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The effect of weight loading and subsequent release from loading on the postnatal skeleton

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ABSTRACT

Introduction: The relationship between load and the structure and mechanical properties of mature bones has been thoroughly described. In contrast, this relationship has been studied much less in immature bones, which consist of bony tissue and cartilaginous growth plate, during the postnatal period. This paper describes the effect of an externally applied load on the bones of young fast-growing chicks; in particular, we examine the effect on the growth plate, which regulates longitudinal bone growth, and the consequences in terms of bone structural and mechanical properties.
Materials and methods: The tibial growth plates from chicks subjected to external load and control chicks, immediately after loading and following 5 days of load release, were studied by histological staining and quantitative PCR. The contralateral tibiae were mechanically tested by three-point bending and their structural features determined by micro-CT.
Results: At the end of the external loading period, the tibias of the experimental group were shorter and their growth plate narrower than in controls. However, at this time point, effects were not yet apparent in the bones' structural or mechanical parameters. After a further 5 days of no external load, bones and growth plates of the experimental group demonstrated the phenomenon of 'catch-up': the thickness of the growth plate exceeded that of the control; however the relative expression of genes controlling chondrocyte differentiation (collagen II and X) did not change, while the expression of factors related to growth-plate ossification (osteopontin, alkaline phosphatase) and cartilage and bone calcification (matrix and bone Gla proteins) was upregulated as a result of the catch-up process. At this time, however, the tibiae of the experimental group showed inferior mechanical and structural properties relative to the control group.
Conclusion: External loading during bone elongation negatively affects the mechanical and structural properties of the skeleton. The effect is first noticeable in the growth plate, which regulates bone growth, and is exhibited in the bone phenotype after a lag period.

Introduction

The morphological, structural and material features of the skeleton in vertebrates are genetically programmed; they can also be modified by epigenetic factors, the most important of these being local tissue stress and strain states [1,2]. These strains are created by intrinsic muscle forces and external loads. The ability of bone tissue to adapt its mass, shape and internal architecture to its mechanical loading environment is a fundamental concept of bone biology [3–6]. Indeed, the idea that bone structure is controlled locally to best fulfill its mechanical function has become a central tenet of orthopedics; furthermore, many paleontological and bioarcheological studies of skeletal material are based on this concept [6].

Many studies have investigated the effect of increased or decreased load on the structure of mature bones, both *in vivo* and *in vitro* [7–13]. Increased load has been shown to cause an increase in bone mass while decreased load (due to disuse by enforced rest, space flight or other unloading protocols) leads to a loss in bone mass. These effects occur through the processes of bone modeling and remodeling, and involve the cells of the bone tissue (osteocytes, osteoblasts and osteoclasts).

Mechanical loads are also known to play an important role during skeletal development in the postnatal period. It is well known, for instance, that various types of physical activity in children affect the geometry and composition of their bones [14–16]. However, studies describing the detailed interaction between loading during this time period and bones' structural and mechanical features are rare. In particular, the mechanism by which such influence is exerted is not

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well understood, and details regarding the relationship between load magnitude and duration and such quantitative properties as strength, stiffness and yield load are currently lacking. Our study sheds some light on this complex biological question.

During the postnatal period, long bones consist of osseous tissue and the cartilaginous growth plate which is responsible for their axial growth. The rate of bone elongation is regulated on one side of the growth plate by the rate of resting-cell division, chondrocyte proliferation and hypertrophy, and on the opposite side by penetration of blood vessels and cartilage resorption at the border of the growth plate and the metaphyseal bone [17–20]. These processes are tightly regulated by systemic (endocrine) and local (paracrine) factors, and determine overall body dimensions as a result of skeletal growth. It is reasonable to assume that mechanical load during this time will affect the chondrocytes in the growth plate and result in morphological and mechanical effects on the developing bone. This study examines these effects.

Animal studies suggest that growth-plate chondrocytes may have a finite proliferative capacity that is gradually exhausted, causing growth to slow and eventually cease altogether [21,22]. It has been shown that conditions that suppress growth-plate chondrocyte proliferation conserve the proliferative capacity of the chondrocytes, thus slowing senescence. Consequently, after transient growth inhibition, growth plates retain a greater proliferative capacity and are less senescent, enabling the phenomenon of catch-up growth [23]. In this way, above-normal growth rates occur when the cause for the growth retardation is removed [24].

The broiler chick was used as the animal model for this study because it is bipedal (compared to most other laboratory animals which are quadrupeds), rendering the results at least partially applicable to humans, and because broilers have been genetically selected for extremely high rates of growth (increasing their weight during the first 5 weeks after hatch by 5000%, from a hatching weight of 40 g to a weight of 2 kg at 5 weeks) [25]. Such 'spurt growth' was generally assumed to occur exclusively in humans during the first year of life [26]. Furthermore, several studies have suggested that the skeleton is more responsive to mechanical stimuli when growth is rapid [27–29], a fact which might accentuate the effects of loading in our broiler model.

In this paper, we describe the use of a previously reported protocol [30] to study the effects of external mechanical loading on the differentiation, mineralization, and ossification processes in the growth plate of fast-growing bones, and on the resulting phenotype of these bones in terms of architecture and mechanical properties. We hypothesize that such loading has deleterious effects on both the growth plate and the bone, and that load removal will be followed by recovery through the phenomenon of catch-up.

Materials and methods

Animals

The experiments were approved by the Ethics Committee for Animal Experimentation, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Israel.

Eighty 1-day-old broiler chicks (Cobb) were obtained from a commercial hatchery (Brown Hatcheries, Hod Hasharon, Israel), raised in constant-temperature battery brooders at 34 °C, and fed an age-appropriate diet, according to National Research Council recommendations, ad libitum. After a 24-h period of adaptation, the chicks were divided into two groups: 'load' (40 chicks) and 'control' (no load) (40 chicks).

Loading model

Two-day-old chicks in the 'load' group were harnessed for 4 days with small bags (2.5×4 cm) filled with sand weighing 10% of their BW, as previously described [30]. At the end of this loading period (when they were 6 days old), 20 chicks from the 'load' group were sacrificed (6-day load, 6dL) while the other 20 chicks were released from loading and allowed to grow for a further 5 days free of load (11 days release from load, 11dRL). Chicks belonging to the control group were raised under the same conditions, but without artificial loads (6-day control, 6dC and 11-day control, 11dC).

At the end of the experiment, chicks were anesthetized by inhalation of isoflurane, USP Terrell (Minrad Inc., USA), followed by cervical dislocation. Tibia lengths were measured with a ruler (±0.1 mm), then prepared for histological assessment or stored at −20 °C for mechanical and architectural evaluation.

Histological staining and in-situ hybridization

The effect of external load on the growth plate was examined by histological staining and *in-situ* hybridization. Growth plates and the adjacent cortical bone tissue were fixed overnight in 4% paraformaldehyde (Sigma Chemical, St Louis, MO, USA) in PBS at 4 °C. The samples were dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in Paraplast. Thin (5 μm) sections were prepared. The sections were stained with Alcian blue and Von Kossa staining (0.6% Alcian blue 8GX in 70% ethanol and 2% silver nitrate exposed to sunlight). Tartrate-resistant acid phosphatase (TRACP) and alkaline phosphatase (ALP) [31] staining were performed as previously described [30].

The sections were hybridized with digoxigenin-labeled antisense probes for collagen (COL) type I, II or X, or with ^{35}S-labeled probes (10 ng) for bone Gla protein (BGP) and matrix Gla protein (MGP)

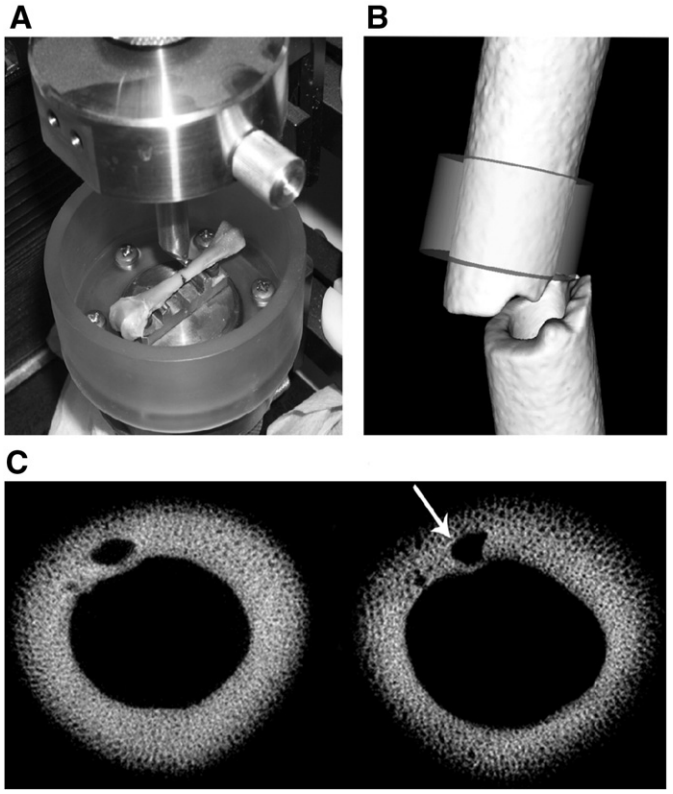

Fig. 1. (A) Three-point bending test set-up with custom-built saline-containing testing chamber. Bones were placed on the two testing supports such that their posterior surface faced the supports, which were equidistant from the ends of the bone. The distance between the supports was set so that both supports contacted the diaphysis. (B) A 1.0-mm transverse section (35 slices) of the diaphysis of the tibia, adjacent to the fracture line, was used to measure the cortical parameters. (C) A typical example of 2D transverse sections of the micro-CT scan to demonstrate the differences in architectural parameters between chicks with (right) or without (left) mechanical load at 11 days of age. Images were reconstructed from the same anatomical region of the tibia by identifying the nutrient foramen (white arrow).

[32,33]. Digoxigenin-labeled probes were detected using a polyclonal anti-digoxigenin antibody attached to ALP [34]. The sections were stained with Methyl green (Zymed, USA) and photographed using bright-field microscopy. The radioactive signal of ^{35}S-labeled probes was intensified using emulsion (Eastman Kodak Company USA): the sections were incubated with the emulsion solution for 1 month in the dark at room temperature. After development, the sections were stained with hematoxylin for nucleus staining and photographed using bright- or dark-field microscopy. No signal was observed in any of the hybridizations with sense probes which were used as controls. Each slide represents three different specimens.

Measurement of ALP activity [31]

ALP activity was measured with *para*-nitrophenyl phosphate (pNP) as the substrate. Protein was extracted from the proximal tibial growth plates of chicks belonging to the no-load group (11dC) and chicks belonging to the load-release group (11dRL). Each sample was pooled from 10 different animals. Protein concentration was measured using the BCA protein reagent kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol. Activity was assayed in 0.5 M Tris–HCl buffer (pH 9.5) supplemented with 0.5 mM pNP and 0.5 mM $MgCl_2$. The mixture was incubated at 37 °C for 10–30 min, and the reaction was stopped by addition of 0.25 vol of 1 M NaOH. Hydrolysis of pNPP was monitored as the change at 410 nm in a spectrometer (BIO-TEK Instruments Inc., Winooski, VT, USA). *Para*-nitrophenol was used as a standard.

RNA isolation and reverse transcription (RT)

Proximal tibial growth plates were isolated and prepared with RNeasy Maxi kit (Qiagen, Germany) according to the manufacturer's protocol in order to prepare RNA samples. Total RNA (1 μg) was reverse-transcribed in a final volume of 20 μl with the Reverse-iT kit (ABgene, UK) following the manufacturer's protocol, using oligo-dT/hexamer primers. Reaction temperatures were 42 °C for 1 h followed by 75 °C for 10 min.

Real-time PCR

cDNA (1 μl) was used for real-time PCR using the fluorescent dye SYBR Green I (Absolute QPCR SYBR Green Mix, ABgene) to monitor DNA synthesis using specific primers as follows:

Gallus gallus ribosomal 18S (normalizing control) primers (forward: 5′-TCCGATAACGAACGAGACTCTG-3′, reverse: 5′-CGGACATC-TAAGGGCATCACA-3′)

COL II primers (forward: 5′-CCAACGACGTGGAGATCAGA-3′, reverse: 5′-CTGCAACCGGTACTCGATCA-3′)

COL X primers (forward: 5′-GATTGCCAGGGATGAAGGG-3′, reverse: 5′- TTTGAGTCCTTGAGGGCCC-3′)

Osteopontin (OPN) primers (forward: 5′-TGGCCAGTGAGCAAAT-CCA-3′, reverse: 5′- ACGTGATCCTGGTGGTACCTG-3′)

MGP primers (forward: 5′-CAGGAGAGGATCAGGGAACG-3′, reverse: 5′-AAGCAGCAGGATAGCCATGG-3′).

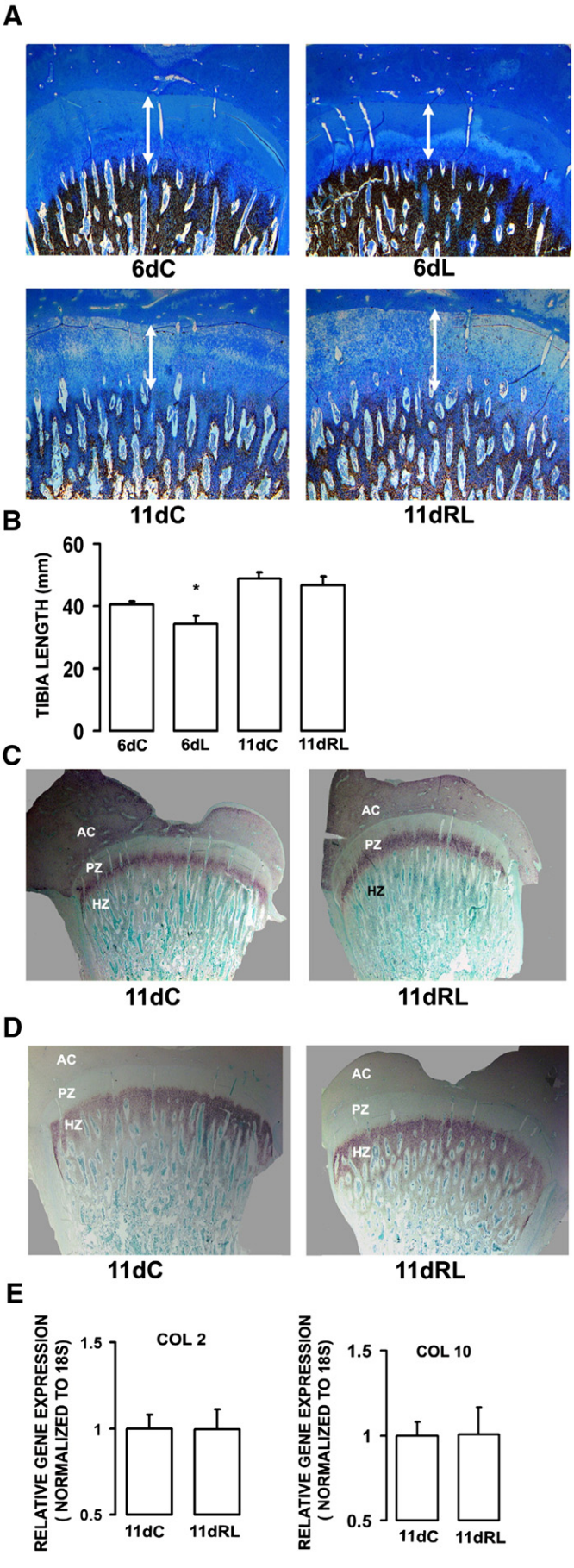

Fig. 2. The effect of mechanical loading and release from loading on bone elongation and growth-plate narrowing and expansion. (A) Histological sections of chick bones stained with Alcian blue/Von Kossa at 7.5× magnification. The arrow marks the nonmineral growth-plate width in chicks with (6dL) or without (6dC, 11dC) mechanical load and chicks after release from load (11dRL). (B) Tibia length of weight-loaded (6dL) chicks differed from that of nonloaded chicks (6dC) at 6 days of age. Chicks after release from load (11dRL) showed no difference from the control group (11dC). *Significantly different at $p<0.05$ ($n=9$). (C, D) Localization of COL II and COL X gene expression was studied by *in-situ* hybridization with digoxigenin-labeled antisense riboprobes. (C) COL II mRNA can be detected in the proliferation zone (PZ) located between the articular cartilage (AC) and the hypertrophic zone (HZ). (D) COL X mRNA can be detected in the HZ. (E) Relative expression of COL II and COL X mRNA in the growth plates measured by real-time PCR analysis ($n=3$).

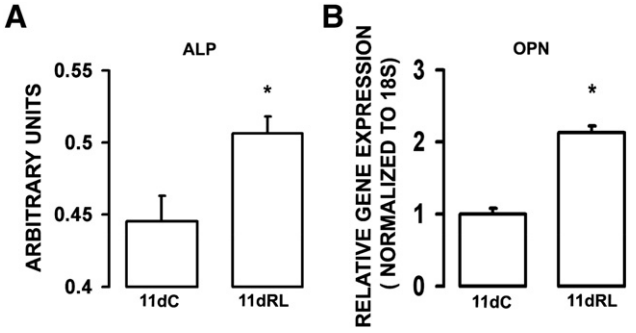

Fig. 3. The effect of mechanical loading and release from loading on growth-plate ossification. Growth plates were dissected from ten different chicks from each group, and RNA and proteins were extracted for quantitative analysis. (A) Alkaline phosphatase (ALP) activity [2] was studied by biochemical analysis, and (B) osteopontin (OPN) relative mRNA expression was measured by real-time PCR analysis. *Significantly different at $p<0.05$ ($n=3$).

The PCR was carried out in an ABI Prism 7300 system (Applied Biosystems, Foster City, CA, USA) using the following cycling protocol: a 95 °C denaturation step for 15 min followed by 40 cycles of 95 °C denaturation (15 s), 60 °C annealing (30 s), and 72 °C extension (30 s). Gene expression was normalized to the housekeeping gene 18S. The amplified PCR product was analyzed with ABI Prism 7300 software (Applied Biosystems). At the end of the real-time PCR run, a melting curve was determined to verify the presence of a single amplicon.

Mechanical testing

To study the consequences of growth-plate changes (and possibly also the direct effects of load on bone tissue) on the mechanical properties of the tested tibiae, mechanical testing of samples was carried out on 10 bones from each of the four groups (6dL, 6dC, 11dRL, and 11dC). Based on methods described previously [35], the bones were tested by three-point bending using a materials-testing machine (Instron materials-testing machine, Model 3345, Norwood, MA, USA) with a custom-built saline-containing testing chamber (see Fig. 1A). On the day of testing, each bone was slowly thawed to room temperature, and placed on two supports with rounded profile (1 mm diameter) such that the supports were located equidistant from the ends of the bone, both contacting the posterior aspect of the diaphysis. The distance between the supports was set to 14 mm in the 6dC and 6dL groups, and to 18 mm in the 11dRL and 11dC groups. Each bone was loaded on its anterior aspect at the point

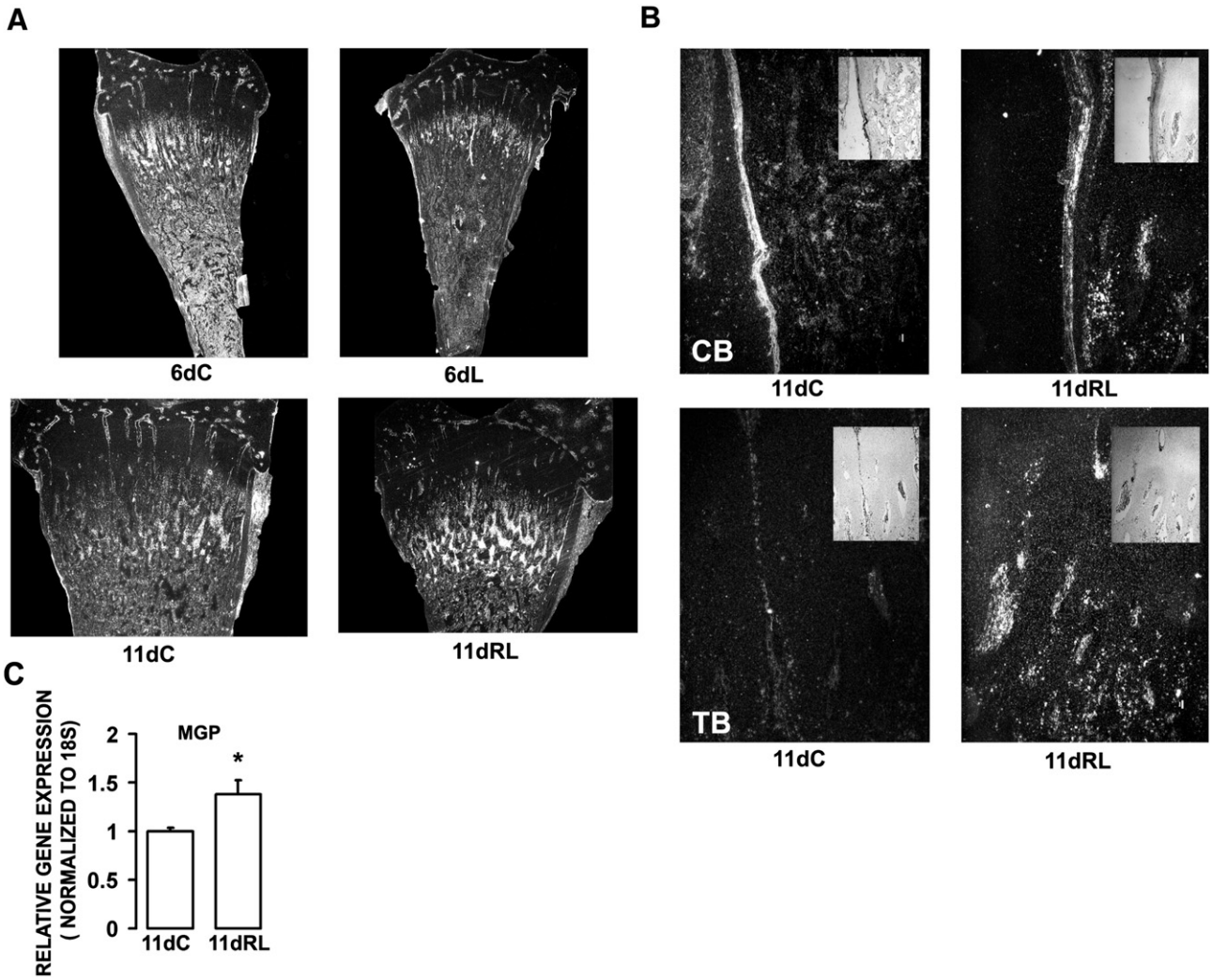

Fig. 4. Effect of mechanical loading and release from loading on gene expression of Gla proteins in the growth plates, chondro-osseous junction and bones of chicks with (6dL) or without (6dC, 11dC) mechanical load and chicks after release from load (11dRL). (A, B) MGP and BGP gene expression were localized by *in-situ* hybridization with ^{35}S-labeled probes. MGP mRNA (A) can be detected in the chondro-osseous junction of the growth plates at 7.5× magnification. BGP mRNA (B) can be detected in the compact bone (CB) and in the trabecular bone (TB) at 40× magnification. (C) MGP relative mRNA expression was measured in growth plates dissected from ten different chicks from 11dC and 11dRL groups by real-time PCR analysis. *Significantly different at $p<0.05$ ($n=3$).

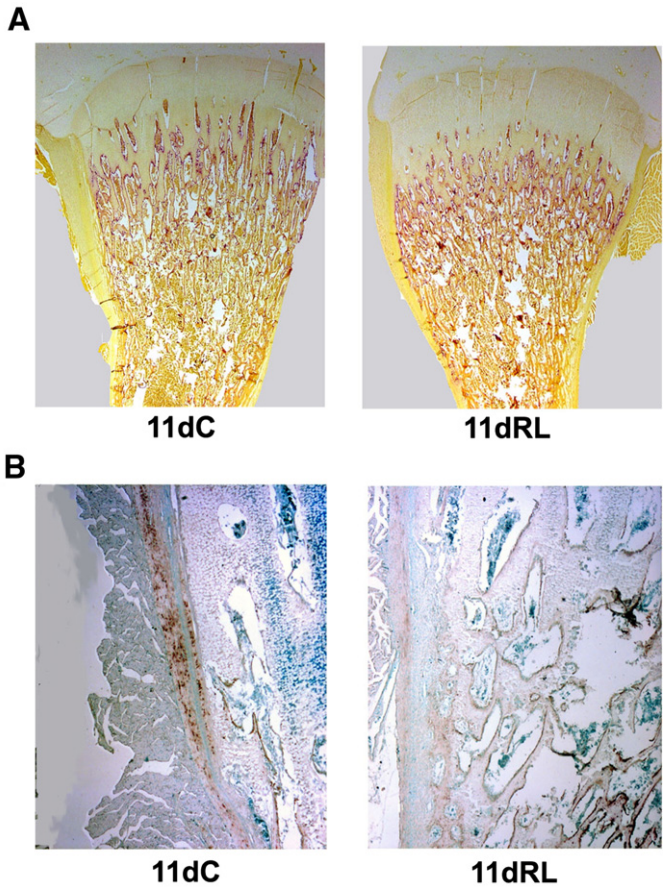

Fig. 5. The effect of mechanical loading and release from loading on bone remodeling of chicks after release from loading (11dRL). (A) TRACP staining of an adjacent section identifies the localization of active osteoclast cells and is shown at 7.5× magnification. (B) COL I mRNA was studied by *in-situ* hybridization with digoxigenin-labeled riboprobes: it can be detected mainly in the compact bone, shown at 40× magnification.

corresponding to the mid-distance between the bottom supports. Loading was conducted at a constant rate (2 mm/min) up to fracture. Force–displacement data were collected by Instron software (BlueHill®) at 10 Hz. The resulting load–displacement curves were used to calculate stiffness, yield load and ultimate load. The stiffness was calculated as the slope of the linear portion of the load–displacement curve [36]. Young's modulus was approximated by using beam theory and the following relationship:

$$E = \frac{S \cdot L^3}{48 \cdot I}$$

where E is the effective Young's modulus (N/mm^2), S is the slope of the linear portion of the load–displacement curve (N/mm), L is the support span (mm) and I is the cross-sectional moment of inertia (mm^4) determined by micro-CT at the area of fracture.

Micro-CT scanning

Differences in the mechanical properties of whole bones are caused by architectural variations and changes in material composition (primarily bone mineral density—BMD). To evaluate the structural and material consequences of external loading, all tibiae that were tested mechanically were also scanned using a micro-CT device (eXplore locus SP, General Electric (GE) London, Ontario, Canada) with custom software version 5.2.2 (MicroView). Four bones were scanned at a time, using a custom-built tube which allowed secure positioning and individual identification of each bone. Medium-resolution scans (28 μm voxel size) were acquired. The X-ray source was set at 80 kV and 80 μA, and 720 projections were acquired over an angular range of 360°. The image slices were reconstructed using Microview reconstruction software, version 2.12. During analysis, the scanned images were rotated so as to align their anatomical and scan axes. As all tibiae fractured at the same location in all bones tested (at the point of load application), a volume adjacent to this area was used for comparative purposes (see Figs. 1B, C). A 1.0-mm-long transverse section of the diaphysis of the tibia, adjacent and proximal to the fracture site, was used to measure the following cortical parameters: volumetric BMD, bone mineral content (BMC), cross-sectional moment of inertia (CSMI), mean cortical thickness, inner and outer perimeter, marrow area and cortical cross-sectional area. Hounsfield units (HU) were calibrated to mineral density (GE Medical) using the same protocol as that used for the experimental samples, and converting the linear attenuation of voxels to mg/cc of hydroxyapatite (HA) accordingly.

Statistical analysis

Statistical analyses of mechanical testing and structural analysis results were conducted in order to find differences between the loaded and control groups. Quantitative data (geometric parameters, mechanical-testing results) were compared between the experimental and control groups at each of the two tested time points (6dL vs. 6dC and 11dRL vs. 11dC) by Student's t test. Level of significance was set at $p \leq 0.05$.

Results

Body weight and bone length

The effects of loading and release from loading were first determined in terms of body weight and total bone length. During the 4-day loading period, the rate of weight gain of chicks from the 6dL group was lower than that of the 6dC group, (15.7±3.2 g/day vs. 23.9±4.9 g/day, respectively). Five days following load release, chicks

Table 1
Microarchitectural characteristics at the midshaft of tibiae from the experimental (6dL, 11dRL) and control (6dC, 11dC) groups

Parameter	Treatment group			
	6dC	6dL	11dC	11dRL
Mean thickness (mm)	0.5642 (0.067)	0.5029 (0.062)	0.9366 (0.104)**	0.8039 (0.089)
Cortical area (mm^2)	3.6484 (0.658)	3.2438 (0.454)	7.7165 (1.119)**	6.2505 (1.035)
Marrow area (mm^2)	2.0963 (0.416)	2.1086 (0.211)	2.713 (0.503)	2.607 (0.356)
CSMI (mm^4)	123.9 (47.7)	104.3 (26.3)	438.5 (111.5)**	343.0 (112.0)
BMD (mg/cc)	1097.5 (57.7)	1090.0 (57.3)	1242.0 (104.8)	1157.2 (70.5)
BMC (mg)	0.1180 (0.017)*	0.1041 (0.010)	0.2935 (0.045)**	0.2248 (0.043)

Results include mean and standard variation (in brackets) (9–10 bones per group). Parameters which were found to differ significantly ($p<0.05$) are followed by an asterisk (between the 6dC and 6dL groups) or by two asterisks (between the 11dC and 11dRL groups). CSMI—cross-sectional moment of inertia, BMD—bone mineral density, BMC—bone mineral content.

in the 11dRL group caught up with those in the control group (11dC) and the weight-gain rate of both groups was not found to be significantly different (33.4±3.9 g/day vs. 32.9±4.1 g/day, respectively). We have previously shown that body-weight reduction of chicks in the experimental group (6dL) was the result of reduced feed consumption rather than energy loss or other metabolic problems [30].

On day 6, the loaded group (6dL) had significantly shorter tibial lengths compared to the nonloaded control chicks (6dC) (34.4±2.7 mm vs. 40.6±1.15 mm, respectively). After the release from loading, the tibial length in the experimental group (11dRL) increased to 46.9±2.7 mm and that of the equivalent control group (11dC) was 48.9±2.1 mm (Fig. 2), a difference which was not statistically significant.

These findings suggest that external loading of the postnatal skeleton inhibits the axial growth of long bones, and that a subsequent load-release period allows accelerated axial growth rate, in essence catching up to control levels.

Effects on the growth plate

External load affects the postnatal skeleton at least in part due to its effect on the various stages of chondrocyte development in the growth plate. A basic parameter that was found to be affected was growth-plate thickness. The thickness of the growth plate was reduced in the 6dL group compared to the 6dC group by 74±4%. However, a dramatic change was seen after 5 days of release from loading: the thickness of the growth plate in the experimental group (11dRL) reached 127±3% that in the control group (11dC) (Fig. 2A). The changes in growth-plate thickness were consistent with the accelerated rate of elongation of the tibiae in the load-release group (11dRL) seen in Fig. 2B, suggesting catch-up growth following load release.

To determine the possible processes taking place in the growth plates and affecting bone properties through loading and release from loading, we studied the expression of marker genes related to chondrogenesis and osteogenesis in the growth plate and the adjacent bone tissue. The expression patterns of COL II (characteristic of resting and proliferating chondrocytes; Fig. 2C) and COL X (typical of hypertrophic chondrocytes; Fig. 2D) demonstrated narrower expression zones in the loaded group (6dL) compared to the control group (6dC). Five days after release from loading, COL II expression was similar in both groups (Fig. 2C; 11dC vs. 11dRL), and COL X expression demonstrated even wider expression zones in the experimental group (11dRL) than in the controls (11dC) (Fig. 2D). Quantification of the expression levels of COL II and X in the growth plates by real-time PCR showed no differences between them (Fig. 2E), suggesting that loading and its release affected both the proliferative and hypertrophic zones of the plate, and the amount of chondrogenic markers was therefore not altered relative to controls. Cartilage ossification was studied by quantitative measurements of ALP activity (Fig. 3A) and OPN mRNA expression (Fig. 3B), using pooled material obtained from 10 different growth plates from each of the different groups. The upregulation of OPN and ALP in the growth plates after load release was associated with accelerated ossification concurrent with the catch-up process.

The effect of external load on mineralization was addressed by studying MGP and BGP, which are known to be involved in this process in cartilage and bone, respectively; MGP is a calcium-binding protein known to inhibit cartilage and vascular mineralization [37] and BGP [38] is expressed mainly by osteoblasts. MGP was detected in the chondro-osseous junction (Fig. 4A). Four days of loading downregulated its expression (6dC vs. 6dL) while release from loading upregulated it, as demonstrated histologically and also by quantitative analysis in these growth plates (Fig. 4C). BGP was affected differently in different fractions of the bone at the end of the catch-up period; in the compact bone (CB), BGP was slightly downregulated as compared to the controls, while in the trabecular bone (TB), it was clearly upregulated (Fig. 4B). The results suggest that these genes are highly responsive to mechanical signals.

The process of bone modeling was also studied. TRACP staining, which marks the presence of active osteoclasts, was detected further away from the growth plate of chicks belonging to the load-release group (11dRL) compared to the control group (11dC) (Fig. 5A). This pattern is consistent with the observed thickness of the growth plate. At the same time, a reduction in the expression of COL I mRNA, (expressed by osteoblasts) was detected in the CB of the experimental group (11dRL) (Fig. 5B), in a manner similar to that observed for BGP at this location.

These results demonstrate that external load applied to chicks during the early postnatal period affects the regulatory mechanisms of all major cells participating in bone growth of both the bone *per se* and its growth plate. Load was found to inhibit the development of the growth plate while 5 days of load removal reversed this process. Loading was also seen to alter the mineralization and ossification processes in the growth plate and adjacent bone.

Mineral density and architectural features

Changes occurring in the regulatory mechanisms are likely to have an effect on the architecture and material properties of the developing bones. This aspect was studied by micro-CT scanning of all bones. Table 1 presents the geometric parameters yielded by micro-CT scanning of the tibiae belonging to the experimental groups (6dL, 11dRL) and control groups (6dC, 11dC). It can be seen that after 4 days of loading, the loaded (6dL) and control groups (6dC) did not differ in most parameters, except for a slight, but nevertheless statistically significant decrease in BMC in the 6dL group. However, after a further 5 days without load, most parameters were lower in the 11dRL group compared to the 11dC group. Specifically, the mean cortical thickness, cortical area, CSMI and mineral content were all significantly lower in the 11dRL group than in the control group. The BMD and bone-marrow area were, however, not significantly different.

These results demonstrate that the application of an external load does negatively influence the architecture (though not mineral density) of the developing bones, but that these effects lag behind the changes occurring in the growth plate. Thus, while the growth plate showed marked catch-up after 5 days of load release, the

Table 2
Results of three-point bending tests on tibiae from the four groups participating in this study (6dL, 6dC, 11dRL, 11dC)

Parameter	Treatment group			
	6dC	6dL	11dC	11dRL
Yield load (N)	24.22 (4.38)	24.09 (1.94)	81.56 (15.79)**	59.63 (18.49)
Ultimate load (N)	27.77 (6.31)	24.48 (7.04)	110.53 (20.68)**	75.05 (22.85)
S (N/mm)	71 (11)	63 (12)	216 (42)**	155 (35)
Young's modulus (GPa)	2.627 (1.032)	2.542 (0.411)	2.069 (0.392)	1.845 (0.468)

Results are reported as mean and standard deviation (in brackets), 9–10 specimens per group. Parameters which were found to differ significantly ($p<0.05$) between the 11dC and 11dRL groups are followed by two asterisks.

architectural features and mineral density of the bones were still inferior to controls at this time point.

Mechanical characteristics

Next, we determined the effects of external loading on the mechanical properties of postnatal bones. Table 2 reports the results of mechanical three-point bending tests for the four study groups. After 4 days of loading, loaded (6dL) and control (6dC) groups did not differ in any of the parameters measured. However after a further 5 days without load, most parameters were significantly lower in the 11dRL group compared to its control (11dC). Specifically, whole-bone stiffness, yield load and ultimate load were lower in the 11dRL group compared to the 11dC group. The only exception was the calculated apparent Young's modulus (a rough estimate of the stiffness of the *material* bone) which was found to be similar in both groups.

These results therefore further support the observed deleterious effect of external load on the mechanical behavior of bones, although this effect is expressed somewhat later than in the growth plate.

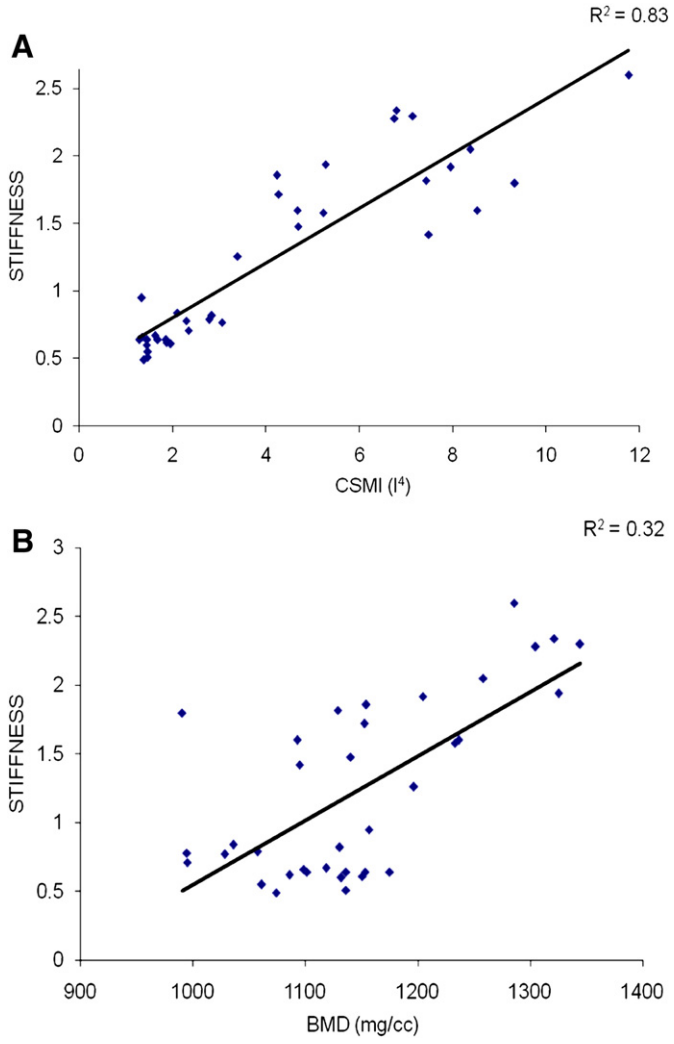

Fig. 6. Correlation between bone stiffness estimated by three-point bending test and the cross-sectional moment of inertia (CSMI) (A) and BMD (B), determined by micro-CT. Bone stiffness was well-correlated to bone structure (CSMI), but poorly correlated to bone quantity (BMD).

Discussion

We show here that skeleton loading and release from loading during the phase of rapid skeletal growth negatively affects the regulatory mechanisms of bone growth. As a result, the mechanical properties, bone architecture and BMC are inferior to controls. In contrast to previous studies which were based on either invasive surgical procedures [39–42] or extreme nonphysiological conditions [43], our model was based on the application of moderate physiological stresses. It is interesting to note that the addition of even such low loads for a short period of time (10% of body weight for 4 days) has a considerable effect on the developing skeleton.

In a previous study we investigated the separate effects of loading, stress and caloric restriction on bone growth and architecture by two additional control groups [30]. The first control group consisted of chicks that were raised under the same conditions as the experimental (loaded) chicks, but fitted with an empty saddle of similar construction as the harness, and weighing only 1% of the chick's body weight (SAD group). This group was used to evaluate the effects of the stress associated with the harnessing procedure, and distinguish them from the effect of loading *per se*. We first evaluated the behavioral response of the chicks and found that both the loaded and SAD groups needed a short acclimation period after being harnessed (about 1 h), but did not exhibit other stress-related behavior such as distress vocalization. They were seen to lie down on the floor of the cage (restricting their movement towards water and food) during the first hour, but then proceeded to behave normally for the rest of the experimental period. Significantly, their bone geometry and growth-plate morphology were similar to those of the control group, suggesting that it is unlikely that the harnessing procedure in itself affects bone growth. A second control group was used to examine whether changes in bone phenotype were the result of loading or decreased weight gain observed in the loaded group [30]. In order to answer this question we studied a group of chicks that was pair-fed for comparison with the loaded group, and parameters such as body weight, feed consumption, feed efficiency and geometric bone parameters were measured. We found that the pair-fed group, despite caloric restriction and subsequent low body weight, had the same geometric bone parameters (length, diameter and growth-plate thickness) like those of the control group, suggesting that the changes we detected in the bones and growth plates of the loaded chicks were mostly the result of loading [30]. Furthermore, no differences in feed efficiency were found between the groups, suggesting that the loaded group had lower body weight as a result of reduced feed consumption rather than energy loss or other metabolic problems.

We analyzed a series of marker genes related to different elements of the endochondral ossification process and showed that despite the changes in growth-plate thickness, the relative quantity of genes controlling chondrocyte differentiation (Col II and X) did not change, while factors related to growth-plate ossification (OPN, ALP) and cartilage and bone calcification (MGP, BGP) were upregulated as a result of the catch-up process. Such effects on the growth plate subsequently cause deterioration of the structural and mechanical characteristics of the skeleton, but only after a lag period.

Vanderwiel and co-workers et al. performed the first experiments examining the effect of adding external weight, using adult rats fitted with a backpack weighing 16% of their body weight [44]. It had been shown that exercise with an additional external load leads to a significant increase in lower-extremity bone mass [44]. We previously reported on a model for studying the effect of external load applied during rapid skeletal development by fitting young chicks with a bag weighing 10% of their body weight [30]. In that study, we found that such loading reduces the length of long bones and the thickness of their growth plates at the end of 4 days of loading (on day 6), as a result of accelerated endochondral ossification [30]. Those findings were also apparent in the current study;

however, the internal geometric parameters and the mechanical properties of the tibiae in the experimental group were not significantly different from those of the control group. The impact of the loading regime on these parameters only became apparent after an additional 5-day period during which both groups (experimental and control) were managed similarly, with no external load. At the end of this release-from-load period, significant differences were found between the two groups in almost every internal structural and mechanical parameter measured. Thus it was shown that tibiae belonging to the experimental group (11dRL) had a thinner cortex, lower BMC and BMD, smaller internal and external diaphyseal diameters and a smaller cortical area than those of the control group (11dC). The 11dRL group also had inferior mechanical properties compared to the control group, as demonstrated by lower stiffness, lower yield load and lower load to fracture.

These phenomena suggest the presence of growth retardation due to external load (shorter bones, narrower growth plates), followed by a catch-up mechanism after load release. Five days after releasing chicks in the experimental group from the load, bone growth was accelerated, as evidenced by the bones' length and proximal tibial growth-plate thickness, which reached the level of the control group and exceeded it, respectively. At this time, however, the effects of the previously applied load became apparent, as manifested by inferior structural parameters (thinner cortices, smaller external diameter) and inferior mechanical properties (lower stiffness, yield load and ultimate load), while the material property (apparent Young's modulus) and BMD remained the same. As shown in Table 1, while the cortical area of the experimental group (11dRL) was significantly smaller than that of the control group (11dC), the marrow area of both groups was not significantly different. This finding suggests that the decreased cortical area in the loaded group was not due to increased bone resorption (by increased activity of osteoclasts in the endosteum), but likely caused by impaired periosteal bone formation by osteoblasts. It is also possible that bone loss in the cortex of tibiae does not occur during the time of increased loading but occurs later when loading is removed.

These effects were also reflected in changes observed in gene-marker expression patterns of COL II and COL X, which were seen to occupy narrower zones in the growth plate of the loaded group immediately post-loading. This suggests that both the proliferative and hypertrophic zones of the growth plate were inhibited by short-term loading. However 5 days after load release, COL II expression in the experimental group had caught up with that of the control group, and COL X expression in the experimental loaded group even exceeded that of the control group. Quantitative analysis of these genes in the growth plates revealed no differences between the treatments, suggesting the occurrence of catch-up growth after transient growth inhibition due to loading. This process of catch-up growth affects both the proliferative and hypertrophic cell populations [23]. During this accelerated chondrogenesis, as illustrated by the presence of more hypertrophic chondrocytes, a higher level of factors related to growth-plate ossification and mineralization, reduced levels of TRACP activity in the chondro-osseous junction and COL I expression in the CB were observed. These findings suggest that an accelerated phase of chondrogenesis might delay the resorption of cartilage and the formation of new bone.

Catch-up growth is a well-known phenomenon in children and growing animals, and is defined as a growth rate above statistical limits of normalcy for age and/or maturity for a limited period of time following a period of growth retardation [24]. Previously listed causes of growth retardation include malnutrition or a variety of diseases such as celiac, growth-hormone deficiency, and Cushing's disease, among others. It has been shown that once the primary cause of the growth retardation is removed, bones undergo an accelerated growth spurt, allowing them to "catch-up" [21,45].

Mechanisms other than external loading could potentially also contribute to, or indeed be responsible for the effects we observed in the bones of the experimental group. For instance, changes in food intake and body weight could affect the growth hormone (GH) or glucocorticoid axes, which could be responsible for decreased bone growth. However, it is not clear whether regulation of bone growth in avian species is mediated through GH or by its receptor levels [46]. Moreover, we showed that caloric reduction in chickens does not affect their bone length and chondrogenesis. Increased serum levels of glucocorticoids due to the stress of harnessing occur only for a short time and thus will probably not affect the chicks much during an extended experiment. Therefore they are unlikely to be the sole cause for such a dramatic phenotype as seen here. Halloran et al. [47] have shown that inhibition of bone formation by skeletal unloading was not a consequence of increased levels of plasma glucocorticoids. They speculated that a local mediator in bone senses mechanical load and transmits that information to the bone-forming cells directly. Still we cannot exclude the possibility that the loading process itself caused mild stress to the chicks, which affected their skeleton to a certain degree.

While the phenomenon of increased growth rate during catch-up has been demonstrated time and again, the mechanical consequences in terms of bone strength and material properties had never been investigated. It is interesting to note that the calculated material stiffness (apparent Young's modulus) of the cortical bone and the mineral density were similar in both groups. These parameters are different from the previously listed mechanical properties of the entire bone organ, which are the combined effect of both material and geometry. This finding suggests that the superior mechanical performance of tibiae from the control group at 11 days is due to the combined differences in geometry (thicker cortex in particular) and mineral content; it also demonstrates that mineralization is the primary factor controlling material stiffness. It is also worth noting that both the mineral content and geometric dimensions increased in chicks between the age of 6 and 11 days. However Young's modulus decreased, suggesting that the rate of increase in mineral content lags behind geometric growth, resulting in a *material* of lower stiffness over time in both groups.

Growth-plate mineralization and ossification are entwined in the process of bone growth, and inhibition of either one of them disrupts the other and inhibits bone elongation [48]. We studied the expression pattern of BGP, COL I [49] and MGP in our model to examine whether the loading/release-from-loading regime affects the expression of these genes. This could serve as a possible link between the phenotype of the growth plate and its consequences with regard to bone mechanical properties.

MGP is a calcium-binding protein that regulates cartilage and vascular mineralization [37]. Its expression pattern was affected by the loading/ release-from-loading regime (downregulated immediately at the end of loading, then upregulated after 5 days of load release). This suggests that accelerated mineralization occurs in the growth plates of the loaded bone, and decelerated mineralization occurs during the catch-up period, when chondrogenesis is the dominant process. BGP was also affected by the loading/release-from-loading protocol. Examination at high magnification revealed that although BGP was upregulated in the mid-metaphyseal region of the bone during the catch-up period, it was downregulated in the CB, at the same site at which COL I (an osteoblast marker) was downregulated. This phenomenon could lead to the speculation that CB mineralization precedes that of the growth plate.

Correlating bone structural parameters and mechanical performance is a major challenge in biological research [35,50,51]. Information on specific architectural parameters which may be responsible for mechanical differences among bones could contribute significantly to our understanding of musculoskeletal pathologies and may serve as a means of treating various bone diseases in the future. Our present analysis indicates that bone structure (CSMI) is a better predictor of bone resistance to bending (stiffness) than BMD (see Fig. 6). These findings are in agreement with those of Warden

et al., who found that bone strength in rats after exercise training was highly related to midshaft minimum moment of inertia (I_{min}), and poorly related to BMC [52].

The effect of external mechanical loading on growing bone is of much interest, in terms of both the basic mechanisms involved and their clinical implications. While much data exist with regard to the effect of loading on mature bones [7–13], its effect on young, growing bones is not well understood. In addition to the basic significance of this question, it is also of practical importance, as evidenced by the sight of school children carrying heavy back-packs to school, and the raising of farm animals that are encouraged to attain extremely rapid weight gain. The effects of mechanical loading on bones during their developmental period, when the cartilaginous growth plate is responsible for rapid elongation, occurs prior to sexual maturation, and may also be relevant to children engaged in intense sports activities during adolescence, children carrying heavy loads and possibly also those suffering from childhood obesity [53].

In conclusion, we show that short-term application of an external load to the postnatal skeleton may negatively affect the regulatory mechanisms of bone growth in the growth plate, and that this process leads to inferior structural and mechanical properties of the bones after a lag period. Load release results in recovery of the regulatory mechanisms through a process of catch-up.

Conflict of interest

None of the authors have any conflict of interest.

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