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Research Article

Hepatic mRNA expression and plasma levels of insulin-like growth factor-I (IGF-I) in broiler chickens selected for different growth rates

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Abstract

The hepatic expression and plasma concentrations of IGF-I were investigated in three broiler chicken strains selected for different growth rates (HP-Hubbard-Pettersen, a fast growing strain; NN-Naked-neck, a strain with an intermediate growth rate and a heterozygous genotype, and C-Caipira, a slow growing crossbred strain). The chickens were studied at 1, 21 and 42 days of age and had free access to food throughout the study. Hepatic IGF-I mRNA expression was assessed by dot blot analysis using a randomly labeled chicken IGF-I cDNA as the probe and plasma IGF-I concentrations were assayed by radioimmunoassay. The hepatic levels of IGF-I mRNA increased from 1 to 21 days of age in all strains, with NN chickens showing a higher ($p < 0.05$) IGF-I expression than the other strains. Plasma IGF-I concentrations increased ($p < 0.05$) with broiler chicken age, but there were no significant differences among the strains. These results indicate that despite differences in the growth rates among the strains, the changes in the expression of IGF-I mRNA in liver and in the plasma levels of IGF-I were independent of broiler chicken strain, but varied with chicken age.

Key words: IGF-I mRNA, broiler chicken, growth performance, plasma IGF-I.

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Introduction

Insulin-like growth factor-I (IGF-I) is a highly conserved, 70 amino acid, single-chain polypeptide that plays an important role in the control of growth and metabolism in chickens and mammals (Dawe *et al.*, 1988; Florini *et al.*, 1996). Growth and differentiation are stimulated when exogenous IGF-I is injected into chicken embryos (Girbau *et al.*, 1987), but after hatching, the gene expression and plasma concentration of IGF-I increase with age and then decline between 6 and 7 weeks of age (Huybrechts *et al.*, 1985; Johnson *et al.*, 1990; McGuinness and Cogburn, 1990). The plasma levels of IGF-I vary with the nutritional state since chickens fed a low protein diet have a low plasma content of this polypeptide (Rosebrough *et al.*, 1992; Rosebrough and McMurtry, 1993).

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Although much is known about the mechanisms involved in the synthesis and secretion of GH and its regulatory effect on IGF-I production, various aspects about the role of GH and its interactions with its receptors require clarification, especially in recently improved broiler chicken strains selected for fast growth or food conversion. Contradictory data have been reported about the positive correlation between IGF-I plasma levels and growth rate. Scanes *et al.* (1989) reported higher IGF-I plasma levels in strains selected for heavy body weight and Beccavin *et al.* (2001) found an association between high growth rates and higher levels of hepatic IGF-I mRNA and circulating IGF-I. However, Goddard *et al.* (1988) and Leenstra *et al.* (1991) found no difference in plasma IGF-I levels in strains selected for food conversion or weight gain.

In this work, we examined the hypothesis that broiler chickens selected for fast growth have higher liver expression of IGF-I mRNA and a higher plasma concentration of this polypeptide during growth.

Material and Methods

Selection, housing and management of chickens

Six hundred one-day-old male and female broiler chicks from three strains (200 birds per strain) were used. The strains were: Hubbard-Pettersen (HP), a commercial strain selected for fast growth, Naked-neck (NN), a strain with the heterozygous genotype *Nana* selected for medium weight gain and Caipira (C), a native crossbred strain with a very low growth rate.

The birds were housed on the floor in separate boxes at a temperature close to thermoneutrality for each age interval. Thus, the initial temperature was 33 °C and decreased at a rate of 2 °C per week to 23 °C at 35 days of age. The birds were fed *ad libitum* with diets containing 3,100 kcal of metabolizable energy (ME)/kg that consisted of corn and soybean meal with a vitamin-mineral supplement (Table 1). From 1-21 and 22-42 days of age, the birds were fed diets containing 21.8% and 19.8% of crude protein (CP), respectively.

The growth curve for each strain was determined by weighing the birds at 1, 7, 21, 35 and 42 days of age. Food intake was also measured, and food conversion was calculated for each strain for the initial (1-21 days), final (22-42 days) and total (1-42 days) periods.

Liver sampling

To assess IGF-I mRNA expression, three males of each strain were sacrificed by cervical dislocation at 1, 21 and 42 days of age and 1 gram of liver was quickly collected and frozen in liquid nitrogen. The samples were stored at -80 °C until total RNA isolation.

Table 1 - Composition (in %) of the diets used.

Ingredients	Starting diet (1-21 days of age)	Final diet (22-42 days of age)
Corn	51.28	58.20
Soybean meal	38.35	32.63
Soybean oil	5.37	4.17
Vitamin-mineral mix[1]	5.00	5.00
Total	100.00	100.00
Calculated values:		
Crude protein (g)	21.85	19.83
Metabolizable energy (kcal/kg)	3099.19	3099.38

[1]Vitamin and mineral mix/kg: vitamin A 176,000IU, vitamin D3 40,000IU, vitamin E 500 mg, vitamin K3 100 mg, vitamin B1 36 mg, vitamin B2 200 mg, vitamin B6 50 mg, vitamin B12 560 mg, niacin 700 mg, biotin 3 mg, pantothenic acid 500 mg, folic acid 30 mg, clostrain chloride 20 mg, iron 1100 mg, copper 300 mg, manganese 1800 mg, zinc 1200 mg, iodine 24 mg, selenium 3 mg, methionine 20 g, calcium 176 g, phosphorus 68 g, sodium 23 g, chloride 36 g, growth promoter 2 g, coccidiostatic 10 g, BHT 1 g.

Blood sampling and IGF-I assay

Blood samples (5 mL) from male chickens (n = 12) were obtained by venipuncture (brachial vein) using a heparinized syringe. The blood was centrifuged at 3,000 rpm and 4 °C for 10 min. The plasma was collected and stored at -20 °C until assayed for IGF-I. Following the precipitation of plasma binding proteins (IGFBPs) with acidified ethanol, plasma IGF-I was quantified using a heterologous radioimmunoassay (RIA) previously validated for chicken plasma (Huybrechts *et al.* 1985).

For IGFBP precipitation, 400 µL of an ethanol-HCl solution (2 N HCl, 95% ethanol) were added to a tube containing 100 µL of plasma. After vortex-mixing, the tubes were incubated at room temperature for 30 min and then centrifuged at 3,000 x g for 30 min, at 4 °C. After centrifugation, an aliquot (200 µL) of each supernatant was collected without disturbing the precipitates and was thoroughly mixed with 80 µL of neutralizing solution (0.855 M Tris) followed by incubation for 60 min at room temperature. After a further centrifugation (3,000 x g, 60 min, 4 °C), a 100 µL aliquot was removed for IGF-I quantification.

For RIA, 1 µg of recombinant human IGF-I (hIGF-I) in 0,1% acetic acid was iodinated with 1 mCi [^{125}I] NaI (Amersham Biosciences) by adding chloramine T (0.1 mg/mL in 0.3 M sodium phosphate buffer, pH 7.6) in a stepwise manner over a period of 4.5 min. The iodination reaction was terminated by adding to the reaction mixture 150 µL of a solution containing 1.0 M NaI, 100 mM KH_2PO_4, 0,1% BSA and 0.02% NaN_3. Iodinated peptide was separated from free ^{125}I by chromatography on a Sephadex G-50 column pre-equilibrated and eluted with 30 mM NaH_2PO_4 containing 0.25% BSA. The specific activity of the purified [^{125}I]-hIGF-I was ~ 320 µCi/µg. Sodium phosphate buffer (30 mM, pH 7.5), containing 10 mM EDTA, 0.02% protamine sulphate and 0.02% sodium azide was used to dilute the primary and secondary antibodies and the hormone standard for the assay. Standard hormone (hIGF-I) and tracer (^{125}I-labelled hIGF-I) were initially dissolved, diluted and stored in phosphate buffer containing 0,1% BSA. The RIA diluent was the phosphate buffer described above containing 0.05% (v/v) Tween 20.

The assay was done under nonequilibrium conditions. On day 1, RIA diluent (250 µL) plus a 50 µL sample of the acid-ethanol extract and 100 µL of tracer containing ^{125}I-labelled hIGF-I (6,000 cpm) were added to RIA tubes containing 100 µL of primary antibody (1:16,000 working dilution), followed by mixing and incubation for 24 h at room temperature. On day 2, secondary antibody (100 µL of goat anti-rabbit IgG diluted 1:25 in phosphate buffer) plus 100 µL of carrier solution (49 mL of RIA buffer, 1 mL of normal rabbit serum, 1.5 g of polyethylene glycol) were added to each tube, followed by vortex-mixing and

centrifugation at 2,000 x g for 30 min at 4 °C. Subsequently, 1 mL of 1 M NaCl and 6% polyethylene glycol were added and incubated at 4 °C for 10 min followed by centrifugation at 2,000 x g for 30 min. The resulting supernatant was aspirated and the pellet counted in a γ-counter. The intra-assay coefficient of variation was 6.9%.

Dot blot analysis of IGF-I mRNA expression

Frozen hepatic tissue (1 g/bird) was homogenized, and total RNA was isolated by the guanidinium thiocyanate method of Chomczynski and Sacchi (1987). Total RNA was quantified by the absorbance at 260 nm and the relative IGF-I mRNA content was determined by dot blot hybridization. Briefly, 20 µg of total RNA were dried under vacuum and dissolved in 25 µL of 50% DMSO (dimethyl sulphoxide), 10 mM sodium phosphate, pH 7.0 and 1.0 M glyoxal. After incubation at 50 °C for 1 h, the samples were placed on dry ice. Two dilutions of 5 µg and 10 µg of RNA were prepared in a final volume of 400 µL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and dotted (Bio-Dot Microfiltration Apparatus, Bio-Rad) onto nylon membranes (Hybond N, Amersham Pharmacia Biotech). The membranes were pre-hybridized for 2 h at 65 °C in a pre-hybridization solution (1% BSA fraction V, 7% sodium dodecyl sulphate, 1 mM EDTA, 0.5 M sodium phosphate, pH 7.2), with 20 mL per 100 cm^2, and hybridized for 16 h at 65 °C with the same solution containing a randomly labeled (Feinberg and Vogelstein, 1983) cDNA probe for chicken IGF-I (cloned into the pGEM-3Z plasmid and kindly provided by Dr. Peter S. Rotwein (School of Medicine, Washington University)). After hybridization, the membranes were washed twice in wash A solution (0.5% BSA, 5% SDS, 1 mM EDTA, 40 mM sodium phosphate, pH 7.2) at 65 °C for 30 min. Autoradiography was done using Kodak X-OMAT X-ray film and Dupont Cronex Lightening Plus intensifying screens. For re-probing, the membranes were washed in 10 mM Tris-HCl at 90 °C for 20 min and then re-hybridized with a cDNA probe for rat 28S RNA under the same conditions as used for IGF-I cDNA. The signals obtained in the dots were quantified by densitometry (GS 300 densitometer, Hoefer Scientific Instruments) and the values were normalized according to the 28S RNA hybridization signal, used as an RNA quantity and quality control for each sample. The densitometric values obtained were within the linear range of the method.

Statistical analysis

The experiments were done using a split-plot design with strains as parcels and age as sub-parcels. The data were analyzed using the general linear model procedure (GLM) and means were compared by the Tukey test using SAS software (SAS Institute, 2000).

Results

Table 2 shows the mean body weight from 1 to 42 days of age for the three strains studied. As expected, broiler chickens of the fast growing strain (HP) grew faster than the NN and C strains (NN also grew faster than C). The hatching body weight was higher ($p < 0.05$) in HP broilers than in the other strains (HP > NN = C). This difference could be attributed not only to genotype, but also to the nutritional status and age of the chickens. From seven days of age, the three strains differed in body weight ($p < 0.05$), with HP birds always being the heaviest, followed by NN and C birds.

The weight gain of the HP strain paralleled the better food conversion values (Table 3), but only during the initial phase (1-21 days), when HP had a significantly lower food conversion than NN birds (1.50 *vs.* 1.65, $p < 0.05$). During the final phase (22-42 days), food conversion was not different (2.18 *vs.* 2.24, $p > 0.05$) between the HP and NN strains, but both were different ($p < 0.05$) from C birds.

Figure 1 shows the autoradiographic signals of the dot blots for hepatic IGF-I mRNA and 28S cDNA, the latter used as an internal control. The dots correspond to ages of 1, 21 and 42 days. The relative densitometric values for the dots at the three different ages (expressed as arbitrary units) are shown in Table 4. Regardless of the broiler strain, hepatic IGF-I mRNA expression increased with age, from hatching to 21 days old. From 21 to 42 days, a decrease ($p < 0.05$) in IGF-I mRNA expression was seen in the NN strain. A comparative analysis among strains showed that NN had a much higher hepatic IGF-I mRNA expression than the other two strains at the age of 21 days (NN > HP > C).

The plasma IGF-I concentrations for the three strains at different ages are shown in Table 4. The broiler strain did not affect the levels of circulating IGF-I but, as observed for IGF-I mRNA expression, plasma IGF-I levels increased from 1 to 21 days of age with no significant change thereafter (days 21 to 42).

Table 2 - Mean body weight (g) of the three strains (Hubbard-Pettersen, Naked-neck and Caipira) during the 42 days of the experiment.

	Strains				
Age (days)	HP[1]	NN	C	F values	CV (%)
1	43.8[a2]	36.9[b]	35.2[b]	83.8	2.58
7	139.2[a]	81.3[b]	69.7[c]	306.8	4.40
21	740.9[a]	333.3[b]	201.7[c]	838.4	4.57
35	1,573.0[a]	656.0[b]	363.1[c]	1,917.7	3.34
42	2,070.5[a]	911.0[b]	527.0[c]	1,352.4	3.74

[1]HP: Hubbard-Pettersen; NN: Naked-neck, C: Caipira. [2]Means followed by the same letter in the rows are not significantly different ($p > 0.05$).

Table 3 - Mean weight gain (g), food intake (g) and food conversion during the initial (1-21 days), final (22-42 days) and total (1-42 days) experimental periods for the three broiler chicken strains used.

Variable	Period (days)	Strains			F value	CV (%)
		HP[1]	NN	C		
Body weight (g)	1-21	697.1[a2]	296.5[b]	167.4[c]	896.7	4.77
	22-42	1,329.6[a]	577.7[b]	325.3[c]	1,547.0	3.57
	1-42	2,020.7[a]	874.1[b]	491.8[c]	1,382.6	3.80
Food intake (g)	1-21	1,044.7[a]	490.1[b]	433.2[c]	688.5	3.93
	22-42	2,893.6[a]	1,296.1[b]	964.0[c]	1,196.6	3.47
	1-42	3,938.2[a]	1,786.2[b]	1,397.2[c]	1,144.6	3.41
Food conversion	1-21	1.50[c]	1.65[b]	2.61[a]	346.0	3.35
	22-42	2.18[b]	2.24[b]	2.97[a]	78.1	4.07
	1-42	1.94[b]	2.05[b]	2.85[a]	146.3	3.06

[1]HP: Hubbard-Pettersen; NN: Naked-neck, C: Caipira. [2]Means followed by the same letter in the rows are not significantly different ($p > 0.05$).

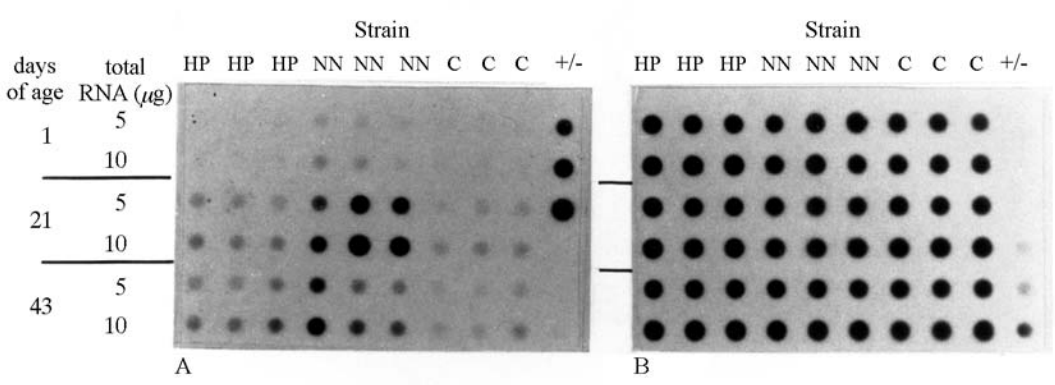

Figure 1 - Liver IGF-I mRNA expression in male broiler chickens. (A) Autoradiograph from liver total RNA dot blot hybridized with chicken IGF-I cDNA probe. (B) Autoradiograph of the same dot blot hybridized with rat RNA 28S cDNA. HP: Hubbard-Pettersen, NN: Naked-neck, C: Caipira. +/-: 1, 2 and 4 ng of positive and negative control (pGEM-3Z plasmid containing the chicken IGF-I cDNA insert and rat 28S RNA cDNA, respectively). Total RNA from three different birds of each strain, at the specified age, was blotted on the membrane.

Table 4 - Relative values for hepatic IGF-I mRNA expression and plasma IGF-I concentrations in the three strains at 1, 21 and 42 days of age.

Age (days)	Strains		
	HP[1]	NN	C
	Hepatic IGF-I mRNA expression (arbitrary units), n = 3		
1	0.707^{Ba2}	1.017^{Ba}	0.707^{Ba}
21	2.193^{Ab}	4.553^{Aa}	1.525^{Ab}
42	2.226^{Ab}	3.023^{Aa}	1.600^{Ac}
	Plasma IGF-I concentration (ng/mL), n = 12		
1	5.20^{Ba}	7.47^{Ba}	4.33^{Ba}
21	30.31^{Aa}	29.68^{Aa}	29.38^{Aa}
42	26.09^{Aa}	30.34^{Aa}	26.98^{Aa}

[1]HP: Hubbard-Pettersen; NN: Naked-neck, C: Caipira. [2]Means with similar capital letters in the columns and lower case in the rows are not significantly different ($p > 0.05$).

Discussion

As expected, the HP and NN birds had better growth performances than the C strain. At 42 days of age, body weight was significantly different among the three strains, thus confirming the growth potential of the genetically improved strains. Despite the increase in body weight, food conversion was not different for HP and NN strains after 21 days. Thus, the regulatory mechanisms involved in growth seem to be dependent on the genetic characteristics of the strains and also on the age of the birds.

Burnside and Cogburn (1992) reported that the hepatic expression of IGF-I mRNA in broiler chickens peaked at 28 days of age, and Beccavin *et al.* (2001) found that high growth rate birds had a five-fold increase in IGF-I expression from the first to sixth week of life. The plasma concentration of IGF-I was reported to increase progres-

sively until the third week of age, and reached a plateau by the seventh week (McGuiness and Cogburn, 1990; Beccavin *et al.*, 2001). As already observed by others (Kikuchi *et al*., 1991; Burnside and Cogburn, 1992; Radecki *et al.*, 1997; Beccavin *et al.*, 2001), the hepatic IGF-I mRNA expression and IGF-I plasma levels in the present study also increased from hatching to day 21, with hepatic expression increasing at least three-fold and plasma levels showing a six- to seven-fold increase. Nevertheless, at 42 days of age, both the expression and plasma IGF-I levels showed no significant increase ($p > 0.05$) when compared to 21 days, although other reports have indicated that this increase persisted until six weeks of age (McGuiness and Cogburn, 1990; Burnside and Cogburn, 1992; Beccavin *et al.* 2001).

The most interesting finding of this study was that the patterns of increase in IGF-I expression and production were the same, irrespective of the broiler chicken strain, except for the NN strain, which had higher values at all ages, but also showed reduced IGF-I mRNA expression from 21 to 42 days of age. Huybrechts *et al.* (1987) and Goddard *et al.* (1988) observed no positive correlation between growth rate and absolute plasma IGF-I levels in chickens. Our results also showed that there was no clear correlation between the growth of the birds and hepatic IGF-I mRNA expression and plasma IGF-I levels.

In mice, complete disruption of the liver IGF-I gene in the post-natal period decreased the circulating IGF-I levels by 75%, while the growth rate was normal (Yakar *et al.*, 1999). According to Oudin *et al.* (1998), there were no differences in the number, affinity or tyrosine kinase activity of IGF-I receptors purified from the muscles of fast and slow growth chickens at one to seven weeks of age. Thus, the difference in body weight among strains may be related to the hypothalamic-pituitary feedback mechanism that interferes with GH synthesis and release. This aspect requires further investigation, as does the genetic variation in muscle IGF-I gene expression, especially since a paracrine action of IGF-I on chicken muscle has been reported (Duclos *et al.*, 1999).

The temporal regulation of IGF-I expression in broiler chickens of unrelated genotypes and different growth rates has been studied (Goddard *et al.*, 1988; Ballard *et al.*, 1990; Johnson *et al.*, 1990), but no influence of strain was reported. In contrast, in work with chickens of different genotypes and high or low growth rates, Scanes *et al*. (1989) found that plasma IGF-I levels were lower in the low compared to high weight strains. Becavin *et al*. (2001) also found that high growth rate birds had higher levels of hepatic IGF-I mRNA and plasma IGF-I. Thus, the increased hepatic IGF-I mRNA expression observed here, which was associated with age but not strain, could be related to the complex regulatory mechanisms of growth in birds. Liver GHR (growth hormone receptor) expression is low after hatching, but increases with age (Burnside and Cogburn, 1992; Scanes *et al.*, 1996; Mao *et al.*, 1998). In fast growing broilers, increased hepatic GHR expression was observed between the first and the fourth week of age and coincided with an increase in IGF-I mRNA expression and plasma IGF-I levels (Leung *et al.*, 1987). The subsequent decrease in plasma GH, also described by McCann-Levorse *et al.* (1993), does not appear to interfere with the hepatic GHR since the number and affinity of these receptors compensates for the low level of circulating GH, thereby maintaining the steady-state expression of IGF-I mRNA.

These results show that the ontogenetic pattern of hypothalamic-pituitary axis activity after hatching is apparently independent of broiler chicken strain, despite the magnitude of the difference in their growth response. Together with previous reports (Leili *et al.* 1997; Beccavin *et al.* 1999, 2001), our findings indicate that additional temporal regulatory mechanisms related to nutrition and age are involved in modulating the expression of hepatic IGF-I mRNA and the consequent increase in plasma IGF-I levels.

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