OLLI LEPPÄNEN

Postnatal Bone Ontogeny

ACADEMIC DISSERTATION
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the Faculty of Medicine of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
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University of Tampere, Medical School
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UKK Institute
Finland

Supervised by
Docent Teppo Järvinen
University of Tampere
Finland
Docent Harri Sievänen
University of Tampere
Finland

Reviewed by
Associate Professor Steven Boyd
University of Calgary
Canada
Professor Petri Lehenkari
University of Oulu
Finland

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 3551 6055
Fax +358 3 3551 7685
taju@uta.fi
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based upon the following original publications, referred to in the text by their Roman numerals (I-IV)

I Leppänen OV, Sievänen H, Järvinen TLN 2008 Biomechanical testing in experimental bone interventions – May the power be with you. J Biomechanics 41(2008):1623–1631

II Leppänen O, Sievänen H, Jokihaara J, Pajamäki I, Järvinen TLN 2006 Three-point bending of rat femur in the mediolateral direction: introduction and validation of a novel biomechanical testing protocol. J Bone Miner Res 21(8)**:**1231-7.

III Leppänen OV, Sievänen H, Pajamäki I, Jokihaara J, Kannus P, Cooper DM, Järvinen TLN Postnatal Ontogeny of Female Bones: How much is attributable to locomotion and estrogen? (submitted)

IV Leppänen OV, Sievänen H, Jokihaara J, Pajamäki I, Kannus P, Järvinen TLN 2008 Pathogenesis of Age-Related Osteoporosis: Impaired Mechano-Responsiveness of Bone Is Not the Culprit. PLoS ONE 2008;3(7):e2540

Formally corrected Tables 4 and 5 for Study IV

ABBREVIATIONS

aBMD	Areal bone mineral density, g/cm^2 or mg/mm^2
ANOVA	Analysis of variance
AP	Anteroposterior
BMC	Bone mineral content, g or mg
BMU	Basic multicellular unit
C	Control
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
cBMD	Cortical bone density, g/cm^3 or mg/mm^3
cCSA	Cortical cross-sectional area of bone, mm^2
CSMI	Cross-sectional moment of inertia, mm^4
CV_{rms}	Average-root-mean-square coefficient of variation
d_{max}	Minimum diameter, mm
d_{min}	Maximum diameter, mm
DXA	Dual energy x-ray absorptiometry
EX	Exercise
F_{max}	Fracture load of bone, N
I_{max}	Maximum principal area moment of inertia, mm^4
I_{min}	Minimum principal area moment of inertia, mm^4
IP3	1,4,5-triphosphate
L+	Loading included
L-	Immobilized
M-CSF	Macrophage colony stimulating factor
minSS	Minimum sample size
ML	Mediolateral
MRI	Magnetic resonance imaging
NO	Nitric oxide
NX	Unilateral sciatic neurectomy
OPG	Osteoprotegerin
OSX	Osterix
OVX	Ovariectomy
PG	Prostaglandin
PKC	Protein kinase C
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor-κB
RANKL	Ligand of the receptor activator of nuclear factor-κB
Runx2	Runt-related transcriptional factor 2

SD	Standard deviation
SEM	Standard error of mean
SHAM	Sham operation
tBMC	Total bone mineral content, g or mg
tBMD	Total bone mineral density, g/cm^3 or mg/mm^3
tCSA	Total cross-sectional area of bone, mm^2
TNFα	Tumor necrosis factor alpha
vBMD	Volumetric bone mineral density, g/cm^3 or mg/mm^3
μCT	Microcomputed tomography

ABSTRACT

The objective of this thesis was to gain further insight into the skeletal ontogeny over the life-span, with a focus on the pathogenesis of age-related osteoporosis. A large portion of the thesis relates to the development of new methods as well as the enhancement of the existing methodology used in experimental bone research. First, referring to the extensive literature survey, it was shown that the breaking load remains the preferable trait for analyzing and reporting the mechanical competence of bones in experimental osteoporosis studies, while the utility of stiffness and energy absorption is seriously challenged due to their poor precision. Secondly, it was shown that the functional adaptation of bones to increased loading is direction-specific, occurring virtually exclusively in the mediolateral direction of the rat femoral midshaft. This finding led to the development (introduction and validation) of a novel testing method for assessing the structural rigidity of the rat femur in this particular direction. The test was used in an experimental study aiming to delineate the respective effects and the possible interaction of locomotive loading and estrogen on the normal development of the rat femoral midshaft. It was shown that the longitudinal growth of rat femur is largely irrespective of locomotive loading or estrogen. However, the phenotype of midshaft geometry was interestingly influenced by both locomotive loading and estrogen. The preform circular cross-section obtained its characteristic elliptic shape as a consequence of locomotive loading. The osteogenic effect of estrogen, in turn, occurred at the endosteal surface of the femur, possibly as this is the most efficient site for mineral metabolism. In the final experiment of this thesis, mature and senescent rats were subjected to a treadmill training intervention, to show that even the bones of very old rats are able to respond appropriately to the increased locomotive loading. Thus, it is unlikely that the pathogenesis of age-related osteoporosis is attributable to a failure in the mechano-sensory system. This finding implies that strengthening of senescent human bones may also be possible – naturally provided that safe and efficient training methods can be developed for the oldest old.

YHTEENVETO

Tämän väitöskirjan tarkoituksena oli selvittää luuston yksilölliseen kehitykseen ja kuormitusvasteeseen vaikuttavia tekijöitä kokeellisin menetelmin. Lisäksi suuri painoarvo asetettiin käytettävien menetelmien validiteetille, minkä varmistamiseksi väitöskirja sisältää sekä mittavan katsauksen olemassa olevaan kirjallisuuteen sekä uuden biomekaanisen testin, jolla rotan reisiluun varren mekaaniset ominaisuudet saadaan tarkoituksenmukaisemmin määritettyä. Katsauksessa osoitettiin, että mittausmenetelmän toistettavuuden määrittäminen ja voimalaskenta ovat erittäin harvinaisia kokeellisissa tutkimuksissa, joissa määritetään kokonaisten luiden biomekaanisia ominaisuuksia. Lisäksi katsauksessa kävi ilmi, että luun lujuuden määrittäminen olemassa olevilla biomekaanisilla testausmenetelmillä on varsin toistettavaa, mutta jäykkyys ja erityisesti energian absorptiokyky ovat huonosti toistettavia asettaen niiden käyttökelpoisuuden kiistanalaiseksi. Luun mineraalimassa, tiheys ja rakenne määritettiin perifeerisellä kvantitatiivisella tietokonetomografialla (pQCT) ja/tai mikrotietokonetomografialla (µCT) ja luun mekaaninen lujuus mekaanisella koestuslaitteella. Rotan reisiluun kolmipistetaivutus osoittautui katsauksessa olevan yleisin luuhun kohdistuva biomekaaninen testi. Väitöskirjan tutkimuksia varten kehitettiin perinteisen etu-taka-suunnassa taivuttavan testin rinnalle sivuttaissuuntainen kolmipistetaivutustesti, joka osoittautui sekä toistettavaksi että päteväksi osoittamaan kuormituksen aiheuttaman luun lujuuden lisääntymän reisiluun varressa. Luun kasvun aikaiseen kehittymiseen vaikuttavien tekijöiden selvittämiseksi rotille suoritettiin toispuoleinen iskias-hermon katkaisu (kuormituksen poistamiseksi) ja osalle rotista munasarjojen poisto (veren estrogeenipitoisuuden alentamiseksi). Tutkimuksessa osoitettiin, että kasvun aikana reisiluun pituuskasvu on pitkälti perimän määräämää. Sen sijaan luun poikkileikkauksen koko ja muoto sekä luun lujuus ja jäykkyys ovat ensisijaisesti kuormituksen aikaansaamia ominaisuuksia. Estrogeenin vaikutus osoittautui putkiluun sisäpinnalla luuta säästäväksi ilmeisenä tehtävänään varastoida sinne mineraalipitoista luuainesta. Toisessa tämän väitöskirjan kokeellisessa tutkimuksessa keski-ikäiset ja vanhat uros- ja naarasrotat asetettiin juoksumattoharjoitusohjelmaan. Tutkimuksen perusteella vanhat eläimet olivat keski-ikäisiä herkempiä reagoimaan lisääntyneeseen kuormitukseen lisäämällä reisiluidensa kaulojen ja varsien poikkileikkausten kokoa, mineraalimäärää ja mekaanista lujuutta. Tämä löydös antaa viitteitä siihen, että ikääntymiseen liittyvä luukato ei ole seurausta luun kuormitusvasteen heikkenemisestä ja että vanhimpienkin yksilöiden luustoon pystytään vaikuttamaan liikunnalla, mikäli käytetään tehokkaita ja turvallisia kuormitusmenetelmiä.

INTRODUCTION

The skeleton forms an essential part of the complex mechanobiological locomotion system of a human body, a “cost-efficient” product of evolution that integrates several vital functions in conjunction with its primary locomotive purpose into a single organ (Einhorn, 1992; Järvinen et al., 2005; Sievänen, 2005). To successfully fulfill their locomotive objective, evolution has equipped the bones with a built-in sensor that perceives the incident loading-induced strain distribution within the bone and subsequently removes bone tissue from sites where the concomitant stresses are marginal while forming new bone tissue at sites subjected to increased stress to maintain their mechanical competence in terms of everyday loading (Frost, 1987a; Thompson, 1919; Wolff, 1892).

The most important feature of bone, its mechanical competence (Järvinen et al., 2005), can ultimately be assessed only by structural strength tests that measure how well the whole bone can bear load. In fact, it has been postulated that there is no alternative to testing whole bone strength, and conclusions regarding bone mechanical function based solely on geometry or bone mineral content are inappropriate and possily misleading (van der Meulen et al., 2001). Accordingly, it is simply not enough that mechanical testing is performed, but it should be carried out appropriately. Although it is among the first methods used for studying bones, there is obviously still room for improvement.

During skeletal ontogeny, both material and architectural properties of bone change in order to meet the biomechanical and endocrinological needs of the individual. A large number of molecular, cellular, and environmental factors have been implicated in the regulation of bone development. Often, events at the organ level are simply presented as the cumulative effect of all factors that individually are known to influence bone development (Schoenau et al., 2003). Thus, although locomotive loading and hormones are universally accepted as the major effectors of skeletal growth, surprisingly little is known about their respective roles in bone ontogeny.

Increased fragility of bones due to osteoporosis has become a major health-care problem in Western societies in recent decades. For example, there were 1.7 million hip fractures alone in the world in 1990 and most recent epidemiological studies suggest that the overall incidence of osteoporotic fractures will increase in the near future (Cummings and Melton, 2002; Kannus et al., 1999), although the latest reports suggest that the most rapid increase in the incidence of hip fractures may be surpassed (Kannus et al., 2006). According to prevailing understanding, postmenopausal osteoporosis (type I osteoporosis) is due to decrease in estrogen level after menopause, and a subsequent decrease in bone density (Riggs et al., 2002; Riggs et al., 2004). Type II osteoporosis, also called

age-related osteoporosis, is postulated to result in declining skeletal mechano-responsiveness (Klein-Nulend et al., 2002; Seeman, 2004).

The purpose of this series of experimental studies was, firstly, to examine the existing literature to obtain a broad overview of the methods used in the experimental bone research. Using this information, a new structural-oriented biomechanical testing method was introduced and used in order to gain insight into research questions concerning bone biology. The second objective was to determine the respective roles of estrogen and locomotion on bone ontogeny, and to follow the skeletal ontogeny into old age with the aim of enhancing our understanding of the pathogenesis of age-related osteoporosis.

REVIEW OF THE LITERATURE

Bone biology

Bone composition and structure

Bone is a connective tissue characterized by a mineralized extracellular matrix, and the resulting hardness of the tissue. Five different cell types are responsible for forming and removing the matrix. Mesenchymal stem cells are multipotent cells that with potential to differentiate to all lineages of mesenchymal tissues, including bone (Pittenger et al., 1999; Zaidi, 2007). Osteoprogenitor cells give rise to osteoblasts that secrete the extracellular matrix of bone. Once the cell is surrounded with the secreted matrix, it is referred to as an osteocyte. Osteoclasts are multinucleated, bone-resorbing cells derived from monocyte stem-cell lineage (Currey, 2002; Forriol and Shapiro, 2005; Ross, 2003).

The major structural components of bone matrix are collagens comprising approximately 85 to 90% of the protein in bone. There are also noncollagenous proteins, such as glycosaminoglycans, glycoproteins and sialoproteins that together with collagens become mineralized to form bone. Most of the mineral phase is composed of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. By weight, the portion of the minerals and inorganic matter is approximately 70%, whereas the proteins constitute 20 to 25% of the weight of the bone. The remainder is made up by water (Buckwalter et al., 1996; Currey, 2002; Einhorn, 1992; Forriol and Shapiro, 2005; Ross, 2003).

Most bones have an outer shell of compact bone tissue enclosing an interior of cancellous bone tissue. The compact part of a bone is very hard and dense and contains cylinders of calcified bone known as osteons or Haversian systems. At the center of each osteon, there is a central canal surrounded by lamellae consisting of calcified bone matrix, and lacunae, which are little spaces for osteocytes. Cortical bone has two surfaces, a fibrous connective capsule covering the outer surface of the bone, known as the periosteum, and the other on its inner surface lining the marrow cavity, known as endosteum. The cancellous bone consists of trabeculae, which are thin, anastomosing spicules of bone tissue. The spaces within the meshwork are continuous and occupied by bone marrow and blood cells. Usually the porosity of bone (i.e. the proportion of the total volume not occupied by bone tissue) is over 50%, and transitional forms between compact and cancellous bones are rare (Carola et al., 1990; Currey, 2002; Ross, 2003).

In the human body, each bone can be classified into six groups according to its shape. Long bones (e.g. femur, humerus) act as levers that are pulled by contracting muscles. Short bones (e.g. scaphoideum) occur only in the regions where limited movement is required, and they are almost completely covered with articular surfaces. Flat bones (e.g. sternum, scapulae) usually facilitate muscle attachments or form a protective enclosure. Irregular bones (e.g. vertebrae) have extensions that usually serve as sites for muscle attachments. Sesamoid bones (e.g. patella) are embedded within certain tendons and they both protect the tendon and help the tendon to overcome compression forces. Accessory bones are excess bones that usually occur when developing bones do not fuse completely. They have only marginal functional role in the human body (Carola et al., 1990; Lindsey, 1996; Ross, 2003).

Long bones have a tubular shaft, called the diaphysis (Figure 1), encompassing a hollow cavity of compact bone tissue filled with marrow. There is hardly any cancellous bone in the diaphysis. The diaphysis continues both cranially and caudally as metaphyses. Metaphysis is made up of the epiphyseal plate and adjacent bony trabeculae of cancellous bone. The proximal and distal ends, or epiphyses, consist chiefly of cancellous bone with a thin outer shell of compact bone (Carola et al., 1990; Ross, 2003).

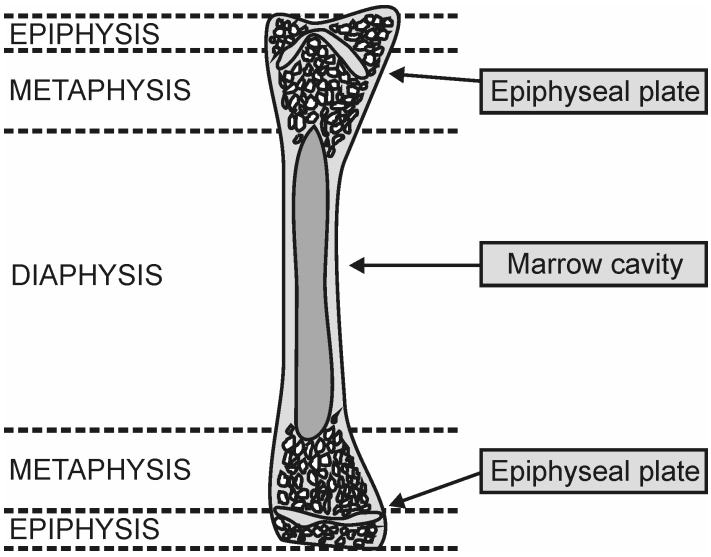


Figure 1. Schematic structure of a long bone.

Function of bones

The skeleton is a mechanically optimized biological system whose composition and organization reflect the functional demand placed upon it (Einhorn, 1992). Since fracture of a bone would have resulted in death in the wild, it is logical that species would develop mechanisms during the evolutionary process that would encourage bone development to produce bones of optimal strength (Schoenau, 2006). By contrast, heavy bones having large surface areas, ideal for mineral metabolism, would increase energy expenditure and decrease running speed and are therefore unlikely to have conferred an evolutionary advantage (Parfitt, 1994; Schoenau, 2006). Therefore, it is now thought, that the most vital function of bone is to withstand loading and enable locomotion (Currey, 2002; Einhorn, 1992; Järvinen et al., 2005). Other functions are protection of internal organs, hematopoesis, and participation in metabolic pathways associated with mineral homeostasis (Einhorn, 1992; Järvinen et al., 2005).

Locomotion in mammals is composed of coordinated moves of several joints. The muscles are responsible for generating the forces for locomotion while bones work as lever arms. For this locomotive function bones have to be stiff, because if bones were floppy, the contractions of muscles would not be converted into the movements of the limbs (Currey, 2002). The strength of the bone is secondary in this function, but inability to bear the load (fracture) would cause a devastating result. For efficient locomotion, the bone material has to be distributed in a way that is a compromise of mass and mechanical competence (Currey, 2003a; Seeman, 2003). This calls for a precise three-dimensional structure and composition of each bone (Currey, 2002; Currey, 2003a; Currey, 2003b; Einhorn, 1992; Frost, 2003; Turner, 2002; van der Meulen et al., 2001). The role of cortical bone is highlighted when assessing the mechanical competence of bone (Einhorn, 1992; Eswaran et al., 2006; Muller et al., 2003). This is especially true in long bone diaphyses, in which the dense cortical bone and hollow marrow cavity provide a stiff and strong tubular structure (Currey, 2001; van der Meulen et al., 2001), but even in the human vertebral body, the thin (<0.5mm) cortical shell has been shown to bear more than half of the load (Eswaran et al., 2006).

Besides locomotion, the mechanical integrity of bone is utilized in protection of the important internal organs. The skull protects the brain, the ribs protect the lungs and heart, and the vertebrae protect the spinal cord (Lindsey, 1996). The hematopoetic cells, which are the constituents of red bone marrow, are enclosed in the medullary cavities of certain bones (Ross, 2003). Bone tissue is also an important reservoir of mineral ions, especially calcium and phosphate; and 99% of total body calcium is deposited in bone (Moe, 2005). Several hormones (e.g. parathyroid hormone, calcitonin, and vitamin D) are responsible for the regulation of blood calcium and phosphorus levels, and in this regulation, bone plays a role as a storage stock of the mineral ions (Ross, 2003). Mineral

homeostasis occurs primarily in cancellous bone, which provides a large surface area well suited to rapid mineral exchange (Mosley, 2000).

Bone turnover

Bone remodeling is the reconstruction of the skeleton by bone resorption followed by bone formation (Orwoll, 2003; Seeman, 2006). The basic multicellular unit (BMU) consisting of osteoclasts, osteocytes, and their progenitors is responsible for this process. The objective of the remodeling process is to renew the existing bone material, because bone loses its competence as it ages (Parfitt, 2003; Seeman, 2006). If bone resorption and formation occur at different locations, the bone morphology is altered. This has been defined as modeling (Frost, 1990). During growth, the purpose of modeling and remodeling is also to achieve peak bone mass and modify the distribution of the bone focally to better accommodate prevailing stresses (Seeman, 2006).

Osteoclasts are responsible for resorption of existing bone. The intensity of bone resorption is dependent on the number and the activity of the osteoclasts at a specific region of bone. The osteoclastogenesis, and a subsequent number of osteoclasts, are a result of several signaling pathways (Zaidi, 2007). The key molecular regulators are macrophage colony stimulating factor (M-CSF), osteoprotegerin (OPG), receptor activator of nuclear factor-κB (RANK), and its ligand (RANKL) (Asagiri and Takayanagi, 2007; Blair et al., 2007; Shinohara and Takayanagi, 2007; Zaidi, 2007). The resorption process itself and the activity of osteoclasts are regulated by released Ca^{2+}, nitric oxide (NO) and several cytokines (e.g. TNFα) (Del Fattore et al., 2008; Robinson et al., 2007; Zaidi, 2007).

The initial step in osteoblastogenesis (and forthcoming bone formation) is the determination of mesenchymal stem cell to become an osteoprogenitor cell, and thereafter an osteoblast. The process is regulated by several hormones and cytokines (e.g. PTH, prostaglandins, interleukins) (Marie, 2008; Zaidi, 2007). Osteoblasts secrete type I collagen and other matrix proteins and are responsible for matrix mineralization. Bone formation is regulated by intrinsic factors, such as Runt-related transcriptional factor 2 (Runx2), Osterix (Osx), and Wnt-β-catenin (Macsai et al., 2008; Marie, 2008; Zaidi, 2007).

To sum up, on the molecular level, the regulation of bone turnover is a mixture of systemic endocrine regulators (hormones), local autocrine and paracrine factors (e.g. cytokines), and neuronal influences (Konttinen et al., 1996).

Bone functional adaptation

On account of remodeling, bones are plastic organs. For almost 200 years, it has been recognized that there is a relationship between physical loading and bone growth (Delpech, 1828; Forriol and Shapiro, 2005). A concept that is thereafter

termed bone functional adaptation (Ruff et al., 2006) was introduced by W. Roux (Roux, 1881). The concept of "Wolff's law" is based on the writings by Julius Wolff (Wolff, 1892) and is commonly considered a synonym for bone functional adaptation, although it should be regarded as a more detailed version of general bone functional adaptation (Ruff et al., 2006). Wolff's law was later simplified by D'arcy Thompson as "form follows function" (Thompson, 1919). Ultimately, the objective of bone functional adaptation is to maintain the inherent safety factor of bone that keeps its fracture risk at an acceptable level (Alexander, 1981; Biewener, 1993).

Mechanotransduction

For successful control of bone biology by mechanical loading-induced stimuli, some mechanism must convert mechanical tissue loads into signals that cells can perceive and respond to (Frost, 1988). Mechanotransduction is a multi-step process that converts the physical stresses into cellular responses (Duncan and Turner, 1995). The first step is mechanocoupling, in which, according to the current understanding, a mechanical strain or deformation either creates canalicular fluid flow that stimulates bone osteoblasts and osteocytes or stretches or compresses the cells directly (Ehrlich and Lanyon, 2002; Forriol and Shapiro, 2005; Frost, 1988; Frost, 2003; Lanyon, 1996; Mosley, 2000). These changes then are sensed by the cell wall processes (Bakker et al., 2001; Burger and Klein-Nulend, 1999; Smalt et al., 1997; Weinbaum et al., 1994). Thereafter, during biochemical coupling, the local mechanical signal is transducted into a biochemical signal. Osteoblasts and osteocytes have been shown to respond to mechanical strain through increases in levels of inositol 1,4,5-triphosphate (IP3), protein kinase C (PKC) activity, prostaglandins (PG), nitric oxide (NO), and cyclic adenosine monophosphate (cAMP) (Ajubi et al., 1999; Carvalho et al., 1994; Forriol and Shapiro, 2005; Jessop et al., 2002; Pitsillides et al., 1995). The biochemical signals are transmitted to the effector cells (osteoblasts and osteocytes) which, eventually, are either directed or inhibited (Forriol and Shapiro, 2005).

Bone mechanostat, mechanoresponsiveness and sensitivity

An appropriate response to mechanical strain is a prerequisite for the success of bone functional adaptation, and several hypotheses have been introduced in order to explain this complex control system. Harold Frost introduced an analogy between strain-adaptive remodeling and a domestic thermostat (or "Mechanostat") that is "off" under circumstances of normal physiological strain and "on" in response to strain magnitudes outside normal physiological thresholds (Frost, 1987a; Frost, 1987b; Frost, 2003) (Figure 2). According to this theory, the mechanostat senses and perceives the incident loading-induced strain distribution within the bone and subsequently removes bone tissue from sites

where the concomitant stresses are marginal while forming new bone tissue at sites subjected to increased stress (Frost, 1987a; Frost, 1987b; Frost, 2003). Thus, the adaptation does not only regulate bone mass but also bone geometry and shape.

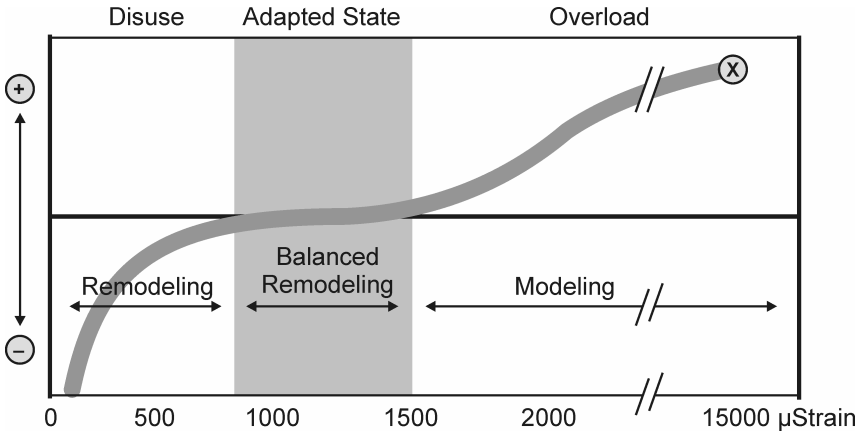


Figure 2. The mechanostat theory. The bone modeling range designated the strain region where mechanically-controlled modeling begins and bone mass is increased, while the remodeling range designates the strain region in which maximal "disuse-mode" remodeling activity occurs and bone is lost. In the adapted state, bone resorption and formation tend to equalize and existing bone mass and strength are maintained.

According to the mechanostat theory, there are genetically preset thresholds for modeling and remodeling. The modeling threshold is proposed to be ~1500 microstrain and the remodeling threshold ~800 microstrain (Frost, 2003). A healthy skeleton is able to withstand loads that are ~6 times greater than the normal voluntary loads, a difference called "safety factor" (Biewener, 1993; Frost, 2003). Functional bone adaptation is a long-term, continuous process occuring over a period of months or years (Frost, 2003; Mosley, 2000).

The comprehensible theory of mechanostat has also been criticized for having flaws, too (Ehrlich and Lanyon, 2002). First of all, it has been claimed that the theory does not regard loading frequency or strain rate as important factors in bone adaptation (Ehrlich and Lanyon, 2002; Lanyon and Rubin, 1984), although experiments have confirmed them to be important determinants of bone adaptation (Rubin and McLeod, 1994; Turner et al., 1994; Turner et al., 1995a). Furthermore, it has been proposed that the theory fails to predict the finding that when the duration of the loading increases, the formation seems to saturate (Saxon et al., 2005; Turner, 1998). Indeed, it has been shown that short periods of exercise, with a 4-8 h rest period between them, are a more effective osteogenic stimulus than a single sustained session of exercise (Burr et al., 2002; Robling et al., 2000; Robling et al., 2001). Also, for an effective homeostatic control mechanism to be established, a suitable and relevant feedback variable

must exist (Lanyon, 1984; Turner, 1991) (Figure 3). According to the current understanding, the variable is the strain that is regulated by incident loading and bone stiffness (Currey, 2002), whereas the mechanostat theory regards bone strength as a endpoint of the homeostatic control machinery (Frost, 2003).

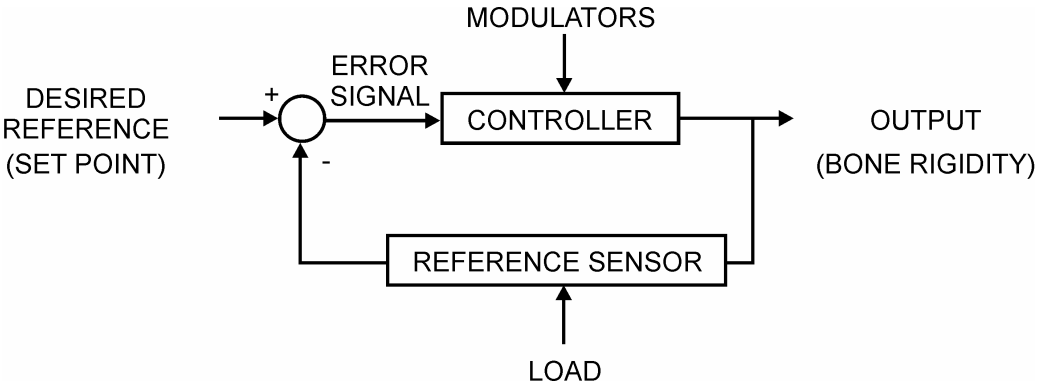


Figure 3. Feedback loop illustrating the function of bone functional adaptation. A control loop, including sensors, control algorithms and actuators, is arranged in such a fashion as to try to regulate a variable at a setpoint or reference value.

Bone mechanoresponsiveness and sensitivity are closely related properties of bone. The terms have been used quite liberally to describe the ability of bone to detect mechanical loading-induced stimuli and then adapt their structural rigidity accordingly. However, in the strictest sense, these two terms depict distinct phases of functional bone adaptation. In essence, bones first need to somehow sense the loading and then to elicit an appropriate response – a structural change, if required, to cope with the loading demands. Thus, mechanoresponsiveness is a parent concept referring to the ability of bone to go through the entire process of functional adaptation. Poorly mechanoresponsive bone is unable to form new bone at sites where it is needed or remove unnecessary bone tissue where stresses are low in magnitude. However, mechanoresponsiveness does not describe which part of the process may cause a possible lack of adaptive response. Mechanosensitivity, instead, refers to mechanosensing and, thus, describes the ability of bone tissue to detect strain stimulus (Pajamäki, 2007). Incorporating this into the mechanostat theory, a possible modulator of the mechanosensory apparatus of the bone could sensitize or desensitize it to mechanical loading by altering the modeling and remodeling thresholds (Frost, 1987b).

Bone architecture (mass, shape, and internal arrangement) at any time will be primarily determined by the individual's genetic inheritance and the response to functional load bearing (Lanyon, 1996). Conversely, it is possible to predict the distribution of functional strain from the geometry of the bone (Lieberman et al., 2004; Ruff et al., 2006).

For example, the long bone cross-sections of newborns are almost totally circular, whereas during growth the cross-section assumes angular or elliptical form (Akkus et al., 2004; Palacios et al., 1992; Sumner and Andriacchi, 1996). According to the theoretical reasoning, this would indicate that the plane of the adaptive response is also the plane of the maximum strain stimulus. Several studies, however, have reported results inconsistent with this logic. Surprisingly, most of the studies (Coleman et al., 2002; Demes et al., 2001; Shahar et al., 2003; Simoes et al., 2000; Taylor et al., 1996) indicate that the maximum strain is in the narrowest plane (I_{min}). There are also studies that report more predictable results, the maximum strain being located on the widest plane (I_{max}) (Main and Biewener, 2004; Taddei et al., 2006). These somewhat contradictory results have been attributed to bias arising from the experiments themselves. At least in adults, strain gauges measure deformations in bones that have already adapted to the mechanical loading (Ruff et al., 2006). In addition, the most osteogenic strains are those that occur under vigorous loading, while most of the above mentioned interventions were monitoring strain distributions during moderate loading (Ruff et al., 2006). As shown previously (Main and Biewener, 2004), the phase of stance (i.e. intensity of loading) alters the orientation of the neutral axis, thus leading to misinterpretations of strain gauge data in terms of *in vivo* loadings (Ruff et al., 2006).

From the clinical point of view, the adaptation of the cross-sectional shape is of great importance, especially in the femoral neck. It has been shown that normal locomotive loading makes the neck to attain a specific cross-sectional shape and cortical properties (Bell et al., 1999a; Bell et al., 1999b; Crabtree et al., 2001; Mayhew et al., 2004; Mayhew et al., 2005; Nikander et al., 2008; Sievänen et al., 2007). A sideways fall on the hip can impose a large stress on the femoral neck from such a direction the cortical bone is not typically adapted to, rendering the bone highly susceptible to fail (Hayes et al., 1996; Lotz et al., 1995).

Apart from the cross-sectional shape, loading has been shown to be responsible for the curvature of long bones (Bertram and Biewener, 1988; Biewener and Bertram, 1994). Loading of long bones is a combination of bending, shear, and compression (Lieberman et al., 2004). In addition, the greatests physiological loads are caused by muscle contractions (Burr, 1997; Schoenau, 2005; Schoenau and Frost, 2002), which makes the mixture of different loads even more complicated. Bending magnifies the effects of forces compared to pure compression, and thus it has been postulated that the curvature is an adaptive means to minimize bending forces and direct loads in predictable fashion (Currey, 2002). Experimental studies (Lanyon and Baggott, 1976;

Lanyon and Bourne, 1979) do not support this hypothesis, although these studies are challenged by the same limitations as described previously (Ruff et al., 2006).

Skeletal ontogeny

Bone development is one of the key processes of intrauterine and postnatal growth (Schoenau et al., 2003). Through a process called skeletal ontogeny, the endocrine, locomotive and protective functions of the bones are integrated into a single organ to sculpt an appropriate size, shape and internal architecture of a given bone site. According to the current understanding, mechanical factors play a fundamental role both in fetal and postnatal skeletal ontogeny (Palacios et al., 1992; Rodriguez et al., 1992; Schoenau et al., 2003).

It has been postulated that biological factors are important only in the initial phases of diaphyseal growth and ossification and their influence disappears over time, whereas mechanobiological influences remain a fundamental influence on bone apposition and resorption throughout life (Carter et al., 1996). The primary bone collar at the midshaft of the anlage will appear and grow with or without the mechanical stimulation associated with fetal muscle contraction, but the normal development of the diaphyseal architecture is critically dependent on forces created by fetal movements (Carter et al., 1996; Gomez et al., 2007). Indeed, it has been shown that paralyzing the muscles of a rat fetus using curare for the entire gestational time will result in the fetus having slender, short bones with reduced mass (Rodriguez et al., 1992), whereas the removal of amniotic fluid (in order to hamper the habitual movements) alone does not affect longitudinal growth (Palacios et al., 1992). After birth, the role of natural movements is especially important for bone girth and cross-sectional characteristics, whereas the long bone length is only weakly influenced by extrinsic regulation (Lanyon, 1980; van der Meulen and Carter, 1995). Structural adaptation of the mature skeleton to changes in locomotive loading are considered an adult manifestation of the same epigenetic factors which are so crucial in normal development (Carter et al., 1996). Thus, the changes in the dimensions of the diaphyseal cross sections during growth can be viewed as a result of (i) mechanically mediated adaptations to locomotive loading, (ii) hormonal regulation, (iii) their possible interactions, and (iv) “genetic growth”, coined by Frank Rauch (Rauch, 2005) and meaning all the remaining factors except the previous.

Along with mechanical loading, adenohypophyseal hormones have been shown to be strong regulators of bone growth (Kim et al., 2003) increasing even the linear growth of long bones (Alippi et al., 2005; Zhang et al., 1999), although these studies were unable to determine the effect of those hormones in non-loaded bones, and thus, for example, the anabolic effect of the growth-hormone to muscles may result in a notable bias. In childhood, bone mass increases with increasing body height and weight (Bailey et al., 1999; Bass et al., 1999). At the

onset of puberty, ~40% of the adult bone mass has been gained and bones expand radially and elongate (Wang, 2005). Skeletal growth is accelerated during puberty (Bailey et al., 1999; Rauch et al., 2001; Wang et al., 2005), and bone modeling activity diverges between male and female. At the endocortical surface, bone is resorbed in males and preserved or formed in females (Libanati et al., 1999; Neu et al., 2001), thus leading to a situation where males have larger bone dimensions than females. In rats, no actual growth spurt can be seen, while the growth is fastest from birth and gradually decelerates achieving the peak bone mass after ~12 months (Warden et al., 2007).

Adaptational lag

In humans, the proper functional adaptation of bone takes 3-6 months (Frost, 1988; Mosley, 2000). Due to the sluggish and "error-driven" nature of adaptation, the development of bone mechanical competence is lagging in relation of the needs of the physical loading during growth (Frost, 2003). The newly formed bone is less stiff than bone tissue that has been completely mineralized (Frost, 1988). This imbalance between bone mechanical competence and habitual loading is called "adaptational lag" (Frost, 2003).

There are several studies that report findings supporting this theory. In human studies, a common finding is that the relation between the peak velocity for bone mass or parameter reflecting bone mechanical competence and lean body mass is lowest during the growth spurt (Crabtree et al., 2004; Heaney et al., 2000; MacKelvie et al., 2002; Rauch et al., 2001; Schiessl et al., 1998). Also, peak stature growth velocity has been shown to precede long bone strength development (Ruff, 2003) and muscle cross-sectional area growth the development of bone mass (Daly et al., 2004; Högler et al., 2008). The same finding has been reported for rats (Wang et al., 2003).

It has been postulated that due to the relative weakness of the bones of rapidly growing individuals, their bones are highly susceptible to fractures (Bailey et al., 1989; Blimkie et al., 1993; Frost, 2003; Heaney et al., 2000), a fact reported as early as the 1960s (Alffram and Bauer, 1962). On the other hand, the relative lower rigidity of bone enables higher strain (Nunamaker et al., 1990) and may account for the superior mechanoresponsiveness during growth spurt compared to postpubertal individuals (Bass et al., 2002; Kannus et al., 1995; MacKelvie et al., 2002).

Growth and mechanoresponsiveness of bone

According to the current understanding, growth is the most opportune time to modify the mass of the skeleton (Haapasalo et al., 1994; Haapasalo et al., 1996; Huddleston et al., 1980; Jones et al., 1977; Kannus et al., 1995; Karlsson et al., 1993; Morris et al., 1997; Parfitt, 1994; Seeman, 2002; Theintz et al., 1993). As mentioned above, in several studies, the mechanoresponsiveness of growing

bone has been shown to be superior to that when growth has stopped (Bass et al., 2002; Heinonen et al., 2000; Kannus et al., 1995; MacKelvie et al., 2002), this being probably due to adaptational lag (Frost, 2003).

In addition, exercise during growth has been shown to be beneficial as a means of optimizing lifelong periosteal bone dimensions, with reduced periosteal bone apposition during growth being implicated in the pathogenesis of fragility fractures later in life (Seeman, 2003; Seeman and Delmas, 2006). In fact, there is evidence that exercise-induced changes in the skeleton during growth could be maintained, even if the exercise is discontinued (Kontulainen et al., 1999; Kontulainen et al., 2001; Warden et al., 2007). However, there is also contradictory evidence showing that the bones of exercised rats did not differ from the bones of control rats after 14 weeks of sedentary life (Pajamäki et al., 2003).

Besides being the optimal time to modify the mass of the skeleton, growth is also an important period for bone geometry (Haapasalo et al., 2000). According to several studies, it has been postulated that there may be a divergence in the adaptation pattern: during the longitudinal growth period, and particularly during puberty, increased loading could produce actual structural changes in bone geometry, whereas additional bone acquired after skeletal maturity would be deposited along the existing bone framework (Forwood and Burr, 1993; Haapasalo et al., 2000; Järvinen et al., 2003c; Kannus et al., 1995). It has also been shown that loading determines the three-dimensional macrogeometry (e.g. curvature of the appendicular bone) during growth (Biewener and Bertram, 1994).

Aging skeleton

Post-menopausal and age-related osteoporosis

Aging is associated with significant bone loss in women and men (Riggs et al., 2002; Riggs et al., 2004). In women, menopause triggers a rapid phase of bone loss that can be prevented by estrogen replacement (Lindsay et al., 1976) and is, thus speculated to be of ovarian function origin (Khosla and Riggs, 2005). At menopause, bone resorption, as assessed by biochemical markers, increases by 90%, while bone formation increases by ~45% (Garnero et al., 1996), leading to a state of imbalance. Estrogen has been shown to suppress RANKL production (Eghbali-Fatourechi et al., 2003), an important regulator of osteoclastogenesis. In addition, estrogen plays a role in the production of other paracrine regulators of osteoclastic and osteoblastic function (Khosla and Riggs, 2005; Manolagas and Jilka, 1995). It has been argued that different estrogen receptor I genotypes may be more sensitive to estrogen than others, resulting in varied responses to the hormone replacement therapy in humans (Leskelä et al., 2006).

As the inhibition of periosteal enlargement at puberty in females is commonly considered to be caused by direct estrogen mediated inhibition of periosteal bone cells, the expansion of the periosteal envelope after menopause (estrogen deficient state) is analogously attributed to the removal of this estrogen-induced constraint on periosteal apposition (Seeman, 2003). It has also been suggested that postmenopausal osteoporosis *per se* is attributable to the estrogen-withdrawal induced desensitation of bones to loading-induced stimuli (Lanyon and Skerry, 2001; Lee et al., 2003). However, it has recently been increasingly acknowledged that bones, as primary locomotive organs, should be considered as structures with the mechanical strength and rigidity representing their ultimate phenotype (Boskey et al., 2003; Seeman, 1997; Seeman, 2002; van der Meulen et al., 2001). It has even been argued that conclusions based solely on observations obtained either from cell culture experiments *in vitro* or from *in vivo* studies assessing bone mass or other surrogates of bone strength are insufficient and likely misleading (Boskey et al., 2003; Järvinen et al., 2005; van der Meulen et al., 2001). Regarding bones, if we do not know whether the bone as an organ has truly strengthened, we have no way of knowing with certainty whether a change in any of the intermediate or surrogate measures of bone strength denote only a transient phenomenon – like a "snap-shot" of a dynamic movement eventually fading away – or actually a strengthened bone structure as a response to the stimulus of interest (Järvinen et al., 2005).

With aging, most men do not develop a hypogonadism that could be compared to women's menopause. However, there is evidence that sex hormones may be responsible for age-related decreases in bone mineral content. The level of estrogen has been shown to positively correlate with bone mineral density (BMD) at various bone sites in old men, whereas testosterone has an inverse correlation (Slemenda et al., 1997). There are also other studies that show the positive correlation between the level of estrogen and BMD in elderly men (Amin et al., 2000; Center et al., 1999; Szulc et al., 2001). According to an experimental study, testosterone enhances the periosteal apposition in rats (Turner et al., 1990), and thus the substantial decrease in the testosterone level during aging (Khosla et al., 1998), may be a relevant factor.

Both in women and men, aging has been shown to decrease the secretion of growth hormone (Ho et al., 1987), which has been shown to affect bone mechanical competence (Halloran et al., 1995). Furthermore, the level of PTH is elevated during aging, and this has been postulated to be at least partly responsible for the increase in bone turnover (Khosla and Riggs, 2005; Ledger et al., 1995).

Aging and mechanoresponsiveness of bone

As described above, several bone deteriorating factors are related to aging. According to the principles of bone functional adaptation, the resulting bone loss should be compensated by newly formed bone if it is to successfully fulfill the needs of habitual loading (Frost, 2003). Thus, the explanation for decreased bone

mass underlies the question whether age-related bone loss is an appropriate response to reduced loading in a less active host, or an aberration in the machinery responsible for mechanoresponsiveness (Klein-Nulend et al., 2002; Lanyon and Skerry, 2001; Lee et al., 2003; Seeman, 2004).

There is an arsenal of controversial studies that support or oppose the hypothesis that aging alters the mechanosensitivity or mechanoresponsiveness of bone. It has been proposed that estrogen deficiency (i.e. menopause) may alter the sensing of mechanical loading, perhaps through effects on osteocytes in bone (Frost, 1999a), thus leading to bone loss. Also, it has been postulated that aging may increase the mechanical loading threshold for bone formation (Turner et al., 1995b). Furthermore, it is well known that the number of osteocytes within the bone tissue decreases with age (Vashishth et al., 2000), but the significance of this decline is uncertain (Turner, 2007). According to an *in vitro* study (Klein-Nulend et al., 2002), the function of human bone osteocytes to detect and respond to pulsating fluid flow is not impaired in cells obtained from old donors. All in all, there is no solid proof that the mechanosensitivity of bone cells *in vitro* is decreased during aging.

Experimental *in vivo* studies have shown that the responsiveness of the aged skeleton is increased (Buhl et al., 2001), reduced (Rubin et al., 1992; Turner et al., 1995b), or unaffected (Järvinen et al., 2003c; Raab et al., 1990; Umemura et al., 1995). Raab et al. (1990) reported a comparable skeletal response to exercise in young and old (2.5 and 25 month-old respectively) rats, but used a different running velocity in the two age groups. Umemura et al. (1995) reached the same conclusion (i.e. the effects of exercise were not limited by age) in their comprehensive comparison of rats of 3, 6, 12, 20 and 27 months of age subjected to both jump training and running. In contrast, Rubin et al. (1992) showed, using an experimental model of externally loadable functionally isolated turkey ulna preparation, that a physical signal clearly osteogenic in the 1-year-old young adult skeleton was hardly acknowledged in the older (3-year-old) bone tissue. Similarly, Turner et al. (1995) observed that both the periosteal and endocortical surfaces of the tibiae of 19-month-old rats were significantly less responsive to mechanical loading than those of 9-month-old rat tibiae. However, the use of historical controls and inappropriate statistical comparisons impair the strength of this latter study.

In a recent study by Järvinen et al. (2003c), the authors showed that the ability of bones of young (5-19 week old) and mature (33-47 week old) male rats to adapt to treadmill-training induced loading was similar, but the adaptive mechanisms differed. In essence, the growing bones displayed primarily geometric changes (increases in cross-sectional size), whereas the adult skeleton responded mainly through an increase in volumetric bone density. However, the major limitation in that study was that the adult animals were still growing at the beginning of the treadmill training, and thus the analysis may have been confounded by the concurrent axial growth. To add yet another dimension to an already confused situation, Buhl et al. (2001) reported that 22-month-old male rats had a greater sensitivity to squatlike-exercise than their younger counterparts (4 and 12-month-old male rats).

Explanations for the increased or decreased responsiveness to loading have been evinced. Old rats usually have lower bone mechanical competence at baseline (Akkus et al., 2004; Buhl et al., 2001) which is most likely due to decreased habitual activity (Peng and Kang, 1984) or other factors leading to loss of bone with aging or "age-related osteoporosis". As a consequence of this age-related bone loss, exogenous loads are distributed over a lesser bone mass, resulting in a greater strain per unit of bone (Buhl et al., 2001). It has been argued that in females, at least, estrogen deposits an extra stock of mineral into premenopausal bones, thus damping the responsiveness to loading in adulthood (Järvinen et al., 2003a; Sievänen, 2005). This damping would cease at menopause when the estrogen levels are low. Lastly, apart from impaired mechanosensitivity (Turner et al., 1995b), the decreased responsiveness may be due to the increased bone stiffness that originates from the known enlargement of the dimension of the bone cross-section (Akkus et al., 2004).

In human studies, the increased loading has been bone preserving rather than bone gaining during aging (Asikainen et al., 2004; Bonaiuti et al., 2002; Kelley et al., 2000; Kelley et al., 2001; Wallace and Cumming, 2000; Wolff et al., 1999). However, the weakness of most of the human studies is that the intensity of loading may be poorly standardized, e.g. because of the decreased physical capacity of elderly people.

There is also evidence that the response to increased loading differs qualitatively between growing and aged bone. According to rodent and human studies (Forwood and Burr, 1993; Järvinen et al., 2003c; Kannus et al., 1995) the additional bone in mature skeleton is deposited along the existing bone structure, whereas during growth the new material is used to increase the dimensions of the bone structure. This has led to a hypothesis that bone obtained in senescence is abolished more easily (Forwood and Burr, 1993; Parfitt, 1994), and although the preservation of existing bone material in elderly has been shown to be challenging (Suominen, 2006), a recent study (Järvinen et al., 2003c) showed no difference in the ability to preserve the newly obtained bone.

Senescent rat as a model of aged skeleton

Several animal species, including rodents, rabbits, dogs, and primates, have been used as animal models in osteoporosis research. The rat is the preferred animal for most researchers (Lelovas et al., 2008), due to its inexpensiveness and ease to house. Also, its life span is relatively short (Table 1) enabling short experiments. In addition, there is a general acceptance of the public to the use of rodents in research (Turner, 2001). Its skeleton has been studied extensively, and although there are several limitations to its similarity to the human condition, these can be overcome through detailed knowledge of its specific traits (Lelovas et al., 2008). Unlike humans, there is no epiphyseal closure in long bones (Bland, 2000). However, there seems to be a period when skeletal growth decelerates markedly occurring at approximately 7 to 8 months in male and female Sprague-Dawley rats (Quinn, 2005). Furthermore, rat skeleton lacks Haversian systems in cortical

bone (Wronski and Yen, 1991). However, it has been stated that the same mechanisms control gains in bone mass and losses, in young and aged rats and humans (Frost and Jee, 1992). Furthermore, they respond similarly in rats and man to mechanical influences, hormones, drugs and other agents (Frost and Jee, 1992).

Table 1. Comparison between human and rat in terms of biological milestones during life.

	Epiphyseal closure (years)	**Senescence (years)**	**Average life span (years)**
Human	20 (Grant, 1972)	51 (Quinn, 2005)	80 (WHO, 2007)
Rat	0.6 (Quinn, 2005) *	1.5 (Meites et al., 1980)	3 (Quinn, 2005)

* Rat has no actual epiphyseal closure but has a dramatic deceleration of linear growth.

Characterization of bone

The primary function of the skeleton is locomotion and, thus, the most important property of bone is the ability to resist mechanical loads (Burr, 1997; Einhorn, 1992; Frost, 1997; Järvinen et al., 2005; Parfitt, 1998). Mechanical tests have commonly been used to determine the mechanical properties of bones (Eckstein et al., 2004; Turner and Burr, 1993; van der Meulen et al., 2001), but naturally due to the destructive nature of the test, they cannot be used for clinical purposes. Fortunately, several non-invasive methods have been developed for both research and clinical work (Cummings et al., 2002; Genant et al., 1996; Sievänen et al., 1998).

Material, texture, mass, and geometry

Bone mechanical competence is compounded of bone material, bulk, texture, and morphology (Järvinen et al., 2005) (Figure 4). In theory, exhaustive data on all these parameters could enable to an accurate estimate of bone mechanical competence, but in reality this is not possible (van der Meulen et al., 2001).

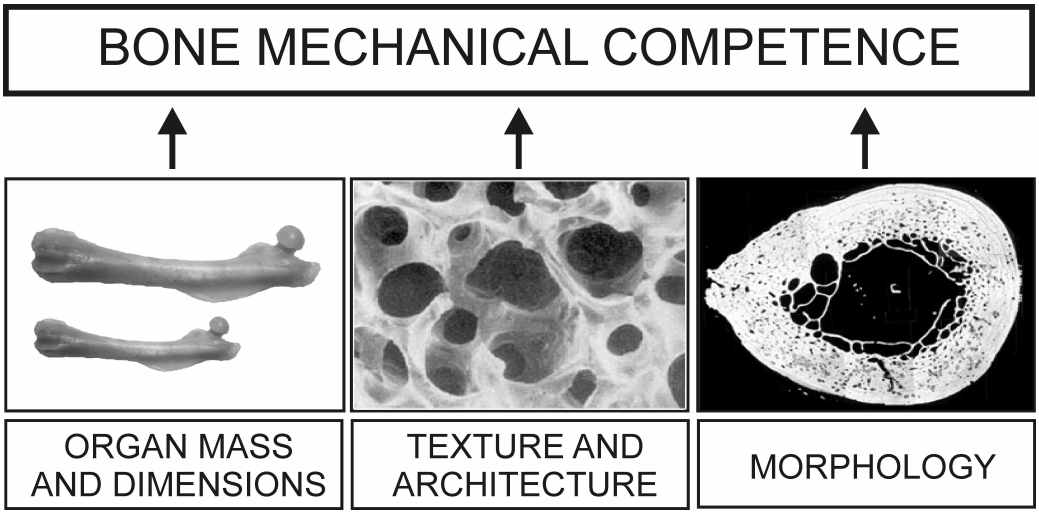


Figure 4. Constituents of bone mechanical competence.

Mineral density of bone is a measure of the amount of bone material. Dual energy X-ray absorptiometry (DXA), peripheral quantitative computed tomography (pQCT), and microcomputed tomography (μCT) can be used to determine a number of different bone mineral densities, and therefore, it is important to understand the differences between total (tBMD) or areal density (aBMD), and true volumetric mineral density (vBMD) or cortical density (cBMD) (Jokihaara, 2007; Schoenau et al., 2004). Using DXA it is not possible to determine vBMD or cBMD, due to the inherent planar nature of the measurement (Genant et al., 1996; Sievänen, 2000; Sievänen et al., 1998). Instead, pQCT enables the determination of properties of cortical bone (cBMD) as well as a lumped single value for the average volumetric density of a certain bone region (vBMD), because a certain volume of solid bone can be included in the analysis (Schoenau et al., 2004). Microcomputed tomography even enables the determination of the density of single trabecula, and is needed in order to determine the microarchitecture of bone.

Bone mass (BMC) is dependent on bone size and bone density (Schoenau et al., 2004; Sievänen, 2000). A classic way to quantify the total mineral content of bone is ashing. DXA offers a useful non-invasive tool for the estimation of BMC, especially for clinical practice (Genant et al., 1996; Johnson and Dawson-Hughes, 1991; Sievänen, 2000; Sievänen et al., 1996; Sievänen et al., 1992). pQCT is also useful in the characterization of bone mineral content (Järvinen et al., 2005; Sievänen et al., 1998).

Bone geometry can be determined with an arsenal of methodologies. The classic way is to use a caliper (Järvinen et al., 1998b) or radiographs (Conlogue and Marcinowski, 1987). For the determination of cross-sectional shape and geometry of an intact bone, pQCT or magnetic resonance imaging (MRI) are needed (Järvinen et al., 2005). Using cross-sectional measures, it is possible to

derive cross-sectional moment of inertia (CSMI or I) values, and certain indexes that help in estimating of bone strength non-invasively (Ferretti et al., 1996; Schoenau et al., 2001).

Biomechanics

Although the surrogates described above to evaluate bone fragility are competent on the theoretical level, direct biomechanical testing of bone provides more information about the mechanical properties of bone and should be regarded as the gold standard (Turner and Burr, 1993). Structural testing refers to the testing of whole bones, whereas bone material testing measures the properties on the tissue level (van der Meulen et al., 2001).

The execution of any mechanical test follows a certain pattern. Load is applied to the specimen until it fractures. During loading, bone experiences stresses and strains. The stresses can be classified to shear, tension and compression (Figure 5). Shear stresses are developed when a region of an object slides relative to an adjacent region. Tension is experienced when the object is stretched and compression results from two forces that are directed towards each other along the same straight line. The three basic stress types may combine as a result of a variety of complex loading configurations and lead to different fracture patterns (Einhorn, 1992; Turner and Burr, 1993).

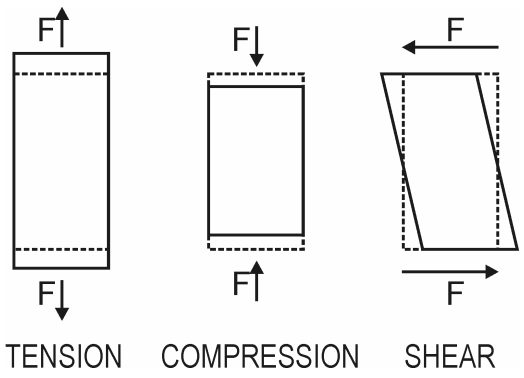


Figure 5. Different types of stress and strain.

The relationship between load applied to a structure and deformation in response to the load is called the load-deformation curve (Figure 6). The curve can be divided into two regions: the elastic region, when the deformations are momentary and the plastic region, where the changes are permanent. The maximum stress the bone can sustain is called ultimate strength (F_{max}), which usually equals the breaking load. This is especially true regarding brittle materials, whereas in ductile materials they can be significantly different.

Stiffness refers to the bone rigidity and can be derived from the linear part of the load-deformation curve as a tangent modulus. The area under the load-deformation curve is a measure of the amount of energy needed to cause a fracture; this is also called toughness. Several other biomechanical traits can be determined from the load-deformation curve, but these three are the most relevant in bone research (Einhorn, 1992; Turner and Burr, 1993).

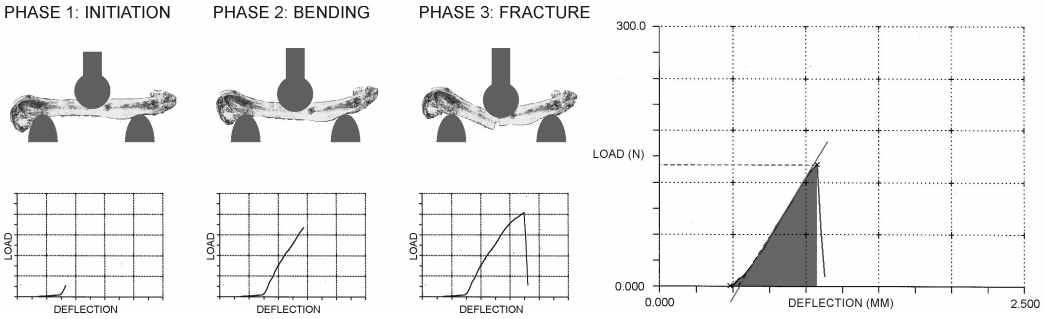


Figure 6. Determination of the breaking load, stiffness, and energy absorption from the load–deformation curve obtained during the three-point bending test of rat femur. The breaking load is determined as the highest point of the curve, the stiffness as the tangent modulus of the linear part of the load-deformation curve. The energy absorption is determined as the area under the load-deformation curve until the point of failure (breaking load).

The testing of bones is a simplification of the actual *in vivo* situation. As stated, the habitual loading is a combination of different stresses (Lieberman et al., 2004), and thus, the testing should be carried out so as to mimic the habitual loading of the bone structure as closely as possible. This applies to the direction of loading and selection of loading type. The most widely used method for the determination of the biomechanical properties of long bones is the bending test. Both three-point and four-point bending tests are useful, although they make an inherent assumption that bone is habitually loaded primarily by bending forces. In the bending test, the bone is placed on two lower supports, and the load is then applied to the bone from above either using one (three-point) or two (four-point) indentors. Another widely used method for testing long bones is torsion testing, during which the bone is rotated in different directions around the longitudinal axis at both ends. The stress is primarily in shear and the benefit of testing is that the results are independent of the orientation of the bone. On the other hand, torsion is uncommon in habitual loading, thus the test does not mimic the natural situation. For short and irregular bone (e.g. vertebrae) compression test is the most commonly used method. The specimen is compressed between two platens until it fractures (Turner and Burr, 1993).

The rate of loading during testing varies quite substantially even in similar test settings. For example, in three-point bending of rat femur, the rate ranges from 0.2 mm/min (Ederveen et al., 2001) to 1.0 mm/sec (Ma et al., 2002).

Considering the profound effect of strain rate on the mechanical properties of bones, this issue is naturally of great significance. However, in this respect, one should keep in mind that bones primarily adapt to dynamic loads during movement and the physiological loading-induced strain rates vary somewhere between 0.01/s and 0.08/s (Turner and Burr, 1993).

Validity of measurements

The standard of any good scientific measurement or test is its validity. Validity consists of both internal and external properties (Mitchell and Jolley, 2001) (Table 2). If a test is internally valid, it measures the desired quality accurately (the mean of a certain variable in multiple measurements is close to the absolute truth) and with good precision (the difference in a certain variable between repeated measurements is minimal). The difference between accuracy and precision has classically been depicted with a model of an archer: an accurate archer hits near the bull's eye but the hits might be spread in all directions quite far from each other, whereas a precise archer hits all the arrows to the same spot, which, however, is not necessarily near the bull's eye. This example, however, is a little misleading with respect to research, as the target is depicted as two-dimensional in nature (the arrows can miss both high/low and left/right). In scientific measurement the results are one-dimensional numbers (the value can only be too large/small). Hence, a more appropriate example would be depicted by a line with different spots (Figure 7).

Table 2. Terms related to the validity of a measurement or test.

Term	**What question does it answer to?**
Internal validity	Are we measuring those things that we think we are measuring?
Accuracy	How close to the truth are our results?
Sensitivity	Is our instrument capable of detecting the magnitude of the predictable effects?
Specificity	Does our instrument measure the desired quality?
Precision	To what degree will further measurements or calculations show the same or similar results…
Inter-observer	…assuming that the performer of the testing will change?
Intra-observer	…assuming that the performer of the testing will remain the same?
External validity	Can we generalize the results that we have obtained to the whole population?

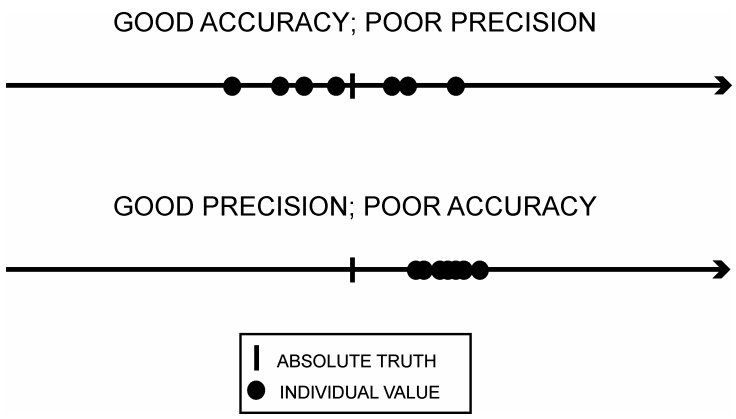


Figure 7. Accuracy vs. precision. Accuracy describes the closeness of individual results to the absolute truth. When all individual results are grouped tightly together, the cluster is considered precise, since they all struck close to the same spot, if not necessarily near the absolute truth.

In addition, in order to be internally valid, the method has to be sensitive enough to be able to detect the anticipated changes (e.g., the diameter of a hair cannot be measured with a ruler) and specific in order to be able to directly measure the desired variable (e.g., the length of a person cannot be estimated by measuring the length of that person's shadow; the strength of a tibia cannot be estimated by measuring the strength of a humerus). External validity refers to the generalizability of the results (Mitchell and Jolley, 2001). For example, one may find that an average cadaver femoral neck can sustain loading up to 3000 N in biomechanical testing. However, this tells little about the femoral neck of average living individuals.

The precision (and validity in general) directly affects the statistical power of the study; i.e., the probability that the given study will show the treatment effect with statistical confidence if the effect truly existed. For obvious scientific and ethical reasons, the sample size of any experiment needs to be planned carefully before starting the study (Eisman, 2006). Ideally the number of animals per group in an experimental osteoporosis study should be large enough to provide a sufficiently high statistical power to show the expected treatment effect (or group-difference). This is crucial, as underpowered studies can seldom address any research question meaningfully, but only lead to inconclusive results, speculation and confusion (Ioannidis, 2005).

In addition, by running more tests on a given data set, there is an increasing likelihood of getting a significant result by chance alone (i.e., in 1 in 21 comparisons; this is what is meant by $p < 0.05$ and 95% confidence limits) (Eisman, 2006). According to the common statistical principles, one has to adjust for the level of Type I error when using the same data set to study several correlations or effects. The famous equation for this is called the Bonferroni

correction (Abdi, 2007). This method only works if all variables are independent; otherwise it is too conservative and possible true findings may remain insignificant. Therefore, more advanced means to adjust the correct alpha error have been developed (Benjamini, 2001).

Summary of the literature

For over 100 years, experimental bone research has broadened our understanding of bone biology. A prerequisite for any research is methodological validity, and experimental bone research is no exception. Some of the criteria for good research practice may have been neglected, primarily due to entrenched tradition. This is especially true regarding the biomechanical testing of whole bones. Indeed, there seems to be an obvious tacit agreement that the precision of the testing method cannot be estimated due to the destructive nature of the test, and a subsequent trend of reporting variables without knowing the precision of the method or sample size needed. However, no proper study of the quality of these studies has been carried out.

Although knowledge about the factors influencing bone ontogeny has increased in recent decades, there are no study settings in the literature that delineate the independent effects of different regulators. According to the current understanding, genetic factors are important only in the initial phases of diaphyseal growth and ossification and its influence disappears over time, whereas locomotion remains a fundamental influence on bone apposition and resorption throughout life. The approach of perceiving bone structure as a direction-specific object, rather than a simple structure whose mechanical properties are independent of the direction of loading, is sparse. A good example is the mechanical testing of rat femoral midshaft, which has been continually tested in anteroposterior direction without justifying the selection of the loading axis.

The current understanding of the pathogenesis of age-related osteoporosis is that it is due to the decreased mechanosensitivity of bones. This assumption is based on the findings of theoretical reasoning, human exercise studies, and a few experimental studies. However, the evidence is not corroborative, and many studies report controversial results.

AIMS OF THE STUDY

The general objective of this thesis was to examine the existing literature to obtain a broad picture of the methods used in experimental bone research. Using this information, the aim was to introduce a new structural-oriented biomechanical testing method which could be used in order to gain insight on interesting research questions of bone biology. Then specifically, the goal was to gain insight into the evolution of bone from postnatal skeletal ontogeny to the other end of the life-span, namely the pathogenesis of age-related osteoporosis. The aims of the individual studies were the following:

1. To evaluate total variation in various biomechanical traits of whole bones of several species and the treatment effects on biomechanical traits in the most common experimental interventions in rats. Thereafter, to devise a scheme to determine the minimum sample size (minSS) needed to detect a treatment effect in biomechanical traits with statistical significance.

2. To develop a method for testing the biomechanical properties of rat femoral midshaft in the mediolateral direction, the apparent primary direction of locomotive loading; and to determine its methodological and biological validity.

3. To delineate the respective effects and the possible interaction of locomotive loading and estrogen on the normal development of the diaphyseal geometry and biomechanical properties of rat femoral midshaft.

4. To evaluate whether the skeleton can maintain its ability to respond to increased loading even in very old age and gain insight into pathogenesis of age-related osteoporosis.

MATERIALS AND METHODS

Animals

A total of 385 rats (231 female and 154 male) of Sprague-Dawley strain were used in the experiments of this thesis (I-IV) (Figure 8, Table 3). The number of rats/groups for each experiment was calculated using power calculation based on the breaking load data gathered for Study I. Animals were housed four animals per cage (I, IV, and the non-operated rats in II) or two animals per cage (III and the operated rats in II) at 20 ^{o}C with a light cycle of 12 hours, and fed standard laboratory chow (Ca^{2+} 0.9%, P 0.7%, and vitamin D 0.6 IU) and water *ad libitum*. In the experiment involving ovariectomized (OVX) rats (III), each cage of OVX rats (two animals per cage) was pair-fed with a cage of control rats with access to food *ad libitum* in order to control for the well-known gain of extra weight associated with OVX (Kalu, 1984; Wronski et al., 1987). The pair-feeding was executed as follows: Each control cage was matched with an OVX cage, the food consumption of the control cage was followed (weighed) every other day and an identical amount of pellets was given to the OVX cage the next day (Järvinen et al., 2003a; Pajamäki et al., 2008). Otherwise the conditions were similar for all animals. All experiments were approved by the Ethics Committee for Animal Experiments of the University of Tampere and the animals maintained according to the guidelines of NIH standards established in the "Guidelines for the Care and use of Laboratory Animals".

Experimental models

Ovariectomy

At the beginning of the neurectomy experiment (III), 3-week-old female rats were randomly subjected to either bilateral sham (SHAM) or ovariectomy (OVX) surgery under fentanyl–midazolam anesthesia using a dorsal approach described previously in detail (Waynforth, 1988). Both ovaries were exposed and removed in the OVX animals. In the SHAM animals, the ovaries were exposed but left intact.

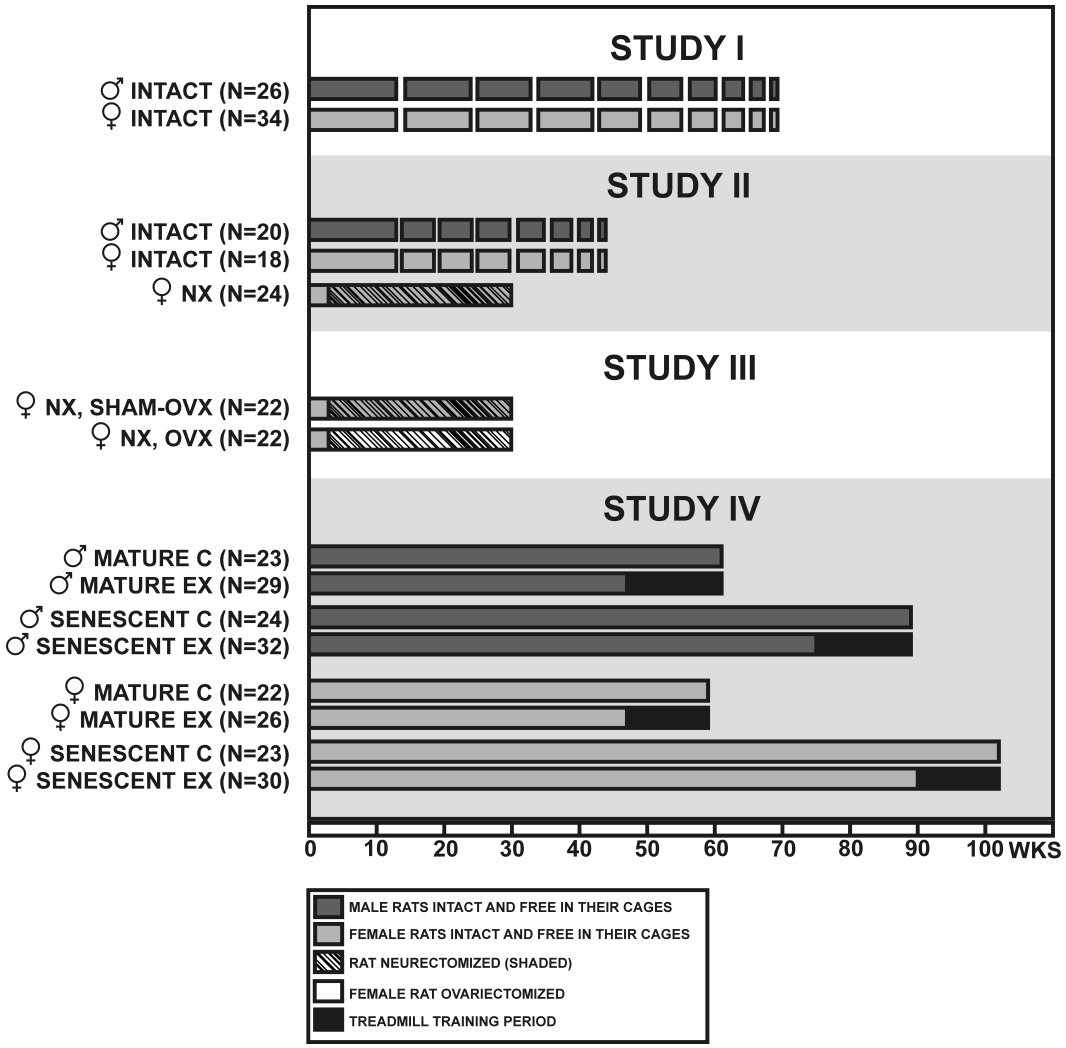


Figure 8. Flowchart of the individual studies. N denotes the number of rats/group. Disconnected bars represent the period when the intermittent sample collection was taking place.

Removal of loading (neurectomy)

In studies involving neurectomy (III and operated rats in II), rats were subjected to unilateral sciatic neurectomy at the age of 3 weeks as described previously (Iwasaki-Ishizuka et al., 2005). Under fentanyl-midatzolam anesthesia, the left sciatic nerve of each rat was exposed through a dorsolateral incision made on the hip and a 1.0-cm section of the nerve was excised. The right limb was left intact to serve as a normally loaded control.

Table 3. Characteristics of the animals used in Studies I-IV. The values are expressed as min-max or mean (SD).

Study	Group	Age, (weeks)	Body weight ENTRY, (g)	Body weight FINAL, (g)	Muscle weight, (g)	Femur length, (mm)	Uterus weight, (g)
I	All rats	17-69	-	383 (105)	-	37.(2.2)	-
	Non-operated rats	14-42	-	381 (105)	-	38.0 (2.2)	-
II	NX rats, L-, tested AP	3-30	66 (6)	333 (58)	0.2 (0.1)	37.0 (1.2)	-
	NX rats, L+, tested AP	3-30	66 (6)	333 (58)	2.3 (0.4)	37.0 (1.2)	-
	NX rats, L-, tested ML	3-30	66 (7)	336 (39)	0.2 (0.1)	37.2 (0.6)	-
	NX rats, L+, tested ML	3-30	66 (7)	336 (39)	2.4 (0.3)	37.2 (0.5)	-
	OVX, L-	3-30	65 (6)	362 (33)	0.3 (0.1)	37.2 (0.9)	0.2 (0.1)
	OVX, L+	3-30	65 (6)	362 (33)	2.5 (0.2)	37.3 (0.8)	0.2 (0.1)
III							
	SHAM, L-	3-30	66 (7)	302 (28)	0.2 (0.1)	36.7 (1.0)	1.2 (0.5)
	SHAM, L+	3-30	66 (7)	302 (28)	2.2 (0.3)	36.6 (1.0)	1.2 (0.5)
	Mature males - C	47-61	566 (45)	574 (45)	3.6 (0.3)	42.0 (1.0)	-
	Mature males - EX	47-61	572 (60)	528 (47)	3.3 (0.4)	41.9 (1.4)	-
	Senescent males - C	75-89	637 (46)	602 (43)	3.2 (0.4)	42.0 (1.2)	-
	Senescent males - EX	75-89	599 (46)	508 (51)	2.8 (0.4)	42.3 (1.4)	-
IV							
	Mature females - C	47-59	291 (23)	307 (35)	2.1 (0.2)	35.9 (0.7)	1.5 0.4)
	Mature females - EX	47-59	300 (27)	312 (31)	2.2 (0.3)	36.6 (0.9)	1.4 (0.3)
	Senescent females - C	90-102	328 (30)	313 (24)	1.9 (0.2)	36.0 (0.9)	1.9 (0.7)
	Senescent females - EX	90-102	334 (31)	298 (20)	1.9 (0.1)	36.0 (0.8)	1.5 (0.4)

Increased loading (exercise)

In the exercise study (IV), the rats were 3 weeks old at the beginning of the study. During the first 2 weeks of the study, all rats ran on a flat-bed treadmill at

a slow speed (10-20 cm/s) for 3 minutes/day on 3 days a week to let the animals adapt the treadmill running and to exclude those animals refusing to run (about 5% of the original population were removed).

Both mature and senescent male and female exercise groups were subjected to a progressive exercise program for 14 and 12 weeks respectively (Tables 4-5). In males, the training began at the age of 47 in the mature exercise group and at 75 weeks in the senescent exercise group. In females, the starting age of training was 47 weeks in the mature group but due to the known increased longevity, postponed till 90 weeks in senescent animals.

The exercise program consisted of progressive running training on a rodent treadmill once a day, 4 days a week. During the first week of the program, the rats ran 5 minutes at a treadmill speed of 20 cm/s and an inclination of 5°. After the first week, the time of the running session was increased to 10 minutes for male rats, while the time was kept at 5 minutes for female rats. The speed of the treadmill and the uphill inclination were gradually increased so that a speed of 30 cm/s was achieved in week 4 and an inclination of 30° in week 9. The total duration of training was 14 weeks for male rats and 12 weeks for female rats.

Table 4. Exercise regimen for male rats in Study IV.

	Age (weeks)				
Week	Mature	Senescent	Duration (min)	Speed (cm/s)	Inclination (deg)
1	47	75	5	20	5
2	|	|	10	20	10
3	|	|	10	20	15
4	|	|	10	30	15
5	|	|	10	30	20
6	|	|	10	30	20
7	|	|	10	30	25
8	|	|	10	30	25
9	|	|	10	30	30
10	|	|	10	30	30
11	|	|	10	30	30
12	|	|	10	30	30
13	|	|	10	30	30
14	60	88	10	30	30

Table 5. Exercise regimen for female rats in Study IV.

	Age (weeks)				
Week	Mature	Senescent	Duration (min)	Speed (cm/s)	Inclination (deg)
1	47	90	5	20	5
2	|	|	5	20	10
3	|	|	5	20	15
4	|	|	5	30	15
5	|	|	5	30	20
6	|	|	5	30	20
7	|	|	5	30	25
8	|	|	5	30	25
9	|	|	5	30	30
10	|	|	5	30	30
11	|	|	5	30	30
12	58	101	5	30	30

Samples

At sacrifice, the rats were killed by carbon dioxide inhalation and the body weights were recorded. The calf muscles (gastrocnemius, soleus, and tibialis plantaris) were carefully prepared and weighed in Study III to ascertain the success of the neurectomy. In all studies, both femora were carefully excised and all surrounding tissues (skin, muscle, and soft tissue) removed. Both femora were then wrapped in saline-soaked gauze bandages, and stored frozen at -20°C in small Ziploc freezer bags. The specimens were frozen and thawed only once before mechanical testing. This kind of storage has been shown not to affect the biomechanical properties of bone (Pelker et al., 1984; Sedlin and Hirsch, 1966). In Study IV, the tibiae were excised and collected for analysis. In addition, in Study III the success of ovariectomy was confirmed by clinically examining the absence of ovarian tissue and measuring the uterine weight.

Bone assessment

On the day of measurements, the bones were slowly thawed at room temperature at least 12 h before actual mechanical testing and kept wrapped in the saline-soaked gauze except during measurements. For each rat, all measurements were performed successively in the same order.

Peripheral quantitative computed tomography (pQCT)

The cross-sections of the femoral midshafts were scanned with peripheral quantitative computed tomography (pQCT, Stratec XCT Research M, software version 5.40B, Stratec Medizintechnik GmbH, Pforzheim, Germany) (Figure 9). For the analysis, the bones were inserted into a specially constructed plastic tube with the shaft in axial direction and one cross-sectional slice from each bone was scanned at 50% of the length of the femur (Järvinen et al., 2003c). The voxel size was 0.070 x 0.070 x 0.5 mm^3 and the scan speed was 3.0 mm/sec. Total bone mineral content (tBMC), total cross-sectional area (tCSA), cortical bone mineral density (cBMD), and cortical cross-sectional area (cCSA) were recorded as given by the pQCT software using contour mode 1 (threshold 214 mg/cm^3), and separation mode 1 for cortical density (threshold 710 mg/cm^3). In our laboratory, the CV_{rms} in the femoral midshaft was 0.9 % for the tCSA, 0.6 % for the cBMD, and 1.5 % for the cCSA (Pajamäki et al., 2003).

For the pQCT assessment of the femoral neck, the bones were inserted with the femoral neck in an axial direction into a specially constructed plastic tube (Järvinen et al., 2003a). The scan line was adjusted to the midneck using the scout view option of the pQCT software. The voxel size, scan speed, and contour and separation modes were same as described above. Total cross-sectional area (tCSA), total bone mineral content (tBMC), and total bone mineral density (tBMD) were determined using the same analysis settings as described above. In our laboratory, the CV_{rms} were 3.9 % for tCSA and 2.1 % for tBMD respectively (Pajamäki et al., 2003).

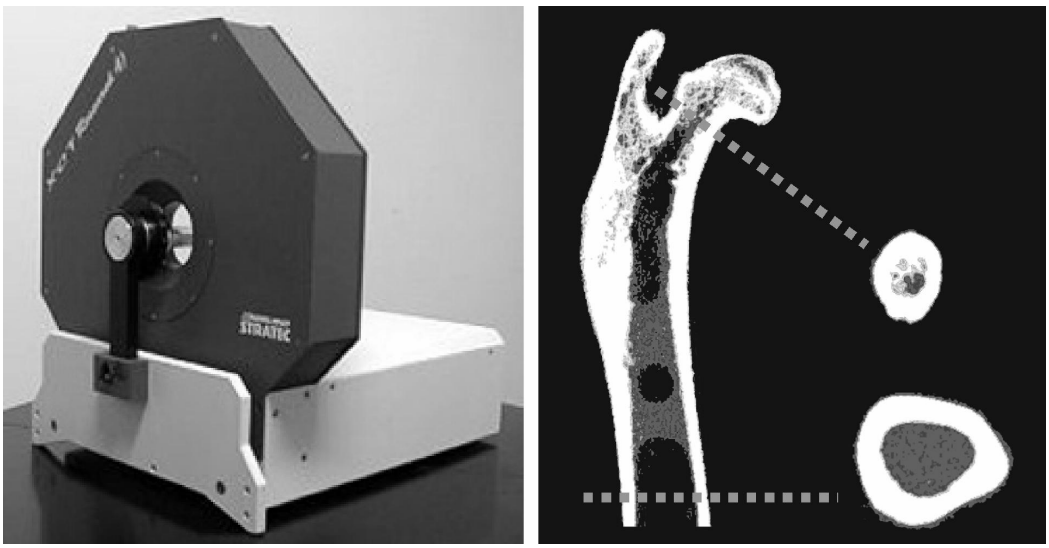


Figure 9. The pQCT device (left). A pQCT derived longitudinal slice of femur and cross-sectional slices of femoral neck and midshaft used for analysis, the zone highlighted with a broken line indicates the scanned region of interest.

Micro-computed tomography (µCT)

In Study IV, the proximal metaphysis of tibiae were scanned using a high resolution micro-computed tomography system (µCT 35; Scanco Medical, Basserdorf, Switzerland) with nominal isotropic resolution of 12 µm (Figure 10). Three-dimensional analysis of trabecular bone was performed on the bone region 1 to 5 mm distal to the growth plate. The trabecular bone compartment was separated from the cortical bone by semi-automatically drawn contours and a global threshold was used to distinguish bone and marrow. The following parameters were determined from the trabecular bone using a direct three-dimensional approach (Hildebrand et al., 1999): total bone marrow volume including the trabeculae (TV; mm^3), trabecular bone volume (BV; mm^3), trabecular bone volume fraction (BV/TV), mean trabecular number (Tb.N; 1/mm), mean trabecular thickness (Tb.Th; mm), and mean trabecular separation (Tb.Sp; mm). For determination of cortical bone porosity, a 0.5 mm thick region of cortical bone at 7 mm distance from the proximal end of tibia was analyzed.

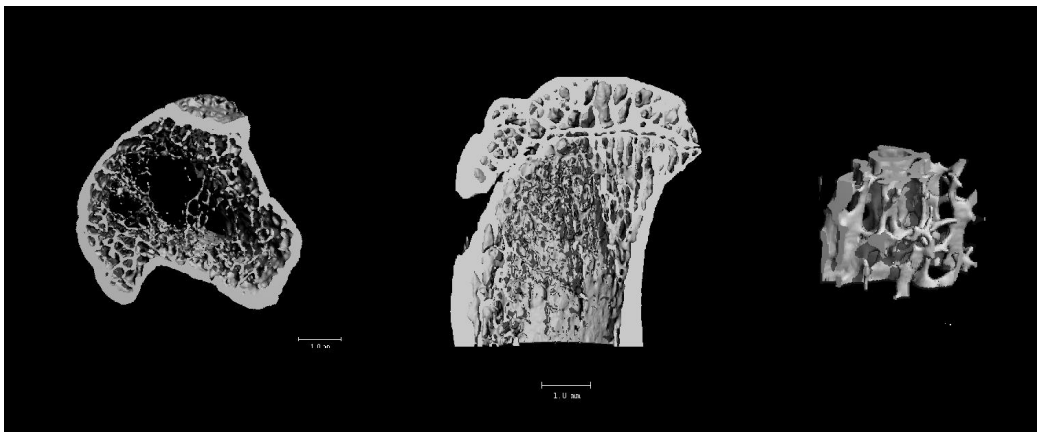


Figure 10. Micro-CT analysis of the proximal tibial metaphysis. Horizontal view (left), sagittal view (middle), and representative trabecular bone block (right).

Geometrical measurements

A digimatic caliper (Mitutoyo 500, Andover, United Kingdom) was used to measure the length of femora in all studies. The coefficient of variation (CV_{rms}) for the determination of the length of the femora is 0.2% (Järvinen et al., 1998b).

Cross-section of the femoral midshafts

For the determination of the cross-sectional diameters, the dimensions were measured using the distance tool of the pQCT software (version 5.40B, Stratec Medizintechnik GmbH, Pforzheim, Germany). In Study II, the cross-sectional moments of inertia (CSMI) were recorded as given by the pQCT software. In Study III, the morphology of the bone cross-section was evaluated using the eccentricity as a measure of how much the given cross-section deviated from being circular. The eccentricity was calculated as follows:

$$Eccentricity = \sqrt{1 - \frac{d_{min}^2}{d_{max}^2}} \qquad (1)$$

where d_{min} is the minimum diameter and d_{max} the maximum diameter of the cross-section. Of note, a zero eccentricity denotes a perfect circle.

In order to evaluate surface changes around the cross-section for both the endosteal and periosteal surfaces, a custom developed macro within the ImageJ 1.40 (http://rsb.info.nih.gov/ij/) image processing platform was used. As with the conventional pQCT analysis a threshold of 214 mg/cm^3 was used to isolate the bone. The centroid of the cross-section and the medial crest of the femoral diaphysis were used to consistently orient the specimens. Rights limbs were inverted in the medial-lateral axis to facilitate direct comparisons with the lefts. Next, maximal (periosteal) and minimal (endosteal) surface radial coordinates were detected within 5 degree steps about the cross-section. Using this approach it was possible to map out mean radial distances for the bone surfaces and make detailed qualitative comparisons between groups. These 72 sets of radial measurements were then collapsed into eight, 45 degree sectors to facilitate qualitative regional comparisons.

Biomechanical testing

Femoral midshaft

In all studies, the AP three-point bending of the femoral shafts was carried out according to the standard protocols (Järvinen et al., 1998a; Järvinen et al., 1998b) (Figure 11). The femur was placed on its posterior surface on the lower supports (stainless-steel plates with rounded edges of 4.0 mm diameter) of the bending apparatus. The supports were placed individually (first just distal to the trochanter minor and the other just proximal to the condyles of the femur). Before the actual testing, a stabilizing preload (10 N) was applied to the medial surface of the femur at a rate of 0.1 mm/s using a steel crossbar fixture (a plate with rounded edges of 10 mm diameter), the plate being oriented perpendicularly

to the long axis of the bone and at the midpoint between the lower supports. The bending load was then applied at a rate of 1.0 mm/s until the failure of the specimen. Breaking load (F_{max}) of the femoral midshaft was determined from the load-deformation curve. In our laboratory, the CV_{rms} of the F_{max} for three-point bending is 5.0% (Järvinen et al., 1998b).

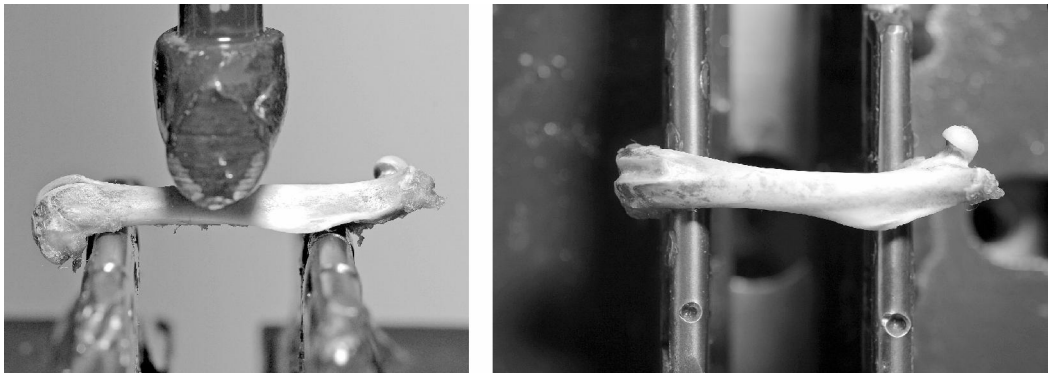


Figure 11. Three-point bending test of the femoral midshaft in conventional anteroposterior direction.

In Studies II and III, the femora were subjected to novel mediolateral (ML) three-point bending using a material testing machine (LR5K, J. J. Lloyd Instruments, Southampton, UK) (Figure 12). The femora were placed on their lateral surface on the lower supports of the bending apparatus. For each bone, these supports were placed individually so that one was under the trochanter major and the other under the distal femur. To prevent the otherwise unavoidable twisting of the bone to the anteroposterior (AP) position during loading, the intercondylar fossa of each femur was gently pressed between the blades of blunt pliers tightly attached to the bending apparatus. After anatomical adjustment of the supports, a bending load using a brass crossbar was applied to the femoral midshaft perpendicularly to the long axis of the bone until the failure of the specimen using the same loading rates as described above. The breaking load (F_{max}), stiffness, and energy absorption of the femoral midshaft were determined from the load-deformation curve.

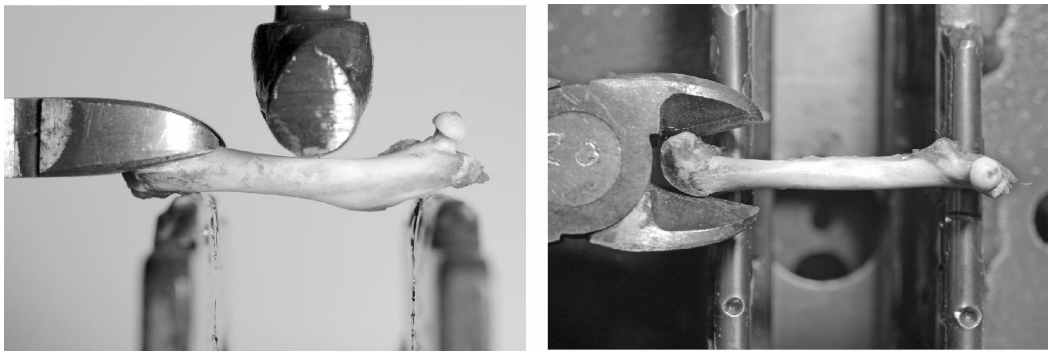


Figure 12. Three-point bending test of the femoral midshaft in novel mediolateral direction.

Femoral neck

After the three-point bending of the femoral shaft, the proximal part of each specimen was collected and the femoral neck subjected to pQCT measurement and subsequently to a compression test using the testing machine. For the compression test, the proximal half of each femur was mounted in a specially constructed fixation device (Sogaard et al., 1994) (Figure 13). The specimen was then placed under the material testing machine, and a vertical load was applied to the top of the femoral head using a brass crossbar until failure of the femoral neck. F_{max}, stiffness, and energy absorption of the femoral neck were determined from the load-deformation curve. In our laboratory, the CV_{rms} of the F_{max} for the femoral neck compression is 6.0% (Järvinen et al., 1998b).

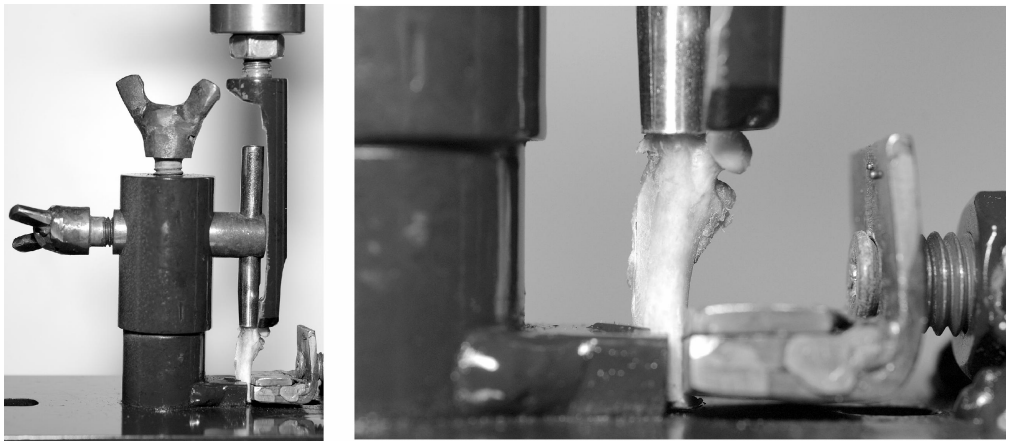


Figure 13. Compression test of the femoral neck.

Literature survey

For Study I, all 3,472 original studies published between 1999 and 2003 in four major journals focused on bone research (*Bone, Calcified Tissue International, Journal of Bone and Mineral Research, and Journal of Orthopaedic Research*) were reviewed. This sample was considered representative of the prevailing status in the field of experimental bone studies. Inclusion criteria were the following: 1) mechanical testing of whole bones was performed; 2) the bone was extracted either from rat, mouse, dog, rabbit, or monkey; and 3) the study included an intact control group. Accordingly, 123 studies were included in the analysis. The number of animals (n), mean and standard deviation (SD) in the control group were collected for breaking load, stiffness, and energy absorption, whenever available.

To obtain appropriate estimates of mean treatment effects in common interventions of experimental osteoporosis research, the focus was set on the commonly used rat model and the data was included only from studies that met the following criteria: 1) femoral shaft 3-point bending test or femoral neck compression test was performed; and 2) the intervention was either ovariectomy, increased activity (climbing, treadmill training, voluntary wheel-running), or inactivity (neurectomy, hindlimb suspension, limb taping). Altogether, data from 40 studies were included.

Statistical analysis

Precision analysis (Studies I and II)

The repeatability of the biomechanical testing of the rat femora was determined by comparing the data from the right and left femora of the non-operated rats. This approach makes an inherent assumption that the right-to-left biomechanical properties and geometry of femora are equal, which may not always be entirely true (Banse et al., 1996; Hanson and Markel, 1994). However, it can be anticipated that, under normal circumstances, there is no systematic difference between the structural and mechanical characteristics of the right and left femora and, thus, the present approach is well grounded. To verify this, for all pQCT and mechanical testing variables, the 95% limits of agreement (i.e., average right-to-left difference ± twice the standard deviation of these side-to-side differences [SD_{meas}]) were determined according to Bland and Altman (Bland and Altman, 1986). If the zero-difference resides clearly within the 95% limits of agreement, it is very unlikely that there would be any true side-to-side difference between the femora. In addition to the above described absolute measure of repeatability, two proportionate measures of precision, the average root mean square coefficient of variation (CV_{rms}) (Gluer et al., 1995) and the reliability coefficient ($R = 100[1-SD_{meas}^2 / SD_{total}^2]$ in percentage), were determined. The advantage of the reliability coefficient over the commonly used CV_{rms} is that R-value takes the total observed variance into account and it can be interpreted as an error-free proportion of the inter-subject variability (i.e., biological variance, SD_{biol}) observed in a given population.

Statistical analysis used in the literature survey (I)

Percentage variation

In Study I, the collected data were employed to determine the total percentage variation ($\overline{\sigma_T}$) in the biomechanical traits for each test and species as described

in Equations 2-4. If the standard error of mean (SEM) was provided instead of SD, the SD was calculated as follows:

$$SD = \sqrt{n} \times SEM \tag{2}$$

Percentage SD (percentage variation, σ_T) was calculated as follows:

$$\sigma_T = \frac{SD}{Mean} \times 100\% \tag{3}$$

The total percentage variation ($\overline{\sigma_T}$) for each type of intervention was calculated as follows:

$$\overline{\sigma_T} = \sqrt{\frac{\sum \sigma_T^2}{k}} \tag{4}$$

where *k* represents the number of independent studies.

Treatment effects

To estimate the pooled treatment effect size (in z-scores and %-values) meta-analytic principles described in Equations 5-13 (Eqs. 2-4 also apply as appropriate) were employed. Bias corrected effect size (ES) and corresponding mean error (SE) were calculated using Hedge's method (either fixed effect model or random effect model) (Cooper and Hedges, 1994) as follows:

$$ES = \frac{Mean_i - Mean_c}{\sqrt{\frac{SD_i^2 \times (n_i - 1) + SD_c^2 \times (n_c - 1)}{n_i + n_c - 2}}} \times \left[1 - \frac{3}{4 \times (n_i + n_c - 2) - 1}\right] \tag{5}$$

and

$$SE = \sqrt{\frac{n_i + n_c}{n_i \times n_c} + \frac{ES^2}{2 \times (n_i + n_c)}} \tag{6}$$

where $Mean_i$ and $Mean_c$ are the means of the biomechanical trait of interest in intervention and control groups respectively, SD_i and SD_c are the standard deviations of these traits, and n_i and n_c the number of rats in the intervention and control groups respectively.

In a fixed effect model, which was used as default method in the determination of pooled effect size, the weighted mean effect size ($\overline{ES}$) was calculated as:

$$\overline{ES} = \frac{\sum(w \times ES)}{\sum w} \tag{7}$$

where the weight (w) for each study was calculated as the inverse of variance:

$$w = \frac{1}{SD^2} \tag{8}$$

The standard error of the mean effect size ($se_{\overline{ES}}$) was then calculated as:

$$se_{\overline{ES}} = \sqrt{\frac{1}{\sum w}} \tag{9}$$

Homogeneity analysis tests whether the assumption that all of the effect sizes estimate the same population mean is a reasonable assumption. Homogeneity test value (Q) was calculated as:

$$Q = \sum(w \times ES^2) - \frac{\left(\sum(w \times ES)\right)^2}{\sum w} \tag{10}$$

If Q was lower than the Chi-squared critical value at k-1 degrees of freedom (k is the number of independent interventions) and p-value of 0.05, the fixed effect model was used. Otherwise, the distribution of effect sizes is assumed to be heterogeneous, and the random effect model was used as described below. In that case, the random effects variance component (v_θ) was first calculated as:

$$v_\theta = \frac{Q - k - 1}{\sum w - \left(\frac{\sum w^2}{\sum w}\right)} \tag{11}$$

where k is the number of independent interventions and w is the weight value for each study (Eq. 8). Then the new weight values (w_i) were calculated for each study as:

$$w_i = \frac{1}{SD^2 + v_\theta} \tag{12}$$

With these new weight values (Eq. 12) the pooled effect size and corresponding mean error were then calculated using Equations 7 and 9. The interpretation of the outcomes from fixed and random effect models does not differ.

Finally, the percentage treatment effect (δ) was calculated as:

$$\delta = \overline{ES} \times \overline{\sigma}_T \qquad (13)$$

Sample size estimation

The minimum sample size (minSS) needed to show a specified treatment effect (δ) in breaking load, stiffness and energy absorption with a statistical significance of p=0.05 (provided that such an effect truly existed) was calculated using an approximation of Neyman´s solution (Snedecor and Cochran, 1967) as follows:

$$\text{minSS} = (7.9)\,\overline{\sigma}_T^2 / \delta^2 \quad \text{for the statistical power of 0.80} \qquad (14)$$

and

$$\text{minSS} = (10.5)\,\overline{\sigma}_T^2 / \delta^2 \quad \text{for the statistical power of 0.90} \qquad (15)$$

Intervention Studies (II, III, and IV)

All data in Studies III and IV, and data obtained from the operated rats in study II, were analyzed using the SPSS for Windows version 13.0 statistical program. Relative exercise (i.e. the percent difference between exercised and control groups), unloading (i.e. the percent difference between neurectomized and control groups), aging effects (i.e. the percent difference between mature and senescent groups), and estrogen (i.e. the percent difference between OVX and SHAM-OVX groups) were tested using analysis of covariance (ANOVA). In the intervention studies (III and IV), all data pertaining to the mechanical competence of the femur (cCSA, tBMC, tCSA, and F_{max}) were statistically controlled for body weight and femoral length using them as covariates (Järvinen et al., 2003a; Järvinen et al., 2003c; Pajamäki et al., 2003), except when comparing intervention groups to the baseline group (in Study III). In all tests, an α level less than 5% ($p < 0.05$) was considered significant.

RESULTS

Bone biomechanical properties in experimental bone research

According to the literature survey, rat is the most common animal in experimental bone interventions employing biomechanical tests, followed by mouse, while the use of other species is rather marginal. All in all, 17 different species were used in the articles reviewed. Of different testing protocols, the femoral shaft three-point bending in the anteroposterior direction is the most common, followed by vertebral compression, femoral neck compression, and three-point bending of tibia. When comparing the total variations between different tests and species, there is an obvious trend: the total variation is smaller for the determination of breaking load than of stiffness, and particularly so in comparison to energy absorption. Also, the testing of the bones of rat, the most commonly used experimental model, showed total variation comparable to the corresponding values of other species (Figures 14-15).

Furthermore, the literature survey was used to obtain the estimates of mean treatment effects in common interventions (ovariectomy, increased activity, and inactivity) (Figure 16). Ovariectomy was shown to result in ~5% mean reduction in the breaking load and stiffness of the femoral shaft, while in the other traits, a statistically significant effect may not be expected, the borderline ~13% reduction in the femoral neck stiffness excepted. Increased activity increases both the breaking load and stiffness of the femoral shaft in the AP direction (no data on energy absorption were available) by ~10 %. It also has a positive effect on the femoral neck breaking load (~6 %) and stiffness (~36%), while the energy absorption appears to decrease correspondingly. Inactivity, in turn, seems not to decrease the mechanical competence of the femoral shaft in the AP direction, while ~30 % reduction in femoral neck breaking load may be expected (no data on other mechanical parameters were available).

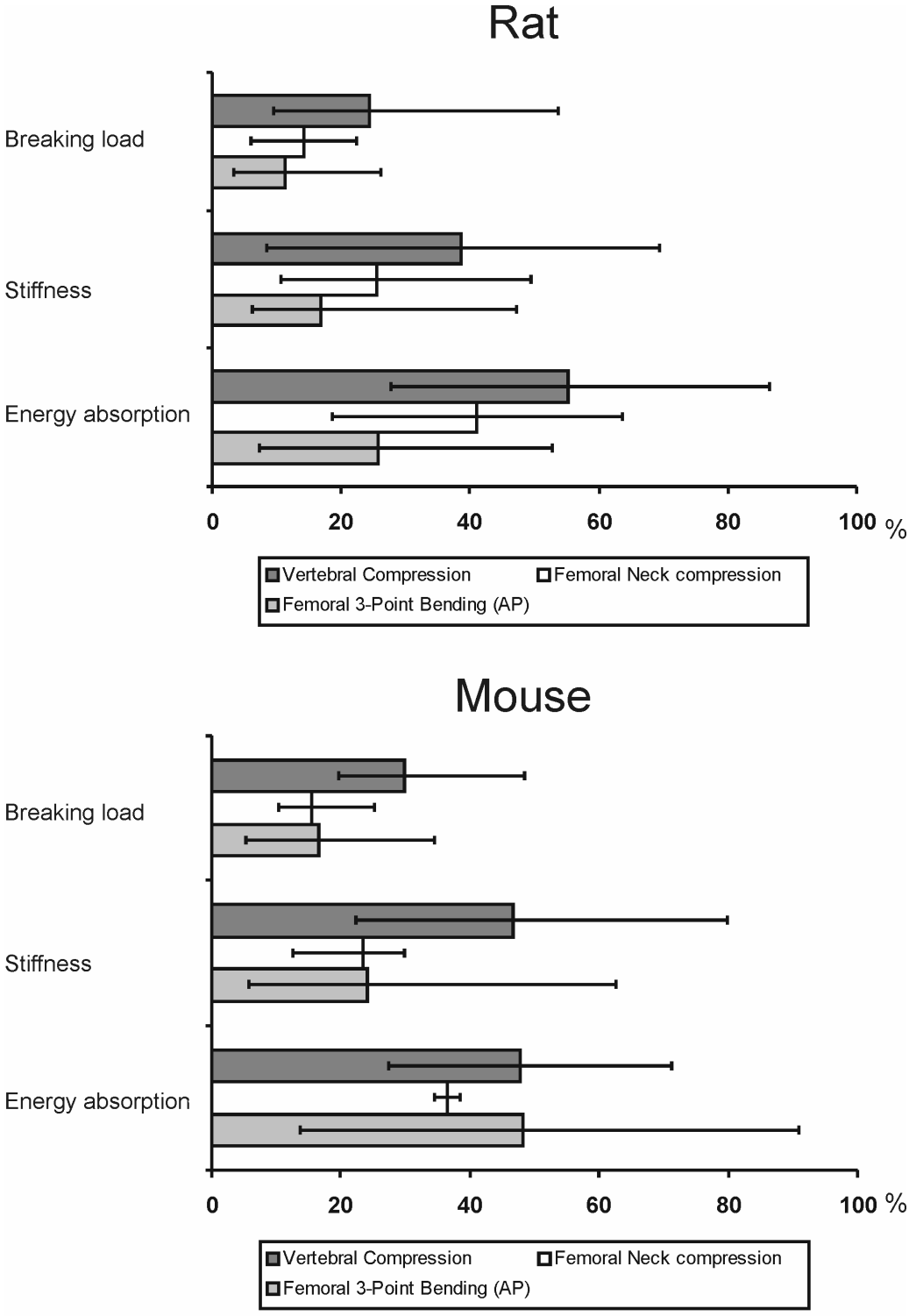


Figure 14. Average percentage total variations in different biomechanical testing settings and in different biomechanical traits in rats and mice. Error bars represent the observed range (min-max).

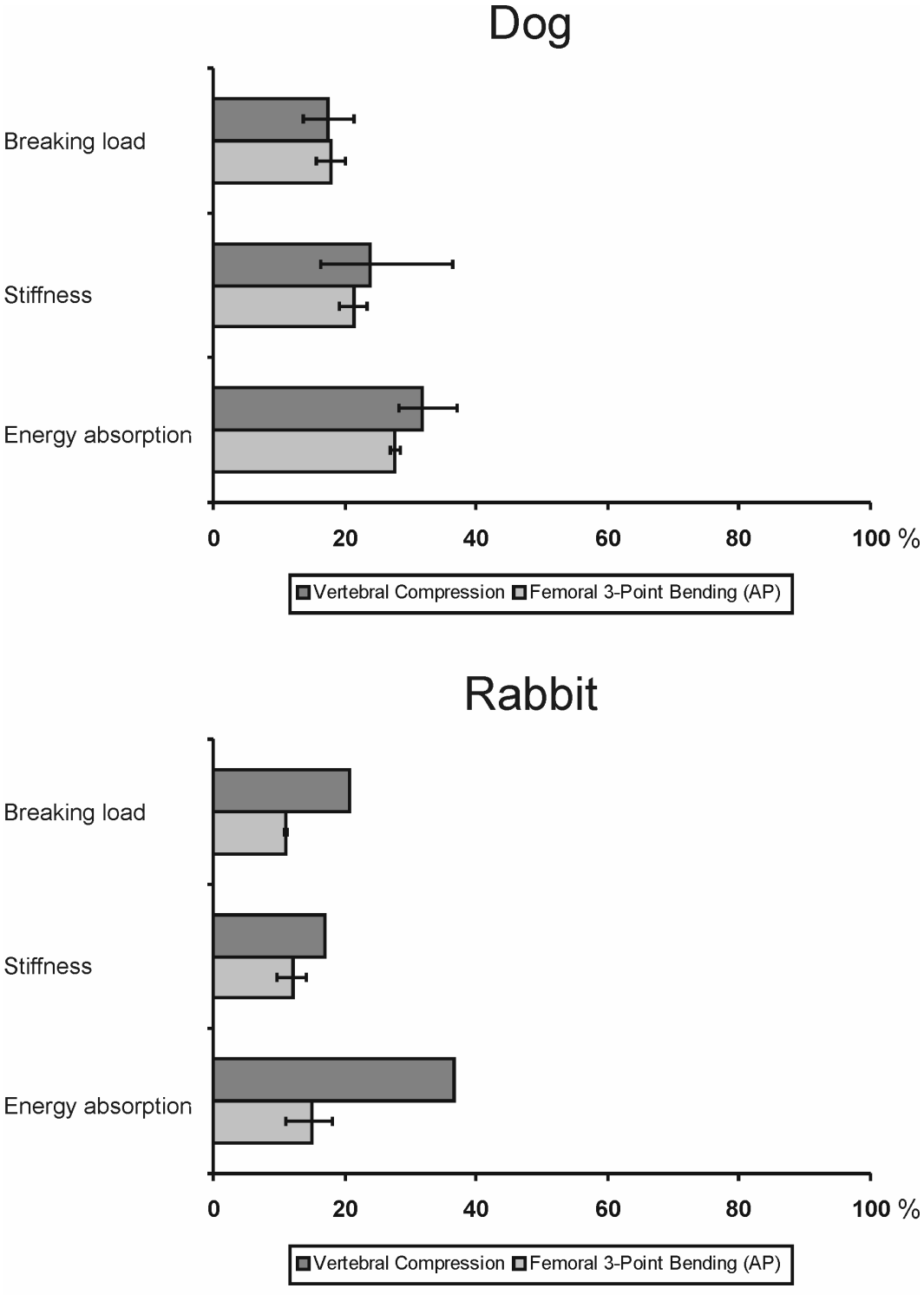


Figure 15. Average percentage total variations in different biomechanical testing settings and in different biomechanical traits in dogs and rabbits. Error bars represent the observed range (min-max).

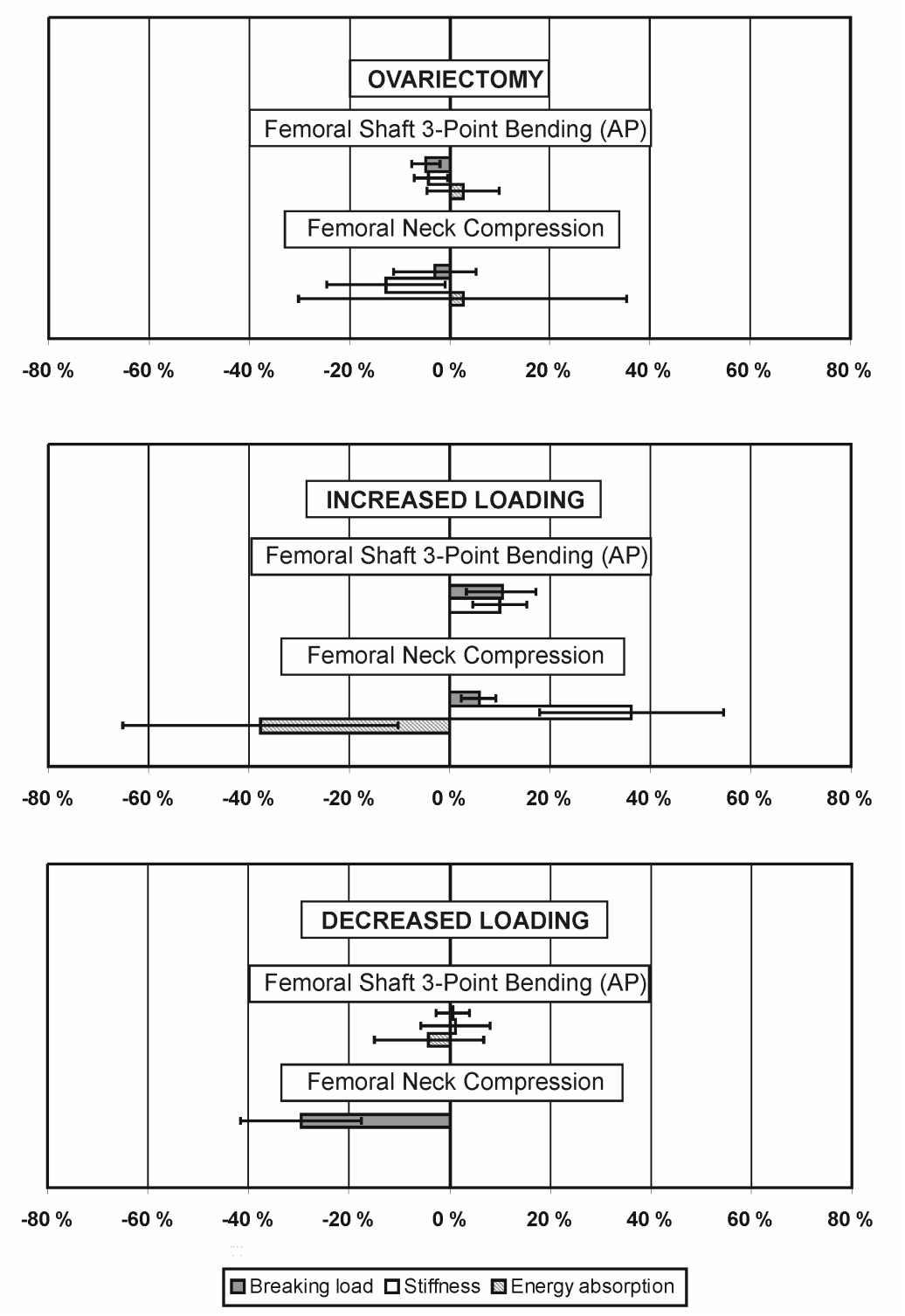


Figure 16. Pooled treatment effect sizes (in %) on breaking load, stiffness, and energy absorption of rat femur in response to ovariectomy, increased activity, or inactivity. The error-bars represent the 95% confidence intervals.

Using the data gathered, the estimation of appropriate sample sizes for the most common skeletal interventions in rats was performed (Figure 17). Due to the lower total variation, the three-point bending of the femoral midshaft in AP direction can be carried out using a significantly smaller minimum sample size (minSS, rats/group) than the femoral neck compression test. Likewise, minSS is considerably higher for stiffness and energy absorption assessment than for breaking load in both testing protocols.

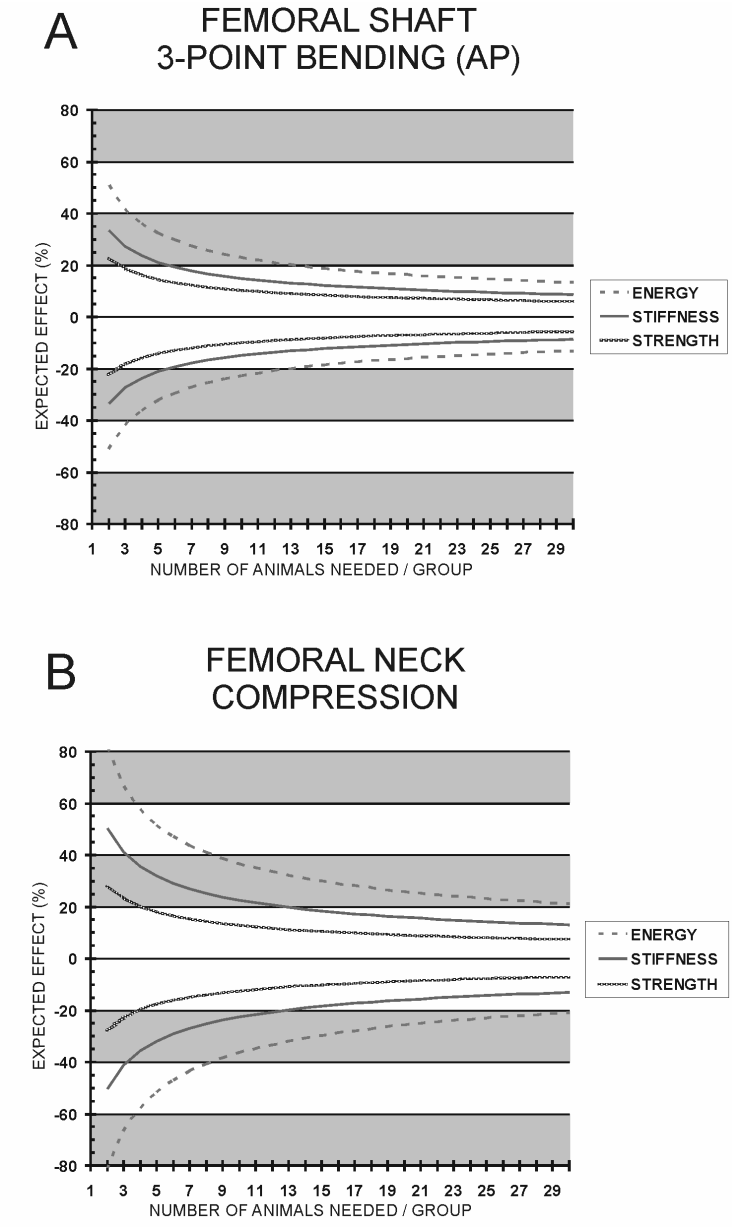


Figure 17. Relationship between the size of the minimum detectable treatment effect (percentage difference) and sample size for breaking load, stiffness, and energy absorption in (A) three-point bending in AP direction, and (B) femoral neck compression test. The statistical power was set at 0.80 and the significance level at $p = 0.05$.

In terms of precision, Studies I and II showed similar results in rat femoral shaft 3-point bending both in AP and ML direction: for breaking load, the CV_{rms} values were 5.1% and 3.8% respectively (Table 6). For the determination of stiffness, the values were 9.7% and 6.6%, and for energy absorption 15.5% and 14.5% respectively. For femoral neck, the CV_{rms} values were 7.6%, 17.9%, and 18.7% in the validation Study (II), for F_{max}, stiffness, and energy absorption respectively.

Table 6. Precision values for breaking load, stiffness, and energy absorption in femoral shaft three-point bending and femoral neck compression tests. The range in parenthesis depicts the 95% confidence interval.

Mechanical trait	**Femoral shaft 3-point bending**		**Femoral neck compression**
	AP (Study I)	ML (Study II)	Study I
	CV_{rms} (%)	CV_{rms} (%)	CV_{rms} (%)
Breaking load	5.1 (3.3-6.5)	3.8 (2.3-5.1)	7.5 (4.1-9.7)
Stiffness	9.7 (6.1-12.3)	6.6 (5.0-8.0)	17.9 (13.5-21.4)
Energy absorption	15.5 (8.7-20.1)	14.5 (10.6-19.1)	18.7 (13.2-23.0)

In the validation study of the ML 3-point bending (II), one bone rotated to the AP position before the actual failure and was, therefore, excluded from the analysis. Accordingly, 37 out of 38 pairs of femora underwent successful testing. Judging by the CV_{rms} values, the ML 3-point bending was more precise than femoral neck compression in the determination of F_{max} and stiffness ($p=0.047$ and $p<0.001$ respectively). In addition, F_{max} was superior to stiffness and, especially, energy absorption ($p<0.05$ in all settings). As readily apparent, the effect of loading was observed only in the ML plane, whereas no difference was observed between the neurectomized and contralateral intact femur when tested in the AP direction. The difference between the two orthogonal testing directions was highly significant ($p<0.01$) for all parameters (Figure 18). Thus, it can be argued that only ML testing was able to detect the habitual loading-induced structural changes in the femoral shaft.

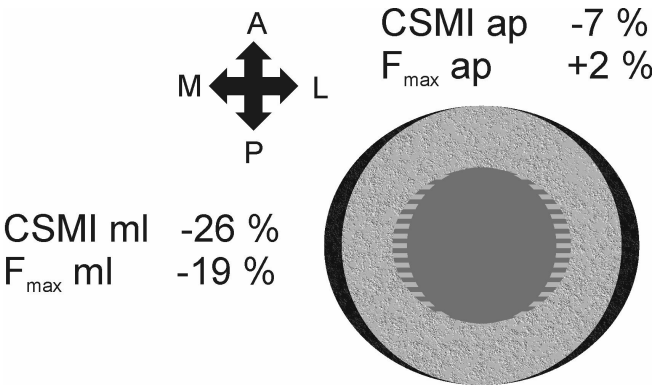


Figure 18. Unloading-induced changes in the femoral midshaft. Both CSMI and F_{max} were decreased in the mediolateral plane, whereas the anteroposterior plane was unaffected.

Skeletal ontogeny

In Study III, relating to the ontogeny of rat femur, one rat in the SHAM group died before the end of the experiment. There was a highly significant difference ($p<0.001$) in mean body weights between OVX and SHAM operated rats despite the pair-feeding. During the 27-week study period, the length of the femur deprived of loading and estrogen increased by 142% ($p<0.001$) and its total mineral content (tBMC) by 687% ($p<0.001$) (Figure 19). Statistically significant increases were also observed in all other structural bone traits. It is noteworthy that the cross-section enlarged relatively symmetrically, maintaining a more circular shape relative to baseline (Eccentricity 0.24 vs. 0.31, no statistical significance).

Judged from the relative longitudinal changes in all bone traits, it is obvious that the effect of loading remained quite modest compared to the concomitant changes due to pure genetic growth. The comparison between the locomotion and genetic growth groups showed that locomotive loading had no effect on longitudinal bone growth, but resulted in significant increases on mediolateral cortical wall thickness (5%; $p=0.010$), mediolateral outer and inner dimension (12%; $p<0.001$ and 29%; $p<0.001$ respectively), total mineral content (8%, $p<0.001$), total cross-sectional area (11%, $p<0.001$), cortical bone area (8%, $p<0.001$), ML tested breaking load (21%, $p<0.001$), and stiffness (21%, $p=0.001$) (Figure 20). It is noteworthy that bone geometry in the anteroposterior plane was not significantly associated with locomotion (Figures 21 and 22), and subsequently no effect was observed in the AP testing. Also, the eccentricity differed significantly (0.44 vs. 0.31 and 0.44 vs. 0.24, $p<0.001$) from the baseline and genetic growth groups respectively.

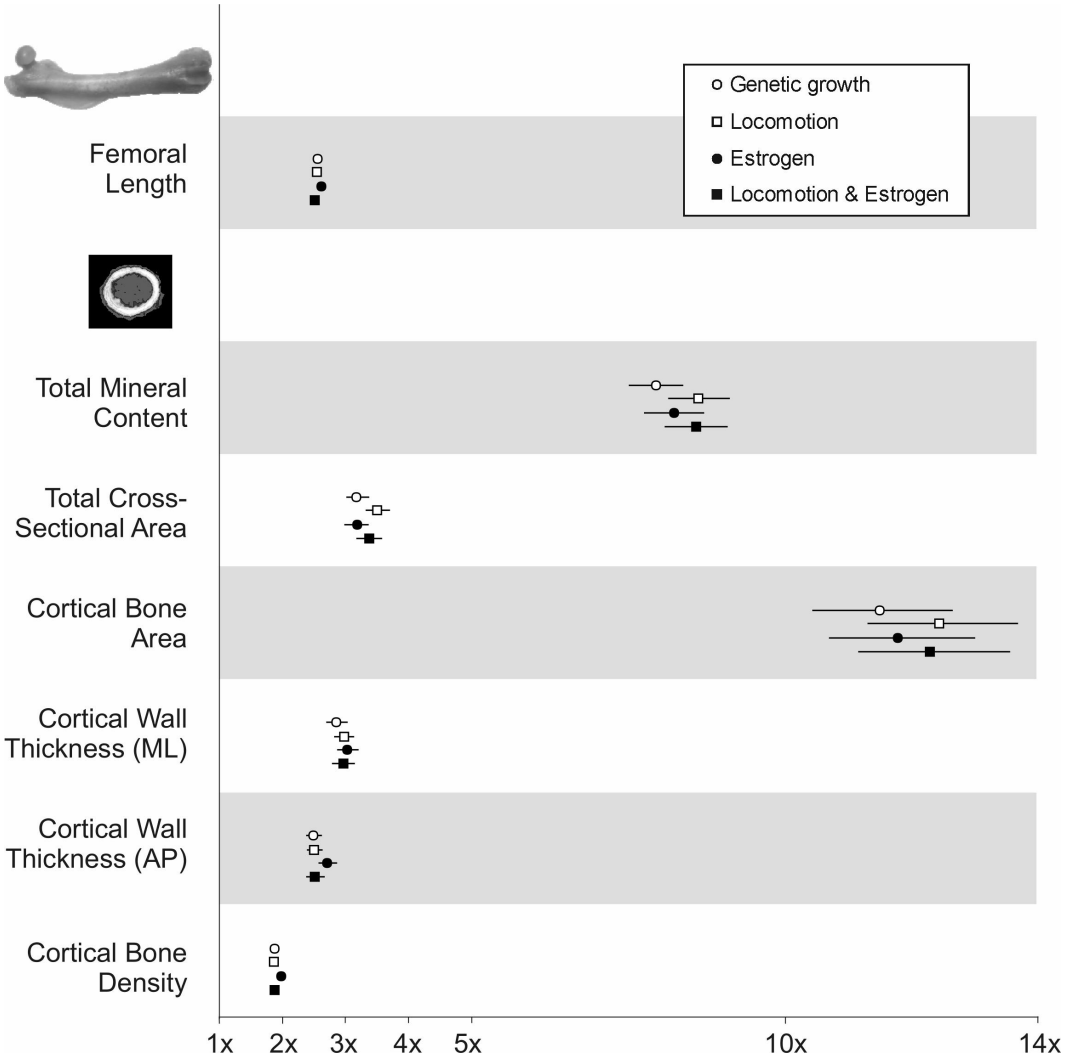


Figure 19. Percentage differences of each group from the baseline group. The whiskers represent the 95% confidence intervals.

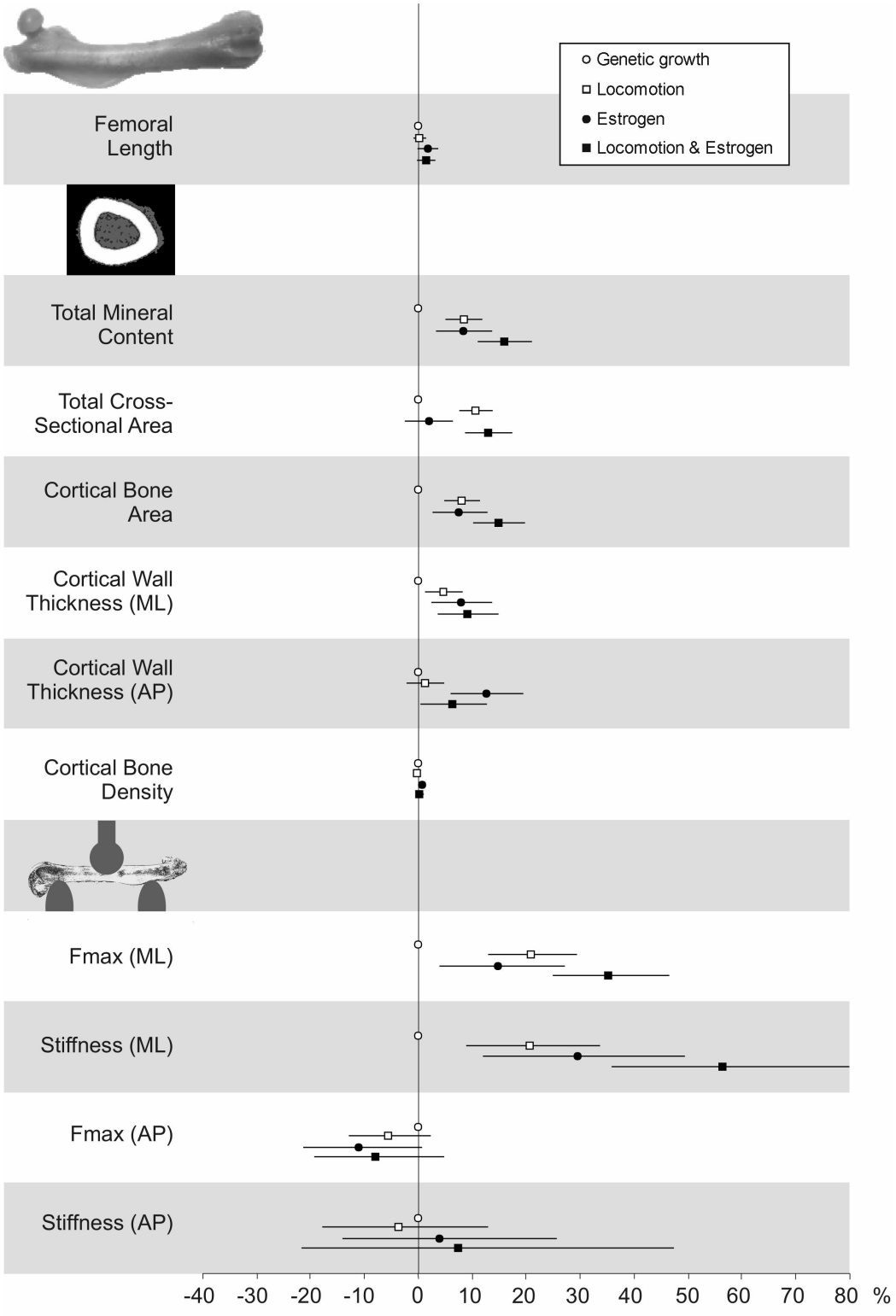


Figure 20. Percentage differences of each group from the genetic growth group (zero line). The whiskers represent the 95% confidence intervals.

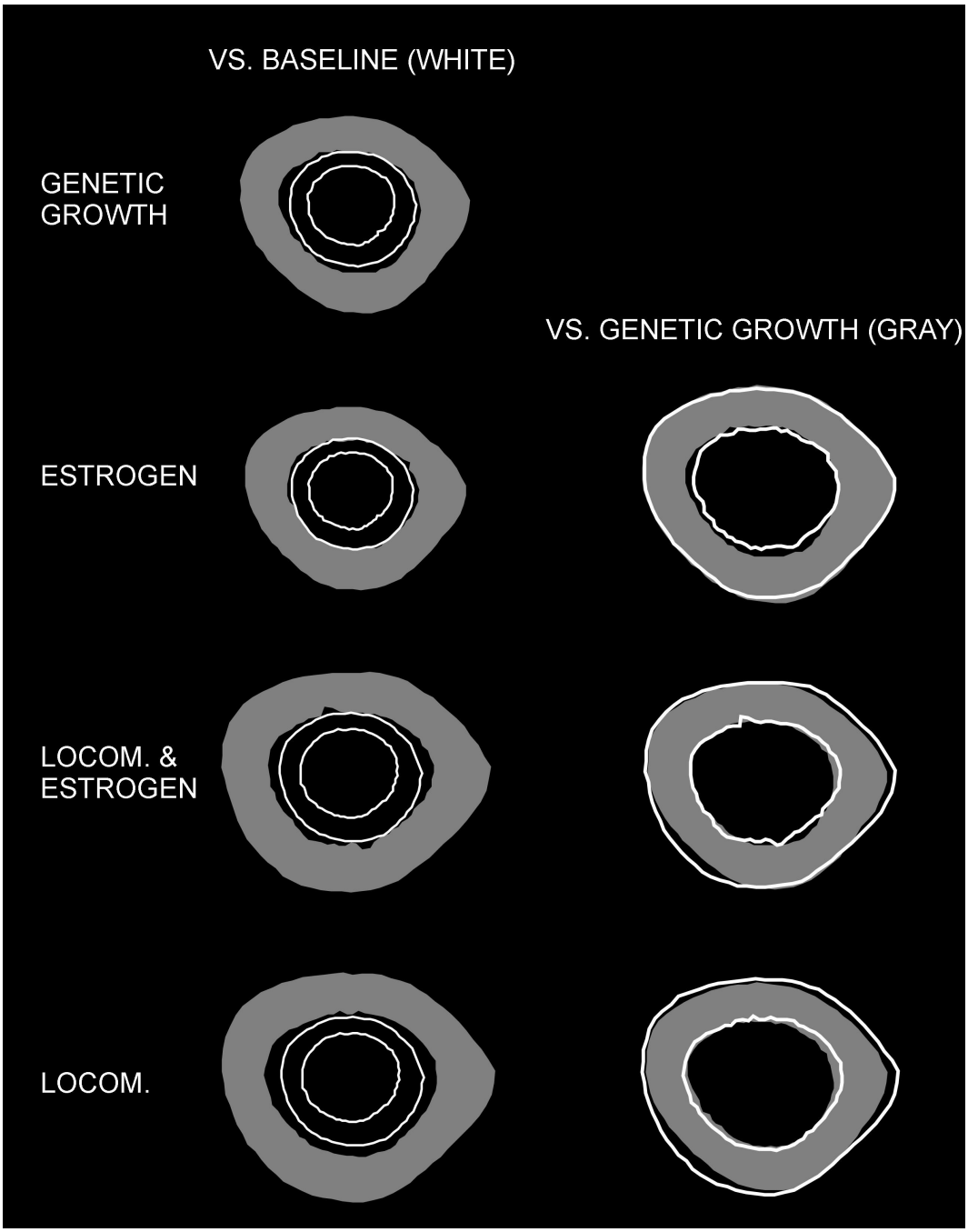


Figure 21. Effect of genetic growth, estrogen, and loading on dimensions of the femoral midshaft cross-section. On the left panel, the white lines depict the cross-sectional surfaces of the bones of the baseline group and gray the cross-sections specifically labeled (genetic growth, estrogen, locomotion&estrogen, or locomotion). On the right panel, the gray color represents the bone cross-section of genetic growth group and white lines the cross-sections specifically labeled.

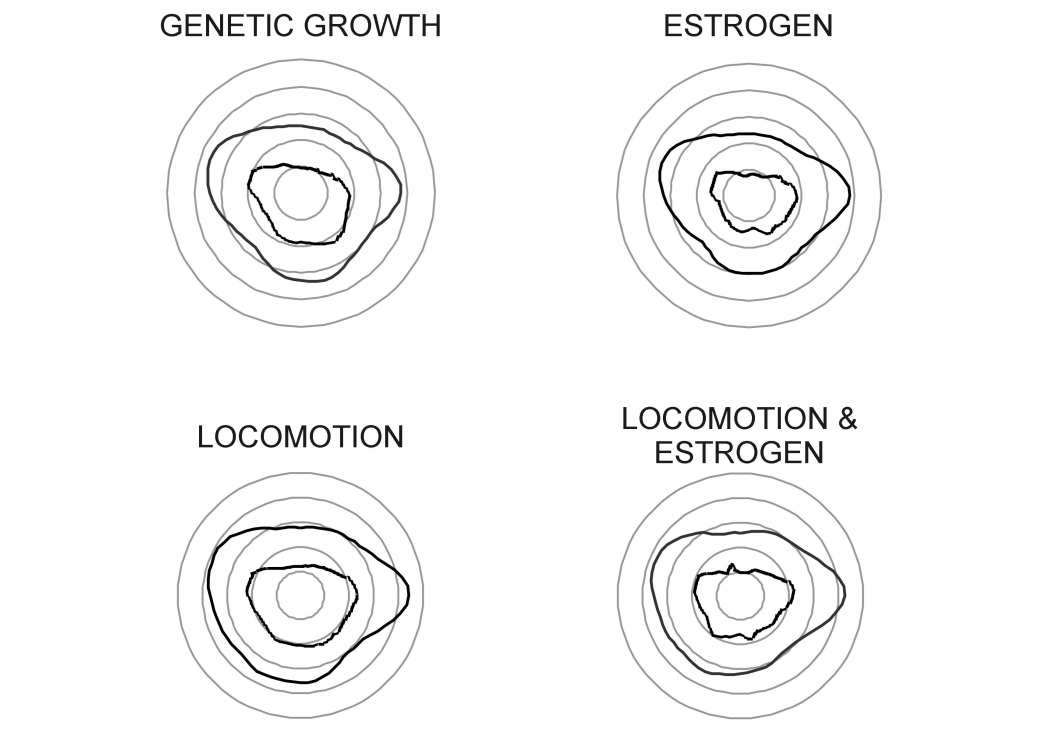


Figure 22. Relative gains and losses around the cross-section for the endosteal and periosteal surfaces when compared to the genetic growth group. Scale: 1 ring = 0.25 mm.

As was the case with locomotion, comparing the changes due to estrogen to those due to genetic growth (baseline vs. genetic growth), one can appreciate that estrogen also has quite a marginal overall effect on bone ontogeny. Between the immobilized femora of OVX and SHAM operated rats (genetic growth vs. estrogen), there were estrogen-associated positive effects in cortical wall thicknesses; 13% ($p<0.001$) in the anteroposterior and 8% ($p=0.005$) in the mediolateral direction. In addition, the anteroposterior inner dimension decreased with the presence of estrogen by 10% ($p<0.001$). Cortical bone density increased by 0.5%; $p=0.017$ and cortical bone area by 8%; $p=0.003$. Estrogen did not affect the eccentricity of the cross-section. The breaking load obtained from the ML testing increased by 15% ($p=0.010$) and stiffness by 29% ($p=0.002$) whereas the AP testing failed to show any difference between contralateral femora.

No statistically significant interaction was found between the effects of locomotion and estrogen in any of the traits measured. The values seemed to have a tendency to be an average of those of the respective individual study groups. The only exception was the breaking load, where the effect was additive, but did not show statistically significant interaction.

Aging and mechanoresponsiveness

In the exercise study (IV), mortality was 28% and 57% among mature and senescent males respectively, while the corresponding rates in females were 21% and 49% (Figure 23). Exercise affected the body weight of male rats at both ages by reducing the body mass: -8.2% ($p=0.005$) and -15.7% ($p<0.001$) in mature and senescent groups respectively. In females weight was not affected, but in mature females the femoral length was 1.7% higher in the exercise group than in the control groups ($p=0.043$).

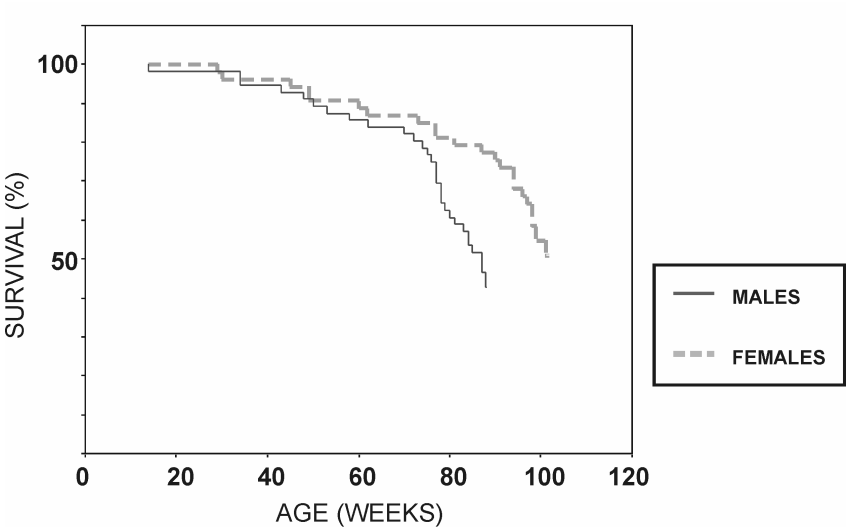


Figure 23. Kaplan-Meier survival curve for the male and female rats in Study IV.

The influence of aging had a tendency to impair the skeletal properties, and statistically significant differences between the mature and senescent control rats were observed in the $F_{max,}$ tBMC and tCSA at the femoral neck in both sexes and in the F_{max} at the femoral midshaft in females. At the femoral midshaft, the tCSA of the male rats and cBMD of the female rats were larger in the senescent rats in compared to the mature animals. In the proximal tibia, the trabecular bone volume fraction (BV/TV) was significantly decreased in the senescent rats when compared to the corresponding mature group both in males and females. In males, Tb.N., Tb.Sp., and cortical porosity also differed significantly between mature and senescent groups, a finding associated with reduced BV/TV indicating deteriorated bone structure among old rats (Figure 24).

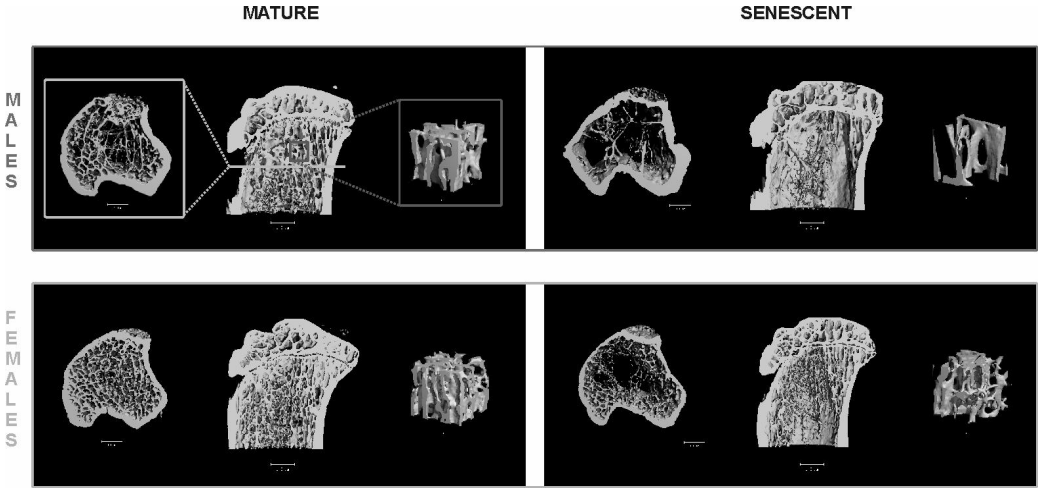


Figure 24. Effects of aging on the trabecular bone texture in the proximal tibial metaphysis. Due to aging, the proportion of trabecular bone of the bone volume (TV/BV) is decreased in males and females. In addition, in males, the number (Tb.N.) and thickness (Tb.Th.) of the trabeculae is decreased, while the distance between individual trabeculae (Tb.Sp.) is increased.

Skeletal responses to increased exercise were rare in mature rats, but numerous in senescents (Figures 25-27). At the femoral neck, statistically significant exercise-induced increases were observed in the tBMC and tCSA in the senescent (+18%, p=0.03 and +19%, p=0.003 in males and +10%, p=0.001 and +10%, p=0.026 in females respectively) animals, but not in the mature group. Total volumetric bone mineral density (tBMD) was increased by exercise in mature female rats (+6%, p=0.001) with a concomitant reduced rate of increase in tCSA (-8%, p=0.018). At the femoral midshaft, the only statistically significant exercise-induced increase was observed in the tCSA of the mature male group (+6%, p=0.018). The senescent females responded to the exercise with increased breaking load (F_{max}) in both femoral neck and femoral midshaft (+16%, p=0.045 and +19% p=0.026 respectively). In males, there was a borderline significant exercise-effect (+18%, p=0.087) on the breaking load of the neck in the senescent group.

An aging effect on bone mechanoresponsiveness (interaction between age and loading) was found at the femoral neck. The exercise effect was evident in the senescent group, being statistically significant for tBMC (p=0.035 and 0.002) and tCSA (p=0.027 and 0.001), in males and females respectively. As a concurrent decrease in tBMD was also observed (p=0.039 and 0.022, in males and females respectively), it seems that the exercise-induced increase was more apparent in the bone size than the corresponding effect on tBMC. Concerning the F_{max}, the average exercise effects were clearly greater in the senescent group, but the group difference was statistically significant only at the femoral midshaft in females (p=0.032).

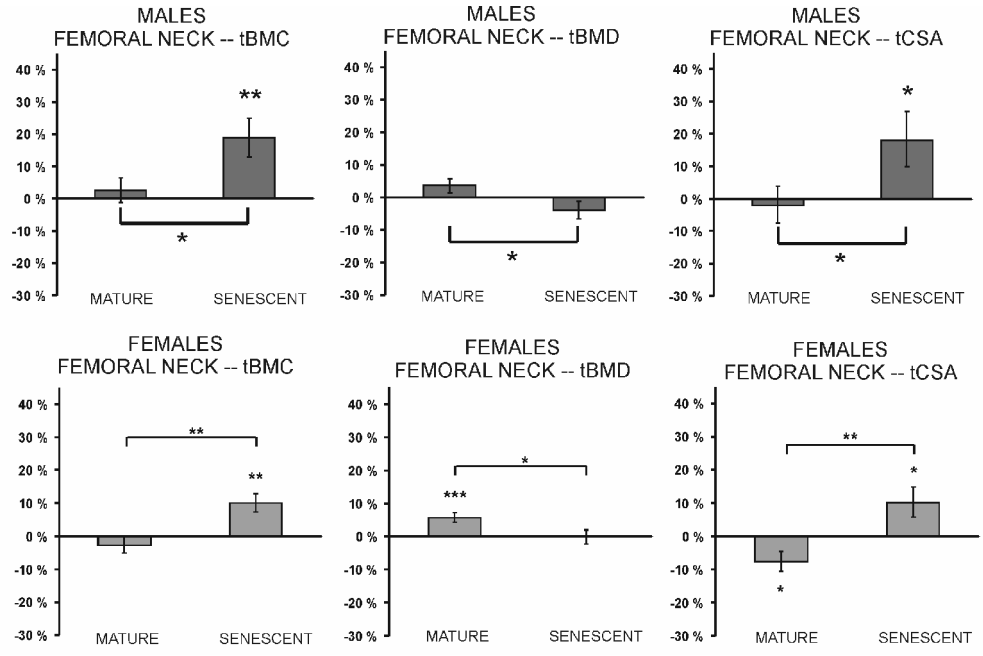


Figure 25. Exercise effect on different bone traits of the femoral neck in mature and senescent rats. Bars represent percent (%) increases (± SEM) of the exercise group compared to the corresponding control group at the end of the treadmill exercise intervention. Significant differences between the exercised rats and their controls, and between the two age groups in the exercise-effect, are indicated: *p < 0.05; **p < 0.01; ***p < 0.001. Results for tBMC and tCSA are adjusted for body weight and femoral length.

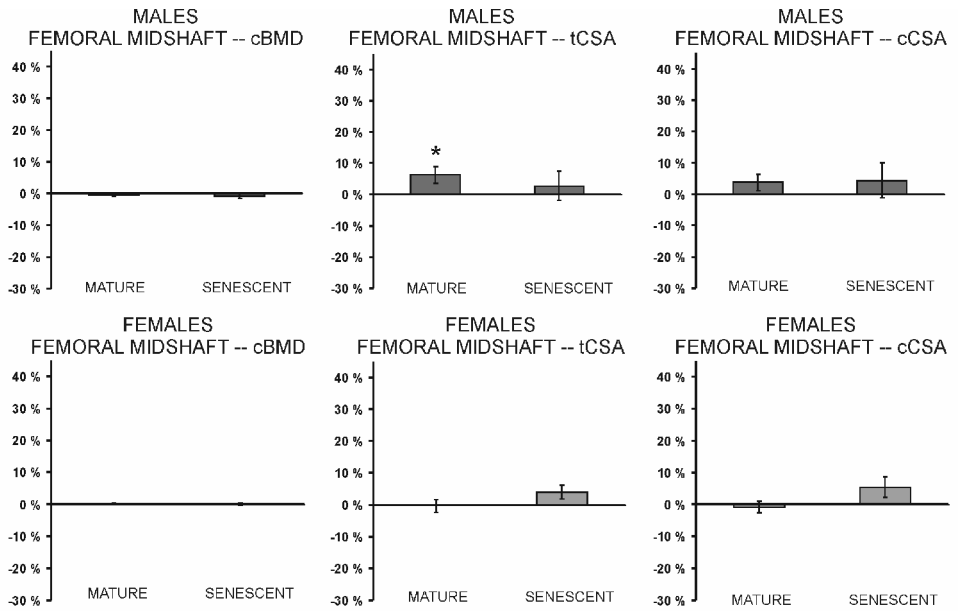


Figure 26. Exercise effect on different bone traits of the femoral midshaft in mature and senescent rats. Bars represent percent (%) increases (± SEM) of the loading group compared to the corresponding control group at the end of the treadmill exercise intervention in the femoral midshaft. Significant differences between the exercised rats and their controls are indicated: *p < 0.05. Results for tCSA and cCSA are adjusted for body weight and femoral length.

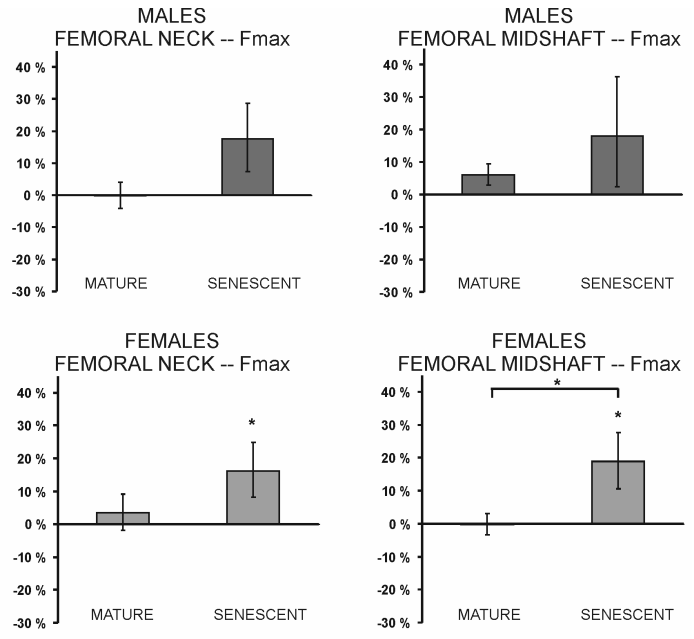


Figure 27. Exercise effect on bone strength (breaking load) of the femoral neck (compression) and midshaft (3-point bending in AP direction) in mature and senescent rats. Bars represent percent (%) increases (± the standard errors of the means, SEM) of the loading group compared to the corresponding control group at the end of the treadmill exercise intervention. Significant differences between the exercised rats and their controls, and between the two age groups in the exercise effect, are indicated: *$p < 0.05$. Results are adjusted for body weight and femoral length.

DISCUSSION

The challenge in all scientific research (and in life in general) is to do the right things, to do these things right, and eventually, to do the right things right. For obvious scientific and ethical reasons, the sample size of any experiment needs to be planned carefully before starting the study (Eisman, 2006). Ideally the number of animals per group in an experimental osteoporosis study should be large enough to provide an adequate statistical power to show the expected treatment effect (or group-difference). This is of great importance as underpowered studies can seldom address any research question meaningfully, but only lead to inconclusive results, speculation and confusion (Ioannidis, 2005). Besides the sensitivity and specificity of the measurement method, the quality of the data depends largely on its accuracy and the precision of the testing method. Precision is consistently required in clinical osteoporosis studies employing bone densitometry, whereas this information is virtually ignored in the biomechanical tests. This is most likely due to the notion that precision analysis is not feasible given the destructive nature of the method. However, a reasonable precision assessment of biomechanical tests can be obtained by characterizing the within-pair variation in the biomechanical and structural parameters of bone. The repeatability affects directly the statistical power of the study; i.e., the probability that the given study will show the treatment effect with statistical confidence if the effect truly exists.

Inspired by the importance of statistical power, all 3,472 original studies published between 1999 and 2003 in four major osteoporosis journals were reviewed. Alarmingly, the sample size calculation was reported in only 2 (1 %) studies (Kaastad et al., 2001; Kurth et al., 2001) out of the 210 studies in which mechanical testing of whole bones was conducted. Furthermore, the survey aimed to identify possible differences between precisions of different biomechanical bone traits. Methodological uncertainties and subsequently poor precision of the stiffness and energy absorption assessments seriously challenge their utility. This is especially true given the observed small effect sizes and large total variations in these traits in response to different interventions. Clearly a substantial proportion (20-50%) of the total variation in measured biomechanical traits may be due to poor precision, and occasionally methodological variation may equal the total variation (II). This leaves one with the illusion that the total variation is attributable solely to the measurement imprecision and there is no room for biological variation at all. Understandably, such a situation is not possible in a biological system. The finding highlights crucial methodological limitations in present biomechanical testing methods, particularly regarding the assessment of stiffness and energy absorption.

Although the stiffness of the whole bone (i.e., its structural rigidity), and energy absorption are very relevant descriptors of bone mechanical competence, it is extremely doubtful whether these traits can essentially enhance the information from that obtained by determining breaking load, because of both their poor precision and correlation with breaking load.

The literature survey is the backbone of this thesis, and it has been used to justify several choices in the subsequent experiments. Although a meta-analytic approach was employed in the analysis of the data surveyed, the study is not a meta-analysis in the most stringent sense. Namely, the inclusion of the studies in the analysis was not systematically executed using Medline and other relevant searches, but rather by reviewing only selected pre-selected journals and volumes. Despite this apparent limitation, the literature survey can be considered extensive and representative. One can also question the validity of the conclusions on predictable magnitudes of effects for different interventions based on the estimates obtained from interventions carried out using rats as experimental animals. It is true that the generalization of these estimates to species other than rat can be questioned. However, the justification relies on the fact that the relative biological variations in biomechanical bone traits were similar between different species. In addition, most of the species are used so seldom in experimental bone research that more reliable information on the average effects cannot be obtained from the existing literature. Related to the same concern, it must be stressed that the most usual interventions chosen for further analysis (ovariectomy and increased and decreased loading) were conducted in quite different study settings. Apparently the duration, intensity, or any other specific feature of the intervention influences the magnitude of the observed treatment effect. In further analyzing the results, it was evident that, for example, the duration of ovariectomy and the age at the time of surgery were either independently correlated with the magnitude of the effect or interacted with each other (Figure 28). Using the random effect model the confounding effect was reduced, but the results must be interpreted with great care.

According to the literature survey (I), the most commonly used method for the characterization of long bone biomechanical properties of the appendicular skeleton is the three-point bending testing of the femoral midshaft in the anteroposterior direction. The arguments favoring the use of this test include the suitable size of the bone, good accessibility during dissection, well-documented and validated testing protocol, and extensive literature for comparative purposes. For obvious anatomical and practical reasons, the femur is usually placed on the testing apparatus on its flat posterior surface and then tested in the AP direction. However, in line with the established functional bone adaptation to loading, bones adapt their structural rigidity and strength to incident loading through changes in the structural particulars (mineral mass, geometry, architecture, material properties). According to this principle, one can assume that the mediolateral direction, given the widest diameter of the elliptic cross-section at the rat midfemur, represents the apparent primary direction of skeletal adaptation to locomotive loading (Ruff et al., 2006). Thus, the conventional AP testing direction of the rat femora can be claimed not to be contextually optimal, but the

testing should rather be carried out in the ML direction. Otherwise, there remains a risk that some essential information regarding the effects of any intervention, particularly that of altered mechanical loading, on the femur structure and mechanical competence might be missed. Of note, in human femoral midshaft, the widest diameter does not run along the mediolateral. In fact, the diameter is greater in anteroposterior direction, although that is neither the greatest diameter (Högler et al., 2008). The four-legged stance, different muscle insertions, and biomechanically distinct way to run and walk are the obvious reasons for the difference between spesies.

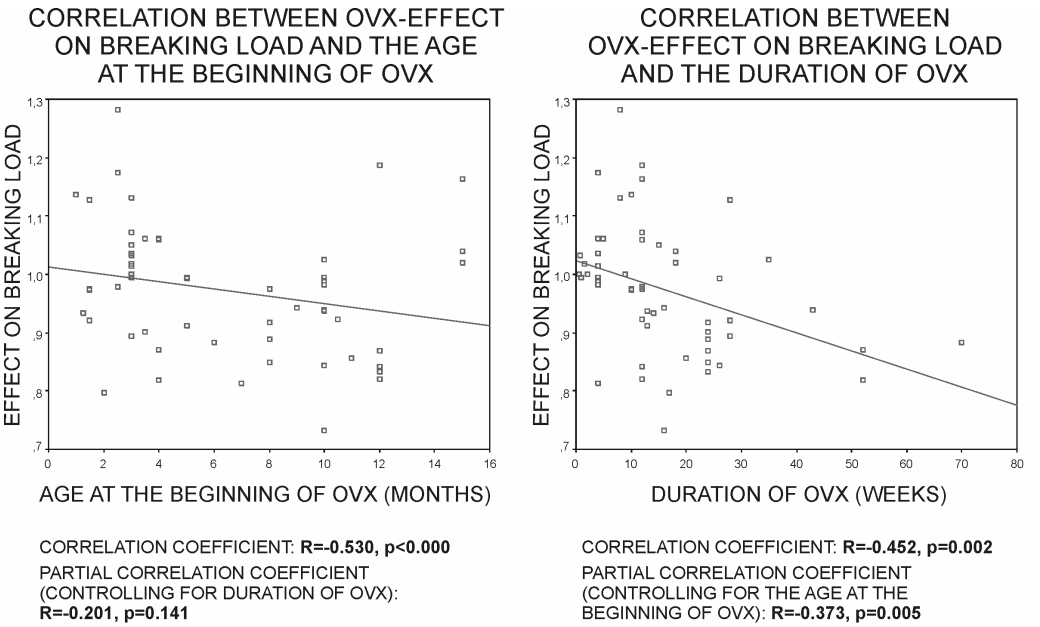


Figure 28. Relationship between age at the beginning of the ovariectomy intervention, duration of the ovariectomy, and magnitude of ovariectomy-induced effect. The statistically significant correlations demonstrate the consequence of the differences between individual study settings.

Although biomechanical testing of bones, like any other *in vitro* measurement, obviously represents a simplification of the actual *in vivo* situation, the intention is to test the skeletal structure of interest as closely as possible in terms of predominant loading environment. To assess the biological validity of the new ML three-point bending of rat midfemur, a comparison was carried out with the conventional AP testing by studying the adaptive responses of the midfemur to altered loading in orthogonal AP and ML directions. According to this biological validation, the ability of the new ML testing was superior in comparison to the conventional AP testing. The second goal of this study was to compare the repeatability of the novel testing protocol to that of the femoral neck compression test, another commonly used test in experimental osteoporosis research. The novel testing method showed better precision than the testing of femoral neck. The CV_{rms} values obtained for the mechanical testing in the ML direction and the femoral neck compression are fully comparable to those reported previously (Table 7).

Table 7. Previously reported precision (CV_{rms}) values in the literature.

Rat	CV_{rms} (%)	Reference
Femur		
Shaft		
3-Point Bending (AP)		
F_{max}	5.0	(Järvinen et al., 1998b)
F_{max}	5.1	Study I
Stiffness	9.7	Study I
Energy absorption	15.5	Study I
3-Point Bending (ML)		
Fmax	3.8	Study II
Stiffness	6.6	Study II
Energy absorption	14.5	Study II
Neck		
Compression		
F_{max}	14.7	(Järvinen et al., 1998b)
F_{max}	7.5	Study I
F_{max}	4.5	(Ammann et al., 2000)
F_{max}	8.2	(Peng et al., 1994)
Stiffness	17.9	Study I
Energy absorption	18.7	Study I
Tibia		
Shaft		
3-Point Bending		
F_{max}	3.3	(Ammann et al., 2000)
F_{max}	5.1	(Peng et al., 1994)
Proximal		
Compression		
F_{max}	5.8	(Ammann et al., 2000)
Lumbar vertebra		
Compression		
F_{max}	4.8	(Ammann et al., 2000)
Mouse		
Femur		
Shaft		
3-Point Bending (AP)		
F_{max}	10.1	(Jämsä et al., 1998)
Stiffness	15.2	(Jämsä et al., 1998)
Ultimate stress	11.1	(Jämsä et al., 1998)
Tibia		
Shaft		
3-Point Bending		
F_{max}	7.3	(Jämsä et al., 1998)
Stiffness	15.0	(Jämsä et al., 1998)

Certain additional concerns regarding biomechanical testing require further consideration. Because of the destructive nature of the biomechanical testing of bones, the precision of the measurement cannot be directly executed in the most stringent sense. Thus, an assumption that there is no systematic difference between contralateral femora had to be made, which may not always totally accurate (Banse et al., 1996; Hanson and Markel, 1994). However, the results of our pQCT analysis showed that no systematic left-to-right difference existed in the study group. Regarding nomenclature, femoral neck compression is a very commonly used biomechanical test. In fact the term, "compression", is somewhat misleading in this context, because the test is more of a cantilever bending test than compression test. However, according to the literature survey (I), several different methods have been used to assess the femoral neck biomechanical properties and all of these protocols have been erroneously called "compression" tests. Indeed, this inappropriate term has become quite established in the literature. Therefore, for the sake of consistency the term "compression test", was also used in this thesis.

Another biomechanical testing related issue is the loading rate. Considering the considerable effect the strain rate has on the mechanical properties of bones, this issue is naturally of great significance. When testing rat bones in experimental studies, the loading rates used vary quite substantially, ranging anywhere from 0.2mm/min (Ederveen et al., 2001) to 1mm/s (Ma et al., 2002). Bones primarily adapt to dynamic loads during movement and the physiological loading-induced strain rates are usually quite high, varying somewhere between 0.01/s and 0.08/s (Turner and Burr, 1993). In terms of the loading rate used in the present studies and typical rat bones dimensions, the estimated strain rate is fairly close to 0.01/s (roughly from 0.01 to 0.03). Thus, the loading rate used in this thesis (1mm/s) could have been even faster without violating the normal situation. Finally, one may wonder as to why three-point bending is used in all studies, although four-point bending is considered to serve a pure bending to a long bone (Turner and Burr, 1993). This decision is mostly practical; the femora of rats are approximately 35mm in length and the distance between supports on average is 22mm. The dimensions are so small that execution of four-point bending might have been impossible. Also, the irregular shape of the bone does not allow the use of four-point bending testing (Turner and Burr, 1993). Unfortunately, no data could be found to compare the precision of the respective testing settings, while the literature survey yielded only one study (Wezeman et al., 2000) in which rat femora were tested using four-point bending. This all said, it is obvious that more development work should be done to promote the use of four-point bending.

Related to Study II, one might wonder if the mediolateral test should be used only in studies related to locomotive loading or whether it should be used universally in all experimental interventions. In Study III, it was shown that ML testing found significant estrogen-induced effect on femoral midshaft that was not seen in AP testing. Also, when comparing the effects reported in the literature survey and the experimental studies of this thesis, there are several interesting findings favoring ML testing. First of all, according to the survey, no

effect in biomechanical traits can be expected from an immobilization intervention in femoral midshaft (in the AP direction). However, in Study III, it was shown that 27 weeks of neurectomy resulted in ~-20% effect in F_{max} and stiffness in the ML direction. Interestingly, the effect of ovariectomy was also found to be greater in Study III when determined in ML direction than in the studies reviewed for the literature survey when the biomechanical properties were determined in the AP direction. However, in light of this thesis alone, it cannot be argued that the method would necessarily be valuable for other interventions than locomotive loading. Furthermore, it has to be noted that although the bone cross-section widened in any direction, the cross-sectional moment of inertia along the opposite axis would increase. This is due to the fact that the new bone cannot be laid down purely one-dimensionally. Unfortunately, due to the chronological order of these studies, the new ML method was not used in Study IV.

The direction specificity in the adaptive response of femoral midshaft to loading was shown when comparing between the loaded and non-loaded bones in Study III. In essence, the data showed that the change in the cross-sectional shape of the bone occurred mainly in the mediolateral axis of the midshaft, while there was nothing particularly remarkable in the cross-sectional dimensions of the bones in the AP axis. Being an adaptive response to loading, this can be interpreted as indication that the ML axis (the axis of largest diameter) represents the primary adaptive of femoral midshaft to prevalent loading. Related to the same phenomenon, it was shown that at birth, the cross-sectional shape of the femoral midshaft was quite symmetric. Quite fascinatingly, the rats subjected to neurectomy showed enlarged, but also essentially round-shaped bones, suggesting that bones deprived of the physiological loading growth remain symmetric in cross-sectional shape. This finding of round cross-sectional geometry of bones without loading stimulus is actually in agreement with the findings in children (Sumner and Andriacchi, 1996).

Although it is increasingly accepted that bones are primarily locomotive organs, biological factors alone (deprived of locomotive loading) are able to, at least partly, drive skeletal ontogeny appropriately (Carter et al., 1996; van der Meulen and Carter, 1995). The paramount role of genetic growth was most prominently seen in longitudinal growth, where the length of the femur increased over 170% even without loading, and in fact, adding loading had no effect on the longitudinal growth of the skeleton; a finding in agreement with earlier studies showing non-existent differences in the length of immobilized bones (Järvinen et al., 2001; Lanyon, 1980; Tuukkanen et al., 1991; Yonezu et al., 2004) but also at least one study reporting growth retardation due to immobilization has been published (Iwamoto et al., 2005). Genetic growth was shown to have a similarly major role in the development of bone mineral content and density, although locomotion and/or estrogen had a small modulatory effect on these bone traits. Although the influence of the two other main regulators of female postnatal ontogeny – locomotive loading and estrogen – were shown to be relatively modest in comparison to the corresponding effects of genetic growth, the changes induced clearly demonstrated how they meticulously sculpt the skeleton

with respect to their evolutionary primary functions (locomotion and reproduction).

The mechanical competence of the skeleton and the related bone structure is not crucial to the function of hormones (e.g. estrogen), which are almost exclusively concerned with maintaining the calcium homeostasis (through nutrition or bone-embedded mineral reservoir, if needed) and coping with physiological needs whenever they emerge (Sievänen, 2005). Thus, it was hypothesized that the alleged modulatory effect of the hormone on the periosteal apposition would be secondary to its effects on bone mineral accrual and subsequent increase in bone rigidity, rather than originating from the direct effect of estrogen *per se*, as commonly believed today (Lanyon and Skerry, 2001; Lee et al., 2003). According to the principles of bone functional adaptation, any change either in the loading subjected on the bone or its strength or structural rigidity necessitates an adaptive response to restore the delicately controlled strain-rigidity equilibrium. Therefore, an estrogen-induced addition of mineral onto the endocortical surface at puberty (Järvinen et al., 2003a; Lauretani et al., 2008) should inherently increase the rigidity of the female bones, and consequently, inhibit periosteal apposition. Using the classic 2 x 2 study design, it was shown (III) that the changes occurring in the morphology of female bones at puberty (restriction of periosteal apposition and endocortical resorption) (Bass et al., 1999; Kim et al., 2003; Libanati et al., 1999) are attributable to an adaptive response of the bones to changes in their strain-rigidity equilibrium, rather than a direct effect of estrogen on the periosteum. The results neither support previous proposals that estrogen *per se* restricts the periosteal apposition (Kim et al., 2003; Turner et al., 1992; Turner et al., 1989; Turner et al., 1987a; Turner et al., 1987b), as no differences were seen in the periosteal diameters between estrogen-deplete and replete bones deprived of loading. This experiment corroborates the previous finding (Pajamäki, 2007).

An interesting observation that could be derived from the data of Study III was that although estrogen and locomotion were similarly anabolic for bone mineral content (+8%), loading had a greater effect on breaking load (+21% vs. +15%, respectively, $p=0.003$) (Figure 29). A possible explanation for this phenomenon is naturally that loading, according to its primary locomotive function, deposits bone in a more mechanically optimal way than estrogen.

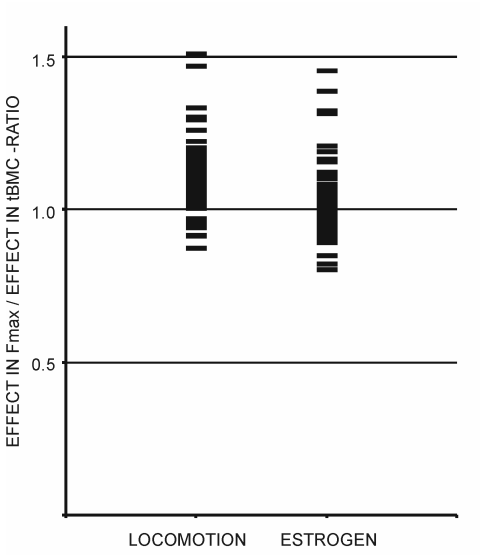


Figure 29. Locomotion has a tendency to be relatively more effective to F_{max} than tBMC when compared to the function of estrogen (p=0.003).

Although Study III was a randomized controlled study, there are certain limitations to be appraised. First of all, in this study, the rats in the genetic growth group cannot be considered as a control group that was free of any extrinsic or intrinsic regulatory factors. Even though the levels of estrogen and physical loading were dramatically decreased, neither of these operations was able totally to remove the effect of the potential regulator. Also, it needs to be conceded that in the present study, the possible effect of bone curvature on the length of the femur could not be assessed. It has indeed been shown that long bones are straighter as a result of disuse (Biewener and Bertram, 1994; Lanyon, 1980; Lanyon and Baggott, 1976). Because no objective measure of straightness was devised, it is possible that unloaded bones were less curved and thus the non-existent difference in bone length may not be entirely true, as more curved bone might have been longer but the difference was not evident in simple axial measurement. However, it should also be noted that there is no major curvature in the rat femur, and thus the potential bias is more relevant in bones with more pronounced curvature (e.g., the tibia). Furthermore, earlier studies have shown that estrogen plays a pivotal role in determining longitudinal bone growth in both genders (Carani et al., 1997; Morishima et al., 1995; Smith et al., 1994; Wronski et al., 1988; Wronski et al., 1989). Ovariectomy has been shown to result in a transient increase in longitudinal bone growth (Wronski and Yen, 1991) which is inhibited by estrogen treatment. These findings suggest estrogen as the principal ovarian hormone active in growth plate cartilage, even though the mechanism of the action of estrogen on growth plate cartilage is poorly understood. In this essence, the present findings are contradictory to the prevailing understanding.

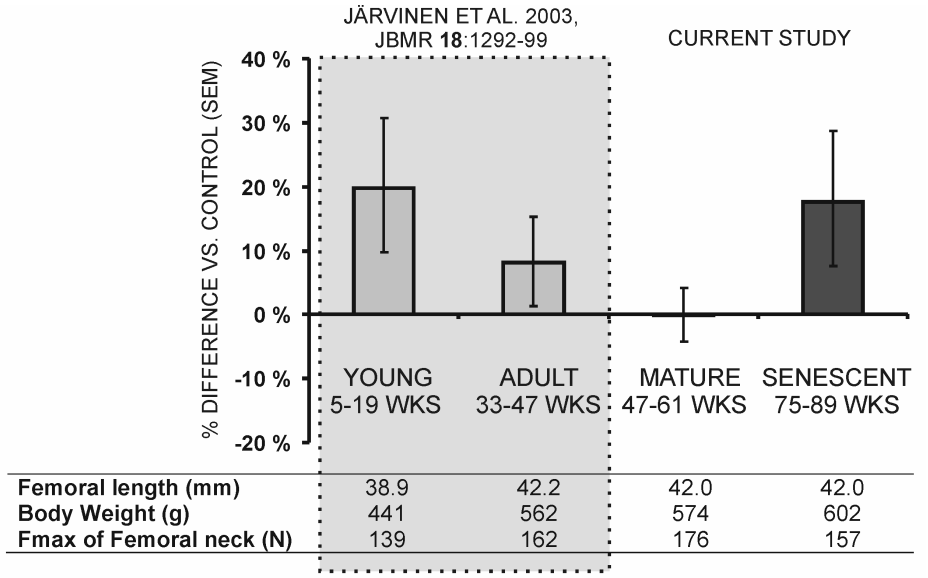
Furthermore, sciatic neurectomy is not a perfect model to investigate responses of bone to unloading due to the alterations to autonomic nervous

supply to the limbs (Gomez et al., 2007). Also, one might criticize the use of the contralateral right hindlimb as a "normally loaded control". In fact, the existing evidence regarding the effect of unilateral hindlimb unloading on the bones of the contralateral limb is somewhat confusing: while some studies have shown that the contralateral "normally" loaded limbs are under the influence of non-physiological overloading (Jee et al., 1991; Shellhart et al., 1992), others have shown signs of unloading (Laitala and Väänänen, 1993; Tuukkanen et al., 1991), and some studies have not reported any overloading or unloading effect (Kannus et al., 1996; Kannus et al., 1994). However, in comparison to the overall effects of loading and estrogen, the potential overloading effect seems modest. Thus, it can be stated that the contralateral (right) limb represents an appropriate control. Also, it should be noted that several other hormones (e.g. hypophyseal hormones) were still effectively and might have affected the bone ontogeny in the genetic growth group, although it is unlikely that this effect was disparate between the groups. In addition, although the study was adequately powered for showing changes resulting in locomotion or estrogen alone, the sample size may not have been large enough to show interaction between these two (Foppa and Spiegelman, 1997; Gauderman, 2002; Luan et al., 2001; Wong et al., 2003).

The objective of Study IV was to evaluate whether the skeleton can maintain its capability to respond to increased loading until a very old age (senescence), since, according to the prevailing understanding, skeletal mechanoresponsiveness declines with age leading to age-related osteoporosis (Klein-Nulend et al., 2002; Seeman, 2004). The findings were somewhat surprising, since senescent animals showed a positive adaptive response to exercise but a much less consistent response was seen in the mature rats subjected to the same exercise regimen. This is in agreement with another experimental study using very old rats (Buhl et al., 2001), but contradicts other experimental studies (Järvinen et al., 2003c; Rubin et al., 1992; Turner et al., 1995b). In an earlier study by our group (Järvinen et al., 2003c), it was shown that the ability of the bones of young (5-19 week old) and mature (33-47 week old) male rats to adapt to treadmill-running induced loading was similar, but the adaptive mechanisms differed; in response to a given exercise, the growing bones primarily increased cross-sectional size, while the mature bones mainly increased bone density. However, when the results were analyzed using analysis of covariance and adjusting the breaking load using body weight and femoral length as covariates, it was obvious that growing rats gained more strength in their femoral necks than mature rats (Pajamäki, 2007).

Study IV compares the exercise-induced changes between mature and senescent bones. By combining the findings of the study by Järvinen et al. (2003c) and the present study (IV), a pattern of exercise-induced responses encompassing the entire rat life span can be illustrated (Figure 30). By doing this, it is evident that the responses to the identical treadmill training program are different between the age groups; growing and senescent animals respond to the exercise program, while the mature animals are indifferent. Although this biphasic pattern in the response relative to the age of the animals may be interpreted as an apparent age-dependent change in the mechanosensitivity of the

bones *per se*, the increased mechanoresponsiveness observed in the adolescent and senescent animals is more likely explained by the lower structural rigidity relative to incident loading in the growing and senescent bones.



Femoral length (mm)	38.9	42.2	42.0	42.0
Body Weight (g)	441	562	574	602
Fmax of Femoral neck (N)	139	162	176	157

Figure 30. Effects of the identical treadmill training regimen in adult, young, mature, and senescent male rats. Data derived by combining past and present (IV) studies.

To comprehend the observed response of the growing bones to increased loading, one has to be familiar with a phenomenon called “adaptational lag” (Frost, 1999b; Frost, 2003) (Figure 31). In brief, during the most rapid period of skeletal growth, the increase in bone size precedes that of consolidation of bone matrix (deposition of mineral), making the growing bones less rigid (more flexible) and thus subjecting them to increased deformations (strains) (Bass et al., 1999; Crabtree et al., 2004; Daly et al., 2004; Frost, 1988; Heaney et al., 2000; Nunamaker et al., 1990; Rauch et al., 2001; Schiessl et al., 1998; Wang et al., 2003), and possibly secondarily increasing the mechanoresponsiveness of growing bone. A virtually identical sequence of events – although opposite in direction and less sudden in nature – has been shown to occur in senescent bones: in essence, aging results in a gradual loss of mineral mainly from the trabecular, endocortical and intracortical compartments of bone and consequently results in a decrease in bone rigidity despite a simultaneous (compensatory) periosteal apposition (Ahlborg et al., 2003; Ruff and Hayes, 1982; Ruff and Hayes, 1988; Seeman, 2003). This age-related osteoporosis was established by showing that the senescent rats displayed clearly impaired bone traits and reduced bone structural strength. The gradual decrease in bone rigidity provides a plausible explanation for the observed increase in the responsiveness of bones to loading in the senescent animals.

While one might disagree with the hypothesis presented (Figure 31) regarding increased mechanoresponsiveness in senescent rats, it is apparent that skeletal responsiveness is not impaired in very old age. This is important when we shift our focus to the second objective of this study – the pathogenesis of age-related osteoporosis. As discussed earlier, the explanation for decreased bone mass of the aged skeleton underlies the question whether age-related bone loss is an appropriate response to reduced loading in a less active host, or an aberration in the mechanisms responsible for mechanoresponsiveness (Klein-Nulend et al., 2002; Lanyon and Skerry, 2001; Lee et al., 2003; Seeman, 2004). Thus, it seems obvious that the finding of a significant adaptive response to increased exercise loading in senescent animals shows that the homeostatic control system of the skeleton functions even in very old age. Therefore, it can be claimed that according to the present study, age-related osteoporosis is mostly due to a decrease in habitual activity rather than a malfunction of the mechanoresponse pathway.

Although one of the major strengths of this thesis is the well validated methodology, there are also methodological limitations that have to be addressed regarding Study IV. First, no direct measurement of the bone strains was performed, although the strains experienced by bones during treadmill training could be determined directly using invasive strain gauges, or indirectly, by a finite element model. Instead, we based our conclusions on a simple engineering principle that equal loading produces less strain in a more rigid bone and vice versa. Secondly, one might be tempted to draw conclusions regarding the potential effect of gender on the mechanoresponsiveness of bones to loading. However, due to the differences in the study designs between males and females (e.g., distinct age at entry of the initiation of exercise in senescent animals and different treadmill training protocols), the results of this study cannot be used for such a purpose. In retrospect, considering the observed lower responsiveness of female bones to increased loading compared to that of males, it could be argued that the protocol should have been identical between genders. However, due to the increased longevity of female rats and the resulting increased frailty, we felt compelled to subject the senescent females to a less physically challenging exercise regimen. Fortunately, the effect of gender on the skeletal responsiveness to loading has been previously assessed (Hoshi et al., 1998a; Hoshi et al., 1998b; Järvinen et al., 2003a; Järvinen et al., 2003b; Kodama et al., 2000; Mosley and Lanyon, 2002; Wallace et al., 2007), suggesting that males are more responsive to loading than females.

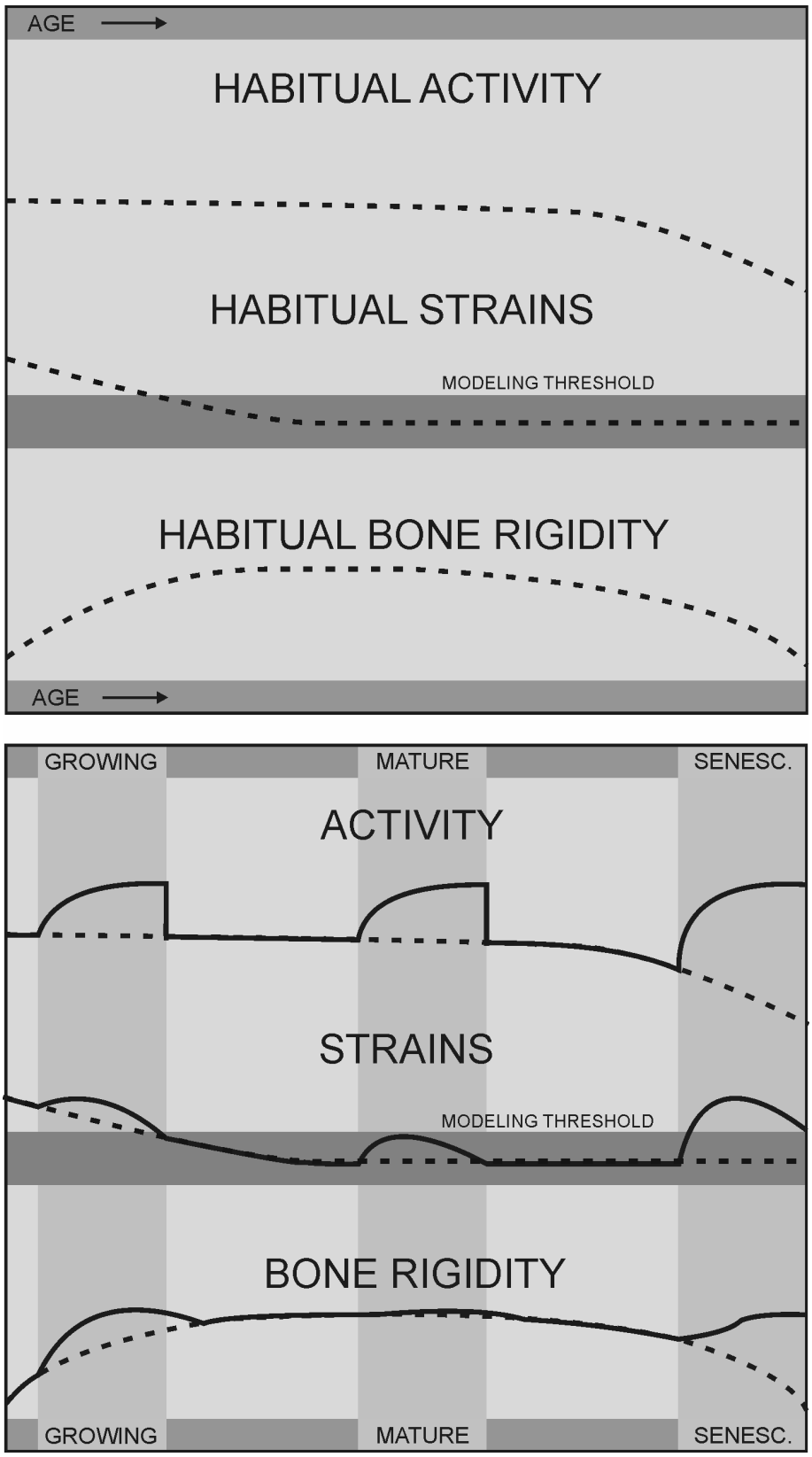


Figure 31. Schematic illustration of habitual activity, strains and bone rigidity in different ages in Study IV. The broken line represents the habitual situation and the solid line is the modulated situation due to the increased loading (exercise) in this experiement.

In addition, it could also be argued that the aged rats used in our experiments (89-week old males and 104-week old females at the end of the experiment) do not represent "true" senescence. However, it is recalled here that the mortality rate of the animals (both controls and exercised) was almost 60% at the end of the study. It can be estimated that the age of our senescent rats corresponded to approximately 75 and over 80 years of age in men and women in Finland, respectively (WHO, 2007). Also, in agreement with the increased mortality, the aged animals displayed deterioration of most bone traits and a decrease in body weight (particularly in males), changes typical of senescence (Frost, 1997). Finally, a limitation concerns the fact that the breaking load of the bone was used as the only index of bone rigidity, the property that, at least in theory, would be the regulator of bone deformation caused by mechanical loading. Orthodoxically, the most appropriate indicator of bone rigidity would be bone stiffness. However, in Studies I and II it was shown that the precision of stiffness evaluation, especially in the femoral neck, is quite poor, resulting in undue variance (noise) in the results. Further, as the breaking load has been shown to correlate well with bone rigidity but displays considerably better precision, it is argued that the breaking load of the whole bone structure represents a viable surrogate for whole bone rigidity in rat experiments. As pointed out earlier, due to the chronological order of the studies of this thesis, the new mediolateral three-point bending test was not used in Study IV. Therefore it is highly likely that some of the loading-induced effects in the biomechanical properties of femoral midshafts might be at least partly missed due to the non-optimal loading direction.

A universal dilemma in all experimental research is the generalization of the experimental model to the clinical counterpart, the human being. In the studies of this thesis, rat was used as an experimental animal because of its numerous benefits as a model for bone research. According to the literature survey (I), rat is the most widely used animal in bone research. Obviously, it is due to its inexpensiveness and ease to house. Also, its life span is relatively short (Frost and Jee, 1992) enabling short experiments. In addition, there is a general acceptance of the public to the use of rodents in research (Turner, 2001). The rat skeleton differs some way from the human skeleton; it lacks Haversian systems in cortical bone (Wronski and Yen, 1991) and skeletal maturation is relatively slower in rats (Olivera et al., 2003), resulting in a long period of linear growth of bones. Also, the orientation of bones and joints differs from corresponding human skeletal structures due to the unique typical posture and locomotion. For example, the loading environment, and subsequently the architecture, is quite different in the proximal femur in rats due to the four-limbed stance compared to the situation in humans whose stance is erect. Despite these warranted concerns, experimental bone studies are needed due to their indubitable advantage, the possibility to obtain reliable information by testing whole bones mechanically.

SUMMARY AND CONCLUSIONS

1. Breaking load remains the preferable trait for analyzing and reporting mechanical response in experimental osteoporosis studies, while the utility of stiffness and energy absorption is challenged. However, if the precision of the latter traits could be improved, their assessment would undoubtedly provide relevant information on bone biomechanics and be of use. The findings underscore the need for larger sample sizes in experimental bone interventions using mechanical traits as primary outcome variables. In addition, quality control of biomechanical tests including the assessment of precision should be much more widely adopted.

2. A new testing method for assessing the structural rigidity of the rat femur in the ML direction, the primary direction of skeletal adaptation to locomotive loading, was introduced and validated. The results not only show that the method is biologically valid and sufficiently precise but also that in the structural testing of rat bones, the determination of bone breaking load yields repeatability superior to that of bone stiffness and energy absorption.

3. The longitudinal growth of rat femur is largely irrespective of locomotive loading or estrogen but the bone ontogeny is somewhat aimless without these factors. As proof of this, the cross-section of unloaded femoral diaphysis is symmetrical in shape, whereas locomotion has a remarkable effect on the cross-sectional size and shape. The osteogenic effect of estrogen is, in turn, marginal at this bone site; the positive influence mainly occurring at the endosteal surface, apparently the most efficient site for mineral metabolism.

4. Concerning the mass, structure, and mechanical competence of rat bones, the homeostatic loading-driven regulatory feedback system maintains its capacity to respond to increased exercise loading even into very old age. Accordingly, it is unlikely that the pathogenesis of age-related osteoporosis is attributable to a failure in this system. Thus, the strengthening of senescent human bones is also possible – naturally provided that safe and efficient training methods can be developed for the oldest old.

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Biomechanical testing in experimental bone interventions—May the power be with you

Olli V. Leppänen[a,b], Harri Sievänen[c,*], Teppo L.N. Järvinen[a,b]

[a]*Medical School and the Institute of Medical Technology, University of Tampere, 33 014 Tampere, Finland*
[b]*Division of Orthopaedics and Traumatology, Department of Trauma, Musculoskeletal Surgery and Rehabilitation, Tampere University Hospital, Tampere, Finland*
[c]*The Bone Research Group, UKK Institute, P.O. Box 30, 33 500 Tampere, Finland*

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Abstract

Total variation in any measured variable, in conjunction with expected treatment effect, defines the minimum sample size (minSS) required to detect the expected effect with statistical confidence should the effect truly exist. A comprehensive literature survey of 3472 original studies was carried out to identify studies with biomechanical testing of whole bones. Total variation in common biomechanical traits and expected treatment effects in typical interventions were statistically determined. According to this survey, total variation in biomechanical traits between different species of experimental animals was similar, justifying the use of rat femur as a model in further analyses. Due to poorer precision, stiffness and energy absorption assessment require substantially larger sample size than breaking load. Due to same reason, minSS for femoral neck compression test is considerably larger than for femoral shaft three-point bending test. For the bending test, minSS to show a 10% treatment effect in the breaking load with 80% statistical power is 11 rats/group, while corresponding minSS is 23 for the stiffness, and 53 for the energy absorption. For the femoral neck compression test, minSSs are 16, 51, and 134 rats/group, respectively. Among the reviewed studies, the mean sample size was 11 animals/group. This underscores the need for considerably larger sample sizes in experimental bone interventions which employ mechanical traits as primary outcome variables. In particular, poor precision and generally small expected treatment effects compromise the utility of stiffness and energy absorption assessments in experimental bone interventions.

Keywords: Sample size; Statistical power; Precision; Mechanical testing; Experimental studies

1. Introduction

Skeleton has primarily evolved to allow efficient *locomotion* (Burr, 1997; Frost, 1997; Parfitt, 1998), and accordingly, bone mechanical competence depicts its ultimate phenotype (Jarvinen et al., 2005; van der Meulen et al., 2001). Biomechanical testing provides a direct method to study mechanical traits of bones among various experimental animals with different testing protocols (Andreassen and Oxlund, 2000; Cullinane et al., 2002; Fleming et al., 2000; Ikeda et al., 2003; Jerome et al., 1999; Judex and Zernicke, 2000; Klein et al., 2001, 2003; Les et al., 2002; Luppen et al., 2002). Besides careful technical execution (Turner and Burr, 1993), general utility of biomechanical testing relies on its precision. Precision is consistently required for studies using bone densitometry, but this is not the case for biomechanical testing of bones. Obviously, precision, in the stringent sense, cannot be determined because of destructive nature of the method. However, precision can be reasonably assessed from within-pair variation in biomechanical and structural traits of bone (Eckstein et al., 2004; Jamsa et al., 1998; Jarvinen et al., 1998b; Leppanen et al., 2006; Peng et al., 1994).

Total variation observed in any trait reflects both biological variation and methodological variation. Precision of the method affects thus the statistical power of the study; i.e., the probability that the study could detect the

*Corresponding author. Tel.: +358 3 282 9111; fax: +358 3 282 9200.
E-mail address: harri.sievanen@uta.fi (H. Sievänen).

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expected treatment effect with statistical confidence if the effect truly existed. For obvious scientific and ethical reasons, the sample size should be planned carefully before starting any experiment (Eisman, 2006), so that the number of animals/group is large enough to provide adequate statistical power. This is crucial as underpowered studies can seldom address any research question meaningfully, but only lead to inconclusive results (Ioannidis, 2005).

Detectable treatment effect is inversely related to sample size, meaning that large samples are needed to reveal small effects (Altman et al., 2001). In practice, appropriate determination of sample size requires realistic estimates of the treatment effect and the total variation in individual responses to given treatment; desired level of statistical significance for the expected results (type I error); and desired statistical power (type II error).

Objectives of the present study were fourfold: (1) to evaluate total variation in various biomechanical traits of whole bones; (2) to estimate treatment effects on biomechanical traits in experimental interventions based on ovariectomy, increased activity, and inactivity using rat femur as a model; (3) to characterize methodological variation in biomechanical testing of rat femur to illuminate contribution of biological and methodological variation to total variation in biomechanical traits; and (4) to devise a scheme for minimum sample size (minSS) needed to detect a treatment effect in biomechanical traits with statistical significance.

2. Materials and methods

2.1. Total variation in biomechanical traits

We reviewed all 3472 original studies published between 1999 and 2003 in *Bone*, *Calcified Tissue International*, *Journal of Bone and Mineral Research*, or *Journal of Orthopaedic Research*. We considered this sample of four major bone journals representative of contemporary status of experimental bone intervention studies. Inclusion criteria were (1) mechanical testing of whole bones was performed; (2) bones were extracted either from rat, mouse, dog, rabbit, or monkey; and (3) the study had an intact control group. Accordingly, 123 studies (see Supplementary data) were included.

Number of animals (n), mean and standard deviation (S.D.) of breaking load, stiffness, and energy absorption were collected for each control group, whenever available. When standard error of mean (S.E.M.) was given instead of S.D., S.D. was calculated as

$$\text{S.D.} = \sqrt{n} \times \text{S.E.M.} \tag{1}$$

Percentage variation (σ_T) in each study and trait was calculated as

$$\sigma_T = \frac{\text{S.D.}}{\text{Mean}} \times 100\%. \tag{2}$$

The mean total percentage variation ($\bar{\sigma}_T$) for each type of intervention and trait was calculated as

$$\bar{\sigma}_T = \sqrt{\frac{\sum \sigma_T^2}{k}}, \tag{3}$$

where k is the number of separate studies.

2.2. Treatment effects on biomechanical traits

To obtain appropriate estimates of treatment effects in typical experimental bone interventions, we chose the common rat model and included studies that met the following criteria: (1) femoral shaft three-point bending test or femoral neck compression test[1] was performed; and (2) intervention was either *ovariectomy*, *increased activity* (climbing, treadmill training, voluntary wheel-running), or *inactivity* (neurectomy, hindlimb suspension, limb taping). Altogether, data from 40 studies (see Supplementary data) were included.

To estimate the effect size of each intervention (in z-scores and %-values) meta-analytic principles described in Eqs. (4)–(12) (Eqs. (1)–(3) also apply as appropriate) were employed (see Appendix A) (Cooper and Hedges, 1994).

2.3. Sample size estimation

The minimum sample size (minSS) needed to show a specified treatment effect (δ) in mechanical traits with statistical significance of $p = 0.05$ (if the effect truly existed) was calculated using an approximation of Neyman's solution (Snedecor and Cochran, 1967) as

$$\text{minSS} = 7.9\frac{\bar{\sigma}_T^2}{\delta^2} \quad \text{for the statistical power of 0.80} \tag{13}$$

and

$$\text{minSS} = 10.5\frac{\bar{\sigma}_T^2}{\delta^2} \quad \text{for the statistical power of 0.90.} \tag{14}$$

2.4. Methodological variation in biomechanical traits

The relationship between total variation (σ_T), biological variation (σ_B) in the tested trait and the precision ($CV\%_{rms}$) is given by

$$\bar{\sigma}_T = \sqrt{\bar{\sigma}_B^2 + CV\%_{rms}^2}. \tag{15}$$

Several studies (Ammann et al., 2000; Jamsa et al., 1998; Jarvinen et al., 1998a, b; Leppanen et al., 2006; Peng et al., 1994) have shown that the precision of biomechanical tests can be assessed using paired specimens. This approach presumes that contralateral bones are equal, which may not always be true (Banse et al., 1996; Hanson and Markel, 1994). However, when the paired bones are extracted from healthy animals developed under normal circumstances, no systematic difference would apparently exist. Thus, the precision was calculated as

$$CV\%_{rms} = \sqrt{\frac{\sum(100 \times (\text{right} - \text{left}/\text{right} + \text{left}))^2}{n}}, \tag{16}$$

where n is the number of femur pairs, and right and left denote the measured values from respective femora.

Precision of femoral shaft three-point bending and femoral neck compression tests was determined using test results from femora of 60 Sprague-Dawley rats (age: 17–69 weeks, and body weight: 240–630 g). Excised and defleshed bones were wrapped in saline-soaked gauze bandages to prevent dehydration and stored at −20 °C. This procedure does not affect bone mechanical properties (Pelker et al., 1984; Sedlin and Hirsch, 1966). The research protocol was accepted by Ethics Committee for Animal Experiments of the University of Tampere. The study conformed to NIH Guide for the Care and Use of Laboratory Animals.

At the testing day, the femora were thawed at the room temperature and kept in the saline-soaked gauzes except during measurements.

[1]Note that the mechanical testing of the femoral neck is rather a cantilever bending test than a compression test. However, the latter term has become established in the literature, and for the sake of consistency, the femoral neck test is called a compression test in the present study.

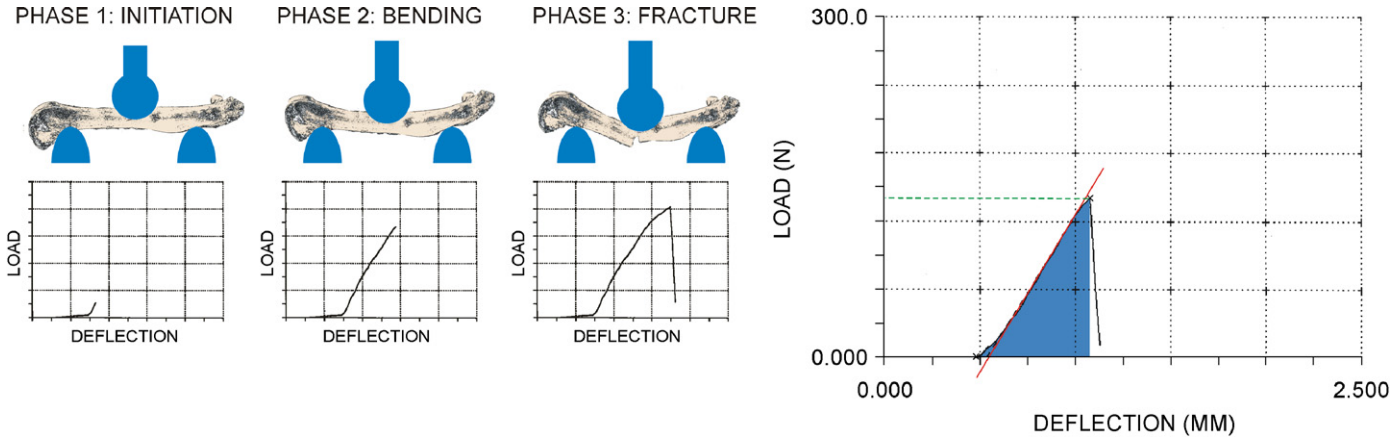

Fig. 1. Determination of the breaking load, stiffness, and energy absorption from the load–deformation curve obtained during the three-point bending test of rat femur. During the testing, the femur was placed on its posterior surface on round-ended lower supports (4 mm in diameter), and the loading force was applied by a round-ended intender (10 mm in diameter). Using the software of the material testing machine, the breaking load was determined as the highest point of the curve, the stiffness as the tangent modulus of the linear part of the load–deformation curve, the determination being started after the force value 60 N was reached. The energy absorption, in turn, was determined as the area under the load–deformation curve until the point of failure (breaking load).

The bone pairs were tested on the same day, and the test order was random. All bones were analyzed by the same operator.

2.5. Mechanical tests

Mechanical testing of the rat femoral shaft and femoral neck was done with a material testing machine (LR5K, J.J. Lloyd Instruments, Southampton, UK) according to our standard protocols (Jarvinen et al., 1998a, b). For the femoral three-point bending test, the femur was placed on its posterior surface on the lower supports of the bending apparatus. A preload (10 N) was first applied to the femoral midshaft and then the bending load was applied at rate of 1.0 mm/s until failure. The breaking load, stiffness, and energy absorption were determined from the load–deformation curve (Fig. 1).

After the three-point bending, the proximal half of the femur was mounted in a fixation device (Sogaard et al., 1994), and a vertical load was applied to the top of the femoral head until the femoral neck failure. Loading parameters and the data collection were equal to those described above for the femoral shaft test.

3. Results

Table 1 gives the total variation in breaking load, stiffness and energy absorption obtained from different testing protocols among different bones and species. Clearly, rat was the most common animal in experimental bone interventions using biomechanical tests of whole bones, followed by mouse; while other species were rarely used. Of different testing protocols, femoral shaft three-point bending was most common, followed by vertebral compression, femoral neck compression, and tibial three-point bending; while torsion and four-point bending tests were rare.

In general, total variation was smaller for breaking load than for stiffness, and particularly for energy absorption among the most common testing protocols. Given the wide ranges, total variation could vary markedly between different studies (Table 1). Of note, the total variation in rat femoral shaft three-point bending and femoral neck compression results were similar to results from other species (Table 1), supporting our choice to use these rat tests as representative models of biomechanical testing.

Table 2 gives the magnitude and variation of mean treatment effects in rat femur mechanical traits in response to different interventions. Ovariectomy appears to result in ~5% reduction in the femoral shaft breaking load and stiffness while in the other traits, significant effects were not apparent, the borderline ~13% reduction in the femoral neck stiffness excluded.

Increased activity appears to increase both the femoral shaft breaking load and stiffness by ~10% (no data of energy absorption were available), and the femoral neck breaking load by ~6% and stiffness by ~36%, while the energy absorption appears to decrease correspondingly.

Inactivity seems not to affect the femoral shaft mechanical traits, while ~30% reduction in the femoral neck breaking load may be expected (no data on other traits were available).

A nomogram for sample size estimation is given in Fig. 2 and summarized in Table 3. Clearly, femoral shaft three-point bending test is useful with smaller minSS (rats/group) than femoral neck compression test. Also, minSS is clearly smaller for breaking load than for stiffness and energy absorption in both tests. For example, minSS to show a 10% difference in the femoral shaft breaking load with 80% power is 11 rats/group, while corresponding minSS is 23 for the stiffness, and 53 for the energy absorption. For the femoral neck compression test, minSSs are 16, 51, and 134 rats/group, respectively.

As a practical example of sample size estimation, ovariectomy can be expected to result in ~5% reduction in femoral shaft breaking load (Table 2). According to Table 3, 42 rats/group would be needed to observe ~5% treatment effect with 80% statistical power. Of note, the

Table 1
Mean percentage total variation and range (in %) in breaking load, stiffness and energy absorption observed in different biomechanical testing protocols of whole bones[a]

Animal	Variable		Femoral shaft three-point bending	Femoral shaft four-point bending	Femoral shaft torsion	Femoral neck compression	Vertebral compression	Tibia three-point bending	Tibia torsion
Rat	Breaking load	Mean (range)	11.4 (3.4–26.1)	20.7	24.0 (18.2–30.6)	14.2 (6.0–22.3)	24.4 (9.5–53.6)	17.6 (6.0–43.1)	21.0 (6.2–26.9)
		n[b]	72	1	3	29	47	17	4
	Stiffness	Mean (range)	16.8 (6.2–47.3)	14.5	18.9 (7.3–28.7)	25.4 (10.6–49.5)	38.5 (8.5–69.3)	15.9 (7.4–24.3)	10.8 (10.7–10.8)
		n[b]	38	1	3	9	31	8	2
	Energy absorption	Mean (range)	25.7 (7.4–52.7)	N.A.	N.A.	41.1 (18.6–63.6)	55.3 (27.8–86.5)	25.1 (18.5–32.3)	43.7 (39.0–47.8)
		n[b]	24	0	0	8	8	5	2
Mouse	Breaking load	Mean (range)	16.6 (5.2–34.5)	12.3 (9.3–15.5)	54.6 (9.6–100.0)	15.4 (10.3–25.2)	29.8 (19.6–48.4)	15.2 (13.6–16.7)	N.A.
		n[b]	24	3	4	6	7	2	0
	Stiffness	Mean (range)	24.1 (5.8–62.7)	20.5 (11.3–28.9)	35.9 (25.7–43.8)	23.5 (12.7–29.8)	46.7 (22.3–79.9)	27.5	N.A.
		n[b]	21	3	2	5	7	1	0
	Energy absorption	Mean (range)	48.3 (13.8–91.0)	31.1 (26.1–35.5)	56.6 (27.0–76.5)	36.5 (34.5–38.5)	47.8 (27.5–71.2)	N.A.	N.A.
		n[b]	10	3	3	2	4	0	0
Dog	Breaking load	Mean (range)	17.9 (15.6–20.0)	N.A.	21.0 (15.3–25.5)	N.A.	17.4 (13.7–21.4)	N.A.	13.3
		n[b]	2	0	2	0	4	0	1
	Stiffness	Mean (range)	21.5 (19.3–23.5)	N.A	21.7 (15.2–26.6)	N.A.	23.9 (16.4–36.5)	N.A.	21.8
		n[b]	2	0	2	0	4	0	1
	Energy absorption	Mean (range)	27.7 (26.9–28.5)	N.A.	40.7	N.A.	31.8 (28.3–37.1)	N.A.	N.A.
		n[b]	2	0	1	0	4	0	0
Rabbit	Breaking load	Mean (range)	11.0 (10.9–11.2)	N.A.	N.A.	N.A.	20.8	18.0 (10.1–22.8)	23.3 (23.0–23.5)
		n[b]	2	0	0	0	1	3	2
	Stiffness	Mean (range)	12.1 (9.7–14.1)	N.A.	N.A.	N.A.	17.0	23.5 (19.6–26.8)	20.9 (19.2–22.5)
		n[b]	2	0	0	0	1	2	2
	Energy absorption	Mean (range)	15.0 (11.1–18.1)	N.A.	N.A.	N.A.	36.7	35.0 (23.1–42.6)	N.A.
		n[b]	2	0	0	0	1	3	0
Monkey	Breaking load	Mean (range)	12.4	N.A.	N.A.	18.2 (15.8–20.4)	18.2 (15.2–21.0)	N.A.	N.A.
		n[b]	1	0	0	2	3	0	0
	Stiffness	Mean (range)	51.0	N.A.	N.A.	23.9	29.4 (22.7–34.7)	N.A.	N.A.
		n[b]	1	0	0	1	2	0	0
	Energy absorption	Mean (range)	24.5	N.A.	N.A.	33.4	107.1 (64.2–137.3)	N.A.	N.A.
		n[b]	1	0	0	1	2	0	0

[a]Analysis was based on the data from intact control groups only.
[b]Number of included studies.

Table 2
Pooled treatment effect sizes (in z-scores and %) on breaking load, stiffness, and energy absorption of rat femur in response to ovariectomy, increased activity, or inactivity

	Effect size				Percentage-transformed effect size				n	Model[a]
	Mean	Standard error	Lower 95% limit	Upper 95% limit	Mean	Standard error	Lower 95% limit	Upper 95% limit		Fixed/ random
Ovariectomy										
Femoral shaft three-point bending (AP)										
Breaking load	−0.40	0.12	−0.64	−0.17	−4.8	1.4	−7.6	−2.0	44	Random
Stiffness	−0.28	0.13	−0.53	−0.03	−4.2	1.9	−8.1	−0.4	21	Random
Energy absorption	0.10	0.14	−0.17	0.38	2.7	3.7	−4.6	9.9	17	Random
Femoral neck compression										
Breaking load	−0.19	0.28	−0.74	0.35	−2.9	4.2	−11.1	5.3	8	Random
Stiffness	−0.54	0.26	−1.05	−0.03	−12.7	6.0	−24.5	−0.8	3	Fixed
Energy absorption	0.09	0.60	−1.09	1.27	2.6	16.7	−30.1	35.3	3	Random
Increased activity										
Femoral shaft three-point bending (AP)										
Breaking load	0.97	0.33	0.32	1.62	10.4	3.6	3.4	17.3	8	Random
Stiffness	0.69	0.19	0.31	1.07	10.0	2.8	4.5	15.5	5	Fixed
Energy absorption	–	–	–	–	–	–	–	–	–	–
Femoral neck compression										
Breaking load	0.40	0.12	0.16	0.64	5.8	1.7	2.4	9.2	12	Fixed
Stiffness	1.58	0.41	0.79	2.38	36.2	9.3	18.0	54.5	2	Fixed
Energy absorption	−1.01	0.38	−1.75	−0.28	−37.8	14.0	−65.2	−10.3	2	Fixed
Inactivity										
Femoral shaft three-point bending (AP)										
Breaking load	0.08	0.22	−0.35	0.51	0.6	1.7	−2.7	3.9	4	Fixed
Stiffness	0.08	0.26	−0.42	0.59	1.1	3.5	−5.8	8.0	3	Fixed
Energy absorption	−0.19	0.26	−0.70	0.32	−4.1	5.5	−14.9	6.8	3	Fixed
Femoral neck compression										
Breaking load	−1.81	0.38	−2.54	−1.07	−29.5	6.1	−41.5	−17.5	2	Fixed
Stiffness	–	–	–	–	–	–	–	–	–	–
Energy absorption	–	–	–	–	–	–	–	–	–	–

[a]See Appendix A for more information about fixed and random-effects models.

same, large sample size may also be sufficient to provide a significant result in the femoral neck stiffness (for which ~10% treatment may be expected).

Precision for breaking load, stiffness and energy absorption assessments are given in Table 4. Applying the $CV\%_{rms}$ values (Table 4) and mean percentage total variation data (Table 1) to Eq. (15), it yields that on average ~20–50% of the total variation in these biomechanical traits are attributable to methodological uncertainties.

4. Discussion

Adequate sample size and sufficient statistical power are prerequisites for any clinical intervention trial for obvious scientific and ethical reasons. Despite being similarly pertinent to all research, however, statistical power has received little attention in experimental bone interventions. According to our recent literature survey (Jarvinen et al., 2005), calculation of sample size was reported only in two out of 210 studies (Kaastad et al., 2001; Kurth et al., 2001) employing mechanical testing of whole bones. However, it is possible that the sample size was calculated in more studies, but was not mentioned. In practice, the calculation of sample size requires reasonable estimates of total variation in the trait of interest and the anticipated treatment effect to given intervention. To facilitate power estimation, we devised a scheme for the minimum sample size required to detect the expected treatment effect with sufficient probability in response to ovariectomy, increased activity, and inactivity; all common interventions in experimental osteoporosis research.

In living organisms, the total variation observed in response to any given intervention stems from two major sources: inherent biological variation between and within individuals, and methodological variation of the test. Of the common mechanical traits, breaking load was most precise, while stiffness and energy absorption were subject to much more methodological variation. Poorer precision may also explain the fact (Tables 1 and 2) that the stiffness and energy absorption results are less reported than breaking load results notwithstanding the apparently

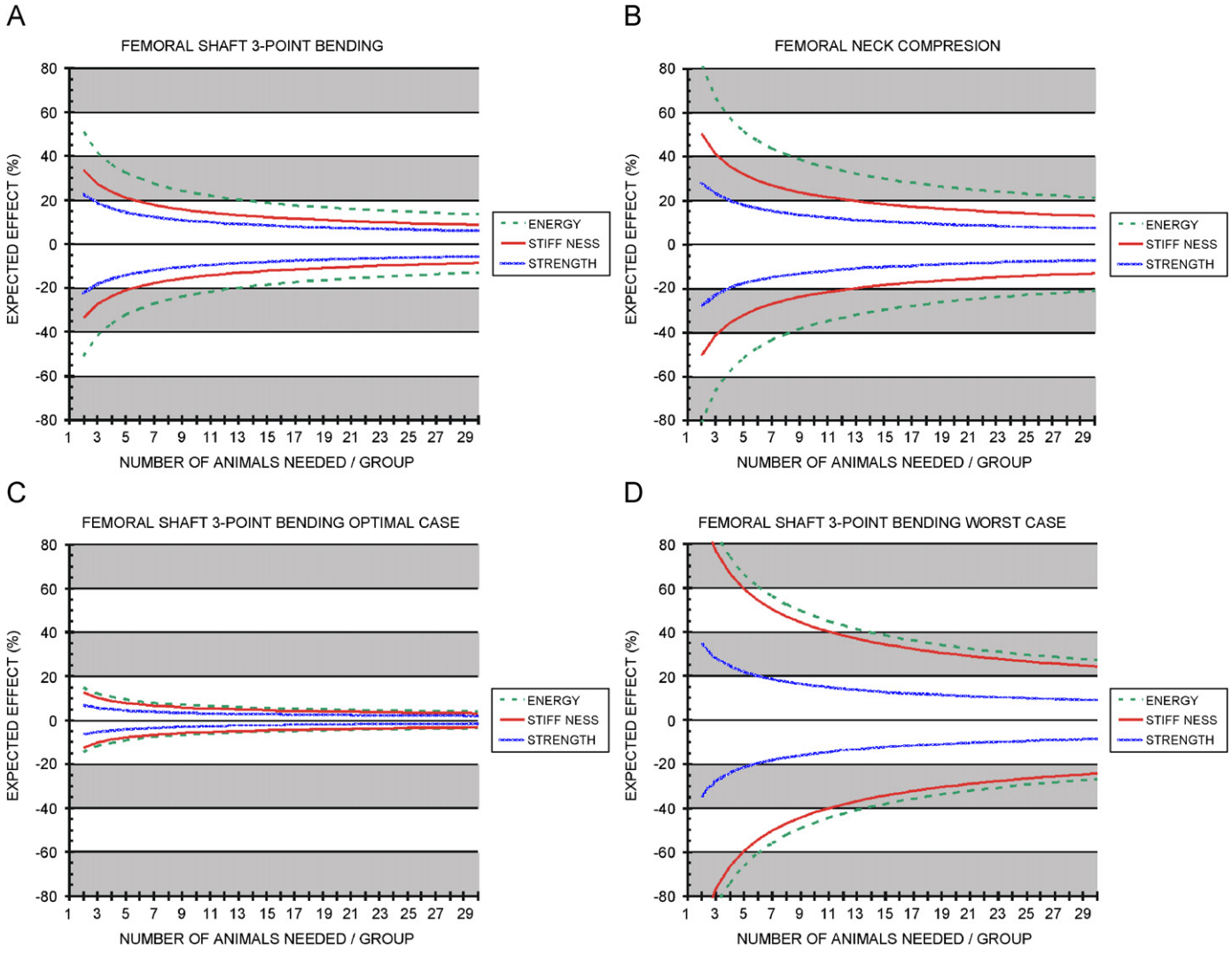

Fig. 2. The relationship between the size of the minimum detectable treatment effect (percentage difference) and sample size for breaking load, stiffness and energy absorption in (A) three-point bending, and (B) femoral neck compression test. In addition, the optimal and worst-case scenarios in three-point bending using the lowest and highest total variations found in the literature are presented in panels (C) and (D), respectively. In all cases, the statistical power was set at 0.80 and the significance level at $p = 0.05$.

complete biomechanical data. It may be so that insignificant results remain unreported. If so, this pinpoints the importance of having precise methods so that relevant information is not missed.

Reliability of biomechanical assessments is affected by many factors (Turner and Burr, 1993). Breaking load, by definition, denotes a single value of the y-axis (magnitude of load) of the load–deformation curve, while stiffness and energy absorption are aggregate measures and thus affected by variation in load and deformation values during the loading. Complex bone geometry, variation in size, spatial variation in material properties, relative variation in intender and support shapes, dimensions and locations in relation to individual bone geometry and material properties can all modify the mechanical behavior of the bone during the test and account for greater methodological variation. In order to optimize precision and information content of biomechanical testing of whole bones, the testing protocol should take into account individual bone anatomy and size so that each bone is subjected to identical loading (e.g., mode, direction, strain rate) from the functional perspective. Given the large number of potential confounding factors in the test situation, the above goal may remain formidable in practice. In any case, employed test protocols should be standardized and their precision properly evaluated and reported.

The present precision data for biomechanical assessment of rat femur (Table 4) are comparable to values reported for breaking load ($CV\%_{rms}$ ~4–5%) and stiffness or energy absorption ($CV\%_{rms}$ ~7–15%) in the femoral shaft bending test (Jarvinen et al., 1998b; Leppanen et al., 2006), and for breaking load ($CV\%_{rms}$ ~5–15%) and stiffness or energy absorption ($CV\%_{rms}$ ~18–19%) in the femoral neck compression (Ammann et al., 2000; Jarvinen et al., 1998b; Leppanen et al., 2006; Peng et al., 1994).

Table 3
Number of animals/group needed to show expected treatment effect (3–20%) at significance level $p<0.05$ statistical power (80% or 90%) in the femur shaft three-point bending test and femoral neck compression test

Expected treatment effect (%)	Variable	Three-point bending		Femoral neck compression	
		Power 0.80	Power 0.90	Power 0.80	Power 0.90
3	Breaking load	115	152	177	236
	Stiffness	248	330	567	753
	Energy absorption	580	771	1483	1971
5	Breaking load	42	55	64	85
	Stiffness	90	119	204	271
	Energy absorption	209	278	534	710
10	Breaking load	11	14	16	22
	Stiffness	23	30	51	68
	Energy absorption	53	70	134	178
15	Breaking load	5	7	8	10
	Stiffness	10	14	23	31
	Energy absorption	24	31	60	79
20	Breaking load	3	4	4	6
	Stiffness	6	8	13	17
	Energy absorption	14	18	34	45

Table 4
Precision analysis based on 60 pairs of rat femora

Mechanical trait	Three-point bending				Femoral neck compression			
	Mean	S.D.	σ_T (%)	Precision ($CV\%_{rms}$)	Mean	S.D.	σ_T (%)	Precision ($CV\%_{rms}$)
Breaking load (N)	149	20	13.4	5.1	141	27	19.1	7.5
Stiffness (N/mm)	226	39	17.3	9.7	256	74	28.9	17.9
Energy absorption (mJ)	62	21	33.9	15.5	44	13	30.2	18.7

σ_T denotes the percentage total variation ($100 \times$ S.D./mean %).

Compared to typical precision (~1–2%) of dual-energy X-ray absorptiometry (DXA) or peripheral quantitative computed tomography (pQCT) in the assessment of bone mineral content or density of rat femur (Ammann et al., 1992; Horton et al., 2003; Jarvinen et al., 1998b; Leppanen et al., 2006; Nagy et al., 2001; Sievanen et al., 1994), the precision of biomechanical testing remained inferior.

As the total variation in common biomechanical traits of rat femur was comparable to respective findings among other species (Table 1), the proposed scheme for sample size calculation may be considered applicable to biomechanical testing of bones in general. However, care must be taken when extending this application to other species than rat or to other tests than femoral shaft three-point bending or femoral neck compression since the number of included studies for certain tests and/or experimental animals was occasionally small (Table 1). Also, given the large range of total variation across studies, the mean total variation obtained from only a few studies may not be representative of the actual situation.

Regarding the statistical power of an experimental bone intervention to detect the expected treatment effect, biomechanical testing is not only hampered by limited precision but also by relatively small responses and slow response times. In addition, duration, intensity, or any other specific feature in the intervention or study group (e.g., age or sex) may influence the magnitude of the treatment effect directly or interactively, and thus, the results may differ substantially between individual studies. Accordingly, the mean effect sizes in the present survey (Table 2) are indicative while they provide reasonable estimates of expected treatment effects. As large samples are necessary to detect small responses with statistical confidence (Altman et al., 2001), experimental osteoporosis interventions using biomechanical test results as primary outcome measures should be long-term and employ large groups of animals—larger than typically used at present. According to present survey, the average sample size (mean $\pm$ S.D.) was 11 ± 5 animals/group. This means that many studies have been underpowered to detect meaningful results with statistical significance.

Besides statistical power, the other objective of this study was to evaluate the utility of different biomechanical traits as primary outcome measures in experimental bone interventions. Poor precision of the stiffness and energy absorption assessments—particularly in conjunction with expected, mainly small responses and large total variation in these traits in response to different interventions (Table 1)—challenge their utility. Clearly a substantial proportion (20–50%) of the total variation in measured biomechanical traits can be attributable to methodological uncertainties, and occasionally methodological variation may equal the total variation (Leppanen et al., 2006). The latter implies no room for biological variation, which is virtually impossible but highlights crucial methodological limitations of present biomechanical assessments, particularly regarding the stiffness and energy absorption.

While the stiffness and energy absorption would be relevant descriptors of whole bone mechanical behavior and competence, it is not self-evident whether these traits, because of poor precision and strong correlation with breaking load on average, can essentially enhance the information obtained from breaking load. The use of stiffness and energy absorption may be argued by significant responses observed in some interventions. However, it is recalled that multiple statistical testing of several outcomes is likely to "reveal" seemingly significant associations (Eisman, 2006). Also, it was recently claimed that most current published research findings can be false, particularly when studies are smaller; when effect sizes are smaller; when there is a greater number and lesser preselection of tested relationships; or where there is greater flexibility in designs, definitions, outcomes, and analytical models (Ioannidis, 2005). This alarming notion is undoubtedly relevant to experimental bone studies and warrants serious consideration.

In conclusion, breaking load remains the preferable trait for analyzing and reporting mechanical response in experimental osteoporosis studies, while the utility of stiffness and energy absorption is challenged. However, if the precision of latter traits could be improved, their assessment would undoubtedly convey relevant information on bone biomechanics and be of use. Our analysis underscores the need for larger sample sizes in experimental bone interventions using mechanical traits as primary outcome variables. In addition, quality control of biomechanical tests including the assessment of precision should be much more widely adopted.

Conflict of interest

We do not possess any financial interests and do not have a conflict of interest.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiomech.2008.03.017.

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Pathogenesis of Age-Related Osteoporosis: Impaired Mechano-Responsiveness of Bone Is Not the Culprit

Olli V. Leppänen[1,2], Harri Sievänen[3], Jarkko Jokihaara[1,2,4], Ilari Pajamäki[1,2], Pekka Kannus[1,2,3], Teppo L. N. Järvinen[1,2]*

1 Medical School and the Institute of Medical Technology, University of Tampere, Tampere, Finland, **2** Division of Orthopaedics and Traumatology, Department of Trauma, Musculoskeletal Surgery and Rehabilitation, Tampere University Hospital, Tampere, Finland, **3** The Bone Research Group, UKK-Institute, Tampere, Finland, **4** Department of Orthopaedics, University of British Columbia, Vancouver, British Columbia, Canada

Abstract

Background: According to prevailing understanding, skeletal mechano-responsiveness declines with age and this apparent failure of the mechano-sensory feedback system has been attributed to the gradual bone loss with aging (age-related osteoporosis). The objective of this study was to evaluate whether the capacity of senescent skeleton to respond to increased loading is indeed reduced as compared to young mature skeleton.

Methods and Findings: 108 male and 101 female rats were randomly assigned into Exercise and Control groups. Exercise groups were subjected to treadmill training either at peak bone mass between 47–61 weeks of age (Mature) or at senescence between 75–102 weeks of age (Senescent). After the training intervention, femoral necks and diaphysis were evaluated with peripheral quantitative computed tomography (pQCT) and mechanical testing; the proximal tibia was assessed with microcomputed tomography (μCT). The μCT analysis revealed that the senescent bone tissue was structurally deteriorated compared to the mature bone tissue, confirming the existence of age-related osteoporosis. As regards the mechano-responsiveness, the used loading resulted in only marginal increases in the bones of the mature animals, while significant exercise-induced increases were observed virtually in all bone traits among the senescent rats.

Conclusion: The bones of senescent rats displayed a clear ability to respond to an exercise regimen that failed to initiate an adaptive response in mature animals. Thus, our observations suggest that the pathogenesis of age-related osteoporosis is not attributable to impaired mechano-responsiveness of aging skeleton. It also seems that strengthening of even senescent bones is possible – naturally provided that safe and efficient training methods can be developed for the oldest old.

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* E-mail: teppo.jarvinen@uta.fi

Introduction

The primary evolutionary function of the bones is to bear the muscle contraction- and gravity-induced mechanical forces exerted on them without breaking, and ultimately, to enable the efficient locomotion of the body [1]. To successfully carry out this locomotive function, the bone tissue is equipped with a mechano-sensory system that facilitates the skeletal adaptation to loading. In essence, bones first *sense* the loading-induced deformation and then elicit a *response* that eventually results in an appropriate modification of the bone structure, if required, to cope with the altered loading milieu (Figure 1A). It has been recently proposed that the pathogenesis of age-related osteoporosis (i.e., the gradual loss of mineral from bones with aging) would be attributable to a failure of this control system [2]: either the mechano-sensitivity of bones is reduced [3,4] or the capacity of bones to respond to loading is weakened. An alternative pathomechanistic theory suggests that bone loss in senescence represents simply an appropriate response to reduced loading in a less active host [4] (Figure 1B).

Regarding the skeletal mechano-responsiveness *per se*, both systemic factors (hormones such as estrogen and growth hormone) [5–12] and local factors (growth factors such as insulin-like growth factor 1 and 2) [13,14] have been shown to have a direct modulatory effect. Also, individual responses to mechanical stimuli have been shown to depend on genetics [15] and gender [16,17], whereas the influence of age on bone mechano-responsiveness has remained controversial [3,18–20]. The accumulation of adipocytes to the bone marrow during aging has been speculated to accelerate endocortical resorption [21], whereas it has been shown that periosteal expansion continues well into old age, particularly in men, implying that the mechanosensory system may be properly functioning [22–24]. Experimental studies have shown that the responsiveness of the aged skeleton is increased [19], reduced [18,25], or unaffected [26–28]. In our previous study [28], we showed that the ability of bones of young (5–19 week old) and mature (33–47 week old) male rats to adapt to treadmill-running - induced loading was similar, but the adaptive mechanisms differed; in response to given exercise, the growing bones primarily

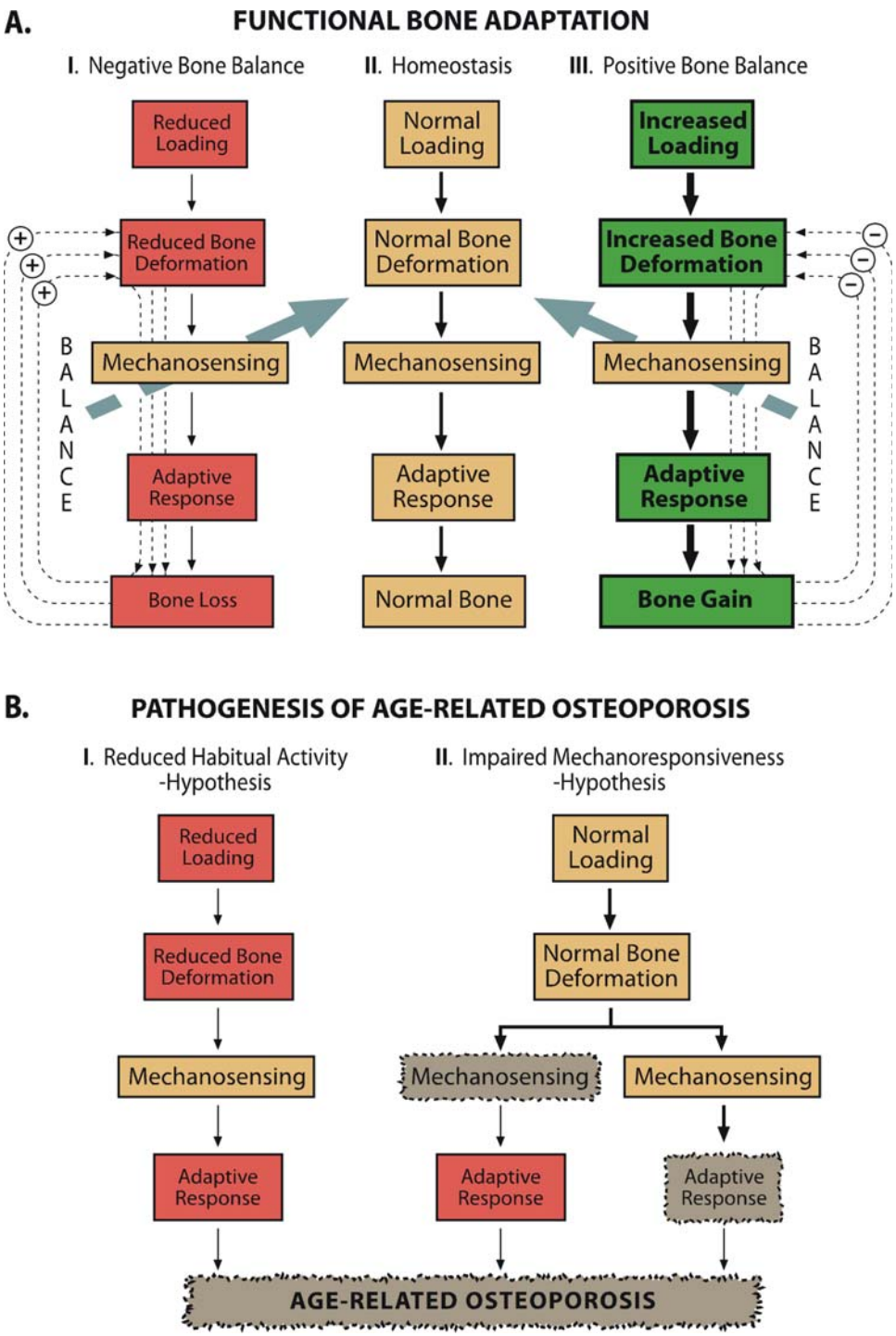

Figure 1. Functional Bone Adaptation (A) and the Proposed Hypothesis for Age-related Osteoporosis (B).
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increased cross-sectional size, while the mature bones mainly increased bone density.

Accordingly, the objective of this study was to evaluate whether the skeleton can maintain its capability to respond to increased loading until very old age (senescence). The timing of the increased exercise loading was chosen to coincide appropriate phases of the rat lifespan: maturity and senescence. The mature rats have stopped the longitudinal growth and reached the peak bone mass, while the senescent rats represented the ultimate group in terms of age as judged from more than 50% mortality among control animals at the end of the experiment.

Materials and Methods

Animals

The sample size used in this study was based on *a priori* knowledge on natural loss of older animals [29,30], the expected loss being 20% and 50% in the mature and senescent age groups,

Table 1. The Number of Rats at Different Period of the Experiment.

	At the Beginning of the Experiment	At the End of the Experiment
MALES		
MATURE CONTROLS	23	16
MATURE EXERCISED	29	22
SENESCENT CONTROLS	24	10
SENESCENT EXERCISED	32	14
FEMALES		
MATURE CONTROLS	22	16
MATURE EXERCISED	26	22
SENESCENT CONTROLS	23	10
SENESCENT EXERCISED	30	17

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Table 2. The Progressive Treadmill Exercise Regimen Used for Male Rats.

Week	Age (weeks)		Duration (min)	Speed (cm/s)	Inclination (deg)
	Mature	Senescent			
1	47	75	5	20	5
2	|	|	10	20	10
3	|	|	10	20	15
4	|	|	10	30	15
5	|	|	10	30	20
6	|	|	10	30	20
7	|	|	10	30	25
8	|	|	10	30	25
9	|	|	10	30	30
10	|	|	10	30	30
11	|	|	10	30	30
12	|	|	10	30	30
13	|	|	10	30	30
14	60	88	10	30	30

doi:10.1371/journal.pone.0002540.t002

respectively, and on the assumed standard deviation of ~11% in the breaking load of rat femur, the primary outcome [31]. To detect a significant ($p<0.05$) 10% loading-induced response in the breaking load of femur in the exercised groups (vs. controls) at 80% statistical power, a minimum of ~15 rats/group was required at the end of the experiment. Accordingly, a total of 108 male rats of the Sprague-Dawley strain were used in the experiment.

The rats were 3 weeks old at the beginning of the study. During the first 2 weeks of the study, all rats ran on a flat-bed treadmill at a slow speed (10–20 cm/s) for 3 minutes/day for 3 days a week to let the animals to adapt the treadmill running and to exclude those animals refusing to run (about 5% of the original population were removed). The rats were then randomly assigned into four groups: "Mature exercised", and "Senescent exercised"; and "Mature control", and "Senescent control" (Table 1, Figure 2). The animals were housed in cages (18×35×55 cm), four animals per cage, at 20°C with a light cycle of 12 h. They were fed standard laboratory chow and water *ad libitum*.

Exercise program

Both mature and senescent exercise groups were subjected to a progressive exercise program for 14 weeks (Table 2). The training began at the age of 47 in the Mature exercise group and at 75 weeks Senescent exercise group, respectively (Table 2, Figure 2). To corroborate (or refute) the findings of male rats, a similar experiment was also carried out using 101 female rats. The determination of sample size, as well as the acclimation and randomization procedures were carried out identically to males, but due to the known increased longevity (increased frailty) of the senescent female rats [32], the training protocol and starting age of training were slightly modified in comparison to males (Table 3, Figure 2).

After the exercise intervention, the exercised animals and their age-matched control animals were euthanized, and body weight and the weight of the uteri, if applicable, were measured. Femora were excised and stored at −20°C in small freezer bags wrapped in saline-soaked gauze bandages to prevent dehydration. This kind of storage has been shown not to affect bone's biomechanical properties [33,34]. Tibiae were excised and dehydrated in an ethanol series (30 and 70% ethanol) and stored in 70% ethanol. The research protocol was accepted by the Ethics Committee for Animal Experiments of the University of Tampere and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland. The study conformed to the NIH Guide for the Care and Use of Laboratory Animals.

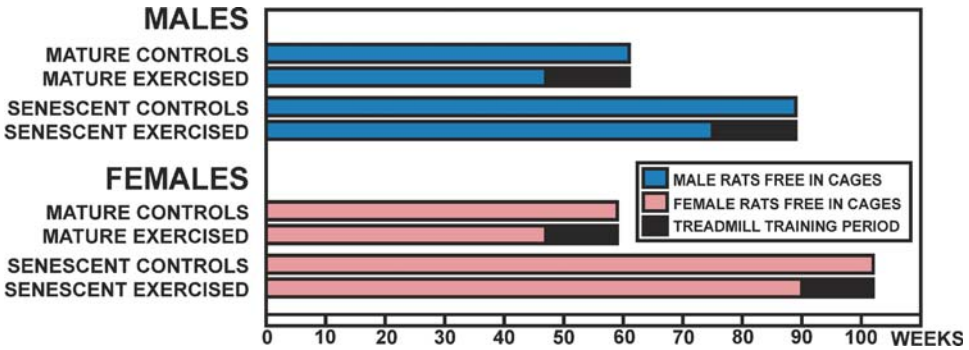

Figure 2. The Design of the Study.
doi:10.1371/journal.pone.0002540.g002

Table 3. The Progressive Treadmill Exercise Regimen Used for Female Rats.

Week	Age (weeks)		Duration (min)	Speed (cm/s)	Inclination (deg)
	Mature	Senescent			
1	47	90	5	20	5
2	|	|	5	20	10
3	|	|	5	20	15
4	|	|	5	30	15
5	|	|	5	30	20
6	|	|	5	30	20
7	|	|	5	30	25
8	|	|	5	30	25
9	|	|	5	30	30
10	|	|	5	30	30
11	|	|	5	30	30
12	58	101	5	30	30

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Bone analysis

At the day of testing, the femora were slowly thawed at the room temperature and kept wrapped in saline-soaked gauzes except during measurements. A digimatic caliper (Mitutoyo 500, Andover, United Kingdom) was used to measure the length of femora. In our laboratory, the coefficient of variation (CV_{rms}) for the determination of the length of the femora was 0.2% [35].

Peripheral quantitative computed tomography

The cross-sections of the femoral diaphysis and neck were scanned with peripheral quantitative computed tomography (pQCT, Stratec XCT Research M, software version 5.40B, Stratec Medizintechnik GmbH, Pforzheim, Germany). For the pQCT assessment of the diaphysis, the femur was inserted into a specially constructed plastic tube with the shaft in axial direction, and one cross-sectional slice was scanned at 50% of the length of the femur [28]. The voxel size was $0.070\times0.070\times0.5$ mm^3 and the scan speed was 3.0 mm/s. Total cross-sectional area (tCSA), cortical cross-sectional area (cCSA), and cortical bone mineral density (cBMD) were evaluated by the pQCT software using contour mode 1 (threshold 214 mg/cm^3) for tCSA and separation mode 1 for cCSA and cBMD (threshold 710 mg/cm^3). In our laboratory, the CV_{rms} in the femoral midshaft were 0.9% for the tCSA, 1.5% for the cCSA, and 0.6% for the cBMD [36].

For the pQCT assessment of the femoral neck, the femur was inserted into a specially constructed plastic tube with the femoral neck in an axial direction [16]. The scan line was adjusted to the midneck using the scout view option of the pQCT software. The voxel size and scan speed were the same as described above. Total cross-sectional area (tCSA), total bone mineral content (tBMC), and total bone mineral density (tBMD) were determined using contour mode 1 (threshold 214 mg/cm^3) for tCSA, tBMC, and tBMD. In our laboratory, the CV_{rms} were 3.9% for tCSA, 2.2% for tBMC and 2.1% for tBMD [36].

Mechanical testing

After the pQCT scanning, the right femora were tested mechanically. A Lloyd material testing machine (LR5K, J. J. Lloyd Instruments, Southampton, UK) was used for the anteroposterior three-point bending of the femoral shaft and compression of the femoral neck according to our standard protocols [35,37].

For the three-point bending, the femur was placed on its posterior surface on the lower supports of the bending apparatus. For each bone, these supports were placed individually (first just distal to the trochanter minor and the other just proximal to the condyles of the femur). After the anatomical adjustment of the supports, a bending load using a brass crossbar was applied to the femoral midshaft perpendicularly to the long axis of the bone until the failure of the specimen. The breaking load (F_{max}) of the femoral midshaft was determined from the load-deformation curve. In our laboratory, the CV_{rms} of the F_{max} for three point bending is 5.0% [35].

After the three-point bending of the femoral shaft, the proximal half of femur was mounted in a specially constructed fixation device [38] and a vertical load was applied to the top of the femoral head using a brass crossbar until failure of the femoral neck. The F_{max} of the femoral neck was determined from the load-deformation curve. In our laboratory, the CV_{rms} of the F_{max} for femoral neck compression is 7.6% [39].

Micro-computed tomography (μCT)

The proximal metaphysis of tibia were scanned using a high resolution micro-computed tomography system (μCT 35; Scanco Medical, Basserdorf, Switzerland) with nominal isotropic resolution of 12 μm. Three-dimensional analysis of trabecular bone was performed on the bone region 1 to 5 mm distal to the growth plate. Trabecular bone compartment was separated from the cortical bone by semi-automatically drawn contours and a global threshold was used to distinguish bone and marrow. The following parameters were determined from the trabecular bone using a direct three-dimensional approach [40]: total bone marrow volume including the trabeculae (TV; mm^3), trabecular bone volume (BV; mm^3), trabecular bone volume fraction (BV/TV), mean trabecular number (Tb.N; 1/mm), mean trabecular thickness (Tb.Th; mm), and mean trabecular separation (Tb.Sp; mm). For determination of cortical bone porosity, a 0.5 mm thick region of cortical bone at 7 mm distance from the proximal end of tibia was analyzed.

Statistical analysis

All data were analyzed using the SPSS for Windows (version 13.0). Relative exercise effects (i.e., the percent difference between exercised and control groups) and aging effects (i.e., the percent difference between mature and senescent groups) were tested using analysis of covariance (ANCOVA), and all data pertaining to mechanical competence of the femur (cCSA, tBMC, tCSA, and F_{max}) were statistically controlled for body weight and femoral length [16,28,36]. In all tests, an α level less than 5% ($p<0.05$) was considered statistically significant.

Results

Mortality was 28% and 57% among Mature and Senescent males, respectively (Table 1, Figure 3). The corresponding rates in females were 21% and 49%, respectively (Table 1, Figure 3). Estimated from this mortality, the age of the senescent groups corresponded to over 75 years old men and over 80 years old women in Finland [41]. Figure 3 shows the weight development curves of the rats in each group. The mean weights of the uteri were similar in all female groups.

Age-related osteoporosis

The influence of aging on bones (Mature vs. Senescent control rats) is summarized in Tables 4 and 5 (grey panels). Senescent control rats had significantly lower F_{max}, tBMC and tCSA at the

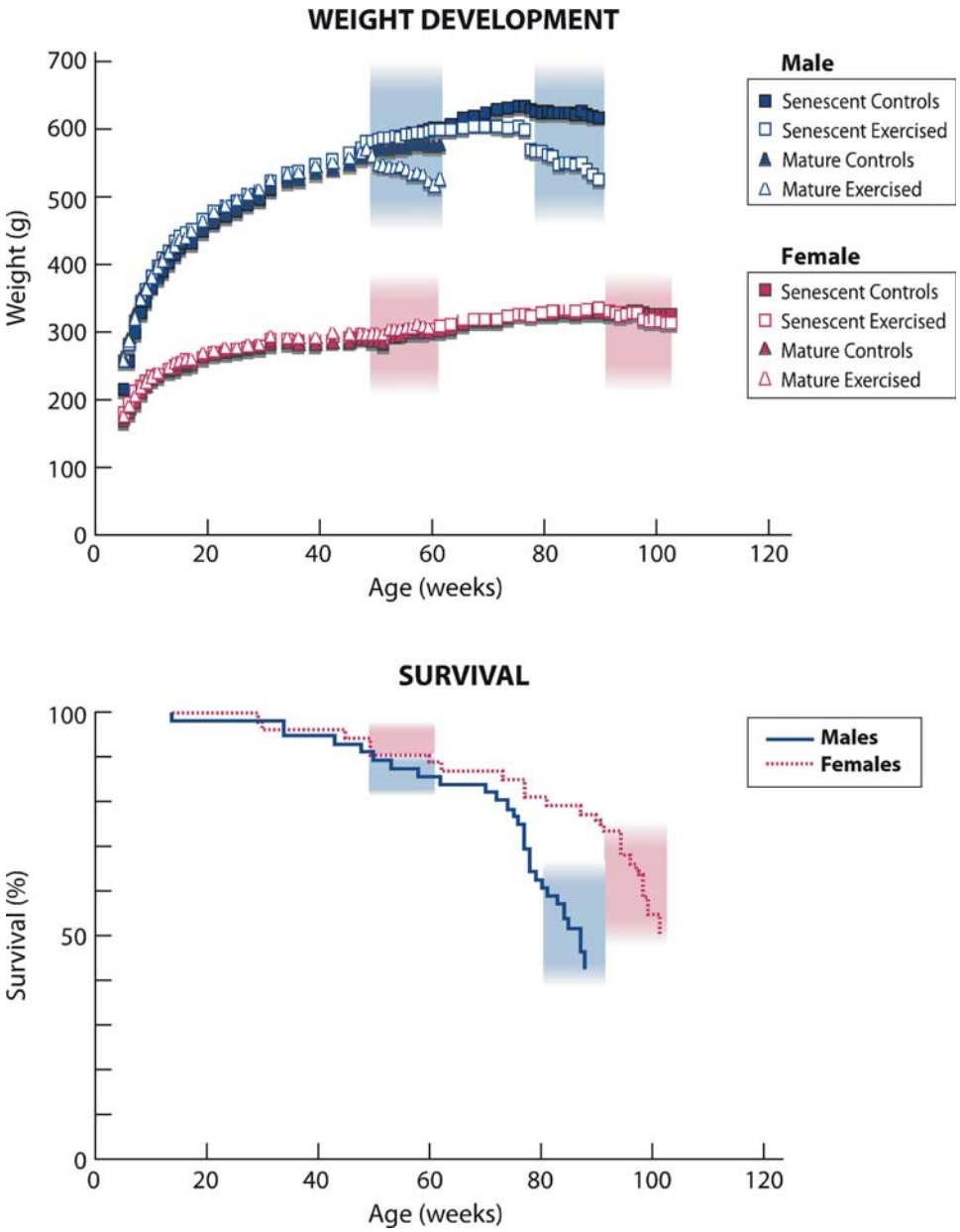

Figure 3. The Body Weight Curves and a Kaplan-Meier Plot Demonstrating the Survival of the Male and Female Rats in This Experiment.
doi:10.1371/journal.pone.0002540.g003

femoral neck in both sexes and F_{max} at the femoral midshaft in females as compared to corresponding Mature control rats. At the femoral midshaft, tCSA of the male rats and cBMD of the female rats were larger in the Senescent groups than in Mature groups. In the proximal tibia, the trabecular bone volume fraction (BV/TV) was significantly decreased in the Senescent rats when compared to the corresponding Mature group both in males and females. In males, also Tb.N., Tb.Sp., and cortical porosity differed significantly between Mature and Senescent groups, a finding in conjunction with reduced BV/TV indicating a deteriorated bone structure among old rats (Figure 4).

Exercise effects

Body weight and femoral length. In males, there was a significant exercise-related decrease in body weight: −8.2% ($p = 0.005$) and −15.7% ($p<0.001$) in Mature and Senescent groups, respectively (Table 4). In females, body weight was not influenced by exercise (Table 5). Femoral length was similar between exercised and control rats in male groups; whereas in Mature females the femur was 1.7% longer in exercise group than in control group ($p = 0.043$).

The geometric, densitometric, and biomechanical bone traits. Skeletal responses to increased exercise among the Mature and Senescent rats are depicted in Tables 4 and 5 and Figure 5. In the Mature groups, significant exercise-induced increases were observed: total cross-sectional area (tCSA) at the femoral diaphysis of the males increased 6% ($p = 0.018$) compared to age-matched controls, and total bone mineral density (tBMD) at the femoral neck of the females increased 6% ($p<0.001$) while its tCSA remained 8% ($p = 0.018$) smaller compared to controls.

Table 4. Descriptive Data of the Biomechanical and Tomographic Measurements and Interaction (Difference Between the Two Age-groups in the Exercise-effect) of the Male Rats.

	MATURE		SENESCENT		Age-related change (p)	Mech.responsiveness vs. Age, Interaction (p)
	CONTROL	EXERCISED	CONTROL	EXERCISED		
	MeanSEM	MeanSEM	MeanSEM	MeanSEM		
BASIC DESCRIPTIVES						
Body weight (g)	57411	52810[b]	60214	50814[a]	0.076	0.032
Femoral length (mm)	42.00.2	41.90.3	42.00.3	42.30.4	0.334	0.235
FEMORAL NECK						
tBMC (mg/mm) *	6.00.1	6.20.1	5.40.2[e]	6.30.2[b]	0.003	0.035
tBMD (mg/cm^3)	104117	107815	105716	101721[f]	0.470	0.039
tCSA (mm^2) *	5.90.2	5.80.2	5.20.3[f]	6.10.2[c]	0.024	0.027
Fmax (N) *	1726	1795	1488[f]	1646	0.018	0.647
FEMORAL MIDSHAFT						
cBMD (mg/cm^3)	14815	14746	14729	14617	0.106	0.769
tCSA (mm^2) *	15.90.3	16.50.3[c]	17.10.4[e]	17.90.4[e]	0.004	0.751
cCSA (mm^2) *	9.40.2	9.70.2	9.30.3	9.90.2	0.759	0.353
Fmax (N) *	1837	1976	1669	1898	0.130	0.756
PROXIMAL TIBIA						
Trabecular TV (mm^3)	57.11.4	57.73.1	59.13.1	62.11.7	0.509	0.591
Trabecular BV (mm^3)	8.480.38	8.921.25	7.220.63	6.390.43[f]	0.080	0.319
Trabecular BV/TV (ratio)	0.150.01	0.150.02	0.120.01[f]	0.100.01[e]	0.031	0.227
Tb.N (1/mm)	2.310.06	2.390.13	1.950.10[e]	1.880.06[e]	0.004	0.391
Tb.Th (mm)	0.0850.001	0.0840.003	0.0860.003	0.0800.003	0.725	0.300
Tb.Sp (mm)	0.420.01	0.410.03	0.510.03[e]	0.530.02[e]	0.003	0.512
Cortical porosity (ratio)	0.0070.001	0.0090.001	0.0170.004[e]	0.0160.002[f]	0.007	0.597

[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ vs. corresponding control group; [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ vs. corresponding Mature group.
*values adjusted with body weight and femoral length; for details, see Statistical analysis.
tBMC, total bone mineral content; tBMD, total bone mineral density; tCSA, total cross-sectional area; Fmax, breaking load; cBMD, cortical bone mineral density; cCSA, cortical cross-sectional area; TV, total bone marrow volume; BV, bone volume; Tb.N, mean trabecular number; Tb.Th, mean trabecular thickness; Tb.Sp, mean trabecular separation.
doi:10.1371/journal.pone.0002540.t004

Among the senescent rats significant exercise-induced between-group effects were observed virtually in all bone traits; both tCSA and bone mineral content (tBMC) at the femoral neck increased 19% ($p = 0.003$) and 18% ($p = 0.030$) in males and 10% ($p = 0.026$) and 10% ($p = 0.001$) in females, respectively. Also, breaking load (F_{max}) both at the femoral neck and femoral diaphysis of senescent females increased 16% ($p = 0.045$) and 19% ($p = 0.026$), respectively; while in the senescent males F_{max} at the femoral neck increased 18% ($p = 0.087$). No differences between exercised and control rats were observed in proximal tibia in any of the bone traits determined using micro-CT analysis.

Age and the mechano-responsiveness of bone

An age-effect on bone mechano-responsiveness (interaction between age and exercise loading) was observed at the femoral neck. The exercise-effect was significantly greater in the Senescent group for tBMC ($p = 0.035$ and $p = 0.002$) and tCSA ($p = 0.027$ and $p = 0.001$) both in males and females, respectively (Tables 4 and 5 and Figure 5). The accompanying significant decrease in tBMD ($p = 0.039$ and $p = 0.022$, in males and females respectively) indicated that the exercise-effect was more pronounced in tCSA than in tBMC. As regards bone strength, the mean exercise-effects on F_{max} were greater in the Senescent group, but the group-difference reached statistical significance only at the femoral diaphysis in females ($p = 0.032$) (Figure 5).

Discussion

Bone functional bone adaptation [42–46] is one of the cardinal principles in skeletal biology depicting a homeostatic feedback system evolved to maintain the skeletal integrity in different loading milieus through appropriate modifications in bone geometry and structure, and/or material properties - with or without changes in bone mass. Accordingly, any substantial change either in the sensitivity of the mechano-sensory system or in the balance between predominant bone loading and coexisting bone rigidity results in an adaptive response to keep the tissue deformations within the predetermined physiological window [42,43,45]. In this context, the occurrence of age-related osteoporosis, or the gradual bone loss with aging, has been attributed to the failure of this mechano-sensory mechanism [3,4]. In our experiment, the senescent rats displayed a clear age-related osteoporosis, manifest as deteriorated bone structure and reduced bone structural strength (Tables 4 and 5). Nevertheless, these animals also showed a positive adaptive response to exercise while much less consistent response was seen in the mature rats

Table 5. Descriptive Data of the Biomechanical and Tomographic Measurements and Interaction (Difference Between the Two Age-groups in the Exercise-effect) of the Female Rats.

	MATURE		SENESCENT		Age-related change (p)	Mech.responsiveness vs. Age, Interaction (p)
	CONTROL	EXERCISED	CONTROL	EXERCISED		
	MeanSEM	MeanSEM	MeanSEM	MeanSEM		
BASIC DESCRIPTIVES						
Body weight (g)	3079	3127	3137	2985	0.634	0.208
Femoral length (mm)	35.90.2	36.60.2[c]	36.00.3	36.00.2	0.901	0.173
Uterus weight (g)	1.50.1	1.40.1	1.90.2	1.50.1	0.123	0.158
FEMORAL NECK						
tBMC (mg/mm) *	5.10.1	5.00.1	4.70.1[f]	5.20.1[b]	0.024	0.002
tBMD (mg/cm^3)	112915	11938[a]	116620	116414	0.155	0.022
tCSA (mm^2) *	4.50.1	4.20.1[c]	4.00.1[f]	4.50.1[cf]	0.015	0.001
Fmax (N) *	1245	1304	1016[e]	1195[c]	0.008	0.226
FEMORAL MIDSHAFT						
cBMD (mg/cm^3)	14862	14882	14974[e]	14994[f]	0.009	0.933
tCSA (mm^2) *	10.70.1	10.70.1	10.80.2	11.10.1[f]	0.648	0.247
cCSA (mm^2) *	6.60.1	6.50.1	6.50.1	6.80.1[f]	0.424	0.055
Fmax (N) *	1444	1464	1235[f]	1444[c]	0.014	0.032
PROXIMAL TIBIA						
Trabecular TV (mm^3)	38.72.0	38.62.4	39.31.4	38.51.2	0.820	0.851
Trabecular BV (mm^3)	9.601.02	9.950.80	7.280.76	7.790.42[f]	0.087	0.904
Trabecular BV/TV (ratio)	0.250.02	0.260.01	0.190.02[f]	0.200.01[e]	0.048	0.666
Tb.N (1/mm)	3.560.29	3.840.14	2.990.15	3.140.10[d]	0.108	0.694
Tb.Th (mm)	0.0770.004	0.0730.001	0.0700.003	0.0730.002	0.204	0.187
Tb.Sp (mm)	0.280.04	0.240.01	0.320.02	0.300.01[d]	0.283	0.704
Cortical porosity (ratio)	0.0060.000	0.0070.001	0.0070.001	0.0060.000	0.205	0.058

[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ vs. corresponding control group; [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ vs. corresponding Mature group.
*values adjusted with body weight and femoral length; for details, see Statistical analysis.
tBMC, total bone mineral content; tBMD, total bone mineral density; tCSA, total cross-sectional area; Fmax, breaking load; cBMD, cortical bone mineral density; cCSA, cortical cross-sectional area; TV, total bone marrow volume; BV, bone volume; Tb.N, mean trabecular number; Tb.Th, mean trabecular thickness; Tb.Sp, mean trabecular separation.
doi:10.1371/journal.pone.0002540.t005

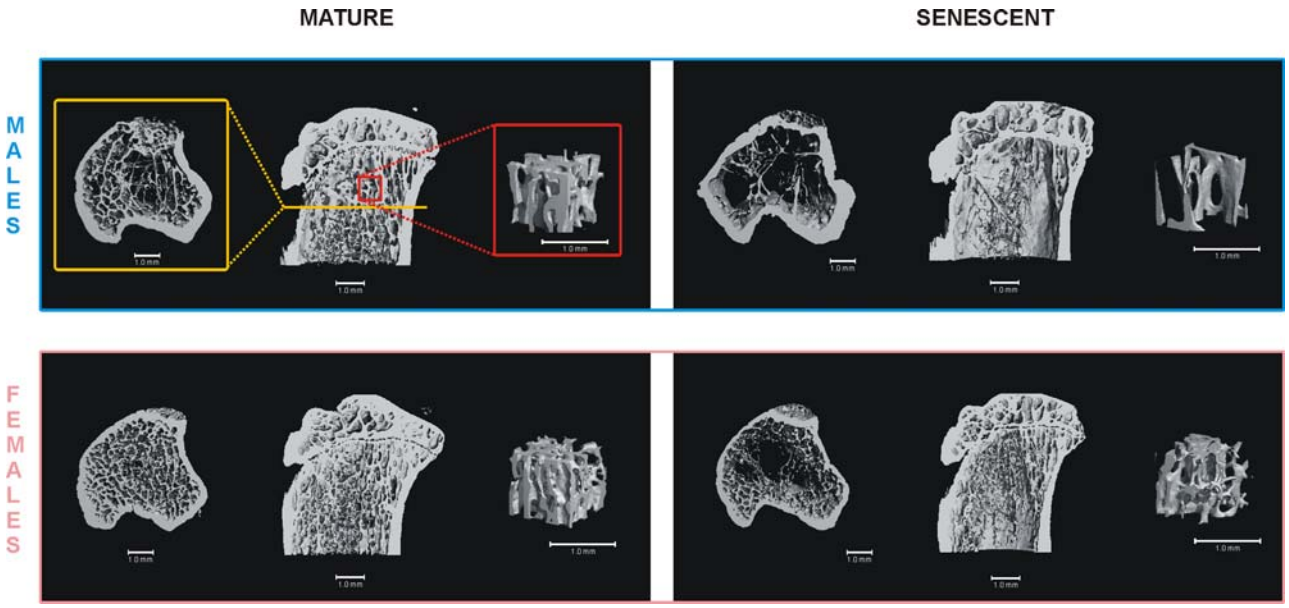

Figure 4. Effects of Aging on the Trabecular Bone Texture in the Proximal Tibial Metaphysis. Due to aging, the proportion of trabecular bone of the bone volume (TV/BV) is decreased in males and females. In addition, in males, the number (Tb.N.) and thickness (Tb.Th.) of the trabeculae is decreased, while the distance between individual trabeculae (Tb.Sp.) is increased.
doi:10.1371/journal.pone.0002540.g004

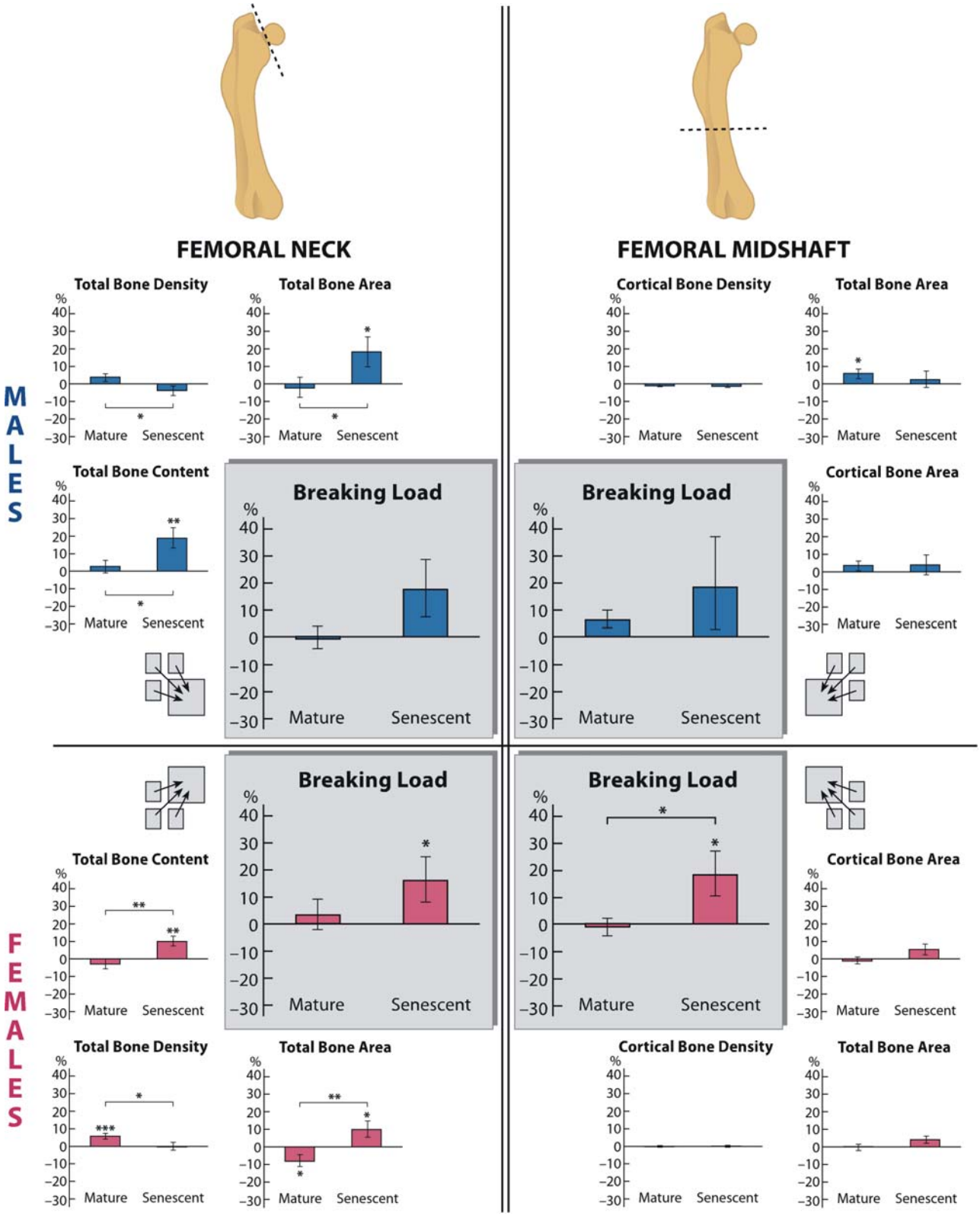

Figure 5. Exercise Effect on Different Bone Traits of the Femoral Neck and Femoral Midshaft in Mature and Senescent Male and Female Rats. Bars represent percent (%) increases (± the standard error of the mean, SEM) of the exercise group compared to corresponding control group at the end of the treadmill exercise intervention in the femoral neck total bone content (tBMC); total bone density (tBMD); total bone area (tCSA); cortical bone density (cBMD); cortical bone area (cCSA); and breaking load (F_{max}). Significant differences between the exercised rats and their controls, and between the two age-groups in the exercise-effect, are indicated: *$p<0.05$; **$p<0.01$; ***$p<0.001$. Results for tBMC, tCSA, cCSA, and F_{max} are adjusted for body weight and femoral length.
doi:10.1371/journal.pone.0002540.g005

subjected to the same exercise regimen (Figure 5). This finding challenges the reduced mechano-sensitivity at senescence as the pathomechanism of age-related osteoporosis.

We therefore speculate that the enhanced mechano-responsiveness among the senescent animals was attributable to the apparent fact that their bones were initially less rigid because of essentially diminished habitual activity in aged rats [47]. However, as a consequence of additional treadmill training, the bones were subjected to increased loading, that being clearly beyond that experienced during normal living in terms of magnitude and intensity. These exercise-induced deformations then resulted in the adaptive response observed in the bones of Senescent animals. In the Mature rats, in turn, their fully developed skeleton and relatively higher habitual activity ensured readily an appropriate mechanical competence for the treadmill running, and there remained only a marginal room to respond to mechanical stimulus caused by additional treadmill training. These observations also suggest a biomechanical explanation for the apparent *direct* modulatory effect of aging on the periosteal apposition: rather than originating from the effect of aging *per se* on the periosteum, it seems that the aging-associated periosteal enlargement is an adaptive response to cope with endocortical loss of mineral (the imminent decrease in bone rigidity). As described above, any change either in the loading subjected on the bone or its strength (structural rigidity) necessitates an adaptive response to restore the delicately controlled stress-strain equilibrium.

Although our study was a randomized controlled trial using rats of controlled genetics, large sample size, long intervention period and well-validated methodology [16,28,36,48,49], it had some limitations that require consideration. First, bone deformations during running were not measured. Instead, our conclusions relied on a simple engineering principle that equal loading imposed on a less rigid bone produces greater deformations and consequently larger response and vice versa. Thus, it needs to be noted that our paper does not deal with the mechano-sensitivity of bones between Mature and Senescent animals. As discussed above, the treadmill training -induced strain stimulus may not have been sufficient for bone formation activity [50,51] for mature animals with inherently more rigid bones, while a more vigorous loading would have been necessary to induce an osteogenic response in mature animals. Here the quite liberally used terms 'mechano-sensitivity' and 'mechano-responsiveness' need to be distinguished from each other. In the most stringent sense, these two terms depict distinct phases of functional bone adaptation -cascade (Figure 1A). It is indeed possible that aging disproportionately affects the skeletal mechano-sensing and responsiveness (Figure 1B) and a failure in the former could be only verified with direct strain measurements; i.e., a similar strain environment would lead to smaller response among old animals than among younger, mature animals. However, notwithstanding this possibility, we highlight that our finding of a significant *adaptive response* to increased exercise loading (i.e., increase in most bone traits, including bone strength) in senescent animals shows that the homeostatic control system of the skeleton functions even in the very old age and the skeletal responsiveness is not impaired.

One might find the lack of exercise-induced increases in bone characteristics in the mature animals somewhat controversial to findings of our previous study [28], in which the exercise-induced benefits were seen among adult male rats (33 to 47-week-old during the study) subjected to the same treadmill training protocol. However, in that study, the adult animals were still growing axially. We therefore feel that the observed difference in the skeletal responsiveness between these two groups of mature animals actually underpins the importance of the longitudinal growth period as an opportune window to enhance of impact of mechanical loading on bone [52–60]. Also, the present senescent rats represent the extreme in terms of age; in agreement with the increased mortality, the aged animals displayed deteriorated bone traits and a decreased body weight (particularly in males) (Figures 3–5 and Tables 4 and 5), all changes characteristic of senescence [61].

The present findings do not allow one to make conclusions about the potential influence of gender on the mechano-responsiveness of bones, since there were apparent differences in the survival and functional capacity of the aged animals rendering the study designs in males and females basically different (distinct age at entry of the initiation of exercise in senescent animals and different treadmill training protocols). In essence, due to the increased longevity of female rats and the resulting increased frailty, we felt compelled to subject the senescent females to a less physically challenging exercise regimen. However, the effect of gender on the skeletal responsiveness to loading has been previously assessed [16,17,62–66], suggesting that males are more responsive to loading than females.

In conclusion, our results demonstrate that concerning the mass, structure, and mechanical competence of rat bones, the homeostatic loading-driven regulatory feedback system maintains its capacity to respond to increased exercise loading even into very old age. Accordingly, it is unlikely that the pathogenesis of age-related osteoporosis would be attributable solely, if at all, to a failure in this system. Thus, our observations suggest that strengthening of senescent human bones is also possible – naturally provided that safe and efficient training methods can be developed for the oldest old.

Author Contributions

Conceived and designed the experiments: TJ HS OL. Performed the experiments: OL JJ IP. Analyzed the data: TJ HS OL JJ. Wrote the paper: TJ HS PK OL JJ IP.

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Table 4. Descriptive Data of the Biomechanical and Tomographic Measurements and Interaction (Difference Between the Two Age-groups in the Exercise-effect) of the Male Rats.

	MATURE		SENESCENT			
	CONTROL	EXERCISED	CONTROL	EXERCISED		
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Age-related change (p)	Mech.responsiveness vs. Age, Interaction (p)
BASIC DESCRIPTIVES						
Body weight (g)	574 ± 11	528 ± 10[b]	602 ± 14	508 ± 14[a]	0.076	0.032
Femoral length (mm)	42.0 ± 0.2	41.9 ± 0.3	42.0 ± 0.3	42.3 ± 0.4	0.334	0.235
FEMORAL NECK						
tBMC (mg/mm) *	6.0 ± 0.1	6.2 ± 0.1	5.4 ± 0.2[e]	6.3 ± 0.2[b]	0.003	0.035
tBMD (mg/cm^3)	1041 ± 17	1078 ± 15	1057 ± 16	1017 ± 21[f]	0.470	0.039
tCSA (mm^2) *	5.9 ± 0.2	5.8 ± 0.2	5.2 ± 0.3[f]	6.1 ± 0.2[c]	0.024	0.027
Fmax (N) *	172 ± 6	179 ± 5	148 ± 8[f]	164 ± 6	0.018	0.647
FEMORAL MIDSHAFT						
cBMD (mg/cm^3)	1481 ± 5	1474 ± 6	1472 ± 9	1461 ± 7	0.106	0.769
tCSA (mm^2) *	15.9 ± 0.3	16.5 ± 0.3[c]	17.1 ± 0.4[e]	17.9 ± 0.4[e]	0.004	0.751
cCSA (mm^2) *	9.4 ± 0.2	9.7 ± 0.2	9.3 ± 0.3	9.9 ± 0.2	0.759	0.353
Fmax (N) *	183 ± 7	197 ± 6	166 ± 9	189 ± 8	0.130	0.756
PROXIMAL TIBIA						
Trabecular TV (mm^3)	57.1 ± 1.4	57.7 ± 3.1	59.1 ± 3.1	62.1 ± 1.7	0.509	0.591
Trabecular BV (mm^3)	8.48 ± 0.38	8.92 ± 1.25	7.22 ± 0.63	6.39 ± 0.43[f]	0.080	0.319
Trabecular BV/TV (ratio)	0.15 ± 0.01	0.15 ± 0.02	0.12 ± 0.01[f]	0.10 ± 0.01[e]	0.031	0.227
Tb.N (1/mm)	2.31 ± 0.06	2.39 ± 0.13	1.95 ± 0.10[e]	1.88 ± 0.06[e]	0.004	0.391
Tb.Th (mm)	0.085 ± 0.001	0.084 ± 0.003	0.086 ± 0.003	0.080 ± 0.003	0.725	0.300
Tb.Sp (mm)	0.42 ± 0.01	0.41 ± 0.03	0.51 ± 0.03[e]	0.53 ± 0.02[e]	0.003	0.512
Cortical porosity (ratio)	0.007 ± 0.001	0.009 ± 0.001	0.017 ± 0.004[e]	0.016 ± 0.002[f]	0.007	0.597

[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ vs. corresponding control group; [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ vs. corresponding Mature group

* values adjusted with body weight and femoral length; for details, see Statistical analysis

tBMC, total bone mineral content; tBMD, total bone mineral density; tCSA, total cross-sectional area; Fmax, breaking load; cBMD, cortical bone mineral density; cCSA, cortical cross-sectional area; TV, total bone marrow volume; BV, bone volume; Tb.N, mean trabecular number; Tb.Th, mean trabecular thickness; Tb.Sp, mean trabecular separation.

Table 5. Descriptive Data of the Biomechanical and Tomographic Measurements and Interaction (Difference Between the Two Age-groups in the Exercise-effect) of the Female Rats.

	MATURE		SENESCENT			
	CONTROL	EXERCISED	CONTROL	EXERCISED		
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Age-related change (p)	Mech.responsiveness vs. Age, Interaction (p)
BASIC DESCRIPTIVES						
Body weight (g)	307 ± 9	312 ± 7	313 ± 7	298 ± 5	0.634	0.208
Femoral length (mm)	35.9 ± 0.2	36.6 ± 0.2[c]	36.0 ± 0.3	36.0 ± 0.2	0.901	0.173
Uterus weight (g)	1.5 ± 0.1	1.4 ± 0.1	1.9 ± 0.2	1.5 ± 0.1	0.123	0.158
FEMORAL NECK						
tBMC (mg/mm) *	5.1 ± 0.1	5.0 ± 0.1	4.7 ± 0.1[f]	5.2 ± 0.1[b]	0.024	0.002
tBMD (mg/cm^3)	1129 ± 15	1193 ± 8[a]	1166 ± 20	1164 ± 14	0.155	0.022
tCSA (mm^2) *	4.5 ± 0.1	4.2 ± 0.1[c]	4.0 ± 0.1[f]	4.5 ± 0.1[cf]	0.015	0.001
Fmax (N) *	124 ± 5	130 ± 4	101 ± 6[e]	119 ± 5[c]	0.008	0.226
FEMORAL MIDSHAFT						
cBMD (mg/cm^3)	1486 ± 2	1488 ± 2	1497 ± 4[e]	1499 ± 4[f]	0.009	0.933
tCSA (mm^2) *	10.7 ± 0.1	10.7 ± 0.1	10.8 ± 0.2	11.1 ± 0.1[f]	0.648	0.247
cCSA (mm^2) *	6.6 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.8 ± 0.1[f]	0.424	0.055
Fmax (N) *	144 ± 4	146 ± 4	123 ± 5[f]	144 ± 4[c]	0.014	0.032
PROXIMAL TIBIA						
Trabecular TV (mm^3)	38.7 ± 2.0	38.6 ± 2.4	39.3 ± 1.4	38.5 ± 1.2	0.820	0.851
Trabecular BV (mm^3)	9.60 ± 1.02	9.95 ± 0.80	7.28 ± 0.76	7.79 ± 0.42[f]	0.087	0.904
Trabecular BV/TV (ratio)	0.25 ± 0.02	0.26 ± 0.01	0.19 ± 0.02[f]	0.20 ± 0.01[e]	0.048	0.666
Tb.N (1/mm)	3.56 ± 0.29	3.84 ± 0.14	2.99 ± 0.15	3.14 ± 0.10[d]	0.108	0.694
Tb.Th (mm)	0.077 ± 0.004	0.073 ± 0.001	0.070 ± 0.003	0.073 ± 0.002	0.204	0.187
Tb.Sp (mm)	0.28 ± 0.04	0.24 ± 0.01	0.32 ± 0.02	0.30 ± 0.01[d]	0.283	0.704
Cortical porosity (ratio)	0.006 ± 0.000	0.007 ± 0.001	0.007 ± 0.001	0.006 ± 0.000	0.205	0.058

[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ vs. corresponding control group; [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ vs. corresponding Mature group

* values adjusted with body weight and femoral length; for details, see Statistical analysis

tBMC, total bone mineral content; tBMD, total bone mineral density; tCSA, total cross-sectional area; Fmax, breaking load; cBMD, cortical bone mineral density; cCSA, cortical cross-sectional area; TV, total bone marrow volume; BV, bone volume; Tb.N, mean trabecular number; Tb.Th, mean trabecular thickness; Tb.Sp, mean trabecular separation.