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ORIGINAL ARTICLE

Bone growth and sexual dimorphism at birth in intrauterine-growth-retarded rats

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Abstract This paper addresses the effect of a reduction of uterine blood flow (RUB) on postcranial bone growth in rats. The objectives were: (1) to discover and characterize the changes evoked by growth retardation through a reduction in placental blood flow, (2) to see if the resulting growth retardation is different in each bone, and (3) to analyze any sex-specific features. RUB was induced by the partial bending of uterine vessels at day 1 of pregnancy. Control and sham-operated animals were also included. The animals were X-rayed at birth. The lengths and widths of the humerus, radius, and femur and pelvic length, interischial, interpubic, and pubic widths were measured. Data were analyzed by ANOVA and LSD post hoc tests. The intersubject analysis showed significant differences between groups and non-significant differences between sexes. In males, sham-operated and RUB showed significant differences in pelvic lengths and widths, and humeral, radial, femoral, and tibial widths. In females, there were significant differences only for humeral widths, radial lengths and widths, and femoral and tibial widths. We conclude that reduced blood flow delays appendicular bone growth as observed at birth. Pelvic length was more affected than that of the limbs. The widths of the pelvic and limbs bones, in turn, were more altered than the lengths, and the growth of the males more than that of the females. Partial bending of uterine vessels compromised postcranial growth, though under such disadvantageous circumstances the females proved to be more capable of growing and thus more resilient than the males.

Keywords Intrauterine growth retardation · Sexual dimorphism · Bone growth

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Introduction

Fetal growth is a complex process that depends on the genetics of the fetus; the availability to the fetus of nutrients and oxygen; the maternal nutrition; and various growth factors and hormones of maternal, fetal, and placental origin (Gicquel and Le Bouc 2006). According to Myatt (2006), placental function evolves in a carefully orchestrated developmental cascade throughout gestation. Disruption of this process can lead to abnormal development of the placental vasculature or of the trophoblast. The timing of a given developmental insult will be critical for consequent placental function and hence the epigenetic

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programming of the fetus. Any disturbance in the placental–fetal circulation will therefore have severe consequences on the supply of important nutrients to the fetus, with intrauterine growth retardation (IUGR) being the end result (Barker 1998; McMillen et al. 2001).

Uterine-artery ligation in the gestational rat is one of the methods most frequently used to study the consequences of uteroplacental insufficiency (Wigglesworth 1964; Oyhenart et al. 1998; Guimarey et al. 2003). Accordingly, the transfer of knowledge gained from animal models (e.g., rats and mice) to humans would further our understanding of intrauterine growth restriction (Chaddha et al. 2004). In this regard, previous studies have shown that IUGR produces significant deficiencies in body and brain weight as well as in bone dimensions measured at birth (Dressino et al. 2002; Oyhenart et al. 2002; Huizinga et al. 2004; Schreuder et al. 2006).

The specific physiological adaptations of the fetus to an adverse intrauterine environment observed to date have included an activation of the fetal hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, an associated increase in circulating cortisol and noradrenaline concentrations, and a consequent constraint of the fetal growth rate. The extent and range of the fetal physiological response to chronic placental insufficiency are determined by the duration of exposure and the degree of severity of the restriction in substrate supply (Morrison 2008). It is clear that the physiological compensations of the fetus in response to a suboptimal intrauterine environment are of critical importance in determining the health and survival of the fetus and of the newborn (McMillen et al. 2001).

Birth weight by itself, therefore, is not a sufficient parameter to identify IUGR. Fetal growth estimation in humans, which takes maternal and fetal characteristics into account, has recently been found to enable a precise evaluation of fetal growth restriction by identifying newborns who have failed to reach their genetic growth potential and those at high risk of an adverse perinatal outcome (Beltrand et al. 2008). Accordingly, environmental stimuli such as nutrition will necessarily influence the ultimate skeletal size of an individual fetus over and above its innate genetic potential (Prader et al. 1963; Dammrich 1991; Loveridge and Noble 1994).

Whereas most studies follow increase in body weight or growth in overall body length in IUGR animal models, only a few have examined the growth of the pelvis and the long-limb bones. This paper thus addressed the effect of a reduction in uterine blood flow (RUB) on postcranial bone growth. The objectives were: (1) to discover and characterize the changes evoked by growth retardation through a reduction in placental blood flow, (2) to see if the resulting growth retardation is different in each bone, and (3) to analyze any sex-specific features.

Materials and methods

Animals

Rattus norvegicus albinus, var. Wistar from the Instituto de Genética Veterinaria (IGEVET) were employed. The animals were kept free of pathogens and treated in compliance with standardized institutional guidelines.

Rats were housed individually in solid stainless steel cages (30.5 × 30.5 × 17.25 cm), which were cleaned three times a week. The room temperature ranged from 21 to 25°C and the photoperiod was 12 h of light, from 6:00 a.m. to 6:00 p.m. Thee rats 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%).

Females (200–250 g body weight) were mated overnight with adult males. The beginning of pregnancy was determined by the presence of spermatozoa in vaginal smears. Pregnant rats were housed in individual steel boxes, fed on stock diet and water ad libitum and assigned to one of three experimental groups: control (C) ($n = 6$), RUB ($n = 5$), and sham-operated (SH) ($n = 5$).

Procedure

Control dams did not receive any treatment. The RUB was induced by the technique originally introduced by Wigglesworth (1964) and subsequently modified by Oyhenart et al. (1998). We anesthetized animals intramuscularly with Ketalar (Parke Davis; 50 µl per 100 g body weight) on the first day of gestation and performed a lower-midline laparotomy while giving supplementary light-ether anesthesia during the surgery. After opening the peritoneal cavity, we exposed the uterus and then ligated the vessels near the lower end of each uterine horn, with a 3-0 silk suture. In order to separate the effects of the surgery from those of vessel bending, the procedure applied to the sham-operated animals was similar to that used for the RUB rats, except that the uterine vessels were left unobstructed. Pregnancy continued until delivery.

Measurements

After delivery, C (20 males and 18 females); RUB (16 males and 14 females) and SH (16 males and 14 females) pups were weighed on a Mettler scale (0.1 mg precision). We took dorsal and lateral radiographs of each animal using a Siemens Heliophos 4 at 240 mA/125 kV (Fig. 1). Shooths were regulated at 50 mA, 0.04 seg, 30–40 kW. A 110-cm focus-film (AGFA Mamoray MR5-II, 18 × 24 cm) distance was used to reduce the magnification effect,

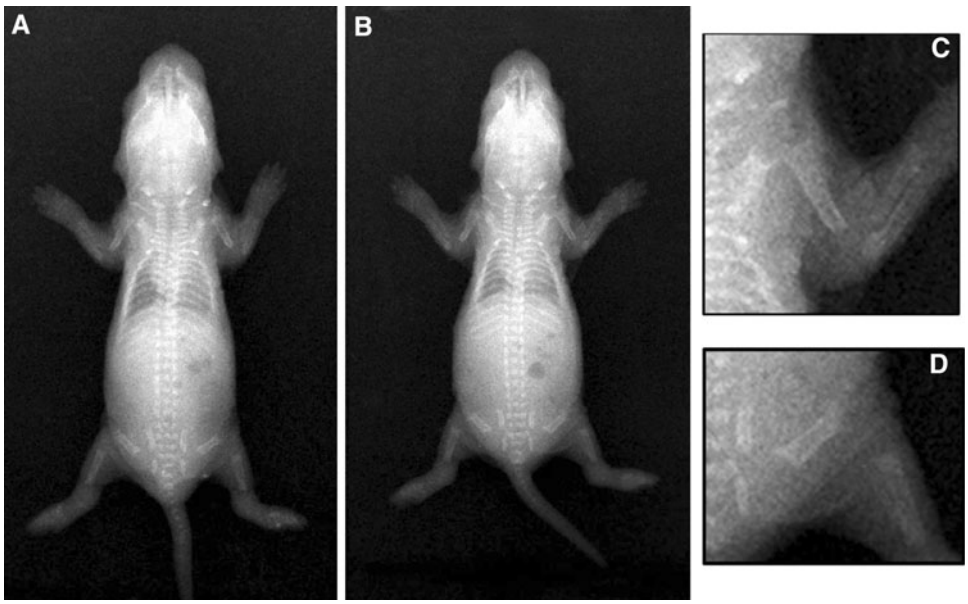

Fig. 1a–d Dorsal radiographs of the newborn rats. **a** Sham-operated pup, **b** reduction of uterine blood flow (RUB) pup. **c**, **d** Enlarged views from **a** and **b**: hindlimb (**c**), forelimb and pelvis (**d**)

calculated as MgC = Bx/Ax, where MgC is the magnification coefficient, Ax a variable measured on the 1st day radiograph and Bx the same variable measured on the skull (Cesani et al. 2006). Light-ether anaesthesia was given during the procedure. The measurements were taken on each radiograph using a Mitutoyo digital caliper (0.05 mm precision). Figure 2 shows a schema of the rat and of the following variables employed:

Pelvis

Pelvic length (PL)	from the anterior tip of the ilium to the posterior tip of the ischium
Interiliac width (IlW)	maximal iliac width
Interpubic width (PW)	minimal internal-pubic width
Interischial width (IsW)	maximal ischial width

Forelimb

Humeral length (HL)	the distance from the head of the humerus to the middle of the condyle
Humeral width (HW)	the humeral width at its midpoint
Radial length (RL)	the distance from the middle portion of the proximal diaphysis to the middle portion of the distal diaphysis
Radial width (RW)	the width of the radius at its midpoint

Hindlimb

Femoral length (FL)	the distance from the head of the femur to the midpoint of the condyle
Femoral width (FW)	the femoral width at its midpoint
Tibial length (TL)	the distance from the middle of the anterior end of the tibia to the midpoint of the malleolus
Tibial width (TW)	the tibial width at its midpoint

Statistical analysis

We estimated the goodness-of-fit for the frequency distributions by the Kolmogorov–Smirnov test for one sample, and found normal distributions in all cases. The data were processed by multifactor analysis of variance (ANOVA). When F values were significant ($P < 0.05$), the treatment and sex comparisons by age were made by the least-square-differences (LSD) and multiple-range tests. Statistical computations were carried out using the program SPSS 7.5.

For graphical comparisons, and according to the following formula, mean values were standardized by the relative difference between mean (RDM %):

$$\mathrm{RDM} = 100 \times (X1 - X2)/X1$$

For treatment comparisons: $X1$ = mean values in the sham-operated group, and $X2$ = mean values in RUB group; while for sex comparisons, $X1$ = mean values in males, and $X2$ = mean values in females. This standardization method has been employed frequently (see Oyhenart et al. 1998). In

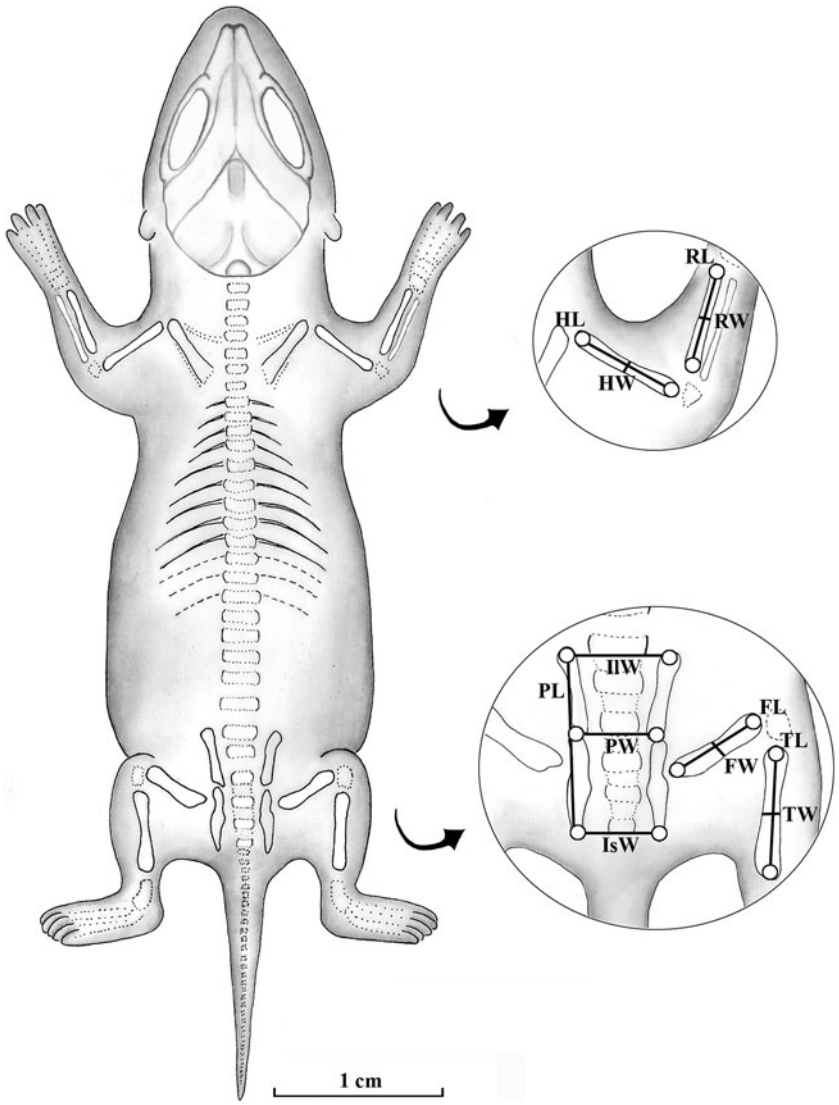

Fig. 2 Schema of the newborn rat showing the measurements employed. Pelvic: *PL* pelvic length, *IlW* interiliac width, *PW* interpubic width, *IsW* interischial width. Forelimb: *HL* humeral length, *HW* humeral width, *RL* radial length, *RW* radial width. Hindlimb: *FL* femoral length, *FW* femoral width, *TL* tibial length, *TW* tibial width

its current form, it reduces any difference to a percent value, expression of which cannot be affected by scaling or sense.

Results

Mean and standard deviation are shown in Table 1.

The *F* values indicated significant differences with respect to treatment in the majority of the variables analyzed. The exception was for PW, HL and RL. The differences between males and females were nonsignificant and interactions between parameters were observed only for IlW and RL (Table 2).

Although the control males and females had significantly greater values than did the sham-operated animals in 30% of the variables analyzed (Table 3), the latter were used as controls since that surgery more closely simulated the experimental intervention. When the Sham-operated and the RUB males were compared, significant differences were seen in the pelvic (PL, IsW, IlW) as well as the humeral, radial, femoral, and tibial widths (HW, RW, FW, TW). In females, there were only significant differences for the humeral width (HW), the radial length and width (RL, RW), and the femoral and tibial widths (FW, TW) (Table 3).

Significant sexual differences in Sham-operated were evident for IlW, while in RUB for PL and RL (Table 4).

Discussion

During the normal growth and development of both man and animals, the bones increase in length as well as in width and undergo progressive changes in their architecture—a process known as skeletal maturation. Each differentiated cell type has it own epigenetic signature, with its maturational destiny reflecting its particular genotype,

Table 1 Mean and standard deviation (SD) for male and female pups

Variable	Treatment											
	Males						Females					
	Control		RUB		Sham-operated		Control		RUB		Sham-operated	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Pelvis												
Pelvic length	6.63	0.38	5.43	0.72	6.20	0.51	6.08	0.46	5.26	0.45	5.8	0.60
Interiliac width	5.07	0.46	4.17	0.57	4.64	0.33	4.69	0.27	4.23	0.18	4.48	0.37
Interpubic width	3.69	0.33	3.13	0.30	3.42	0.24	3.50	0.27	3.19	0.31	3.16	0.39
Interischial width	4.19	0.11	3.60	0.33	3.98	0.16	4.03	0.17	3.63	0.28	3.67	0.32
Forelimb												
Humerus length	5.08	0.50	4.17	0.54	4.60	0.44	4.92	0.47	4.26	0.60	4.28	0.66
Humerus width	1.18	0.08	0.94	0.16	1.15	0.13	1.18	0.11	0.94	0.09	1.15	0.16
Radius length	4.42	0.21	3.89	0.46	3.84	0.43	4.05	0.35	3.47	0.32	3.94	0.43
Radius width	1.04	0.06	0.61	0.14	0.91	0.12	0.97	0.11	0.56	0.12	0.84	0.14
Hindlimb												
Femur length	4.09	0.24	3.23	0.44	3.58	0.30	3.76	0.49	3.28	0.46	3.44	0.50
Femur width	1.37	0.15	1.00	0.20	1.23	0.11	1.31	0.16	0.99	0.10	1.19	0.08
Tibia length	4.76	0.29	3.92	0.59	4.33	0.53	4.58	0.56	4.04	0.55	4.32	0.59
Tibia width	1.23	0.12	0.91	0.19	1.11	0.11	1.17	0.11	0.94	0.12	1.12	0.10

RUB reduction of uterine blood flow

Table 2 Multiway variance analysis for treatment, sex and interaction factors

Variable	Treatment		Sex		Treatment × sex	
	F value	*P*	*F* value	*P*	*F* value	*P*
Pelvis						
Pelvic length	19.00	0.000**	3.48	0.067 ns	0.63	0.430 ns
Interiliac width	8.50	0.005**	3.74	0.058 ns	5.41	0.024*
Interpubic width	2.72	0.104 ns	1.35	0.250 ns	4.03	0.050 ns
Interischial width	12.54	0.001**	0.26	0.609 ns	1.21	0.277 ns
Forelimb						
Humeral length	2.46	0.123 ns	0.65	0.423 ns	2.01	0.161 ns
Humeral width	32.82	0.000**	0.00	0.990 ns	0.00	0.990 ns
Radial length	3.69	0.060 ns	2.15	0.148 ns	5.96	0.018*
Radial width	74.04	0.000**	3.17	0.081 ns	0.10	0.751 ns
Hindlimb						
Femoral length	5.44	0.023*	0.13	0.723 ns	0.71	0.403 ns
Femoral width	36.63	0.000**	0.45	0.504 ns	0.22	0.643 ns
Tibial length	5.60	0.021*	0.15	0.697 ns	0.21	0.648 ns
Tibial width	27.74	0.000**	0.41	0.522 ns	0.05	0.831 ns

ns non-significant

*$P < 0.05$, **$P < 0.01$

developmental history, and environmental influences, and is ultimately reflected in the phenotype of the cell and of the organism. Some cells undergo major epigenetic reprogramming during fetal development. The proper, or improper, handling of this highly sensitive period may have significant short-term and even long-term effects on the newborn and its progeny (Nafee et al. 2008).

Even under extreme conditions, such as those marked by the cessation of weight gain, the body maintains a priority for longitudinal skeletal growth (McCance 1960; Stewart et al. 1975; Widdowson and McCance 1963). Nevertheless, the development of the skeleton is critically affected by malnutrition, and accordingly several studies have examined the effect of nutritional deficiencies on bone growth

Table 3 Treatment differences in males and females pups

Variable	Comparison (C–SH)						Comparison (SH–RUB)					
	Males			Females			Males			Females		
	Mean difference	SE	*P*	Mean difference	SE	*P*	Mean difference	SE	*P*	Mean difference	SE	*P*
Pelvis												
Pelvic length	0.43	0.18	0.152 ns	0.28	0.19	0.655 ns	0.78	0.19	0.001**	0.54	0.20	0.084 ns
Interiliac width	0.20	0.08	0.117 ns	0.36	0.08	0.001**	0.38	0.08	0.000**	0.04	0.09	0.997 ns
Interpubic width	0.27	0.10	0.115 ns	0.64	0.19	0.014*	0.29	0.11	0.087 ns	−0.03	0.12	1.000 ns
Interischial width	0.42	0.13	0.022*	0.21	0.14	0.659 ns	0.48	0.14	0.011*	0.25	0.15	0.540 ns
Forelimb												
Humeral length	0.48	0.18	0.093 ns	0.03	0.04	0.989 ns	0.43	0.19	0.208 ns	0.02	0.20	1.000 ns
Humeral width	0.03	0.04	0.979 ns	0.11	0.13	0.965 ns	0.21	0.04	0.000**	0.21	0.05	0.000**
Radial length	0.58	0.12	0.000**	0.11	0.13	0.965 ns	−0.06	0.13	0.998 ns	0.47	0.14	0.014*
Radial width	0.13	0.04	0.011*	0.13	0.04	0.024*	0.30	0.04	0.000**	0.28	0.04	0.000**
Hindlimb												
Femoral length	0.51	0.14	0.004**	0.32	0.15	0.255 ns	0.35	0.14	0.159 ns	0.16	0.15	0.894 ns
Femoral width	0.14	0.05	0.051 ns	0.13	0.05	0.151 ns	0.23	0.05	0.000**	0.19	0.05	0.008**
Tibial length	0.43	0.17	0.146 ns	0.26	0.18	0.717 ns	0.41	0.18	0.226 ns	0.28	0.20	0.714 ns
Tibial width	0.12	0.04	0.059 ns	0.05	0.05	0.880 ns	0.19	0.05	0.001**	0.18	0.05	0.006**

ns non-significant

*$P < 0.05$; **$P < 0.01$

Table 4 Sexual differences in Sham-operated and RUB pups

	Sham-operated			RUB		
	Mean difference	SE	*P*	Mean difference	SE	*P*
Pelvis						
Pelvic length	0.40	0.19	0.304 ns	−1.21	0.18	0.000**
Interiliac width	0.31	0.09	0.007**	−0.03	0.09	0.999 ns
Interpubic width	0.25	0.11	0.224 ns	−0.07	0.11	0.991 ns
Interischial width	0.17	0.14	0.857 ns	−0.06	0.14	0.998 ns
Forelimb						
Humeral length	0.32	0.19	0.567 ns	−0.09	0.19	0.998 ns
Humeral width	0.00	0.05	1.000 ns	0.00	0.05	1.000 ns
Radial length	−0.11	0.14	0.971 ns	0.42	0.14	0.029*
Radial width	0.07	0.04	0.558 ns	0.05	0.04	0.855 ns
Hindlimb						
Femoral length	0.13	0.15	0.950 ns	−0.05	0.15	0.999 ns
Femoral width	0.04	0.05	0.975 ns	0.01	0.05	1.000 ns
Tibial length	0.01	0.19	1.000 ns	−0.12	0.19	0.986 ns
Tibial width	−0.02	0.05	1.000 ns	−0.03	0.05	0.988 ns

ns non-significant

*$P < 0.05$, **$P < 0.01$

during gestation (Cameron and Eshelman 1996). Diverse forms of retarded skeletal growth have been reported, depending on the type of malnutrition and/or its intensity, as well as on the period in which the stress was applied. Our results here have shown that impaired placental blood supply delays postcranial bone growth in fetuses as manifest at birth. Mughal et al. (1989) reported that a reduced placental blood supply causing experimental fetal-growth retardation also gave rise to a reduced placental calcium-ion transport that is proportional to the reduction in fetal body size. With respect to infants that are small for their gestational age, Namgung and Tsang (2000) concluded that, in theory, a reduced uteroplacental blood flow may produce a diminished transplacental mineral supply and

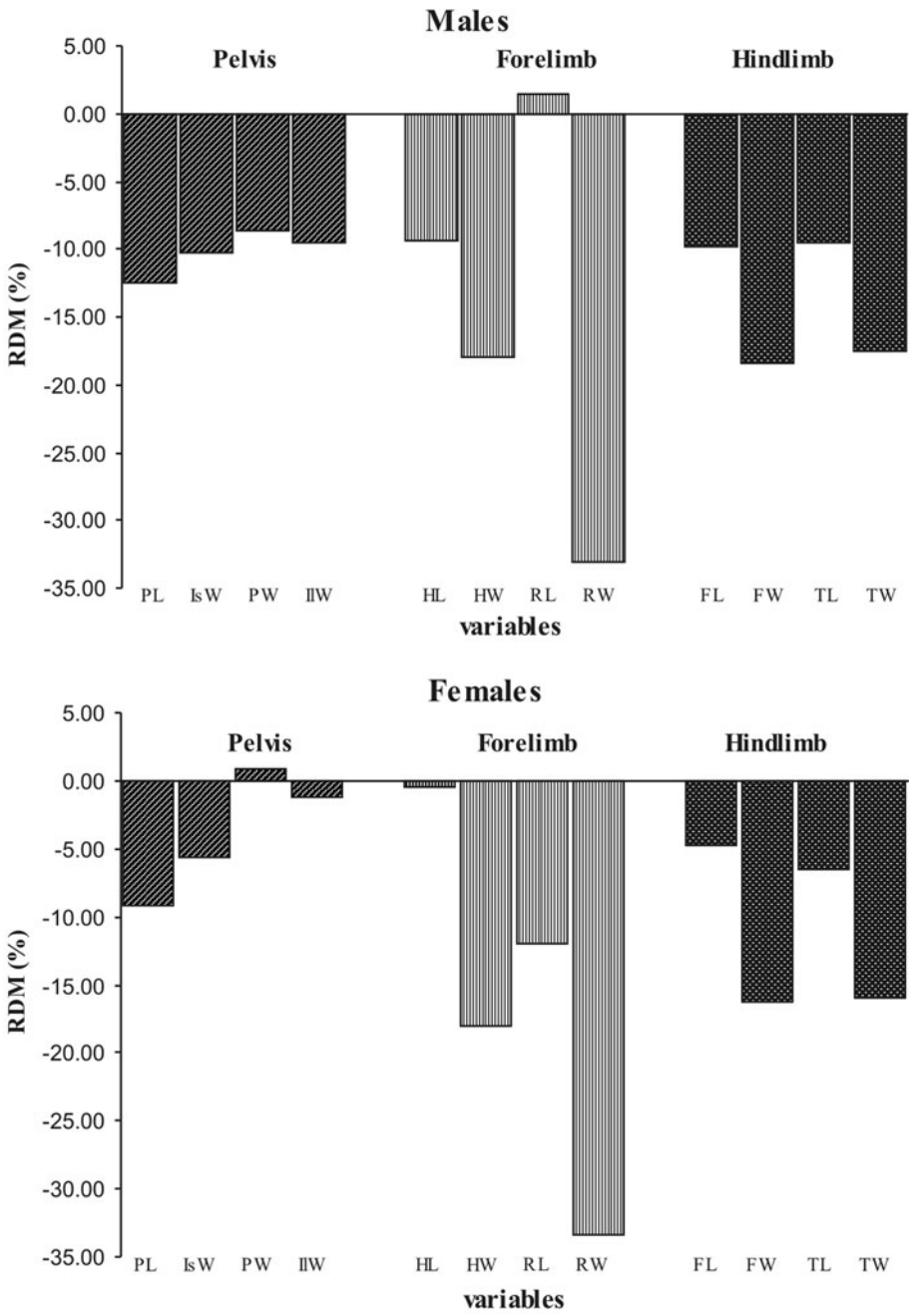

Fig. 3 Percentage mean differences (%) between Sham-operated and RUB animals. *PL* Pelvic length, *IsW* interischial width, *PW* interpubic width, *IlW* interiliac width, *HL* humeral length, *HW* humeral width, *RL* radial length, *RW* radial width, *FL* femoral length, *FW* femoral width, *TL* tibial length, *TW* tibial width

therefore a decrease in fetal-bone formation. Here, though, the experimental stress applied had a differential effect on each bone measured. For example, pelvic length was shorter in the RUB than in the sham-operated rats (a reduction of 13 and 9% in males and females, respectively), while the lengths of the fore and hind limbs were unaffected (Fig. 3). These changes in body proportions confirm previous results. When IUGR was induced during the last trimester of pregnancy, Oyhenart et al. (2002) also found that the length of the pelvis was relatively more affected than that of the limb bones.

By contrast, in both the pelvis and the limbs, the widths of the bones were more affected than the lengths. Accordingly, Adams and Berridge (1969) had reported that they had no doubt that there was less trabecular bone than normal in the metacarpals of children with kwashiorkor, and that these changes in the amounts of cortical and trabecular bone resemble those found by Adams (1969) in the long bones of animals with experimentally induced protein deficiency. Moreover, the widths of the bones analyzed had also shown a starvation-associated variation. In the present study, the forelimb widths of both sexes evinced the most

pronounced growth retardation (HW 18%, RW 33%), followed next by those of the hindlimb (FW 17%, TW 16%). Finally, the widths of the pelvic bones likewise showed marked degrees of growth retardation, but that differed between males (IsW 10%, PW 9%, IlW 10%) and females (IsW 6%, PW 1%, IlW 1%) (Fig. 3). The mechanisms involved in this aspect of the differentiation process are difficult to explain. Nevertheless, any reasonable conjecture must take into account the fact that the pelvis is a complex structure, critical for two significant functions in mammals: locomotion and parturition.

Sexual dimorphism exists between the sexes of any species in the form of differences in either the shape or the size of a given structure. While in mammals dimorphism with respect to some characters already exists at birth (Oyhenart et al. 1998), the difference between the sexes more typically develops over the course of the postnatal phase of ontogeny (Berdnikovs et al. 2007). The sexual dimorphism of the human pelvis is intimately linked to its adaptive functions. Although comparative studies support the parturition explanation for the adult pelvic-shape dimorphism, little is known about differences between the sexes in the postnatal growth and differentiation of the pelvis. Bernstein and Crelin (2005) reported that normal pelvic dimorphism in rats is the result of the male pelvis acquiring morphological features during postnatal development that differ from those of the female, with the first appearance of dimorphic differences occurring at 32 days of age. This late differentiation of the pelvic bones would provide a reasonable explanation for the scarce sex differences that we found in both the sham-operated and the RUB animals.

According to Desai et al. (1996), during periods of inadequate nutrition, selective changes in the growth rates of specific organs might differ between the sexes. As an example, the growth of the tibia in rats may be influenced by sex, breed, or strain as well as by nutritional status (Cameron and Eshelman 1996; Miller and German 1999). Accordingly, in our experiment, females were more resistant to prenatal stress; they manifested higher pelvic size and greater lengths and widths in the femur and tibia. These results add further evidence to previous reports of sex differences seen at the time of birth in the response to intrauterine stress (Oyhenart et al. 1998; Dressino et al. 2002). In summation, according to the terminology of Tanner, female growth could be termed "better canalized"—that is, females appear to exhibit a greater capacity to maintain homeostasis in a relatively constant fashion throughout prenatal growth than do males (Tanner 1962).

We conclude that a reduced placental blood supply in rats delays appendicular-bone growth at birth. Pelvic length is more affected than the lengths of the limbs. By contrast, the widths of the pelvis and of the limb bones are more affected than their lengths, and the growth retardation of males is more pronounced than that of females.

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