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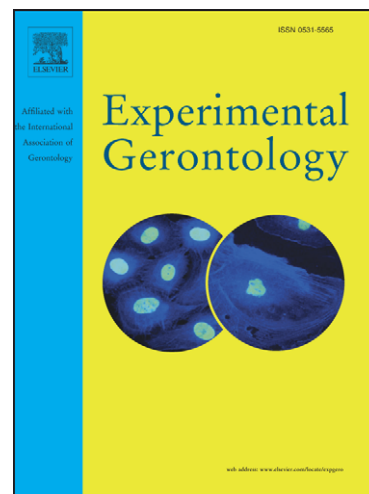
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**TO EXPERIMENTAL GERONTOLOGY,  
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**New title:**

**LONG-TERM VOLUNTARY EXERCISE OF MALE MICE INDUCES MORE BENEFICIAL EFFECTS ON CANCELLOUS AND CORTICAL BONE THAN ON THE COLLAGENOUS MATRIX**

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**ABSTRACT:**

The effects of lifelong physical exercise on the composition, structure and mechanical properties of bone are not well understood. Earlier, we found that voluntary physical exercise improved various properties of bone in maturing male mice up to 6 months of age. In the present study, we extended the previous study to 18 months. Half of the mice (total N=144) had access to running wheels while half were kept sedentary. The collagen network was assessed biochemically and by tensile testing of decalcified bone. The mineralized femur was analyzed with pQCT and 3-point-bending of the diaphysis and neck-strength-test. The proximal tibia was analyzed with microCT. The bone collagen revealed inferior tensional properties with aging and the mineralized femur demonstrated decreased stiffness with age. In the running mice, tensile properties and the BMD were reduced at 18 months of age compared to the sedentary mice. In contrast, the stiffness of both the diaphysis and femoral neck was higher, and trabecular architecture and structure were improved in the running mice. In summary, the results suggest that lifelong exercise training of male mice results in more beneficial effects on intact mineralized bone in both the diaphysis and epiphysis than on bone collagenous matrix.

## INTRODUCTION

Peak bone mass, mineral content and mechanical strength of the human skeletal system is reached after 25 to 30 years. Thereafter the mineral density and the mechanical properties of the bone are gradually impaired (Kahn et al., 2001; Matsushita et al., 1986). *Bone mass in aging adults may decrease more rapidly with unfit physical conditions or lifestyle (Karlsson, 2004)*. Lifelong physical exercise can reduce the rate of bone mineral decline and prevent or delay the onset of osteoporosis (Bailey and Brooke-Wavell, 2008).

Most previous studies have addressed the role of decreased bone volume, architecture and mineral content during aging (Beck et al., 1993; Karlsson et al., 2005). Other studies indicate that the relative composition of mineral and collagen matrix is altered during aging, in favor for more mineral (Boskey and Mendelsohn, 2005; McCreadie et al., 2006). The collagen matrix is most likely affected during aging (Bailey and Knott, 1999; Viguet-Carrin et al., 2006). The amounts of intra- and intermolecular enzymatic hydroxylysylpyridinoline (HP), lysylpyridinoline (LP) and non-enzymatic pentosidine (PEN) cross-links are believed to change with age (Bailey and Knott, 1999; Banse et al., 2002; Knott and Bailey, 1998). For example, an increase of PEN due to reduced rate of collagen turnover may be partly responsible for the age-related decrease of mechanical strength of the collagen network (Wang et al., 2002). Physical exercise could significantly affect the time schedule of the development of bone properties, collagen orientation and cross-links.

Several studies have investigated the effect of short exercise periods on bone mineral content in rodent models (Kodama et al., 2000; Mori et al., 2003; Plochocki et al., 2008). However, the documentation regarding the effect of long-term exercise and timing of exercise is limited (Hoshi et al., 1998; Jarvinen et al., 2003; Pajamaki et al., 2003). Hoshi et al. showed that 60 weeks of voluntary exercise of female mice leads to increased bone density and breaking

force in the diaphysis of the femur (Hoshi et al., 1998). Järvinen et al. showed that short term exercise in young and adult male rats resulted in similar response (Jarvinen et al., 2003). Contrary, using the turkey ulna model, *it has been reported that bones of aged animals respond to mechanical stimulation less efficiently than those of young animals (Rubin et al., 1992). In female rats, increased loading at an early age resulted in increased bone mineral density and fatigue resistance at older age (Warden et al., 2007).* These studies did not investigate bone properties that could be specifically related to the collagen matrix.

In a previous study, we examined the effects of exercise during growth and maturation in male mice, *i.e.* one to six months old, and found positive effects of exercise on properties of both the collagen network and the intact bone (Isaksson et al., Accepted). In the runners, the development of the mechanical properties of both intact mineralized bone and the collagenous matrix was faster than in the sedentary group. Hence, they showed improved properties already at two and four months of age. In the present study, we continue this by investigating changes in the diaphysis and epiphysis of the mineralized bone, as well as the collagen matrix of bone at senescence (18-month-old animals), and whether increased mechanical loading through voluntary exercise can modulate age-related changes that occur between 6 and 18 month of age. We hypothesized that age-related changes in the diaphyses and the epiphyses of mineralized bone and the collagen matrix will be influenced by lifelong voluntary exercise and the timing of that exercise.

## **MATERIALS AND METHODS**

### ***Experimental conditions***

Male C57BL/6J mice were obtained (n = 144) from Harlan CPB (Rijswijk, The Netherlands) and were cared for in the National Laboratory Animal Center, Kuopio, Finland. The room temperature was kept at  $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and the humidity at  $50\% \pm 20\%$ . Twelve hours light and

twelve hours dark rhythm was maintained. Commercial R36 mouse food (Lactamin AB, Stockholm, Sweden) and drinking water was given *ad libitum* until sacrifice (Lapvetelainen et al., 1997). The study protocol was approved by the Animal Care and Use Committee, University of Kuopio.

At the age of 1 month, the mice were randomly divided into a total of six experimental groups and placed in individual cages. The groups were abbreviated 'S' for sedentary and 'R' for runners, i.e. voluntary exercise groups. The mice were euthanized at 6 or 18 months of age, each group with 24 animals (Figure 1a). Note that the 6-month-old animals (R6 and S6) were introduced in the previous study (Isaksson et al., Accepted). Four groups of 18-month-old animals were included. The first two groups were merely sedentary or running groups (S18 and R18), whereas the last two groups were 18-month-old animals that experienced mixed exercise protocols. They were able to voluntary exercise in the running wheel during growth and maturation (from 1 to 6 months), but not thereafter (R6S18), or during adult life (from 6 to 18 months), after being sedentary during the growth and maturation period (S6R18) (Figure 1a). The running mice had free access to running wheels (width 8 cm and diameter 23 cm) within their cages (25 x 50 x 15 cm). The average daily running distances, running speeds, and total active running time were monitored as previously described (Lapvetelainen et al., 1997). The mice were weighed weekly until the age of 9 weeks and thereafter at monthly intervals. The mice were sacrificed at respective experimental time points by an overdose of CO<sub>2</sub>. The femur, tibia and humerus bones were carefully dissected free from soft-tissues, and stored at -20°C prior to further analyses (Figure 1b).

### ***Collagen matrix***

#### *Tensile testing of collagen network in the humeral mid-shaft*

The left humerus bones of 20 mice per group were decalcified in 0.5M EDTA for 14 days, and thereafter stored in cryogenic vials in PBS buffer at  $-20^{\circ}\text{C}$  until tensile testing. Prior to the mechanical testing, the specimens were thawed in a water bath ( $30^{\circ}\text{C}$ ) for 15 min. After thawing the specimens were kept on an ice bath to ensure stable test conditions for each specimen. A linear servo-motorized material testing device equipped with sample holders, Newport PM500-C Precision Motion Controller, Newport PM1A1798 Actuator and 1000g load cell (Honeywell Sensotec 31/1426-02) was used. The specimens were placed in clamps with a 1 mm initial distance at the mid-diaphysis between the clamps. After fixation and equilibration to zero load, the specimens were stretched at a rate of 1 mm/s until failure. Time, force, and actuator displacement were recorded during the tests. From these data, the force  $F$  and deformation  $d$  and strain  $\epsilon$  at failure were recorded. The stiffness  $k$  was defined as a slope of the linear part of the force-displacement curve and the absorbed energy  $W$  was calculated by integrating the force-displacement curve from 0 to breaking point. Young's modulus  $E$  was defined as the slope of the linear part of the stress-strain curve. The cross-section of the bone was assumed to be ellipsoidal, and the cross-sectional area at the failure site was determined using a stereomicroscope equipped with a drawing mirror (Helminen et al., 1983).

#### *Biochemical analysis*

High-pressure liquid chromatography (HPLC) was used for biochemical assessment of collagen content and collagen cross-linking. The mid-diaphyseal samples were cut from the decalcified left humerus of 14 animals per group after the tensile testing of the collagen network (see above). The samples were weighed, freeze-dried and hydrolyzed in 6M HCl. The amount of hydroxyproline in hydrolysate was estimated using external standard. The amount of collagen was calculated assuming 300 mol of hydroxyproline per 1 mol of collagen (Bank et al., 1998). This information was then used to calculate the collagen content of the samples and is given in nmol per wet weight of tissue. Amount of the enzymatic collagen



cross-links, *i.e.* HP, LP, and non-enzymatic collagen cross-link PEN were measured using reversed-phase HPLC analysis (Bank et al., 1998; Black et al., 1988). HP, LP, and PEN cross-links were determined as described by Bank et al. (1998) with the help of HP, LP and PEN standards that were kind gifts of Dr. Jeroen de Groot, TNO, Leiden, The Netherlands. The quantities of cross-links are expressed either as pmol (HP, LP) or fmol (PEN) per wet weight of tissue or mmol (HP) or  $\mu\text{mol}$  (PEN) per mol collagen.

### ***Diaphyseal bone***

#### *pQCT measurements*

Bone mineral density (BMD,  $\text{mg}/\text{cm}^3$ ) of the left femur of 20 mice per group was measured with a Stratec XCT 960A instrument (Software version 5.20, Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). One cross-sectional slice (length 1.25mm) was scanned at midshaft of each bone with a voxel size of  $0.092 \times 0.092 \times 1.25 \text{ mm}^3$ . The location of the midshaft was determined from the scout view of the pQCT device. Mean volumetric cortical bone mineral density (BMD) and cross-sectional cortical area at midshaft (CSA) were measured with the attenuation threshold values of  $0.690 \text{ mg}/\text{cm}^3$  and  $0.464 \text{ mg}/\text{cm}^3$  to enhance the accuracy of both density and area measurements (Hangartner, 2007; Jamsa et al., 1998a; Ward et al., 2005). The measurement was repeated once, and the average of the two measurements was used for analyses.

#### *Three-point-bending of femur*

Following the pQCT measurements, the left femurs were subjected to mechanical testing. A previously described custom-made testing machine was used (Jamsa et al., 1998a). Three-point-bending strength was measured with a constant span length of 6.5mm. The press head as well as the two support points were rounded to avoid shear load and cutting. The bone was positioned horizontally with the anterior surface upwards, centered on the supports, and the

pressing force was directed vertically to the mid-shaft of the bone, where the pQCT measurements were performed. Each bone was compressed with a constant speed of 0.155 mm/sec until failure. The measured data was converted to a load-displacement curve. The breaking (ultimate) force was defined as the highest force in the load-displacement curve. Stiffness was calculated as the slope of the linear (elastic) part of the load-displacement curve.

### ***Epiphyseal bone***

#### *Strength of the femoral neck*

The femoral neck of the left femur from 14 animals per group was subjected to mechanical testing after the 3-point-bending test. The proximal half of the broken left femur was placed axially in a support and the femoral neck was compressed axially at a constant speed of 0.155 mm/s until failure (Jamsa et al., 1998b; Jamsa et al., 1999). The load was applied to the femoral head, using a concave loading cup. The strength of the femoral neck was determined as the maximal load at failure and the stiffness was calculated as the slope of linear part of the load-displacement curve.

#### *Micro-computed tomography (microCT)*

Structural properties of the trabecular bone in the proximal left tibiae were determined with a high resolution microCT system (SkyScan 1172, Aartselaar, Belgium) from 6 animals per group from 6- and 18-month-old sedentary and runner groups (S6, R6, S18, R18). An isotropic voxel size of 10 $\mu$ m was used. The samples were immersed in PBS buffer in a specimen tube and fixed to the sample holder of the microCT system. Image reconstruction was done with NRecon (Skyscan, v 1.5.1.4). The regions of interests (ROI) were chosen to capture the trabecular bone in the subchondral and metaphyseal regions. The subchondral trabecular bone area was defined as the trabecular bone proximal to the growth plate and the metaphyseal trabecular bone was chosen to begin distal to the growth plate and to continue for

50 slices. The data sets were thresholded by assuming bone to have values above 100 (in gray scale 0-255) and analyzed with CTAn program (Skyscan, Aartselaar, Belgium). Trabecular bone volume fraction (BV/TV, %), mean trabecular thickness (Tb.Th,  $\mu\text{m}$ ), trabecular number (Tb.N  $\text{mm}^{-1}$ ) and trabecular separation (Tb.Sp,  $\mu\text{m}$ ) were quantified (Hildebrand and Ruegsegger, 1997; Odgaard et al., 1997).

### *Statistical Analysis*

The differences between the experimental groups (runners vs. sedentary, 6- and 18-month-old mice) were analyzed. To account for the effect of body weight on the mechanical characteristics (Hollinger, 2005), the generalized linear model with analysis of covariance (GLM, ANCOVA) was used with body weight as the covariant when comparing sedentary to running animals. When GLM was used, the normality of the residuals was confirmed by histogram plotting. Nonparametric Mann-Whitney U test were conducted when the normality of the data could not be confirmed. Additionally, Pearson correlation coefficients were used to highlight relationships between parameters that are related to the collagen matrix analysis in all 18-months-old animals. Statistical analysis was performed with the SPSS software, version 14 (SPSS Inc., Chicago, IL), and  $p < 0.05$  was considered statistically significant.

## **RESULTS**

All animals gained weight continuously, but the runners gained significantly less weight than the sedentary animals (Figure 2a). The weight differences peaked at 10 months of age (16% leaner,  $p < 0.001$ ), and after 18 months, the runners were 9% leaner than the sedentary animals ( $p < 0.001$ ) (Figure 2a). The immediate effects of exercise on body weight were observed in the groups that changed their exercise protocol at 6 months of age. The S6R18 group got access to running wheels at 6 months of age, and their body weight was reduced with 9% in one month, whereas the group that lost their possibility to exercise (R6 S18) continued to increase

in weight. Both sedentary groups (S18 and R6S18) and the runner groups (R18 and S6R18) had similar body weights at 18 months of age, the runners being significantly leaner (Figure 2a).

The mice were exercising most actively between the second and fourth months of age (Figure 2b), and ran up to 5 km per night. During the subsequent time period, the daily running distance slowly decreased. The daily running distance was significantly reduced after nine months compared to the distance ran at two and three months of age ( $p < 0.05$ ). *There were no significant differences between the groups that started running after 6 months of age (S6R18) and the group that was exercising the entire experimental time (R18), but on average, the S6R18 group exercised 1 km more per night than the R18 group.*

### ***Collagen matrix***

#### ***Biochemical analysis***

The amount of collagen in the decalcified bone showed no significant differences between 6 and 18 months of age (Figure 3a). The enzymatic collagen cross-links LP and HP showed similar trends as the collagen content (Figure 3b-c). The amount of HP in mouse bone was 4-5 times higher than LP. The amount of the non-enzymatic cross-link PEN, however, increased significantly between 6 and 18 months of age (Figure 3d). No significant differences were observed between the running and sedentary animals. However, there appeared to be a trend that the amounts of cross-links (HP, LP and PEN) were lower in the exercised animals compared to the sedentary animals. The ratios of HP/collagen and PEN/collagen in the four 18-month-old groups demonstrated no significant differences between the groups (Figure 3e-f).

#### ***Tensile testing of collagen network***

The force needed to break the collagen structure decreased significantly between 6 and 18 months of age in the running group ( $p < 0.05$ ), and there was a decreasing trend in the sedentary group (Figure 4a). The cross-sectional area increased significantly between 6 and 18 months of age in all groups ( $p < 0.001$ ) (Figure 4c). In the running group, the energy required to break the humerus was significantly lower at 18 months compared to 6 months, but not in the sedentary animals ( $p < 0.05$ ) (Figure 4d).

When comparing the effect of exercise regimens on bone in 18 months old animals, it was observed that the force needed to break the collagen structure was lower in the running animals (R18) compared to the other three groups at 18 months of age (Figure 4a). Both groups that were running during aging had lower tissue deformations at failure, but only the S6 R18 group was statistically lower than the sedentary S18 group ( $p < 0.05$ ) (Figure 4b). The S6 R18 group also had lower cross-sectional areas than the other groups ( $p < 0.05$ ) (Figure 4c). The energy required to break the humerus during tensional test was lower in the running group (R18) compared to the other three groups at 18 months of age (Figure 4d). However, there were no statistical differences between any 18-month-old groups in terms of stiffness or modulus (Figure 4e-f).

### ***Diaphyseal bone***

#### *pQCT and 3-point-bending mechanical test*

The pQCT measurements of bone mineral density (BMD) and cross-sectional area (CSA) of the femur displayed significant increases between 6 and 18 months of age in the sedentary animals ( $p < 0.001$ ) (Figure 5a-b), but in the other groups only an upward trend could be demonstrated. The force required to break the mid-diaphyses of the femur was significantly higher after 18 months compared to 6 months in the sedentary group ( $p < 0.05$ ), while it remained about the same level in the running group. On the other hand, between 6 and 18

months of age, the stiffness showed an increasing trend in the running group at the same time as it actually decreased significantly in the sedentary group ( $p < 0.05$ ).

When comparing the effects of different exercise regimens on 18-month-old animals, the running group (R18) and the R6 S18 group both had lower BMD and CSA compared to the sedentary (S18) group ( $p < 0.05$ ) (Figure 5a-b). The force required to break the mid-diaphysis in 3-point-bending of the femur was similar in sedentary and running animals, but significantly lower in both mixed groups (R6S18, S6R18) ( $p < 0.05$ ) (Figure 5c). However, stiffness was higher in groups that were running during aging compared to the sedentary groups (R18 vs. S18,  $p < 0.05$ ) (Figure 5d). In the sedentary animals the BMD of femoral diaphysis increased with aging (from S6 to S18), however, the value of bone stiffness decreased ( $p < 0.05$ ).

### ***Epiphyseal bone***

#### *Femoral neck*

Between 6 to 18 months, the force and stiffness appeared to remain unaltered in the sedentary group, while there was an increasing trend in the running group (Figure 5e-f). The breaking force and the stiffness of the femoral neck were higher in runners than in sedentary mice ( $p = 0.057$  and  $p = 0.062$ ) at 18 months of age (Figure 5e-f). This was also true for the S6R18 group that was exercising during adult life and aging.

#### *MicroCT*

The analyses of the trabecular architecture in both the metaphyseal and subchondral regions of the tibia showed declining structural properties between 6 and 18 months of age (Figure 6). The BV/TV decreased significantly between 6 and 18 months of age in both the metaphyseal and subchondral bone area ( $p < 0.005$ ). Additionally, during aging Tb.Sp increased and Tb.N

decreased in both the metaphyses and subchondral regions ( $p < 0.005$ ). When the trabecular architecture of the running and sedentary animals was compared with each other (R6 vs. S6 and R18 vs. S18), the exercise groups showed improved structural properties both in the metaphyseal and subchondral regions of the tibia in comparison to the sedentary groups (Figure 6). The subchondral and metaphyseal regions of running animals displayed higher BV/TV and Tb.Th both after 6 and 18 months than the sedentary animals ( $p < 0.05$ ), and lower Tb.Sp at 18 months ( $p < 0.05$ ).

## DISCUSSION

The aim of the present study was to investigate if lifelong mechanical loading improves bone functional properties at old age. Therefore, we evaluated the effect of aging (change between 6 months and 18 months) and lifelong voluntary exercise on both intact mineralized bone and demineralized bone, i.e. its collagenous matrix, in a mouse model. Additionally, the effect of timing of the exercise training was studied, by comparing the effect of exercise during growth to that during aging. The findings presented in this study suggest that different matrix components and anatomical locations respond differently to long-term exercise (Table 1).

### *Aging in sedentary animals*

Novel observations regarding effects of aging on bone collagen network properties were demonstrated by analyzing the changes occurring between 6 and 18 months. No differences in the collagen content or its enzymatic cross-links were observed. However, the amount of the non-enzymatic cross-link PEN increased between 6 and 18 months of age. PEN cross-links are advanced glycation end products, which are formed by the process of non-enzymatic glycation (Sell et al., 1991; Vlassara, 1994). This process is entirely time-dependent and occurs in collagen molecules because of their exceptionally long lifetime. Hence, the accumulation of PEN depends on the turnover rate of a protein or tissue and thus the

metabolic activity of that tissue (Verzijl et al., 2000). Therefore, the increased PEN level with aging in this study most likely reflects decreased remodeling and turnover rates in bone with increased age. Our findings are consistent with literature that indicates that PEN increases with age in human bone (Knott and Bailey, 1998; Wang et al., 2002). The resistance to tension of the collagen network in decalcified bone significantly decreased between 6 and 18 months of age. The modulus and stiffness of the decalcified humerus diaphysis decreased by 55% and 45%, respectively, and the force and the energy needed to break the tissue decreased, although not significantly (Figure 4). Based on 3-point-bending, the stiffness of mineralized femoral bone decreased with 20% between 6 and 18 months of age. Previous studies also suggest that BMD decreases with age, whereas in our study the BMD actually increased in the sedentary group ( $p < 0.05$ ). This result may partly be explained by the almost 25% increase in body weight between 6 and 18 months of age. It is known that size and body weight highly influences BMD and mechanical properties of bone in small animals (Hollinger, 2005).

### *Effects of exercise*

The effect of lifelong exercise on the bone properties appears more complex. In this study, we found no differences in the amount of collagen or its cross-links between the runners and the sedentary mice, whereas the tensile properties of the collagen network displayed lower force and energy at failure in the runners at 18 months (Table 1). However, the stiffness and modulus were similar in both runners and sedentary animals. In contrast to the organic bone phase, the mechanical properties of calcified bone both from the diaphysis and epiphyses showed consistently a more positive response to exercise at old age. Even though the radiographic parameters BMD and CSA were lower in exercising mice than in sedentary mice after 18 months, the bending force and stiffness values were at similar or higher level (Figure 5, Table 1). There was also a trend that the animals that had been running for the last year (S6



R18) displayed higher stiffness. The changes in the mechanical properties of intact bone are consistent with those published earlier. However, Hoshi et al. (1998) also found an increase in bone mineral density, which was not observed in this study. We propose that the reduced BMD of the compact bone of runners and also the high force and stiffness values represent real differences due to the use of the running wheel (Hollinger, 2005). Additionally, our study confirms previous studies showing that lifelong voluntary exercise in rodents leads to leaner animals (Hoshi et al., 1998). *It is known that physical exercise-training reduces body weight gain and that de-training accelerates the gain. We also observed that body weight was suppressed during the exercise training period, and was quickly induced when exercise started (S6R18), and disappeared when the possibility to exercise was removed (R6S18) (Figure 2a).*

### **Correlation analysis**

Based on Pearson correlation analysis in all 18 month-old animals, there were no significant linear correlations between the weight of the animals and the properties related to the collagen network. However, the weight of the animals correlated significantly with BMD, force and stiffness of intact bone. Significant correlations were found between the tensile stiffness of the collagen network and the stiffness of mineralized bone of the femoral neck ( $r^2 = 0.47$ ,  $p < 0.001$ ) (Figure 7a). Also, a negative correlation was found between the collagen network displacement and the amount of LP cross-links ( $r^2 = 0.59$ ,  $p < 0.001$ ) (Figure 7b). In addition, the amount of LP cross-links were negatively related to the 3-point-bending force ( $r^2 = 0.57$ ,  $p < 0.05$ ) (Figure 7c).

In our previous study, we found that voluntary running exercise improved properties of bone and its collagen network in young mice (Isaksson et al., Accepted). In that study, the mechanical properties of the bone during maturation developed earlier and reached higher

levels in the exercise group than in the sedentary group. However, as shown in this study, the improved properties of the collagen network due to voluntary exercise were not maintained to the age of 18 months. On the other hand, the intact bone remained stiffer and potentially stronger in the exercise group at 18 months of age. This suggests that in the diaphysis exercise at adult age modulates predominately the mineralization and affects the collagen network to a lesser degree. Exercise had most beneficial effects on the strength and architecture of trabecular bone. *Exercise training appeared to suppress the age-related trabecular bone loss.* Interestingly, most of the effect of exercise on the proximal femur took place between 6 and 18 months of age while the improvement of the properties of trabecular network of the proximal tibia was demonstrated between 1 and 6 months of age. Overall, the present observations support the idea that different bones, their anatomical parts (diaphysis, epiphysis, femoral neck) and components (compact bone, trabecular bone, collagen network) respond differently to physical exercise. Schriefer et al. (2005) presented the view that the mechanical properties of mineralized mice bone are somewhat dependent on the anatomical location (Schriefer et al., 2005). Therefore, the bones can respond to exercise differently (Wallace et al., 2007).

In summary, this study shows that lifelong voluntary wheel running had no significant effect at old age on bone collagen content and network stiffness and modulus and had actually a negative effect of collagen strength. Hence, the hypothesis that exercise leads to improved collagen network properties at old age was not confirmed in this study. However, exercise displayed positive effects on the intact femoral neck, stiffness of diaphysis and the trabecular architecture of epiphyseal bone. These results suggest that increased physical activity at old age, in addition to reduced body weight, can improve intact trabecular bone strength, but not diaphyseal bending strength or the tensile strength of the organic matrix.

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**FIGURE LEGENDS**

**TABLE 1:** Summary of the results. The development of properties between 1-6 months of age (<sup>1</sup> based on previous study by Isaksson et al. (Accepted)), and between 6-18 months of age (pooling runners and sedentary mice), and the comparison between the runners and the sedentary mice at 6 and 18 months of age. — represents no difference, ↗ represents a trend ( $p < 0.10$ ) and ↑ represents significantly higher ( $p < 0.05$ ) values in the runners compared to the sedentary mice.

**FIGURE 1:** Experimental setup of the study **(a)**. Sedentary (S) and running (R) mice were euthanized at 6 and 18 months of age. In two groups, the animals were exercising either during the maturation period from 1 to 6 months and thereafter living in cages without running wheels (R6 S18) or only during the aging phase from 6 to 18 months after prior sedentary life from 1 to 6 months (S6 R18). The humerus was decalcified and the femur and tibia were intact (mineralized) **(b)**. The analyses that were conducted on the diaphyses or the epiphyses are indicated at each bone.

**FIGURE 2:** Weight and running distances. All animals gained weight during the experiment **(a)**. The runners gained less weight than the sedentary animals. The maximum difference was observed at 10 months of age (\*  $p < 0.05$ , \*\*\*  $p < 0.001$ , non-parametric Mann-Whitney U-test). The daily running distance increased between the second and fourth months of age (5 km per night), and thereafter slowly decreased until 18 months of age **(b)**. There were no differences between different running groups, but on average the S6 R18 group ran 1 km more per night than the R18 group.

**FIGURE 3:** High performance liquid chromatography (HPLC) measurements. HPLC of bone collagen/wet weight of decalcified diaphysis of humerus **(a)**, enzymatic LP (lysylpyridinoline) cross-links **(b)**, enzymatic HP (hydroxylysylpyridinoline) cross-links **(c)**, and non-enzymatic PEN (pentosidine) cross-links **(d)**. Collagen content, LP and HP remained constant between 6 to 18 months, while the amount of PEN increased (Mann-Whitney U test between time points, † control  $p < 0.05$ , ‡ running,  $p < 0.05$ ). No statistical differences between the sedentary and running groups were found (non-parametric Mann-Whitney U-test). The same was true for HP/Collagen **(e)** and PEN/Collagen **(f)**. After 6 and 18 months, there appeared to be a trend towards lower total amounts of all three cross-links in running mice compared to sedentary mice.

**FIGURE 4:** Tensile testing of decalcified diaphysis of humerus bones from 6- and 18-month-old mice. The force **(a)** and displacement **(b)** needed to break the tissue were recorded. The bone cross-sectional area was measured **(c)** and the energy used **(d)**, stiffness of the tissue **(e)** and Young's modulus **(f)** were calculated (mean  $\pm$  SEM). Significant changes during aging and between exercise groups are marked, based on the GLM with body weight as a covariate (\*  $p < 0.05$ ).

**FIGURE 5:** Properties of femoral bone from pQCT measurements from 6- and 18- month-old mice show the bone mineral density (BMD) **(a)** and cross-sectional area (CSA) **(b)**, the force **(c)** and stiffness **(d)** from 3-point-bending test and the force **(e)** and stiffness **(f)** from the femoral neck test (mean  $\pm$  SEM). Significant changes during aging and between exercise groups are marked, based on the GLM with body weight as a covariate (\*  $p < 0.05$  and \*\*  $p < 0.005$ ).



**FIGURE 6:** MicroCT analyses of tibia from subchondral (left) and metaphyseal (right) regions of 1-, 6-, and 18-month-old mice show increased trabecular bone volume fraction (BV/TV %) (**a-b**), increased mean trabecular thickness (Tb.Th,  $\mu\text{m}$ ) (**c-d**), increased trabecular number (Tb.N  $\text{mm}^{-1}$ ) (**e-f**) and decreased trabecular separation (Tb.Sp,  $\mu\text{m}$ ) (**g-h**) in the running mice compared to the sedentary mice, based on non-parametric Mann-Whitney U test (\*  $p < 0.05$ , and \*\*  $p < 0.005$ ). Additionally, comparison between time points showed significant effects of aging (Mann-Whitney U test between time points, control †  $p < 0.05$ , ††  $p < 0.005$  and running ‡  $p < 0.05$ , ‡‡  $p < 0.005$ ).

**FIGURE 7:** Correlation analyses, based on all 18-month-old mice, between collagen tensile stiffness of decalcified humerus and mineralized neck stiffness of femur (**a**), tissue collagen displacement and LP cross-links of humerus (**b**) and force from 3-point-bending test of femur and LP/collagen of decalcified humerus (**c**) were moderate, but significant (\*  $p < 0.05$ , \*\*  $p < 0.001$ ).

Figure 1

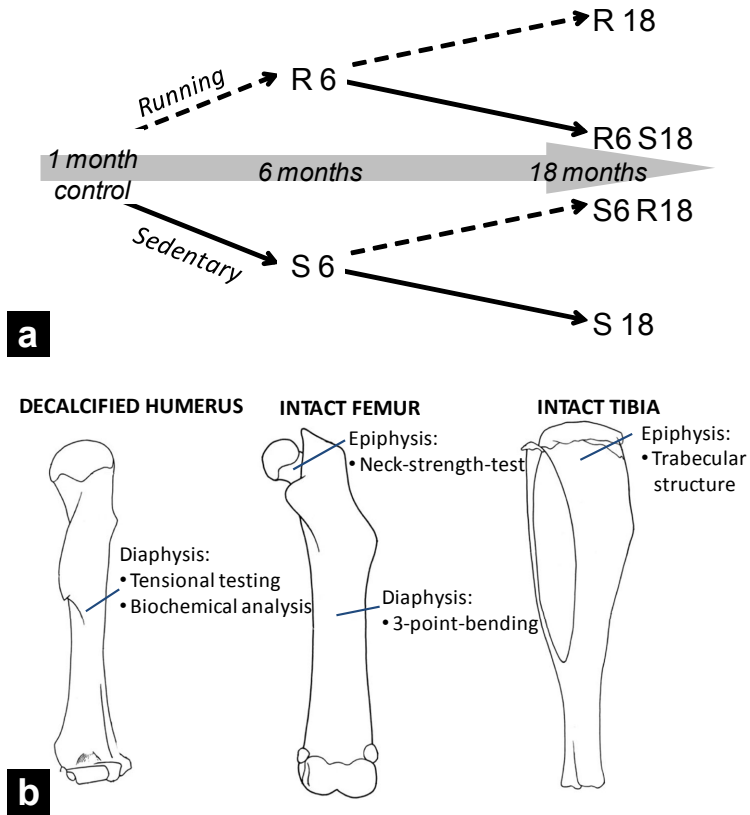


Figure 2

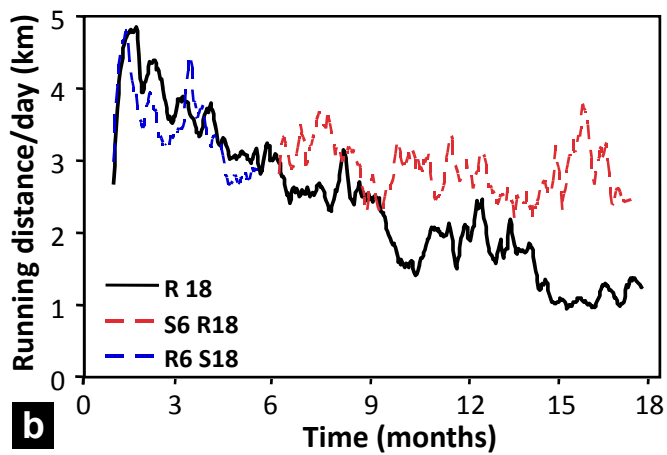
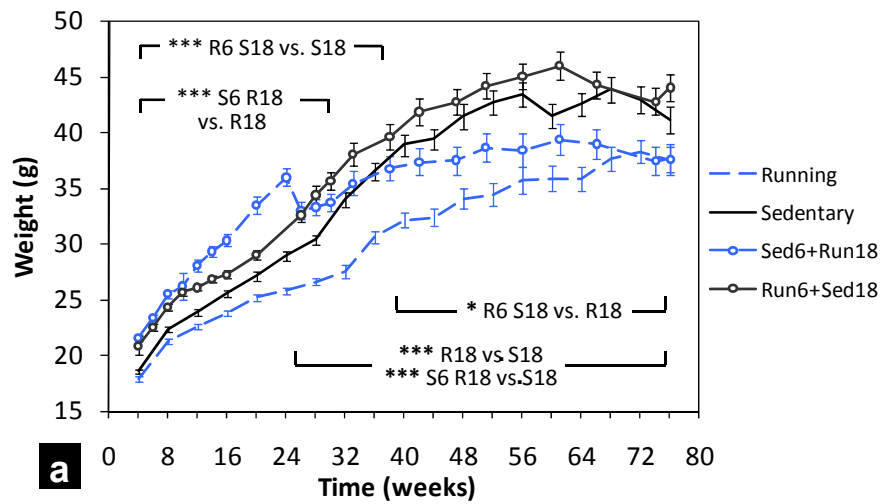


Figure 3

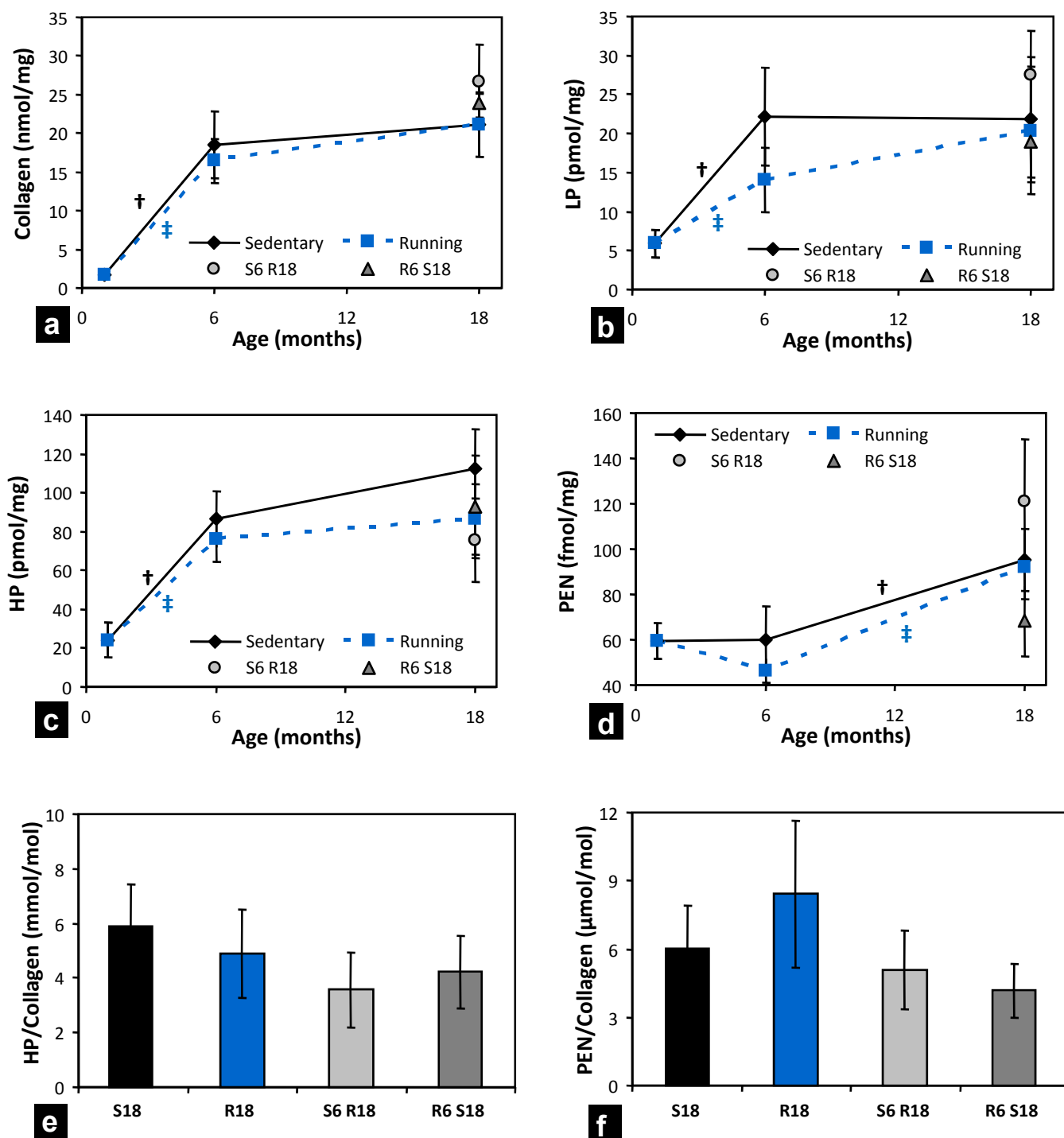


Figure 4

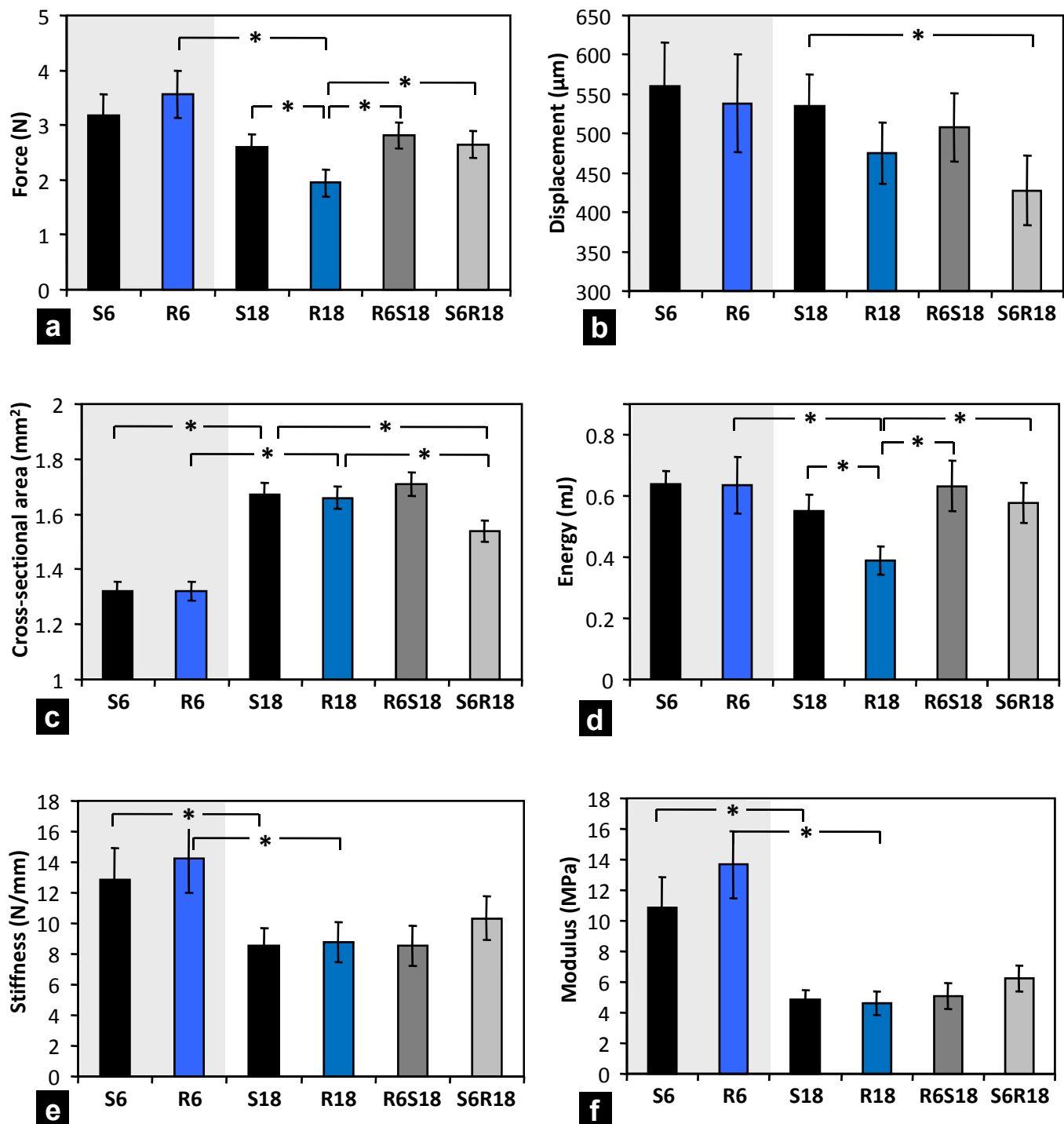


Figure 5

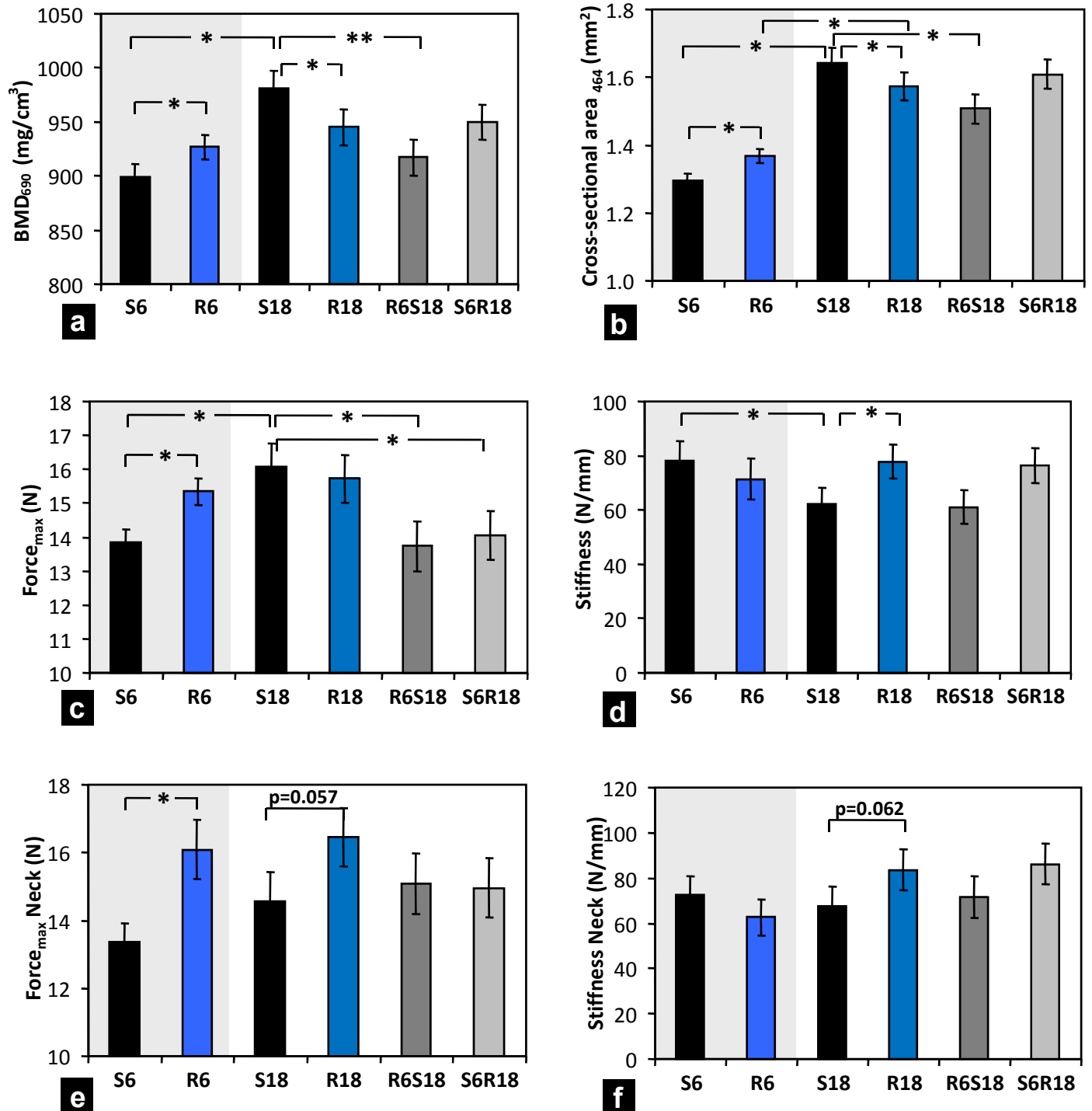


Figure 6

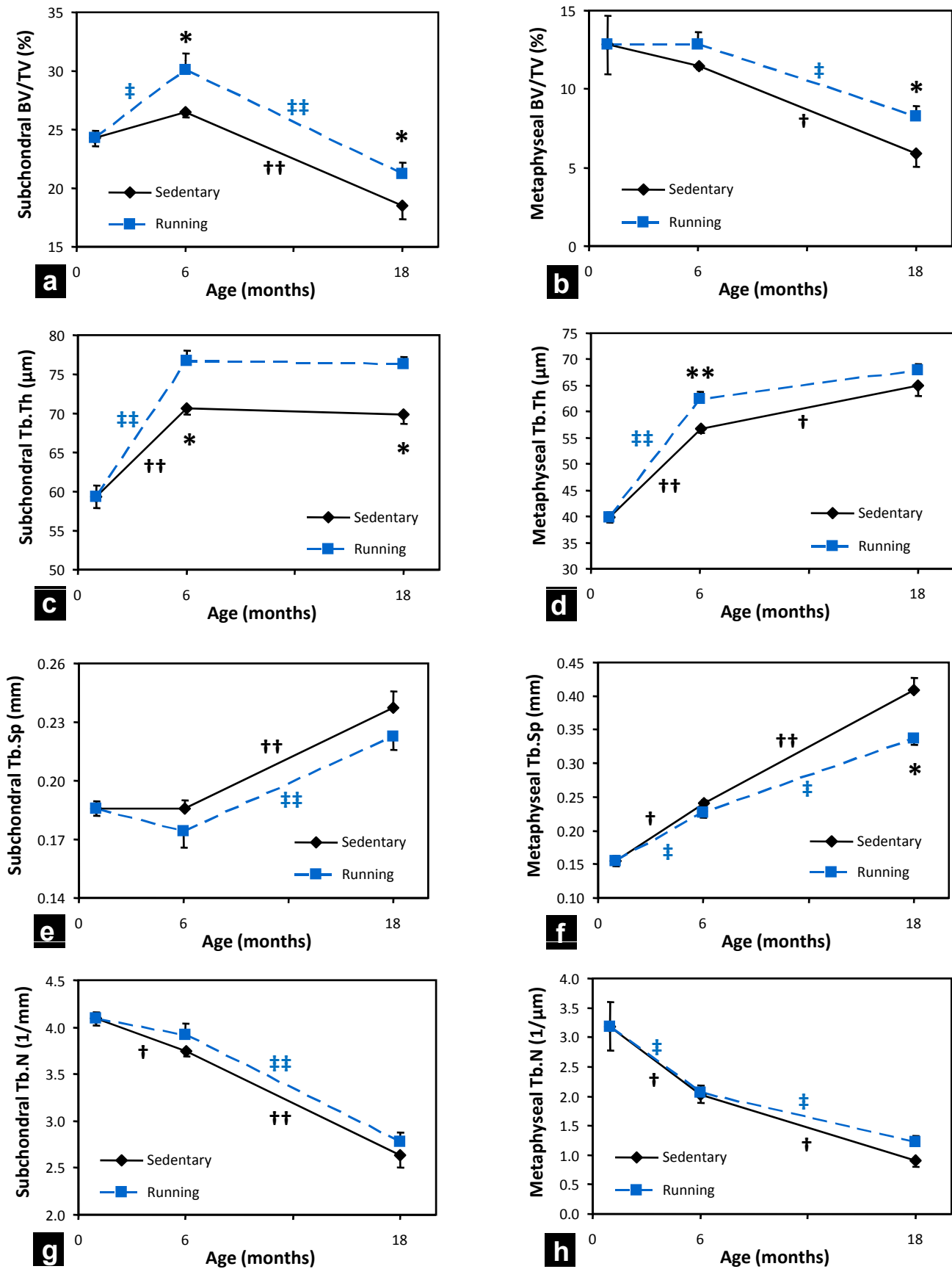


Figure 7

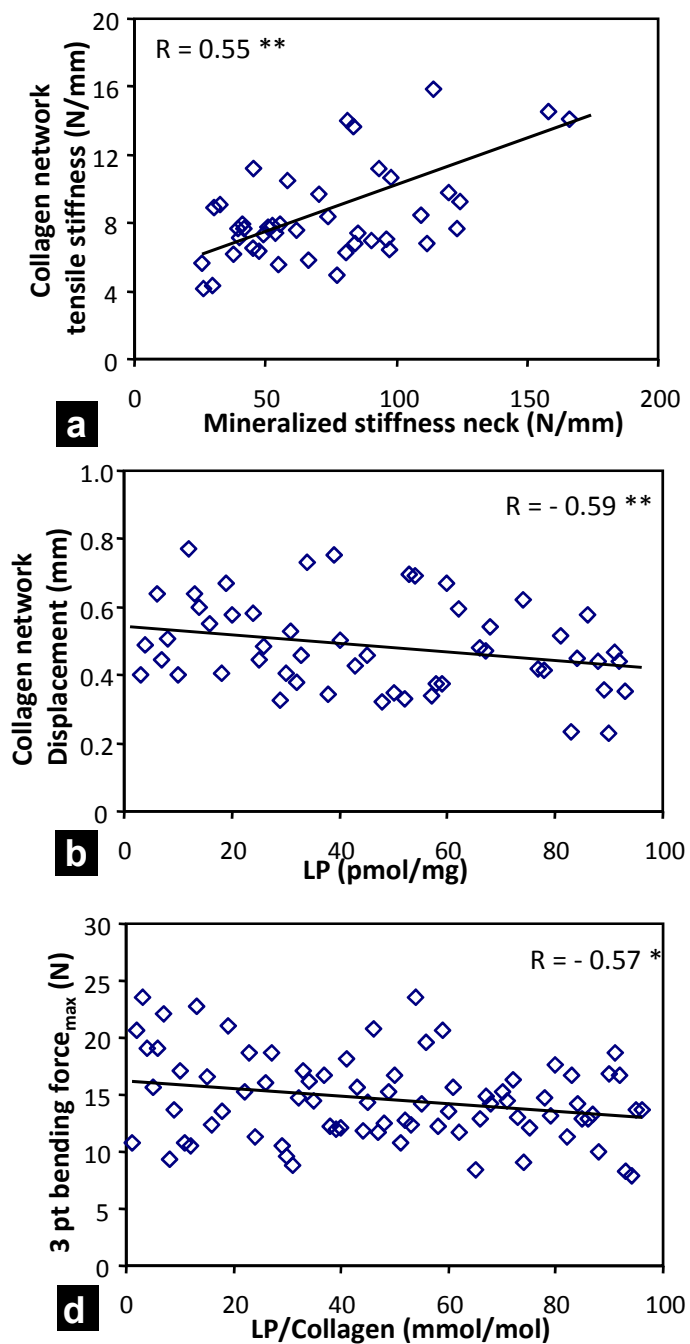




TABLE 1

			Change between:		Running vs. sedentary	
			1 - 6 mo	6 - 18 mo	6 mo	18 mo
Collagen	Collagen		↑ <sup>1</sup>	—	—	—
	Biochemistry humerus	HP	↑ <sup>1</sup>	—	—	—
		LP	↑ <sup>1</sup>	—	—	—
		PEN	—	↑	—	—
		Force	↑ <sup>1</sup>	↓	↗ <sup>1</sup>	↓
	Tensile test tibia	Energy	↑ <sup>1</sup>	↓	—	↓
		Stiffness	↑ <sup>1</sup>	↓	—	—
		Modulus	↑ <sup>1</sup>	↓	—	—
Mineralized diaphyseal bone	pQCT femur	BMD	↑ <sup>1</sup>	↑	↑ <sup>1</sup>	↓
		Area	↑ <sup>1</sup>	↑	↑ <sup>1</sup>	↓
	3 pt bending femur	Force	↑ <sup>1</sup>	↗	↑ <sup>1</sup>	—
		Stiffness	↑ <sup>1</sup>	—	—	↑
Mineralized epiphyseal bone	Neck strength femur	Force	↑ <sup>1</sup>	—	↑ <sup>1</sup>	↗
		Stiffness	↑ <sup>1</sup>	—	—	↗
	MicroCT proximal tibia,	BV/TV	↑	↓	↑	↑
		Tb.Th	↑	—	↑	↑
		Tb.Sp	—	↑	↘	↘