

# Bone quality is affected by food restriction and by nutrition-induced catch-up growth

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## Abstract

Growth stunting constitutes the most common effect of malnutrition. When the primary cause of malnutrition is resolved, catch-up (CU) growth usually occurs. In this study, we have explored the effect of food restriction (RES) and refeeding on bone structure and mechanical properties. Sprague–Dawley male rats aged 24 days were subjected to 10 days of 40% RES, followed by refeeding for 1 (CU) or 26 days long-term CU (LTCU). The rats fed *ad libitum* served as controls. The growth plates were measured, osteoclasts were identified using tartrate-resistant acid phosphatase staining, and micro-computed tomography (CT) scanning and mechanical testing were used to study structure and mechanical properties. Micro-CT analysis showed that RES led to a significant reduction in trabecular BV/TV and trabecular number (Tb.N), concomitant with an increase in trabecular separation (Tb.Sp). Trabecular BV/TV and Tb.N were significantly greater in the CU group than in the RES in both short- and long-term experiments. Mechanical testing showed that RES led to weaker and less compliant bones; interestingly, bones of the CU group were also more fragile after 1 day of CU. Longer term of refeeding enabled correction of the bone parameters; however, LTCU did not achieve full recovery. These results suggest that RES in young rats attenuated growth and reduced trabecular bone parameters. While nutrition-induced CU growth led to an immediate increase in epiphyseal growth plate height and active bone modeling, it was also associated with a transient reduction in bone quality. This should be taken into consideration when treating children undergoing CU growth.

## Key Words

- ▶ catch-up growth
- ▶ food restriction
- ▶ micro CT
- ▶ mechanical testing

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## Introduction

Although there are numerous genetic and environmental factors that may affect growth, malnutrition, marked by various nutrient deficiencies, is considered to be a leading cause of low weight and short stature. In the developing

countries, an average 33% of all children younger than 5 years of age suffer from linear growth retardation or stunting due to chronic malnutrition, caused by food shortage as well as by infectious diseases (Walker *et al.* 2007).

Malnutrition may also occur in developed countries, mostly due to chronic disease. Catch-up (CU) growth occurs after the resolution of a growth-inhibiting condition and has been found to be characterized by a growth velocity above the normal statistical limits for age and/or maturity (Boersma *et al.* 2002). Some excellent examples for nutrition-induced CU growth are in childhood celiac disease (Boersma *et al.* 2002), as remarkable CU growth has been documented shortly after the onset of a gluten-free diet (Boersma *et al.* 2002).

The skeleton undergoes rapid structural adaptations to the demands of the growing body, manifested by elongation and bone modeling, together with changes in the bone mineral composition, cortical and trabecular architecture, and altered mechanical properties. An adequate supply of numerous nutritional factors is required for this process, some of which, such as proteins, lipids, and carbohydrates, constitute the 'building materials' while others play regulatory roles.

To study the effect of food restriction (RES) and CU growth on bones, we used young, rapidly growing rats. In this experimental model, 40% RES was initiated on 24 days old rats and continued during the linear growth period for 10 days. In our previous studies, we have shown that the most dramatic changes in weight gain and a significant increase in epiphyseal growth plate (EGP) height were observed after only 1 day of refeeding (Even-Zohar *et al.* 2008). The changes in the EGP height in response to nutritional manipulation were found to result from differences in both cell number and extracellular matrix production, as exemplified by changes in gene expression, with the most affected genes being those associated with matrix formation (Even-Zohar *et al.* 2008). It was therefore reasoned that these changes are likely to have an effect on the architecture and material properties of the developing bones.

In this study, we aimed to analyze the changes occurring in the long bones during RES and nutrition-induced CU growth, using micro-computed tomography (CT) analysis (Bouxsein *et al.* 2010) and mechanical testing (Shipov *et al.* 2010).

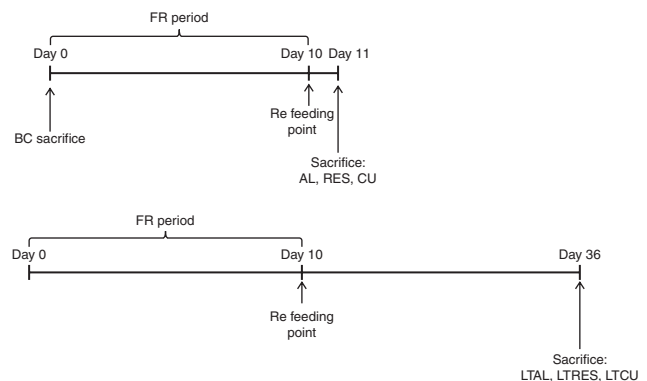
## Materials and methods

### Animals

Male Sprague–Dawley rats, 21 days old, were purchased from Harlan (Jerusalem, Israel) and housed individually at the animal care facility of the Felsenstein Medical Research Center in separate cages to facilitate the monitoring of

their food consumption. The animals were allowed to adapt to these conditions for 3 days before the beginning of the experiment. One group of animals was killed at the beginning of the experiment (24 days old; BC;  $n=6$ ). The data obtained from these young animals served to assess the effect of age and weight on bone quality. Other rats were divided into three groups: one was given an unlimited amount of food (complete diet for rats and mice, 3.4 kcal/g, provided by Teklad, South Easton, MA, USA) (*ad libitum* (AL) group), and two received 60% of the same chow (RES group and CU group); all animals had unlimited access to water. The 40% restriction was calculated on the basis of a previous study wherein animals were housed individually and the amount of food consumed each day was measured, together with the animals' weight and weight gain (Even-Zohar *et al.* 2008). The RES was maintained for 10 days. At that point, while the RES group was kept restricted, the CU group was given normal chow *ad libitum*. To study the effect of long-term refeeding, the CU period was extended to 26 days (long-term CU (LTCU)); AL and RES groups were monitored throughout this extended period as well (long term *ad libitum* (LTAL) and long term food restricted (LTRES) respectively) (Fig. 1). After 1 or 26 days of refeeding, animals were killed by CO<sub>2</sub> inhalation. Throughout the study, animals were observed daily, and all remained bright, alert and active, with no evidence of any disorder. The Tel Aviv University Animal Care Committee approved all procedures.

After killing the animals, the tibias and one humerus of each animal were carefully removed, measured, and fixed in 4% neutral buffered formalin for 24 h, followed by demineralization in 20% EDTA for 6 weeks for histological and morphological studies. The other humerus was cleaned and stored at  $-20^{\circ}\text{C}$  until analyzed by micro-CT and three point bending.



**Figure 1** Schematic representation of the timeline of the study.

## Micro CT

Bones were stored in saline-soaked gauze, at  $-20^{\circ}\text{C}$ . They were thawed on the day of being scanned in high humidity (moistened and wrapped in ceram-wrap and then placed in the holder for scanning).

The region of the proximal metaphysis to mid-diaphysis of all humeri were scanned with a SkyScan 1174 X-ray-computed microtomograph scanner (SkyScan, Aartselaar, Belgium), equipped with a CCD detector. Scanner source parameters used were 50 kV and 800  $\mu\text{A}$ . The bone samples were scanned using an 0.25 mm aluminum filter, 4000 ms exposure time, and at 11.1  $\mu$  pixel size resolution. For each specimen, a series of 900 projection images was obtained, with a rotation step of  $0.4^{\circ}$ , two frames averaging, for a total  $360^{\circ}$  rotation. Flat field correction was performed at the beginning of each scan for a specific zoom and image format. A stack of 2D X-ray shadow projections was reconstructed to obtain images using NRecon Software (SkyScan), and subjected to morphometric analysis using CTAn Software (CTAn Software, v.1.11, SkyScan). During reconstruction, dynamic image range, post-alignment value, beam hardening, and ring-artifact reduction were optimized for each experimental set. Analysis of the diaphyseal cortical region was performed on 200 slices, similarly selected for all samples, corresponding to a section of mid-diaphysis of 2.764 mm long. The following cortical parameters were measured: volumetric bone mineral density (BMD ( $\text{mg}/\text{mm}^3$ )), polar moment of inertia ( $\mu\text{m}^4$ ), mean cortical thickness ( $\mu$ ), and cortical cross-sectional area ( $\mu^2$ ). Cortical cross-sectional area ( $\mu^2$ ) was calculated by the software individually for each slice, and mean values are presented.

In addition, two phantoms of known mineral density (0.25 and 0.75 g/cc) supplied by the manufacturer of the scanner (SkyScan) were also scanned, using the same scanning parameters. This allowed calibration of the attenuation levels directly to BMD values. For the trabecular region, a total of 150 slices, corresponding to a segment 2.073 mm long, were selected. The selection of region of interest (ROI) was consistent in all bones analyzed. Adaptive threshold was used (this is a software option that helps overcome errors resulting from partial volume effects, it calculates density gradients to determine if a particular pixel is bone or not). Morphometric analysis was performed using SkyScan Software (CTAn Software, v.1.11, SkyScan). 3D images (CTM file format) were constructed from the cortical and trabecular regions of interest, utilizing Marching Cubes 33 algorithm, using SkyScan Software (CTVol, SkyScan) (Reich *et al.* 2010, Idelevich *et al.* 2011).

## Mechanical testing

Following micro-CT scanning, mechanical tests were carried out on the diaphyseal region of the humeri. The loading system included a stainless-steel test chamber so that mechanical testing could be performed on samples immersed in physiologic solution. An axial-motion DC motor (PIM-235.2DG, Physik Instrumente GmbH, Karlsruhe, Germany) moved a metal shaft into the testing chamber in small sub-micron steps while being able to apply substantial force ( $>100\text{ N}$ ). The metal shaft was connected in series to a load cell (AL311, Sensotec, Honeywell, Morriston, NJ, USA,  $\pm 0.4\text{ N}$ ), which was attached in turn to a movable anvil that was designed to contact the bone sample. The bones were tested by the three-point bending method while being fully immersed in physiologic saline. Each bone was placed on two supports having rounded profiles (0.5 mm radius) to limit stress concentration at the point of load application, so that the supports were equidistant from the ends of the bone, and both contacted the posterior aspect of the diaphysis. The distance between the supports was 8 mm. Each bone was loaded on its anterior aspect, by a moving prong with rounded profile, at the mid-point between the bottom supports, and at the mid-point along its length.

Force and displacement data were collected at 50 Hz, using custom-written software (NI Labview, Austin, TX, USA). The resulting load-displacement curves were used to calculate whole-bone stiffness (slope of the linear portion of the load-displacement curve), ultimate load and load to fracture (Lanyon *et al.* 1982).

## Tartrate-resistant acid phosphatase staining for osteoclasts

Tibias were fixed overnight in 4% formalin (Sigma Chemical) in PBS at  $4^{\circ}\text{C}$ , dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in Paraplast. Thin (6  $\mu\text{m}$ ) sections were prepared. Staining for osteoclasts was done with para-nitrophenylphosphate (PNPP) (Leukocyte Acid Phosphatase Kit, Sigma Chemical). Quantitation of tartrate-resistant acid phosphatase (TRAP)-stained sections was performed on digital images in Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA). Only cells at the lower border of the EGP were counted because dramatic differences were observed at this area. At least two sections from each animal were analyzed. The sections were visualized using an Olympus Bx52 light microscope and photographed using Camera Olympus DP71 (Japan).

## Histology

The humeri were fixed as described for tibias. Thin (6  $\mu\text{m}$ ) horizontal cross-sectional sections were prepared and stained with H&E or Masson's trichrome staining (Sigma Chemical). The sections were visualized as described earlier.

## Chemical analysis of serum samples

While killing the animals, blood was collected by cardiac puncture, serum was separated and kept at  $-20^\circ\text{C}$  until analyzed using an Immunolite automated analyzer. Serum leptin and total insulin-like growth factor 1 (IGF1) levels were measured using commercial ELISA Kits (Rat Leptin Kit; Millipore (Billerica, MA, USA) and Rat IGF1 Kit (cat no. MG100), Quantikine; R&D Systems (Minneapolis, MN, USA) respectively); bone-specific alkaline phosphatase (ALP) was measured using a commercial Rat C-terminal Peptide of Bone-specific Alkaline Phosphates ELISA Kit (Cusabio Biotech Co. Ltd, Wuhan, Hubei Province, China).

## Statistical analyses

Results are expressed as mean  $\pm$  s.d. Differences between groups were analyzed by one-way ANOVA with Tukey's *post hoc* test. A *P* value below 0.05 was considered significant.

## Results

### Effect of RES on morphological parameters

The effects of RES and short-term nutrition-induced CU growth on weight, bone, and EGP length are given in Table 1 (and see also Even-Zohar *et al.* (2008)).

The height of the EGP measured from the reserve zone to the ossification front of the metaphyseal bone, which was significantly smaller in the RES group ( $P < 0.01$ ), increased significantly already after 1 day ( $P < 0.05$ ) while

the length of the humerus was still not significantly increased. There were significant differences in humerus length between the RES and AL groups ( $P < 0.05$ ), and between the CU and AL groups ( $P < 0.05$ ) (Table 1).

After 26 days of refeeding, the weight of the animals (LTCU) and the length of their humeri were significantly increased, but were still lower compared with the control group (LTAL) (Table 1). While the EGP in the control LTAL group was already reduced (compared with AL) due to age, the height of the humeri EGP in the LTCU group was significantly larger compared with the LTRES and was not significantly different from that of the LTAL EGP (Table 1 and Fig. 2).

In this study, we also examined a group of 24 days old rats (BC), finding significant differences in tibia length between the BC group and all other rat groups, indicating that under these conditions of RES the bones continued to grow, although in a slower pace (data not shown).

### Effect of age on cortical and trabecular bone architecture

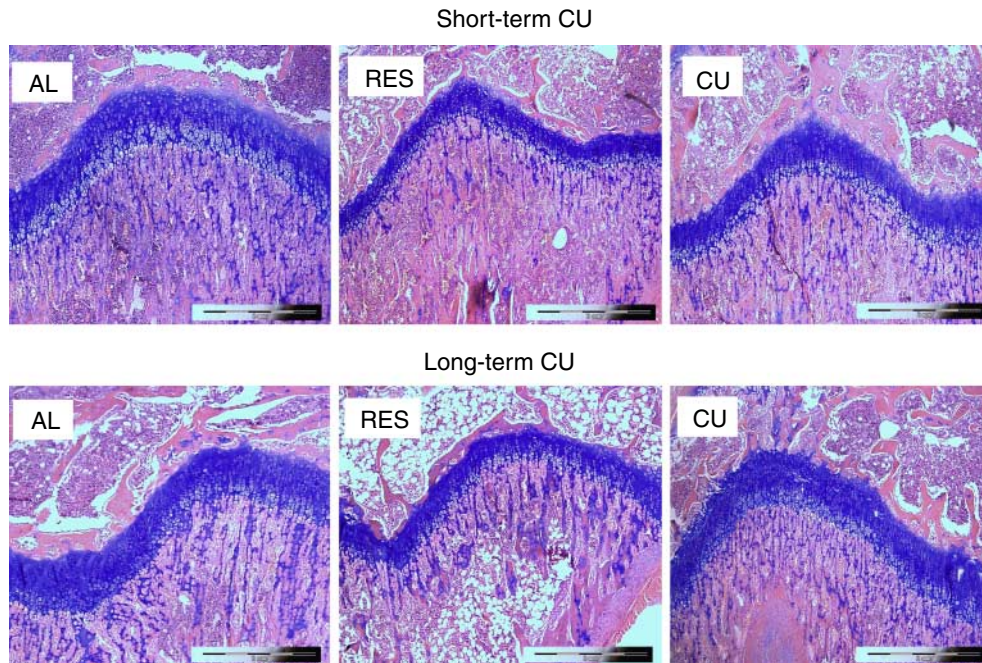
To examine the effect of age on bone structure and strength, the parameters of the bones from the BC, AL, and LTAL groups were compared. No significant differences were found between the groups in BMD. In other cortical bone parameters, including cortical area/total area (Ct.Ar/Tt.Ar), cortical thickness, and mean polar moment of inertia, there were significant differences between the BC group and all other groups of rats, which were killed at an older age ( $P < 0.05$ ) (Table 2 and Fig. 3).

In the trabecular bone, percent bone volume (BV/TV) and trabecular thickness (Tb.Th) were greater in the BC group than in all the other groups, with no significant change in trabecular number (Tb.N) at the short-term experiment (AL) but with a significant reduction by 60 days of age (LTAL). Trabecular separation (Tb.Sp) was significantly lower in the BC rats (Fig. 3). These results indicate that in the stage of skeletal maturation examined in this study, modeling processes resulted in mechanically

**Table 1** Morphology parameters of the animal groups. Rats were divided into the following groups: rats fed *ad libitum* (AL, LTAL); 40% food-restricted (RES, LTRES) or food restricted followed by refeeding for 1 (CU) or 26 days (LTCU). Results are presented as mean  $\pm$  s.d.

	AL	RES	CU	LTAL	LTRES	LTCU
Weight (g)	116.7 $\pm$ 8.00	60.95 $\pm$ 3.27 <sup>*,‡</sup>	71.75 $\pm$ 2.27 <sup>*,†</sup>	268 $\pm$ 16.9 <sup>*,†,‡</sup>	67.5 $\pm$ 2.5 <sup>*,§</sup>	224.9 $\pm$ 11.3 <sup>*,†,‡,§,  </sup>
Humerus length (mm)	19.42 $\pm$ 0.52	18.48 $\pm$ 0.55 <sup>*</sup>	18.1 $\pm$ 0.69 <sup>*</sup>	24.7 $\pm$ 0.48 <sup>*,†,‡</sup>	18.97 $\pm$ 0.32 <sup>*,§</sup>	23.57 $\pm$ 0.27 <sup>*,†,‡,§,  </sup>
Humeral EGP height ( $\mu\text{m}$ )	420 $\pm$ 22	280 $\pm$ 10 <sup>*,‡,§,  </sup>	344 $\pm$ 50 <sup>*,†,  </sup>	343 $\pm$ 22 <sup>*,†,  </sup>	183 $\pm$ 23 <sup>*,†,§</sup>	368 $\pm$ 12 <sup>†,  </sup>

\* $P < 0.05$  compared with AL (*ad libitum*); <sup>†</sup> $P < 0.05$  compared with RES (food-restricted). <sup>‡</sup> $P < 0.05$  compared with CU (1 day catch-up). <sup>§</sup> $P < 0.05$  compared with LTAL (long-term *ad libitum*). <sup>||</sup> $P < 0.05$  compared with LTRES (long-term food-restricted). LTCU, long-term CU.

**Figure 2**

Representative epiphyseal growth plates from rats of the six groups stained with hematoxylin/eosin/alcian blue to show the specific margins of the growth plate (magnitude  $\times 4$ ), at least six sections from each group

were analyzed. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JOE-14-0486>.

improved cortical bone (thicker and less porous), while the cancellous regions show lower BV/TV and thinner trabeculae.

### Effect of RES on cortical and trabecular bone architecture

Comparison of the bones of the AL group with those of the RES group showed that while cortical BMD was not significantly affected by this protocol of RES (Table 2), cortical thickness, and mean polar moment of inertia, were all significantly less in the RES group as well as in the LTRES (Fig. 3). Tb.Th was not affected, while Tb.N was significantly lower with RES, concomitant with an

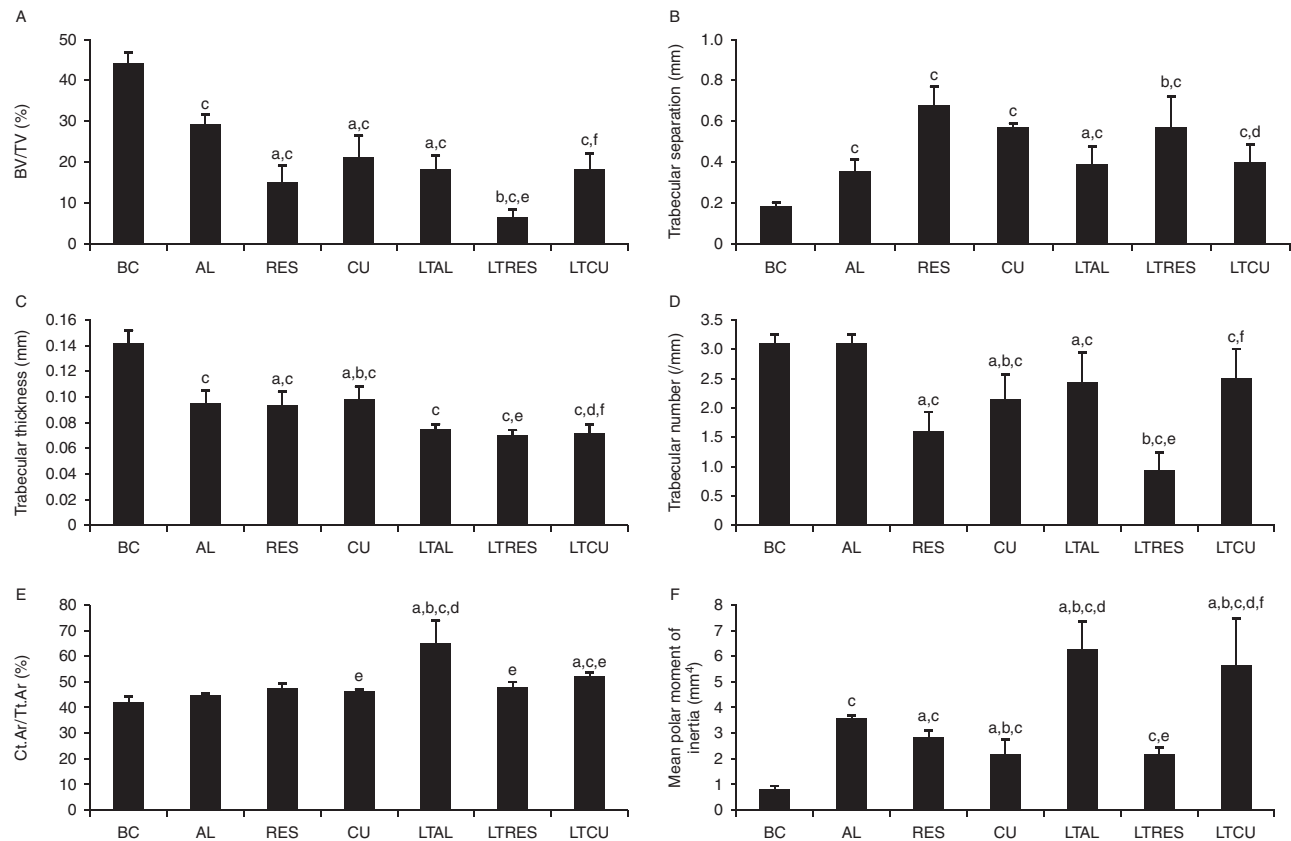
increase in Tb.Sp in both short- and long-term protocols (Fig. 3). There was no significant difference between the groups in Ct.Ar at the short-term protocol, but a significant difference was observed after long-term restriction (Table 2), while trabecular BV/TV was significantly lower in both the short-term and the long-term protocols (Fig. 3).

Interestingly, both age and RES increased Tb.Sp; however, while age induced a reduction in Tb.Th, RES led to a lower Tb.N. Together these results demonstrate that while 10 days of restriction are not sufficient to affect the cortical bone parameters, they have dramatic effect on the trabecular bone.

**Table 2** Analysis of the cortical region

	BC	AL	RES	CU	LTAL	LTRES	LTCU
BMD (gr/cm <sup>3</sup> )	0.869 ± 0.08	0.859 ± 0.03	0.845 ± 0.03	0.813 ± 0.02	1.15 ± 0.25	0.81 ± 0.13	0.83 ± 0.11
Cortical area (mm <sup>3</sup> )	1.15 ± 0.08	2.4 ± 0.06 <sup>†</sup>	2.2 ± 0.12 <sup>†</sup>	1.9 ± 0.2 <sup>†</sup>	3.32 ± 0.19 <sup>*,†,‡,§,¶</sup>	1.8 ± 0.11 <sup>‡,  </sup>	3.15 ± 0.42 <sup>*,†,‡,§,  ,¶</sup>
Cortical thickness (mm)	0.224 ± 0.01	0.301 ± 0.01 <sup>†</sup>	0.286 ± 0.01 <sup>*,‡</sup>	0.286 ± 0.01 <sup>†</sup>	0.30 ± 0.01 <sup>†</sup>	0.26 ± 0.02 <sup>*,‡,  </sup>	0.31 ± 0.02 <sup>‡,¶</sup>

\* $P < 0.05$  compared with AL (*ad libitum*). <sup>†</sup> $P < 0.05$  compared with RES (food-restricted). <sup>‡</sup> $P < 0.05$  compared with BC (24 days old, baseline control). <sup>§</sup> $P < 0.05$  compared with CU (1 day catch-up). <sup>||</sup> $P < 0.05$  compared with LTAL (long-term *ad libitum*). <sup>¶</sup> $P < 0.05$  compared with LTRES (long-term food-restricted). LTCU, long-term CU.



**Figure 3**

Effect of food restriction and refeeding on architectural features. Bones obtained from rat of all groups were examined using micro-CT: (A) trabecular percent bone volume (BV/TV). (B) Trabecular separation. (C) Trabecular thickness. (D) Trabecular number. (E) Cortical area (Ct.Ar)/total area (Tt.Ar). (F) Mean polar moment of inertia. Results are

presented as mean  $\pm$  s.d.; <sup>a</sup> $P < 0.05$  compared with AL (*ad libitum*); <sup>b</sup> $P < 0.05$  compared with RES (food-restricted); <sup>c</sup> $P < 0.05$  compared with BC (24 days old, baseline control); <sup>d</sup> $P < 0.05$  compared with CU (1 day catch-up); <sup>e</sup> $P < 0.05$  compared with LTAL (long term *ad libitum*); and <sup>f</sup> $P < 0.05$  compared with LTRES (long-term food restricted). LTCU, long-term CU.

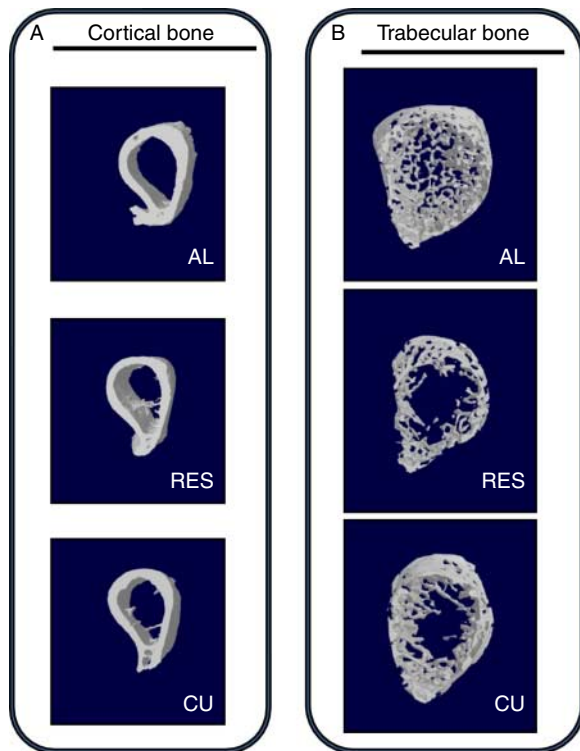
### Effect of CU growth on bone structure and bone parameters

Comparing the bones of the CU group with those of the RES group showed that after 1 day of refeeding, cortical bone parameters were not significantly affected (Table 2, Figs 3 and 4) except for the mean polar moment of inertia, which was less than that of the RES group. However, 26 days of refeeding led to a slight increase in Ct.Ar/Tt.Ar and a significant increase in cortical thickness and in the mean polar moment of inertia (compared with RES). Restoring the regular food supply led to an inversion of the effect of RES on trabecular architecture, with less Tb.Sp and a significantly higher Tb.N in the CU group than in the RES group in both short- and long-term experiments (Figs 3 and 4). Trabecular percent bone volume (BV/TV) tended to increase already after 1 day of refeeding (Fig. 3) and was completely corrected after 26 days (Fig. 3).

The animals in the LTCU group were not significantly different from those of the control LTAL.

### Effect of nutritional manipulation on bone modeling

As the effect of refeeding on bone structure was very rapid, we studied both bone resorption and bone formation at 1 day of refeeding. Specific staining for active osteoclasts (TRAP) – which have the unique ability to resorb mineralized bone – demonstrated a similar number in the BC and AL groups (BC 25.33; AL 27; NS), but a significant increase in the RES group (RES 55.32;  $P < 0.0001$ ). Interestingly, as shown in Fig. 5A, B and C, osteoclasts in the RES and CU bones were seen to concentrate in the lower boundary of the growth plate, suggesting an enhanced resorption in both of these groups. The addition of food to the restricted animals did not significantly affect either the number of the TRAP-positive cells or their localization (CU 57.3).



**Figure 4**

Effect of food restriction and refeeding on architectural features. Bones obtained from short-term AL, RES, and CU animals were examined using micro-CT: (A) micro-CT 3D image of the cortical bone. (B) Micro-CT 3D image of the trabecular bone from the AL and RES and CU groups, illustrating smaller bone volume in the RES group that increases in the CU group. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JOE-14-0486>.

Bone formation was followed by measuring collagen fibers production (see blue staining in the mid part of the sections Fig. 5D and F compared with Fig. 5E). The sections presented in Fig. 5D, E and F shows that collagen fibers were very abundant in sections taken from AL rats, but could not be seen under these conditions in sections taken from animals under RES (Fig. 5E). Already after 1 day of refeeding, there was a significant staining of the collagen content between the trabeculae in the CU group. These results clearly indicate that the thickening occurred both from rapid deposition of collagen fibers and mineralization.

Bone-specific ALP binds to collagen type 1 and prepares the skeletal matrix for mineralization by hydrolyzing organic phosphates, increasing the local concentration of phosphate, and encouraging deposition of hydroxyapatite. The two most significant sources of serum ALP are bone and liver. We measured bone-specific ALP (B-ALP) and found a significant reduction in the level

of B-ALP in the RES group, with a reduction of 90% in the RES animals, and 86% in LTRES (Table 3). A significant increase was observed already after 1 day of refeeding, similar results were obtained with LTCU, although the levels of the LTAL and LTCU tended to be lower compared with the short-term group, probably due to the increase in age.

These results show that while RES leads to a reduction in bone growth, CU growth is associated with an immediate increase in EGP height and bone construction.

#### Effect of age and RES on mechanical parameters

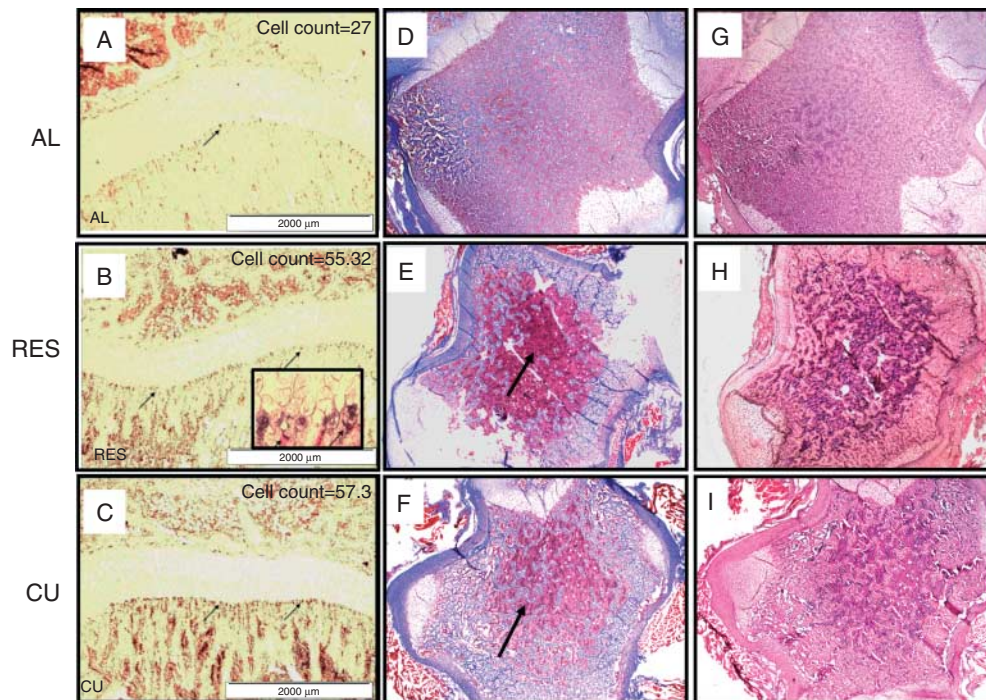
Age was found to affect the mechanical properties of the bones, with an increase in ultimate load, and load to fracture (Table 4), suggesting an improvement in bone mechanical performance with age (BC vs AL vs LTAL).

Assessment of the effect of RES and refeeding on the mechanical characteristics showed that whole-bone stiffness was not significantly affected by the RES in both protocols; in contrast, the load to fracture that was shown not to be significantly different in the RES group compared with the AL group after 11 days of restriction became significantly lower in the LTRES by 36 days of RES (Table 4); the ultimate load tolerated by the bones was significantly reduced already after 11 days of restriction and was further reduced with prolonged restriction. Interestingly, the bones of the short-term CU group showed no improvement in any of the mechanical parameters, but rather a further deterioration, with the load required for bone to fracture significantly lower than in the RES animals. These results demonstrate that 1 day of nutrition-induced CU growth, which did cause an improvement in some of the architectural parameters, was not enough to improve the mechanical performance of the bone, and even increased bone fragility.

This reduction in bone quality, however, seems to be transient and reversible, as after 26 additional days of non-restricted eating, all mechanical parameters of the LTCU group tested were lower, although not significantly different from the LTAL, control group (Table 4, LTAL vs LTCU; NS).

#### Effect of nutritional manipulation on serum components

As the most dramatic effects were observed after 1 day of refeeding, we analyzed several serum components at this time point. There were no significant differences in calcium, phosphate, cholesterol, and HDL-cholesterol levels in all the groups (data not shown).

**Figure 5**

Histological staining. (A, B and C) Humeri sections were stained using TRAP staining, specific for active osteoclast cells in bones. The majority of the staining is localized in the lower boundary of the EGP in all bones (AL, RES, and CU animals; magnitude  $\times 12.5$ ; the small square in B is magnified by  $\times 100$ ); at least six sections from each group were analyzed. Arrows point to

positively stained osteoclasts. (D, E, F, G, H and I) Humeri from AL, RES, and CU rats were taken for histological staining by Masson's trichrome (D, E and F) or H&E (G, H and I) stains (magnitude  $\times 40$ ). Blue stain indicates collagen fibers (arrows); at least four sections from each group were analyzed.

We next measured the serum levels of two growth-stimulating factors known to be affected by nutritional status, IGF1, and leptin. Serum analysis of IGF1 and leptin levels showed that the level of IGF1 was significantly reduced by calorie restriction (Table 3; by  $\sim 80\%$ ;  $P < 0.05$ ). One day of refeeding was enough to increase the level of IGF1 back to normal (CU vs AL; NS), similar results were obtained with long-term data, although the levels of IGF1 were somewhat higher in all groups, possibly due to the fact that these animals have already reached puberty. Leptin levels showed a similar behavior indicating immediate effects on metabolism (CU vs AL; NS), the level of leptin was significantly increased with age

(LTAL vs AL;  $P < 0.05$ ) (Table 3) concomitant with the increase in body weight (Table 1).

## Discussion

To the best of our knowledge, this is the first study showing the immediate effect of nutrition-induced CU growth on bone structure and mechanical characteristics in bones of young, fast-growing rats.

We have previously found that 40% RES for 10 days after weaning led to a significant reduction in total body weight as well as in bone and EGP lengths; nutrition-induced CU growth was associated with a rapid increase in

**Table 3** Serum components analyzed in the animal groups

	AL	RES	CU	LTAL	LTRES	LTCU
Bone ALP (ng/ml)	6936 $\pm$ 1900	696 $\pm$ 149*	5716 $\pm$ 711 <sup>†</sup>	5535 $\pm$ 696 <sup>†</sup>	771 $\pm$ 157 <sup>*,†,§</sup>	4731 $\pm$ 520 <sup>*,†,  </sup>
IGF1 (ng/ml)	1303 $\pm$ 348	230 $\pm$ 53*	932 $\pm$ 180 <sup>†</sup>	1723 $\pm$ 177 <sup>†,‡</sup>	371 $\pm$ 82 <sup>*,†,§</sup>	1790 $\pm$ 240 <sup>*,†,‡,  </sup>
Leptin (ng/ml)	1.2 $\pm$ 0.2	<0.022*	1.44 $\pm$ 0.3 <sup>†</sup>	3.03 $\pm$ 0.7 <sup>*,†,‡</sup>	<0.022 <sup>*,†,§</sup>	2.13 $\pm$ 0.3 <sup>*,†,§,  </sup>

\* $P < 0.05$  compared with AL. <sup>†</sup> $P < 0.05$  compared with RES. <sup>‡</sup> $P < 0.05$  compared with CU. <sup>§</sup> $P < 0.05$  compared with LTAL. <sup>||</sup> $P < 0.05$  compared with LTRES.



**Table 4** Mechanical parameters of the animal groups

	BC	AL	RES	CU	LTAL	LTRES	LTCU
Stiffness (N/mm)	0.10±0.03	0.26±0.04 <sup>†</sup>	0.24±0.04 <sup>†</sup>	0.20±0.03 <sup>†</sup>	0.15±0.07 <sup>†</sup>	0.15±0.06 <sup>*,†,‡</sup>	0.18±0.07 <sup>†</sup>
Ultimate load (N)	32.12±5.57	64.55±7.60 <sup>†</sup>	49.95±8.78 <sup>*,†</sup>	42.66±4.94 <sup>*</sup>	65.2±10.65 <sup>†,‡,§</sup>	41.76±2.2 <sup>*,§</sup>	57.81±7.8 <sup>†,§,¶</sup>
Load to fracture (N)	24.84±4.47	54.54±13.58 <sup>†</sup>	47.85±9.08 <sup>†</sup>	26.62±1.80 <sup>*,†</sup>	58.94±10.93 <sup>†,§</sup>	38.95±3.94 <sup>¶</sup>	48.46±10.6 <sup>†,§</sup>

\* $P < 0.05$  compared with AL (*ad libitum*). <sup>†</sup> $P < 0.05$  compared with RES (food-restricted). <sup>‡</sup> $P < 0.05$  compared with BC (24 days old, baseline control). <sup>§</sup> $P < 0.05$  compared with CU (1 day catch-up). <sup>¶</sup> $P < 0.05$  compared with LTAL (long term *ad libitum*). <sup>||</sup> $P < 0.05$  compared with LTRES (long-term food-restricted). LTCU, long-term CU.

weight and EGP height (already after 1 day), followed by a later increase in bone length (7 days) (Gat-Yablonski *et al.* 2008). In this study, the analysis was extended to include 26 days of CU and detailed morphology by micro-CT and mechanical studies of the bones. Micro-CT has become the 'gold standard' for the evaluation of bone morphology and micro-architecture in rodents *ex vivo*, as it enables direct 3D measurement of trabecular and cortical morphology (Bouxsein *et al.* 2010).

Compared with AL controls, RES rats had lower serum leptin, IGF1, and B-ALP, all of which increased significantly after 1 day of food replenishment. RES did not affect BMD and cortical Ct.Ar/Tt.Ar, but significantly reduced trabecular BV/TV and Tb.N and increased separation. Histological examination indicated reduced collagen fibers content in the mid part of the bones of the RES animals and immunohistochemistry revealed an increase in osteoclast number at the margin of the EGP.

The Masson's Trichome staining of the collagen fibers show that during CU growth, already after 1 day of refeeding, there is a significant deposition of collagen fibers, in contrast to previous state of RES. Similar results regarding the specific and significant effect of RES on collagen production were previously shown also by Spanheimer *et al.* (1991) (collagen production in fasted and RES rats: response to duration and severity of food deprivation). In this study, the authors followed collagen production by monitoring [<sup>3</sup>H] proline incorporation into collagen proteins and they have shown that 40% of RES compared with the *ad libitum* diet (control) specifically reduced the level of collagen production, probably by affecting its synthesis (Spanheimer & Peterkofsky 1985, Spanheimer *et al.* 1991). In addition, similar to our study, they have shown a reduction in the serum level of IGF1. *In vitro*, IGF1 in physiological concentrations was previously shown to induce collagen production in chondrocytes (Willis & Liberti 1985) and in bones (Canalis 1980). Our results clearly show similar changes in IGF1 levels, with a

significant reduction in RES rats and a rapid increase already after 1 day of refeeding.

The sections used for the Masson's Trichome staining were cut from an area in which the EGP is not present, and the differences in the intensity of the staining stem from type 1 collagen, which is predominant in the bones. Our results all together indicate that there is a reduction in the level of collagen in the bone during RES and a rapid synthesis of bone already after 1 day of refeeding, as shown by both collagen staining and B-ALP.

Examination of a group of young rats killed at the beginning of the study enabled us to follow the effect of age on bone parameters during the 'childhood' period of fast linear growth (24–35 and 60 days). There was significant increase in most of the cortical and trabecular parameters as well as the mechanical properties of the bones, including mean polar moment of inertia, even in RES rats.

RES led to significant reduction in multiple parameters of cortical and trabecular bones, with the most dramatic effect seen in the trabecular components already after a short term of 11 days RES. Caloric restriction was shown to be associated with bone loss in middle-aged humans (Villareal *et al.* 2006) and late-middle-aged rodents (LaMothe *et al.* 2003); however, no changes in BMD were observed in this study, maybe due to the differences in age.

Interestingly, mechanical analysis showed that although stiffness was not affected, the load required for fracture, which was slightly reduced after 11 days of RES, continued to decrease and was significantly lower than that of the control group after 36 days of RES. In addition, we noted a significant reduction in the ultimate load of the bone. All these results suggest increased fragility of the bones in the RES group. In a similar study conducted on fast-growing young mice reported by Devlin *et al.* (2010) there was a significant reduction in ultimate force with a concomitant reduction in stiffness. The difference in the

effect on bone stiffness reported by Devlin *et al.* may be due to the differences in bones studied, species studied, or the different restriction protocol. Thus, we agree with their statement that 'the skeletal consequences of CR vary depending on the age at onset, duration, and severity of CR' (Devlin *et al.* 2010).

In recent years, the effect of RES on bone quality was assessed in several studies using micro-CT scanning and mechanical testing. However, most of these studies were carried out on adult animals and used diverse protocols, including different species (mostly mice and rats) of various ages, sex and strains, different extent, and duration of the restriction (from 4 weeks to 4 months) with matched or non-matched intake of micronutrients (Talbot *et al.* 2001, LaMothe *et al.* 2003, Lambert *et al.* 2005). These variations make it quite difficult to make valid comparisons. Our results are, however, in line with the most common observations that cortical bone was less significantly affected by RES than the trabecular bone.

Recently, it has become apparent that the skeleton is actively involved in regulatory processes due to its capability to store and release chemical elements. One likely explanation for the deterioration in bone quality when nutrition is limited may be that RES causes increased resorption of bone material to compensate for nutritional deficiencies (Ferguson *et al.* 1999). Indeed, in our study, serum calcium and phosphate levels were similar in both groups despite the fact that the RES group was provided with only 60% of the food intake by the AL group, with no fortification. It is of interest that our study as well as others (Hamrick *et al.* 2008, Devlin *et al.* 2010) showed a significant increase in osteoclast number in RES animals; their localization at the margins of the EGP suggests increased bone resorption.

The most important observation, in our opinion, is the deterioration of bone quality observed after 1 day of CU growth. In previous publications, we showed that during the CU growth there was a dramatic change in EGP height, cell number, and gene expression (Even-Zohar *et al.* 2008). The increase in collagen fiber content, B-ALP level, and trabecular parameters already after 1 day of CU indicated that active bone-growing processes were occurring. It was previously (Devlin *et al.* 2010) shown that trabecular architecture was more dynamically regulated than cortical bone; indeed an immediate increase in trabecular bone construction was noted, while the mechanical parameters of the bones showed either no change compared with the bones of the restricted group, or a further reduction. This effect may be due to the significant increase in production of cartilage matrix

occurring in EGP cells, probably, at the expense of the modeling process. The results of the three-point bending tests performed on whole bone are affected by both the geometry of the whole bone and the mechanical properties of the material from which it is made. Our data clearly show that the geometry and composition of the cortical part of bones tested was affected by both RES (lower cortical thickness and mean polar moment of inertia) and by refeeding (mineral density, which although it was not significantly different from the AL and RES groups by the selected statistical criteria, still showed a tendency to be lower; Ct.Ar showed a similar tendency; and mean polar moment of inertia was significantly reduced compared with AL and RES). We therefore believe that the cumulative effect of BMD and geometry (and perhaps also unmeasurable aspects like collagen arrangement) are responsible for the effect on the mechanical parameters measured by the three-point bending test.

These results are supported by another study carried out by our research group, in which the effect of weight load on the growth plate of chicks was analyzed (Reich *et al.* 2010). Using a completely different system for growth restriction and CU growth, it was found that during CU growth, concomitantly with an increase in EGP height, there was deterioration of the structural and mechanical characteristics of the skeleton suggesting that accelerated mineralization occurred in the EGP of the affected bones and that decelerated mineralization occurred during the CU period, when chondrogenesis is the dominant process. These results suggest that the process may be more general than at first thought as it took place in both rats and chicken in very different experimental set-ups (Reich *et al.* 2010). Of interest in this respect is the recent report on preterm infants, in which there was a significant decrease in bone strength during the first weeks of life, despite overall growth and weight gain (Eliakim *et al.* 2009).

Systemic factors previously shown to be most affected by nutritional status are IGF1 and leptin. IGF1 serves as both the main mediator of GH action and as a GH-independent growth factor, and is a well-known growth factor for both chondrocytes and osteoblasts. IGF1 concentrations are responsive to changing nutritional status and intake of amino acids and free fatty acids (Mosier & Jansons 1976, Hermanussen *et al.* 1996), and serum IGF1 levels were reduced by RES in all animals studied (Lowe *et al.* 1989, Gat-Yablonski *et al.* 2008, Devlin *et al.* 2010, Pando *et al.* 2012). The reduced serum IGF1 level is likely to contribute to the cortical thinning observed, as a similar phenotype was also observed in liver-specific IGF1 deficient lid/lid mice (Yakar *et al.* 2002).

Calorie restriction also leads to significant changes in adipocyte number and activity. Adipose tissue produces numerous adipokines, some of which have been previously shown to affect bone growth and mass accrual, and may directly regulate bone formation by affecting osteoblasts and osteoclasts. These may include leptin (Gat-Yablonski & Phillip 2008, Karsenty & Oury 2010), adiponectin (Challa *et al.* 2010), estrogen (Grodin *et al.* 1973), and interleukin 6 (Peruzzi *et al.* 2012). A significant reduction in the level of leptin upon RES with an immediate increase in its level upon food replenishment was shown in this study as well as in previous ones (Gat-Yablonski *et al.* 2004, Devlin *et al.* 2010). The effect of leptin on bone quality is controversial (Turner *et al.* 2013). Several genetic models show that leptin is a powerful inhibitor of bone mass accrual (Karsenty & Oury 2010), while others showed the opposite (Turner *et al.* 2013). It may have differential effects on cortical and trabecular bone as decreased cortical thickness and increased trabecular bone volume were observed in leptin-deficient *ob/ob* mice. However, in our study the significant reduction in leptin level during RES was associated with decreased cortical thickness as well as a reduction in trabecular BV/TV and Tb.N. As direct infusion of leptin into the bone was shown to increase osteoblast activity (Evans *et al.* 2011, Turner *et al.* 2013), the increase in leptin levels upon refeeding may be associated with the rapid increase in B-ALP.

Leptin stimulated bone elongation (Gat-Yablonski *et al.* 2004). A direct link between leptin and linear growth was suggested by findings that leptin administration to *Ob/Ob* mice corrected their metabolic abnormalities and also led to a significant increase in femoral length (Steppan *et al.* 2000, Iwaniec *et al.* 2007). Leptin was also found to directly stimulate GH secretion (Carro *et al.* 1997, Jin *et al.* 1999, Goldstone *et al.* 2002, Accorsi *et al.* 2007) and significantly improve structural properties and elongation rate of bones in an IUGR model (Bar-El Dadon *et al.* 2011). Furthermore, in our previous studies on CU growth, it was observed that leptin treatment can increase longitudinal growth and EGP height in RES rodents (Gat-Yablonski *et al.* 2008).

Long-term follow-up of the animals, for 26 days of non-restricted food consumption, led to correction of all mechanical and architectural parameters, similar to the results reported in the study published by Boyer *et al.* (2005) in which they examined a 4 and 8 weeks of CU growth and found no significant difference in bone strength between the control and the CU groups. Thus, the reduction in bone quality is a transient and reversible phenomenon, and

further studies to identify the precise time point of the correction may be required. It will be interesting to follow studies of BMD and strength in children following CU growth to validate our findings in children.

How can we relate these findings to children? It is quite inaccurate to compare rat's life with humans. In a recent summary published by Sengupta (2013) it is said: 'it could be easily noticed that rats have a brief and accelerated childhood in respect of humans. Rats develop rapidly during infancy... and reach puberty at an average age of 50 days after birth (P50)'. Other studies also showed that rats begin puberty at the average age of 50 days, depending on the specific strain.

We have begun the experiment as soon as these animals could use solid food as a sole source of food (day 21), and allowed them to acclimatize to solitary stay in cages before the beginning of the experiment (3 days). At the age of 24 days (the first day of the experiment) rats are at their linear growth stage, similar to childhood phase in humans. In the short-term experiment, the rats reached the age of 34 days, thus they were before puberty, while at the end of the long-term experiment they were at the age of 60 days, they have already reached puberty. However, the most dramatic effect on the deterioration of bone quality was observed after 1 day of refeeding, in which the animals are in their 'childhood' phase, thus it seems that sex hormones have no effect on this phenomenon. Furthermore, at the termination of the long-term experiment, the animals reach their target height and show complete CU growth, again suggesting that sex hormones probably have no significant effect on this model.

To conclude: our findings, suggesting that nutrition-induced CU growth may be associated with an immediate reduction in bone quality, suggest the importance of taking appropriate measures to avoid bone fractures in vulnerable children undergoing CU growth during the first period.

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#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

R P, E M-O, R S, and G G-Y designed research; R P, A I, B S, and M M conducted research; R P performed statistical analysis; R P, E M-O, R S, and

G G-Y wrote paper; E M-O, R S, M P, and G G-Y had primary responsibility for final content. All authors read and approved the final manuscript.

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