Growth Hormone Size Variants: Changes in the Pituitary During Development of the Chicken (44464)

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Abstract. **There is considerable evidence for the existence of structural variants of growth hormone (GH). The chicken is a useful model for investigating GH heterogeneity as both size and charge immunoreactive-(ir) variants have been observed in the pituitary and plasma. The present study examined the size distribution of ir-GH in the pituitary gland of chicken, from late embryogenesis through adulthood. Pituitaries were homogenized in the presence of protease inhibitor, and the GH size variants were separated by SDS-PAGE, transferred by Western blotting, immunostained with a specific antiserum to chicken GH, and quantitated by chemiluminescence followed by laser densitometry (chemiluminescent assay). Under nonreducing conditions ir-GH bands of 15, 22, 25, 44, 50, 66, 80, 98, 105 and >110 kDa were observed. Both the relative proportion of the GH size variants and the total pituitary content varied with developmental stage and age. The proportion of the 15-kDa fragment was greatest in the embryonic stage, and then it decreased. The proportion of the monomeric 22-kDa form was lowest at 18 days of embryogenesis (dE) and highest at 20 dE. In contrast, the high MW forms (≥66 kDa) were lowest in embryos, and they increased ($P < 0.05$) after hatching. The 22-, 44-, 66-, and 80-kDa forms were assayed for activity by radioreceptor assay following isolation by semipreparative SDS-PAGE. Only the 22-kDa GH variant showed radioreceptor activity. Under reducing conditions for SDS-PAGE, ir-GH bands of 13, 15, 18, 23, 26, 36, 39, 44, 48, 59 and 72 kDa were oberved, but most of the high MW form disappeared. There was a concomitant increase in the proportion of the monomeric band and of several submonomeric forms. The present data indicate that the expression, processing, and/or release of some if not all size variants are under some differential control during growth and development of the chicken.**

[P.S.E.B.M. 2000, Vol 223]

There is considerable evidence for the existence of structural variants of GH. This may be due to gene duplication (e.g., human GH-N and GH-V forms) (1) or alternative splicing of the mRNA as in the case of 20 kDa human GH (1) and also perhaps eel GHI and GHII (2). Multiple forms of GH are also due to post-translational modification of GH due to deamidation, proteolytic cleavage, cleavage and reduction, glycosylation, phosphorylation, and aggregation (1, 3, 4). Some of these post-translationally modified forms exhibit differences in biological activity (e.g., human (5), bovine (6), chicken (7)). The chicken appears to be a useful model for the investigation of GH variants as both charge and size ir-variants (including oligomers) have been observed in the pituitary gland (7–9) as have glycosylated (10, 11), phosphorylated (12, 13), and cleaved forms (14). One isomer has been isolated that presumably correponds to the nontransformed monomer (15). This monomeric variant exhibits a range of biological activities including increasing the rate of both IGF-I secretion by hepatocyte *in vitro* and lipolysis *in vitro*

This is a paper of the Journal Series of the New Jersey Agricultural Experiment Station (project 06140) supported by the Hatch Act and State Funds (C.G.S.) and also by grants from The Rockefeller Foundation, The Fogarty International Center (NIH), DGAPA-UNAM (México, PAPIIT IN206196), and CONACYT(3455-PN) to C.A.

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Received March 25, 1999. [P.S.E.B.M. 2000, Vol 223]
Accepted July 30, 1999.

0037-9727/00/2231-0067$14.00/0

by adipose tissue together with shifting the ratio of T_4 to T_3 *in vivo* (indicating stimulation of monodeiodination) (15). There is also some evidence that the synthesis or content of GH variants by the chicken pituitary gland varies during post-hatch growth as indicated by isoelectric focusing followed by Western blotting (9) or by incorporation of ^{14}C-leucine into two ir-GH bands (16) as separated by disc polyacrylamide electrophoresis (17). There is circumstancial evidence for ontogenic shifts of GH variants in the turkey pituitary gland. The content of GH was higher when measured by radioreceptor assay than by radioimmunoassay in young turkey poults but was lower when measured by radioreceptor assay than by radioimmunoassay in almost full-grown turkeys (18).

In the present study, a chemiluminescence assay has been developed for GH size variants in the chicken pituitary gland, and this has been used to examine the changes in size variants of GH through late embryonic development and throughout post-hatch growth/maturation into adulthood. The chicken shows a simple overall ontogenic pattern of GH secretion, with plasma concentration of GH increasing during the late embryonic period and immediately following hatch, high concentration in the young chick, and a decline to the low adult concentration occurring sometime prior to even the beginning of sexual maturation (19, 20, 21).

Materials and Methods

Biological Materials. Birds were obtained as fertilized eggs or day-old chicks from Avian Services (Frenchtown, NJ) and reared with free access to water and commercial feed. Pituitary glands were collected from white Leghorn chickens at different ages: 18 and 20 days of embryonic development (18dE, 20dE), 1 day post-hatch (1d), and at 2, 4, 8, 12, and 52 weeks old (2w, 4w, 8w, 12w, A or adult). The glands were immediately frozen on dry ice after disection, weighed and stored at –20°C until used. The tissue used in Studies 1 and 2 had the following weights: 18dE: 3.71 ± 0.01 mg; 20dE: 1.94 ± 0.16 mg; 1d: 6.8 ± 0.42 mg; 2w: 4.97 ± 0.72 mg; 4w: 9.82 ± 1.40 mg; 8w: 11.85 ± 0.97 mg; 12w: 13.0 ± 0.41 mg; A: 18.2 ± 2.16 mg/gland (Mean ± SEM, n = 4).

Preparation of Pituitary Extracts. Glands were homogenized in a solution of 0.75 m*M* phenylmethylsulfonyl fluoride (PMSF) (to prevent proteolysis) in H_2O, pH 9.5 (150 µl/gland) employing a glass microtissue grinder (Konte, Vineland, NJ) on ice. In preliminary studies, it was found that the presence of additional protease inhibitor [pepstatin (0.7 µg/ml), leupeptin (0.5 µg/ml), pefablock (1 m*M*), phosphoramidon (50 µg/ml), and E64 (1 µg/ml)] did not influence either the electrophoretic pattern or the relative quantities of any of the GH size variants. The pituitary homogenates were subsequently agitated on a magnetic stirrer for 30 min. After centrifugation (12,000 r.p.m. at 4°C for 5 min), the supernatants were collected, and protein content was determined by the Bio-Rad micromethod (Bio-Rad, Richmond, CA).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Aliquots corresponding to 20 µg total protein were analyzed by SDS-PAGE on 1-mm–thick 12% gel (Mini-Protean, Bio-Rad) under nonreducing conditions (NRC) or under reducing conditions (RC) (in the presence of 5% β-mercaptoethanol) using the buffer system of Laemmli (22), and run at 100 V in the stacking gel and 150 V in the separating gel. A control corresponding to 500 ng of native cGH (purified (23)) was included in one lane in every gel. Pre-stained molecular weight markers from Bio-Rad were employed to determine the relative Mr of the GH-like bands.

Western Blotting. After electrophoresis the gels were equilibrated in transfer buffer (25 m*M* Tris, 192 m*M* Gly, 20% methanol, pH 8.3) and transferred to Immun-lite membranes (Bio-Rad) at 180 mA for 45 min (24) to develop the immunostained bands by chemiluminescence. After transfer the membranes were washed with 20 m*M* Tris, 500 m*M* NaCl, pH 7.5 (TBS) for 5 min and then blocked with 5% nonfat dry milk (Bio-Rad) in TBS for 2 hr at 37°C. After washing the membranes with TBS for 15 min, they were incubated with chicken GH primary antibody (25) (1/4000 in 1% nonfat dry milk in 0.05% Tween-20 in TBS, T-TBS) at room temperature overnight. The membranes were rinsed for 15 min (3x) with TBS and then incubated with a secondary antibody (goat anti-rabbit IgG) conjugated to alkaline phosphatase (Bio-Rad), 1/3000 in 1% nonfat dry milk in T-TBS for 1 hr. The blots were washed 3x with T-TBS and once with TBS (15 min each) and then incubated for 5 min in the substrate solution (3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)phenyl-1,2-dioxetane disodium salt [AMPPD, 180 µl/100 ml] in 0.1 *M* diethanolamine, 1 m*M* $MgCl_2$, pH 10, Bio-Rad Immun-Lite chemiluminescent assay kit. Luminograms were obtained after exposing the blots to Kodak X-Omat AR5 X-ray films (Eastman Kodak 165–1454, Rochester, NY) for 1 min. The films were then developed (Kodak GBX 190–0984) and fixed (Kodak GBX 190–2485).

To quantify GH size variants in the chemiluminescent assay, a standard curve with different amounts of purified cGH (in the range of 37.5–750 ng) was constructed. Figures 1A and 1B show, respectively, the luminogram obtained after Western blotting and the dose-response relationship between chemiluminescence and GH dose. Analysis of the individual size variants present in the standard yielded linear dose-response curves (e.g., 22K: $y = 0.258x + 0.079$, $r^2 = 0.98$; 44K: $y = 0.175x + 0.187$, $r^2 = 0.99$; 60K: $y = 0.06x - 0.051$, $r^2 = 0.93$; 80K: $0.064x + 0.061$, $r^2 = 0.94$; 98K: $y = 0.063x + 0.012$, $r^2 = 0.99$; 105K: $y = 0.049x + 0.026$, $r^2 = 0.99$). When total area sum (AUxmm) was plotted versus total protein, a linear dose-response was also observed ($y = 0.0178x + 0.3141$, $r^2 = 0.995$) with 1 ng equivalent of cGH corresponding to 0.01761 ± 0.00086 AUxmm (mean ± SEM, n = 8). The chemiluminescence assay showed good reproducibility with a coefficient of variation of 8.5% for the standard cGH.

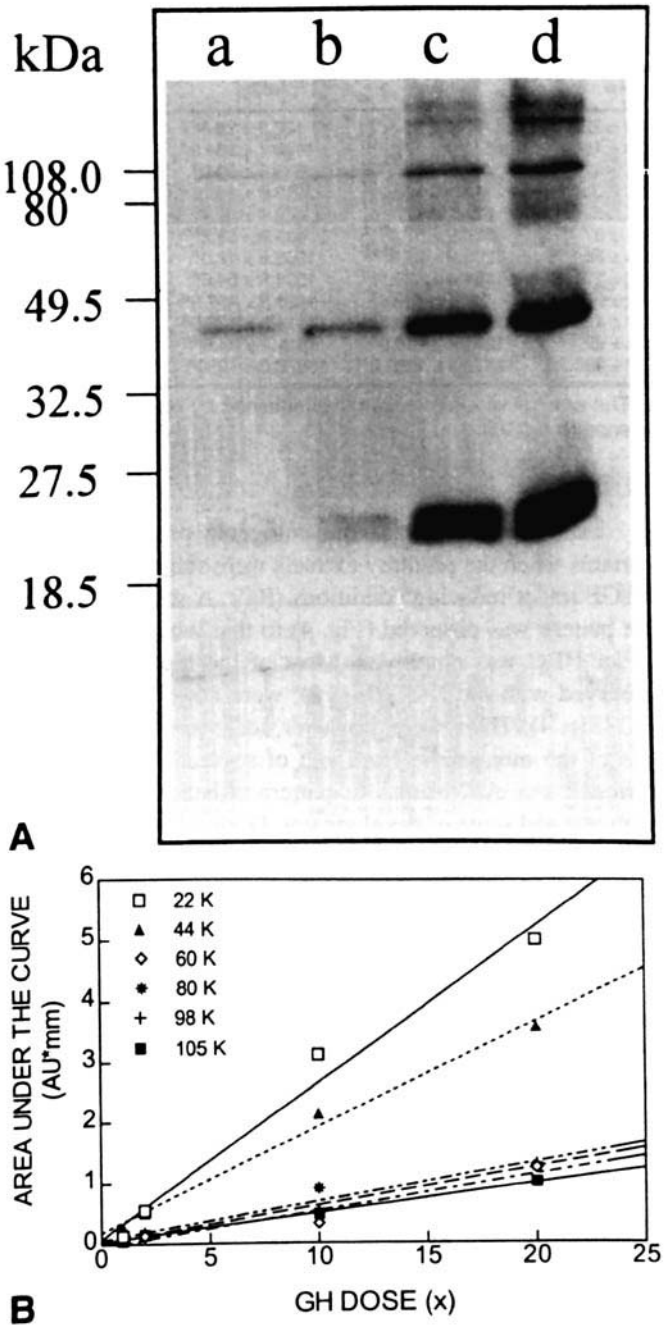

Figure 1. Dose-response relationship between GH concentration and chemiluminescence. (A) Representative autoluminogram of standard chicken GH after SDS-PAGE (NR), Western blot, and chemiluminescent assay. The cGH doses were: [a] 37.5 ng (1x); [b] 75 ng (2x); [c] 375 ng (10x); [d] 750 ng (20x). (B) Plot of the area under the curve (AU*mm) as determined by chemiluminescence, autoluminogram, and densitometry vs standard GH dose.

The same samples employed above were analyzed by SDS-PAGE under reducing conditions (boiling in the presence of 5% β-mercaptoethanol) to study if the GH variants or the ontogenic pattern changed when disulfide bridges were reduced. These samples were also developed with the chemiluminescent assay described above.

Densitometry. The ir-cGH-like bands in the X-ray films were analyzed in a laser densitometer (Pharmacia-LKB, Bromma, Sweden) employing GelScan XL 2.1 software.

Isolation of GH Size Variants. The 22-, 44-, 66-, and 80-kDa GH variants were isolated by semipreparative SDS-PAGE of pituitary-derived chicken GH. The GH variants were electroluted from the gel using a Hoeffer Scientific electroluter. The protein content of the samples was estimated by absorbance at 280 nm.

Radioreceptor Assay of GH Size Variants. The GH size variants (22, 44, 66 and 80 kDa GH) were subjected to radioreceptor assay (26) using recombinant chicken GH (donated by American Cyanamid, Princeton, NJ) as the tracer, following iodination by the Iodogen (Pierce) method and a liver membrane preparation (48,000*g*) from adult male chickens (strain white Leghorn) (26).

Statistical Analysis. Data (content of each GH variant as ng per gland or the proportion of the variant as a percentage of total GH immunoreactivity) from four separate electrophoresis runs each with different pituitary samples were analyzed by one-way ANOVA. Differences between the groups were determined by Fisher's LSD range test.

Results

Changes in the Pituitary Content and in the Proportion of GH Size Variants During Development and Growth as Determined by Chemiluminescence Assay. The GH size variants in pituitary homogenates were separated by SDS-PAGE under NRC and quantified by chemiluminescence assay following Western blotting. Figure 2 shows a typical luminogram: ir-GH bands of 15, 22, 25, 44, 50, 60, 66, 80, 98, 105 and >110 kDa being observed. Table I summarizes the ontogenic changes in the pituitary content of ir-GH variants. When the total pituitary content for the ir-GH variants was analyzed, the

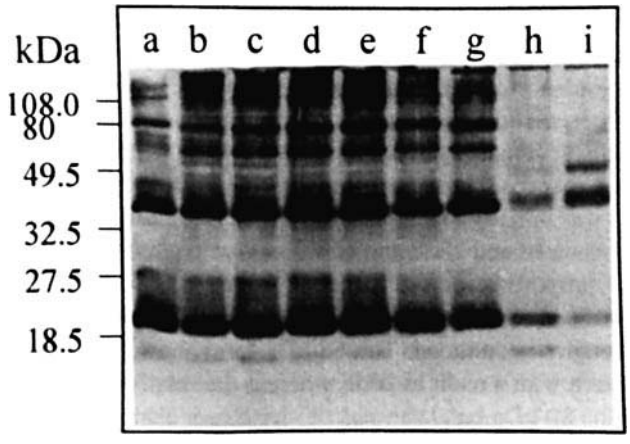

Figure 2. Representative Western blot of chicken pituitary GH size variants during ontogeny (NRC). (A) Pituitary extracts were submitted to SDS-PAGE under nonreducing conditions, and the ir-GH bands were developed with a chemiluminescent assay. Lanes: [a] standard cGH (500 ng); [b] Adult; [c] 12 weeks; [d] 8 weeks; [e] 4 weeks; [f] 2 weeks; [g] 1d; [h] 20dE; [i] 18 dE.

Table I. Ontogenic Profile of the Pituitary Content of GH Size Variants as Separated by PAGE Under NCR and Quantitated by Chemiluminescence Assay*

MW	18dE	20dE	1d	2w	4w	8w	12w	A
15	15.6 ± 1.3^{b}	17.1 ± 0.7^{b}	12.4 ± 1.9^{b}	27.6 ± 7.6^{b}	80.3 ± 22.4^{ab}	165.0 ± 53.3^{a}	122.9 ± 29.6^{ab}	161.4 ± 39.3^{a}
22	35.4 ± 2.9^{c}	123.3 ± 13.1^{c}	381.9 ± 94.6^{c}	1876.8 ± 107.8^{b}	2787.0 ± 156.9^{b}	3542.4 ± 371.7^{ab}	4496.7 ± 364.0^{a}	4497.8 ± 649.5^{a}
25	3.2 ± 1.3^{d}	8.7 ± 1.8^{d}	28.2 ± 7.1^{d}	155.2 ± 31.2^{cd}	344.2 ± 51.3^{bc}	447.5 ± 42.5^{b}	633.8 ± 39.8^{a}	706.2 ± 126.5^{a}
44	127.5 ± 10.4^{c}	56.2 ± 9.7^{c}	323.1 ± 73.7^{c}	1443.5 ± 261.6^{b}	1935.3 ± 307.9^{b}	2112.1 ± 173.2^{b}	3013.3 ± 196.8^{a}	2923.2 ± 369.8^{a}
50	1.7 ± 0.4^{d}	12.8 ± 2.9^{d}	80.4 ± 16.1^{d}	370.5 ± 21.4^{c}	482.3 ± 54.0^{c}	627.1 ± 82.4^{bc}	762.4 ± 66.8^{b}	1061.7 ± 103.6^{a}
60	70.7 ± 6.8^{cd}	20.2 ± 2.5^{d}	66.5 ± 8.0^{cd}	217.0 ± 30.3^{bc}	259.4 ± 37.7^{b}	324.9 ± 67.4^{b}	431.9 ± 59.2^{ab}	475.9 ± 65.3^{a}
66	33.5 ± 7.2^{e}	21.4 ± 2.8^{e}	140.2 ± 20.4^{e}	700.1 ± 52.9^{d}	1002.0 ± 86.6^{cd}	1203.9 ± 85.8^{bc}	1655.6 ± 12.0^{a}	1639.6 ± 159.1^{a}
80	31.6 ± 9.6^{c}	29.8 ± 2.0^{c}	214.6 ± 46.3^{c}	745.8 ± 122.3^{b}	1148.2 ± 171.0^{ab}	1161.4 ± 128.5^{ab}	1508.8 ± 54.6^{a}	1445.5 ± 296.6^{a}
98	17.1 ± 5.2^{b}	20.2 ± 7.1^{b}	213.6 ± 18.8^{b}	827.5 ± 50.9^{ab}	1288.2 ± 250.9^{a}	1531 ± 439.8^{a}	1626.6 ± 437.8^{a}	1304.5 ± 186.2^{a}
105	11.1 ± 2.9^{c}	15.8 ± 1.9^{c}	159.6 ± 40.8^{c}	766.8 ± 135.3^{b}	967.4 ± 116.3^{ab}	991.0 ± 143.7^{ab}	1114.3 ± 136.6^{a}	985.7 ± 63.8^{ab}
>110	30.8 ± 6.6^{c}	36.4 ± 6.1^{c}	217.7 ± 36.6^{c}	815.5 ± 153.6^{b}	904.4 ± 82.1^{ab}	1208.9 ± 188.0^{a}	1011.7 ± 34.6^{ab}	980.2 ± 135.5^{ab}
Sum	378.2 ± 43.2^{e}	361.9 ± 20.0^{e}	1838.2 ± 313^{e}	7946.5 ± 631.9^{d}	11198.7 ± 936.7^{cd}	13314.8 ± 1252.6^{bc}	16378.0 ± 1060^{a}	16181.7 ± 1507^{ab}

Note. Ir-GH variants were developed with the chemiluminescence assay. The amount of each variant was obtained by comparison with a standard preparation of pure cGH. Different letters mean a statistical difference ($p < 0.05$).
* Equivalent ng ir-cGH/gland, X ± SEM, $n = 4$.

same general developmental pattern was found for each (Table I). For most of the variants their total glandular content was lowest during the embryonic stage and 1d post-hatch showing a significant increase at 2w of age ($P < 0.01$) and then progressively increasing until adulthood. In view of the consistency in the developmental pattern for each variant, it was decided to determine whether there were ontogenic changes in the proportion of the GH variants. This was the case (Fig. 3). It is readily apparent that the proportion of the 15-kDa variant was greatest ($P < 0.001$) in the embryonic stage and then decreased considerably. The proportion of the 22-kDa monomeric variant was lowest at 18dE (9.5%), peaked (34.1%) at 20dE ($P < 0.001$), and after a slight decrease to 19.5% at 1d there was an increase in later ages ($P < 0.01$). The 25-kDa form showed a distinct pattern with increasing proportion at the older age. The 25-kDa form may represent a proteolytically cleaved GH whereas the 15-kDa variant is generated as a result of reduction of the cleaved form. Thus, both were initially produced by proteolytic cleavage. An estimate of the changes in this can be obtained by combining data on the 15- and 25-kDa forms. The proportion of the two forms combined were highest at 20dE (7.2%) and then decreased to 2.3% at 2w, showing a light increase thereafter. The 44-kDa form (presumed to be the dimer) had it highest proportion ($P < 0.001$) at 18dE (34%) and after decreasing to about 15% at 20dE, it remained approximately constant with no significant changes thereafter. The proportion of the 50-kDa variant was initially low at 18dE, and then increased ($P < 0.001$) between 18 and 20dE and between 12w to adult. In contrast, the proportion of the 60-kDa variant was highest ($P < 0.001$) at 18dE and then showed a consistent decrease through to adulthood. The 66-kDa form showed a biphasic pattern with a nadir at 20dE whereas the relative proportion of the 80-kDa band showed no significant change. The 98-, 105- and 110-kDa GH-like ir-bands showed a similar ontogenic pattern with their lowest proportion during the embryonic stage and peaking between 1d–2w of age ($P < 0.01$, $P < 0.001$, and $P < 0.05$, respectively) and then smoothly decreasing toward adulthood.

Table II summarizes the ontogenic profile of ir-GH variants when the pituitary extracts were submitted to SDS-PAGE under reducing conditions (RC). A strikingly different pattern was observed (Fig. 4) to that when SDS-PAGE under NRC was employed. Most of the high-MW forms, observed with the NRC (Fig. 2), were not observed under RC (Fig. 4). There were, however, increases in the intensities of the monomeric band and of several submonomeric forms. It was evident that the pattern of bands also changed with age and stage of development. During late embryogenesis, the most conspicuous GH variants corresponded to the following Mr: 15, 23, 44, 48 and 59 kDa. Additional variants of Mr 13, 18, 26, 36, 39 and 72 kDa were only oberved in post-hatching ages.

Radioreceptor Assay of GH Size Variants. Size variants of GH (22, 44, 66, and 80 kDa GH) were isolated by semipreparative SDS-PAGE under NRC. When subjected to radioreceptor assay, only the 22-kDa variant inhibited binding of the ^{125}I-GH to the liver membrane preparation (Fig. 5). The other variants showed no GH radioreceptor activity (<1% recombinant cGH) (Fig. 5).

Discussion

Molecular and functional heterogeneity of pituitary hormones has been recognized in different vertebrate species. It is now accepted that GH shows both size and charge variants thus constituting a family of closely related proteins. However, the functional role of these variants is not clear yet, and the physiological significance is still questioned. There has been some evidence suggesting that the GH variants may have different bioactivities [e.g., bGH (27), hGH (28), cGH (7)] or potency (29). Moreover, GH variants appear to be secreted into the bloodstream [e.g., hGH (4), cGH (30)]. At present there is little evidence in support of the independent/differential control of GH variant synthesis and/or release. We attempted to address this problem by studying any variation in the proportion of the GH variants during ontogeny, since the pattern of total GH synthesis and secretion is relatively simple in the chicken model (18–21, 31).

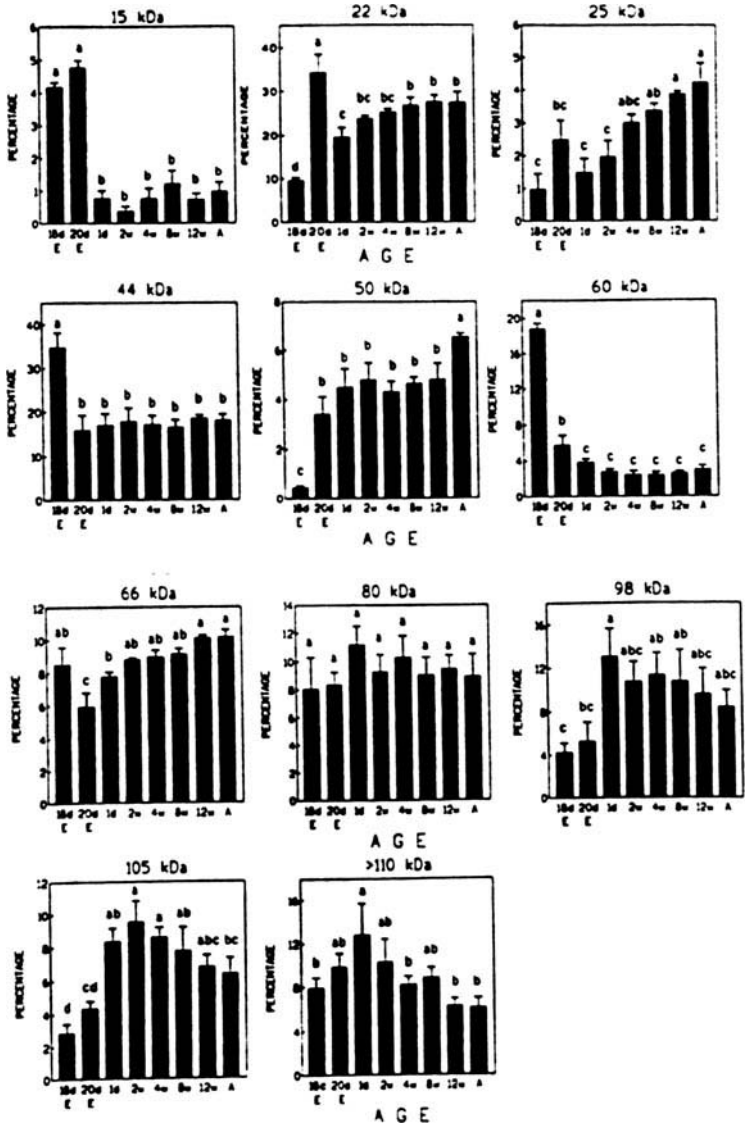

Figure 3. Changes in the proportion of GH variants (as separated by SDS-PAGE under NRC and quantitated by chemiluminescence assay) in the chicken pituitary gland. After Western blots were quantitated by chemiluminescence, the proportion of the area corresponding to each variant was expressed as a percentage of the total ir-GH ($n = 4$). Bar with different letters differ ($P < 0.05$).

Table II. Ontogenic Profile of the Pituitary Content of GH Size Variants as Separated by SDS-PAGE Under Reducing Conditions and Quantified by Chemiluminescence Assay*

MW	18dE	20dE	1d	2w	4w	8w	12w	A
13	0^{b}	0^{b}	0^{b}	649.9 ± 504.1^{ab}	857.6 ± 298.8^{ab}	2190.1 ± 909.6^{a}	1117.3 ± 374.2^{ab}	857.2 ± 696.5^{ab}
15	141.7 ± 31.6^{c}	194.9 ± 25.8^{c}	179.7 ± 48.3^{c}	731.5 ± 108.8^{bc}	1261.4 ± 146.7^{ab}	1689.2 ± 352.5^{a}	1754.5 ± 308.6^{a}	1777.6 ± 631.3^{a}
18	0^{d}	16.6 ± 3.8^{d}	109.4 ± 40.6^{d}	521.3 ± 274.0^{cd}	1421.4 ± 251.6^{bc}	2014.3 ± 487.8^{ab}	2654.1 ± 505.8^{a}	2141.0 ± 683.7^{ab}
23	326.0 ± 72.8^{d}	771.7 ± 100.3^{d}	1599.5 ± 296.4^{d}	4772.1 ± 1281^{c}	7546.7 ± 389.9^{b}	9588.7 ± 964^{b}	12292.6 ± 403.4^{a}	12844.0 ± 1499.7^{a}
26	0^{b}	34.1 ± 21.4^{b}	163.3 ± 38.0^{b}	689.5 ± 269.9^{b}	2049.7 ± 193.5^{a}	2489.3 ± 487.5^{a}	2932.5 ± 248.1^{a}	2934.9 ± 791.7^{a}
36	0^{c}	0^{c}	34.8 ± 20.7^{c}	153.2 ± 76.4^{c}	528.4 ± 112.0^{b}	761.4 ± 173.9^{b}	1421.9 ± 116.1^{a}	1461.3 ± 178.1^{a}
39	352.5 ± 22.3^{b}	186.9 ± 84.8^{b}	25.6 ± 25.6^{b}	112.6 ± 112.6^{b}	271.1 ± 90.5^{b}	878.9 ± 206^{a}	1145.8 ± 191.4^{a}	1141.8 ± 334.5^{a}
44	297.9 ± 76.4^{a}	131.7 ± 39.5^{b}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
48	0^{d}	144.9 ± 17.7^{d}	629.0 ± 226.1^{d}	2098.5 ± 1033.1^{cd}	4311.2 ± 707.7^{bc}	5456.3 ± 1164^{ab}	6961.5 ± 903.1^{a}	6958.2 ± 1512.3^{a}
59	125.8 ± 16.1^{d}	149.8 ± 16.0^{d}	316.7 ± 44.1^{d}	791.8 ± 153.1^{cd}	1390.4 ± 257.2^{bc}	1732.6 ± 453.3^{abc}	2710.9 ± 714.6^{ab}	2229.6 ± 472.5^{a}
72	0^{d}	0^{d}	0^{d}	0^{d}	195.1 ± 137.8^{cd}	505.9 ± 214.5^{bc}	821.9 ± 248.6^{ab}	1091.2 ± 333.3^{a}
Sum	1418.2 ± 163.1^{d}	1630.5 ± 119.0^{d}	3058.4 ± 659.8^{cd}	10663.4 ± 3367^{c}	19909.6 ± 1767^{b}	27373.2 ± 4328^{ab}	33884 ± 2595^{a}	33450 ± 5530^{a}

Note. Different letters mean a statistical significance.
* (equivalent ng ir-cGH/gland, X ± SEM, $n = 4$).

The size variants of chicken GH were initially analyzed by SDS-PAGE under NRC to leave intact such interactions as disulfide bonds that may otherwise be broken by the addition of β-mercaptoethanol. It has been shown that much (although certainly not all) of the oligomeric GH disappears upon reduction, thus suggesting that disulfide linkages are important in the formation of high-MW forms of GH. The number of ir-GH size variants in the chicken pituitary observed in the present study by SDS-PAGE under nonreducing conditions (Fig. 2) is distinctly different from that reported previously by Houston and Goddard (9) where only three size variants were observed but is more in agreement

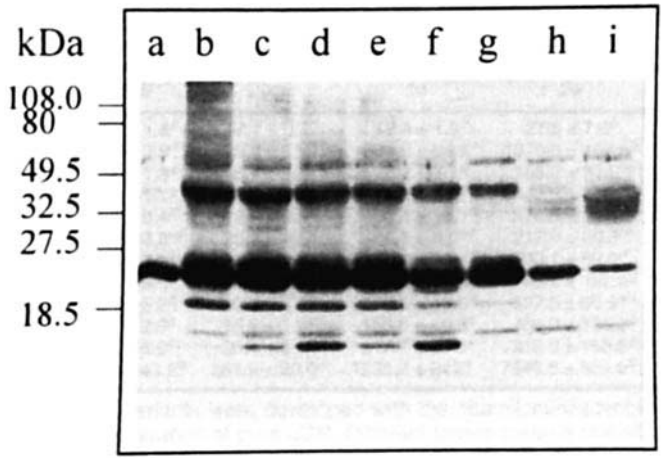

Figure 4. Representative Western blot of chicken pituitary GH size variants during ontogeny (RC). (A) Pituitary extracts were submitted to SDS-PAGE under reducing conditions and the ir-GH bands were developed with a chemiluminescent assay. Lanes: [a] standard cGH (500 ng); [b] Adult; [c] 12 weeks; [d] 8 weeks; [e] 4 weeks; [f] 2 weeks; [g] 1d; [h] 20dE; [i] 18 dE.

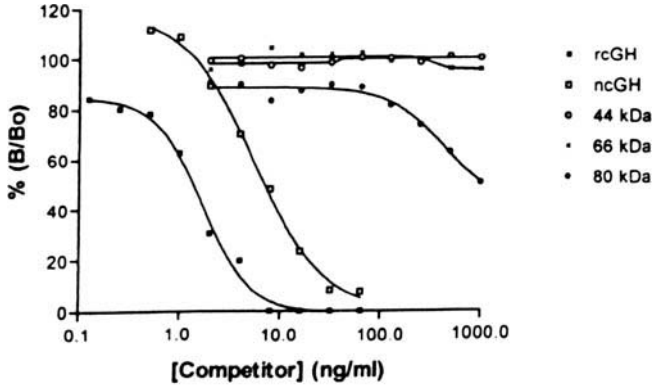

Figure 5. Comparison of the ability of chicken GH size variants to inhibit binding of ^{125}I-cGH to a chicken liver membrane preparation. The different GH variants (22, 44, 66, and 80 kDa) were obtained by electroelution from SDS-PAGE slabs and then tested for binding on chicken liver membranes (26). (rcGH: recombinant chicken GH; ncGH: native cGH purified from pituitary tissue).

with the pattern obtained by Montiel *et al.* (30) with both chicken pituitary extracts and sera. The present data closely resemble the pattern obtained for rat GH where 11 GH variants with apparent MW ranging from 14 to 88 kDa have been described (32). The 22 kDa, 44 kDa, 66 kDa and 80 kDa may be presumed to represent, respectively, monomer, dimer, trimer, and tetramer (the first three having been noted previously) (9). The 25-kDa form may represent a "two-chain" cleaved GH with the 15-kDa band a fragment generated by reduction of the cleaved form (14). The 29-kDa band observed under reducing conditions may be equivalent to the glycosylated form of GH previously reported (10, 11). The 50-, 60-, 98-, and 105-kDa forms may represent oligomers. Similar such forms of GH have previously been observed in the human (4) and the rat (32). Finally the 110-kDa band might either be a very complex GH oligomer, a form bound to binding protein(s), or simply a GH aggregate that was precipitated and did not enter the separating gel since it remained in the origin.

A 15-kDa form of GH has been observed previously in pituitary extracts from young (6-week-old) or adult chickens by SDS-electrophoresis under reducing conditions (with β-mercaptoethanol) but not under nonreducing conditions. This is not surprising in view of the very low levels of 15-kDa GH in the pituitary. This 15-kDa form of chicken GH may be equivalent to the 16-kDa form of rat PRL (33, 34). There is growing evidence that this 16-kDa rat PRL not only is synthesized and secreted by the pituitary gland but also shows distinctly different biological activities to that of the monomeric 23-kDa form (34, 35). It is interesting to note that the proportion of ir-15 kDa GH is greater in the embryonic chicken pituitary, decreasing thereafter. Moreover there is also a reduction in the relative proportion of the 25-kDa form between embryonic development and 2 weeks post-hatch (Fig. 3). These data sugget that there is a decline both in reduction and in proteolytic cleavage during the first 2 weeks of post-hatch growth.

The ontogenic changes in relative proportion of the GH size variants may have implications to biological functioning of GH as some variants may have different inherent biological activities. The 44-, 66-, and 80-kDa ir-GH variants have very low GH radioreceptor activity (Fig. 5). Thrombin cleavage of cGH produces a 25-kDa form that on reduction yields a 15-kDa form in a manner that may be analogous to the generation of the 15- and 25-kDa forms in the pituitary. Both the thrombin-cleaved and the 15-kDa product generated by its reduction have very low (<1% of GH) radioreceptor activity (Aramburo C, Scanes CG, unpublished data). In the case of bGH, a 15-kDa form (recombinant 1–132) has low GH radioreceptor activity, whereas the 22-kDa form show high activity (36). This is similar to the situation with dimeric human GH, which has been reported to have much lower somatotropic activity than monomeric GH (37, 38). Moreover, high concentrations of big-big forms of human GH are associated with inappropriately low levels of IGF-I (39). In addition, elevated circulating concentrations of non-22 kDa human GH are correlated to reduced growth rate (40, 41). In contrast, the high-MW variant of rat GH has been reported to be more potent in a somatotropic (tibia) bioassay than the GH monomer (29). It has also been found that culture media from rat heavily granulated somatotrophs (releasing larger forms of GH) (32) is very potent in GH tibia assay activity (42). Likewise, implantation of densely granulated (but not lightly granulated) somatotroph into the cerebral ventricle of hypophysectomized rat restores animal growth (43). If it is assumed that the 26-kDa form is equivalent to the glycosylated form, this is biologically active (44). However, if the radioreceptor data are accepted for cGH, then both the low (15 kDa) and the large (>40 kDa) form have low biological activities, at least in terms of binding to the adult liver receptor. It would therefore appear that the already very low ir-GH content of the chick embryo pituitary gland (31) may, in

fact, be an overestimate of GH biological activity in view of the relatively low proportion of the 22-kDa form and the high proportion of the 15-, 44-, and 60-kDa forms (it cannot be precluded that 15-kDa GH has biological activity but is acting *via* another receptor in an analogous manner to 16-kDa prolactin) (35). These data on the ontogenic shifts in GH variants in the chicken may provide an explanation for the observed discrepancies (lack of any correlation) between GH levels in the pituitary gland during growth and development of the turkey as determined by two assay methods, radioimmunoassay and radioreceptor assay (18). It might be speculated that only one GH variant is secreted in birds. This is supported on circumstancial grounds by the observation that there is a very close relationship (high correlation) between serum GH concentrations during growth and development in the turkey (18). However, in the 7-week-old broiler Montiel *et al.* (30) have shown that the majority of the variants existing in the pituitary are also present in the circulation. These facts deserve further investigation to elucidate their physiological significance.

In conclusion, the present data support the hypothesis that the synthesis/release of different GH variants is under differential control as their proportions change during development and growth. Analogous shifts in both glycosylated and nonglycosylated forms of both PRL and GH in plasma and pituitary have been observed during development/growth and other physiological states (e.g., pregnancy and lactation) in pig (45, 46). In view of the evidence of difference in biological activities for variants of GH and PRL (5–7, 28, 35, 45–48), it is a distinct possibility that GH and PRL actually each represent a spectrum of hormonal activities.

The authors thank Dr. Fernando A. Barrios and Leopoldo González-Santos (Image Analysis Unit) for digitization of the figures.

1. Lewis UJ. Variants of growth hormone and prolactin and their post-translational modifications. Ann Rev Physiol **46**:33–42, 1984.
2. Kishida M, Hirano T, Kubota J, Hasegawa S, Kawauchi H, Yamaguchi K, Shirahata K. Isolation of two forms of growth hormone secreted from eel pituitaries *in vitro*. Gen Comp Endocrinol **65**:478–488, 1987.
3. Lewis UJ, Singh RNP, Tutwiler GF, Sigel MB, VanderLaan EF, VanderLaan WP. Human growth hormone: A complex of proteins. Recent Prog Horm Res **36**:477–508, 1980.
4. Baumann G. Growth hormone heterogeneity: Genes, isohormones, variants, and binding proteins. Endocr Rev **12**:424–447, 1991.
5. Singh RNP, Seavey BK, Rice VP, Lindsey TT, Lewis UJ. Modified forms of human growth hormone with increased biological activities. Endocrinology **94**:883–891, 1974.
6. Hart IC, Blake LA, Chadwick PME, Payne GA, Simmonds AD. The heterogeneity of bovine growth hormone: Extraction from the pituitary of components with different biological and immunological properties. Biochem J **218**:573–581, 1984.
7. Arámburo C, Campbell RM, Scanes CG. Heterogeneity of chicken growth hormone (cGH): Identification of lipolytic and non-lipolytic variants. Life Sci **45**:2201–2207, 1989.
8. Arámburo C, Montiel JL, Perera G, Navarrete S, Sánchez R. Molecular isoforms of chicken growth hormone (cGH): Different bioactivities of cGH charge variants. Gen Comp Endocrinol **80**:59–67, 1990.
9. Houston B, Goddard C. Molecular forms of growth hormone in the chicken pituitary gland. J Endocrinol **116**:35–41, 1988.
10. Berghman LR, Lens P, Decuypere E, Kühn ER, Vandesande F. Glycosylated chicken growth hormone. Gen Comp Endocrinol **68**:408–414, 1987.
11. Arámburo C, Navarrete S, Montiel JL, Sánchez R, Berghman LR. Purification and electrophoretic analysis of glycosylated chicken growth hormone (G-cGH): Evidence of G-cGH isoforms. Gen Comp Endocrinol **84**:135–146, 1991.
12. Arámburo C, Donoghue D, Montiel JL, Berghman LR, Scanes CG. Phosphorylation of chicken growth hormone. Life Sci **47**:945–952, 1990.
13. Arámburo C, Montiel JL, Proudman JA, Berghman LR, Scanes CG. Phosphorylation of prolactin and growth hormone. J Mol Endocrinol **8**:181–191, 1992.
14. Arámburo C, Scanes CG. Endogenous proteolytic cleavage of growth hormone. (abstract) Program of the 73rd Annual Meeting of The Endocrine Society, Washington DC, p232, 1991.
15. Houston B, O'Neill IE, Mitchell MA, Goddard C. Purification and biological activity of a single charge isomer of pituitary-derived chicken growth hormone. J Endocrinol **125**:207–215, 1990.
16. Hoshino S, Suzuki M. Immunochemical determination of pituitary growth hormone separated by polyacrylamide disc gel electrophoresis in normal and dwarf chickens. Poultry Sci **61**:813–815, 1982.
17. Hoshino S, Yamamoto K. Synthesis and release of growth hormone, prolactin, and other proteins from the anterior pituitary of normal and dwarf chickens. Gen Comp Endocrinol **32**:7–16, 1977.
18. Proudman JA, Krishnan KA, Maruyama K. Ontogeny of pituitary and serum GH in growing turkeys as determined by radioimmunoassay and radioreceptor assay. Poult Sci **74**:1201–1208, 1995.
19. Harvey S, Scanes CG, Chadwick A, Bolton NJ. Growth hormone and prolactin secretion in growing domestic fowl: Influence of sex and breed. Br Poult Sci **20**:9–17, 1979.
20. Vasilatos-Younken R, Zarkower PG. Age-related changes in plasma ir-growth hormone secretory patterns in broiler pullets. Growth **51**:171–180, 1987.
21. Scanes CG. The physiology of growth, growth hormone, and other growth factors in poultry. CRC Crit Rev Poult Biol **1**:51–105, 1987.
22. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227**:680–685, 1970.
23. Arámburo C, Carranza M, Sánchez R, Perera G. Partial biochemical and biological characterization of purified chicken growth hormone (cGH): Isolation of cGH charge variants and evidence that cGH is phosphorylated. Gen Comp Endocrinol **76**:330–339, 1989.
24. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci U S A **76**:4350–4354, 1979.
25. Arámburo C, Sánchez R, Fenton B, Perera G, Valverde-RC. Desarrollo de un radioinmunoensayo homólogo y específico para la determinación de hormona de crecimiento de pollo (cGH). Vet Mex **20**:397–405, 1989.
26. Krishnan KA, Proudman JA, Bahr JM. Avian growth hormone receptor assay: Use of chicken and turkey liver membranes. Mol Cell Endocrinol **66**:125–134, 1989.
27. Hart IC, Chadwick PME, Boone TC, Langley KE, Rudman C, Souza LM. A comparison of the growth-promoting, lipolytic, diabetogenic, and immunological properties of pituitary and recombinant-DNA-derived bovine growth hormone (somatotropin). Biochem J **224**:93–100, 1984.
28. Frigeri LG, Peterson SM, Lewis UJ. The 20,000-dalton variant of human growth hormones: Lack of some early insulin-like effects. Biochem Biophys Res Commun **91**:778–782, 1979.
29. Ellis S, Grindeland RE, Nuenke JM, Callahan PX. Isolation and prop-

erties of rat and rabbit growth hormones. Ann N Y Acad Sci **148**:328–342, 1968.
30. Montiel JL, Berghman LR, Arámburo C. Identification of growth hormone molecular variants in chicken serum. Gen Comp Endocrinol **88**:298–306, 1992.
31. McCann-Levorse LM, Radecki SV, Donoghue DJ, Malamed S, Foster DN, Scanes CG. Ontogeny of pituitary growth hormone (GH) and GH mRNA in the chicken. Proc Soc Exp Biol Med **202**:109–113, 1993.
32. Farrington M, Hymer WC. Growth hormone aggregates in the rat adenohypophysis. Endocrinology **126**:1630–1638, 1990.
33. Clapp C. Analysis of the proteolytic cleavage of prolactin by the mammary gland and liver in the rat: Characterization of the cleaved and 16K forms. Endocrinology **121**:2055–2064, 1987.
34. Clapp C, Sears PS, Russell DH, Richards J, Levay-Young BK, Nicoll CS. Biological and immunological characterization of cleaved and 16K forms of rat prolactin. Endocrinology **125**:2892–2898, 1988.
35. Ferrara N, Clapp C, Weiner R. The 16K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells. Endocrinology **129**:896–900, 1991.
36. Scanes CG, Arámburo C, Campbell RM, Radecki SV. Chemistry and physiology of poultry growth hormone. In: Sharp P, Ed. Avian Endocrinology. Bristol: J Endocrinol Ltd, pp262–274, 1993.
37. Lewis VJ, Peterson SM, Bonewald LF, Seavey BK, Vander Laan WO. An interchain disulfide of human growth hormone. J Biol Chem **252**:3697–3702, 1977.
38. Becker GW, Bowsher RB, MacKeller WC, Poor ML, Tackitt PM, Riggin RM. Chemical, physical, and biological characterization of a dimer of biosynthetic human growth hormone. Biotechnol Appl Biochem **9**:478–487, 1987.
39. Arosio M, Nissim M, Ballabio M, Orefice R, Bazzoni N, Faglia G. Size heterogeneity of circulating growth hormone in acromegaly: "Big-big" GH forms are associated with inappropriately low IGF-I levels. Acta Endocrinol **125**:150–159, 1991.
40. Boguszewski CL, Hansson C, Boguszewski MC, Rosberg S, Wikland KA, Carlsson B, Carlsson LM. Circulating non-22 kDa growth hormone isoforms in healthy children of normal stature: Relation to height, body mass, and pubertal development. Eur J Endocrinol **137**:246–253, 1997.
41. Boguszewski CL, Jansson C, Boguszewski MC, Rosberg S, Carlsson B, Albertsson-Wikland K, Carlsson LM. Increased proportion of circulating non-22 kilodalton growth hormone isoforms in short children: A possible mechanism for growth failure. J Clin Endocrinol Metab **82**:2944–2949, 1997.
42. Tietjen GH, Grindeland RE, Hymer WC, Vasques M, Holley DC. Somatostatin (SRIF) and growth hormone releasing factor (rGRF-43) differentially regulate immunoassayable and bioassayable growth hormone secretion from two populations of rat somatotrophs. (abstract) Program of the 71st Annual Meeting of The Endocrine Society, Seattle, WA, p219, 1989.
43. Hymer WC, Wilbur DL. Dispersed pituitary cells: Their use *in vitro* and *in vivo*. In: Mahesh V, Muldoon T, Saxena B, Sadler W, Eds. Functional Correlates of Hormone Receptors in Reproduction. Amsterdan: Elsevier/North-Holland, pp13–44, 1989.
44. Berghman LR, Darras VM, Huybrechts LM, Decuypere E, Kühn ER. The glycosylated chicken growth hormone variant stimulates 5'-monodeiodination activity in the chick embryo. Med Sci Res **17**:391–392, 1989.
45. Sinha YN, Klemcke HG, Maurer RR, Jacobsen BP. Ontogeny of glycosylated and nonglycosylated forms of prolactin and growth hormone in porcine pituitary during fetal life. Proc Soc Exp Biol Med **194**:293–300, 1990.
46. Sinha YN, Klemcke HG, Maurer RR, Jacobsen BP. Changes in the glycosylated and nonglycosylated forms of prolactin and growth hormone in lean and obese pigs during pregnancy. Endocrinology **127**:410–418, 1990.
47. Stroud CM, Deaver DR, Peters JL, Loeper DC, Toth BE, Derr JA, Hymer WC. Prolactin variants in ram adenohypophyses vary with season. Endocrinology **130**:811–818, 1992.
48. Briski KP, Swanson GN, Sylvester PW. Size and charge heterogeneity of pituitary and plasma prolactin in the male rat. Neuroendocrinology **63**:437–445, 1996.