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Original article

Effect of the sex-linked dwarf gene on thyrotrophic and somatotrophic axes in the chick embryo

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Summary — Plasma concentrations of thyroxine (T4), triiodothyronine (T3), reversed triiodothyronine (rT3), and insulin-like growth factors I and II (IGF-I, IGF-II) together with peripheral 5'-monodeiodination activity were measured in both normal and sex-linked dwarf embryos between day 14 of incubation and day 1 posthatch. Plasma T4 levels increased gradually during embryonic development while T3 concentrations remained low until day 20, when a sharp increase was observed. rT3 levels also increased from day 14 and dropped on day 20 when T3 levels started to increase. 5'-monodeiodination activity was high on day 14 of incubation, decreased thereafter, and showed an increase at the time of air sac penetration together with increased T3 levels. At this stage, differences between normal and dwarf embryos were observed; the latter had lower nonsignificant 5'-Monodeiodination activity and lower ($P < 0.01$) plasma T3 levels. Plasma IGF-II levels were high during the whole embryonic period studied. Dwarf embryos had lower ($P < 0.05$) IGF-II levels at the time of hatching. IGF-I levels were high on days 14 and 16, declined afterwards, and started to increase again around hatching. With the exception of T3 and IGF-II levels, introduction of the dwarf gene did not cause major changes in the hormonal parameters studied. This may explain the identical body weight at hatching.

chicken — dwarf — embryo — thyroid — IGF-I — IGF-II

Résumé — Effet du gène de nanisme lié au sexe sur l'axe thyrotrope et l'axe somatotrope de l'embryon de poulet. *Les concentrations plasmatiques de la thyroxine (T4), de la triiodothyronine (T3), de la «triiodothyronine reverse» (rT3) et des facteurs de croissance «insulin-like growth factor» I et II (IGF-I et IGF-II) sont estimées ainsi que la 5'-monodeiodination périphérique (5'-D), chez des embryons de poulet nains et normaux. Les mesures commencent le 14e jour de l'incubation et continuent jusqu'au jour suivant l'éclosion. Au cours du développement embryonnaire, la concentration de la T4 augmente graduellement. Celle de la T3 reste basse jusqu'au 20e jour. Une augmentation rapide est alors observée. Le taux de la rT3 augmente graduellement du 14e jour au 18e jour. On constate au 20e jour une diminution transitoire qui va de pair avec l'augmentation de la concentration de la T3. L'activité 5'-D est élevée le 14e jour de l'incubation, elle diminue ensuite et se maintient à un niveau faible entre le 16e et le 20e jour. Elle augmente de nouveau au moment de la pénétration de la chambre à air. Ce phénomène est suivi d'un accroissement du taux de la T3. Des différences entre les embryons nains et normaux sont*

constatées à ce stade, l'activité de la 5'-D (non significative) et la concentration en T3 (P < 0,01) sont plus basses chez les embryons nains que chez les normaux. Pendant toute la période embryonnaire, la concentration de l'IGF-II est élevée. Au moment de l'éclosion, les embryons nains ont une concentration de l'IGF-II plus basse (P < 0,05). Aux 14e et 16e jours, la concentration de l'IGF-I est élevée, elle diminue au cours des jours suivants et passe par un minimum avant l'éclosion. L'introduction du gène de nanisme n'a pas d'effets spectaculaires sur les paramètres hormonaux suivis lors du développement embryonnaire, à l'exception de la concentration en T3 et en IGF-II vers la fin de l'incubation, ce qui peut expliquer le poids corporel identique au moment de l'éclosion.

poulet — nanisme — thyroïde — IGF-I — IGF-II

Introduction

The effect of the sex-linked dwarf gene on growth and hormonal parameters controlling growth has been studied in detail in chickens. During the fast growth period (1—7 wk), depressed levels of triiodothyronine (T3) and insulin-like growth factor I (IGF-I) have been reported (Scanes *et al.*, 1983; Huybrechts *et al.*, 1986, 1987), together with normal or elevated levels of growth hormone (GH). However, no attention has been paid to the effects of the dwarf gene on hormonal parameters during embryonic development.

In the avian embryo, hormonal parameters change dramatically during incubation (for review see Scanes *et al.*, 1987). Plasma concentrations of thyroxine (T4) rise exponentially between days 8 and 20 of incubation (Decuypere *et al.*, 1979; Thommes and Hylka, 1977; Gaspard *et al.*, 1981), while plasma triiodothyronine (T3) levels remain low until day 19 of incubation, when they increase rapidly until hatching (Thommes and Hylka, 1977; Decuypere *et al.*, 1981). At the same time, reversed triiodothyronine (rT3) levels are high on day 17 and decline thereafter to reach minimal levels at hatching (Hylka *et al.*, 1986).

GH levels, on the other hand, are not detectable until day 17 of incubation (Harvey *et al.*, 1979) and their role in embryonic growth is therefore not well understood. Since it is considered that GH exerts an effect on growth through insulin-like growth factors (IGF), it was more interesting to look at IGF levels during embryonic development. Gaspard *et al.* (1981) could indeed detect IGF activity between days 13 and 17 of incubation.

In view of the importance of thyroid hormones, GH, and IGF in controlling growth, it seemed important to follow changes in these parameters during embryonic development in both normal and dwarf embryos. Special attention was given to changes in both IGF-I and insulin-like growth factor II (IGF-II) since reports of these growth factors in chick embryos are rare. Also the change in 5'-monodeiodination (5'-D) that occurred on day 20 were more closely examined. Both T3 and liver 5'-D activity increase rapidly at that stage of development, while 5'-D activity is depressed in dwarfs (Scanes *et al.*, 1983) and cannot be stimulated by GH or prolactin (Kühn *et al.*, 1986).

Materials and Methods

All the animals came from mating between heterozygous males and dwarf dams of a medium—heavy laying strain kindly provided by Dr Mérat (INRA, France). To distinguish dwarf and normal embryos, a marker for the dwarf

gene, the S gene for feather color, was introduced; this resulted in brown-feathered dwarf and white-feathered normal embryos. The gene did not interfere with the hormonal parameters studied, as concluded in a preliminary growth experiment. The eggs were incubated at 37.5°C in a forced-draught incubator with constant light and a relative humidity of 50%.

With heparin as anticoagulant, blood samples were taken by cardiac puncture on days 14, 16, 18, 19, 20, and 21 of incubation and by decapitation day 1 posthatch. Additional embryos were taken on days 16, 20, and 21 for the determination of plasma IGF-II levels. In a second experiment, the changes occurring on day 20 were studied in more detail. For this purpose, eggs were taken at regular intervals during day 20 of incubation and divided into two groups, one with air chamber penetration (20 ip) and the other in which this had not yet started (20 np). On day 21, only eggs with signs of external pipping (21 ep) were used. The plasma was separated and stored frozen at –20°C until assay. In both experiments, the livers were removed and frozen for measuring 5'-D activity.

Plasma T4 levels were measured by means of a commercial kit (Abbott Diagnostic Division) with intra- and interassay variabilities of 3.2% and 3.3% respectively. Plasma rT3 was measured with an RIA-mat rT3 kit (Mallinckrodt Diagnostica) with an intra-assay variability of 7% and an interassay variability of 10%. For the determination of T3, a commercially available T3 antiserum (Mallinckrodt Diagnostica), at a dilution of 1/12500 (40 µl), was added to 40 µl of plasma and 50 µl of tracer (10 000 cpm, Amersham IM 321). After incubation overnight, 50 µl of a 1% bovine gamma globulin solution and 250 µl of 20% PEG were added. This mixture was allowed to stand at room temperature a further 30 min before the tubes were centrifuged and the pellets counted. The intra-assay variability for this assay was 2.9%.

Plasma IGF-I levels were measured in acid-extracted plasma samples by use of a heterologous radioimmunoassay (Huybrechts *et al.*, 1985a). The antiserum (UBK 487) directed against human IGF-I was kindly provided by the National Institute of Diabetes, Digestive and Kidney Diseases (Bethesda, MD). This antiserum had 0.5% cross-reactivity with IGF-II and a minimal cross-reactivity with insulin at 10^{-6} molar. Intra- and interassay variabilities of acid-treated samples were 6.5% and 15%, respectively. IGF-II levels were determined by means of a heterologous radioimmunoassay (Buonomo, 1987) with an intra-assay variation of 4.9% and an interassay variation of 7.6%. Prior to assay, all samples were diluted (1:5 vol/vol) with formic acid/EtOH (12.5%:87.5% vol/vol) and incubated for 30 min at room temperature. After centrifugation at 10 000 x *g* for 5 min, a sample of the supernatant was removed and lyophilized. Recovery determined by measuring the quantity of [^{125}I]-labeled IGF-II remaining in a control sample before and after the extraction procedure, was approximately 96%. All the samples were then reconstituted in assay buffer (0.03% M sodium phosphate, 0.01 M EDTA, 0.02% protamine sulfate, 0.05% Tween 20, pH 8.0) and analyzed at a final dilution of 1:10. All the samples were assayed in duplicate for IGF-II concentration with recombinant bovine IGF-II (>97% pure; Monsanto Co.) as the standard and iodinated tracer. IGF-II was iodinated using lactoperoxidase. The antiserum used was a monoclonal antibody raised against human IGF-II (Amano Intl. Ez. Co.). This antibody did not cross-react with recombinant human IGF-I (Algen), bovine IGF-I (Monsanto), or with either bovine (Sigma) or chicken (Litron Laboratories) insulin at levels more than 100-fold in excess. Serial dilution of acid—ethanol-extracted chicken sera collected from normal chicks was parallel to the bovine IGF-II standard curve, and recovery standard in the presence of the equivalent of 1 µl of extracted chicken sera was 101%.

Monodeiodination activity was measured according to the method of Visser *et al.* (1979) with some modifications. Livers (0.5 g) were homogenized in 1 ml of a 0.15 M phosphate buffer (pH 6.5). After centrifugation, 50 µl of the supernatant were incubated at 37°C with 10 µl of T4 (4 µM) and 200 µl of dithiothreitol (DTT, 3 mM) for 1 h. To stop the reaction, the tubes were transferred to an ice bath and 1 ml of ice-cold Brij 35 (0.625%, Sigma) was added to each tube. The T3 produced per hour was measured with the T3 RIA as described before but with the standard solutions diluted in the same Brij 35 solution as the samples. Protein content of the supernatant was measured with Coomassie brilliant blue G-250 as an indicator and bovine serum albumin as standard in order to express the 5'-D activity as nanograms of T3 produced per milligram protein per hour.

All the results were expressed as the mean ± SEM with the number of animals given in the figures. Statistical analyses were done by

analysis of variance (ANOVA) followed by the least significant difference test when F was significant (Snedecor and Cochran, 1967).

Results

Plasma T4 levels gradually increased from day 14 to day 19 of incubation in both genotypes (Fig. 1a). On day 20 plasma concentrations were somewhat lower: 68% and 64% of day 19 levels for normal and dwarf embryos, respectively. At the moment of external pipping, the levels increased again. Throughout embryonic development no difference in T4 levels was found between the two genotypes. Plasma T3 levels remained low until day 19 when a first increase was detected (Fig. 1b). The major increase in T3 occurred on day 20 and was found only in birds that had already penetrated the air chamber (20 ip). At this stage, a difference between the two genotypes was found, with the dwarfs having a delayed increase in plasma T3 concentration ($P < 0.01$). Less than 24 h later, at the time of hatching, the levels were again not statistically different.

The increase in plasma T3 levels at the end of incubation coincided with a rise in 5'-D activity in normal birds (Fig. 1c; Table I). No significant differences were found between the two genotypes, although results from the second experiment (Table I) showed a trend towards an increase in 5'-D activity in normal birds. Differences between the two experiments (Fig. 1c *vs.* Table I) as to the T3 produced were caused by determining the enzyme activity in the livers of two different sets of animals and in two different *in vitro* incubations, but both showed the same general increase in 20 ip normal birds. Surprisingly high levels of 5'-D activity were found on day 14 of incubation in both genotypes. At that moment, this high 5'-D activity was not reflected in high plasma T3 levels.

Plasma rT3 levels increased from day 14 to day 18 of incubation, then dropped until day 20 and peaked again at hatching (Fig. 1d). The drop on days 19 and 20 coincided with the rise in both plasma T3 and liver 5'-D activity. After hatching levels decreased and this process was faster in normal than in the dwarf birds ($P < 0.05$). No difference between the genotypes was found in rT3 levels during embryonic development.

Plasma IGF-I levels were high on days 14 and 16 of incubation in both genotypes, then gradually declined to reach minimal values on day 20 (Fig. 1e). During hatching, IGF-I concentrations

Table I. 5'D activity in livers of normal (Dw) or dwarf (dw) embryos on day 20 of incubation with (ip) or without (np) penetration of the air chamber.

	np	*ip*
Dw	2.91 ± 0.27 (33)	4.84 ± 0.49 (26) *
dw	3.09 ± 0.29 (30)	3.65 ± 0.36 (23)

Results are expressed as ng T3/mg protein produced per hour in an *in vitro* incubation mean ± SEM *(N)*.
* $P < 0.001$ (ANOVA within genotype).

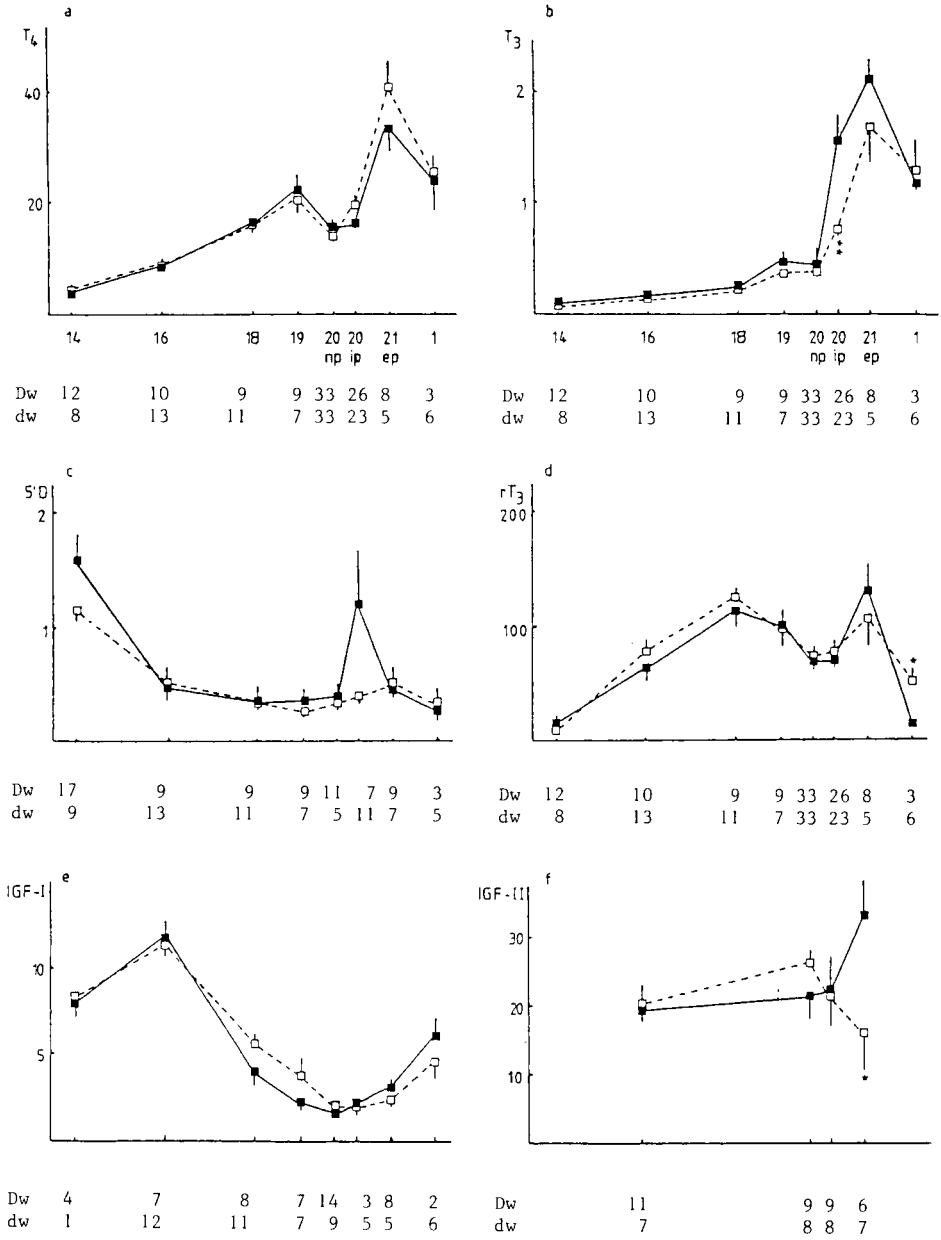

Fig. 1. Plasma T4 (**a,** ng/ml), T3 (**b,** ng/ml), rT3 (**d,** pg/ml), IGF-I (**e,** ng/ml), and IGF-II (**f,** ng/ml) levels and 5'-monodeiodination activity (**c,** ng T3/h/mg protein) during embryonic development in normal (■—■) and dwarf (□—□) chicks.
* $P < 0.05$; ** $P < 0.01$ (ANOVA between genotypes). Number of animals are given under each figure.

increased again. At the same time, IGF-II levels were constant between days 16 and 20 (Fig. 1f). A difference in IGF-II levels between the two genotypes occurred only at hatching, with the levels lower in the dwarf birds ($P < 0.05$). IGF-II levels were high in comparison with IGF-I levels found during incubation.

Discussion

The data on T4 and T3 concentrations are in agreement with previous observations (Thommes and Hylka, 1977, Decuypere *et al.*, 1979; Scanes *et al.*, 1987). The drop in T4 levels on day 20 was also noted by Decuypere *et al.* (1982), although it occurred somewhat later between internal and external pipping, and was also found in the recalculated values presented by Scanes *et al.* (1987). The rise in plasma T3 levels on day 20 has also been described previously (Scanes *et al.*, 1987); it coincided with a clear drop in rT3 levels at that time. Similar observations were made by Hylka *et al.* (1986), although they did not find a peak at hatching, but rather a gradual increase in plasma rT3 levels during the first 3 to 4 wk posthatch; this is in contrast with the results of Premachandra *et al.* (1977).

The effects on plasma thyroid hormone levels observed on day 20, when the air chamber was penetrated, are related to changes in 5'-D activity (Decuypere *et al.*, 1982). During this period, increased 5'-D activity was found; this is in agreement with the results of Borges *et al.* (1980) and of Galton and Hiebert (1987), who also found increased 5'-D activity towards the end of incubation. On day 14 of incubation, 5'-D activity was high although plasma T3 levels remained low. This might indicate a high enzyme activity because of low substrate (T4) availability or high T3 consumption (perhaps intracellular) at that moment of development; this could be related to the high IGF-I levels observed at the same period since a relationship between T3 and IGF-I levels was proposed earlier (Binoux *et al.*, 1985; Huybrechts *et al.*, 1988). The 5'-D activity to produce rT3 seemed to start only after day 14 since rT3 levels increased only after that day. The high 5'D activity on day 14 was also observed by Darras (personal communication) in a commercial laying strain (Hisex, Euribrid) but not by Borges *et al.* (1980), who found a gradual increase in 5'-D activity towards the end of incubation.

Plasma levels of IGF-I were high between days 14 and 16 of incubation. This is in agreement with the results of Gaspard *et al.* (1981) who found bioassayable IGF activity only between days 13 and 17. During the same period and even earlier, high concentrations of type I IGF-I receptors were found in chick embryo brain (Bassas *et al.*, 1985), indicating that high plasma levels can exert growth-promoting activity. The rise in plasma IGF-I levels around hatching is in agreement with previous results (Scanes *et al.*, 1987) and can be linked to an increase in GH levels at that moment (Harvey *et al.*, 1979; Huybrechts *et al.*, 1985b). Plasma IGF-II levels were high in comparison with IGF-I and did not change during the embryonic period studied. This might reflect another mechanism controlling the synthesis and the release of IGF-II in comparison with IGF-I. In mammals, placental GH has been put forward as the control mechanism for placental IGF-II production (Caufriez *et al.*, 1987). The high levels of IGF-II could be related to their importance in neonatal life since IGF-II is considered to be important at that

stage in mammals (Adams *et al.,* 1983). Evidence that IGF-I and II promote growth at that early stage can be found in the occurrence of collagen synthesis (Gaspard *et al.,* 1981) and the increase in fibroblast proliferation (Haselbacher *et al.,* 1980) during the same period.

The introduction of the sex-linked dwarf gene had only minor effects on the hormonal parameters studied during embryonic development in comparison with the effects observed posthatch. On day 20 a delay in the rise in plasma T3 levels was found and this delay is reflected in the 5'-D activity at that moment, which tended to be lower in dwarf embryos. It is interesting to note that Kühn *et al.* (1986) observed a lack of stimulation of 5'-D activity in dwarf embryos with either GH or prolactin. The delay observed in the present work could also be explained by the same phenomenon since this increase in both the 5'-D activity and the plasma T3 levels occurred at the time when the GH levels started to increase (Scanes *et al.,* 1987). Considering that diminished hepatic GH binding has been observed in growing (Leung *et al.,* 1987) and adult (A. Vanderpooten, personal communication) dwarf chickens, both the delayed increase on day 20 and the lack of reponse after GH stimulation might be explained by this lower number of hepatic GH receptors. In dwarf embryos, IGF-II levels were significantly lower at the moment of hatching. To our knowledge, no data are yet available confirming that this difference lasts during posthatch growth.

It is clear that the effect of the dwarf gene was not expressed profoundly during embryonic development, which could explain the identical body weight at hatching. Major effects of the dwarf gene only became evident around or after hatching, and it is possible that the increase in GH levels which then occurred was not recognized by dwarf birds because of their lowered hepatic GH binding. This in turn would cause the observed differences in 5'-D activity and IGF-I and IGF-II levels after hatching.

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