

Bone status in an animal model of chronic sub-optimal nutrition: a morphometric, densitometric and mechanical study

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In children, inappropriate eating habits can induce a disease known as nutritional dwarfing (ND). Due to the link between nutritional condition and bone growth, the effects induced by a 20% reduction of food intake on bone competence were assessed in an animal model of ND. Bone status during catch-up growth was also analysed. Male Wistar rats were divided into control (C) and ND groups. C rats were fed *ad libitum*. ND received 80% of the diet consumed by C for 4 weeks (T4); thereafter, they were fed *ad libitum* for 8 weeks. Results, expressed as mean (SEM) for ND v. C, were as follows. At T4, body weight (g) and length (cm) and femur weight (g) and length (mm) were 97.35 (SEM 5.89) v. 199.07 (SEM 9.24), 16.91 (SEM 0.41) v. 20.26 (SEM 0.31), 0.30 (SEM 0.01) v. 0.46 (SEM 0.01) and 23.09 (SEM 0.29) v. 26.98 (SEM 0.26), respectively ($P < 0.001$); bone mineral content (g) and density (g/cm^2) were 0.014 (SEM 0.002) v. 0.030 (SEM 0.002) and 0.061 (SEM 0.004) v. 0.080 (SEM 0.003), respectively ($P < 0.001$); load-bearing capacity (N), yielding load (N) and elastic stiffness (N/mm) were 25.06 (SEM 1.24) v. 50.34 (SEM 2.94), 23.72 (SEM 1.02) v. 46.97 (SEM 1.75) and 65.98 (SEM 4.42) v. 115.07 (SEM 3.85), respectively ($P < 0.001$); cross-sectional area (mm^2) and moment of inertia (mm^4) were 2.86 (SEM 0.19) v. 4.54 (SEM 0.17) and 1.27 (SEM 0.08) v. 3.03 (SEM 0.16), respectively ($P < 0.001$). Significant effects were not evident in material properties. Parameters assessed normalized during re-feeding. These results suggest that the impaired mechanical femur competence in ND rats could be due to an altered bone mass and architectural distribution rather than to intrinsic quality. Re-feeding caused a reversal of the effects of food restriction on growth and bone parameters in ND rats.

Weanling rat: Food restriction: Densitometry: Mechanical performance

Body growth and development are complex processes that depend on multiple factors, including heredity, gender, endocrine and nutritional status, and physical activity (Javaid & Cooper, 2002). Nutrition is probably the most important factor influencing overall growth (Galler & Probert, 1981). Chronic undernutrition is one of the most important causes of metabolic and neuroendocrine dysfunction with an evident growth failure (Cameron *et al.* 1993; Compagnucci *et al.* 2002–03). Furthermore, both animal and human studies indicate that protein–energy malnutrition can be detrimental for both acquisition of bone mass during growth and its conservation during adulthood. Consequently, malnutrition during development can increase the risk of osteoporosis, bone fragility and possible fractures (Bonjour *et al.* 2001; Leonard & Zemel, 2002).

Bone status depends on peak bone mass achieved, rate of bone loss and maintenance of bone micro-architecture, all related to genetics, body weight and composition, neuroendocrine activity, and quality and quantity of food consumption throughout life (Eaton-Evans, 1994; Cummings *et al.* 1985).

Different animal models have been used to study the effect of altered nutrition on postnatal skeletal growth, bone density and biomechanical parameters. In general, the authors assessed

the effect of either a protein-deficient diet fed *ad libitum* (Ferretti *et al.* 1988, 1991) or severe or chronic limitation of energy requirements for variable periods of time in infantile rats (Ndiaye *et al.* 1995a). Similar treatments have also been applied to adult animals (Lee *et al.* 1986; Lane *et al.* 1995; Talbott *et al.* 1998; Bourrin *et al.* 2000).

The growing rat shows a markedly diminished bone mass with alterations of morphometric and biomechanical variables in the femoral diaphysis (Ferretti *et al.* 1988), whereas a decrease in bone mineral density highly correlated with weight loss and an increase in skeletal fragility were found in adult rats (Talbott *et al.* 2001).

In man, certain parental misconceptions, health practices and beliefs concerning what constitutes a healthy diet for infants can restrict their nutrition to the point of inducing a nutritional disease known as nutritional dwarfing (ND; Lifshitz & Moses, 1989). Although poverty and food shortage in developing countries remain the most common cause of ND, inappropriate eating habits can lead to children's failure to thrive among the middle-to-upper socio-economic class (Pugliese *et al.* 1987; Lifshitz & Moses, 1988). ND refers to a pattern of growth defined by the anthropometric indices of the Wellcome classification

system (Keller & Fillmore, 1983), characterized by subnormal body and length growth where weight-for-height deficit and alterations in the biochemical markers of malnutrition are not evident (Lifshitz *et al.* 1987; Sandberg *et al.* 1991). In our laboratory, we have developed a nutritional stress model in weanling male rats placed on a 20% restricted balanced diet for a long time, that closely resembles human ND (Friedman *et al.* 1998). Significant decreases in growth rate, body fat mass and serum luteinizing hormone, follicle-stimulating hormone, testosterone and leptin levels, with altered sperm quantity and morphology, were evident in ND animals (Compagnucci *et al.* 2002–03).

Because there is a link between nutritional status and bone growth during critical periods of body growth, the aim of the present research was to assess the effects induced by the nutritional stress of a 20% reduction of food intake, started immediately after the weaning period, on morphometric, densitometric and mechanical parameters in the femur of ND male rats. The effects of nutritional stress on appendicular bone status during catch-up growth were also assessed.

Materials and methods

Animals

Weanling male Wistar rats (21–22 days old; mean initial body weight 45.47 (SEM 4.39) g) were provided by the Animal Resources of the Department of Biochemistry, School of Dentistry, University of Buenos Aires, Argentina. Animals were housed in galvanized cages with meshed floors and kept under 12 h light–12 h dark cycles. Room temperature was maintained at $21 \pm 1^\circ\text{C}$ with 50–60% humidity. The experiment was conducted in accordance with the principles and procedures outlined in the National Institutes of Health guide for the care and management of laboratory animals, and approved by the University of Buenos Aires Ethic Committee.

Diet

Animals were fed with a standard diet (Purina chow) of the following composition (g/100 g): protein, 20.6; lipids, 5.67; fibre, 6.0; Ca, 1.3; P, 0.8; ashes, 5.01; water, 8.21; dextrin, balance.

Experimental design

A total of sixty male rats were randomly assigned to two groups of thirty animals each: control (C) and experimental (ND). C rats were fed freely with the standard diet. ND rats received, for 4 weeks, 80% of the amount of food consumed by C the previous day, corrected by body weight (food intake in g/100 g body weight per d). The age at which the animals were food-restricted was based on clinical paediatric findings related to infant feeding practices such as hypocaloric foods that are given to infants post-lactation (Kaplan & Toshima, 1992; Akesson *et al.* 2000). After 4 weeks of food restriction, the ND group was fed freely with the same standard diet eaten by the C group for 8 weeks. All rats had free access to water. Body weight was recorded every day in the morning before food distribution, and length, every 4 d. Growth data were compared over time. Dietary intake was registered daily. Ten rats from each group were sacrificed under anaesthesia every 4 weeks. Immediately, both femurs from each animal were dissected avoiding periosteal lesion and weighed, and their

length was determined with a digital calliper. Left femurs were used in the determination of bone mineral content (BMC) and bone mineral density (BMD). Right femurs were used for mechanical studies. Additionally, ten rats were sacrificed for initial measurements on the day the experiment began (day 0).

Nutritional status

Anthropometry. Body weight and length were recorded serially during the experimental period (daily and 4 d, respectively), after a 2–4 h fasting period. A Mettler PC 4000 scale (Zurich, Switzerland) was used to measure body weight with an accuracy of ± 1 mg. For length measurements, animals were anaesthetized slightly with diethyl ether in an anaesthetic induction chamber. Body length was determined with a scaled ruler in mm from the nose tip to the last hairs of the tail base.

Diet intake. Food consumption was measured by using special feeders, which allowed the recovery of spilled food. Food intake was weighed daily with a Mettler scale (accuracy ± 1 mg) and expressed as kJ/100 g body weight per d for each rat.

Evaluation of femur morphology

Femur length was measured with a digital calliper from the tip of the greater trochanter to the distal surface of the lateral-medial condyle. A Mettler PE 600 scale (Zurich, Switzerland) was used to measure femur weight expressed in g.

Bone mass assessment

BMC, bone area and BMD of the femur were determined using a bone densitometer (LUNAR DPX-L) and specific software for small animals designed by LUNAR General Electric Medical Systems (Madison, WI, USA). The DPX-L uses a constant potential X-ray source combined with the K-absorption edge with effective energies between 38 and 70 keV. All measurements were carried out with a fine-diameter collimator on the X-ray output. Results are expressed as g (BMC) or g/cm^2 (BMD). Precision, expressed as a CV, was 1.38 (SD 0.54) % for BMC and 0.72 (SD 0.34) % for BMD. These measurements are precise and sensitive enough to detect small changes in bone mass over short periods of time (Ammann *et al.* 1992; Hassager & Christiansen, 1995; Paniagua *et al.* 1998).

Biomechanical tests on femur

The whole bones were submitted to a three-point bending test in a computerized Instron Universal Testing Machine (model 4442; Canton, MA, USA). In this procedure of bone breaking, the force was delivered to the midshaft by a cross-head at a constant speed. The breaking force was applied perpendicularly to the long axis of the bone at midshaft. Bones were placed lying horizontally with the anterior aspect facing down on two supports equidistant from their ends, separated by a constant distance, and loaded (50 N) centrally at a speed of 5 mm/min. Femurs were broken from the anterior to posterior plane. The plots of load *v.* deformation (*W–d*) obtained were analysed to determine bone mechanical properties. These plots recorded both the elastic (linear) and plastic components, separated by the yield point. The following structural (whole-bone) mechanical properties were determined:

- (1) Load-bearing capacity (W_f) or fracture load (N), which expresses bone strength in a broad sense.
- (2) Yielding load (W_y), load at the yield point (N), an expression of bone maximum elastic strength.
- (3) Yielding deformation (d_y), arrow of the arch formed by the bending bone at the yield point (mm), an index of maximum elastic deformation.
- (4) Bone stiffness in elastic conditions or load-to-deformation ratio (W_y/d_y), determined graphically at any point over the linear portion of the curve (N/mm).

Because bone segments between the supports were closely comparable to hollow, elliptically shaped cylinders, the micromorphometry of the horizontal and vertical external (H and B) and internal (h and b) diameters of the fracture sections enabled us to calculate the following geometric properties:

- (1) Cross-sectional cortical bone area, $A = 3.14(HB - hb)/4$ (mm^2).
- (2) Second moment of inertia of the cross-section in relation to the horizontal axis, $I_x = 3.14(H^3B - h^3b)/64$ (mm^4).

A stress-strain curve was determined from the previous force-deformation curve using engineering formulae (Turner & Burr, 1993). Material properties of the bone tissue were calculated as follows:

- (1) Yield stress, the force per unit cross-sectional area at the yield point, $\sigma_y = W_yLB/8I_x$ (N/mm^2), where L is the distance between the supports, an estimation of tissue strength.
- (2) Young's modulus of elasticity, $E = W_yL^3/48d_yI_x$ (N/mm^2), represented by the slope of the stress-strain curve within the elastic region, which is an estimator of tissue intrinsic stiffness.

Statistical analysis

Results are expressed as mean and standard error of the mean. Data were analysed by one-way ANOVA. Differences between means were analysed by the Student-Neuman-Keuls multiple comparison test. Differences were considered significant if $P < 0.05$ (Sokal & Rohlf, 1994). Analyses were performed using the Graphpad Prism (version 3.0) statistical package (Graphpad Software, San Diego, CA, USA).

Results

As shown in Fig. 1, ND rats' food consumption was 20% less than that of C rats during the food restriction period. When the ND group was fed *ad libitum*, a hyperphagic response was evident during the first 4 weeks of the re-feeding period ($P < 0.001$), with values reaching those of C rats at the third week of the re-feeding period.

Food restriction induced a highly significant ($P < 0.001$) decrease in growth rate in ND rats. After 4 weeks of food restriction, the reduction in body weight and length were 46.6% and 17.6%, respectively. There was a catch-up growth during re-feeding that reached the same values as for C animals at week 11 (Fig. 2).

Femoral growth was negatively affected ($P < 0.001$) in ND rats at the end of the restrictive period (4 weeks). Femoral weight and length were, respectively, 34.7% and 15% lower in ND rats than in the C group. During re-feeding, no significant differences

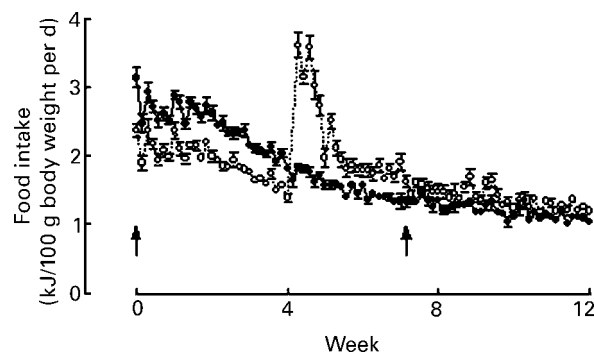


Fig. 1. Food consumption at different times after weaning (week 0) of control (—●—) and experimental (·····○·····) rats. Values are means, with standard errors of the mean represented by vertical bars, for ten animals per group. Mean values between the arrows are significantly different between groups ($P < 0.05$).

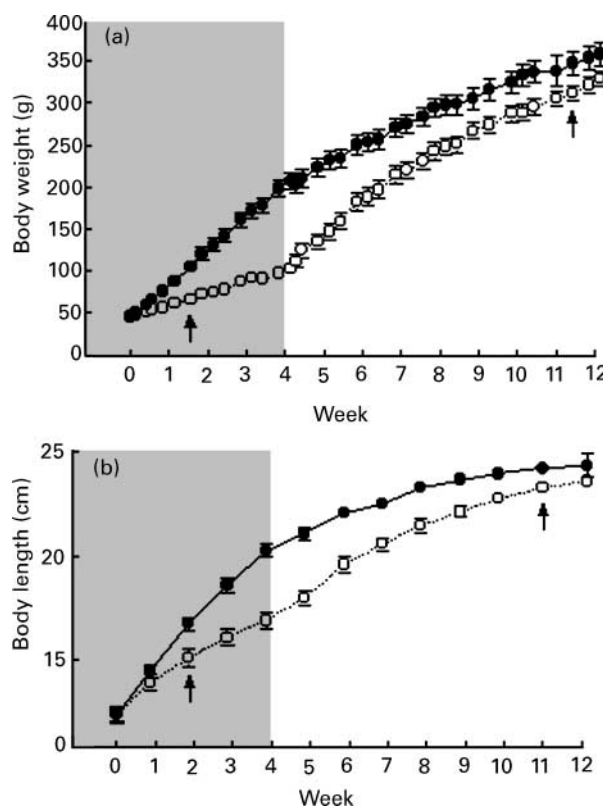


Fig. 2. Changes in body weight (a) and body length (b) at different times after weaning (week 0) for control (—●—) and experimental (·····○·····) rats. Values are means, with standard errors of the mean represented by vertical bars, for ten animals per group. Shaded area represents period of food restriction. Mean values between the arrows are significantly different between groups ($P < 0.05$).

between ND and C rats were seen at weeks 8 and 12 of the experimental period (Table 1).

After the first 4 weeks of the experimental period, BMC and BMD were 46% and 24% lower ($P < 0.001$), respectively, in ND rats compared with C rats. A high correlation was found between BMC and BMD *v.* body weight (r^2 0.82 and 0.79, respectively). When these parameters were corrected for bone

Table 1. Changes in femoral weight, length, bone mineral content (BMC), bone mineral density (BMD), BMC and BMD corrected for femur weight at different times after weaning (week 0) in control (C) and experimental (ND) rats
(Mean values and standard error of the mean for ten animals per group)

	Week 4				Week 8				Week 12			
	C		ND		C		ND		C		ND	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Femur weight (g)	0.46	0.01	0.30*	0.01	0.70	0.04	0.66	0.04	0.86	0.04	0.79	0.03
Femur length (mm)	26.98	0.26	23.09*	0.29	30.69	0.25	30.35	0.30	34.54	0.38	34.00	0.45
BMC (g)	0.030	0.002	0.014*	0.002	0.129	0.024	0.109	0.024	0.184	0.016	0.168	0.008
BMD (g/cm ²)	0.080	0.003	0.061*	0.004	0.14	0.006	0.131	0.006	0.163	0.007	0.153	0.006
BMC/femur weight	0.053	0.959	0.035	0.961	0.153	0.885	0.141	0.887	0.186	0.879	0.177	0.881
BMD/femur weight	0.143	0.953	0.164	0.952	0.17	0.882	0.171	0.881	0.165	0.883	0.181	0.879

Mean value is significantly different from that of the control at the same time point: * $P < 0.001$.

weight, significant differences between groups were not evident ($P > 0.05$) at any time point. In addition, a highly significant increase ($P < 0.001$) in both variables was evident in C and ND rats with time (Table 1).

Structural properties, i.e. load-bearing capacity (W_f), yielding load (W_y) and diaphyseal stiffness (W_y/d_y), were significantly ($P < 0.001$) and negatively affected in ND rats after food restriction (Fig. 3). However, at week 8, ND structural parameters achieved C values, showing that the maximum force required to fracture the bone at midshaft, maximal load resistance in elastic conditions and slope of the $W-d$ curve during bone elastic behaviour did not differ between the two groups. Moreover, there was a significant increase ($P < 0.001$) in values of structural parameters in C and ND animals with time.

On the other hand, no significant effects of food restriction were evident between groups in terms of yield stress (σ_y) and modulus of elasticity (E), which are parameters of material bone quality, at any time point (Fig. 4).

Geometrical properties, i.e. cross-sectional cortical area (A) and moment of inertia of the fracture section (I_x), were significantly reduced ($P < 0.001$) after 4 weeks of food restriction in the post-weaning period (Fig. 5). The correlation between I_x and A v. body weight was r^2 0.74 and 0.60, respectively. A high correlation was also found between W_f and W_y v. I_x (r^2 0.76 and 0.66, respectively). In agreement with the effects on structural properties, no significant differences were observed in geometrical properties between groups at week 8.

Discussion

The present study analysed the effects induced by global mild chronic food restriction (20% reduction of food intake) and rehabilitation on skeletal growth and densitometric and mechanical behaviour of an appendicular bone (femur) in weanling male rats. The nutritional stress model used herein was developed in weanling male rats in our laboratory (Friedman *et al.* 1998) and resembles the sub-optimal nutrition observed in children who consume inappropriate diets with insufficient total energy to sustain normal growth and weight gain.

Many factors are determinants of bone quality in both the axial and appendicular skeleton, of which nutritional status is one of the most important. Protein-energy malnutrition during

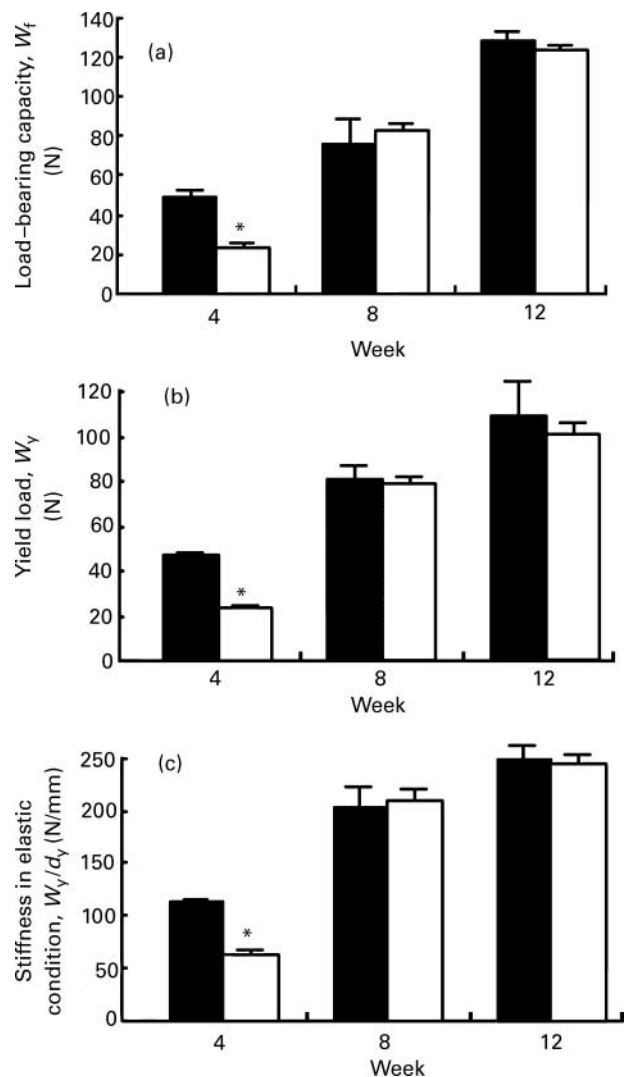


Fig. 3. Load-bearing capacity (a), yield load (b) and stiffness in elastic conditions (c) of the femoral diaphysis at different times after weaning (week 0) in control (■) and experimental (□) rats. Values are means, with standard errors of the mean represented by vertical bars, for nine or ten animals per group. Mean value is significantly different from that of the control at the same time point: * $P < 0.001$.

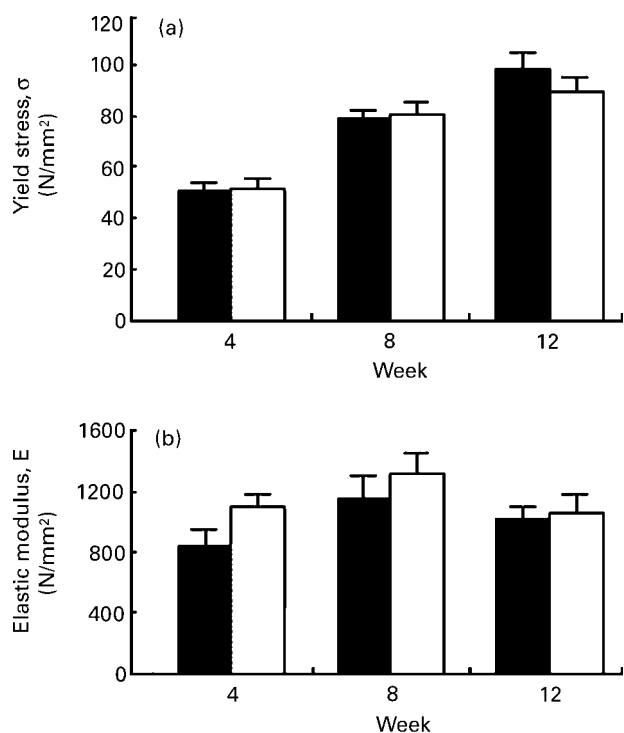


Fig. 4. Yield stress (a) and elastic modulus (b) of the femoral diaphysis at different times after weaning (week 0) in control (■) and experimental (□) rats. Values are means, with standard errors of the mean represented by vertical bars, for nine or ten animals per group.

development, mostly during critical periods of body growth, contributes to longitudinal growth failure with subsequent risk of osteoporosis and bone fragility later in life (Bonjour *et al.* 2001). It is well known that the restriction of energy intake reduces bone formation in both malnourished children (Ndiaye *et al.* 1995b) and undernourished rats (Ndiaye *et al.* 1995a; Rhee *et al.* 2002).

In the present study, the level of food restriction imposed was severe enough to decrease normal growth rate in ND animals. This negative effect was more evident in body weight, considered an expression of overall body growth, than in body length, which gives information on longitudinal bone growth. These observations could be due to the combined effect of mild sub-optimal intake in addition to the length of the restriction. A similar decrease was observed in femoral growth in the experimental group. However, when restriction ceased, complete catch-up (Boersma & Wit, 1997) of the mentioned parameters was evident in ND rats. A normal growth velocity pattern from the post-weaning period to adulthood (Friedman *et al.* 1999) was observed in the control rats.

Since measurement of bone mass is essential in the diagnosis of bone quality and it correlates highly with body weight (Lee *et al.* 1986; Lane *et al.* 1995; Nguyen *et al.* 1998; Talbott *et al.* 2001; Leonard & Zemel, 2002), in the present study bone densitometry was performed in order to predict bone status in ND animals (Slosman *et al.* 1992; Griffin *et al.* 1993).

The significant differences found in BMC and BMD in post-weaning male rats after 4 weeks of food restriction could probably be associated with body weight loss and/or related to impaired bone quality. However, these negative effects on bone

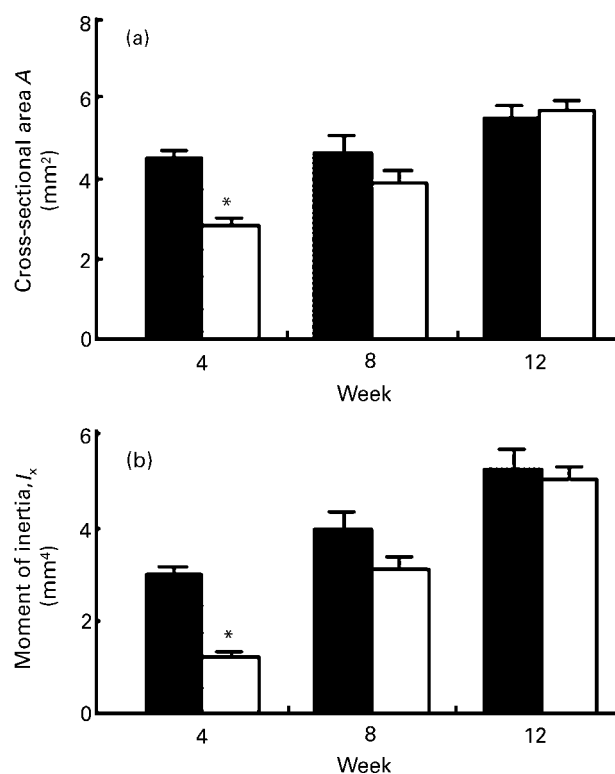


Fig. 5. Cross-sectional cortical area (a) and cross-section moment of inertia (b) of the fracture section of the femoral diaphysis at different times after weaning (week 0) in control (■) and experimental (□) rats. Values are means, with standard errors of the mean represented by vertical bars, for nine or ten animals per group. Mean value is significantly different from that of the control at the same time point: * $P < 0.001$.

status were completely suppressed after re-feeding, suggesting a reversible response of bone mass to 20% reduction of food intake, started immediately after weaning, in male rats. However, the lack of differences in BMC and BMD corrected for bone size suggests that bone mass is adequate for bone weight achieved in ND rats.

It is well known that the mechanical properties of bone as a whole are determined by both geometry (bone architecture) and material properties (bone tissue; Turner & Burr, 1993). Changes in the mechanical effectiveness (structural properties) of a solid body of bone could be due to changes in mass and its spatial distribution (geometry) and/or intrinsic mechanical quality of its constitutive substance (material properties; Ferretti, 1997). Our results showed that the mechanical strength of diaphyseal femurs (W_f , W_y) was negatively affected in response to external loading after 4 weeks of food restriction in the post-weaning period. In congruence with the effects of food restriction on mechanical properties, the cross-sectional parameters, A and I_x , were significantly reduced in ND rats compared with control animals of the same age. Cross-sectional moment of inertia and area depended significantly on body weight, as seen by the correlation between I_x and A v. body weight. Assuming that rat models usually show the effects of treatments on cortical bone modelling in long bones, changes in cross-sectional moment of inertia should be interpreted as reflecting an additional influence of body weight on bone modelling. These findings are consistent with another study (Ferretti *et al.* 1988)

that reported impairment of mechanical performance (structural properties) and geometrical properties in a different model of nutritional stress. However, in the present study, the material quality of bone (σ_y , E) was not affected by food restriction, in agreement with other authors (Ferretti *et al.* 1991). The lack of difference in material properties, as expressed by yield stress and modulus of elasticity, between ND and control animals after food restriction suggests that the impaired performance of diaphyseal shafts of ND animals should be regarded as resulting predominantly from changes in spatial distribution of bone material, rather than bone mass quantity and intrinsic quality. This suggestion is supported by the high correlation found between BMC and BMD *v.* body weight, and W_f and W_y *v.* I_x .

All parameters assessed normalized according to catch-up growth, allowing appropriate bone mechanical competence of the appendicular skeleton to be attained during re-feeding. The reversibility of the effects on bone status observed in this animal model of ND suggests that mild chronic food restriction does not produce long-term side-effects on bone growth and competence.

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