

# Bone growth in IUGR rats treated with growth hormone: A multivariate approach

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## SUMMARY

The aim of this study was to assess the morphological changes (in size and shape) in Intrauterine Growth Retarded (IUGR) rats treated with growth hormone.

Wistar albino rats were divided into the following groups: Control, Sham-operated, Intrauterine growth retarded, and Intrauterine growth retarded injected with growth hormone. Intrauterine growth retardation was induced by partial bending of uterine vessels on day 14 of pregnancy. After weaning, all groups received a stock diet *ad libitum*. At 84 days of age, the animals were X-rayed on the dorsal and lateral planes. Cranial and postcranial measurements were taken on each radiograph. Data were standardized and processed by principal component and discriminant analysis.

Both the first component and discriminant function revealed size differences between sexes and treatments. Males were larger than females. Sham males were the largest, followed by intrauterine growth retarded animals treated with growth hormone and intrauterine growth retarded rats. Sham and intrauterine growth retarded females treated with growth hormone were similar and larger than their IUGR counterparts. The second and subsequent components and discriminant functions described shape variations. Sham and intrauterine growth retarded animals treated with growth hormone formed a single cluster separated from intrauterine growth retarded rats.

Intrauterine growth retardation produces a differential reduction of bone dimensions, i.e. a modification of allometric growth. Growth hormone seems to promote harmonic growth recovery (size and shape) only in females.

**Key words:** Intrauterine growth retardation – Growth hormone – Catch up growth

## INTRODUCTION

Intrauterine growth retardation (IUGR) elicits a decrease in fetal growth rate that prevents an infant from obtaining his or her complete growth potential. There are two main patterns of intrauterine growth retardations (IUGR). If fetal growth is impaired during the first or second trimester, the infant will have symmetric growth restriction. This proportional lack of growth is caused by reduced fetal cellular proliferation of all organs and occurs in approximately 20% to 30% of IUGR infants (Lin et al., 1991). In contrast, asymmetric growth, in which an infant has a smaller abdominal size compared to head size, will occur if the decrease in growth velocity happens in the last trimester. This head-sparing phenomenon is the most common form of IUGR (~70%-80%) (Lin et al., 1991) and is attributed to the ability of the fetus to adapt, redistributing its cardiac output to the spleen, adrenal, coronary, and cerebral circulations (Brodsky and Christou, 2004). Most disproportionately growth-retarded

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newborns fail to catch up growth in 8-10% of young adults (Leger et al., 1997; Albertsson-Wikland et al., 1998).

In mammals, the postnatal period is an opportunity to recover any growth deficit. Parent-related variables (Karlberg and Albertsson-Wikland, 1995), prematurity and very low birth weight (Leger and Czernichow, 1994), disturbances of hormone production to hormonal unresponsiveness of target cells (Albertsson-Wikland, 1989), and post natal nutrition (Stanley and Speidel, 1985) have been proposed as possible factors predicting postnatal growth. According to Williams (1981), catch up is either complete or incomplete and this seems to depend on the amount of time available for recovery and the degree of growth retardation that occurs. Previous studies have demonstrated that IUGR caused by bilateral uterine vessel ligation during the last week of pregnancy leads to a persistent failure to catch up during lactation or in the post lactation period (Houdijk et al., 2000, Oyhenart et al., 2002; Oyhenart et al., 2003).

It is well established that the GH axis is an important mediator of the interaction between genetic potential and environmental factors (Bauer et al., 1985). In a previous study we demonstrated that GH stimulates body weight catch up growth in IUGR rats (Guimarey et al., 2003). However, while catch up appears to be a whole body response, the different parts of the body seem to respond in an individual way (Oyhenart et al., 2002).

Most morphological studies about catch up growth are focused on maturation and final height. However, there is a lack of studies concerning the effects of IUGR on allometric bone growth. The current analysis assesses the morphological changes (in size and shape) in IUGR rats treated with GH.

## MATERIAL AND METHODS

The animals employed in the present experiment were *Rattus norvegicus albinus*, var. Wistar, brought from the Comisión Nacional de Energía Atómica in 1997. They were grown as an out bred colony in the animal house of the Centro de Investigaciones en Genética Básica y Aplicada (CIGABA) for about twelve generations up to the experiment. The out bred condition was assured by maintaining a minimal stock of two hundred rats free of experimentation. Periodic genetic monitoring was carried out to avoid bottlenecks or similar effects. The animals were kept free of pathogens and treated in compliance with standardized institutional guidelines.

Rats were individually housed in solid stainless-steel cages (12"x12"x6.8"), which were cleaned three times a week. Room temperature

ranged from 21 to 25°C and the photoperiod was 12h of light, from 6:00 a.m. to 6:00 p.m. Animals were fed on a pelleted and sterilized commercial stock diet containing proteins (23%), carbohydrates (44%), lipids (11%), water (8%), fiber (5%), mineral mixture (3%) and vitamin mixture (1%).

Adult male and female rats were mated overnight. The beginning of pregnancy was determined by the presence of spermatozoa in vaginal smears. Pregnant rats were housed in individual steel boxes, fed on a stock diet *ad libitum* and assigned to one of three experimental groups: Control (C), IUGR, and Sham-operated (SH). Control dams did not receive any treatment. A lower midline laparotomy was performed in the mothers of the IUGR group on day 14th of gestation. Ether anaesthesia was given during surgery. The uterine vessels near the lower end of each uterine horn were partially ligated with a 3-0 silk suture (Oyhenart et al., 1998). Pregnancy was allowed to proceed until delivery. The SH dams were subjected to a laparotomy, without vessel bending, in order to isolate the effects of surgery from those of vessel ligation.

After delivery, IUGR (37 males and 31 females) and SH (15 males and 15 females) pups were cross-fostered to a well-nourished control dam. A standard diet was available *ad libitum* to mothers. Control pups did not receive any treatment (16 males and 15 females). The IUGR group was divided into two subgroups: non-treated IUGR (14 males and 14 females) and IUGR+GH injected subcutaneously with GH (3.0 mg/kg/day of Genotropin®) between day 21 (weaning) and day 60 (23 males and 17 females). Sham-operated pups were injected only with hormonal diluent (same doses and periodicity as in IUGR+GH group).

After weaning, all groups received a stock diet *ad libitum*. At 84 days of age, all the animals were X-rayed under light-ether anaesthesia on the dorsal and lateral planes. The following measurements were taken on each radiograph: *Cranial measurements*: neurocranial and splanchnocranial length, width, and height; *Postcranial measurements*: femur, tibial, and humeral length and width, vertebral length and upper, middle and lower pelvic widths.

Except for tibial width, all measurements were normally distributed. Data were processed by Principal Component Analysis (PCA) and Discriminant Analysis (DA). PCA is a multivariate method that can identify redundancy or correlations among a set of measurements or variables for the purpose of data reduction. DA is an inferential statistical technique employed to classify individuals or experimental units in two or more populations defined in a single way. This analysis built discriminant functions, which are linear

combinations of the original variables. The new variables or functions maximize the between-group variance and minimize the within-group. Because the functions are orthogonal and independent, their contributions to discrimination among groups are not overlapped (Johnson, 2001).

In order to determine the dimensionality of the model, the standardized eigenvalue method and screen plot graphics were employed. Statistical work was performed with the MVSP 3.13b package and SPSS 7.0.

RESULTS

Because of the similarity between the control and sham-operated animals, the latter were taken as a comparative reference.

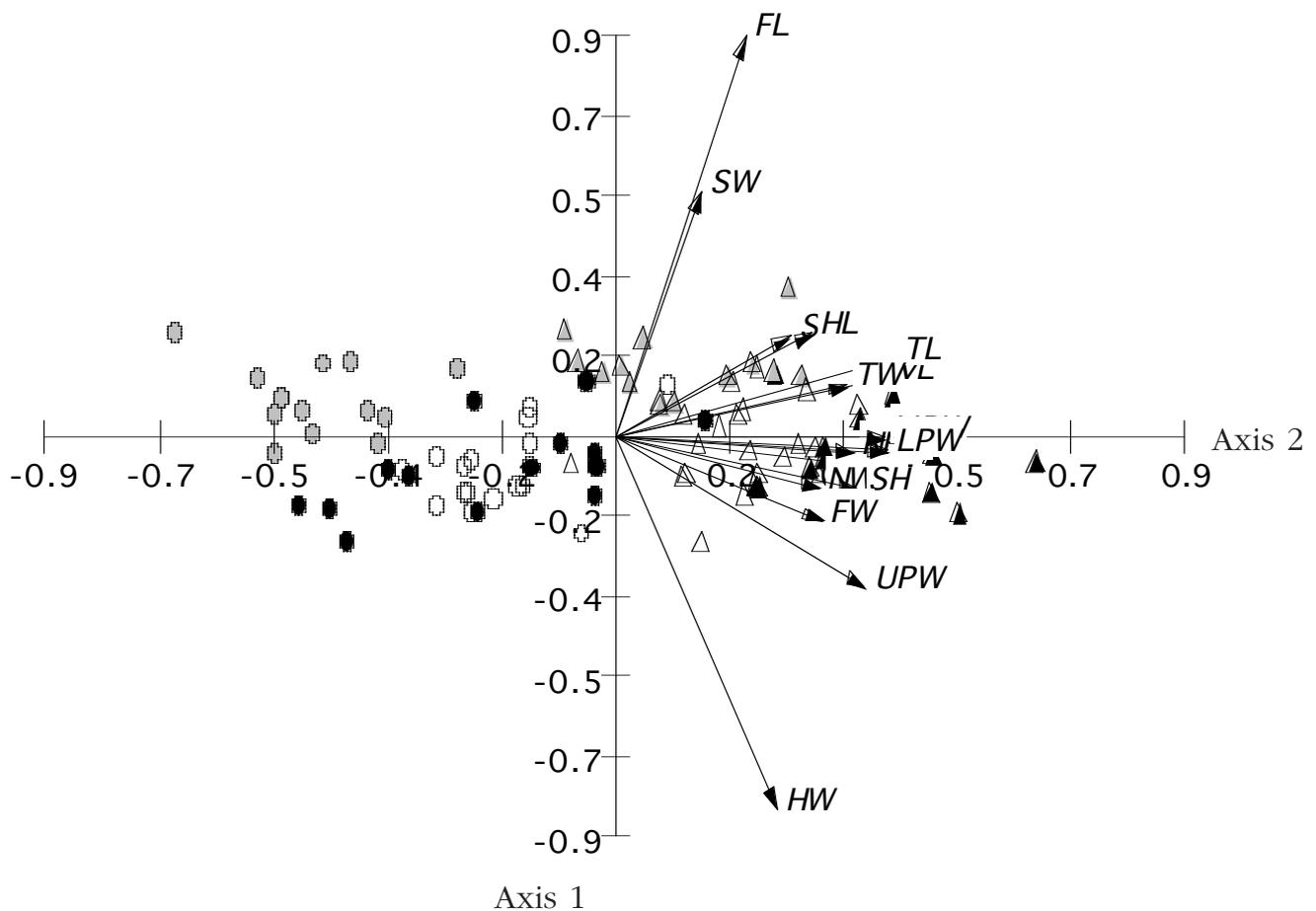
*Principal Components Analysis*

In both males and females, vertebral length showed greater variance than the other variables, even after being log-transformed. In order to avoid the primacy of this variable on the first component, the pooled analysis was performed by standardizing data.

The first axis of PCA captured 54.2% of variability, showing an eigenvalue of 9.2%. All the variables were positively correlated (Table 1). The greatest variation corresponded to sexual differences since males were larger than females. The remaining variation was seen among treatments. Sham males were the largest, followed by

**Table 1.-** Principal components analysis.

	Axis 1	Axis 2	Axis 3
<i>Eigenvalue</i>	9,22	1,28	0,99
<i>Variance explained (%)</i>	54,22	7,53	5,84
<i>Cumulative variance(%)</i>	54,22	61,75	67,59
<i>Variable loadings</i>			
NL: neurocranial length	0,27	-0,05	-0,07
NW: neurocranial width	0,22	-0,09	-0,34
NH: neurocranial height	0,26	-0,03	0,12
SL: splanchnocranial length	0,19	0,15	-0,08
SW: splanchnocranial width	0,10	0,37	-0,80
SH: splanchnocranial height	0,26	-0,09	0,06
HL: humeral length	0,21	0,16	-0,07
HW: humeral width	0,18	-0,57	-0,20
FL: femur length	0,14	0,61	0,30
FW: femur width	0,22	-0,13	-0,01
TL: tibial length	0,30	0,12	0,08
TW: tibial width	0,25	0,08	0,21
VL: vertebral length	0,29	0,09	-0,08
PL: pelvic length	0,29	-0,02	0,08
UPW: upper pelvic width	0,27	-0,23	0,09
MPW: upper pelvic width	0,29	0,01	0,05
LPW: lower pelvic width	0,29	-0,02	0,11



Vector scaling: 0.55

**Fig. 1.-** Plots of principal component scores: Axis 1-Axis 2. SH (black), IUGR (grey) and IUGR+GH (white); males (triangles), females (circles).

IUGR+GH and IUGR. Sham and IUGR+GH females were similar and larger than their IUGR counterparts (Fig. 1). The second axis explained 7.5% of variance, showing bipolar loadings (positive and negative correlations) (Table 1). The Sham and IUGR+GH rats formed a single cluster separated from the IUGR group (Fig. 1). The third axis explained only 5.8% of variation and did not show a clear separation of groups (Table 1, Fig. 2). Given the scarce contribution to the total variance and low eigenvalues, the remaining axes were excluded.

*Discriminant analysis*

The general model was statistically significant (Wilks' Lambda: 0.0056; F: 85.37;  $P < 0,0000$ ). The five discriminant functions were also significant and explained 66, 84, 93, 97 and 100% of the total cumulative variance (Table 2).

The correlation coefficients for each variable on the first three functions are listed in table 3. The first function was defined –in decreasing order- by TL, PL, UPW, MPW, FW, HL and NW. All of them were negatively correlated and revealed strong differences between sexes (Table 3, Fig. 3). The second one –defined by UPW (negatively correlated), TL and SW (positively correlated)- discriminated the IUGR rats

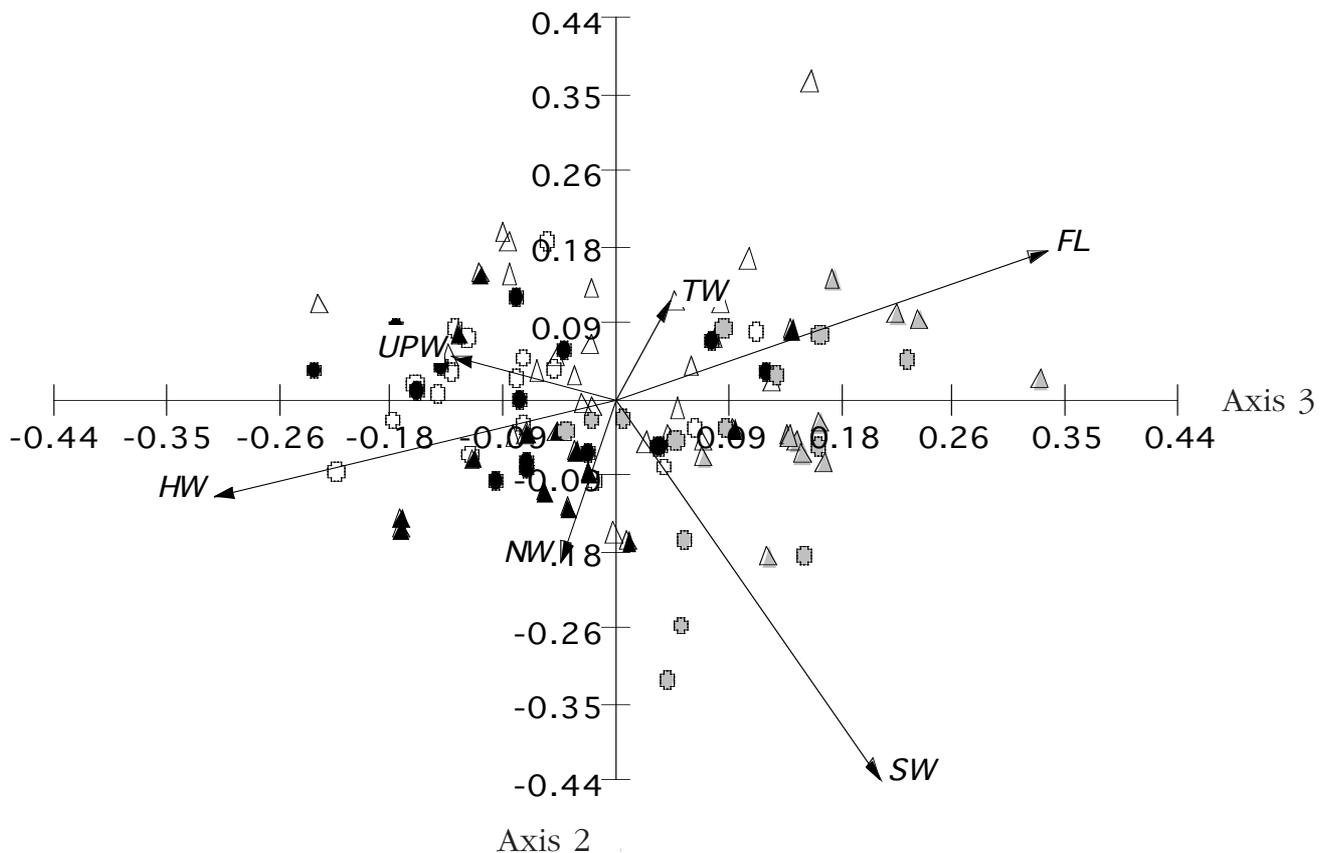
from the remaining groups (Table 3, Fig. 3). The third function was defined by FW, MPW, NW, HL, SW and SL, which were negatively correlated. This function only separated the Sham and IUGR+GH males (Table 3, Fig. 4).

**Table 2.-** Discriminant functions: eigenvalues and variance explained.

Function	Eigen-value	Variance (%)	Wilks'	p-level
1	9,2	66,0	0,006	0,000
2	2,5	18,0	0,057	0,000
3	1,5	9,0	0,201	0,000
4	0,6	4,0	0,458	0,000
5	0,4	3,0	0,731	0,013

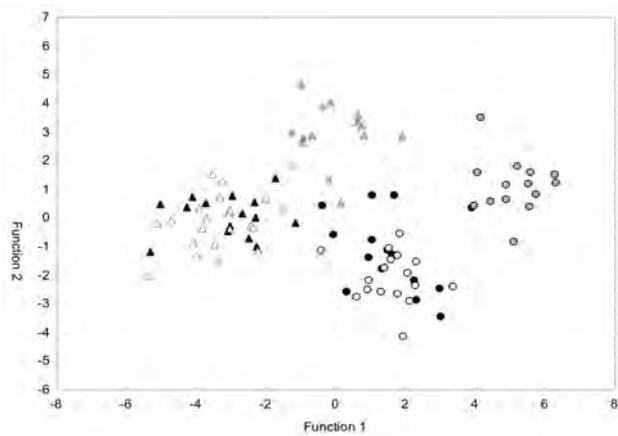
**Table 3.-** Correlation coefficients for each variable on the first three discriminant functions.

Variable	Function 1	Function 2	Function 3	Wilks/Lambda	p-level
NL	-0,173	-0,221	0,041	0,006	0,218
NW	-0,012	-0,128	-0,258	0,007	0,008
NH	-0,238	-0,226	0,069	0,006	0,137
SL	0,167	0,285	-0,160	0,007	0,011
SW	0,586	0,428	-0,268	0,010	0,000
SH	0,089	0,000	0,015	0,006	0,344
HL	0,216	0,101	-0,491	0,007	0,005
HW	-0,138	-0,383	0,027	0,006	0,053
FL	0,119	0,150	0,416	0,006	0,063
FW	-0,055	-0,223	-0,783	0,008	0,000
TL	-0,746	0,658	0,492	0,011	0,000
TW	-0,163	-0,093	-0,194	0,006	0,243
VL	0,098	0,198	-0,002	0,006	0,433
PL	-0,481	-0,017	0,274	0,007	0,035
UPW	-0,125	-0,873	0,260	0,008	0,000
MPW	0,212	0,461	-0,569	0,007	0,028
LPW	-0,349	-0,124	-0,021	0,006	0,111

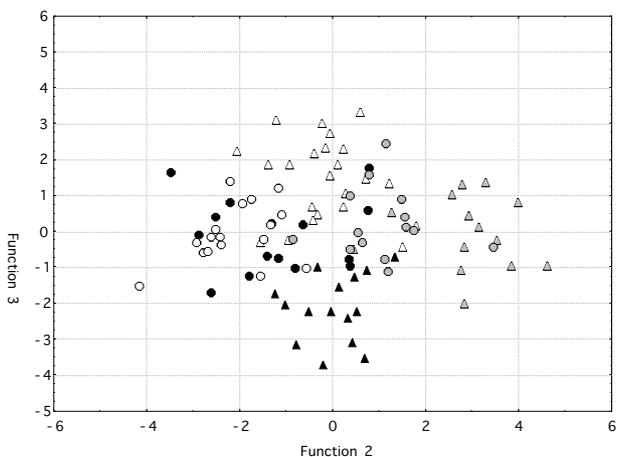


Vector scaling: 0.55

**Fig. 2.-** Plots of principal component scores: Axis 2-Axis 3. SH (black), IUGR (grey) and IUGR+GH (white); males (triangles), females (circles).



**Fig. 3.-** Plots of discriminant analysis: Function 1 vs. Function 2. SH (black), IUGR (grey) and IUGR+GH (white); males (triangles), females (circles).



**Fig. 4.-** Plots of discriminant analysis: Function 2 vs. Function 3. SH (black), IUGR (grey) and IUGR+GH (white); males (triangles), females (circles).

## DISCUSSION

PCA and DA methods are commonly used by biologists to analyze morphological distances between species, populations, sexes, etc. In the present study, these methods were introduced in order to analyze many variables at the same time and estimate differences in absolute (size) and relative (shape) growth among groups.

Almost all the variation in body size was explained by the first PCA axis. This component revealed a clear separation of the sexes and contributed to the differentiation of the treatments. While sexual dimorphism was expressed as a larger body size of the males, differences among groups were represented -in decreasing magnitude- by the Sham, IUGR+GH, and IUGR non-treated. The variable loadings of component I were all positive, with a narrow range of values, indicating that each dimension contributed equally to differences in body size (Fig. 1). Thus, the first discriminating function separated males from females and IUGR animals from the other groups, confirming the size effect as the main

element responsible for discrimination among groups (Fig. 3). The sexual differences revealed the typical pattern of sexual dimorphism in rat size. (Orden, 1997; Orden et al., 1999). The size differences among treatments reflect the influence of uteroplacental blood supply on prenatal growth (Dressino et al., 2002; Oyhenart et al., 2003).

The body shape variation was seen in component II, in which IUGR rats were grouped towards positive values and the other groups towards negative values (Fig. 1). Similar results were obtained by the second discriminant function (Fig. 3), confirming a strong effect of IUGR on body proportions, i.e. by altering the relative growth of different bones (Woodall et al., 1996; Oyhenart et al., 2002). In the IUGR animals, appositional growth (bone widths) was more affected than linear growth (bone lengths). These findings are in agreement with those previously reported by Oyhenart et al. (2002), who found that the widths of long bones remained significantly retarded in IUGR rats and also by Reichling and German (2000) in rats with protein deficit. These results also suggest that nutritional rehabilitation of IUGR animals is not enough to overcome the growth restriction.

The growth retardation was "reversed" by GH treatment. This hormone promoted a complete catch up in females but not in males. This dimorphic response to GH has been found in several studies. Thus, Oyhenart et al. (2003) found that GH enhanced weight gain in IUGR female rats and neurocranial growth in males. Also, Guimarey et al. (2003) found an earlier and stronger effect of GH on body weight in IUGR female rats as compared to males. According to Rol de Lama et al. (2000), there are sexual differences in the growth rate of peripubertal rats. Males have a maximal growth rate and GH administration seems unable to increase it. In contrast, females have a minimal growth rate, which can be enhanced by GH. However, the physiological causes underlying such mechanisms are not well known.

In sum, both PCA and DA indicated that IUGR caused a differential reduction of bone dimensions, i.e. a modification of allometric growth. GH promoted harmonic growth recovery (absolute and relative size). However, this recovery depended on sex, since a complete catch up was only observed in females.

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