Increasing plasma thyroxine levels during late embryogenesis and hatching in the chicken are not caused by an increased sensitivity of the thyrotropes to hypothalamic stimulation

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Abstract

The hatching process in the chicken is accompanied by dramatic changes in plasma thyroid hormones. The cause of these changes, though crucial for hatching and the onset of endothermy, is not known. One hypothesis is that the pituitary gland becomes more sensitive to hypothalamic stimulation during this period. We have tested whether the responsiveness of the thyrotropes to hypothalamic stimuli changes throughout the last week of embryonic development and hatching by studying the mRNA expression of receptors involved in the control of the secretory activity of this cell type. We used a real-time PCR set-up to quantify whole pituitary mRNA expression of the β subunit of thyrotrophin (TSH-β), type 1 thyrotrophin-releasing hormone receptor (TRH-R1), corticotrophin-releasing hormone receptors (CRH-R1 and CRH-R2) and somatostatin subtype receptor 2 (SSTR2) on every day of the last week of embryonic development, including the day of hatch and the first day of posthatch life. The thyrotrope-specific expression was investigated by a combination of *in situ* hybridization with immunohistochemistry at selected ages. Although TSH-β mRNA levels increased towards day 19 of incubation (E19), the expression of CRH-R2 and TRH-R1 mRNA by the thyrotropes tended to decrease during this period, suggesting a lower sensitivity of the thyrotropes to the stimulatory factors CRH and TRH. CRH-R1, which is not involved in the control of TSH secretion, increased steadily throughout the period tested. The expression of SSTR2 mRNA by the thyrotropes was low during embryonic development and increased just before hatching. We have concluded that the sensitivity of the pituitary thyrotropes to hypothalamic stimulation decreases throughout the last week of embryonic development, so that the higher expression of TSH-β mRNA around E16–E19, and hence the increasing plasma thyroxine level, is unlikely to be the result of an increased stimulation by either TRH or CRH.

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Introduction

During the last developmental stages of the chicken embryo, when the bird changes from a well-protected environment to a more hazardous life outside the egg, some major physiological changes occur. These include yolk sac retraction, transition from chorioallantoic to pulmonary respiration and functional maturation of the lung, development of endothermy and hatching (Decuypere *et al.* 1990). The onset of breathing starts at the moment the beak penetrates the inner shell membrane delineating the air chamber, a process called internal pipping. Several hours after breathing is established, the chick opens the eggshell (i.e. external pipping). Parallel with these physiological changes, plasma levels of several hormones, and not in the least thyroid hormone (TH) levels, undergo profound changes (Decuypere *et al.* 1990, Kühn *et al.* 1993). The transition from allantoic to pulmonary respiration is characterized by an increase of plasma thyroxine (T_4) levels and an even more dramatic surge of plasma 3,5,3′-tri-iodothyronine (T_3). The underlying cause of augmented TH secretion shortly before hatching is unknown. Some authors have speculated that elevated thyroidal and pituitary sensitivity to thyrotrophin (TSH) and thyrotrophin-releasing hormone (TRH) respectively, coupled with increased thyroid size, is responsible for the changes in T_4 secretion (Scanes *et al.* 1987). However, TRH is not the only factor controlling TSH secretion in birds. Corticotrophin-releasing hormone (CRH) is also a potent stimulator of TSH secretion (Geris *et al.* 1996, 2003*b*), whereas somatostatin (SRIH) was shown to inhibit TSH release at the level of the pituitary (Geris *et al.* 2003*a*, 2003*b*). The inhibition of TSH release by SRIH is thought to be at least partially mediated by the type 2 SRIH receptor (SSTR2) (De Groef *et al.* 2003*a*, Geris *et al.* 2003*a*), while the type 2 CRH receptor

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(CRH-R2) was shown to be responsible for CRH-induced TSH secretion (De Groef *et al.* 2003*b*). The stimulating effects of TRH on thyrotropes are mediated through the type 1 TRH receptor (TRH-R1) (De Groef *et al.* 2003*a*).

Changes in the sensitivity of the thyrotropes to the above-mentioned hypothalamic factors could be part of the underlying causes of the rising plasma T_4 levels during the last week of embryonic development in the chicken. Yet nothing is known about differences in the thyrotropes' responsiveness at different embryonic ages. To test this hypothesis, we have measured plasma hormone concentrations (T_4, T_3 and corticosterone) during the last week of chicken embryonic development and determined whether the responsiveness of the thyrotropes to TRH, CRH or SRIH changes, by quantification of whole pituitary and thyrotrope-specific TRH-R1, CRH-R2 and SSTR2 mRNA expression. Pituitary TSH-β and CRH-R1 mRNA were also measured.

Materials and Methods

All experiments were approved by the Ethical Committee for Animal Experiments of the K.U. Leuven.

Animals, sampling and tissue processing

Fertilized eggs from broiler chickens (first experiment Ross and second experiment Cobb) were purchased from Broeierij Vervaeke (Tielt, Belgium) and Avibel (Halle-Zoersel, Belgium) respectively. The eggs were incubated in a forced-draft laboratory incubator at 37·8 °C with increasing humidity and ventilation from day 14 on, and with continuous lighting. The eggs were turned 45 ° every hour. The start of incubation was called day 1 (E1). Posthatch chicks were kept in a warmed cage with water and food available *ad libitum*.

Blood and tissue samples were taken daily from day 14 of incubation (E14) onwards. On E20, a distinction was made between non-pipping (NP) and internal pipping (IP) embryos. Samples were also taken from newly hatched (C0) and 1-day-old chicks (C1). Blood samples were collected by heart puncture (embryos) or by decapitation (posthatch chicks). Smaller plasma volumes were pooled, resulting in ten to 26 samples per condition. Plasma was stored at − 20 °C until assayed. In the first experiment, five pituitaries were pooled per age on dry ice (three pools per age), rapidly frozen in liquid nitrogen and stored at − 80 °C for RNA isolation. In the second experiment, six pituitaries per age were fixed overnight in 4% paraformaldehyde in PBS at 4 °C, cryoprotected overnight at 4 °C in the same solution containing 30% sucrose, and subsequently stored at − 80 °C until sectioning. Twenty micrometre cryosections of the pituitaries were kept at − 80 °C in a cryoprotectant solution containing 30% (v/v) ethylene glycol and 30% (v/v) glycerol in 0·1 M phosphate buffer.

Radioimmunoassays

Plasma was assayed for T_3, T_4 and corticosterone by RIA. Specifications for the TH RIAs have been described previously (Van der Geyten *et al.* 2001). Corticosterone was determined using a commercial RIA kit (ICN Biomedicals, Costa Mesa, CA, USA) adapted and validated for use in chicken plasma (Darras *et al.* 1996). All hormone values are expressed as means ± S.E.M. Statistical analysis of the data was done by the general linear model of SAS (1985 edition), followed by Scheffé's test.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated from three pituitary pools per age using the RNAgents Total RNA Isolation System (Promega) according to the manufacturer's guidelines. RNA was treated with DNase I using the DNA-Free kit (Ambion, Austin, TX, USA). Approximately 1 µg RNA was heated for 5 min at 72 °C with oligo(dT) primer and then reverse transcribed to cDNA in a volume of 20 µl containing reaction buffer (50 mM Tris–HCl, pH 8·3, 50 mM KCl, 10 mM $MgCl_2$, 0·5 mM spermidine and 10 mM dithiothreitol), 1 mM of each dNTP, ribonuclease inhibitor (10 U) and avian myeloblastosis virus reverse transcriptase (2·5 U) (Roche Diagnostics). The mixture was incubated for 1 h at 42 °C.

Primers for real-time PCR amplification were designed using ABI Prism Primer Express 2·0 software (Applied Biosystems, Warrington, Cheshire, UK); primer sequences and accession numbers are listed in Table 1. All the primers were designed to be intron spanning, except for the SSTR2 primers, as the SSTR2 gene consists of a single exon. The absence of non-specific amplification was confirmed by using the selected primers in a standard PCR protocol and analyzing the PCR amplification products by agarose gel electrophoresis. Each real-time PCR amplification reaction mixture had a final volume of 25 µl and contained either 2 ng (TSH-β, β-actin) or 50 ng (receptors) of cDNA (5 µl), 300 nM forward and reverse primers (0·75 µl each), 1 × SYBR Green PCR Master Mix (Applied Biosystems; 12·5 µl) and 6 µl DNase-free water. Real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). After 2 min at 50 °C and 10 min at 95 °C, samples went through 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A dissociation protocol followed the amplification programme to detect non-specific amplification. Each individual sample was measured in triplicate. For each gene, a non-template control was added as a negative control. The range of the amplification start cycles was 10–17 for β-actin (1/125 dilution), 10–16 for TSH-β

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Table 1 Primers used for real-time PCR analysis and their position within the cDNA

Gene	Primer	**Position** (bp)
TSH-β AF033495	F 5′-CCACCATCTGCGCTGGAT-3′ R 5′-GCCCGGAATCAGTGCTGTT-3′	148–165 275–257
TRH-R1 Y18244	F 5′-GACTGTGAATTCCAAGATGACTAACAAG-3′ R 5′-TTGACAACCACCAGTGTTCGA-3′	735–762 872–852
CRH-R1 L41563	F 5′-CTACGGCGTGCGGTACAAC-3′ R 5′-TCCCGTTAGCGAGGCATTC-3′	368–386 426–408
CRH-R2 AJ557031	F 5′-AGCTGCGGAAATGGGTCTT-3′ R 5′-GCCCACGCAACGATGATT-3′	665–683 731–714
SSTR2 AY029228	F 5′-GGCTTCCTGGTGCCTCTCA-3′ R 5′-GGAGGAGCCCACCCTGAT-3′	14–32 103–86
β-actin L08165	F 5′-ATGGCTCCGGTATGTGCAA-3′ R 5′-TGTCTTTCTGGCCCATACCAA-3′	103–121 222–202

The EMBL accession number is indicated under each gene. F, forward primer; R, reverse primer.

(1/125), 14–18 for TRH-R1 (1/5), 20–23 for CRH-R1 (1/5), 22–26 for CRH-R2 (1/5) and 22–27 for SSTR2 (1/5). The relative efficiencies of the target and reference (β-actin) amplification were checked and the $\Delta\Delta C_T$ method for the relative quantitation was found to be valid. The comparative $\Delta\Delta C_T$ method uses arithmetic formulas for relative quantification of gene expression. Derivation of the formulas and validation tests have been described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). Statistical analysis of data was done by the general linear models of SAS (1985 edition), followed by Scheffé's test.

Combined in situ *hybridization and immunohistochemistry*

Combined *in situ* hybridization and immunohistochemistry was performed as described in detail earlier (De Groef *et al.* 2003*a*,*b*). *In situ* hybridization with ^{35}S-labelled riboprobes was used to visualize TRH-R1, CRH-R2 and SSTR2 mRNA expression. Thyrotropes were immunostained using a homologous anti-TSH-β antiserum (anti-Tb3550, 1:4000) (Iwasawa *et al.* 2002). Slides were analyzed using a Leitz DM RBE microscope equipped with a colour video camera (Optronics Engineering, Goleta, CA, USA) and attached to a computer-aided image analysis system (Bioquant; R and M Biometrics, Nashville, TN, USA). The number of silver grains per area of thyrotropes was determined for 30 cell groups in three different animals as the number of overlaying pixels with a brightness exceeding a predetermined threshold (Arckens *et al.* 1998). As the average area covered by a single thyropic cell (cell size) does not vary amongst the ages tested, the number of pixels overlaying a certain area of thyrotropic cells could be compared between the different ages and is representative for the expression level of the average thyrotrope. Scheffé's test was used to analyze the data obtained statistically.

Results

Plasma hormone levels

In agreement with earlier measurements (Thommes & Hylka 1978, Darras *et al.* 1992, Kühn *et al.* 1993, Gregory *et al.* 1998), plasma T_4 levels rose gradually from E15 until E20 (Fig. 1A). The highest plasma T_4 values were measured around E20. After hatching, T_4 decreased markedly. Plasma T_3 values remained very low until E19–E20/NP and then increased dramatically (Fig. 1B). The IP process and hatching were accompanied by significantly higher T_3 levels. In 1-day-old chicks, plasma T_3 concentrations were still elevated. Plasma corticosterone levels increased fourfold between E14 and E16 and remained high until E20/IP (Fig. 1C). After pipping, corticosterone values dropped to their original values around E14.

Hypophysial TSH and receptor mRNA expression

TSH-β mRNA expression increased significantly between E14 and E19 (Fig. 2A). After E19, TSH-β mRNA levels decreased to reach minimal values around the period of hatching. Whole pituitary TRH-R1 mRNA expression did not change significantly throughout the last week of embryonic development and the perinatal period (Fig. 2B). Combined *in situ* hybridization and immunohistochemistry experiments were carried out in parallel with the RT-PCR analyses to compare the

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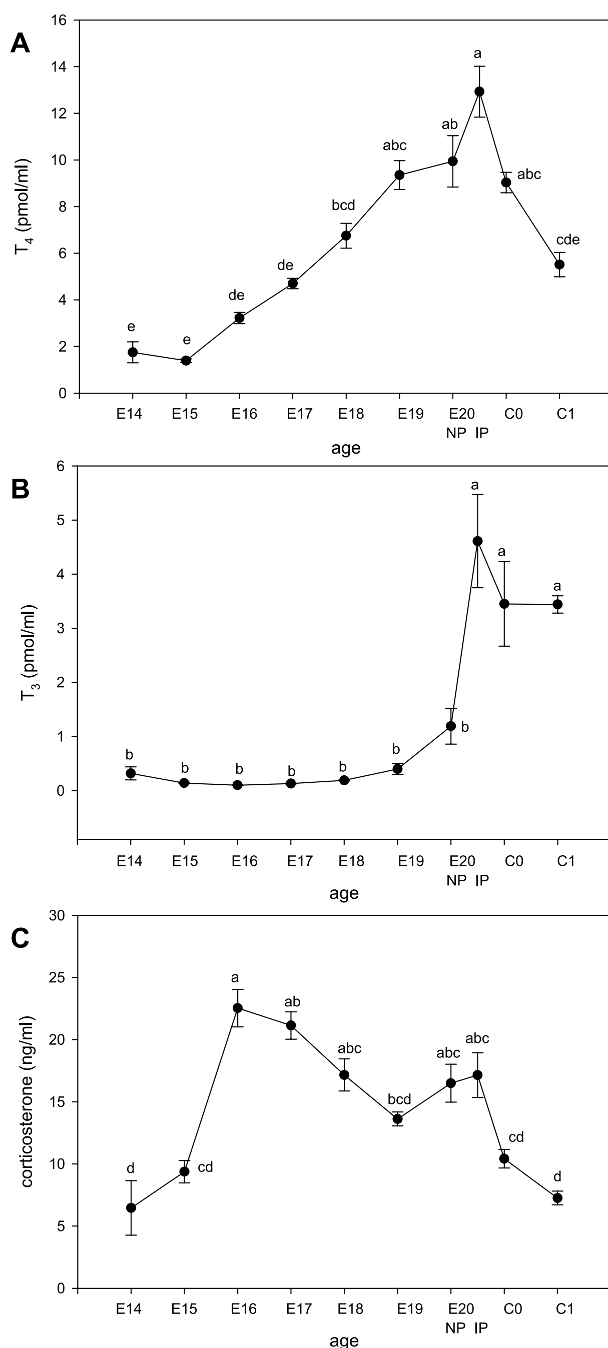

Figure 1 Plasma (A) T_4, (B) T_3 and (C) corticosterone levels during the last week of embryonic development and hatching in the chicken. Data shown are means ± S.E.M. n=10–26. Data without a common letter are significantly different (P<0·05, Scheffé's test).

observed changes in whole pituitary RNA to the effects at the level of the thyrotropes (Table 2). The (non-significant) transient increase in whole pituitary TRH-R1 mRNA that occurred on E19 was not supported by the *in situ* hybridization signal on the thyrotropes. On E19, as well as on C1, TRH-R1 mRNA expression on thyrotropes was significantly lower than on E15 and E16. An example of the result of combined *in situ* hybridization and immunohistochemical staining is given in Fig. 3.

CRH-R1 mRNA levels showed a linear increase between E14 and C1 (Fig. 2C). In addition, pituitary CRH-R2 mRNA levels showed striking changes during the last week of embryonic development in the chicken (Fig. 2D). CRH-R2 expression decreased between E14 and E17, and dropped even lower towards E20/IP. After IP was accomplished, CRH-R2 mRNA levels rose steeply to C1. CRH-R2 mRNA expression on thyrotropes, as determined by *in situ* hybridization, was comparable with whole pituitary changes: CRH-R2 expression was significantly lower on E20/NP compared with E16 and C0, the latter values being similar (Table 2).

Finally, SSTR2 mRNA expression was found to remain low during most of the last week of embryonic development (Fig. 2E). A marked increase in SSTR2 mRNA levels occurred between E19 and E20/IP and levels reached maximal values in the newly hatched and 1-day-old chicks. Using *in situ* hybridization, thyrotropes were found to express little SSTR2 mRNA on E16, but levels had increased significantly in E20/NP and C0 animals (Table 2). The expression levels on days E20/NP and C0 did not differ significantly.

Discussion

The marked changes in pituitary TSH-β and receptor expression during the last week of chicken embryonic development and hatching are likely to be correlated – consequentially and/or causatively – to the concomitant changes in plasma hormones. Gregory and colleagues (1998) reported low TSH-β mRNA amounts up to E19. The present study showed a slightly different pattern, with TSH-β mRNA levels being elevated in the period between E16 and E19. Serum T_4 levels were similar, but temporally lagging behind those of pituitary TSH-β mRNA, supporting a role for TSH in stimulating the increase in T_4 production and secretion towards hatching. This assumption is further supported by the ontogenic changes of chicken thyrotrope number, which increases gradually to reach a maximum around E18–E20 (Nakamura *et al.* 2004, Muchow *et al.* 2005).

Hypophysial CRH-R2 mRNA expression showed a reverse relationship with plasma T_4 concentrations, suggesting that the expression of CRH-R2 mRNA is under the inhibitory control of T_4. CRH-R2 is expressed by the thyrotropes in the chicken pituitary gland and is responsible for the mediation of CRH-induced TSH release (De Groef *et al.* 2003*b*). The control of CRH-R2 expression by T_4 might therefore represent a negative feedback mechanism to regulate TSH secretion. CRH-R1 mRNA levels,

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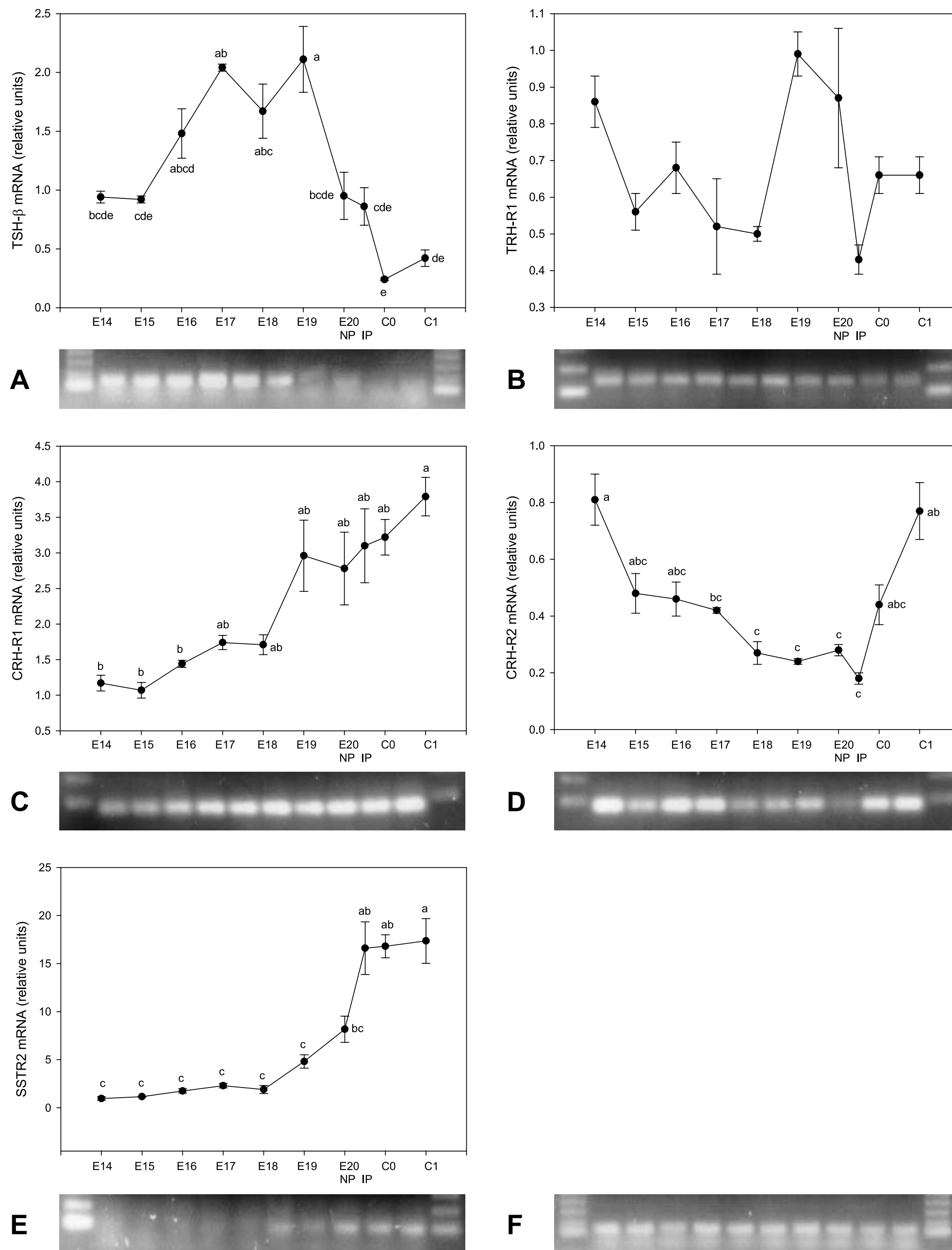

Figure 2 Whole pituitary expression of (A) TSH-β mRNA, (B) TRH-R1 mRNA, (C) CRH-R1 mRNA, (D) CRH-R2 mRNA (E) and SSTR2 mRNA. Relative units were calculated using the $\Delta\Delta C_T$ method with β-actin mRNA as a reference. Data shown are average values ± S.E.M. $n=3$ pools of five pituitaries. Data without a common letter are significantly different ($P<0{\cdot}05$, Scheffé's test). No statistical differences were found between the TRH-R1 mRNA values (B). Under each graph is a photograph showing the equivalent result of a standard PCR reaction using the same primers and amplification temperatures as in the real-time analysis (20–30 cycles). On the left and right of each photograph is a DNA marker the lowest band of which has a length of 100 bp. (F) β-actin (control gene).

on the other hand, increased gradually and showed no correlation with the components of the thyroidal axis. CRH-R1 mRNA expression is indeed confined to corticotropes mediating the stimulatory effect of CRH on adrenocorticotrophin (ACTH) synthesis and/or secretion (De Groef *et al.* 2003*a*,*b*). Ontogenic changes in TSH-β

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Table 2 Quantification of silver grains (*in situ* hybridization signal) over thyrotropes at selected ages

	Age	Pixels/1000 μm^2
TRH-R1	E15	540 ± 32^a
	E16	667 ± 42^a
	E19	233 ± 27^c
	C1	370 ± 33^b
CRH-R2	E16	273 ± 23^a
	E20/NP	63 ± 15^b
	C0	226 ± 26^a
SSTR2	E16	75 ± 11^b
	E20/NP	205 ± 20^a
	C0	272 ± 24^a

Data are expressed as pixels per 1000 μm^2 of thyrotrope. Values are means ± S.E.M. n=30 cell groups in three different embryos. For a given receptor, data with a common letter are not significantly different (P<0·05; Sheffé's test). Values can only be compared with the same type of receptor.

and ACTH secretion have not been measured yet because of the lack of sufficiently sensitive antibodies.

Pituitary SSTR2 mRNA expression was found to increase dramatically prior to hatching. SRIH is an inhibitor of both TRH- and CRH-induced TSH secretion in the chicken (Geris *et al.* 2003*a*, 2003*b*). The increased SSTR2 mRNA expression as well as elevated circulating T_3 levels near the end of embryonic development might be responsible for the coinciding decrease in TSH-β mRNA levels. However, the *in situ* hybridization experiment also hints that the increase in SSTR2 mRNA expression is not merely due to an increased expression by the thyrotropes alone. Somatotropes, in particular, might be largely responsible for this phenomenon, as this cell type has been shown to express SSTR2 as well (De Groef *et al.* 2003*a*). Differentiation of functional chicken somatotropes occurs between E14 and E16 (Porter *et al.* 1995). Between E20 and C1, when whole pituitary SSTR2 mRNA expression suddenly rises, the number of

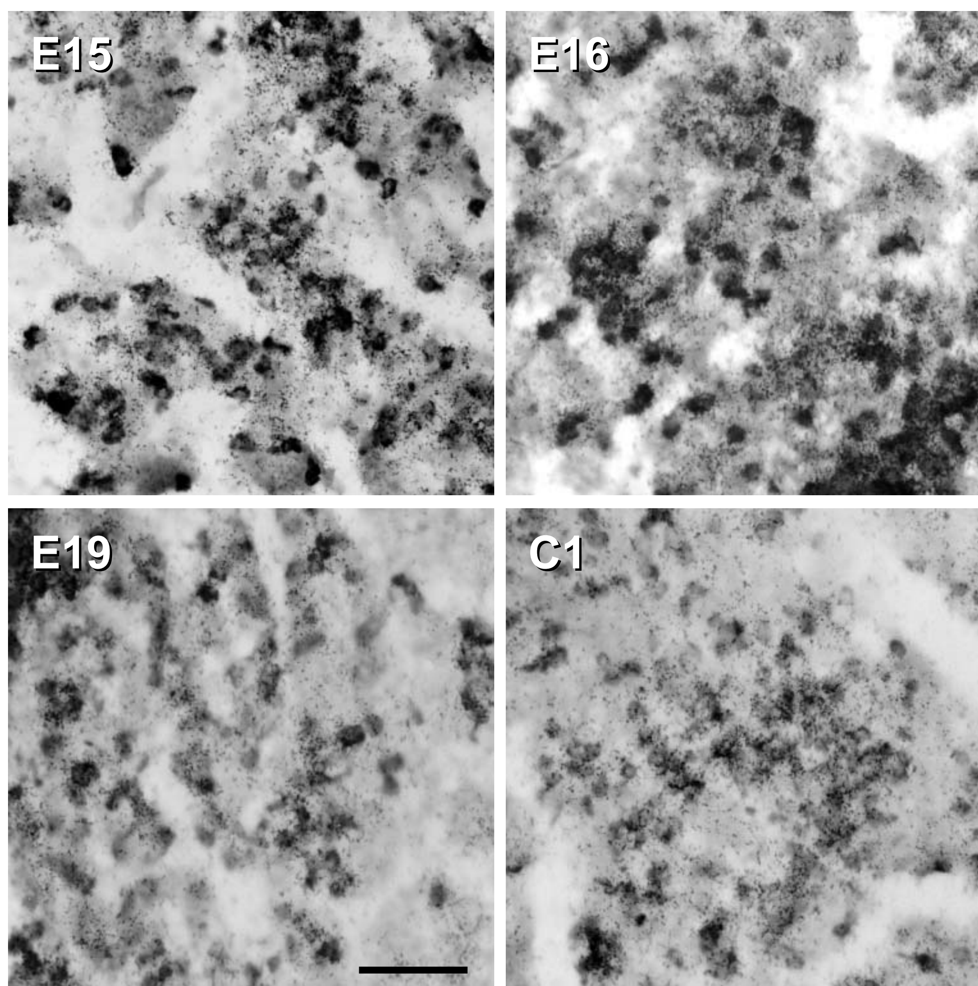

Figure 3 Results of combined *in situ* hybridization (TRH-R1 mRNA) and immunohistochemistry (TSH-β) on pituitaries from chicken embryos (E15, E16, E19) and 1-day-old chicks (C1). Bar=0·05 mm.

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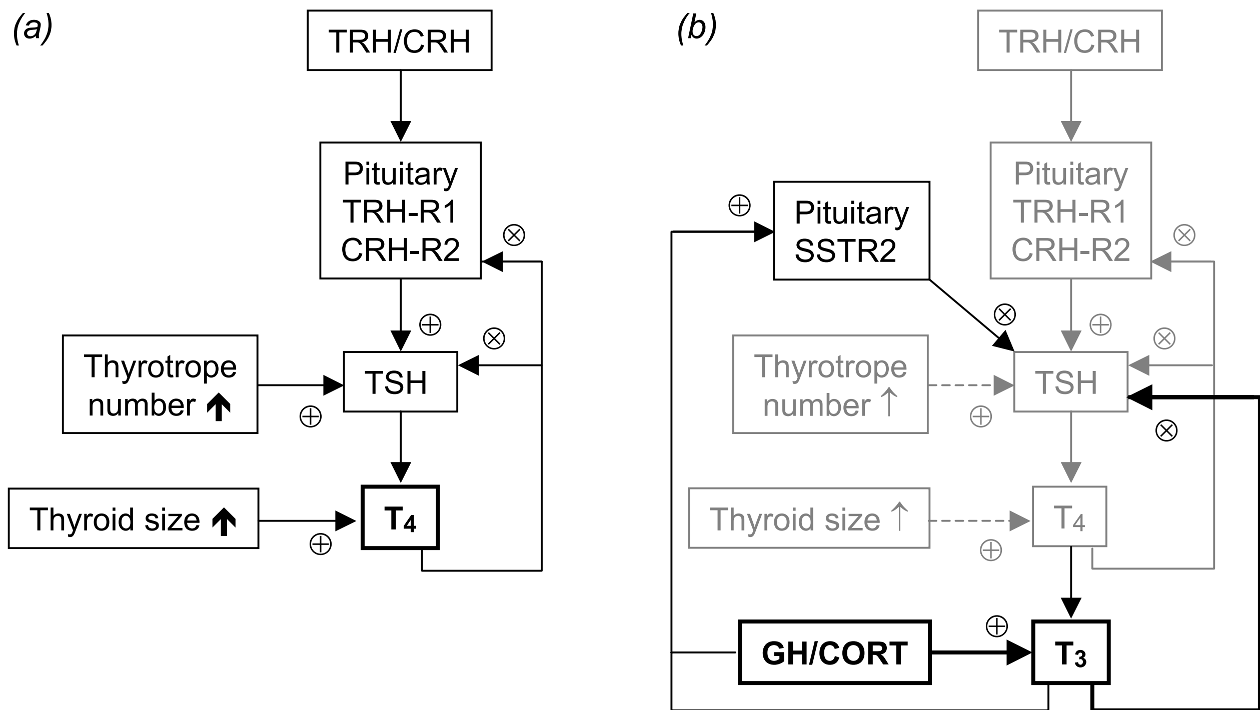

Figure 4 Hypothetical scheme of some of the interactions controlling the changes in the thyroidal axis *(a)* before and *(b)* around pipping. GH and corticosterone (CORT) increase plasma T_3 levels by decreasing hepatic T_3 inactivation through type III iodothyronine deiodinase (Darras *et al.* 1992, 1996). ⊕, stimulation and ⊗, inhibition. Dashed lines represent presumably decreasing influences.

somatotropes as well as plasma growth hormone (GH) levels increase enormously (Darras *et al.* 1992, Malamed *et al.* 1993, Geris *et al.* 1998). While GH-releasing hormone is thought to be the primary regulator of GH secretion around the time that somatotropes differentiate, TRH may assume a greater regulatory role as development proceeds (Dean *et al.* 1997). Likewise, the slightly higher whole pituitary content of TRH-R1 mRNA on E19, coinciding with low TRH-R1 mRNA expression by the thyrotropes, presumably represents an increased expression in other cell types such as somatotropes or lactotropes. A possible scheme explaining the present findings is shown in Fig. 4.

The question remains as to what triggers the increased thyroidal activity during the last week of chicken embryonic development. An increasing sensitivity of the thyroid gland to TSH stimulation can be excluded, since the expression of thyroidal TSH receptor mRNA decreases between E14 and E15 and remains low until C1 (S V H Grommen, S Taninchi, T Janssen, S Takeuchi, S Takahashi, V M Darras & B de Groef, unpublished results). A lack of a negative feedback inhibition within the thyroidal axis during the embryonic period does not seem likely, since Muchow and colleagues (2005) showed that pituitary thyrotropes are under a tonic inhibition by endogenous THs prior to hatching. These authors have suggested hypothalamic feed-forward stimulation as the predominant cause of increased thyroidal axis activity at the end of embryonic development. In the present study, the sensitivity of the thyrotropes to CRH and TRH, as evidenced by CRH-R2 and TRH-R1 mRNA expression respectively, decreased rather than increased during the last week of embryonic development, which might be due to a negative feedback effect exerted by the increasing T_4 levels. However, this could be compensated for by an increased secretion of hypothalamic-releasing factors. The decreasing CRH content of the median eminence towards E20 is suggestive of such an increased CRH secretion (Vandenborne *et al.* 2005). Other factors worth considering are the increasing number of thyrotropes in the pituitary (Nakamura *et al.* 2004, Muchow *et al.* 2005) and the increasing size of the thyroid gland. Finally, it is highly probable that the observed changes are also related to changes in the expression and activity of other components mediating TH action, such as iodothyronine deiodinases, TH transporters, TH receptors and cofactors.

To summarize, using a real-time PCR approach, combined with *in situ* hybridization, we have shown that the expression of receptors for hypothalamic factors by the thyrotropes changes significantly during the last week of embryonic development and hatching in the chicken. The rising plasma T_4 concentrations seem to exert a negative feedback effect on CRH-R2 and possibly TRH-R1 mRNA expression by the thyrotropes. Despite this lower

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thyrotrope sensitivity to hypothalamic stimulation, hypophysial TSH-β mRNA levels increase towards E19 and decrease considerably during the pipping and hatching process. The spectacular surge in SSTR2 mRNA expression near the end of the incubation period might be largely caused by the increasing number of somatotropes, but may also contribute to the decreasing TSH-β mRNA levels in this stage of development.

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