Van Herck et al., 2013). We also expected the effect of iodine restriction to be more pronounced as the exposure duration to the treatment becomes longer. For example, iodine stores may deplete with time in the I- diet, thus decreasing circulating and/or yolk iodine and THs with time in the I- group but not in the I+ group. In addition, laying several clutches under limited iodine availability may further deplete iodine and TH stores. We expected therefore yolk iodine and THs to decrease with clutch order in the I- diet. In general, and in line with previous studies (McNabb, Dicken & Cherry, 1985; McNichols & McNabb, 1987), we may expect limited dietary iodine to have a stronger effect on T_4 than on T_3, as T_4 needs one more atom of iodine and is much less biologically active.

Material and methods

Study species and housing conditions

The experiment was conducted in 2018 on 38 pairs of wild-type rock pigeons (*Columba livia*). Rock pigeons lay two eggs per clutch, with a 48-hour interval between the two eggs. In addition, rock pigeons can lay multiple clutches in a single breeding season (Johnston & Janiga, 1995). The birds were identified by unique ring code and combination and were housed in a large outdoor aviary (45 m long x 9 m wide x 4 m

high) in Groningen, the Netherlands, divided in 4 equal compartments (2 compartments per treatment, n = 9–10 breeding pairs per compartment, see below). The aviary included enough nest boxes and nesting material for all the breeding pairs. Before the experiment, all birds were fed a standard diet for pigeons (seed mixture Kasper™ 6721 + seed mixture Kasper™ 6712 + pellets P40 Kasper™ 6700). Standard food, water and grit were provided ad libitum. Before the experiment, 18 eggs were collected from unidentified females under standard diet and used for analyses of yolk iodine (see statistical section below).

Experimental design

Iodine restricted and supplemented diet

We provided the experimental birds with either an iodine restricted (I-, n = 19 pairs) or an iodine supplemented (I+, n = 19 pairs) diet until all eggs and blood samples were collected. Egg collection was ended around three weeks after the initiation of second clutches, leading to a total of approx. 10 weeks (see below for more details). The restricted diet contained 0.06 mg of iodine/kg of food (Altromin™ C1042) and the supplemented diet was the same food supplemented with 3 mg of iodine/kg by the manufacturer. Therefore, both diets had exactly the same composition of all essential micro- and macronutrients except iodine. The restricted treatment corresponds to about 10% of the iodine content in the standard pigeon diet (0.65 mg/kg), and approximately 20% of the minimum iodine requirement for ring doves (0.30 mg I/kg) according to Spear and Moon (1985). In addition, this restricted treatment corresponds to a low iodine treatment (0.05 mg/kg) used in a previous experiment on Japanese quails (McNabb, Blackman & Cherry, 1985) that induced a significant decrease in circulating and yolk iodine. The supplemented treatment (3 mg/kg) corresponds to ten times the minimum requirements estimated by Spear and Moon (1985). Since rock pigeons are 2 to 3 times larger than ring doves, our supplemented treatment was actually 3 to 5 times the minimum requirements of our study species, after correcting for body mass. In McNabb and colleagues (1985a), the maximal dietary iodine (1.2 mg I/kg feed) was ca. eight times the sufficient iodine concentration required for Japanese quails (0.15 mg I/ kg feed) and the authors observed no detrimental effects of this high dose. As our supplemented treatment was lower than that of McNabb and colleagues (1985a), we expected no detrimental effect of our supplemented treatment either. Food, water, and grit were provided ad libitum throughout the experiment.

Timeline of the experiment

Nest boxes were opened and nesting material was provided two weeks after the experimental diet was introduced to stimulate egg laying. Egg laying usually starts

within a week of nest-box opening. Based on Newcomer (1978), who fed hatchling chicken with low iodine diet (0.07 mg I/kg feed), we could expect thyroid iodine content to be the lowest from 10 days onwards after introducing the experimental diet. The first eggs (i.e., from the first experimental clutches) were collected 3 weeks after the introduction of the experimental diet and were collected over 12 days. On average, the eggs from 1st clutches were laid 26.4 days (SD = 2.9) days after the onset of the experimental diet. Freshly laid eggs were collected, replaced by dummy eggs to avoid nest desertion. Second clutches were initiated by removing dummy eggs approximately 2 weeks after the completion of the first clutch (i.e., 5 weeks after the start of the experimental diet), and eggs collected over a period of 18 days. On average, the eggs from 2nd clutches were laid 53.5 days (SD = 3.3) after the onset of the experimental diet. We also collected some late first clutches (on average 52.6 (SD = 6.5) days after the onset of the experimental diet).

Egg and blood sample collection

Table 1 summarises the number of samples collected. We collected both eggs from first and second clutches of females fed with restricted or supplemented iodine diets. We also collected blood samples after clutch completion from the two experimental groups (I- and I+). The second set of samples (i.e. eggs and blood) was collected to test for the effect of exposure duration of the treatment (see timeline below). Eggs and blood samples were collected in the exact same manner in the first and second clutches. Freshly laid eggs were collected and were stored in a –20°C freezer. Not all females laid complete clutches of 2 eggs, and several females did not lay an egg at all. Females were captured during incubation in the nest boxes, and blood samples (ca. 400 µl) were taken from the brachial vein after clutch completion (average (SD) = 4 (4.7) days after clutch completion, range = 0–21 days). Unfortunately, we could not blood sample the females that did not lay eggs, as this would have caused serious disturbance to all the birds in the same aviaries as we had to catch them by hand netting in the large aviary. Half of the blood sample (ca. 200 µl) was taken with heparinised capillaries for plasma extraction (for TH analyses) and stored on ice until centrifugation. The other half of the sample was taken with a sterile 1 ml syringe (BD Plastipak ™) and let to coagulate for 30 min at room temperature before centrifugation for serum extraction (for iodine analyses). Previous studies measured iodine in serum samples (McNabb, Blackman & Cherry, 1985; McNichols & McNabb, 1987), therefore we decided to measure iodine in the serum for comparable results. Whole blood samples were centrifuged at 3 500 RPM (ca. 1164 G-force) for 5 min to separate the plasma from red blood cells (RBCs), and at 5 000 RPM (ca. 2376 G-force) for 6 min to separate the serum from RBCs. After separation, all samples (plasma, serum and RBCs) were stored in a –80°C freezer for analyses of THs and iodine.

Table 1: Summary of the egg and blood samples collected.

		Untreated	**I-**		**I+**	
		1st clutches	1st clutches	2nd clutches	1st clutches	2nd clutches
Egg samples	Complete clutches	NA	6	6	12	5
	Incomplete clutches	NA	5	2	6	2
	Total number eggs	17	17	14	30	12
Blood samples	Number of females	NA	10	8	14	7

Each group included 19 females in total. 6 females could not be captured and sampled (1 in I- and 5 in I+ group).

Hormone and iodine analyses in plasma and yolk samples

Eggs were thawed, yolks separated, homogenised in MilliQ water (1:1) and a small sample (ca. 50 mg) was used for TH analysis. Yolk and plasma THs were analysed using nano-LC-MS/MS, following Ruuskanen et al. (2018, 2019). TH concentrations, corrected for extraction efficiency, are expressed as pg/mg yolk or pg/ml plasma.

Yolk and serum iodine (ICP-MS, LOD of 3 ng/g of yolk and 1.5 ng/ml of serum) analyses were conducted by Vitas Analytical Services (Oslo, Norway). Yolk iodine was measured in a sample of ca. 1 g of yolk, and serum iodine was measured in a sample of ca. 0.2 ml of serum.

Statistical analysis

General information

Data were analysed with the software R version 4.0.2 (R Core Team, 2021). To test for the effect of iodine restriction on egg laying, we compared the number of females that laid first clutches in both groups, and the total number of eggs laid in first clutches with two Pearson's chi-squared tests. The rest were fitted with linear mixed models (LMMs) using the R package lme4 (Bates et al., 2015) and p-values of the predictors and interactions were calculated by model comparison using Kenward-Roger approximation with the package pbkrtest (Halekoh and Højsgaard, 2014). The response variables were plasma THs concentrations (T3, T4), serum iodine, and concentrations of yolk THs and

yolk iodine. Relevant interactions between predictors were added in a full model and removed when non-significant to estimate the main effects. Post-hoc tests of interactions were performed with the package phia (de Rosario-Martinez, 2015). Model residuals were inspected for normality and homogeneity with the package DHARMa with 1,000 simulations (Hartig, 2020). When either of the assumptions was violated, the response was ln-transformed (see Tables) and in these cases the model residuals showed the required distributions. Estimated marginal means (EMMs) were calculated from the models using the package emmeans (Lenth, 2019). When the response was transformed, the EMMs were calculated on the back-transformed data. When presenting the results, we used the language of "statistical clarity" instead of "statistical significance", as the latter may interfere with the meaning of "biological significance" (Dushoff et al., 2019).

Although the treatment started for all females on the same date, each female, at the time of egg laying, was exposed to the experimental diet for different durations because of the varying laying dates between females. This may influence the effects of iodine manipulation on circulating and yolk iodine and THs. However, because the second clutches were laid after a longer exposure to the treatments than were first clutches (average exposure duration (SD) 2^{nd} clutches = 53.5 (3.3) days; 1^{st} clutches = 30.9 (10.6) days), clutch order and exposure duration (i.e., the number of days between the onset of the experimental diet and laying date) were confounded. Therefore, we used two separate models, one with exposure duration and another one with clutch order. We controlled for egg order (i.e. first or second egg in a clutch) in our models for yolk THs initially since a previous study in the rock pigeons showed a non-significant trend for higher yolk T_3 concentrations in the second eggs (Hsu et al., 2016). However, we detected no such effect in our models (all $F < 0.57$, all p-values > 0.45) and thus egg order was excluded from the final models.

Model specification

Circulating iodine (ln-transformed) and T_3 and T_4 concentrations were analysed by fitting an LMM with treatment (I- or I+), exposure duration, completeness of a clutch as a categorical variable (complete or incomplete, i.e., to further test for the effect of number of eggs laid), and the two-way interactions between treatment and exposure duration or completeness as fixed factors. Female identity (for iodine and T_4, but not T_3 because of singularity: variance estimate collapsed to 0) and hormone extraction batch (for T_3 and T_4) were added as random intercepts. Estimates and p-values of the main effects for T4 were obtained using the lmerTest package (Kuznetsova et al., 2017) using Kenward-Roger method for denominator degrees of freedom. However, this package presents t values instead of F values (provided by the pbkrtest package), hence Table 2 shows F statistics for circulating iodine and T3, and t statistics for circulating T4.

Yolk components (iodine, T_3, T_4) were analysed in two different sets of models. The first set of models, for yolk iodine only, compared untreated eggs (collected before the start of the treatment) to the eggs collected in the two treatments (I+ and I-). This way, we could test whether yolk iodine differed between untreated and experimental eggs. Here we only used experimental first clutches as only one clutch of eggs per untreated female was collected. The model only included treatment (a three-level categorical predictor: untreated, I+ and I-) as the predictor.

The second set of models tested the effect of iodine treatment, exposure duration to the treatment or clutch order on yolk iodine and THs. Here we included both 1st and 2nd clutches from iodine treatments, but no eggs from untreated females. Yolk iodine (ln-transformed) was first analysed in a LMM that included treatment as a categorical variable, exposure duration (days since the start of the experiment), completeness of a clutch (complete or incomplete) and the two-way interactions between treatment and exposure duration or completeness, and female identity as a random intercept. This LMM somewhat violated the assumption of homogeneity of variances between the groups because of the larger variance in yolk iodine in the I+ group. Nevertheless, such a violation should not undermine our results as a recent paper demonstrated that LMMs are fairly robust against violations of distributional assumptions (Schielzeth et al., 2020). Yolk iodine was also analysed in a similar model in which exposure duration was replaced with clutch order (1st or 2nd clutch, categorical variable), both as a main effect and in the interaction with treatment. Yolk T_3, T_4 (ln-transformed) were analysed using the same models (with exposure duration or clutch order) as for yolk iodine. Hormone extraction batch was added as a random intercept for yolk T_3 and T_4.

Results

Circulating iodine and TH concentrations

In line with our expectations, there was a clear effect of iodine treatment on circulating iodine concentrations: serum iodine was about 75% lower in the I- group than in the I+ group (raw data average (SE), I- = 11.1 (1.2) ng/ml serum, I+ = 44.0 (4.7) ng/ml serum; Table 2, Fig. 1). The effects of clutch completeness, exposure duration and their interactions with treatment on serum iodine were statistically unclear (Table 2).

Plasma T3 was not affected by supplementation or restriction of iodine, nor by exposure duration (Table 2; Fig. 2A). There was a statistically significant interaction between iodine treatment and exposure duration of the treatment on plasma T4 (Table 2). A post-hoc test of the interaction showed that plasma T4 increased with time in the I+ group but not in the I- group (adjusted slope±s.e.m. I+ = 0.24±0.07, $\chi^2 = 12.9$, Holm-adjusted $p < 0.001$; adjusted slope±s.e.m. I- = 0.05±0.06, $\chi^2 = 0.56$, Holm-adjusted $p = 0.46$; Fig. 2B). Yet, the large confidence intervals warrant due caution in interpreting this

interaction. There were no clear effects of clutch completeness and its interaction with treatment on plasma THs (Table 2).

Egg iodine and egg TH concentrations

Untreated eggs vs 1st clutches of the iodine treatments

In line with our prediction, eggs from the I- group had ca. 87% lower iodine levels than eggs from the I+ group and 50% lower iodine levels than untreated eggs; eggs from the I+ group had ca. four times higher iodine levels than untreated eggs (back-transformed EMMs (SE), untreated = 29.7 (4.1) ng/g yolk, I- = 16.1 (2.0) ng/g yolk, I+ = 123.4 (11.9) ng/g yolk; (overall test: LM, $F = 91.3$, $p < 0.001$); post-hoc Tukey comparisons, all $|t| > 3.38$ and all $p < 0.004$, Fig. 3).

Effect of exposure duration and clutch order (experimental eggs from 1st and 2nd clutches)

Also in this dataset, yolk iodine concentration was 87% lower in eggs of I- treated females than in I+ females (mean (SE), I- = 16.03 (0.52) ng/g yolk, I+ = 117.48 (12.36) ng/g yolk; Table 3, Fig. 4), but longer exposure duration, clutch completeness or clutch order had no clear effect on yolk iodine concentration (Table 3).

Yolk T_3 was not affected by iodine treatment (EMMs (SE) T_3: I- = 2.67 (0.20) pg/mg yolk, I+ = 2.95 (0.20) pg/mg yolk), but showed a slight increase over the exposure duration and with clutch order (Table 3; Fig. 5A). Yolk T_4 was not affected by iodine treatment (back-transformed EMMs (SE) T_4: I- = 7.13 (0.59) pg/mg yolk, I+ = 7.48 (0.60) pg/mg yolk), exposure duration of the treatment, or by clutch order (Table 3; Fig 5B). Finally, there were no clear effects of clutch completeness and its interaction with treatment on yolk THs (Table 3).

Egg production

Overall, fewer females in the I- group laid their first clutches (complete or incomplete) than those in the I+ group (I- = 11 out of 19 females, I+ = 18 out of 19 females; $\chi^2 = 5.24$, $p = 0.02$; Table 1). This resulted in fewer eggs produced in the I- group than in the I+ group (I- = 17 eggs, I+ = 30 eggs; $\chi^2 = 8.03$, $p = 0.005$; Table 1). Focusing on the complete clutches, half as many females in the I- group laid complete clutches compared to those in the I+ group, yet the difference between both groups was unclear (I+ = 12/19 females, I- = 6/19 females; $\chi^2 = 2.64$, $p = 0.10$; Table 1).

Table 2: Results of the LMMs on circulating THs and iodine from rock pigeon females treated with an I- or I+ diet.

Predictor	**Ln-Iodine**			**T_4**			**T_3**		
	Estimate±s.e.m.	F_{ddf}	p	Estimate±s.e.m.	t_{ddf}	p	Estimate± s.e.m.	F_{ddf}	p
Treatment (I+)	1.43±0.15	$93.6_{17.0}$	**<0.001**	-6.89 ±5.44*	$-1.27_{25.3}$	0.07	-0.02±0.37	$0.002_{32.0}$	0.96
Exposure duration	0.005±0.004	$1.63_{12.0}$	0.23	0.04±0.06*	$0.58_{12.4}$	0.47	0.002±0.01	$0.04_{32.0}$	0.85
Complete clutch (Yes)	-0.01±0.15	$0.01_{22.5}$	0.93	-0.19±3.62	$-0.05_{29.3}$	0.37	0.35±0.41	$0.73_{32.0}$	0.40
Treatment (I+) * exposure duration	-0.003±0.008	$0.10_{11.1}$	0.75	0.21±0.09	$2.23_{15.7}$	**0.04**	-0.004±0.02	$0.03_{30.0}$	0.87
Treatment (I+) * Complete clutch (Yes)	-0.05±0.33	$0.02_{20.7}$	0.89	-4.30±4.78	$-0.84_{30.0}$	0.41	-0.33±0.86	$0.15_{30.1}$	0.70

LMM on plasma T_4 included batch of hormone extraction and female identity as random intercepts. LMM on plasma T_3 only included batch as the random intercept. LMM on serum iodine only included female identity as the random intercept. Interactions were included in the full models and tested one by one by comparison with a model without the interaction of interest. P-values and estimates±s.e.m. for main effects were calculated from a model without interactions, except for the model on circulating T_4. For this model, the significant interaction was retained in the model to calculate the estimates±s.e.m. and p-values of the main effects. Circulating iodine was ln-transformed to achieve homogeneity of the model residuals. Ndf = 1. See Table 1 for sample sizes. * Because of the present interaction between treatment and exposure duration, the estimates±s.e.m. of the main effects here represents the values when the other factor was 0.

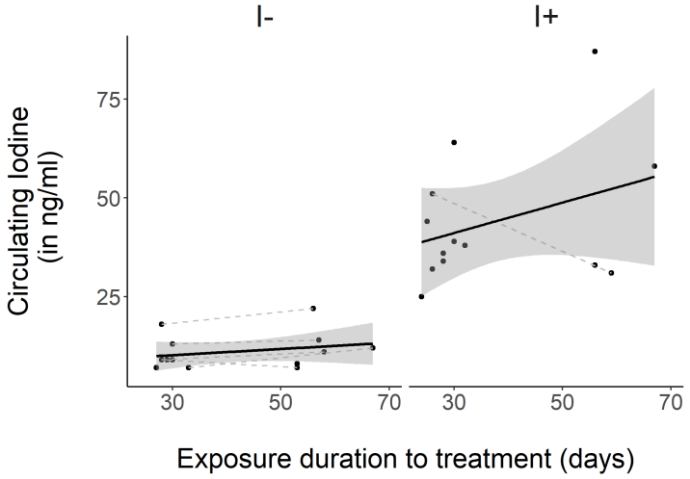

Figure 1: Circulating iodine in rock pigeon females treated with an I- or I+ diet. Black lines and shadow areas represent average values and 95% CI within each group, and grey dashed lines connect blood samples from the same females. Some females were only captured once, hence not all dots are connected. See Table 1 for sample sizes.

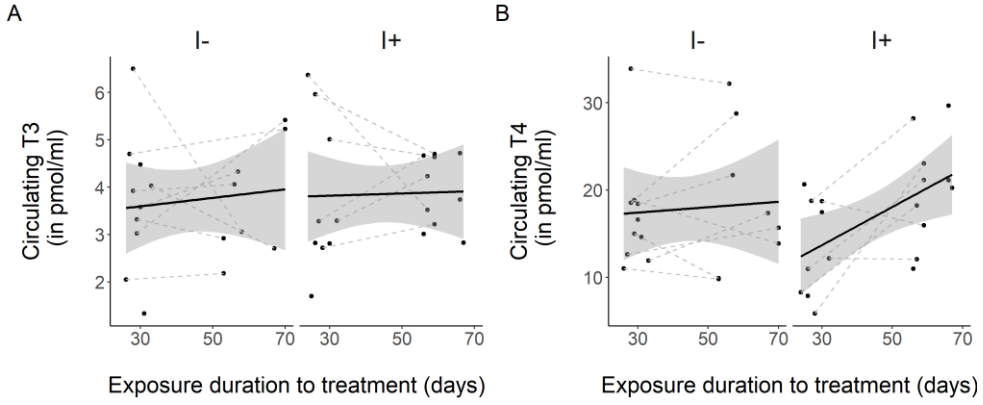

Figure 2: Circulating T_3 (A) and T_4 (B) in rock pigeon females treated with an I- or I+ diet. Black lines and shadow areas represent average values and 95% CI within each group, and grey dashed lines connect blood samples from the same females. Some females were only captured once, hence not all dots are connected. See Table 1 for sample sizes.

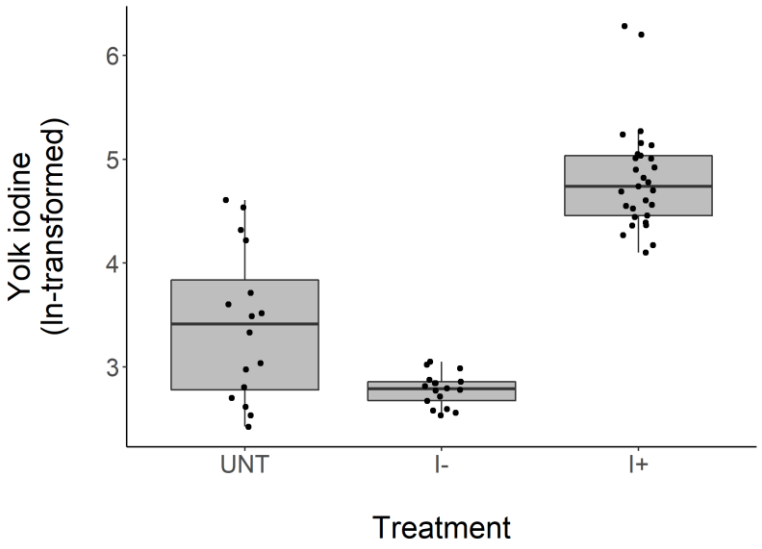

Figure 3: Yolk iodine in eggs from 1st clutches laid by rock pigeon untreated females (UNT), females treated with an I- or an I+ diet. See Table 1 for sample sizes.

Table 3: Results of the LMMs on yolk iodine (a), T_4 (b) and T_3 (c) in eggs from 1st and 2nd clutches laid by rock pigeon females treated with an I- or I+ diet.

(a) Predictor	**Ln-Iodine**		
	Estimate (SE)	F_{ddf}	p
Treatment (I+)	2.04 (0.15)	$183.82_{21.5}$	**<0.001**
Exposure duration	0.001 (0.002)	$0.14_{47.3}$	0.71
Complete clutch (Yes)	-0.03 (0.13)	$0.07_{60.4}$	0.80
Treatment (I+) * exposure duration	0.007±0.005	$1.70_{45.4}$	0.20
Treatment (I+) * complete clutch (Yes)	0.09±0.26	$0.11_{57.3}$	0.74
Treatment (I+)	2.04 (0.15)	$180.55_{21.9}$	**<0.001**
Clutch order (2)	-0.02 (0.07)	$0.09_{43.9}$	0.76
Complete clutch (Yes)	-0.02 (0.13)	$0.03_{60.5}$	0.87
Treatment (I+) * clutch order (2)	0.08±0.14	$0.35_{42.4}$	0.55
Treatment (I+) * complete clutch (Yes)	0.11±0.27	$0.16_{57.6}$	0.69

(b) Predictor	**Ln-T_4**		
	Estimate (SE)	F_{ddf}	p
Treatment (I+)	0.05 (0.08)	$0.36_{21.3}$	0.56
Exposure duration	-0.001 (0.001)	$0.49_{44.6}$	0.49
Complete clutch (Yes)	-0.04 (0.07)	$0.38_{57.7}$	0.54
Treatment (I+) * exposure duration	0.001±0.003	$0.16_{42.8}$	0.69
Treatment (I+) * complete clutch (Yes)	-0.06±0.14	$0.15_{55.4}$	0.70
Treatment (I+)	0.04 (0.08)	$0.31_{21.7}$	0.58
Clutch order (2)	-0.02 (0.04)	$0.30_{41.3}$	0.59
Complete clutch (Yes)	-0.05 (0.07)	$0.42_{57.7}$	0.52
Treatment (I+) * clutch order (2)	0.02±0.07	$0.10_{40.4}$	0.76
Treatment (I+) * complete clutch (Yes)	-0.05±0.14	$0.12_{55.3}$	0.72

(c) Predictor	**T_3**		
	Estimate (SE)	F_{ddf}	p
Treatment (I+)	0.28 (0.18)	$2.40_{18.4}$	0.14
Exposure duration	0.01 (0.005)	$7.59_{52.3}$	**0.01**
Complete clutch (Yes)	-0.15 (0.20)	$0.54_{49.2}$	0.47
Treatment (I+) * exposure duration	0.01±0.01	$1.30_{48.9}$	0.25
Treatment (I+) * complete clutch (Yes)	0.20±0.41	$0.24_{50.3}$	0.61
Treatment (I+)	0.32 (0.18)	$3.31_{18.8}$	0.09
Clutch order (2)	0.36 (0.13)	$7.18_{47.8}$	**0.01**
Complete clutch (Yes)	-0.14 (0.20)	$0.46_{49.1}$	0.50
Treatment (I+) * clutch order (2)	0.39±0.26	$2.13_{46.2}$	0.15
Treatment (I+) * complete clutch (Yes)	0.15±0.40	$0.13_{50.4}$	0.71

Two different LMMs (separated by the dashed line) were computed, one with exposure duration, and the other one with clutch order. LMMs on yolk THs included batch and female identity as random intercepts. LMM on yolk iodine only included female identity as the random intercept. P-values and estimates (SE) for main effects were calculated from a model without the interaction. Interactions were added one by one and tested by comparison with the model without any interactions. Yolk iodine and T_4 were ln-transformed to achieve homogeneity of the model residuals. Ndf = 1. See Table 1 for sample sizes.

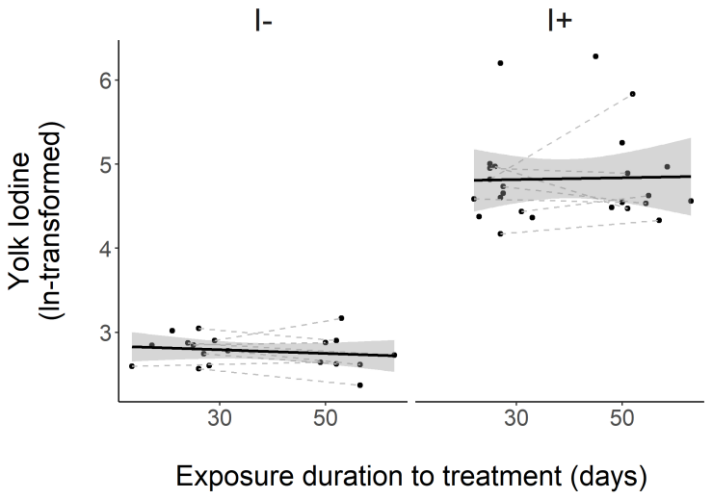

Figure 4: Yolk iodine in eggs from 1st and 2nd clutches laid by rock pigeon females treated with an I- or I+ diet. Eggs from the same female and the same clutch were averaged. Black lines and shadow areas represent average values and 95% CI within each group, and grey dashed lines connect clutches from the same females. Some females did not lay two clutches, hence not all dots are connected. See Table 1 for sample sizes.

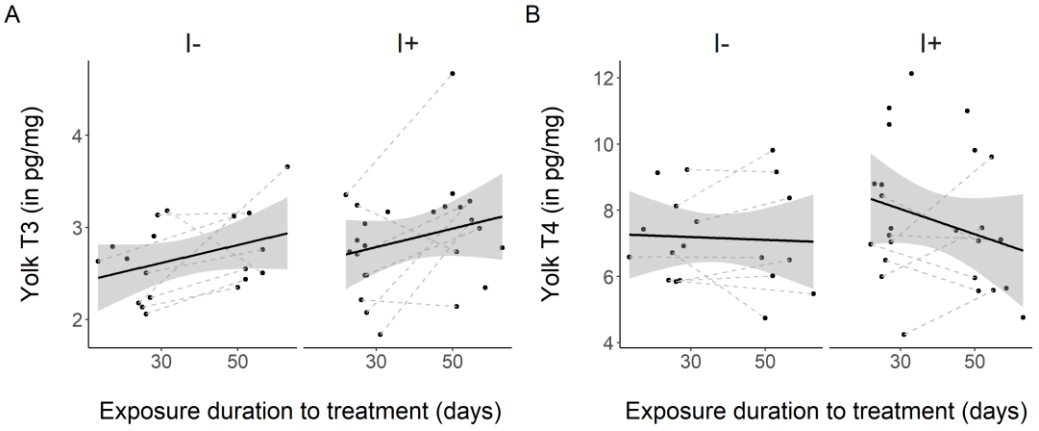

Figure 5: Yolk T_3 (A) and T_4 (B) in eggs from 1st and 2nd clutches laid by rock pigeon females treated with an I- or I+ diet. Black lines and shadow areas represent average values and 95% CI within each group, and grey dashed lines connect clutches from the same females. See Table 1 for sample sizes.

Discussion

In this study we tested whether dietary iodine limits mothers' circulating TH concentration, TH transfer to the yolk, and egg production. To our knowledge, our study is the first to investigate the potential trade-off between circulating and yolk THs induced by low dietary iodine. We found that fewer females laid first clutches under the iodine-restricted diet compared to the females under the iodine-supplemented diet, resulting in a lower total number of eggs laid. We found that the iodine restricted diet decreased circulating and yolk iodine levels, though circulating and yolk THs were unaffected. Longer exposure to restricted iodine had no clear effect on circulating or yolk iodine and THs. Finally, we observed a slight increase in plasma T_4 (in the I+ group only) and in yolk T_3 across time that was unrelated to the dietary iodine and is likely explained by seasonal changes or clutch order effects. Yet, because exposure duration to the treatment and clutch order are partly confounded, our experimental design does not allow us to fully disentangle both variables.

Does restricted iodine induce a cost and a trade-off between circulating and yolk THs?

Our iodine-restricted diet successfully decreased circulating iodine concentrations compared with the supplemented diet. Despite this effect, we observed no differences in circulating TH concentrations. This is consistent with a previous study that showed that Japanese quails under limiting iodine availability maintained normal circulating THs concentrations (McNabb, Blackman & Cherry, 1985). However, a similar study on ring doves found a decrease in circulating T_4 concentrations with no changes in circulating T_3, suggesting increased peripheral conversion from T_4 to T_3 to maintain normal T_3 levels (McNichols & McNabb, 1987). The causes for discrepancies between our study and that of the dove study (McNichols & McNabb, 1987) are not clear. One potential explanation is that, in our study, we could only sample the females that laid eggs and thus apparently managed to maintain normal circulating THs despite restricted iodine whereas those that did no lay eggs may have suffered from low circulating TH concentrations.

In the yolk, like in the circulation, restricted dietary iodine decreased yolk iodine but not yolk TH concentration, in contrast to our predictions. The result of decreased yolk iodine is in line with a previous study on quails, which found that mothers fed with low dietary iodine produced eggs with low iodine but did not report yolk TH concentrations (McNabb, Blackman & Cherry, 1985). Low egg iodine concentration in turn disturbs thyroid function in embryos and hatchlings (McNabb, Dicken & Cherry, 1985; Stallard & McNabb, 1990). Circulating TH concentrations of embryos, however, were not affected by low egg iodine (McNabb, Dicken & Cherry, 1985; Stallard & McNabb, 1990).

Contrary to previous studies that manipulated dietary iodine, we found that limited iodine availability hampered egg production, with 40% fewer females producing eggs in the I- group compared to the I+ group. However, females that managed to maintain normal circulating THs were also able to lay eggs with normal yolk THs, similar to the study by McNabb and colleagues (1985). At the moment it is unclear why some females were affected and others not, but a potential explanation may be for example individual differences in the ability to store iodine. Two other studies found that administration of methimazole, a TH-production inhibitor, ceased egg laying in Japanese quails (Wilson & McNabb, 1997), and reduced egg production in chickens (Van Herck et al., 2013). These results suggest that our restricted diet might have induced hypothyroidism in some females, thus preventing them from laying eggs.

Therefore, we did not show evidence for a cost of restricted iodine in females that managed to lay eggs. Those females did not appear to face any trade-off between allocating iodine and THs to either self or their eggs. Yet, 40% of the females under the restricted diet paid a cost in terms of egg production. Whether this effect is due to limited production of THs is as yet unclear, but these females may have faced a trade-off between maintaining normal circulating THs and yolk TH deposition. Our results suggest that females would favour investing in eggs with normal yolk THs, but when facing suboptimal environmental conditions they may prefer to reduce egg production.

Is there a regulatory mechanism to cope with the cost of restricted iodine and its associated trade-off?

The fact that some females did not lay egg, supposedly to maintain normal circulating THs, suggests that mothers are not able to regulate yolk TH deposition independently from their own circulating THs, as recently proposed by Sarraude et al. (2020c). This is contradictory to previous studies showing evidence of independent regulation (Wilson & McNabb, 1997; Van Herck et al., 2013). However, the latter two studies induced supraphysiological hypo- or hyperthyroidism to the birds, which may explain such discrepancies.

Interestingly, we found that our restricted diet reduced egg production. As discussed above, this may be due to a hypothyroid condition that prevented females from laying eggs. There is, to our knowledge, no evidence that iodine is directly involved in egg production (e.g., follicle maturation, yolk formation, shell formation). However, previous studies have shown detrimental effects of limited iodine availability on embryos and hatchlings. This may have evolved to protect embryos from exposure to too low iodine and/or TH concentrations. In breeding hens, restricted dietary iodine can decrease egg hatchability (Rogler et al., 1959, 1961b) and retard embryonic development (Rogler et al., 1959, 1961b; McNabb, Dicken & Cherry, 1985), and can induce thyroid gland hypertrophy in embryos and hatchlings (Rogler et al., 1961a; McNabb et al.,

1985b; but see Stallard & McNabb, 1990). As producing such low-quality eggs and offspring is a waste of resources, decreasing egg production under low iodine availability seems adaptive. Thus, such regulation may have evolved to protect embryos from exposure to too low iodine and/or TH concentrations. Overall, our results suggest that mothers in the restricted group appear to prioritise self-maintenance and offspring quality over offspring quantity.

Restricted iodine and trade-offs in wild populations

Our low-iodine diet (0.06 mg I/kg food) is comparable to what birds may sometimes experience in the wild. Although relevant data are scarce, estimates of iodine content in food items such as barley and maize grains, wheat, or rye is highly variable, ranging from 0.06 to 0.4 mg I/ kg (Anke, 2004). Insectivorous species may also encounter iodine deficiency as the iodine content in insects vary from <0.10 up to 0.30 mg I/kg (Anke, 2004). As such low iodine availability can also be found in the wild, it is therefore relevant to study whether mothers may face trade-offs in iodine or TH allocation during the breeding season, or whether it influences egg laying itself. Yet, our study did not show evidence for the existence of a trade-off between circulating and yolk THs when environmental iodine is limited. Nevertheless, mothers may face a trade-off between allocating resources to themselves or producing eggs of sufficient quality.

In conclusion, we found that restricted dietary iodine did not decrease circulating or yolk THs despite reduced circulating and yolk iodine, and thus could not find evidence of a trade-off between allocating THs to self or to the eggs. Restricting iodine intake even further might help revealing such a trade-off. However, reducing iodine too much would render mothers hypothyroid, likely stopping egg production. Our results provide evidence that restricted availability of iodine induces a cost on egg production. Thus, mothers may not be able to regulate yolk TH transfer but may be able to regulate egg production when facing limited iodine. Our results also indicate that females under limited iodine availability may prioritise their own metabolism over reproduction or avoid exposing their offspring to detrimentally low iodine and/or THs. These explanations serve as interesting hypotheses for future research to further explore the consequences of limited iodine in wild populations.

Acknowledgments

We thank Martijn Salomons and the animal caretakers for helping us maintaining the colony. We are also grateful to Gerard Overkamp and Asmoro Lelono for their help with blood sampling. We also thank Bonnie de Vries and Tiphaine Bailly for their help with egg dissection.

Ethics

All the procedures were approved by the Centrale Commissie Dierproeven (AVD1050020185444) and the Animal Welfare Body of the University of Groningen (18544-01-001).

Chapter 3

Testing different forms of regulation of yolk thyroid hormone transfer in pied flycatchers

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Abstract

Hormones transferred from mothers to their offspring are considered a maternal tool to prepare progeny for expected environmental conditions, increasing maternal and offspring fitness. To flexibly influence offspring, mothers should be able to transmit the hormonal signals independent of their own hormonal status. However, the ability to regulate hormone transfer to the next generation is under debate. We studied the transfer of thyroid hormones (THs) to eggs in a bird model. We elevated thyroxine (T_4, the prohormone for the biologically active triiodothyronine, T_3) during egg-laying using T_4 implants in females of a wild population of pied flycatchers (*Ficedula hypoleuca*), and measured the resulting plasma and yolk T_4 and T_3 levels. We found an increase in plasma and yolk T_4 and no changes in plasma or yolk T_3 concentrations, leading to a decrease in yolk T_3/T_4 ratio in response to the T_4 treatment. The yolk T_3/T_4 ratio was similar to the plasma ratio in females during the yolking phase. This suggests that mothers are not able to regulate TH transfer to yolk but may regulate the T_4-to-T_3 conversion to avoid potential costs of elevated exposure to the active hormone to herself and to her progeny. The absence of regulation in hormone transfer to eggs is in contrast to our predictions. Future studies on deiodinases activity that converts T_4 to T_3 in maternal and embryonic tissues may help our understanding of how mothers regulate circulating THs during breeding, as well as the embryos' role in converting maternal T_4 to its biologically active T_3 form during development.

Introduction

Maternal effects are the non-genetic influences of a mother on her progeny and are thought to be adaptive (Mousseau & Fox, 1998b; Moore, Whiteman & Martin, 2019; Yin et al., 2019). Maternal hormones transferred to the next generation are a potential prenatal pathway for mothers to shape their offspring phenotype (Groothuis et al., 2005, 2019; Ruuskanen & Hsu, 2018). Transfer of maternal hormones may be adaptive when the environment of the offspring can be predicted by the mothers ("anticipatory maternal effects", Marshall & Uller, 2007). Mothers transfer thyroid hormones (THs), which have so far received little attention compared to glucocorticoids and androgens (Ruuskanen & Hsu, 2018). THs are produced by the thyroid gland and are present in two main forms: thyroxine (T_4) and triiodothyronine (T_3). T_4, a precursor of T_3, is converted to T_3 in tissues. T_3 exerts most of the TH action, as a result of its much greater affinity to TH receptors than T_4 (ca. 50-fold greater, Zoeller et al., 2007), although T4 can also exert non-genomic actions (Davis, Goglia & Leonard, 2016). Thyroid hormones have pleiotropic effects that serve several biologically important functions across vertebrates, including growth, reproduction and metabolism (Ruuskanen & Hsu, 2018; Behringer et al., 2018).

Regulatory mechanisms of maternal hormone transfer are essential to minimise physiological trade-offs between optimal hormone exposure in the mother versus that in the offspring (Groothuis & Schwabl, 2008). For example, circulating THs increase with low temperature to stimulate metabolism and hence heat production (McNabb & Darras, 2015). If elevated maternal circulating THs increase yolk THs, this could stimulate embryo metabolism, which could be detrimental if the resources in the egg are insufficient to cope with the increased metabolism. The evidence for a regulatory mechanism for several hormones, including corticosterone and THs, is mixed (Groothuis & Schwabl, 2008), but such regulation could take place at the circulating level in the mothers and/or at the follicle level. Regulation at the follicle level may happen by controlling the transfer or conversion of THs or by producing THs independently from the thyroid gland. These mechanisms have been suggested to exist in human ovaries (Rae et al., 2007; Monteleone, Faviana & Artini, 2017). Such regulatory mechanisms may allow mothers to regulate the deposition of THs in their eggs independently from their own circulating TH levels. This would free mothers from the possible constraint to optimise their own circulating levels of THs and the levels in their eggs independently of each other. A few studies in birds have shown some preliminary evidence that mothers may indeed be able to regulate yolk TH transfer. In Japanese quails, a low-dose oral administration of T_4 resulted in an increase in yolk T_3 but not in circulating T_3, whereas T_4 increased in both tissues (Wilson & McNabb, 1997). Administration of T_3 in turn increased plasma T_3 but not yolk T_3 (Wilson & McNabb, 1997). Furthermore, artificial

blocking of TH production in hens led to a decrease in yolk T_3 but not in plasma T_3, while T_4 decreased in both tissues (Van Herck et al., 2013). These studies on domesticated precocial birds respectively induced hyperthyroidism for 3 to 6 weeks and hypothyroidism for 16 weeks. Such long exposure to hormone concentrations outside of the natural range may have triggered responses one would not observe under natural variations. Therefore, there is a need for complementary studies under shorter time scales and within the physiological range of wild altricial species.

In this experiment we tested whether mothers are able to regulate their transfer of yolk THs, at the circulating and/or at the follicle level. We experimentally manipulated TH levels with T_4 implants using a within-subject design in female pied flycatchers (*Ficedula hypoleuca*) during egg-laying and collected plasma samples and unincubated pre- and post-implantation eggs for the analysis of T_3 and T_4. Implanting the prohormone T_4 enabled us to test the possible differential conversion of this hormone to the biological active T_3 in the mother as a regulatory mechanism to protect herself or the egg from increased exposure to these hormones. It also allowed us to test whether mothers can regulate the transfer of hormones to the egg. First, if the implant successfully increased circulating T_4, we would expect a higher availability of the substrate (i.e. the prohormone T_4) in tissues and subsequent conversion to T_3. Some of the increased T_3 in tissues may be released back into the circulation, leading to increased plasma T_3 (e.g., Escobar-Morreale et al., 1995). Alternatively, females may buffer the increase in plasma T_4 by downregulating the conversion of T_4 to T_3, thus yielding no increase in tissue or plasma T_3. Second, we predicted that if mothers were able to regulate yolk TH transfer independently from their circulating levels, only one of these two compartments (i.e. plasma or yolk) would be affected by exogenous T_4, or one would be more affected than the other (Groothuis & Schwabl, 2008). In this case, the T_3/T_4 ratio may be different between the two tissues. Conversely, if mothers were unable to regulate yolk TH transfer, one would expect both plasma and yolk THs to vary in the same direction and with a similar magnitude in response to exogenous T_4 (Groothuis & Schwabl, 2008). Thus, the T_3/T_4 ratio would not differ between the tissues. If implants increased plasma THs (i.e. T_4 and/or T_3), they may also have increased female metabolism, thus affecting their body mass and percentage of red blood cells (haematocrit). One would expect a decrease in body mass if resources are not sufficient, and an increase in haematocrit as a result of increased energy expenditure, although the latter relationship may not be so straightforward (Fair, Whitaker & Pearson, 2007).

Material and methods

The experiment was conducted in 2016 and 2017 in Turku, Finland (60°26'N, 22°10'E). The study species, the pied flycatcher Ficedula hypoleuca (Pallas 1764), generally lays

a single clutch of 6 to 7 eggs. Into egg-laying females we inserted either a T_4 implant (10 µg, hereafter T4) or a control implant (hereafter CO) (Innovative Research America, Sarasota, FL, USA). The amount of T_4 was aimed to mimic natural T_4 production and was designed to release the hormone steadily over 21 days (see below for more details on the dose and implantation).

Preparation of T_4 implants and implantation

Two types of sterile implants (ca. 3 mm of diameter) were used for this experiment: ready-made T_4 pellets (10 µg, hereafter T_4 implants) and respective controls (CO) which were identical, but without T_4 (both implants from Innovative Research America, Sarasota, FL, USA). The amount of T_4 in the implants was based on the natural production rate of T_4 measured in chickens, quail and pigeons (1–3 µg T_4/100 g of body mass per day (McNabb & Darras, 2015) and adjusted to the average body mass of pied flycatchers. The T_4 was embedded in a matrix that is designed to steadily release the hormone for 21 days.

Before implantation between the scapula, the skin was disinfected with a cotton pad dipped in 70% ethanol. An incision was made with a 18G needle (BD Microlance ™) and the implant was inserted and pushed away from the incision to avoid losing the implant. The wound was sealed with veterinary tissue adhesive (3M Vetbond™), which is commonly used in experiments with pit tags and shown to have no effects on birds.

Experimental design - captive females

First, to validate that implants increased circulating THs in a short time window after implantation, we conducted an experiment with female pied flycatchers in captivity in 2016. As the yolk formation takes approximately 3.5 to 4 days in passerines (Williams, 2012), implants inserted during egg-laying need to increase hormone levels within days to enable quantification of their effect on newly formed eggs. We captured egg-laying female flycatchers from the wild population and housed them on a natural photoperiod and ad libitum food for the validation experiment as repeated disturbance during egg-laying in the wild could cause nest desertion. Because of the similar treatment and breeding stage, we expect the data on wild-caught captive birds to be similar to plasma levels of wild birds in the main experiment (see below). On the 4th day after capture, each female received either a subcutaneous control or T_4 implant (n = 4 per group). Blood samples were taken before the implant was inserted, and at 24 hours and 72 hours after the implant (between 09.30 h and 11.30 h). After the last blood sample, females were released to the site where they were captured. Circulating TH levels in response to the implants are presented in Table 1.

Experimental design - wild females

In the wild population, the experiment was conducted in 2016 and 2017. The first egg of a clutch was collected freshly on the day it was laid and replaced by a dummy egg as a within-clutch control (hereafter "pre-implant"). On the morning that the second egg was laid (07:00–09:00 h), females were captured and weighted (± 0.1 g), and received a T_4 (n = 11) or a control implant (n = 10) as above. The last egg of the clutch (mean egg rank (SD) = 6 (0.25), hereafter "post-implant") was collected (on the day it was laid), as it is mostly formed under the influence of the hormone implant (see above). In total, we collected 26 eggs from 13 clutches (13 pre- and 13 post-implant eggs) in the T_4 implant group, and 22 eggs from 11 clutches (11 pre- and 11 post-implant eggs) in the control group. Early in the incubation, on average 1.3 days (SD = 1.0) after the last egg was laid, females were blood sampled (8 a.m.–12.30 p.m.) for the analysis of circulating T_4 and T_3 (n = 11 for T_4 implant and n = 10 for control, respectively, blood sample analysis failed for 3 females) as well as haematocrit (proportion of red blood cells obtained by centrifugation of the capillaries). Body mass was also recorded (± 0.1 g) to analyse potential body mass loss following the insertion of the implant. During blood sampling, the implants were still visible, which indicates that the implants were still releasing T_4 at that time.

Hormone analysis

Blood samples (ca. 40 µl) were taken from the brachial vein. Plasma was collected via centrifugation and frozen at -20°C until analysis. Eggs were thawed, the yolks separated and homogenised in MilliQ water (1:1) and a small sample (ca. 50 mg) was used for TH analysis. Yolk and plasma THs were analysed using nano-LC-MS/MS, following Ruuskanen et al. (2018, 2019). TH concentration, corrected for extraction efficiency, is expressed as pg/mg yolk or pg/ml plasma.

Table 1: Circulating thyroxine (T_4), triiodothyronine (T_3) and T_3/T_4 ratio in captive female pied flycatchers in response to T_4 or control implants.

	Control implant			**T_4 implants**		
Time (h)	0	24	72	0	24	72
n	3	4	4	3	3	3
T_4 (in pg/µl)	4.9 (1.7)	3.5 (1.3)	4.9 (1.9)	5.3 (2.6)	28.9 (18.0)	8.4 (3.3)
T_3 (in pg/µl)	0.7 (0.5)	0.5 (0.4)	0.9 (0.5)	0.7 (0.1)	0.7 (0.2)	1.1 (0.2)
T_3/T_4 ratio	0.16 (0.10)	0.20 (0.19)	0.17 (0.09)	0.18 (0.14)	0.04 (0.04)	0.15 (0.09)

Females were sampled prior to insertion of the implant, and 24 and 72 h later. Thyroid hormone (TH) analysis of one sample (time 0 in control group) failed as a result of too low a plasma volume. TH data are means (SD).

Statistical analysis

Data were analysed with the software R version 3.6.2 (R Core Team, 2021). Linear mixed models (LMMs) were fitted using the R package *lme4* (Bates et al., 2015). P-values were obtained by model comparison using Kenward-Roger approximation from the package *pbkrtest* (Halekoh & Højsgaard, 2014). Estimated marginal means and standard errors (EMMs ± SE) were derived from models using the package *emmeans* (Lenth, 2019). Effect size estimates (Cohen's *d)* obtained from marginal means were computed with the package *emmeans*. Effect size estimates obtained from the raw data were calculated with the package *effsize* (Torchiano, 2020). Model residuals were checked for normality and homogeneity with the package DHARMa (Hartig, 2020).

Yolk THs were ln-transformed to achieve normal distribution of the residuals. Yolk TH concentrations and T_3/T_4 ratio were analysed by fitting linear mixed models that included the treatment (i.e., T_4 or control implant) as the predictor, hormone levels in the pre-implant egg and year as covariates, and the hormone assay as a random intercept. Pre-implant egg was added to account for the variation in yolk THs among females.

Plasma TH levels of the incubating females were analysed using linear regressions with the treatment as a fixed factor and body mass, ambient temperature and time of the day as covariates, as these covariates are known to influence circulating levels (McNabb & Darras, 2015). Covariates were centred and scaled. Year was not included in the model as it covaried with ambient temperature (variance inflation factor, VIF > 2), and the latter is known to affect circulating THs (McNabb & Darras, 2015). Plasma T_3/T_4 ratio was ln-transformed and analysed with an identical linear regression to that of plasma THs.

Female haematocrit and loss of body mass were analysed by fitting linear models with treatment as the fixed factor, and year and clutch size (as a proxy for reproductive investment) as covariates.

Effect sizes (Cohen's *d*) of the treatment on yolk and wild female plasma THs were estimated from marginal means. Effect size estimates of the treatment on the T_3/T_4 ratio in the yolk and in captive female plasma were computed from the raw data. To avoid nest abandonment, we did not blood sample wild females during egg laying. Therefore, we used the data from captive birds in the following way: Plasma samples from captive females averaged over day 1 and 3 after the implantation (reflecting the yolking phase of the last egg in wild birds) were compared with the post-implant last eggs collected from wild females.

Results

Yolk THs of pre-implant eggs (first eggs of a clutch) did not differ between females with control or T_4 implants (mean yolk T_4 (SE), control = 8.29 (0.61) pg/mg yolk vs T_4 implant = 7.63 (0.43) pg/mg yolk; mean yolk T_3 (SE), control = 2.85 (0.20) pg/mg yolk vs T_4 implant = 2.94 (0.38) pg/mg yolk; all $t \leq 0.89$ and all $p \geq 0.39$). After receiving a T_4 implant, females produced eggs with ca. two times higher yolk T_4 concentration than control-implanted females (Estimated marginal means, EMMs ± SE: post-implant treated egg = 17.14 ± 2.07 pg/mg yolk vs post- control implant egg = 8.54 ± 1.08 pg/mg yolk, Table 2, Figs. 1A, 2A). Pre-implant yolk T_4 did not predict post-implant yolk T_4 (Table 2). In contrast, post-implant yolk T_3 did not differ between the groups (mean (SE), control = 2.34 (0.27) pg/mg yolk vs T_4 implant = 2.72 (0.29) pg/mg yolk; Table 2, Figs. 1B, 2A), but pre-implant yolk T_3 predicted post-implant yolk T_3 (Table 2).

Regarding circulating THs, captive females implanted with a T_4 implant had higher circulating T_4 than captive control females during the first 3 days after the implant (Table 1). There was a similar, but non-significant, trend in wild female plasma T_4 early in the incubation (when egg laying was finished, ca. 10 days after implantation) (Table 2). Plasma T_3 and T_3/T_4 ratio were not affected by the implants in wild females (Table 2).

The treatment decreased the T_3/T_4 ratio in the post-implant egg (Table 2) and the trend was similar for plasma levels in captive females sampled during egg formation (Table 1, Fig. 2B). The effect sizes for T_3/T_4 ratios in plasma and in yolk were largely overlapped, suggesting no clear difference between the two tissues (Fig. 2B).

The T_4 implant did not affect female body mass loss or haematocrit (Table 3). Female body mass loss and haematocrit did not differ between the sampling years (Table 3). Clutch size was not associated with female body mass loss but was positively related to haematocrit (Table 3).

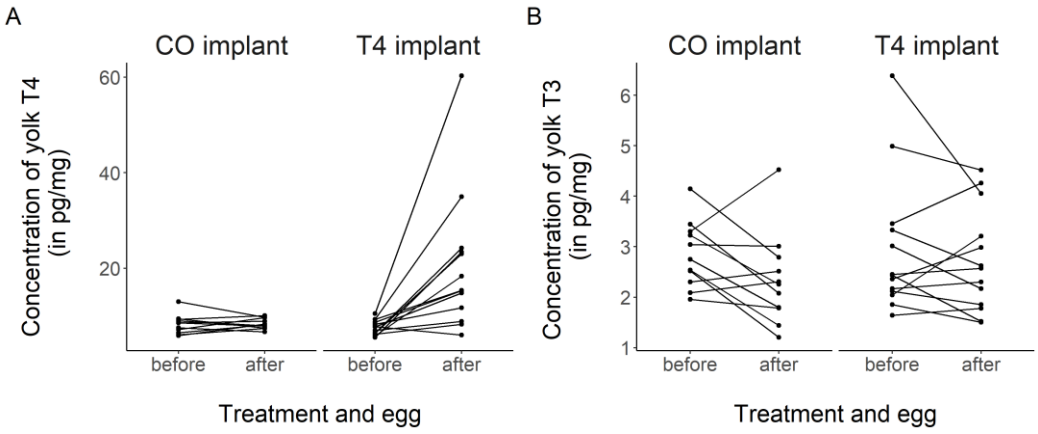

Figure 1: Experimental manipulation of thyroid hormone (TH) levels in female pied flycatchers. Concentrations of thyroxine (T_4; A) and triiodothyronine (T_3; B) in eggs of female pied flycatchers implanted with a control implant (control implant, n = 11 pre-implant and 11 post-implant eggs), or 10 µg T_4 implant (T_4 implant, n = 13 pre-implant and 13 post-implant eggs). "Before" and "after" respectively refer to eggs collected before or after the females had received an implant.

Table 2: Linear models of yolk and plasma THs in wild female pied flycatchers in response to T4 implants

	Estimate (SE)	F_{ddf} **or** t	**p-value**
(A) Yolk THs			
Yolk T_4			
Implant (T4)	0.75 (0.19)	$14.96_{17.6}$	**0.001**
Pre-implant egg	0.07 (0.06)	$1.09_{20.0}$	0.31
Year (2017)	0.09 (0.20)	$0.18_{18.2}$	0.67
Yolk T_3			
Implant (T4)	0.12 (0.11)	$1.09_{17.1}$	0.31
Pre-implant egg	0.20 (0.05)	$13.33_{18.1}$	**0.002**
Year (2017)	-0.01 (0.12)	$0.01_{17.8}$	0.92
T_3/T_4 ratio			
Implant (T4)	-0.12 (0.03)	$12.28_{17.6}$	**0.003**
Pre-implant egg	-0.01 (0.11)	$0.01_{19.3}$	0.91
Year (2017)	-0.01 (0.03)	$0.10_{18.2}$	0.76
(B) Plasma THs			
Plasma T_4			
Implant (T4)	1.16 (0.75)	1.54	0.14
Body mass	-0.90 (0.38)	-2.33	**0.03**
Temperature	0.11 (0.39)	0.29	0.78
Time	-0.46 (0.39)	-1.80	0.26
Plasma T_3			
Implant (T4)	0.14 (0.15)	0.95	0.36
Body mass	-0.02 (0.08)	-0.26	0.80
Temperature	-0.05 (0.08)	-0.26	0.58
Time	-0.09 (0.08)	-1.16	0.26
Plasma T_3/T_4 ratio			
Implant (T4)	-0.13 (0.29)	-0.45	0.66
Body mass	0.23 (0.15)	1.54	0.14
Temperature	-0.10 (0.15)	-0.64	0.53
Time	0.02 (0.15)	0.15	0.89

(A) Full linear mixed models of yolk THs in response to T4 implants in wild pied flycatchers (T_4 implant n = 13 pre-implant and n = 13 post-implant and n = 13 eggs; control n = 11 pre-implant and n = 11 post-implant eggs). Hormone assay was included as a random intercept. F-value denominator degrees of freedom (ddf) are shown; numerator degrees of freedom (ndf) = 1 in each case. (B) Full linear models of plasma THs in response to T_4 implants, in wild female pied flycatchers (T4 implant n = 11 females; control n = 10 females). Covariates were centred and scaled. P-values were obtained by separate t-test for each predictor. Significant p-values are shown in bold.

Table 3: Linear models of body mass loss and haematocrit in wild female pied flycatchers in response to T4 implants

	Estimate (SE)	*t*	p-value
Body mass loss			
Implant (T4)	-0.16 (0.56)	-0.28	0.79
Year (2017)	0.04 (0.65)	0.06	0.95
Clutch size	-0.24 (0.35)	-0.68	0.51
Haematocrit			
Implant (T4)	0.03 (0.02)	1.53	0.14
Year (2017)	0.04 (0.02)	1.52	0.15
Clutch size	0.04 (0.02)	2.66	**0.02**

T_4 implant n = 11 females; control n = 10 females. Significant p-value is shown in bold

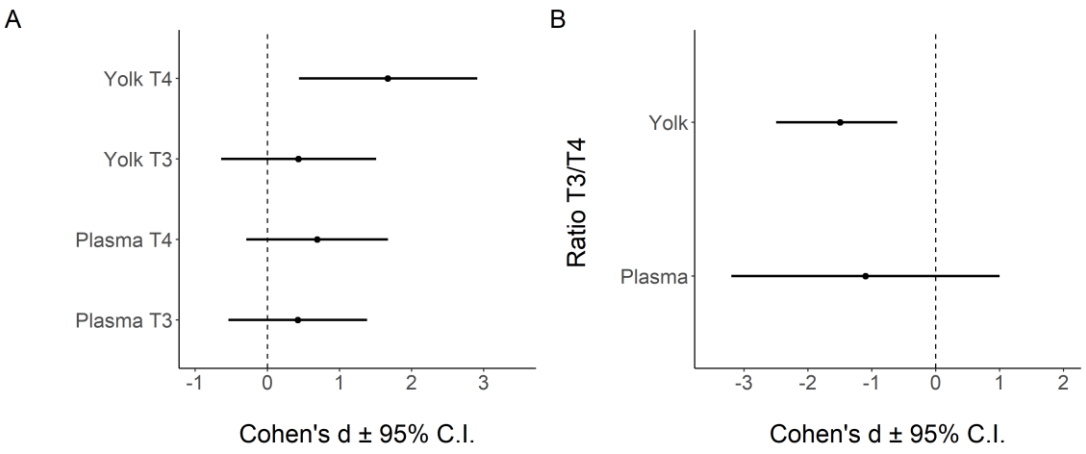

Figure 2: Yolk and plasma levels of T_3 and T_4 in captive and wild females. (A) Cohen's *d* and 95% CIs for yolk (post-implant egg) and plasma (wild females) T_3 and T_4 calculated from the marginal means of the respective models. Cohen's *d* and 95% CIs for plasma of captive females were calculated from the raw data. D1 = one day after implantation; D10 = ten days after implantation. (B) Cohen's *d* and 95% CIs for the T_3/T_4 ratio in the yolk (post-implant egg) and plasma (captive females) calculated from the raw data. D2 = two days after implantation, obtained by averaging the values for day 1 and day 3 after implantation. This period overlaps with the timing of yolk formation of the post-implant eggs, and thus reflects the circulating TH levels during yolk formation.

Discussion

To our knowledge, this study is the first one to manipulate circulating thyroid hormones (THs) of a wild bird species during egg-laying, to study potential regulation of the maternal TH transfer at the level of mothers' circulation and at the follicle level. Contrary to previous studies on other maternal hormones (e.g., steroid hormones), we looked not only at the response in the implanted hormone T_4, but also at its more active metabolite T_3. To our knowledge, studies that manipulated a prohormone and measured the change in its active metabolites have rarely been conducted. We detected no effects of exogenous T_4 on female haematocrit or body mass. We found an increase in plasma and yolk T_4 in response to exogenous T_4. This result would indicate an absence of regulation in the transfer of T_4 from mothers to their eggs, supporting the epiphenomenon hypothesis of Groothuis and Schwabl (2008), namely that yolk hormones merely reflect or mirror the maternal circulating levels. Yet there was a brief peak in T_4 levels with relatively high plasma T_4 levels occurring during the yolking phase (Table 1), and we cannot fully exclude the potential explanation that any regulatory mechanism would have failed under high T_4. We predicted that elevated plasma T_4 would increase plasma or yolk T_3, the more potent hormone, because of the increased amount of its precursor, T_4. However, we observed no changes in circulating or yolk T_3. Our study therefore probably failed to induce a trade-off in mothers between plasma and yolk T_3, and we thus cannot conclude on the presence or absence of a regulatory mechanism for maternal transfer of yolk T_3. The unchanged plasma T_3 concentrations, together with the rapid decrease in plasma T_4 after implantation observed in captive females (Table 1), suggest a change in the peripheral TH metabolism to quickly remove excess T_4. In rats, hyperthyroidism increases the conversion of T_4 and T_3 into inactive metabolites (Bianco et al., 2002). Likewise, increased circulating T_4 rapidly decreases the conversion of T_4 into T_3 (Bianco et al., 2002). Both mechanisms prevent the production of T_3, which may explain why we observed no increase in plasma T_3. Because plasma T_3 is known to positively correlate with basal metabolic rates in wild birds (Chastel, Lacroix & Kersten, 2003; Welcker et al., 2013; Elliott et al., 2013), these mechanisms may allow individuals to cope with elevated THs and could be important tools for mothers to protect themselves and their progeny from the potentially detrimental consequences of elevated T_3. As T_3 binds with 50 times greater affinity to TH receptors than does T_4 (Zoeller, Tan & Tyl, 2007), it is reasonable to expect a stricter regulation of the more potent hormone. This is indeed in line with previous studies reporting that yolk T_3 showed low within-individual variation, contrary to yolk T_4 (Hsu et al., 2019b), and that yolk T_3 was heritable whereas yolk T_4 was not (Ruuskanen et al., 2016b). To ascertain the hypothesis of a regulation of the T_4-to-T_3 conversion, one should analyse the expression and activity of different

enzymes involved in TH metabolism in response to exogenous THs, both in mothers and in embryos.

In addition to regulating their own plasma levels of THs, we hypothesised that mothers may be able to regulate the exposure of the developing follicles to THs. Elevated yolk THs (within the natural range) can affect hatching success, offspring growth and metabolism as found by studies in altricial species (Ruuskanen et al., 2016; Hsu et al., 2017, 2019). We found no evidence for such a regulatory mechanism, as the T_3/T_4 ratio appeared not to differ between female plasma at the time of yolking (data from captive birds) and yolk. This result is contrary to that of Wilson and McNabb (Wilson & McNabb, 1997), where yolk T_3, but not circulating T_3 was increased in response to long-term T_4 administration. This contradiction may be caused by different time scales between the two studies, the method of administration and/or the timing of sampling. In our study, the peak in T_4 rapidly decreased after implantation. Conversely, Wilson and McNabb administrated exogenous T_4 for longer periods of time, which might have forced females to deposit T_3 in their eggs to maintain normal plasma T_3.

We found no evidence for regulation of plasma T_4 concentration or T_4 transfer to the yolk, suggesting that yolk T_4 levels reflect circulating levels in the mother. Nevertheless, we found evidence that females regulated plasma T_3 concentration, while the results on yolk T_3 regulation remain inconclusive because of the lack of changes in plasma T_3. Whether the potential regulation of plasma T_3 is due to changes in the T_4-to-T_3 conversion and whether it has been selected to benefit the mother or the offspring is as yet unclear. This could be tested by elevating plasma and yolk T_3 and measuring whether potential detrimental effects are larger in the mother or the offspring. Further studies could also aim at investigating the changes in TH metabolism (enzyme production and activity) in response to increased hormones levels.

Acknowledgements

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Ethics

The experiments were conducted under licence from the Animal Experiment Board of the Administrative Agency of South Finland (ESAVI1018/04.10.07/2016) and South-Western Finland Centre for Economic Development, Transport and Environment (VARELY/412/2016).

Chapter 4

Testing the short- and long-term effects of elevated prenatal exposure to different forms of thyroid hormones

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Abstract

Maternal thyroid hormones (THs) are known to be crucial in embryonic development in humans, but their influence on other, especially wild, animals remains poorly understood. So far, the studies that experimentally investigated the consequences of maternal THs focused on short-term effects, while early organisational effects with long-term consequences, as shown for other prenatal hormones, could also be expected. In this study, we aimed at investigating both the short- and long-term effects of prenatal THs in a bird species, the Japanese quail *Coturnix japonica.* We experimentally elevated yolk TH content (the prohormone T_4, and its active metabolite T_3, as well as a combination of both hormones). We analysed hatching success, embryonic development, offspring growth and oxidative stress as well as their potential organisational effects on reproduction, moult, and oxidative stress in adulthood. We found that eggs injected with T_4 had a higher hatching success compared with control eggs, suggesting conversion of T_4 into T_3 by the embryo. We detected no evidence for other short-term or long-term effects of yolk THs. These results suggest that yolk thyroid hormones are important in the embryonic stage of precocial birds, but other short- and long-term consequences remain unclear. Research on maternal thyroid hormones will greatly benefit from studies investigating how embryos use and respond to this maternal signalling. Long-term studies on prenatal THs in other taxa in the wild are needed for a better understanding of this hormone-mediated maternal pathway.

Introduction

Maternal effects represent all the non-genetic influences of a mother on her offspring and have received increasing attention in evolutionary and behavioural ecology. Through maternal effects, mothers can influence the fitness of their progeny by adapting their phenotype to expected environmental conditions ("adaptive maternal effects" in Marshall & Uller, 2007; Mousseau & Fox, 1998), and this view is now also incorporated in the human disease literature (Gluckman, Hanson & Spencer, 2005). Maternal hormones transferred to the offspring can mediate important maternal effects. Historically, research on maternal hormones has mostly focused on steroid hormones (Groothuis et al., 2005; von Engelhardt & Groothuis, 2011). While research on maternal thyroid hormones has emerged between the 80s and the 90s in several taxa (mammals, Morreale De Escobar et al., 1985; fish, Brown et al., 1988; birds, Wilson & McNabb, 1997), these hormones are still underrepresented in the literature on hormone-mediated maternal effects (reviewed in Ruuskanen & Hsu, 2018).

Thyroid hormones (THs) are metabolic hormones produced by the thyroid gland and are present in two main forms: the prohormone thyroxine (T_4) and the biologically active form triiodothyronine (T_3). THs play a crucial role in various aspects of an individual's life, e.g. development, metabolism and reproduction, across vertebrates, including humans (Morreale de Escobar, Obregon & Escobar del Rey, 2004; Krassas, Poppe & Glinoer, 2010). In humans, physiological variation of maternal THs (i.e. no clinical symptoms in both mothers and foetuses) is found to be associated with infant birth weight and IQ in older children (Medici et al., 2013; Korevaar et al., 2016). In other vertebrates, THs in general play a role in brain development and neuronal turnover (mammals, Morreale de Escobar, Obregon & Escobar del Rey, 2004; birds, McNabb, 2007). THs control the endothermic heat production, and are therefore important in thermoregulation in homeothermic species (mammals, Danforth & Burger, 1984; birds, McNabb & Darras, 2015).

THs can act, in concert with other hormonal axes, as mediators of life stage transitions across vertebrates (reviewed in Watanabe et al., 2016). The interaction between thyroid hormones and corticosteroids on amphibian metamorphosis is a well-known example of such effect on life stage transition (Kikuyama et al., 1993; Wada, 2008). THs are involved in gonadal development, and hyperthyroidism tends to accelerate maturation (Holsberger & Cooke, 2005), and coordinate the transition between reproduction and moult (McNabb & Darras, 2015). Administration of exogenous THs is known to stop egg laying and induce moult in birds (Sekimoto et al., 1987; Keshavarz & Quimby, 2002). THs are also involved in the photoperiodic control of seasonal breeding (Dardente, Hazlerigg & Ebling, 2014). For example, thyroidectomised starlings transferred to long photoperiods became insensitive to future

changes in photoperiod, and short photoperiod did not induce gonadal regression (Dawson, 1993).

While there has been recent research effort on the influence of maternal THs on offspring traits across vertebrate taxa, there are still substantial gaps in our knowledge. Manipulating yolk hormones within the natural range of a species is necessary to better understand the role of maternal THs in an eco-evolutionary context. In humans, studies have essentially looked at the consequences of clinical hyper- or hypothyroidism (but see Medici et al., 2013). Research in fish has applied supra-physiological doses for aquaculture purposes (Brown et al., 2014). However, these studies do not give information on how variations within the natural range of the species would shape offspring phenotype and affect its fitness, in turn influencing evolution. While recent literature on birds has shown that even physiological variations of prenatal THs can have phenotypic consequences (Ruuskanen et al., 2016; Hsu et al., 2017; 2019; Sarraude et al., 2020a), this view is still underrepresented in maternal THs research.

Besides, research on maternal thyroid hormones up to date has mainly investigated the short-term effects of prenatal THs on developing fish (Brown et al., 1988; Raine et al., 2004) and amphibians (Duarte-Guterman et al., 2010; Fini et al., 2012) and pre-fledging birds (Ruuskanen et al., 2016; Hsu et al., 2017, 2019; Sarraude et al., 2020). So far, only a study on rock pigeons has looked at the influence of yolk THs on post-fledging survival and found no effect (Hsu et al., 2017a). None of these studies in any taxa investigated the potential organisational effects of prenatal THs on life-history stage transitions in adult life. Early exposure to elevated THs may affect the hypothalamic-pituitary-thyroid (HPT) axis (humans and mice: Alonso et al., 2007; Srichomkwun et al., 2017; Anselmo et al., 2019), via epigenetic modifications for example, such as those induced by adverse early life conditions (Jimeno et al., 2019) or yolk testosterone (Bentz, Becker & Navara, 2016).

Oviparous species, such as birds, are suitable models for studying the role of maternal hormones on the progeny because embryos develop in eggs outside mothers' body. The content of an egg cannot be adjusted by the mother after laying, which facilitates the quantification of hormones transmitted by the mothers. In addition, the measurement and experimental manipulation of maternal hormones in the egg after it has been laid is not confounded by maternal physiology. These advantages combined with their well-known ecology and evolution, birds have become the most extensively studied taxa in research on the function of maternal hormones (Groothuis et al., 2019).

Previous studies on prenatal THs in birds focused only on altricial species (great tits, Ruuskanen et al., 2016; rock pigeons, Hsu et al., 2017; collared flycatchers, Hsu et al., 2019, pied flycatchers, Sarraude et al., 2020a). Embryonic development differs substantially between altricial and precocial species. In the latter, embryonic development is more advanced than in the former. In addition, precocial embryos start

their endogenous production of TH around mid-incubation, considerably earlier than their altricial counterparts, in which endogenous TH production begins only after hatching (McNabb, Scanes & Zeman, 1998). While embryonic hormone production may limit the influence of maternal hormones, prenatal hormones have been shown to affect chick endogenous production and sensitivity (Pfannkuche et al., 2011). Overall, exposure to maternal hormones may be of different importance in these two developmental modes.

Previous research has studied the effects of T_3 only (Raine et al., 2004; Walpita et al., 2007; Fini et al., 2012) or a combination of T_3 and T_4 (Ruuskanen et al., 2016; Hsu et al., 2017, 2019; Sarraude et al., 2020a), where the effects of the two forms cannot be separated. Although T_3 is the biologically active form that binds to the receptors, both T_3 and T_4 are deposited in eggs (Prati et al., 1992) and T_4 may be converted to T_3 via deiodinases from the mother or the developing embryo (Van Herck et al., 2015) or may still exert non-genomic actions (reviewed in Davis et al., 2016). Manipulating yolk T_4 and T_3 independently would help understanding the relative contribution of these two hormones.

In this study, we aimed at assessing the effects of maternal THs on development and life-history traits in a precocial bird species, the Japanese quail (*Coturnix japonica*). We manipulated eggs with either an injection of T_4 or T_3 separately, a combination of both hormones, or a control injection of the vehicle saline solution. First, we hypothesise that elevation of yolk THs in Japanese quails positively affects hatching success, as found in two studies on collared flycatchers and rock pigeons (Hsu et al., 2017; Hsu et al., 2019, but see Ruuskanen et al., 2016 and Sarraude et al., 2020a). Second, elevation of yolk THs is predicted to increase the proportion of well-developed embryos before hatching, as found in rock pigeons (Hsu et al., 2017a). We therefore looked at the age at mortality in unhatched eggs. Third, we expect elevated yolk THs to affect chick growth (in body mass, tarsus and wing length) either positively (Wilson & McNabb, 1997; Hsu et al., 2019; weak effect in Sarraude et al., 2020), negatively (Hsu et al., 2017a), or in a sex-specific manner (Ruuskanen et al., 2016). Prenatal THs may exert most of their effects in the offspring early life; this is why we separately tested both posthatch morphological traits and the growth curve. Similarly, we also independently analysed morphological traits at adulthood, as these traits may affect the fitness of an individual. For example, small adult females may lay smaller eggs and larger males may be more dominant. Fourth, we predict that yolk THs will have organisational effects on life-history stage transitions; that is, age at sexual maturity and male gonadal regression (using cloacal gland size as a proxy), and moult when birds are exposed to short photoperiod. Based on the literature mentioned above we expect elevated yolk THs to advance the timing of puberty, gonadal regression, and moult. The rate of moult should also be influenced, with birds receiving experimental TH elevation moulting faster.

Previous studies have reported that gravid female three-spined sticklebacks (*Gasterosteus aculeatus*) exposed to predatory cues produced eggs with higher corticosterone (Giesing et al., 2011), disturbed embryonic transcriptome (Mommer & Bell, 2014), offspring with altered anti-predator behaviour (Giesing et al., 2011) and modified cortisol response in adulthood (Mommer & Bell, 2013). We may therefore expect elevated yolk THs to similarly induce long-term behavioural changes in response to environmental cues (i.e., photoperiod), via organising effects during the embryonic development. We also explored the effects of yolk THs on reproductive investment in females, another important fitness aspect. Finally, yolk THs may increase oxidative stress due to their stimulating effects on metabolism.

Material and Methods

Overview of the method

Japanese quails are easy to maintain in captivity, and their short generation time makes it a good model to investigate the long-term effects of maternal hormones. Rearing birds in captivity allowed us to apply a powerful within-female experimental design (i.e., knowing which chick hatched from which egg which is not feasible in field studies), thus reducing the effect of random variation among females. Moreover, studying the role of natural variation of prenatal THs in precocial species may give additional information to previous studies in altricial species. Finally, Japanese quail is a commonly used model in maternal hormone research with substantial literature available (e.g. McNabb, Blackman & Cherry, 1985; McNabb, Dicken & Cherry, 1985; Wilson & McNabb, 1997; Okuliarova et al., 2011).

We injected unincubated eggs from Japanese quails maintained in captivity with either T_4 or T_3 alone, a combination of both hormones, or a saline (control) solution. This design allowed us to explore the effects of T_4 and T_3 separately, which has not been done in previous studies. The elevation in yolk THs remained within the natural range of this species, a crucial condition to obtain relevant results for an eco-evolutionary context. We measured traits known to be influenced by circulating and yolk THs: hatching success, age at embryonic mortality, growth, transition between life-history stages (i.e., reproductive state and moult) and oxidative stress.

Parental generation and egg collection

The parental generation was composed of adult Japanese quails provided by Finnish private local breeders that were kept in two acclimated rooms. Twenty-four breeding pairs were formed by pairing birds from different breeders. Individuals were identified using metal leg rings. The floor was covered with 3–5cm sawdust bedding. A hiding place, sand and calcium grit were provided. Each pair was housed in indoor aviary

divided into pens of 1 m² floor area. The temperature was set to 20°C with a 16L:8D photoperiod (light from 06.00 to 22.00). Food (Poultry complete feed, "Kanan Paras Täysrehu", Hankkija, Finland) was provided *ad libitum* and water was changed every day.

Pairs were monitored every morning to collect eggs for 7 days. Eggs were individually marked (non-toxic marker), weighed and stored in a climate-controlled chamber at 15°C and 50% relative humidity. On the last day of collection, a total of 4 to 8 eggs per pair were injected with a solution (see next section).

Preparation of the solution, injection procedure and incubation

The preparation of hormone solution and the procedure of injection were based on previous studies (Ruuskanen et al., 2016; Hsu et al., 2017b). In brief, crystal T_4 (L-thyroxine, ≥ 98% HPCL, CAS number 51-48-9, Sigma-Aldrich) and T_3 (3,3',5-triiodo-L-thyronine, > 95% HPCL, CAS number 6893-02-3, Sigma-Aldrich) were first dissolved in 0.1M NaOH and then diluted in 0.9% NaCl. The injection of thyroid hormones resulted in an increase of two standard deviations (T_4 = 8.9 ng/egg, equivalent to 1.79 pg/mg yolk; T_3 = 4.7 ng/egg, equivalent to 1.24 pg/mg yolk), a recommended procedure for hormone manipulation within the natural range (Ruuskanen et al., 2016; Hsu et al., 2017b; Podmokła, Drobniak & Rutkowska, 2018). The control solution (CO) was a saline solution (0.9% NaCl). The concentrations of the hormone solutions were based on previous measurements of 15 eggs from the same flock (content per egg (SD) T_4 = 15.3 (4.4) ng, T_3 = 7.6 (2.3) ng; concentrations (SD), T_4 = 4.20 (0.89) pg/mg yolk, T_3 = 2.10 (0.62) pg/mg yolk).

Hormone injections were performed at room temperature in a laminar hood. Eggs were put sideways, allowing yolks to float up to the middle position. Before injection, the shell was disinfected with a cotton pad dipped in 70% EtOH. We used a 27G needle (BD Microlance ™) to pierce the eggshell and then used a 0.3 ml syringe to deliver 50 µl of the respective hormone solution or control. After injection, the hole was sealed with a sterile plaster (OPSITE Flexigrid, Smith&Nephew).

In total, 158 eggs were injected and divided as follows over the treatments: T_3 treatment (N = 39); T_4 treatment (N = 39); T_3+T_4 treatment (N = 40); and control, CO (N = 40). To balance the genetic background of the parents and the effect of storage, each egg laid by the same female was sequentially assigned to a different treatment and the order of treatments was rotated among females. After injection, eggs were placed in an incubator at 37.8°C and 55% relative humidity. Until day 14 after starting incubation, eggs were automatically tilted every hour by 90°. On day 14, tilting was halted and each egg was transferred to an individual container to monitor which chick hatched from which egg. On day 16 after injection, (normal incubation time = 17 days), the temperature was set to 37.5°C and the relative humidity to 70%. Eggs were checked for

hatching every 4 hours from day 16 onwards. Four days after the first egg hatched, all unhatched eggs were stored in a freezer and dissected to determine the presence of an embryo. The age of developed embryos was assessed according to Ainsworth et al. (2010).

Rearing conditions of the experimental birds

In total, 66 chicks hatched (N = 10 CO, 15 T_3, 20 T_4 and 21 T_3T_4), yielding a rather low overall hatching success (ca. 40%). Among the unhatched eggs, 33.7% (31 out of 92) had no developed embryos, and these were evenly distributed between the treatments (CO = 9/40, T_3 = 8/39, T_3T_4 = 8/40, and T_4 = 6/39 eggs). Discarding the unfertilised eggs gives an overall hatching success of ca. 51%. Previous studies on Japanese quails have reported comparable hatching success, even in unmanipulated eggs (e.g. 40% in Okuliarová, Škrobánek & Zeman, 2007; ca. 60% in Pick et al., 2016 and in Stier, Metcalfe & Monaghan, 2019). In addition, the injection procedure itself is also known to reduce hatching success to some extent (Groothuis & von Engelhardt, 2005). Twelve hours after hatching, the chicks were marked by a unique combination of coloured rings and nail coding and transferred to two cages of 1 m² floor area and ca. 30 cm height (ca. 30 chicks/cage, sex and treatments mixed together). The chicks were provided with heating mats and lamps as extra heat sources for the first two weeks. The chicks were fed with sieved commercial poultry feed ("Punaheltta paras poikanen", Hankkija, Finland), and provided with Calcium and bathing sand. Two weeks after hatching, the chicks were separated in four 1 m² cages (ca. 30 cm high) of about 16 individuals. Around 3 weeks after hatching, coloured rings were replaced with unique metal rings. On week 4 after hatching, birds were transferred to eight pens of 1 m² floor area (average of 7.1 birds/pen, range = 4–9), under the same conditions as the parents. Around the age of sexual maturity (ca. 6–8 weeks after hatching), the birds were separated by sex in twelve 1 m² pens (average of 4.8 birds/pen, range = 4–5). The chicks were under the same photoperiod as the adults (i.e., 16L:8D).

Monitoring of growth and reproductive maturation

Body mass and wing length were measured twelve hours after hatching. Tarsus was not measured because it bends easily, resulting in inaccurate measures and potential harm for the young. From day 3 to day 15, these three traits were monitored every 3 days. From day 15 to day 78 (ca. 12 weeks), chicks were measured once a week. Body mass was recorded using a digital balance to the nearest 0.1 g. Wing and tarsus lengths were respectively measured with a ruler and a calliper to the nearest 0.5 mm and 0.1 mm. The sample size for the growth analysis was 7 CO, 11 T_3, 18 T_4 and 21 T_3T_4. From week 6 to week 10, we monitored cloacal gland development and foam production in 28 males. Cloacal glands were measured every other day with a calliper to the nearest 0.1 mm as a

proxy for testes development and sexual maturation (Biswas et al., 2007). Foam production (by gently squeezing the cloacal gland) was assessed at the same time and coded from 0 (no foam) to 3 (high production of foam), as a proxy for cloacal gland function (Cheng et al., 1989a; Cheng et al., 1989b). The same observer performed all measurements. We collected eggs produced by 10-week-old females over a 6-day period and recorded their mass to the nearest 0.1 g. We collected on average 5.7 eggs (range = 4–7) per female from 28 females.

Monitoring of cloacal gland regression and moult

In Japanese quails, exposure to short photoperiod and cold temperature triggers reproductive inhibition and postnuptial moulting (Tsuyoshi & Wada, 1992). Thyroid hormones are known to coordinate these two responses (see introduction). When the birds reached the age of ca. 7 months, we exposed them to short photoperiod (8L:16D, i.e., light from 08.00 to 16.00) with a 12:12-h cycle of normal (20°C) and low (9°C) temperature (low temperature was effective from 18.00 to 06.00). Cloacal gland regression (as a proxy for testes regression) was monitored every other day for 2 weeks with a calliper by measuring the width and length to obtain the area of the gland to the nearest 0.1 mm^2 (N = 26 males; 4 CO, 4 T_3, 8 T_4 and 12 T_3T_4). Primary moult was recorded from a single wing by giving a score to each primary from 0 (old feather) to 5 (new fully-grown feather) following Ginn and Melville (1983) (N = 54 males and females; 7 CO, 11 T_3, 16 T_4 and 20 T_3T_4). The total score of moulting was obtained by adding the score of all feathers.

Oxidative status biomarker analyses

Two blood samples were drawn, when birds were 2 weeks (N = 58 chicks) and 4 months old (N = 55 adults), respectively. The sample size per treatment was 7 CO, 11 T_3, 17 T_4 and 20 T_3T_4. 200 µl of blood was collected from the brachial vein in heparinized capillaries and directly frozen in liquid nitrogen. Then, the samples were stored at -80°C until analyses. We measured various biomarkers of antioxidant status; the antioxidant glutathione (tGSH), the ratio of reduced and oxidised glutathione (GSH:GSSG) and activity of the antioxidant enzymes glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) from the blood. Measuring multiple biomarkers of oxidative and antioxidant status allows a broader understanding of the mechanism, and the interpretation of the results is more reliable if multiple markers show similar patterns. The GSH:GSSG ratio represents the overall oxidative state of cells and a low ratio reveals oxidative stress (Hoffman, 2002; Isaksson et al., 2005; Lilley et al., 2013; Rainio et al., 2013; Halliwell & Gutteridge, 2015). GPx enzymes catalyse the glutathione cycle, whereas CAT and SOD directly regulate the level of reactive oxygen species (ROS) (Ercal, Gurer-Orhan & Aykin-Burns, 2001; Halliwell & Gutteridge, 2015). The

methodology for measuring each biomarker is described in detail in Rainio et al. (2015). All analyses were conducted blindly of the treatment following Ruuskanen et al (2017).

Statistical analysis

Data were analysed with the software R version 3.5.3 (R Core Team, 2021). In this study, two different statistical approaches were used: null-hypothesis testing with Generalised Linear Mixed Models (GLMMs) and Linear Mixed Models (LMMs), and multimodel inference with Generalised Additive Mixed Models (GAMMs). GAMMs were used to analyse the data on body and cloacal gland growth to account for its non-linear pattern (see *Growth*). In this analysis, we preferred multimodel inference as GAMMs generate many candidate models that cannot be directly compared (e.g., by the Kenward-Roger approach). Instead, candidate models were ranked based on their Akaike Information Criterion (AIC) values. Models with a $\Delta AIC \leq 2$ from the top-ranked model were retained in the set of best models. Akaike weights of all models were calculated following (Burnham & Anderson, 2002), and evidence ratios of the top-ranked models were calculated as the weight of a model divided by the weight of the null model (Burnham, Anderson & Huyvaert, 2011). To estimate the effect of the predictors, we computed the 95% confidence intervals from the best models using the *nlme* package (Pinheiro et al., 2018). GLMMs and LMMs were fitted using the R package *lme4* (Bates et al., 2015), and GAMMs were fitted using the package *mgcv* (Wood, 2017). P-values for GLMMs were obtained by parametric bootstrapping with 1,000 simulations and p-values for LMMs were calculated by model comparison using Kenward-Roger approximation, using the package *pbkrtest* in both cases (Halekoh & Højsgaard, 2014). Post-hoc Tukey analyses were conducted with the package *multcomp* (Hothorn, Bretz & Westfall, 2008). Model residuals were checked visually for normality and homoscedasticity. Covariates and interactions were removed when non-significant ($\alpha = 0.05$).

Effect size calculations (Cohen's d and 95%CI) were performed with the website estimationstats.com (Ho et al., 2019) and statistical power analyses were performed using t-tests for independent means with GPower (Faul et al., 2009) with the effect size values calculated. When presenting and discussing our results, we use the language of statistical "clarity" rather than statistical "significance" as suggested by Dushoff et al. (2019).

Hatching success

To analyse hatching success, each egg was given a binary score: 0 for unhatched egg and 1 for hatched egg. A GLMM was fitted with a binomial error distribution (logit link) and mother identity as a random intercept and the 4-level treatment as the predictor. Egg mass might affect hatchability and was therefore added as a covariate in both models.

The potential effect of storage duration on hatchability (Reis, Gama & Soares, 1997) was accounted for by including laying order as a covariate in both models. This covariate allowed us to control for the age of the egg as well.

Duration of embryonic period, age at embryonic mortality and early morphological traits

Duration of embryonic period and early morphological traits (mass and wing length at hatching, and tarsus length at day 3) were modelled with separate LMMs. Treatment, sex of the individuals and egg mass were included as fixed factors. Laying order was added as a covariate to account for potential effects of storage duration on hatching time and on chick weight (Reis, Gama & Soares, 1997). Mother identity was included as a random intercept.
The data for embryonic age had a skewed distribution and residuals were not normally distributed and heterogenous, which violated LMM assumptions on residual distribution. We therefore performed a simple Kruskal-Wallis test.

Growth

As growth curves typically reach an asymptote, we fitted non-linear GAMMs to these curves. Growth in body mass, tarsus and wing length were analysed in separate GAMMs. Growth was analysed until week 10 after hatching as all birds appeared to have reached their maximum body mass and tarsus and wing length. The data are composed of repeated measurements of the same individuals over time; therefore, we first corrected for temporal autocorrelation between the measurements using an ARMA(1,1) model for the residuals (Zuur et al., 2009). Second, as mothers produced several eggs, the models included nested random effects, with measured individuals nested into mother identity, allowing for random intercepts. GAMMs allow modelling the vertical shift of the curves (i.e., changes in intercepts) and their shape. Treatment and sex were included as predictors. A smoothing function for the age of the birds was included to model the changes in the growth curves and was allowed to vary by sex or treatment only, or none of these predictors. The interaction between sex and treatment was not analysed due to low statistical power. Additive effect of treatment and sex was tested for the intercept but could not be computed for curve shape. All combinations of the relevant predictors were tested for both shape parameters (i.e., intercept and curve shape).

Prenatal THs may exert most of their effects in the offspring early life; this is why we additionally tested hatchlings morphological traits apart from the growth curve. Likewise, we also analysed separately morphological traits at adulthood (ca. 9 weeks old), as these traits may condition the fitness of an individual. Because of sex differences and low sex-specific sample sizes, we standardised the measures within sex and regressed the standardised responses against treatment in a linear regression.

Table 1: Loadings of the different antioxidant biomarkers on the principal components 1 and 2.

Factor loadings	PC1 (34.0%)	PC2 (26.2%)
CAT	-0.49	0.14
SOD	0.20	-0.71
GST	-0.65	-0.10
GP	0.04	-0.63
tGSH	-0.60	-0.26

Reproductive maturation, regression and investment

Due to low sample sizes in these sex-specific responses, we could not perform robust statistical analyses. We therefore present these analyses and results in the supplementary material and only briefly discuss them (Figs. S6 to S9; Table S3).

Moult

Two parameters of moult were analysed in separate LMMs: the timing of moult (i.e., the moult score after one week of short photoperiod), and the rate of moult (i.e., how fast birds moulted). Both models included treatment and sex as fixed factors, and mother identity as a random intercept. The rate of moult was tested by fitting an interaction between treatment and age. This model also included the main effect of age and individual identity, nested within mother identity, as a random intercept to account for repeated measures. Estimated marginal means and standard errors (EMMs ± SE) were derived from the model using the package *emmeans* (Lenth, 2019).

Oxidative stress

A principal component analysis (PCA) was first performed on measured antioxidant markers (SOD, CAT, GPx, tGSH and GST), to reduce the number of metrics for subsequent analyses. The first and the second principal components (PCs) explained together 60.2% of the variance (Table 1). PC1 and PC2 were then used as dependent variables in separate LMMs. LMMs included the treatment, sex and age of individuals (2 weeks and 4 months old) as fixed factors and the 2-way interactions between treatment and sex, and treatment and age. Mother and individual identities, to account for repeated measures, were added as random intercepts. Malondialdehyde (MDA) is a marker of oxidative damage, which is a different measure from antioxidant activity, and was therefore analysed in a separate LMM using the same parameters as for PC1 and PC2, adding the batch of the assay as an additional random intercept. The marker of cell

oxidative status (GSH:GSSG ratio) was analysed with the same model used for PC1 and PC2.

Results

Effects of prenatal THs on hatching success and age of embryo mortality

There was a clear effect of elevated prenatal THs on hatching success (GLMM, $p = 0.03$, Fig. 1). Tukey post-hoc analysis revealed that hatching success in the T_3T_4 (66%) group was statistically higher than in the CO group (32%) (Tukey $z = 2.77$, $p = 0.03$) . There was a non-significant trend between the T_4 (61%) and the CO groups ($z = 2.37$, $p = 0.08$). There were no clear differences in hatching success between the T_3 (48%) and the CO group ($z = 1.25$, $p = 0.45$), or between the hormone treatments (all $z < 1.61$, all $p > 0.37$). Dissection of the unhatched eggs showed that age of embryo mortality did not differ between the treatments (Kruskal-Wallis $\chi^2 = 7.22$, $df = 3$, $p = 0.07$; Fig. S1). Finally, the manipulation of yolk THs did not affect the duration of embryonic period (LMM, $F_{3,42.0} = 0.57$, $p = 0.64$, Fig. S2). Sex of the embryo or egg mass (LMM sex, $F_{1,49.7} = 2.63$, $p = 0.11$; LMM egg mass, $F_{1,19.3} = 0.01$, $p = 0.92$) were also not associated with the duration of the embryonic period. Laying order (i.e., the effect of storage duration) was not correlated with any of the responses (all $p \geq 0.25$).

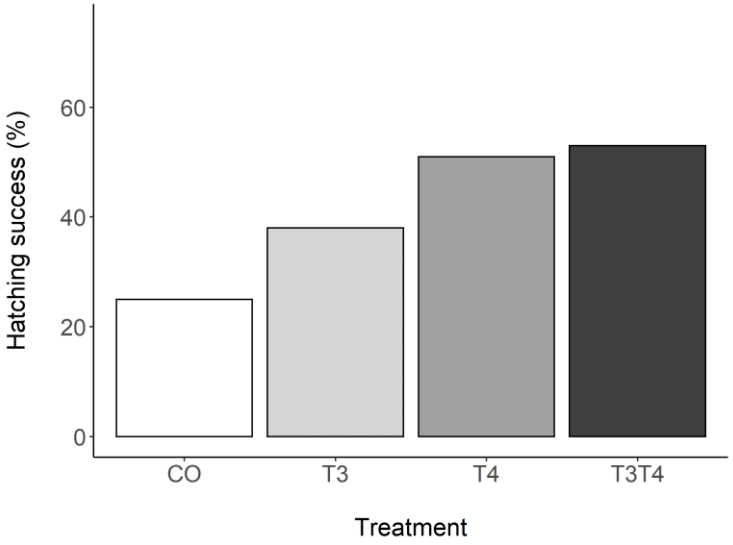

Figure 1: Percentage of hatching success according to yolk TH manipulation treatments: N = 40 CO, 39 T_3, 39 T_4 and 40 T_3T_4. CO control, T_4 (thyroxine) = injection of T_4, T_3 (triiodothyronine) = injection of T_3, T_3T_4 = injection of T_3 and T_4.

Table 2: Cohen's d, 95% CIs and achieved statistical power for post-hatching and adult morphological measures (body mass, wing and tarsus length).

	Hatchlings			Adults		
Contrast	Cohen's d	95% CI	Statistical power (1-β)	Cohen's d	95% CI	Statistical power (1-β)
			Body mass			
CO-T3	0.22	-0.70;1.09	0.13	-0.01	-1.23;1.05	0.05
CO-T4	0.17	-0.79;1.11	0.11	-0.46	-1.54;0.57	0.26
CO-T3T4	0.06	-1.03;1.05	0.07	-0.48	-1.61;0.48	0.28
T3-T4	-0.09	-0.81;0.66	0.08	-0.51	-1.32;0.22	0.36
T3-T3T4	-0.24	-1.0;0.52	0.17	-0.53	-1.28;0.19	0.40
T4-T3T4	-0.16	-0.80;0.49	0.13	-0.01	-0.66;0.65	0.05
			Wing length			
CO-T3	-0.41	-1.33;0.36	0.23	-1.07	-2.09;0.36	0.67
CO-T4	-0.79	-1.62;0.10	0.57	-0.72	-1.52;0.20	0.47
CO-T3T4	-0.56	-1.31;0.13	0.37	-0.81	-1.52;0.11	0.56
T3-T4	-0.41	-1.09;0.31	0.31	0.12	-0.54;0.91	0.09
T3-T3T4	-0.20	-0.80;0.54	0.14	0.09	-0.49;0.91	0.08
T4-T3T4	0.19	-0.44;0.86	0.15	-0.03	-0.67;0.62	0.06
			Tarsus length			
CO-T3	-0.58	-1.29;0.33	0.33	-0.10	-1.25;1.08	0.07
CO-T4	-0.88	-1.93;0.08	0.61	-0.67	-1.59;0.31	0.43
CO-T3T4	-0.92	-2.02;0.06	0.68	-0.78	-1.73;0.09	0.54
T3-T4	0.01	-0.91;1.02	0.05	-0.68	-1.41;0.08	0.53
T3-T3T4	0.05	-0.91;1.07	0.06	-0.79	-1.44;-0.11	0.67
T4-T3T4	0.06	-0.69;0.79	0.07	-0.11	-0.73;0.54	0.10

95% CIs were calculated by bootstrap resampling with 5,000 resamples. CO = control, T4 (thyroxine) = injection of T4, T3 (triiodothyronine) = injection of T3, T3T4 = injection of T3 and T4.

Effects of prenatal THs on growth

Mass at hatching was not influenced by the elevation of prenatal THs (LMM, $F_{3,35.0}$ = 0.81, p = 0.50, Fig. S3). Mass at hatching was positively correlated with egg mass (LMM, Estimate±SE = 0.72±0.10 g, $F_{1,24.1}$ = 46.9, p < 0.001). Although we detected no clear differences on hatchling morphological traits (body mass, wing and tarsus length) due to prenatal THs (all p > 0.12), the calculated effect sizes (Cohen's d[95%CI]) and achieved statistical power yielded additional information regarding the potential effects of prenatal THs (Table 2). For body mass, the effect sizes were low and the achieved statistical power was very low. For wing length, the effect sizes were moderate and the achieved statistical power was low. For tarsus length, the effect sizes were moderate to large and the achieved statistical power was low to moderate. Similarly, adult morphology was not affected by the treatment (all p > 0.13), but effect sizes indicate small to large effects of prenatal THs (Table 2). For body mass, the effect sizes were small and the achieved power was low. For wing length, the effect sizes were large and the achieved power was moderate. For tarsus length, the effect sizes were small to large and the achieved power was moderate to high.

Regarding body mass growth, the top-ranked model showed that the curve shape and the intercept differ according to sex (Table 3). After 10 weeks, females had a larger body mass than males (mean±SE females = 214.4±5.7 g, males = 172.4±4.5 g, Fig. 2), which was supported by the 95% CIs (Table 4). Based on model selection we conclude that the treatment had no effect on body mass growth (Table 3).

For wing growth, the top-ranked model ($\Delta AIC \leq 2$) included sex in the intercept, while treatment was not included in the best supported model (Table S1). The 95% CIs (Table 3) confirmed that males had a lower wing length than females (Fig. S4).

Concerning tarsus growth, the models within $\Delta AIC \leq 2$ included no predictors for the curve shape but included treatment for the intercept (Table S2). The 95% CIs of the parameter estimates from these models suggested that there was a slight negative effect of T_3T_4 treatment on tarsus growth (Table 4, Fig. S5). However, as the estimates were close to 0 (Table 4) and evidence ratios showed that the model with treatment as a predictor was only 3.5 times more supported than the null model (Table S2), we conclude that the effect of THs on tarsus length is likely to be very small. Likewise, the second model for tarsus length included sex as a predictor for the intercept, but its 95% CIs overlapped with 0 (Table 4). We therefore conclude that sex had no effect on tarsus growth.

Table 3: Results of the Generalised Additive Mixed Models (GAMMs) on body mass growth, with sex and treatment fitted either as intercept, curve shape or both (all combinations tested).

Model	Intercept	Curve shape	ΔAIC	df	Weight
1	Sex	Sex	0.0	11	0.8430
8	Treatment + sex	Sex	3.5	14	0.1497
3	-	Sex	9.9	10	0.0061
2	Treatment	Sex	13.2	13	0.0012
11	Sex	-	77.6	9	<0.001
9	Treatment + sex	-	81.6	12	<0.001
12	-	-	91.2	8	<0.001
10	Treatment	-	95.0	11	<0.001
5	Sex	Treatment	147.9	15	<0.001
7	Treatment + sex	Treatment	151.7	18	<0.001
6	-	Treatment	161.2	14	<0.001
4	Treatment	Treatment	165.5	17	<0.001

A total of 12 GAMMs were fitted and ranked based on their AIC, from the lowest to the highest. Weight: Akaike's weights.

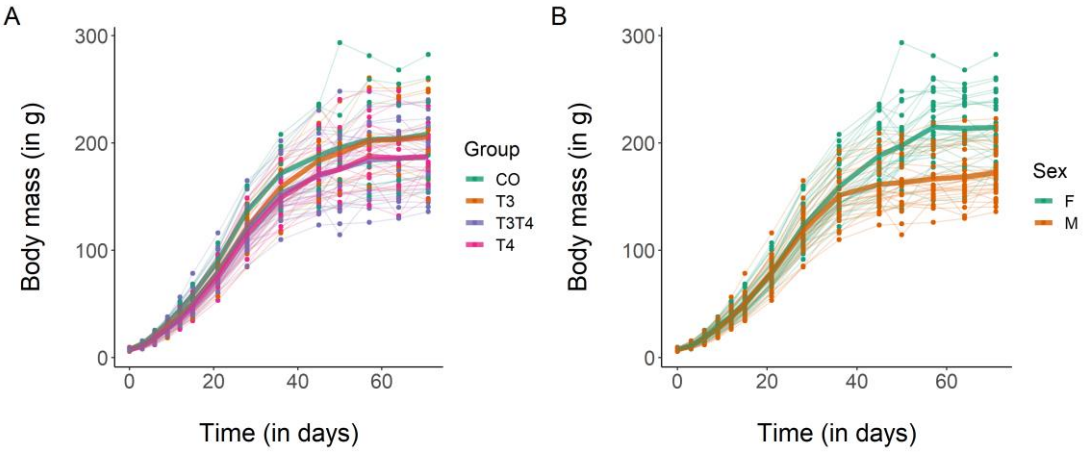

Figure 2: Growth curves in body mass of Japanese quails hatching from eggs treated with either T_3, T_4, a combination of both hormones, or a control solution. See Fig. 1 for a description of the treatments. Each line represents an individual bird, while thick coloured lines represent mean values. A: Growth curve according to yolk TH manipulation. N = 7 CO, 11 T_3, 18 T_4 and 21 T_3T_4. B: Growth curve according to sex. N = 29 females and 28 males.

Table 4: 95% confidence intervals of the predictors in the top-ranked models according to AIC values (see Tables 2, S1 and S2).

Curve parameter	Predictors	Lower limit	Estimate	Upper limit
(A) Body mass (Model 1)				
Intercept	**Sex (M)**	-19.7	-12.6	-5.5
Curve shape	**Sex (F)**	9.9	20.0	30.0
Curve shape	**Sex (M)**	14.3	24.5	34.7
(B) Wing length (Model 11)				
Intercept	**Sex (M)**	-2.3	-1.2	-0.1
Curve shape	**Age**	26.4	28.7	31.0
(C) Tarsus length (Model 10)				
Intercept	Treatment (T_3)	-0.8	0.02	0.8
Intercept	**Treatment (T_3T_4)**	-1.5	-0.8	-0.1
Intercept	Treatment (T_4)	-1.3	-0.6	0.2
Curve shape	**Age**	10.5	11.1	11.8
Tarsus length (Model 9)				
Intercept	Treatment (T_3)	-0.9	-0.07	0.7
Intercept	**Treatment (T_3T_4)**	-1.5	-0.8	-0.1
Intercept	Treatment (T_4)	-1.4	-0.6	0.1
Intercept	Sex (M)	-0.8	-0.3	0.3
Curve shape	**Age**	10.5	11.1	11.7

Predictors in bold have confidence intervals that do not overlap with 0. For the intercept, the reference groups are female and CO for the predictors sex and treatment, respectively.

Effects of prenatal THs on postnuptial moult

As expected, birds started to moult soon after being exposed to short photoperiod, with an average increase of moult score by 6 per week (SE = 0.2, $F_{1,254.0}$ = 827.4, p < 0.001, Fig. 3). The first moult score (assessed one week after switching to short photoperiod) was not affected by the treatment (LMM, $F_{3,42.7}$ = 0.36, p = 0.78), but was influenced by sex, with females having a higher score than male (EMMs ± SE: female = 21.4 ± 1.6, male = 7.2 ± 1.7; LMM $F_{1,45.3}$ = 41.9, p < 0.001). Yolk TH elevation did not affect the rate of moult (LMM interaction treatment × time, $F_{3,251.0}$ = 0.59, p = 0.62, Fig. 3).

Effects of prenatal THs on oxidative stress

The elevation of yolk THs had no effect on PC1 or PC2 of antioxidants at either 2 weeks ("chicks") or 4 months ("adults") old (LMM on PC1, $F_{3,40.3}$ = 2.40 , p = 0.08; LMM on PC2, $F_{3,42.2}$ = 0.92, p = 0.44, treatment × age, F < 0.91, p > 0.44). The age of the birds had a highly significant effect on PC1, with chicks generally having higher antioxidant capacities (CAT, GST and tGSH) than adults (LMM, Estimate±SE = -1.34±0.19, $F_{1,49.2}$ = 52.1, p < 0.001). All the other predictors had no effect on either PC1 or PC2 (all F < 2.93 and all p > 0.09).

The marker of oxidative damage, MDA, was affected by the elevation of yolk THs (LMM, $F_{3,43.6}$ = 3.08, p = 0.04, Fig. 4). Tukey post-hoc analysis showed that the T_4 group had higher MDA values than the T_3 group (Estimate±SE = 0.01±0.004, Tukey contrast p = 0.01), but none of the groups differed from the control (Tukey p-values > 0.19). However, this result became non-significant when removing the outlier in the T_4 group (LMM, $F_{3,43.1}$ = 2.68, p = 0.06). MDA levels were not affected by the age or the sex of individuals (LMM age, $F_{1,54.4}$ = 0.30, p = 0.59; LMM sex, $F_{1,42.0}$ = 1.47, p = 0.23).

The marker of cell oxidative balance, GSH:GSSG, was not influenced by the yolk THs nor by the sex of the birds (LMM treatment, $F_{3,33.0}$ = 0.85, p = 0.48; LMM sex, $F_{1,40.6}$ = 0.57, p = 0.45). However, chicks had a higher GSH:GSSG ratio than adults (LMM, Estimate±SE = 0.17±0.04, $F_{1,50.0}$ = 18.3, p < 0.001).

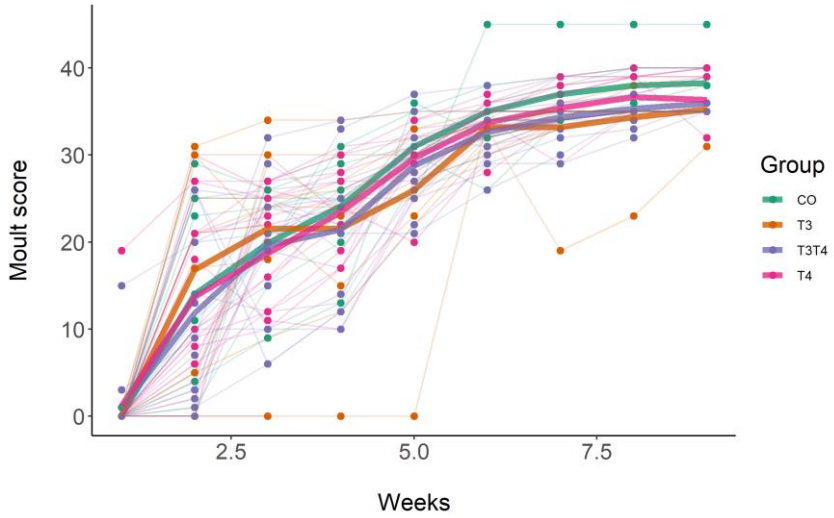

Figure 3: Primary moult score in 7-month-old Japanese quails according to yolk TH manipulation treatments: N = 7 CO, 11 T_3, 16 T_4 and 20 T_3T_4. See Fig. 1 for a description of the treatments. Measures were taken once a week after switching from long photoperiod (16L:8D) to short photoperiod (8L:16D, switch = time point 0 on x-axis). Each line represents an individual bird, while thick coloured lines represent group mean values.

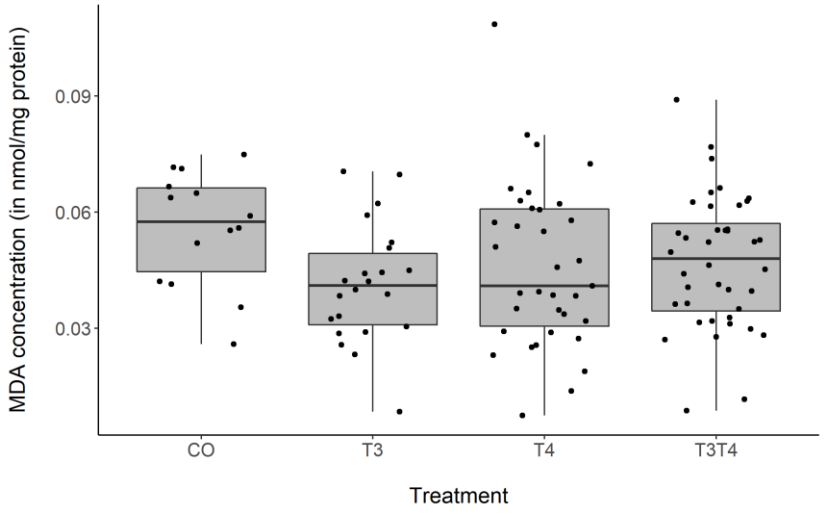

Figure 4: MDA concentration according to yolk TH manipulation treatments, samples from two ages pooled: N = 7 CO, 11 T_3, 17 T_4 and 20 T_3T_4. See Fig. 1 for a description of the treatments. Boxplots show median and quartiles.

Discussion

The aim of this experimental study was to investigate the potential short-term and organisational effects (with long-term consequences) of maternal thyroid hormones (THs) in a precocial species, the Japanese quail, by experimental elevation of THs in eggs. Our study is the first to investigate the effects of yolk T_3 and T_4 separately, within the natural range of the study model. In addition we studied both short- and long-term effects on embryonic development, growth, life stage transitions and oxidative stress. We detected a positive effect of yolk THs on hatching success. All other response variables studied were not clearly affected by elevated prenatal THs.

Effects of prenatal THs on hatching success and embryonic development

The overall low hatching success, and especially in the control group, forces us to interpret these results with caution. In addition, we cannot exclude that our results may be partly due to selective disappearance of lower quality embryos in the control group and with injected THs helping lower quality chicks to hatch. This might have biased the results after hatching, but is still a relevant effect of the hormone treatment. We found that hatching success almost doubled when the eggs received an injection of both T_4 and T_3, or an injection of T_4 only. Previous similar studies reported comparable effects of yolk THs in rock pigeons (Hsu et al., 2017a) and in collared flycatchers (Hsu et al., 2019). In these studies, injections consisted of a mixture of both T_3 and T_4. Given that mostly T_3 binds to receptors, these results suggest that embryos likely express deiodinase enzymes to convert T_4 to T_3, and/or yolk may contain maternally derived deiodinase mRNA, as injection with T_3 only did not differ from control. Indeed, deiodinase expression has previously been characterised in chicken embryos already 24h after the onset of incubation (Darras et al., 2009). An old study found that injecting T_4 close to hatching can advance hatching time, which suggests that yolk THs may help embryos overcoming hurdles close to hatching (Balaban & Hill, 1971). In contrast with our study, two similar studies in altricial species detected no increased hatching success due to the injection of THs (Ruuskanen et al., 2016; Sarraude et al., 2020a). The dissimilarities between the studies may come from inter-specific differences in terms of utilisation of yolk THs by the embryos or from context-dependent effects (e.g. due to other egg components). Further comparative and mechanistic studies could help understanding the dynamic of yolk THs during incubation.

Increased yolk THs did not influence age of embryo mortality. Similar to our study, Ruuskanen et al. (2016) did not find any difference in the timing of mortality in great tit embryos. Conversely, the study on rock pigeons found that yolk THs increased the proportion of well-developed embryos (Hsu et al., 2017a). Similar to our result on

hatching success, yolk TH effects on embryonic development may differ in a species-specific manner.

Our results on hatching success may partly be attributed to yolk THs balancing the negative effects of injections on embryonic survivability. Further studies may aim at understanding the contribution of THs to counteract the effect of injection. To do so, such studies may use an non-invasive method to manipulate yolk THs (e.g., egg-dipping method as in Perrin et al. 1995), in addition to injected controls, like in our study.

Effects of prenatal THs on growth

We found no apparent influence of yolk THs on growth, contrary to our expectations based on the recent literature. Other comparable studies found either a positive (Hsu et al., 2019; weak effect in Sarraude et al., 2020a), a negative (Hsu et al., 2017) or a sex-specific effect (Ruuskanen et al., 2016) of yolk THs on growth. This notable difference may be due to the captive conditions experienced by the Japanese quails in our study, with unrestricted access to food and water. Although the pigeon study also provided ad libitum food, parents still needed to process food before feeding their nestlings in the form of crop milk, whereas precocial quails have no such limitation. In addition, the Japanese quail has been domesticated for many generations, and probably selected for rapid growth for economic reasons. Whole-genome sequencing in chickens showed that domestication I nduced a strong positive selection on genes associated with growth (Rubin et al., 2010). Interestingly, that study also found a strong selection for a locus associated with thyroid stimulating hormone (TSH) receptor. TSH controls most of the TH production by the thyroid gland (McNabb & Darras, 2015), and this artificial selection may overshadow the effects of natural variations of prenatal THs on growth. Besides, the low number of individuals in the control and T_3 groups (7 and 11, respectively) limited the statistical power to detect differences between all the treatments. Indeed, we were able to detect small to moderate negative effects of yolk THs on morphological traits at hatching and in adulthood. Such negative effects, although small, may still be biologically relevant. Repeating the study with a larger sample size may allow us to ascertain the effects of yolk THs on growth in precocial study models. Research on the influences of prenatal THs on growth will also benefit from experimental studies on wild precocial species.

Effects of prenatal THs on postnuptial moult

Short photoperiod in combination with cold temperature triggered primary moult, as expected. However, we detected no effect of yolk THs on the timing or speed of moult. Thyroid hormones are important in moult and feather growth (reviewed in Dawson, 2015). For example, thyroidectomised birds fail to moult after being exposed to long photoperiods (Dawson, 2015). In addition, thyroidectomised nestling starlings failed to

grow normal adult plumage and grown feathers presented an abnormal structure (Dawson et al., 1994). By removing the thyroid gland, these two studies implemented extreme pharmacological protocols that differ drastically from our injection of physiological doses. In addition, our experimental design, increasing TH exposure (vs decreased TH exposure in the above-mentioned studies), may have different consequences. For example, there may be a threshold above which any additional hormones may not affect moult.

Overall, our results show no support for the hypothesis of organising effect of prenatal THs on life stage transitions. Yet, due to small sample sizes in sex-specific analyses (i.e., male gonadal maturation and regression, and female reproductive investment), there remains a relatively high uncertainty about the potential organising effects of prenatal THs. Replicate studies with larger samples sizes and different study models will reduce this uncertainty.

Effects of prenatal THs on oxidative stress

In contrast to our predictions, elevated yolk THs did not affect oxidative status during chick or adult phase. We found no changes in antioxidant activities in relation to yolk THs and no imbalance in the oxidative cell status. Nevertheless, T_4 birds had a higher level of oxidative damage on lipids than T_3 birds, but this was a weak effect driven by one outlier. The lack of effects on chick oxidative status among the treatment groups could be explained by the absence of treatment effects on growth, given that high growth rates usually result in higher oxidative stress and damage (e.g. Alonso-Alvarez et al., 2007). In turn, the lack of treatment effects on adult oxidative status may suggest no organisational effects of prenatal THs on adult metabolism. Two recent studies in altricial species also found no influence of yolk THs on nestling oxidative stress (Hsu et al., 2019; Sarraude et al., 2020a), yet telomere length, a biomarker of aging was affected (Stier et al., 2020). Our study shows for the first time that prenatal THs have no influence on adult oxidative stress either. The previous study focused on a limited set of biomarkers: one antioxidant enzyme, oxidative damage on lipids and oxidative balance. In the present study, we measured 7 biomarkers, thus providing broader support to the absence of effects of prenatal THs on post-natal/hatching oxidative stress.

Conclusion

To our knowledge, this study is the first one to experimentally investigate the consequences of natural variations of maternal THs not only early but also in adult physiology and postnuptial moult in any vertebrate. Furthermore, this study explored for the first time the effects of maternal T_3 and T_4 separately. We found no evidence for differential effects of maternal T_4 and T_3, while an effect of T_4, alone or in combination with T_3, on hatching success suggests that T_4 is converted into T_3, the biologically active

form during embryonic development. Contrary to similar studies on wild altricial species, we found no influence of maternal THs on growth. Further research on embryos utilisation of maternal THs may help understand the differences observed between precocial and altricial species. Studies in other vertebrates are urgently needed to understand the potential organising effects of maternal THs with long-term consequences.

Acknowledgements
We thank Sophie Michon for her help on setting up the parental generation. We also thank Ido Pen for consultation and help with statistical analysis, and Esther Chang for her help throughout the writing phase.

Ethics
The study complied with Finnish regulation and was approved by the Finnish Animal Experiment Board (ESAVI/1018/04.10.07/2016). In case of signs of harassment or disease, birds were placed in quarantine and monitored daily until they had recovered. Criteria for humane endpoints were defined as follow: passive behaviour, loss of appetite, loss of 30% of body weight, moving abnormally, trouble breathing. If we observed no clear improvement after two days, we would consult the veterinarian. A bird would be euthanised if it does not show signs of improvement in the next two days, though some judgement can be applied based on the alleged cause. One male was euthanised before the end of the experiment due to severe head injury. At the end of the experiment, all birds were euthanised by decapitation for collection of tissue samples (not used in this study).

Supplementary information

Effects of prenatal THs on early development

Age of unhatched embryos

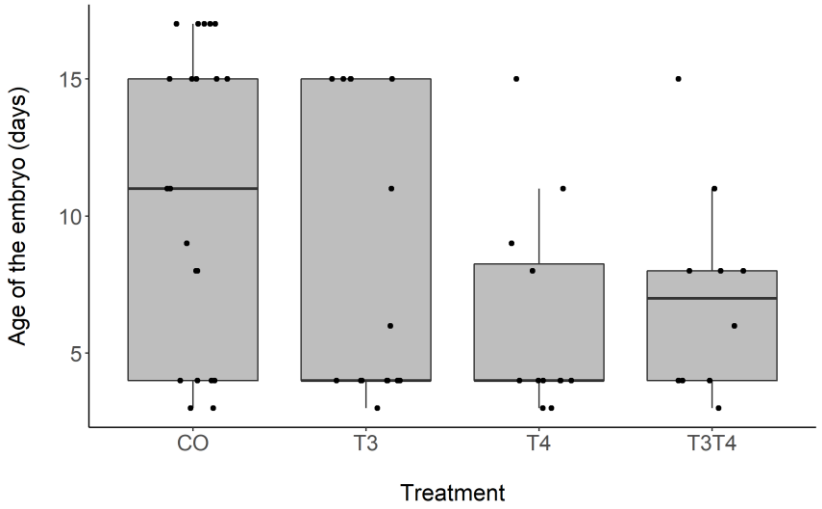

Figure S1: Boxplot of the age of unhatched embryos in Japanese quail eggs according to yolk TH manipulation treatments: CO (N=21), T_3 (N=16), T_4 (N=12), T_3T_4 (N=10). CO = control, T_4 (thyroxine) = injection of T_4, T_3 (triiodothyronine) = injection of T_3, T_3T_4 = injection of T_3 and T_4. Boxplot shows median and quartiles.

Duration of embryonic period

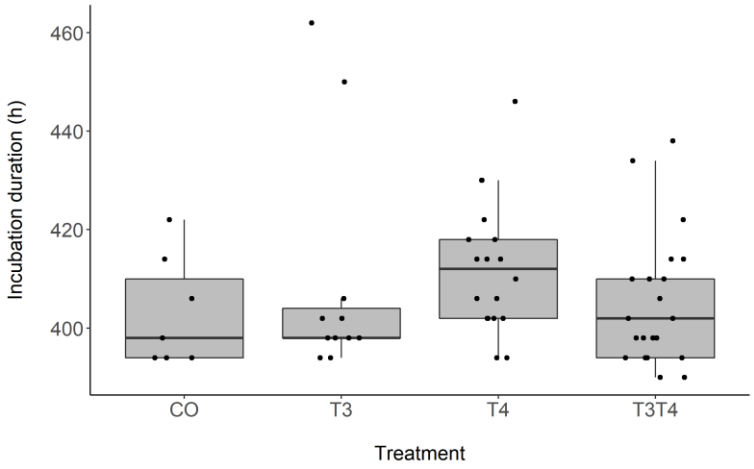

Figure S2: Boxplot of the duration of the embryonic period in Japanese quail eggs according to yolk TH manipulation treatments: CO (N=10), T_3 (N=15), T_4 (N=20), T_3T_4 (N=21). See Fig. S1 for a description of the treatments. Boxplot shows median and quartiles.

Mass at hatching

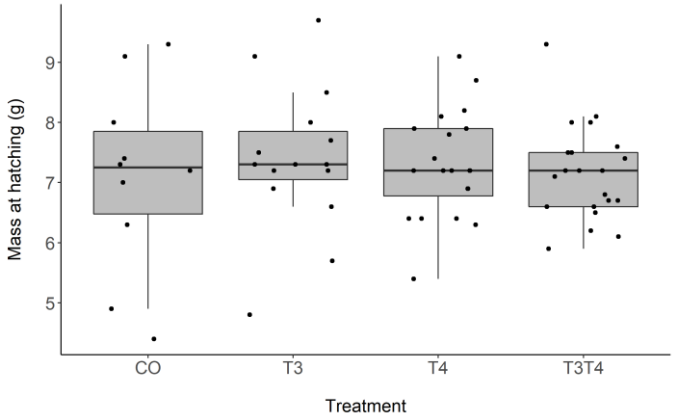

Figure S3: Boxplot of the mass at hatching in Japanese quail chicks according to yolk TH manipulation treatments: CO (N=10), T_3 (N=15), T_4 (N=20), T_3T_4 (N=21). See Fig. S1 for a description of the treatments. Boxplot shows median and quartiles.

Effects of prenatal THs on tarsus and wing growth

Table S1: Results of the Generalised Additive Mixed Models (GAMMs) on wing length, with sex, treatment and their interaction fitted either as intercept, curve shape or both (all combinations tested). A total of 12 GAMMs were fitted and ranked based on their AIC, from the lowest to the highest. Weight: Akaike's weights. ER: the evidence ratio of the weight of the top-supported model divided by the weight of the null model (model 12).

Model	Intercept	Curve shape	ΔAIC	df	Weight	ER
11	Sex	-	0.0	9	0.60	4
9	Treatment + Sex	-	2.2	12	0.21	-
12	-	-	2.8	8	0.15	-
10	Treatment	-	5.4	11	0.04	-
1	Sex	Sex	29.3	11	<0.001	-
3	-	Sex	30.0	10	<0.001	-
8	Treatment + Sex	Sex	31.2	14	<0.001	-
2	Treatment	Sex	32.4	13	<0.001	-
5	Sex	Treatment	102.6	15	<0.001	-
7	Treatment + Sex	Treatment	104.1	18	<0.001	-
6	-	Treatment	105.1	14	<0.001	-
4	Treatment	Treatment	107.4	17	<0.001	-

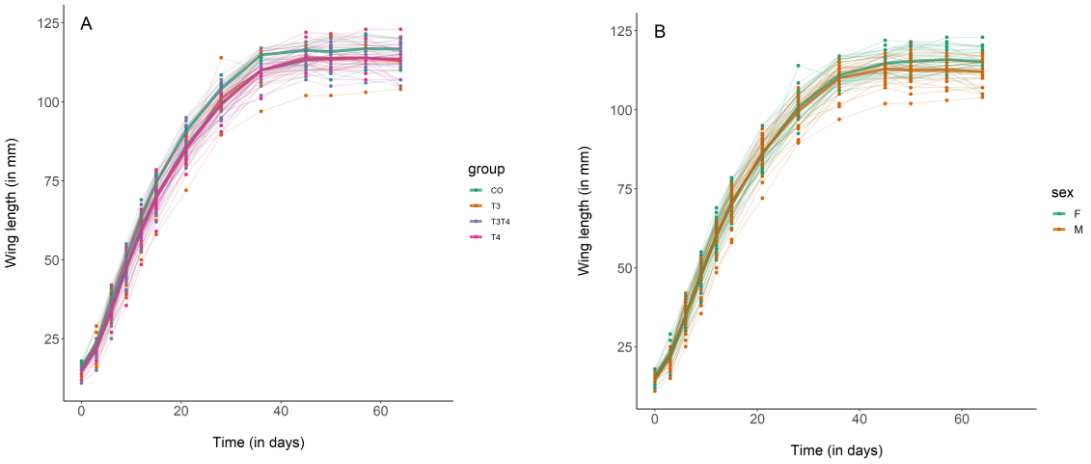

Figure S4: Growth curves in wing length of Japanese quails hatching from eggs treated with either T_3, T_4, a combination of both hormones, or a control solution. See Fig. S1 for a description of the treatments. Each line represents an individual bird, while thick coloured lines represent mean values. A: Growth curve according to yolk TH manipulation. N = 7 CO, 11 T_3, 18 T_4 and 21 T_3T_4. B: Growth curve according to sex. N = 29 females and 28 males.

Table S2: Results of the Generalised Additive Mixed Models (GAMMs) on tarsus length, with sex, treatment and their interaction fitted either as intercept, curve shape or both (all combinations tested). A total of 12 GAMMs were fitted and ranked based on their AIC, from the lowest to the highest. Weight: Akaike's weights. ER: the evidence ratio of the weight of the models within $\Delta AIC \leq 2$ divided by the weight of the null model (model 12).

Model	Intercept	Curve shape	ΔAIC	df	Weight	ER
10	Treatment	-	0.0	11	0.46	3.5
9	Treatment + Sex	-	1.0	12	0.28	2.2
12	-	-	2.5	8	0.13	-
11	Sex	-	2.5	9	0.13	-
2	Treatment	Sex	21.4	13	<0.001	-
8	Treatment + Sex	Sex	22.8	14	<0.001	-
3	-	Sex	23.5	10	<0.001	-
1	Sex	Sex	24.2	11	<0.001	-
4	Treatment	Treatment	91.3	17	<0.001	-
7	Treatment + Sex	Treatment	92.4	18	<0.001	-
6	-	Treatment	943.8	14	<0.001	-
5	Sex	Treatment	94.1	15	<0.001	-

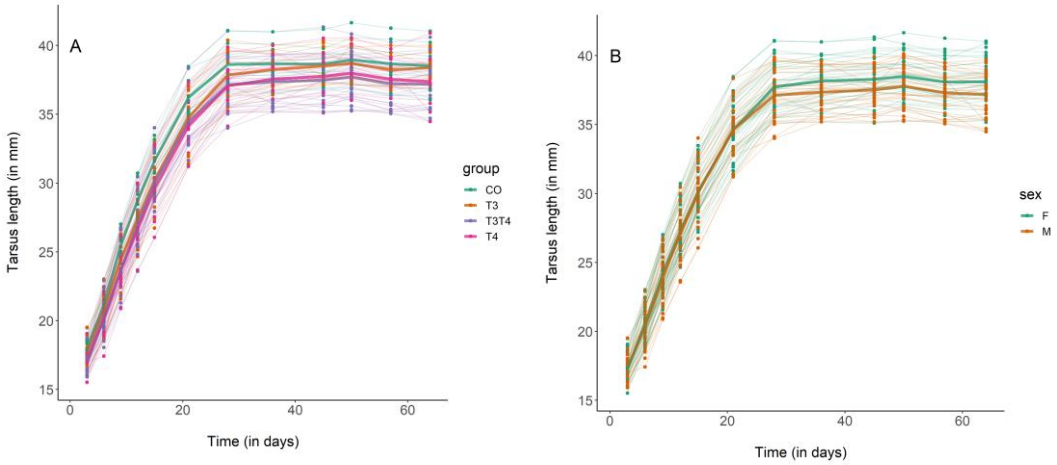

Figure S5: Growth curves in tarsus length of Japanese quails hatching from eggs treated with either T_3, T_4, a combination of both hormones, or a control solution. See Fig. S1 for a description of the treatments. Each line represents an individual bird, while thick coloured lines represent mean values. A: Growth curve according to yolk TH manipulation. N = 7 CO, 11 T_3, 18 T_4 and 21 T_3T_4. B: Growth curve according to sex. N = 29 females and 28 males.

Reproductive maturation and regression, and female reproductive investment

Statistical analyses

Male reproductive maturation was assessed by measuring the growth of the cloacal gland and foam production from week 4 to week 10 after hatching. Cloacal gland growth is non-linear and was then fitted with a GAMM with the intercept and the curve shape varying according to the treatment, similarly as for body mass. The model included a residual autocorrelation structure AR-1 to account for repeated measurements (Zuur et al., 2009).

Foam production was fitted with an LMMs with treatment as a predictor and body mass as a covariate. Two- and three-way interactions between these three parameters were tested, but not presented as statistically non-significant. Mother identity was added as a random intercept, and individuals were nested within mothers and allowed to vary both in the intercept and in the slope (i.e., random slope).

Female reproductive investment in eggs was assessed by multiplying the mean egg mass by the number of eggs laid by 2-month-old females over 6 days (N = 27). Data were analysed by separate LMMs with treatment as the fixed factor and mother identity of the parental generation as a random intercept. Female body mass two days before egg collection was added as a covariate.

Cloacal gland regression in males was analysed with an identical LMM as for foam production.

Effects of prenatal THs on prenuptial life stage transition and reproductive investment

Cloacal gland growth did not differ according to the treatment, as the null model was the only one within $\Delta AIC \leq 2$ (Table S3, Fig. S6). Likewise, the elevation of yolk THs did not affect foam production (LMM, $F_{3,17.2} = 0.82$, p = 0.50, Fig. S7), while body mass had a positive association with foam production (LMM, Estimate = 0.01±0.003, $F_{1,28.6} = 10.6$, p = 0.003). Manipulation of prenatal THs had no effect on female reproductive investment in eggs (LMM, $F_{3,14.8} = 0.30$, p = 0.83, Fig. S8). Taken together, male gonadal development and female reproductive investment were not affected by elevated yolk THs. However, the low sample sizes in the control groups do not allow us to make robust comparisons.

Table S3: Results of the Generalised Additive Mixed Models (GAMMs) on cloacal gland growth, with treatment fitted either as intercept, curve shape or both. A total of 4 GAMMs were fitted and ranked based on their AIC, from the lowest to the highest. Weight: Akaike's weights.

Model	Intercept	Curve shape	ΔAIC	df	Weight
4	-	-	0.0	7	1
3	-	Treatment	31.2	13	<0.001
1	Treatment	Treatment	35.1	16	<0.001
2	Treatment	-	71.0	10	<0.001

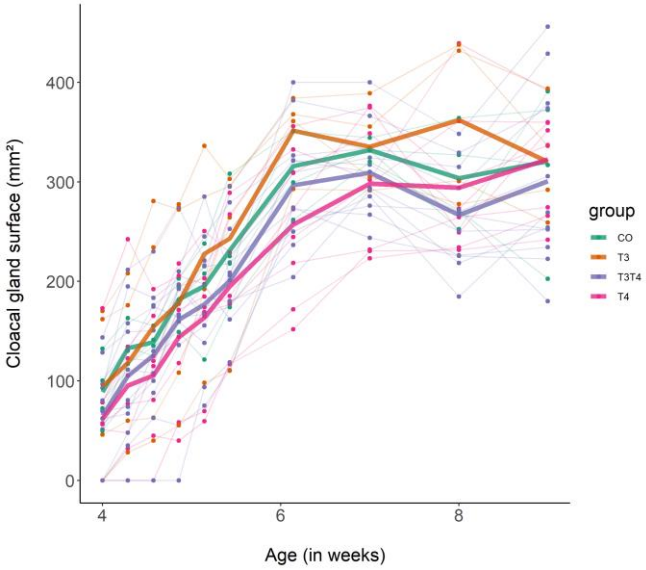

Figure S6: Cloacal gland growth in male Japanese quails according to yolk TH manipulation treatments: CO (N = 4), T_3 (N = 4), T_4 (N = 8), T_3T_4 (N = 12). See Fig. S1 for a description of the treatments. Measures were taken every second day from week 4 to week 6 after hatching, and then once a week until week 10. Each line represents an individual bird, while thick coloured lines represent mean values.

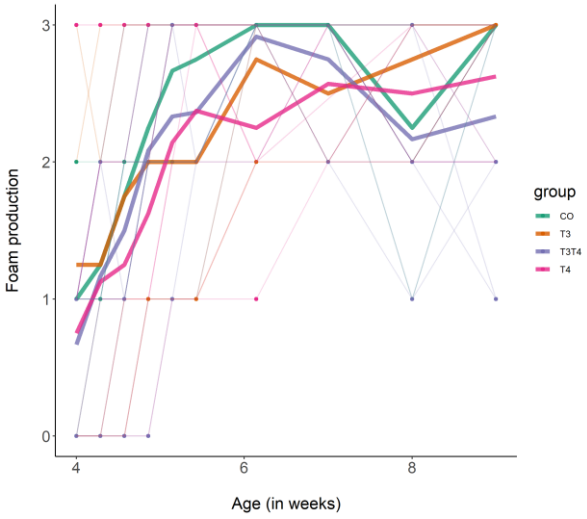

Figure S7: Foam production in male Japanese quails according to yolk TH manipulation treatments: CO (N=4), T_3 (N=4), T_4 (N=8), T_3T_4 (N=12). See Fig. S1 for a description of the treatments. Measures were taken every second day from week 4 to week 6 after hatching, and then once a week until week 10. Each line represents an individual bird, while thick coloured lines represent mean values.

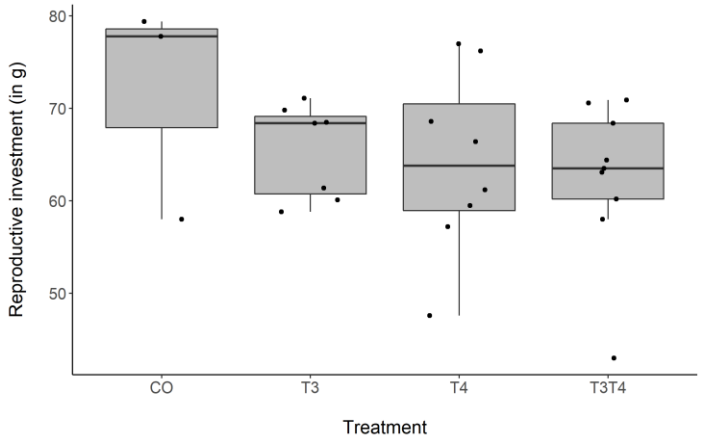

Figure S8: Boxplot of the reproductive investment by 2-month-old female Japanese quails according to yolk TH manipulation treatments: CO (N=3), T_3 (N=7), T_4 (N=8), T_3T_4 (N=8). See Fig. S1 for a description of the treatments. Egg investment is calculated as the number of eggs produced during 6 days multiplied by the average egg mass. Boxplot shows median and quartiles.

Effects of prenatal THs on gonadal regression

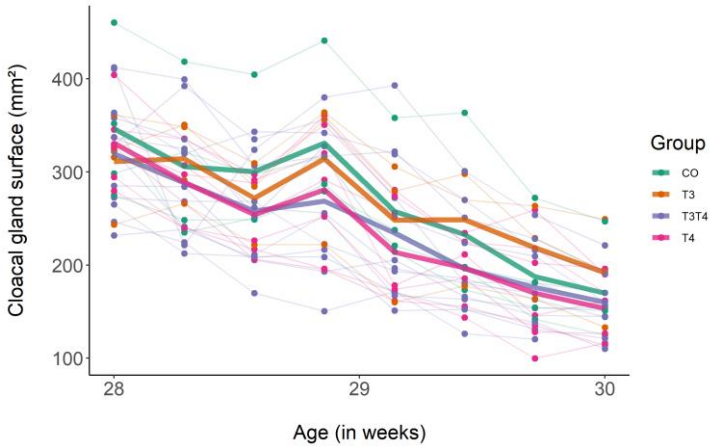

Figure S9: Cloacal gland regression of 7-month-old male Japanese quails according to yolk TH manipulation treatments: CO (N = 4), T_3 (N = 3), T_4 (N = 7), T_3T_4 (N = 12). See Fig. S1 for a description of the treatments. Measures were taken every second day after switching from long photoperiod (16L:8D) to short photoperiod (8L:16D, switch = time point 0 on x-axis). Each line represents an individual bird, while thick coloured lines represent group mean values.

As expected, cloacal gland regressed rapidly after switching to short photoperiod (LMMs, Estimate±SE = -11.61±0.47 mm², $F_{1,183.5}$ = 637.58, p < 0.0001, Fig. S9). We detected no effect of yolk thyroid hormones on cloacal gland regression (LMM interaction treatment × time, $F_{3,183.3}$ = 1.76, p = 0.16; treatment $F_{3,15.9}$ = 0.58, p = 0.64, Fig. S9), while we found a significant positive correlation with body mass (LMM, Estimate±SE = 1.32±0.26 mm², $F_{1,53.2}$ = 18.20, p < 0.0001). To sum up, male gonadal regression was not affected by elevated yolk THs. However, the low sample size in the control group does not allow us to make robust comparisons.

Chapter 5

Manipulation of prenatal thyroid hormones does not affect growth or physiology in nestling pied flycatchers

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Abstract

Hormones transferred from mothers to their offspring are thought to be a tool for mothers to prepare their progeny for expected environmental conditions, thus increasing fitness. Thyroid hormones (THs) are crucial across vertebrates for embryonic and postnatal development and metabolism. Yet, yolk THs have mostly been ignored in the context of hormone-mediated maternal effects. In addition, the few studies on maternal THs have yielded contrasting results that could either be attributed to species or to environmental differences. In this study, we experimentally elevated yolk THs (within the natural range) in a wild population of a migratory passerine, the European pied flycatcher *Ficedula hypoleuca*, and assessed their effects on hatching success, nestling survival, growth, and oxidative status (lipid peroxidation, antioxidant enzyme activity and oxidative balance). We also sought to compare our results with those on a closely related species, the collared flycatcher *Ficedula albicolis* that has strong ecological and life-history similarities with our species. We found no effects of yolk THs on any of the responses measured. We could only detect a weak trend on growth: elevated yolk THs tended to increase growth during the second week post hatching. Our results contradict the findings of previous studies including those in the collared flycatcher. However, differences in fledging success and nestling growth between both species in the same year suggest a context-dependent influence of the treatment. This study should stimulate more research on maternal effects mediated by thyroid hormones, and their potential context-dependent effects.

Introduction

Maternal effects are all the non-genetic influences of a mother on her offspring and receive increasing attention in evolutionary and behavioral ecology (Moore, Whiteman & Martin, 2019; Yin et al., 2019). Via maternal effects, mothers may influence the fitness of their progeny by adapting their phenotype to expected environmental conditions (Mousseau & Fox, 1998a; "adaptive maternal effects" in Marshall & Uller, 2007), and a recent meta-analysis found strong support for adaptive effects (Yin et al. 2019). Maternal effects are observed in plants, invertebrates and vertebrates, and can have many possible mediators (Danchin et al., 2011; Kuijper & Johnstone, 2018). One intriguing pathway is via the hormones transmitted from the mother to her progeny. These hormone-mediated maternal effects have been found to profoundly influence offspring phenotype in many different taxa (e.g. in mammals, Dantzer et al., 2013; birds, von Engelhardt & Groothuis, 2011; reptiles, Uller et al., 2007 and invertebrates, Schwander et al., 2008). Most studies in the field of hormone-mediated maternal effects have focused on steroid hormones, such as glucocorticoids and androgens (Groothuis & Schwabl, 2008; von Engelhardt & Groothuis, 2011). However, mothers transfer other hormones to their embryo (Williams & Groothuis, 2015), including thyroid hormones (THs; Ruuskanen & Hsu, 2018).

THs are metabolic hormones produced by the thyroid gland and are present in two main forms: thyroxine (T_4) and triiodothyronine (T_3). T_3 has a greater affinity with thyroid hormone receptors and is therefore responsible for most of the receptor-mediated effects. T_4, on the other hand, is mostly a precursor of T_3, although it may carry non-genomic effects (i.e. independent of TH receptors) (Davis, Goglia & Leonard, 2016). Thyroid hormones have pleiotropic effects that serve several biologically important functions across vertebrates (Ruuskanen & Hsu, 2018), and have been studied previously to some extent in various taxa (e.g. in birds, Wilson & McNabb, 1997; fish, Brown et al., 1988 and amphibians, Duarte-Guterman et al., 2010). In early life, they participate in the maturation of multiple tissues (e.g. birds, McNabb & Darras, 2015; mammals, Pascual & Aranda, 2013), and interact with growth hormones to increase growth (e.g. structural growth: Wilson & McNabb, 1997; McNabb & Darras, 2015). THs also regulate metabolism, and, during adult life, are necessary for normal reproductive functions (e.g. birds, McNabb & Darras, 2015; mammals, Norris & Carr, 2013). In wild bird species, plasma THs correlate positively with metabolic rate (Welcker et al., 2013; Elliott et al., 2013), and studies on mammalian model species found mechanistic evidence on the influence of THs on metabolism (Mullur, Liu & Brent, 2014). THs can alter the concentration of sodium and potassium in the cells (Haber & Loeb, 1986; Ismail-Beigi, Haber & Loeb, 1986), hence requiring ATP consumption to restore a normal gradient, which in turn stimulate metabolism (Mullur et al., 2014).

THs could further influence cell oxidative status, a biomarker that may underlie life-history trade-offs and ageing (Metcalfe & Alonso-Alvarez, 2010) via multiple pathways. Oxidative stress occurs when the reactive oxygen species (ROS) production exceeds the capacity of antioxidant defenses (Monaghan, Metcalfe & Torres, 2009). It results in oxidative damage on, for example, DNA, lipids and proteins (Monaghan, Metcalfe & Torres, 2009). As previous studies have shown that accelerated growth could increase oxidative stress (e.g. Alonso-Alvarez et al., 2007; Stier et al., 2014), the stimulating effects of THs on growth and metabolism likely contribute to the production of ROS, hence increase oxidative stress (Asayama et al., 1987; Villanueva, Alva-Sanchez & Pacheco-Rosado, 2013).

Studies on the effect of maternal thyroid hormones on offspring development in wild animals are scarce. In humans and rats, hypothyroid condition of the mother impairs brain development and cognition in her children (Moog et al., 2017). A potential problem here is that in mammalian species, maternal thyroid variation or manipulation inevitably influences other aspects of maternal physiology, which confounds the direct effects on the offspring. Oviparous species, such as birds, are therefore suitable models for studying the role of maternal hormones on the progeny because embryos develop in eggs outside the mother's body and maternally derived hormones are deposited in egg yolks (Prati et al., 1992; Schwabl, 1993). This allows the measurement and experimental manipulation of maternal hormone transfer to be independent of maternal physiology. Birds, with their relatively well-known ecology and evolution, have become the most extensively studied taxa in research on the function of maternal hormones (Groothuis et al., 2019).

Maternal thyroid hormones have long been detected in egg yolks of chicken (Hilfer & Searls, 1980; Prati et al., 1992) and Japanese quail (Wilson & McNabb, 1997). To date, only three studies have investigated the effects of physiological variation in yolk THs on offspring development (great tits, *Parus major*, Ruuskanen et al., 2016; rock pigeons, *Columba livia*, Hsu et al., 2017; collared flycatchers, *Ficedula albicollis,* Hsu et al., 2019). These studies revealed potential biological relevance and fitness consequences but also some discrepancies on the role of yolk THs. For example, yolk THs improved hatching success in rock pigeons (Hsu et al., 2017) and in collared flycatchers (Hsu et al., 2019) but had no effect in great tits (Ruuskanen et al., 2016). Moreover, TH injection in great tit eggs increased offspring growth in males but decreased it in females (Ruuskanen et al., 2016). Conversely, yolk THs decreased growth during the second half of the nestling phase in rock pigeons (Hsu et al., 2017), whereas they increased early growth, but decreased later postnatal growth in collared flycatchers (Hsu et al., 2019). Finally, great tits showed no response to elevated yolk THs in resting metabolic rate (RMR) (Ruuskanen et al., 2016), whereas RMR was increased in females but decreased in males rock pigeon hatchlings (Hsu et al., 2017). These studies suggest that yolk THs may exert costs and benefits on the offspring in a species-specific manner.

Another non-mutually exclusive hypothesis is that yolk THs may have context-dependent effects if the costs and benefits of THs differ across environments. For example, if prenatal THs increase RMR (as suggested by Hsu et al., 2017), the elevated RMR may lead to increased growth in benign conditions, but decreased growth when resource availability is poor (Auer et al., 2015). Therefore, further studies on other species and contexts are needed to understand these contradicting findings.

Moreover, the study on collared flycatchers is the only one so far that investigated the association between yolk THs and oxidative stress in offspring (Hsu et al., 2019). This study surprisingly showed no adverse effect of yolk THs on whole blood oxidative damage or oxidative balance, despite the early growth-enhancing effects in the same study (Hsu et al., 2019). This absence of influence on oxidative stress contradicts the general knowledge of THs, with hyperthyroid tissues exhibiting higher oxidative damage in mammals (liver and heart, Venditti et al., 1997; brain, Adamo et al., 1989), calling for additional studies to confirm or contradict these findings.

To explore the origin of the discrepancies between previous studies (i.e. species- or context-dependency), we conducted a similar experiment as Hsu et al. (2019) in a closely related species with a similar ecological niche, the pied flycatchers (*Ficedula hypoleuca*). Pied and collared flycatchers are sister species that have very similar life-histories, reproductive ecology and morphology, and can also hybridize (Lundberg & Alatalo, 1992). Importantly, the similarity between the two species offers us an opportunity to explore the potential role of the environment in modulating the effect of maternal hormones, which may contribute to explain the discrepancies of TH-related effects in the previous studies. To this end, we manipulated the concentrations of yolk THs in a wild population of pied flycatchers by injecting a combination of T_4 and T_3 in their eggs. We ensured that the treatment was within the physiological range. As proxies of environmental quality, we also collected data on temperature, precipitation, and fledging success of pied flycatchers. These data were then compared with those collected previously for collared flycatchers (Hsu et al., 2019). If the environmental contexts were similar between the two studies, we would expect to observe similar effects of elevated yolk THs, namely enhanced embryo development, hatching success, body mass and structural growth. By contrast, if the environmental context and the effects of elevated yolk THs differed between the studies, it would lend some support for the potential of context-dependent modulation. Finally, elevated yolk THs may result in higher oxidative stress (a general trend from the literature, e.g. Villanueva et al., 2013) either directly via the stimulating effects of THs on metabolism or indirectly via increased growth, or no association with oxidative stress may be shown at all (as suggested by Hsu et al., 2019).

Material and Methods

Study site and study species

The experiment was conducted during the spring 2017 in Turku, South-West of Finland (60°26'N, 22°10'E). The study species is the pied flycatcher, a small (ca. 15 g) migratory passerine that breeds in Finland from May to July. Pied flycatchers are secondary cavity nesters that also breed in artificial nest boxes. At this latitude, females generally lay a single clutch of 5 to 8 eggs.

Nest monitoring and experimental design

Yolk thyroid hormone concentrations were elevated via injections into unincubated eggs using a between-clutch design (i.e. all eggs of the same clutch received the same injection). In total, 29 clutches (170 eggs) received a thyroid hormone injection (hereafter TH-treatment), and 28 clutches (169 eggs) received a control injection (hereafter CO-treatment). In two nests, one in each treatment, none of the eggs hatched due to desertion before incubation. These two clutches were therefore removed from the analysis. The final sample size is 28 TH-nests (166 eggs) and 27 CO-nests (164 eggs).

Nest boxes were monitored twice a week during nest construction until egg laying. On the morning when the fifth or sixth egg was laid, all eggs were temporarily removed from the nest for injection, replaced with dummy eggs and returned after injection. Nests were then visited every following morning to inject freshly laid eggs until clutch completion, marked by the absence of freshly laid eggs and females incubating their eggs. Females generally start incubating their eggs after the last egg has been laid.

The clutches were randomly assigned to one of the treatments. In addition, the treatments were alternated across clutches to balance the order of the treatments within a day. Similarly, we also balanced the treatments across the laying period. There was no difference in the average (± SD) laying date from May 1 (TH = 27.00 ± 2.64 vs. CO = 27.19 ± 2.65, Wilcoxon unpaired test, $W = 402.5$, $p = 0.68$), nor in the average (± SD) clutch size (TH = 5.93 ± 0.81 eggs vs. CO = 6.07 ± 0.78 eggs, Wilcoxon unpaired test, $W = 439.5$, $p = 0.26$).

Preparation of the solution and injection procedure

The thyroid hormone solution (TH solution) was composed of a mix of T_4 (L-thyroxine, ≥ 98% HPLC, CAS number 51-48-9, Sigma-Aldrich) and T_3 (3,3',5-triiodo-L-thyronine, >95% HPLC, CAS number 6893-02-3, Sigma-Aldrich), first dissolved in 0.1M NaOH and then diluted in 0.9% NaCl. The concentration of each hormone was based on hormone measurements in 15 pied flycatcher eggs, from 15 clutches, collected during the spring 2016 in Turku. The average (± SD) hormone contents of these eggs was as

follows: T_4 = 2.307 ± 0.654 and T_3 = 0.740 ± 0.238 ng/yolk. We injected twice the standard deviation of each hormone (1.308 ng/yolk of T_4 and 0.477 ng/yolk of T_3), a standard and recommended procedure for hormone manipulation within the natural range (Ruuskanen et al. 2016; Hsu et al. 2017; Podmokła et al. 2018). The control solution (CO) was a saline solution (0.9% NaCl).

Before the injection, the shell was disinfected with a cotton pad dipped in 70% alcohol. The injection procedure consisted of four steps. First, a disposable and sterile 25G needle (BD Microlance ™) was used to pierce the shell. To locate the yolk, the egg was lit by a small torch from underneath. Second, the injection of 5µl was performed with a Hamilton® syringe (25 µl, Hamilton Company) directly into the yolk. Third, the hole in the shell was sealed with a veterinary tissue adhesive (3M Vetbond ™) and the eggs were marked with a permanent marker (Stabilo OHPen universal). Finally, all eggs of a clutch were returned to the nest at the same time, and the dummy eggs removed.

Nestling growth monitoring and blood sampling

Nests were checked daily for hatching two days before the expected hatching date. The date of hatching for a particular nest was recorded as the day the first hatchlings were observed (day 0). Two days after hatching, nestlings were coded by clipping down feathers to identify them individually. Nestlings were ringed at day 7 after hatching. Body mass (0.01 g) was recorded at day 2, 7 and 12 after hatching. Tarsus (0.1 mm) and wing length (1 mm) were recorded at day 7 and 12. At day 12, blood samples from all nestlings were also collected (ca. 40 µl) from the brachial vein in heparinized capillaries and directly frozen in liquid nitrogen for analyses of oxidative stress biomarker and molecular sexing. All nestlings from the same nest were sampled within 20 min. Samples were stored at -80°C until analyses. Finally, fledging was monitored from day 14 after hatching. Fledging date was recorded when all the nestlings had fledged from the nest, and fledging success (fledged/not) was scored for each hatchling.

Finally, we collected data on temperature (hourly averages) and precipitation from the European Climate Assessment & Dataset (ECA&D, Klein Tank et al., 2002), and calculated the daily averages and length of periods of continuous rain, a key factor affecting mortality in flycatchers (Siikamäki, 1996; Eeva et al., 2002). Temperature data (hourly averages) were extracted from a station located approximately 3 km away from our field site. To compare environmental conditions between the collared flycatcher study by Hsu et al. (2019) and our study, we also collected similar data for the study period from a field station close to the collared flycatcher population (See Figs. A1 and A2 and Table A3, available online). In addition, we used overall fledging success as a proxy for environmental quality. In both populations, nest predation and adult mortality rates are low and are not main determinants of fledging success (Doligez & Clobert, 2003; B. Doligez and S. Ruuskanen, personal communication). Thus, fledging success

may be a good indicator of environmental conditions during the nestling phase. The data in Hsu et al. (2019) and in our experiment were collected on the same year (2017), and both nest-box populations were located in mixed forest habitats.

Sexing method

DNA extraction procedure from the blood cells followed Aljanabi and Martinez (1997), using approximately 5 µl of whole-blood samples. The method of sexing followed that described by Ruuskanen and Laaksonen (2010) with minor changes on the PCR condition: 5 µl QIAGEN multiplex PCR kit + 0.1 µl of each primer (20 µM) + 1.8 µl H2O + 3 µl DNA, yielding 10µl for the final PCR volume. The initial denaturation was at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 90 s, and 72 °C for 60 s. The samples were then held at 72 °C for 10 min and 20 °C for 5 mins. PCR products were analyzed with 3% agarose gel under 100 V for 90 min.

Oxidative stress analysis methods

Samples from two individuals per clutch were analyzed. Whenever possible, one male and one female were chosen of approximately the same body mass since body mass is known to covary with oxidative status (Rainio et al., 2015). The average difference in mean body mass between the chicks selected for oxidative stress analysis within each clutch is -0.01 g (SD = 0.43; range = -1.80–0.77 g). If samples could not be taken for both sexes from a clutch, then two individuals of the same sex were selected. In total, 103 nestlings were included in the analysis (TH, N = 27 nests and 50 nestlings; CO, N = 27 nests and 53 nestlings).

Three biomarkers of oxidative status were measured: the activity of the antioxidant enzyme glutathione S-transferases (GSTs), the ratio of reduced and oxidized glutathione (GSH:GSSG ratio) and lipid peroxidation (using malonaldehyde, MDA, as a proxy) (Sheenan et al., 2001; Halliwell & Gutteridge, 2015). GST enzymes catalyze the conjugation of toxic metabolites to glutathione (Sheenan et al., 2001; Halliwell & Gutteridge, 2015). In normal cells, GST activity is expected to be lower than in damaged cells (Rainio et al., 2013). The GSH:GSSG ratio represents the overall oxidative state of cells, and a low ratio reveals oxidative stress (e.g. Rainio et al., 2013; Halliwell & Gutteridge, 2015; Rainio et al., 2015). Lipid peroxidation is commonly measured with the thiobarbituric acid reactive substances test (TBARS, Alonso-Alvarez et al., 2008; Halliwell & Gutteridge, 2015). This test relies on the ability of polyunsaturated fatty acids contained in cell membranes to readily react with oxygen radicals by donating a hydrogen atom. The fatty acid radical is unstable, and a chain of reactions occurs. Malonaldehyde is an end product of this reaction (Marnett, 1999) and thus used as a measure of lipid peroxidation.

Whole blood was first thawed and then diluted in 0.9% NaCl to achieve protein concentrations ranging 4–13 mg/ml. Overall protein concentration (mg/ml) was measured using a bicinchoninic acid protein assay (Thermo Scientific) with a bovine serum albumin standard (Sigma-Aldrich). The methodology for measuring GST and the GSH:GSSG ratio followed Rainio et al. (2015). The marker of lipid peroxidation, MDA, was analyzed using a 384-plate modification of TBARS-assay described by Espín et al. (2017). All biomarkers enzyme activities were measured in triplicate (intra-assay coefficient of variability [CV] <10% in all cases).

Statistical analysis

Data were analyzed with the software R version 3.5.3 (R Core Team 2019). General and generalized linear mixed models (LMMs and GLMMs, respectively) were performed using the R package *lme4* (Bates et al., 2015). All mixed models included nest as a random intercept. P-values in LMMs were obtained by model comparison using Kenward-Roger approximation from the package *pbkrtest* (Halekoh & Højsgaard, 2014). The significance of the predictors in GLMMs was determined by parametric bootstrapping with 1,000 simulations using the package *pbkrtest*. Model residuals were checked for normality and homogeneity by visual inspection. Significant interactions were further analyzed by post-hoc comparison with the package *phia* (de Rosario-Martinez, 2015). Estimated marginal means and standard errors (EMMs ± SE) were derived from models using the package *emmeans* (Lenth, 2019).

To analyze hatching success, a dummy code was given to each egg: 0 for unhatched egg and 1 for hatched egg. A GLMM was performed with a binomial error distribution (logit link). Treatment was included as the predictor and two covariates were included: the average temperature over the egg laying period and clutch size. Fledging success was coded similarly: 0 for dead and 1 for fledged nestling. A similar GLMM was fitted, with treatment as a predictor and the average temperature over the nestling period and brood size at day 2 as covariates.

Duration of the embryonic period and duration of the nestling phase were fitted in separate linear models with treatment as the fixed effect, and the average ambient temperature over these two phases as covariates to control for potential temperature-related effects (Olson, Vleck & Vleck, 2006; Salaberria et al., 2014). Laying date and brood size were added as additional covariates for nestling phase duration as they both may influence nestling growth and thereby nestling phase duration (Williams, 2012).

Early body mass (i.e. at day 2 after hatching) was analyzed separately from growth during the second week post-hatching (i.e. from day 7 to day 12) for two reasons. First, variation in the former may represent better the influence of maternal THs on prenatal development, while the variation in the latter also reflects the influence during the postnatal stage when the yolk that contain the hormones is totally consumed. Second,

including the three time points in a single model created a non-linear growth curve, hampering proper statistical analyses. The model to analyze early body mass included laying date and mean temperature between hatching and day 2 as covariates. To analyze growth between day 7 and 12, we used the scaled mass index by Peig and Green (2009), a recommended method to estimate changes in body condition. The SMI was calculated as follows:

$$\mathrm{SMI}_i = M_i \times (L_0/L_i)^b$$

where M_i and L_i are body mass and tarsus length of the individual i, respectively. L_0 is the mean value of tarsus length for the whole population (L_0 = 17.0 mm, N = 228), and b is the slope estimate of a regression of ln-transformed body mass on ln-transformed tarsus length (b = 1.83). Furthermore, we analyzed growth in wing and tarsus length separately, given that THs may also influence structural size (e.g. Wilson & McNabb, 1997), independently of mass. Models to analyze the morphological variables included sex as a fixed factor to test for potential sex-dependent effects of THs, as found by Hsu et al. (2017) and Ruuskanen et al. (2016). Treatment and age were added as fixed factors together with their 2- and 3- way interactions with sex. Brood size at day 2, laying date and average temperature were included as covariates. Individual identity was added as a random intercept to account for repeated measures.

The models to analyze growth (SMI and structural size) included age and treatment as fixed factors. Brood size at day two, laying date and average daily temperature (between day 3 and 7 for measurements at day 7, and between day 8 and 12 for measurements at day 12) were added as covariates, and nestling identity as an additional random intercept to account for repeated measures.

The models of oxidative stress biomarkers (i.e. GST activity, MDA concentration and GSH:GSSG ratio) included treatment and sex as the predictors, and brood size at day 2, laying date and mean daily temperature as covariates. Body mass at day 12, which was the day of blood sampling, as an additional covariate, because body mass is known to be associated with oxidative status (e.g. Rainio et al., 2015). In a separate model, body mass at day 12 was replaced with growth rate (in grams/day) between day 7 and 12, to test the association of growth rate on oxidative stress. Assay number was also added as a random intercept to account for inter-assay variation. Response variables were log-transformed to achieve normal distribution of the residuals.

Results

Hatching and fledging success, duration of embryonic and nestling periods

Hatching success (TH = 75.3% vs. CO = 76.8%) and fledging success (TH = 92.2% vs. CO = 92.3%) were similar between the two treatments (GLMMs, $p > 0.71$). Hatching success was not affected by clutch size or by ambient temperature during incubation (GLMMs, both $p > 0.09$). Likewise, fledging success was not correlated to brood size at day 2 or ambient temperature (GLMMs, both $p > 0.10$). Duration of the embryonic period did not differ between the groups ($t = -0.59$, $p = 0.56$). Injection of yolk THs did not affect the duration of the nestling period either ($t = -1.01$, $p = 0.32$). Likewise, there was no association between laying date or brood size at day 2 and the duration of the nestling period (all $t < 1.87$, $p > 0.07$). Finally, there was no association between temperature and duration of embryonic or nestling periods (all $p > 0.09$).

Growth

Experimental elevation of yolk thyroid hormones did not affect early postnatal body mass (day 2 Estimated marginal means [EMMs] ± SE: CO = 3.63 ± 0.13 g vs TH = 3.50 ± 0.13 g), and neither did sex (Table 1). We detected a tendency of an interaction between treatment and age on nestling scale mass index (SMI) between day 7 and 12 that just did not reach statistical significance ($p=0.07$, Table 1). Although the interaction was not significant, we performed post-hoc analyses to explore the trend further. We found that TH-treated nestlings tended to grow faster than control nestlings during the second week post-hatching (adjusted slopes ± SE = 1.32 ± 0.11 for CO nestlings, 1.61 ± 0.12 for TH nestlings, $\chi^2 = 3.41$, Holm-adjusted $p = 0.06$, Fig. 1). Though, there was no significant difference in mass index between the treatments at day 7 ($\chi^2 = 0.06$, Holm-adjusted $p = 0.81$) or day 12 ($\chi^2 = 1.04$, Holm-adjusted $p = 0.62$), indicating that the interaction likely originates from small differences in the opposite directions at day 7 and day 12 between TH and control groups. On average, males had a slightly higher mass index than females between day 7 and day 12 (EMMs ± SE: males = 80.78 ± 0.59, females = 79.86 ± 0.61, Table 1). For structural size measurements, tarsus and wing lengths, however, no effects of yolk TH treatment were detected (Table 1). Ambient temperature was negatively correlated with SMI, and positively associated with wing length (Table 1).

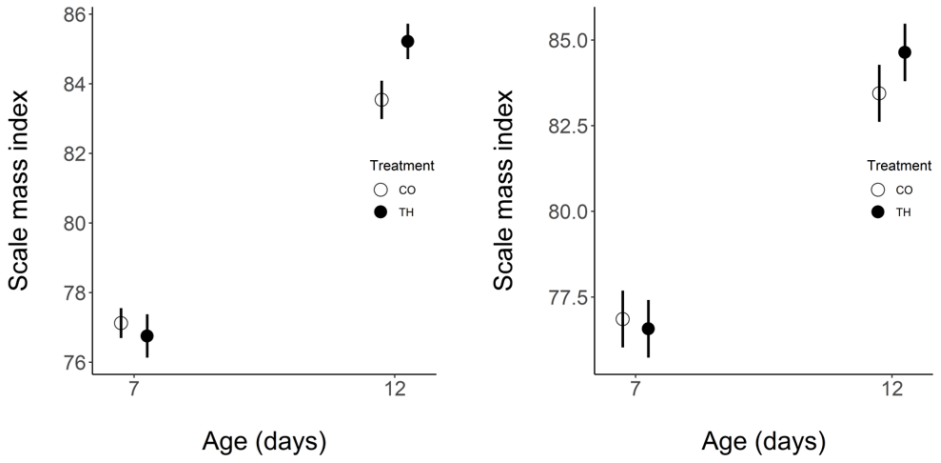

Figure 1: Scale mass index raw data (mean ± SE, left) and marginal means (right) at day 7 and day 12 after hatching in TH-treated nestlings (N = 125) and controls (N = 126). The interaction between the treatment and age of the nestlings approached significance (p = 0.07). Empty circle: CO = control injection; solid circle: TH = yolk thyroid hormone elevation.

Oxidative stress and oxidative damage

Experimental elevation of yolk THs did not affect antioxidant enzyme activity (mean ± SE: CO and TH nestlings = 0.006 ± 0.0003 pmol GST/min/mg protein), oxidative damage on lipids (mean ± SE: CO = 0.051 ± 0.003 and TH = 0.053 ± 0.004 nmol MDA/mg protein) or oxidative status (mean ± SE: CO = 3.86 ± 0.47 and TH = 4.51 ± 0.73 GSH:GSSG ratio) (Table 2). None of the other predictors or covariates (i.e. sex, body mass, growth rate, temperature and brood size) were associated with these oxidative stress biomarkers, except laying date, which was negatively correlated with MDA concentration (Table 2).

Environmental context

Patterns of temperature and precipitation during the different stages of breeding are shown in Figures A1 and A2 for pied flycatchers and collared flycatchers. There were only minor differences in mean temperature across the stages and species: For pied flycatchers, the average temperatures over the laying, incubation and nestling periods were 12.14 °C, 13.43 °C and 15.02 °C respectively, and for collared flycatchers 12.48 °C, 13.67 °C and 14.56 °C. Likewise, the number of days with rain was rather similar (Table A3) and we could not reliably associate peaks in precipitation with peaks in nestling mortality (Fig. A4). Importantly, however, collared flycatchers experienced lower fledging success (ca. 75%, Hsu et al. 2019) compared to pied flycatchers (ca. 90%) during the study year, whereas both species have similar fledging success of about 90% when the environmental conditions are good (Qvarnström et al., 2009), suggesting that the collared flycatchers experienced harsher environmental conditions than the pied flycatchers.

Table 1: Full linear mixed models of morphometric measures in response to yolk thyroid hormone elevation, in nestling pied flycatchers (sample sizes: TH = 125; CO = 126).

Predictors	Body mass at day 2 (g)			Scale mass index from day 7 to 12			Wing length from day 7 to 12 (mm)			Tarsus length from day 7 to 12 (mm)		
	E (SE)	F_{ddf}	p	E (SE)	F_{ddf}	p	E (SE)	F_{ddf}	p	E (SE)	F_{ddf}	p
Treat (TH)	-0.15 (0.17)	$0.86_{49.0}$	0.36	-3.06 (2.61)	$0.007_{51.6}$	0.94	0.08 (0.92)	$0.10_{50.0}$	0.75	0.07 (0.22)	$0.44_{49.6}$	0.51
Sex (Male)	0.04 (0.05)	$0.58_{182.5}$	0.45	0.58 (2.21)	$4.6_{188.2}$	0.03	0.51 (0.69)	$1.15_{183.2}$	0.29	0.02 (0.18)	$0.03_{190.3}$	0.86
Age (12 days)	-	-	-	1.30 (0.17)	$330.4_{226.0}$	<0.001	4.72 (0.05)	$39241_{226.0}$	<0.001	0.29 (0.01)	$2174.0_{226.0}$	<0.001
Laying date	0.004 (0.04)	$0.01_{52.9}$	0.92	-0.07 (0.22)	$0.1_{56.8}$	0.77	0.05 (0.11)	$0.17_{55.2}$	0.69	0.001 (0.02)	$0.004_{57.2}$	0.95
Brood size	0.16 (0.07)	$5.46_{56.6}$	0.02	-0.90 (0.47)	$4.3_{61.3}$	0.04	0.36 (0.24)	$2.27_{58.3}$	0.14	0.08 (0.05)	$3.24_{62.4}$	0.08
Temperature	-0.11 (0.06)	$3.18_{49.9}$	0.08	-0.97 (0.17)	$33.1_{236.9}$	<0.001	0.22 (0.05)	$19.2_{227.8}$	<0.001	-0.004 (0.01)	$0.10_{231.7}$	0.75
Treat × sex	-	-	-	1.15 (3.20)	$0.1_{187.0}$	0.76	-0.66 (0.99)	$0.08_{182.1}$	0.78	-0.14 (0.26)	$0.07_{189.2}$	0.80
Treat × age	-	-	-	0.36 (0.24)	$3.2_{224.2}$	0.07	-0.03 (0.07)	$0.06_{224.1}$	0.81	-0.01 (0.02)	$0.30_{224.1}$	0.59
Sex × age	-	-	-	0.03 (0.22)	$0.03_{224.0}$	0.86	-0.04 (0.07)	$0.001_{224.0}$	0.98	-0.0004 (0.02)	$0.18_{224.0}$	0.67
Treat × age × sex	-	-	-	-0.10 (0.32)	$0.1_{223.0}$	0.72	0.08 (0.10)	$0.70_{223.0}$	0.41	0.01 (0.02)	$0.23_{223.0}$	0.63

E (SE): Estimate ± SE. P-values and ddf were obtained using the Kenward-Roger approximation. Ndf = 1. P-values were obtained by removing each predictor one by one from the model, except for the main effects of treatment, sex and age, which were removed from models without their interactions, otherwise the models were not nested

Table 2: Full linear mixed models of oxidative stress biomarkers in response to yolk thyroid hormone elevation, in nestling pied flycatchers at day 12 after hatching (sample sizes: TH=50; CO=53).

Predictors	MDA concentration (nmol/mg protein)			GSH:GSSG ratio			GST activity (pmol/min/mg protein)		
	E (SE)	F_{ddf}	p	E (SE)	F_{ddf}	p	E (SE)	F_{ddf}	p
Treat (TH)	0.004 (0.074)	$0.003_{41.3}$	0.96	0.13 (0.16)	$0.63_{44.6}$	0.43	-0.07 (0.06)	$1.21_{44.6}$	0.28
Sex (Male)	-0.03 (0.07)	$0.18_{55.1}$	0.67	0.11 (0.14)	$0.55_{55.4}$	0.46	-0.07 (0.06)	$1.73_{57.4}$	0.19
Mass at day 12	-0.06 (0.04)	$2.78_{45.2}$	0.10	-0.07 (0.08)	$0.62_{60.6}$	0.43	0.02 (0.03)	$0.46_{49.4}$	0.50
Temperature	-0.006 (0.019)	$0.09_{42.3}$	0.76	-0.003 (0.039)	$0.008_{44.4}$	0.93	0.001 (0.016)	$0.003_{43.8}$	0.95
Laying date	-0.04 (0.02)	$5.40_{40.2}$	0.03	0.02 (0.03)	$0.51_{49.8}$	0.48	0.005 (0.014)	$0.13_{42.9}$	0.72
Brood size	-0.06 (0.03)	$3.13_{49.9}$	0.08	0.001 (0.072)	$0.0002_{59.1}$	0.99	0.001 (0.028)	$0.002_{52.7}$	0.97
Growth rate	-0.15 (0.15)	$0.94_{51.2}$	0.34	0.23 (0.35)	$0.42_{66.3}$	0.52	-0.06 (0.13)	$0.17_{58.0}$	0.68

E (SE): Estimate ± SE. Response variables were log-transformed to achieve normal distribution of the residuals. P-values and ddf were obtained using the Kenward-Roger approximation. Ndf=1. To examine the association between growth rate (between day 7 and day 12) and oxidative status, we further replaced body mass with growth rate in the reduced model while keeping all other predictors constant. The dashed line indicates that growth rate was tested in another model than body mass at day 12. P-values of the predictors were obtained by removing these predictors individually from the full model.

Discussion

We replicated an experimental study on the effect of egg thyroid hormones on offspring development in collared flycatchers in a closely related and ecologically similar species, the pied flycatcher while at the same time monitoring environmental factors. This would allow us to study the generality of results found earlier but also potential environmentally dependent hormone effects.

Overall, our results on pied flycatchers differ substantially from those on collared flycatchers (Hsu et al., 2019). We found no effect of prenatal THs on hatching success or growth (in body mass, body condition or structural growth), whereas Hsu et al. (2019) found an increase in hatching success and in early growth but decreased growth during the second week of the nestling period. Because these two species are closely related and display ecological similarities (Lundberg & Alatalo, 1992), we predicted that such discrepancies in the results could arise if THs influence growth differently in different environmental conditions. We observed that fledging success, a proxy for environmental harshness, was lower in collared than the pied flycatcher experiment. Yet, temperatures and rainfall did not generally seem to differ across the studies, suggesting that other environmental factors may interact with yolk THs. Furthermore, collared flycatchers generally have a slightly higher early body mass (Qvarnström et al., 2009) and a higher fledging mass (Myhrvold et al., 2015) than pied flycatchers. Yet, when comparing the present study with Hsu et al. (2019), collared flycatchers had a lower early body mass than pied flycatchers and a similar body mass close to fledging, suggesting poorer growth of collared flycatchers during the study year. Prenatal environmental conditions (i.e. during egg laying and incubation) were rather similar between the two species, and thus cannot explain why yolk THs enhanced hatching success and early body mass in collared flycatchers (Hsu et al., 2019) but not in pied flycatchers (this study). More experimental studies on the context-dependent effects of yolk THs are thus needed.

Despite no clear differences in temperature and precipitation, the lower growth and survival of nestling collared flycatchers suggest that the environmental conditions may have been harsher in this population than in the pied flycatcher population. Such environmental conditions may have contributed to the contrasting results on the effects of yolk THs on postnatal growth. We can speculate that a potential underlying mechanism is linked to metabolic rates. Hsu and colleagues suggested that prenatal THs increase basal metabolic rates (Hsu et al., 2017). Increased basal metabolic rates may lead to decreased postnatal growth in harsh conditions, such as those for the collared flycatcher population, but have no effect or even increase growth when resource availability is good, as is the case for the pied flycatcher population. Nevertheless,

despite the high degree of ecological similarity between the two species, the possibility that species difference actually explained the contrasting results remains to be examined.

We observed no effect on antioxidant enzyme activity (GST) or in the oxidative balance (GSH:GSSG) and no increase in oxidative damage in lipids (MDA) in response to elevated yolk THs. The earlier study on collared flycatchers reported similar levels of oxidative stress biomarkers and found no increase in oxidative stress in response to elevated prenatal THs (Hsu et al., 2019). These results may suggest that egg THs do not affect the oxidative status of nestlings as it would be expected from the literature. However, the absence of detrimental consequences on oxidative stress may be due to the experimental design of both studies, with an increase in yolk THs within the natural range of the species. Thus, individuals may have been able to raise their antioxidant capacities (other than those measured in this study) to avoid oxidative damage. That said, physiological elevation (i.e. within the natural range) of yolk THs was necessary to get ecologically relevant results.

Furthermore, due to fieldwork constraints, there are some limits to our approach. We measured a limited number of markers of oxidative status at a single time point in one tissue, and therefore lack an overview of the variation that may have happened over the course of the whole nestling phase, also in other tissues and for other biomarkers. Further studies with more comprehensive measures of oxidative stress would help understanding the relationship between yolk THs and oxidative stress.

In conclusion, this study shows no convincing effect of yolk THs on nestling development. We found that yolk THs did not increase growth and incurred no extra oxidative damage, nor affected nestling survival. Our results differ from a study on a closely related species, suggesting that the role of prenatal THs may differ according to the environment experienced by the progeny. The study adds to the small body of literature on so far largely neglected thyroid hormone-mediated maternal effects. Research on maternal THs would greatly benefit from further studies with the same species in different, experimentally manipulated, contexts. It would also profit from comparative studies on species with different life-histories that are likely to influence the effects induced by exposure to maternal THs.

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Ethics
The study complied with Finnish regulation and was approved by the Finnish Animal Experiment Board (ESAVI/2389/04.10.07/2017) and by the Finnish Ministry of Environment (VARELY580/2017).

Supplementary material

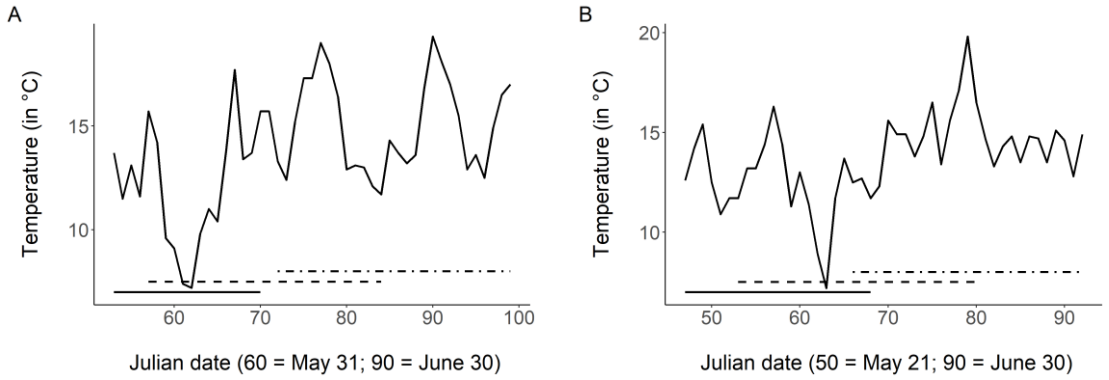

Figure A1: Average daily temperature experienced by pied flycatchers in Turku, Finland (left) and by collared flycatchers in Gotland, Sweden (right). Solid, dashed and dash-dotted lines represent egg laying, incubating and nestling periods, respectively. In Turku, average temperatures over the different periods were as follow: 12.14°C, 13.43°C and 15.02°C. In Gotland, average temperatures were: 12.48°C, 13.67°C and 14.56°C.

Table A1: Number of days with rain in both sites experienced by pied flycatchers in Turku and by collared flycatchers in Gotland, during the different periods.

	Turku	Gotland
Number of days with rain:		
Egg laying	6	6
Incubation	12	8
Nestling	9	10
Number of consecutive days with rain:		
Egg laying	4 (2 * 2 days)	2
Incubation	5 (2 days + 3 days)	5 (2 days + 3 days)
Nestling	5 (2 days + 3 days)	6 (2 * 3 days)

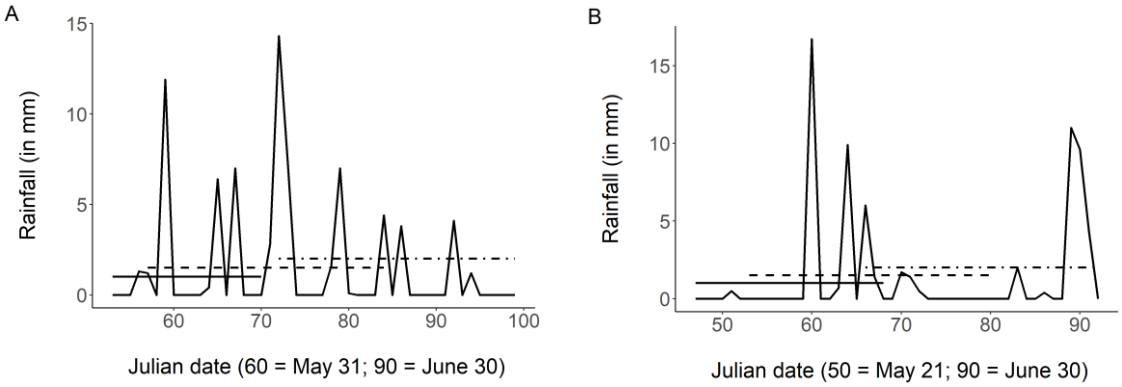

Figure A2: Daily precipitation experienced by pied flycatchers in Turku, Finland (left) and by collared flycatchers in Gotland, Sweden (right). Solid, dashed and dash-dotted lines represent egg laying, incubating and nestling periods, respectively.

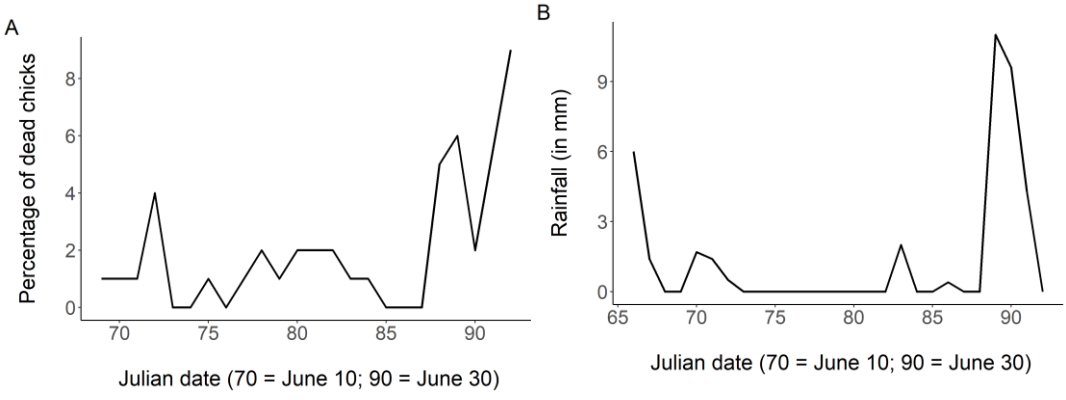

Figure A3: Percentage of dead nestlings in collared flycatchers (left) and daily precipitation in the same population (right). These graphs show no convincing overlapping between the events of continuous rain and the peaks in nestling mortality.

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English summary

Phenotypic plasticity is defined as the ability of a genotype to produce several phenotypes depending on the environmental conditions. Phenotypic plasticity enables individuals to rapidly adjust to changing environments and can even subject to natural selection. Parental effects are a form of phenotypic plasticity, in which the phenotype of the parents influence the phenotype of the offspring. Parental effects are widespread across many taxa and can be adaptive. Although both parents can exert parental effects, most of the literature so far has focused on the mother's influence on offspring phenotype, i.e., maternal effects.

Maternal hormones are one possible pathway for maternal effects and have received increasing attention in the last 20 years. So far, most of the research on maternal hormones has focused on steroid hormones: androgens and glucocorticoids. There is now ample evidence that hormones of maternal origin can affect offspring phenotype. Other hormones, such as thyroid hormones, have been widely overlooked in the context of hormone-mediated maternal effects. Thyroid hormones (THs) are crucial hormones playing important roles in vertebrates (e.g., embryonic development, juvenile growth, metabolism and reproduction) and in invertebrates (mostly on growth and metamorphosis). TH production by the thyroid gland consists mostly of T_4, the prohormone, which is then converted in the tissues into T_3, the active form. T_3 is considered the most active form as it binds with a greater affinity to TH receptors. Iodine is a key component of TH production that cannot be synthesised by an organism and must therefore be found in the environment. This makes THs interesting candidates to study whether maternal hormone deposition can be costly for the mothers.

Oviparous species such as birds are good models to study maternal hormones as embryos develop in a sealed compartment and rely exclusively on maternal signalling during their development. In addition, it makes it easier to measure and manipulate yolk hormones independently of maternal physiology.

In my thesis I aimed at answering several questions on the causes and consequences of variation in maternal THs in avian eggs. I studied whether (i) iodine can limit TH production and transfer to the yolk; (ii) mothers are able to regulate yolk TH deposition independently from their circulating THs; (iii) prenatal THs can have long-lasting effects on offspring as well as short-term effects; and (iv) prenatal THs can have context-dependent effects. I found that (i) iodine availability may limit egg production in some females; (ii) females may not be able to regulate yolk TH deposition independently from the circulation levels; (iii) prenatal THs do not appear to have long-term effects, while short-term effects seem inconsistent with previous similar studies; (iv) prenatal THs do not seem to interact with ambient temperature during the nestling phase.

Overall, my thesis sheds light on a crucial hormonal signal that has received little attention in the context of maternal hormones. It should stimulate future research

on prenatal THs that would tackle essential questions such as the potential adaptive value of variation in prenatal THs or the embryonic response to this maternal signalling.

Tiivistelmä suomeski

Translation by Suvi Ruuskanen

Ilmiasun joustavuus määritellään genotyypin kykynä tuottaa erilainen ilmiasu ympäristöolosuhteista riippuen. Ilmiasun joustavuus mahdollistaa yksilöiden sopeutumisen muuttuviin ympäristöolosuhteisiin, ja se voi olla myös luonnonvalinnan kohteena. Vanhempainvaikutukset ovat ilmiasun joustavuuden muoto, jossa vanhempien ilmiasu vaikuttaa jälkeläisten ilmiasuun. Vanhempainvaikutukset ovat yleisiä kaikissa eliöryhmissä ja voivat olla adaptiivisia. Vanhempainvaikutukset voivat välittyä joko isältä tai äidiltä, mutta suurin osa kirjallisuudesta on keskittynyt äitivaikutuksiin.

Äidiltä välittyvät hormonit ovat yksi mahdollinen äitivaikutusten välittäjämekanismi, joka on saanut huomiota erityisesti viimeisten 20 vuoden aikana. Suurin osa tähänastisesta kirjallisuudesta on keskittynyt steroidihormoneihin (androgeenit ja glukokortikoidit): äidiltä välittyvien hormoneiden vaikutuksesta jälkeläisten ilmiasuun on nyt laajaa tutkimusnäyttöä. Toiset hormonit, kuten kilpirauhashormoni, ovat vähemmän tutkittuja äitivaikutusten välittäjiä. Kilpirauhashormonit ovat tärkeitä selkärankaisille (mm. alkionkehitys, poikasten kasvu, metabolia ja lisääntyminen), mutta myös selkärangattomille (kasvu ja metamorfoosi). Kilpirauhanen tuottaa tyroksiinia, hormonin esiastetta, joka muunnetaan kudoksissa trijodityroniiniksi. Trijodityroniinia pidetään biologisesti aktiivisena muotona, sillä se kiinnittyy hormonireseptoreihin. Jodi on kilpirauhashormonin keskeinen osa, jota organismit eivät voi itse tuottaa, vaan ovat riippuvaisia ympäristön jodista. Tämän vuoksi on kiinnostavaa tutkia, onko äidiltä jälkeläisille välittyvä kilpirauhashormoni kustannus äidille.

Linnut ovat hyvä malliorganismi äitihormonien tutkimukseen, sillä alkiot kehittyvät munissa riippumattomissa äidin fysiologian lyhytaikaisista muutoksista. Munien hormonitasoja voidaan myös mitata ja manipuloida ilman, että äidin fysiologia muuttuu. Väitöskirjassani pyrin vastaamaan useisiin kysymyksiin äidiltä välittyvien kilpirauhashormonien vaihtelun syistä ja seurauksista linnuilla. Tutkin (i) voiko jodi rajoittaa äidin kilpirauhashormonituotantoa ja munan hormonitasoja; (ii) kykenevätkö emot säätelemään munien hormonitasoa riippumatta omasta hormonitasoistaan; (iii) onko alkionkehityksen kilpirauhashormonitasolla lyhyt- tai pitkäkestoisia vaikutuksia jälkeläisten ilmiasuun; ja (iv) riippuvatko alkionkehityksen kilpirauhashormonitason vaikutukset poikasiin niiden kasvuympäristöstä. Havaitsin, että (i) jodin saatavuus rajoittaa munantuotantoa joillakin naarailla; (ii) naaraat eivät kykene säätelemään munan hormonitasoa omista hormonitasoistaan riippumattomasti; (ii) alkionkehityksen aikainen kilpirauhashormoni ei näytä aiheuttavan pitkäkestoisia muutoksia jälkeläisten ilmiasuun, ja lyhytkestoiset vaikutukset ovat vaihtelevia eri tutkimuksissa; ja (iv) alkionkehityksen aikainen kilpirauhashormoni ei näytä vaikuttavan poikasten kehitykseen lämpötilasidonnaisesti.

Väitöskirjani valottaa tärkeän, mutta eko-evolutiivisessa kontekstissa vähän tutkitun hormonaalisen signaalin merkitystä äitivaikutusten välittäjänä. Väitöskirjani toimii inspiraationa tuleville tutkimuksille varhaiskehityksen kilpirauhashormonista,

esimerkiksi hormonin adaptiivisesta vaihtelusta ja alkioiden vasteesta tähän äidin signaaliin.

Nederlandse samenvatting

Translation by Martje Birker

Fenotypische plasticiteit wordt gedefinieerd als het vermogen van een genotype om verschillende fenotypen te produceren, afhankelijk van de omgevingsomstandigheden. Fenotypische plasticiteit stelt individuen in staat zich snel aan te passen aan veranderende omgevingen en kan zelfs onderhevig zijn aan natuurlijke selectie. Parental effects zijn een vorm van fenotypische plasticiteit, waarbij het fenotype van de ouders het fenotype van het nageslacht beïnvloedt. Ouderlijke effecten zijn wijdverbreid in veel taxa en kunnen adaptief zijn. Hoewel beide ouders ouderlijke effecten kunnen uitoefenen, heeft de meeste literatuur zich tot nu toe gericht op de invloed van de moeder op het fenotype van het nageslacht, d.w.z. maternale effecten.

Maternale hormonen zijn een mogelijke route voor maternale effecten en hebben de afgelopen 20 jaar steeds meer aandacht gekregen. Tot nu toe heeft het meeste onderzoek naar maternale hormonen zich gericht op steroïde hormonen: androgenen en glucocorticoïden. Er is nu voldoende bewijs dat hormonen van maternale oorsprong het fenotype van nakomelingen kunnen beïnvloeden. Andere hormonen, zoals schildklierhormonen, zijn wijdverbreid over het hoofd gezien in de context van hormoongemedieerde maternale effecten. Schildklierhormonen (TH's) zijn cruciale hormonen die een belangrijke rol spelen bij gewervelde dieren (bijv. embryonale ontwikkeling, juveniele groei, metabolisme en voortplanting) en bij ongewervelde dieren (meestal bij groei en metamorfose). TH-productie door de schildklier bestaat voornamelijk uit T4, het prohormoon, dat vervolgens in de weefsels wordt omgezet in T3, de actieve vorm. T3 wordt als de meest actieve vorm beschouwd omdat het zich met een grotere affiniteit aan TH-receptoren bindt. Jodium is een sleutelcomponent van de TH-productie die niet door een organisme kan worden gesynthetiseerd en daarom in het milieu moet worden aangetroffen. Dit maakt THs interessante kandidaten om te onderzoeken of maternale hormoonafzetting kostbaar kan zijn voor de moeders.

Oviparous soorten zoals vogels zijn goede modellen om maternale hormonen te bestuderen, aangezien embryo's zich ontwikkelen in een afgesloten compartiment en tijdens hun ontwikkeling uitsluitend afhankelijk zijn van maternale signalen. Bovendien maakt het gemakkelijker om dooierhormonen te meten en te manipuleren, onafhankelijk van de fysiologie van de moeder.

In mijn scriptie heb ik mij gericht op het beantwoorden van een aantal vragen over de oorzaken en gevolgen van variatie in maternale TH's in vogeleieren. Ik heb onderzocht of (i) jodium de productie van TH en de overdracht naar de dooier kan beperken; (ii) moeders zijn in staat om dooier-TH-afzetting onafhankelijk van hun circulerende TH's te reguleren; (iii) prenatale TH's kunnen zowel langdurige als kortetermijneffecten hebben op het nageslacht; en (iv) prenatale TH's kunnen contextafhankelijke effecten hebben. Ik ontdekte dat (i) de beschikbaarheid van jodium de eierproductie bij sommige vrouwtjes kan beperken; (ii) vrouwtjes zijn mogelijk niet in staat om dooier-TH-afzetting onafhankelijk van de circulatieniveaus te reguleren; (iii) prenatale TH's lijken geen langetermijneffecten te hebben, terwijl kortetermijneffecten inconsistent lijken met

eerdere vergelijkbare onderzoeken; (iv) prenatale TH's lijken geen interactie te hebben met de omgevingstemperatuur tijdens de nestfase.

Over het algemeen werpt mijn proefschrift licht op een cruciaal hormonaal signaal dat weinig aandacht heeft gekregen in de context van maternale hormonen. Het zou toekomstig onderzoek naar prenatale TH's moeten stimuleren dat essentiële vragen zou aanpakken, zoals de mogelijke adaptieve waarde van variatie in prenatale TH's of de embryonale respons op deze maternale signalering.

Résumé en français

La plasticité phénotypique se définit par la capacité d'un génotype à produire plusieurs phénotypes différents selon les conditions environnementales. La plasticité phénotypique permet aux individus de rapidement s'acclimater à un environnement changeant et peut être soumise à la sélection naturelle. Les effets parentaux sont une forme de plasticité phénotypique dans laquelle le phénotype des parents influence le phénotype de sa progéniture. Les effets parentaux peuvent être adaptifs dans certaines conditions et on les retrouve dans de très nombreux taxons. Même si les deux parents peuvent exercer des effets parentaux, la littérature scientifique s'est principalement concentrée sur l'influence de la mère sur sa progéniture, c'est-à-dire les effets maternels.

Les mères peuvent influencer leur progéniture grâce à la transmission d'hormones, que l'on appelle alors « hormones maternelles ». Ces dernières font l'objet d'un intérêt croissant depuis les 20 dernières années. Jusqu'à présent, la plupart de la recherche sur les hormones maternelles s'est concentrée sur les hormones stéroïdiennes, à savoir les hormones androgènes et les glucocorticoïdes. Il est maintenant clair que ces hormones d'origine maternelle peuvent influencer le phénotype des descendants. D'autres hormones, telles que les hormones thyroïdiennes, ont été largement ignorées dans le contexte des effets maternels. Les hormones thyroïdiennes (HT) remplissent d'importantes fonctions chez les vertébrés (par ex. développement embryonnaire, croissance des jeunes, métabolisme et reproduction) et les invertébrés (principalement croissance et métamorphose). La production d'HT par la thyroïde consiste essentiellement en T_4, la pro-hormone qui est ensuite convertie dans les tissues en T_3, la forme active. T_3 est considérée comme la forme active car elle s'attache aux récepteurs à HT avec une bien plus grande affinité que T_4. La production d'HT nécessite un composant essentiel, l'iode. Cet élément ne peut pas être synthétisé par un individu, qui doit donc le trouver dans son environnement. Cette particularité fait des HT un intéressant candidat pour étudier le potentiel coût que représente le transfert d'hormones pour une mère.

Les espèces ovipares comme les oiseaux sont un bon modèle pour étudier les hormones maternelles. En effet, les embryons se développent dans un compartiment scellé et ne peuvent compter que sur les informations transmises par la mère. De plus, il est relativement facile de mesurer et manipuler les hormones contenues dans les œufs sans interférer avec la physiologie des mères.

Le but de ma thèse est de répondre à plusieurs questions sur les causes et conséquences de la variation d'hormones maternelles thyroïdiennes dans les œufs d'espèces aviaires. J'ai étudié si (i) l'iode peut limiter la production d'HT et leur transfert dans les œufs ; (ii) les mères peuvent réguler le dépôt d'HT dans les œufs indépendamment de leurs concentrations d'HT dans le sang ; (iii) les HT prénatales peuvent avoir des effets à long terme sur la progéniture, ainsi que des effets à court terme ; et (iv) les HT prénatales peuvent avoir des effets contexte-dépendant. J'ai trouvé

que (i) la disponibilité d'iode peut limiter la production d'œufs pour certaines femelles ; (ii) les femelles ne sont peut-être pas capables de réguler le transfert d'HT indépendamment de leur circulation ; (iii) les HT prénatales n'ont pas d'effets à long terme, et les effets à court terme semblent en contradiction avec les résultats d'études similaires précédentes ; (iv) les HT prénatales ne semblent pas interagir avec la température ambiante.

En conclusion, ma thèse met en lumière une hormone essentielle qui a, jusqu'à présent, été peu représentée dans le domaine des hormones maternelles. J'espère que mes résultats encourageront de futures recherches sur les HT prénatales, leur valeur adaptative ou la capacité des embryons à répondre à cette source d'information d'origine maternelle.

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