Doctoral Thesis

A combined experimental and computational model for genetic control of micro structural bone adaptation

Author(s):
Webster, Duncan James

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A Combined Experimental and Computational Model for Genetic Control of Micro Structural Bone Adaptation

A dissertation submitted to the ETH Zurich for the degree of Doctor of Science

presented by Duncan James Webster MEng University of Bath born 20th July, 1976 Doncaster, South Yorkshire, United Kingdom

accepted on the recommendation of Prof. Dr. Ralph Müller, examiner Prof. Dr. Franz Weber, co-examiner

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Summary

Osteoporosis is a disease characterized by an excessive decrease in bone mass leading to an increased susceptibility to skeletal fracture and deformation. To treat the disease medical research is attempting to target genes which define osteoporosis using the mouse as a model. Owing to the recent deciphering of the mouse genome and the high homology that exists between the human and mouse genomes, inbred strains of mice represent ideal models for genetic studies. Using the mouse to identify genes implicated in the bone remodeling process could therefore lead to advances in understanding which may enable the precise regulation of genes and proteins responsible for particular bone phenotypes i.e. bone mineral density or bone strength. One interesting phenotype under investigation is the response of bone to mechanical loading or its ‘Mechano-sensitivity’. Mechanical loading is the most important physiological/environmental factor regulating bone mass and shape. It has been demonstrated in humans that cyclic overloading enhances bone mass in both cortical and trabecular components. An understanding of the biological pathways (from gene expression to protein function) governing load stimulated bone formation could provide opportunities to mimic or augment bone mechano-sensitivity using pharmacological agents thereby leading to the development of novel strategies in the management of osteoporosis. The goal of this thesis was therefore to establish an in vivo model in two genetically distinct mouse strains which exhibit contrasting degrees of mechano-sensitivity so as to facilitate the study of load regulated genes in both cortical and trabecular bone. Furthermore we also sought to investigate the quantitative relationship between the micro-mechanical environment and subsequent bone morphology so as to better understand the drivers of load induced bone formation.

To investigate the genetic regulation of mechanical loading, a Caudal Vertebrae Axial compression Device (CVAD) was developed to mechanically stimulate the fifth caudal vertebrae (C5) of C57BL/6 (B6) and C3H/Hej (C3H) female mice via two pins inserted into the adjacent vertebrae (C4 and C6). Preliminary in vitro studies showed that the device was capable of delivering dynamic load profiles consisting of 14 bouts of 3’000 cycles at a frequency of 10Hz and maximum amplitude of 8N without compromising the structural integrity of the proposed loading configuration and with errors of less than 1%. The attachment of micro-strain gages to the cortical shell also showed that the applied dynamic load was successfully transmitted to the target vertebrae, despite the interconnecting, viscoelastic vertebral discs.
In vivo loading trials were performed in which both C3H and B6 strains were loaded dynamically (frequency: 10Hz, 3 times a week for 4 weeks) with amplitudes of 0N, 2N, 4N and 8N. For a load of 8N, at trabecular sites bone volume density (BV/TV) increased by 25.9% and 14.2%, relative to the 0N group in B6 and C3H strains respectively (P < 0.05) as measured by µCT. In the case of B6 mice this was accompanied by a significant global increase of 21.9% (P < 0.001) in trabecular thickness (Tb.Th) and local significant increases of up to 14.6% (P < 0.001) in trabecular number (Tb.N) suggesting that a fully anabolic model was obtained. At cortical sites a near significant 11% (P =0.06) global increase in bone volume (BV) was reported by the B6 loading group together with significant local changes of up to 17.6% (P < 0.001). No significant changes were reported at cortical sites for the C3H strain. This study showed that both in terms of absolute and percentage increases in bone mass B6 mice were more responsive to mechanical loading.

To investigate the relationship between the micromechanical environment and subsequent bone formation a combined experimental and computational approach was used. Here successfully validated FE models were used to determine both cortical and trabecular micro-structural stresses and strains specific to the target vertebra. Mean regional strains from the target vertebrae were then correlated with a mean regional bone formation index, determined for the second loading study using B6 mice. For a load of 8N strong linear correlations were found to exist between the bone formation index and strain energy density (SED) for cortical bone ($R^2 = 0.82$, $P < 0.001$), however surprisingly no correlations existed for trabecular bone ($R^2 = -0.072$). Whilst contesting current theory and suggesting different mechanisms drive cortical and trabecular bone formation this study highlighted the disadvantages of cross sectional morphological data sets and the potential weakness of modeling the target vertebrae as a homogeneous, isotropic material.

In conclusion, by demonstrating that both mechanical load and genetic background regulate the degree of bone adaptation in vivo, the work of this thesis has successfully established a model for the study of load induced bone adaptation. This will facilitate future studies aimed at investigating the biochemical pathways involved in bone formation.
Zusammenfassung


Das Ziel dieser Arbeit war deshalb die Etablierung eines Modells im lebenden Organismus in zwei genetisch unterschiedlichen Mausstämmen, welche gegensätzliche mechano-sensitive Eigenschaften aufweisen. Dies erleichtert die Untersuchung von Genen, welche durch Belastungen reguliert sind, sowohl im kortikalen wie auch im trabekulären Knochen.

Zusätzlich gilt ein Augenmerk der Untersuchung der quantitativen Beziehung zwischen der micro-mechanischen Umgebung und der sich anpassenden Knochenmorphologie. Damit können die antreibenden Komponenten der belastungsinduzierten Knochenumwandlung besser verstanden werden. Um die genetische Regulation der mechanischen Belastung zu untersuchen wurde ein Gerät zur axialen Kompression der Schwanzwirbel entwickelt (Caudal Vertebrae Axial compression Device; CVAD). Dieses ermöglicht die mechanische Stimulation des fünften
Schwanzwirbels (C5) und wurde an den weiblichen Mäusen der Stämme C57BL/6 (B6) und C3H/Hej (C3H) mittels zweier in die anliegenden Wirbelkörper eingeführten Nadeln angewendet. Vorausgehende in vitro Studien zeigten die Eignung des Gerätes zur Überbringung der dynamischen Belastungsprofile, welche aus 14 Wiederholungen zu je 3000 Zyklen bei einer Frequenz von 10 Hz und einer maximalen Amplitude von 8N bestehen. Dabei wird die strukturelle Integrität der gewünschten Belastungsconfiguration nicht beeinträchtigt und der Fehler beträgt weniger als 1%.

Dehnungsmessstreifen, welche direkt auf die kortikale Oberfläche geklebt wurden, zeigten zudem, dass die dynamische Belastung, trotz der angrenzenden, viskoelastischen Bandscheiben, erfolgreich auf den gewünschten Wirbelkörper übertragen wurde.

Dynamische Belastungsversuche wurden in vivo an beiden Mausstämmen C3H and B6 durchgeführt (Frequenz: 10Hz, dreimal die Woche während 4 Wochen). Dabei wurden Amplituden von 2N, 4N und 8N verwendet. Bei einer Belastung von 8N erhöhte sich die trabekuläre Knochendichte (BV/TV), gemessen mittels µCT, um 25.9% und 14.2% in den Stämmen B6, respektive C3H (P < 0.05). Für den B6 Mausstamm war die Veränderung begleitet von einer signifikanten globalen Zunahme der trabekulären Dicke (Tb.Th) um 21.9% (P < 0.001) und einer signifikanten Zunahme der lokalen Anzahl Knochenbälkchen von bis zu 14.6% (P < 0.001), was auf ein vollkommen anabolisches Modell hindeutet. Für den kortikalen Bereich konnte für die belastete B6 Gruppe eine annähernd signifikante globale Zunahme des Knochenvolumens (BV) von 11% (P = 0.06) und eine signifikante lokale Zunahme um 17.6% (P < 0.001) gefunden werden. Keine signifikanten Veränderungen konnte im kortikalen Knochen für den C3H Stamm gefunden werden. Diese Studie zeigte, dass bezüglich absoluter und prozentualer Zunahme der Knochenmasse die B6 besser auf mechanische Belastungen ansprechen als C3H Mäuse.

kortikalen Knochen überzeugende lineare Korrelationen zwischen dem Knochenentwicklungsindex und der spezifischen Verzerrungsenergie (SED) gefunden werden ($R^2 = 0.82, P < 0.001$). Erstaunlicherweise fand man aber für den trabekulären Knochen keine Korrelationen ($R^2 = -0.072$).

Währendem sich diese Studie mit bestehenden Theorien auseinandersetzt und auf verschiedene Mechanismen eingeht, welche die kortikale und trabekuläre Knochenbildung antreiben, zeigt sie auch deutlich die Nachteile von morphologischen Querschnittsdatensätzen und die mögliche Schwäche der homogenen und isotropen Materialmodellierung der Wirbelkörper auf. Zusammenfassend hat diese Doktorarbeit erfolgreich ein in vivo Modell hervorgebracht, welches zukünftige Studien zur Erforschung der biochemischen Pfade der Knochenbildung, erleichtern wird.
Chapter 1
Chapter 1

1. Introduction

Osteoporosis is a disease characterized by an excessive decrease in bone mass leading to an increased susceptibility to skeletal fracture and deformation, symptoms which can have a dramatic, negative impact on the quality of a person’s life, and which in more extreme cases can lead to death. A common misconception about this disease is that it is considered to only afflict females, but the prevalence in men also increases exponentially with age. The rise in hip fracture rate occurs about 10 years earlier in women than in men. By the age of 90, about 17% of males have had a hip fracture, compared to 32% of females. Additional to the obvious costs on health, osteoporosis is a global problem and carries with it significant socio-economic costs. This is illustrated by the IOF audit report “Call to Action” published in 2001, which claims that osteoporosis costs national treasuries in the EU over 4.8 billion Euro annually in hospital healthcare alone. Clinically approved strategies aimed at treating the disease employ hormonal based medications which disrupt the bone remodeling process via provocation of bone forming cells or the inhibition of bone resorbing cells (1-24). All however have limited effects and in some cases there are negative consequences (25,26). Medical research is now attempting to target genes which define osteoporosis using the mouse as a model system for human diseases. Owing to the recent deciphering of the mouse genome and the high homology that exists between the human and mouse genomes (27), inbred strains of mice represent ideal models for genetic studies. Using the mouse to identify genes implicated in the bone remodeling process could therefore lead to advances in understanding which may enable the precise regulation of genes and proteins responsible for particular bone phenotypes i.e. bone mineral density or bone strength. One interesting phenotype under investigation is the response of bone to mechanical loading or its ‘Mechano-sensitivity’. Mechanical loading is the most important single physiological/environmental factor regulating bone mass and shape (28). It has been demonstrated in humans that cyclic overloading enhances bone mass in both cortical and trabecular components. An understanding of the biological pathways (from gene expression to protein function) governing load stimulated bone formation could provide opportunities to mimic or augment bone mechano-sensitivity using pharmacological agents thereby leading to the development of novel strategies in the management of osteoporosis. In vivo models which mechanically
stimulate mice tibia have already been established, furthermore attempts have been made to identify mechano-sensitive genes by comparing the genomes of two genetically distinct strains of mice which exhibit complementary levels of mechano-sensitivity (29-31), however the primary focus so far was on cortical bone and not trabecular bone which has been shown to have a more enduring sensitivity to mechanical stimulation in humans (28,32). A rat model has been established where an appreciable dose response has been observed in both components of bone by cyclically compressing the 8th Caudal vertebrae via two metal pins inserted into the adjacent vertebrae (33). However the rat genome is not very well characterized. The goal of this thesis is therefore to establish a similar in vivo model in the mouse which will facilitate the study of load regulated genes in both cortical and trabecular bone. Hence this thesis can be described by 3 specific aims:

1.1. Specific aims

1. **The design and validation of a mechanical loading device:** A target caudal vertebra will be cyclically compressed via pins inserted into adjacent vertebra (figure 1.1). The magnitude and frequency of compression shall be controlled by a loading device which will be designed specifically for this application in the mouse.

Figure 1.1: Fluoroscopic image of a mouse graphically edited to demonstrate how a single caudal vertebra will be compressed via the application of a cyclic compressive force on two pins.
2. **Characterization of two complimentary in vivo mouse models for the study of load induced bone adaptation:** The effect of pinning on the developmental morphometry of the target vertebrae shall be characterized in two genetically distinct biological strains of inbred mice. Once established, we will aim to establish a significant dose response following intensive bouts of mechanical loading in both cortical and trabecular components for at least one biological strain. The influence of the genetic background on mechano-sensitivity shall then be studied by mechanical stimulation of the second biological strain.

3. **The prediction of load-regulated micro-structural bone adaptation using a combined experimental and computational approach:** The exact spatial relationship between the micromechanical environment and consequent bone adaptation remains poorly understood. A quantitative understanding of how the mechanical environment regulates bone adaptation, combined with knowledge of the associated biochemical pathways would lead to an improved understanding of bone formation and bone quality maintenance. Whilst it is beyond the goal of this thesis to discover the biochemical pathways involved in bone formation our final aim is to establish the relationship between micromechanical strains and bone adaptation using a combined experimental and computational approach. Here Finite Element models of the target vertebrae will be developed and used to compute the micro-structural strains and stresses associated with loading, correlations with micro-structural bone adaptation will then be tested for. The aim is to develop a computational tool capable of predicting global and local bone morphometry in response to mechanical loading.

**Quantitative of bone morphometry**

In order to be able to measure changes in bone architecture, the use of a quantitative method of bone assessment is mandatory. Quantitative bone morphometry has been traditionally assessed in two dimensions where the structural parameters are measured in histological sections and the third dimension is extrapolated on the basis of stereology.
Introduction

The conventional histomorphometry typically entails substantial specimen preparation, including embedding in a plastic resin, sectioning and staining. While offering high spatial resolution and image contrast, this established technique is both tedious and time-consuming. Particularly limiting is the destructive nature of the procedure, excluding the specimen from use for other measurements such as analysis in different planes, which is highly desirable in view of the anisotropic structure of cancellous bone (36-38). µCT is an established, alternative technique to non-destructively image and quantify trabecular bone in three dimensions. The current system, which will be employed here, is based on a compact cone beam tomograph, also referred to as ‘desktop µCT’, and provides isotropic spatial resolutions up to 6 µm. Desktop µCT has been used extensively to study the micro-structural organization of bone and other biomaterials (39-46). It has allowed investigators to gain new insights into trabecular bone micro-architecture and the influence of age-related bone loss on bone architecture (47,48). More recently this system has been used to precisely assess quantitative traits in genetic studies (49-51) and to monitor the changes in bone properties using molecular (52,53) and gene therapy (54) in the mouse model.

Suitable inbred mouse strains

Inbred strains of mice are developed by repeated matings between siblings for at least 20 consecutive generations, which result in mice with almost identical genetic backgrounds. Inbred strains can therefore be used to identify the gene or combinations of genes responsible for particular phenotypes. C57/BL6 (B6) and C3H/Hej (C3H) inbred mice are of particular interest as they exhibit a number of contrasting phenotypes specific to both cortical and trabecular bone. B6 mice are also considered to be more responsive to mechanical loading. Several studies show the mechano-sensitivity of cortical bone in both strains to be complimentary (55-58). Breeding strategies have therefore been employed together with quantitative trait loci (QTL) analysis in an effort to refine the search for the chromosomal regions responsible for the complementary phenotypes (59,60). It therefore seems feasible that these two complimentary strains represent ideal candidates for the study of load regulated bone adaptation using the proposed modality of mechanical stimulation which will enable the both cortical and trabecular bone to be
investigated in a single bone. A preliminary study which compared the trabecular content of the caudal vertebra in 12 week old C3H/Hej mice against that of 12 week old C57BL/6 mice, has shown the 5th caudal vertebrae to be the optimal target vertebra. Using µCT, the trabecular content of five caudal vertebrae (C4, C5, C6, C7 and C8) from male and female mice belonging to both inbred strains were scanned using µCT (µCT40 Scanc0, Basserdorf, Switzerland) at 20µm resolution (Figure 1.2). Analyses of these inbred strains show the most mechno-sensitive biological strain (B6) to have significantly more cancellous bone. When considering the fact that ultimately mRNA transcripts will be isolated from cell population’s specific to both trabecular and cortical bone it is clear that the C6 caudal vertebra would offer the greatest yield. However that would mean pinning C7 which lies within the main body of the mouse, C5 must therefore be taken as the target vertebrae.

![Figure 1.2: Trabecular bone volume density (Trab BV/TV) of 5 caudal vertebrae from male and female C3H/Hej and C57BL/6 inbred mouse strains.](image)

**Quantification of the micro-mechanical environment**

The exact spatial relationship between the micromechanical environment and consequent bone adaptation remains poorly understood. It is widely believed that a quantitative understanding of bone adaptation mechanisms will contribute to the fundamental knowledge of bone formation, bone quality maintenance, prevention and treatment of age related fractures, as well as improvements in many orthopedic implants. Several computational models (61-64) using different mechanical parameters as inputs have attempted to provide theoretical frameworks which explain load induced adaptations.
Operating on the assumptions that bone formation is driven either by micro crack propagation interrupting communication between osteocytes (65,66), or by the shear stresses imposed by fluid flow through canaliculi (67), they employ absolute values of mechanical strains or strain gradients as their primary inputs. However, whilst they mimic bone adaptation when qualitatively compared to in vivo bone adaptation, many lack quantitative, in vivo validation. One study in particular has attempted to address this issue for trabecular bone and has correlated strain energy densities with increased bone formation indices as measured by histology. Guo et al. (68) used finite element analysis to quantify the rat tail model (discussed previously) for the study of trabecular bone adaptation. They scanned vertebrae using µCT at 34 µm resolution and converted the resulting images to 3D FE models by mapping each bone voxel to an eight node brick element. Using this model, correlations between the trabecular bone mechanical microenvironments and bone formation indices were observed during the first two weeks of loading (69). However, there were limitations in their approach; only half of the vertebra was modeled about the mid-sagittal plane and the distal ends of the vertebrae neglected (figure 1.3a), thereby excluding the growth plates (an important structural component through which mechanical forces are transferred). In this thesis, similar FE techniques will be used to model the whole caudal vertebrae together with the application of more realistic boundary conditions.

Figure 1.3: (a) Contour plot of the axial strain distribution in a single vertebra. (b) An FE model used to characterize femoral bone strength in an F2 population of mice (produced from B6 and C3H mice).
This will permit a more accurate characterization of the spatial relationship between mechanical micro-strains and bone adaptation for both cortical and trabecular components in 3 dimensions. To further illustrate the power of FE methodology, micro-structural finite element (FE) models were used to determine bone strength directly from bone structure for a large numbers of mice femora (unpublished results). This will provide the phenotypic data required to identify the QTL’s responsible for bone strength in an F2 population obtained when crossing B6 and C3H biological strains (figure 1.3b).

1.2. Outline of the thesis

This thesis is composed of 6 chapters. In order to put the novelty of the proposed research project into context, chapter 2 will introduce the basic physiology of bone, the fundamentals of cell biology relevant to gene expression and the techniques applied to identify particular genes. With a basic understanding of the fundamental concepts, the strategies for finding the genetic determinants of osteoporosis will then be discussed. This latter point will emphasize the power of the mouse as a model for human diseases, demonstrating the potential of this project to deliver results which could lead to the effective treatment of osteoporosis. Chapter 3 presents the hardware and software which constitute the design of the force controlled device able to deliver precise mechanical loads to the caudal vertebrae of mice via the proposed loading configuration. This chapter is organized into sections which describe all aspects of the system. First of all the design of a specialized surgical tool able to insert the metallic pins is discussed. Here the influence of pin-type, pin dimensions, pin position and partial constraining of the tail are all investigated to optimize the mechanical stability of the loading configuration. Proceeding sections present the physical make-up of the device along with the fundamental principles of operation. This includes descriptions of the electronic hardware which controls the device along with the software environments and specially designed programs which enable the user to accurately define, apply and assess the applied loading protocol. Validation and optimization procedures are then outlined. To finalize the validation of the device and develop a tool capable of determining the load induced micromechanical environment in the target vertebra the final section of this chapter
provides the description of a combined experimental and computational approach used to characterize the relationship between the input force and output vertebral mechanical strains when loading the caudal vertebrae in-vitro. In addition to validating the loading device this combined approach will also validate an FE model of the complete caudal vertebrae which will eventually be used to quantify and predict load related changes in cortical and trabecular bone micro-architectures. Chapter 4 presents the results of in vivo studies in which the methodology and device, developed in chapter 3, are deployed to mechanically stimulate the caudal vertebrae of two complementary inbred mouse strains. In an effort to construct a computational model capable of predicting load induced morphometrical changes in the caudal vertebrae. Chapter 5 investigates the relationship between load induced bone adaptation and the associated micro-mechanical environment in the most responsive biological strain using the FE model established in Chapter 4. Finally, the synthesis in Chapter 6 brings together the results and discusses the benefits and limitations of the presented work outlining future steps which should be taken to further advance this field of research.
References


Introduction


Introduction
Chapter 2
Background
2. Background

This section has been adapted from the 4th edition of ‘Molecular biology of the cell’ (1) and provides an overview of the physiology of bone.

2.1. Bone

Bone is a specialized connective tissue that together with cartilage constitutes the skeletal system. These tissues provide our bodies with a rigid structure which together with muscle facilitates stable locomotion. Like reinforced concrete the bone matrix is predominantly a mixture of tough fibers (type I collagen fibrils) which resist pulling forces and solid particles (Calcium phosphate as hydroxyapatite crystals) which resist compression. For all its rigidity bone is by no means an inert substance and can be considered as a living organ. Running throughout the hard extra cellular matrix are channels and cavities occupied by living cells which account for about 15% of the weight of compact bone. These cells are engaged in the life long process of remodeling the bone: one class of cells (osteoclasts) demolishes old bone matrix while another (osteoblasts) deposits new bone matrix in the interior of the bone.

Unlike soft tissues, which can grow by internal expansion, bone can grow only by apposition, that is, by the laying down of additional matrix and cells on the free surfaces of existing bone. During development this process must occur in co-ordination with the growth of other tissues, in such away that the pattern of the body can be scaled up with out its proportions being radically disturbed. For most of the skeleton, and in particular for the long bones of the limbs and the trunk, the coordinated growth is achieved by a complex strategy. A set of minute “scale models” of these bones are first formed out of cartilage. Each scale model then grows, and as new cartilage is formed, the older cartilage is replaced by bone. Cartilage growth and erosion and bone deposition are so ingeniously coordinated during development that the adult bone is almost the same shape as the initial cartilaginous model at infancy.
Macroscopic organization

Anatomically, two types of bones can be distinguished in the skeleton: flat bones (skull bones, scapula, mandible and ilium) and long bones (tibia, femur, humerus etc). Examination of a long bone (Figure 2.1) shows three distinct zones, the two wider extremities (the epiphyses) and a more or less cylindrical tube in the middle (the midshaft or the diaphysis). The external shell of the long bone is made from a thick dense layer of calcified tissue, referred to as the cortex or compact bone which becomes progressively thicker in the diaphysis. Towards the extremities the cortex becomes progressively thinner and the internal space is filled with a boney mesh, referred to as cancellous bone, spongy bone or trabecular bone. The spaces enclosed by these trabeculae are filled with hematopoietic bone marrow which also fills the medullary cavity of the diaphysis. Both Cortical and Trabecular bone are constituted of the same cells and the same matrix elements The surfaces of the bone which are exposed to surrounding soft tissues (The Endosteal surface and Periosteal surface) are lined with osteogenic cells organized in layers, the periosteum and endosteum.
Microscopic organization

The bone matrix is secreted by osteoblasts that lie on the surface of existing matrix and deposit fresh layers of bone. This freshly formed material consists mainly of type I collagen and is referred to as osteoid. This is quickly converted into hard bone matrix by the deposition of calcium phosphate crystals. Some of the osteoblasts become caught up in their own secretion and are subsequently imprisoned in the calcified matrix; these “Imprisoned” osteoblasts are now referred to as osteocytes, and have no opportunity to divide although it continues to secrete further matrix in small quantities around itself. These osteocytes occupy small cavities or lacuna, in the matrix. However they are not isolated. Tiny channels, or canaliculi, radiate from each lacuna and contain cell processes from the resident osteocyte, enabling it to form gap junctions with adjacent osteocytes (Figure 2.2). Although the network of osteocytes do not themselves secrete or erode quantities of matrix, they probably play an important part in controlling the activities of the cells that do. While new bone is formed by osteoblasts, it is eroded by osteoclasts (Figure 2.3). These large multinucleated cells originate from hemopoietic stem cells in the bone marrow. The precursor cells are released as monocytes into the blood stream and collect at sites of bone resorption, where they fuse to form multinucleated osteoclasts, which cling to faces of the bone matrix and eat it away.

Figure 2.2: Deposition of bone matrix by osteoblasts. Osteoblasts lining the surface of bone secrete the organic matrix of bone (osteoid) and are converted into osteocytes as they become embedded in the matrix. The osteoblasts themselves are thought to derive from osteogenic stem cells that are closely related to fibroblasts.

Figure 2.3: An osteoclast shown in cross section. This multinucleated cell erodes bone matrix. The ruffled border is a site of secretion of acids and hydrolyses.
Osteoclasts are capable of tunneling into compact bone, forming cavities which are then invaded by other cells. A blood capillary grows down the centre of these tunnels, the walls of which become lined with a layer of osteoblasts (Figure 2.4). These osteoblasts lay down concentric layers of new matrix, which gradually fill the cavity leaving only a narrow canal surrounding the new blood vessel. Many of the osteoblasts become trapped and survive as concentric rings of osteocytes. At the same time as some tunnels are filling up new tunnels are being bored out by osteoclasts, cutting through older concentric systems. The results of this perpetual remodeling are displayed in the layered patterns of matrix observed in compact bone (Figure 2.5). The micrograph shows the outlines of tunnels formed by osteoclasts and then filled in by osteoblasts during successive rounds of bone remodeling. This section has been prepared by grinding. The hard matrix has been preserved, but not the cells. Lacunae and canaliculi that were occupied by osteocytes are clearly visible. The alternating bright and dark concentric rings correspond to an alternating orientation of the collagen fibers in the successive layers of bone matrix. It is important to note how older systems of concentric layers of bone have been partly cut through and replaced by newer systems.

Through remodeling, bone is able to adjust its structure in response to changes in its mechanical environment. This adaptive behavior implies that the deposition and erosion of the matrix are somehow controlled by local mechanical stresses, but the mechanisms involved are not understood. In the context of osteoporosis there is excessive erosion of
the bone matrix. (Figure 2.6), Knowledge of how the remodeling process is controlled would give further insight into factors which trigger osteoporosis and therefore lead to more effective treatments or preventative measures.

![Figure 2.6: Left, healthy lumbar vertebra with normal trabecular structure. Right, Lumbar vertebra from an osteoporotic patient with perforations and disintegration of the trabecular network.]

### 2.2. Cell biology

This section has been adapted from the 4th edition of ‘Molecular biology of the cell’ (1) and provides an overview of the relevant aspects of cell biology. The accurate characterization of the biochemical pathways will not form part of this project, however once a reliable mouse model has been correctly established, additional research can then start to investigate these pathways, this project will focus only on the genetic control of micro-structural bone adaptation by investigating micro-structural adaptation in inbred strains of mice.

**DNA, genes and chromosomes**

Life depends on the ability of cells to store, retrieve and translate genetic instructions to make and maintain a living organism. This hereditary information is passed on from a cell to its daughter cells at cell division and from one generation of an organism to the next through the organism’s reproductive cells. These instructions are stored within every living cell as its **genes**, which are the elements containing the information that determine the characteristics of a species as a whole and the individuals with in it. The genes
themselves are made up of DNA (deoxyribonucleic acid) and it is this molecule which provides the instructions to direct the formation of life.

The fundamental building block of DNA is a sugar phosphate molecule which combines with one of four nucleotides adenine (A), cytosine (C), guanine (G) or thymine (T). These molecules combine to form a DNA chain or DNA strand (figure 2.7a). Two DNA chains unite in complementary fashion to form a DNA ladder (figure 2.7b) which assumes the form of a double helix, energetically the most favorable position. Owing to chemical affinity Adenine always pairs with Thymine and Guanine always pairs with Cytosine. The shape and chemical structure of the base pairs can be seen in figure 2.7c.

The structure of DNA, more specifically the sequence of nucleotides along the DNA double helix encodes the information for life. Each base, A, C, T or G can be considered as a letter in a four-letter alphabet that spells out biological messages. The reason why organisms differ from one another is because each organism contains different nucleotide sequences which spell out different biological messages. The way in which these messages are read and used to direct the development of life and define its characteristics is both complex and fascinating. The following paragraphs attempt to summarize the entire process to give the reader a basic understanding;

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**Figure 2.7: DNA and its building blocks.**

- **a)** Nucleotide base
- **b)** Double stranded DNA
- **c)** Chemical structure of Bases

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The nucleotide sequences provide the instructions to build a secondary nucleotide sequence in the form of an RNA (Ribonucleic acid) molecule, which is essentially a disposable copy of one segment of a particular stretch of DNA. This new copy of instructions is then read and used to synthesize proteins, molecules which perform particular cellular functions and which ultimately put into effect the directives specified by DNA (figure 2.8).

The underlying mechanisms which perform this process are complex. In the nucleus of the Cell where the DNA resides, molecular machinery (The DNA Helicase) actively breaks the hydrogen bonds between the base pairs, and unzips the DNA Helix, to give access to another piece of machinery (the RNA polymerase) which then reads particular segments of the exposed base pairs and translates them into RNA strands. These RNA’s are then transported out of the nucleus where they are the acted upon by the Ribosome which reads the RNA sequence and then builds the protein specified by the sequence. Figure 2.9 visualizes this molecular machinery and is intended to give the reader an impression of the mechanistic way in which DNA is processed.

![Figure 2.8: Schematic of the DNA to protein pathway.](image)

![Figure 2.9: (a) A DNA Helicase opening up the DNA for RNA transcription. (b) Micrograph of multiple RNA polymerases transcribing RNA.](image)
Considering this basic description of the DNA-protein pathway, the definition of the word *gene* can be more precisely defined as the nucleotide sequence in DNA which characterizes a certain type of protein. There are many different protein molecules produced by each cell, and, leaving out water, they form most of the cells mass. It is important to note that all cells in an organism, even though they may have different functions contain the same DNA, hence only certain genes are expressed in some cells and not in others. It is this difference which effectively determines a cells lineage, i.e. Bone cell, skin cell, etc. However there are large proportion of the genes which are expressed in all cells, these are called ‘housekeeping’ genes and are genes which have been conserved throughout millions of years of evolution and which are shared by all organisms. These genes are responsible for the production of proteins which interact to create among other things, the molecular machinery used to splice open the DNA and induce RNA translation. Another set of important genes, again common to all cells produce protein complexes which enable DNA to replicate itself. In order for an organism to develop and grow cells must proliferate, and to enable those proliferating cells to do their job, they must inherit instructions from the mother cell. DNA therefore contains the instructions for the molecular machinery which unzips the DNA ladder (DNA Helicase) and which scans along the exposed base pairs, in a similar fashion to the RNA polymerase, but produces a high fidelity copy of the complete length of DNA (The DNA polymerase). This brief and over simplified description of DNA demonstrates its remarkable nature, it is both fascinating and incomprehensible to think that the arrangement of innate, seemingly lifeless molecules possesses an ‘apparent intelligence’, to produce elements which act upon themselves to direct the formation of individual cells into a configuration which describes not only a human being, but all other life which lives on this planet.

DNA is packaged into Chromosomes. DNA is ‘wound up’ by molecular machinery (protein complexes) such that it is condensed into a very small space. DNA in this conformation is referred to as chromatin, the total sum of which constitutes a chromosome. The entire genome or entire sequence of nucleotides is distributed between these chromosomes, the number of which is specific to each organism. As might be expected, a correlation exists between the complexity of an organism and the number of
its genes in its genome. For example the human genome has DNA composed of $3.2 \times 10^9$ nucleotide pairs, which in turn describes approximately 30,000 genes, all of which are distributed between 24 chromosomes. Figure 2.10 shows the organization of genes on a human chromosome.

Chromosomes from many organisms (including humans) contain, in addition to nucleotide sequences which describe functional genes, large segments of interspersed DNA that does not seem to carry critical information. As can be seen from figure 2.10, 80% of a single gene contains nucleotide sequences which are not translated into RNA, these regions are known as introns, and separate the useful regions which are translated into RNA, known as exons. It is also important to note the orange section of the gene, which represents the sequence which is associated with the control of this particular gene. This control sequence is recognized by various proteins which either induce or block the recruitment of an RNA polymerase to begin RNA transcription. The signals which initiate the recruitment of these proteins to the control sequence are not yet fully understood, but it is clear that an intelligent feedback system within the cell exists and
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tells the cell when and how much of a particular gene to express. It has also been shown that it is not as simple as having a control region just before the gene of interest. Sequences of DNA which lie at great distances from a particular gene, both up stream or down stream have been shown to control the gene from distance. In some cases the DNA forms a loop to bring this distant control region close to the gene and so initiate or block transcription. This could be one explanation for why there are long sequences of non-coding DNA, they simply facilitate the flexibility required to bring two distant regions into contact. Figure 2.11 shows the structure of two human genes and the arrangement of exons and introns. The small β-globin gene encodes one of the subunits of the oxygen-carrying protein hemoglobin and contains only 3 exons. The much larger Factor VIII gene contains 26 exons and codes for a protein that functions in the blood-clotting pathway.

![Figure 2.11: Structure of two human genes showing the arrangement of exons and introns.](image)

**Genetic recombination**

Genetic recombination is a process responsible for the genetic diversity between individuals. Most cells in the human body contain two sets of chromosomes, 12 from the mother and 12 from the father. These are called somatic cells and are said to be diploid, i.e. they contain two sets of chromosomes. It is therefore logical that the gametes (sperm and female egg cells) are haploid, i.e. they contain only one set of chromosomes. When somatic cells divide they produce and exact copy of all 24 chromosomes and pass this information onto the daughter cells, a process known as mitosis. In contrast haploid sex cells produced from diploid cells must contain half the number of chromosomes as they must be endowed with either the maternal or the paternal copy of each gene and not both. This process is known as meiosis and occurs via two cell divisions (figure 2.12)
In stage one both maternal and paternal chromosome homologues of a diploid cell are faithfully replicated, giving 24x2 sets of chromosomes. (For simplicity figure 2.12 shows only two pairs of chromosomes). By a mechanism that is not yet fully understood the duplicated copies pair with their original chromosomes and then pair with the other homologous pair, i.e. the male pair combine with the corresponding female homologues, forming structures containing four sets of chromosomes. At this point, genetic recombination occurs where segments from homologous chromosomes are exchanged. It is the chromosomal crossing over during in meiosis which is responsible for the genetic diversity amongst organisms within a particular species. It is also a process which greatly complicates genetic analysis, as shall be seen in the proceeding paragraphs. Following recombination cells division occurs producing two sets of diploid cells. Formation of the actual gamete nuclei can now proceed through a second cell division without further DNA replication. Meiosis thus consists of a single phase of DNA replication followed by two cell divisions. Four haploid cells are therefore produced from each cell that enters meiosis (figure 2.12).

This is not the only example of recombination, recombination also occurs in somatic cells, not to facilitate genetic diversity, but to correct any errors that may have occurred in DNA replication, i.e. via the miss-pairing of two non-complementary bases. Site specific recombination can also occur and is a tool used by many viruses which insert their own genetic sequence into the DNA helix and so use the cells machinery to
manufacture their life threatening proteins. AIDS is one such example. Again recombination is facilitated by molecular machinery (proteins, enzymes, coded for by the DNA itself) which is able to recognize specific DNA sequences and break open the chain for insertion and then re-seal the break, another example which underlines the apparent intelligence of DNA. Such techniques have also been reproduced in-vitro and allow biologists to insert or knockout genes of interest, so their function, or lack or function can be analyzed in situ. For example suppose a gene was identified which was suspected to play an important part in regulating bone density, mammalian models which have the gene knocked out could then be used to accurately characterize its influence. This forms the basis for transgenic technologies which shall be discussed later in relation to the mouse.

Gene identification

Ultimately the purpose of investigating DNA is to determine how genes and the proteins they encode function in the intact organism. Knowledge of this will then permit the treatment of many diseases via pharmacological intervention which may inactivate, replace or modify a particular gene or inhibit the normal function of an expressed protein. In order to identify a genes function a common approach is to work backward from the phenotype (the appearance or behavior of the individual) to the genotype, i.e. determining which genes are responsible for the observed characteristic. For example one could begin by isolating mutants which display the desired defect, then via comparison of the different genomes of mutant and non mutant the gene responsible for the defect can be identified. This is the fundamental principle of comparative genomics.

Comparative genomics is a powerful tool used for the identification of DNA sequences that are responsible for a particular phenotype and is not only limited to the study of mutants. As shall be seen later mice strains exhibiting certain desirable phenotypes i.e. high bone density are crossed with another complementary inbred strain in order to produce a second generation which expresses the desired phenotype at different levels. Via the use of genetic markers and Quantitative Trait Loci Analysis the chromosomal region responsible for the phenotype can be roughly identified. To illustrate the importance of markers consider the human genome: 99.9% of the human genome is
identical when comparing individuals in a population; however there are single nucleotide differences at various points. These are referred to as single-nucleotide polymorphisms (SNPs). These differences can lie within non-coding regions of DNA or are part of gene responsible for the variation in phenotypes across a population, which could be a disease. By using these polymorphic points as ‘genetic’ markers, a map of the human genome can be constructed. These maps can then be used to identify the possible location of a gene responsible for a particular defect. For example consider the progeny of two parents, one who has a disease and the other healthy. Owing to recombination during meiosis some of the progeny will not express the disease whilst some will, i.e. some of the progeny will have inherited the SNP responsible for the disease. By comparing the genomes of the mutant parent, with the mutant progeny candidate SNP’s can be identified. This would be simple if genomes where virtually identical and say only 3 SNPs existed for an entire species (figure 2.13).

![Diagram illustrating in a much simplified case how the region of DNA responsible for a particular disease can be determined by correlating SNP’s with the observed phenotype.](image)

However in reality $10^6$ SNPs exist and during recombination a large percentage will have been inherited by the progeny, hence in order to refine the search for the candidate SNP the comparison must include the progeny which have not inherited the disease. Hence the identification of the responsible SNP becomes a question of statistical confidence values, i.e. how well a particular SNP correlates with the observed phenotype. This forms the
basis of Quantitative trait loci analysis, which employs statistical algorithms to correlate genetic sequences with a particular phenotype across a large population of the progeny from two specifically selected parental strains, see later sections.

**Genetic manipulation techniques**

Whilst the characterization and identification of a phenotype such as a disease was always relatively easy, up until now it was not possible to attribute the phenotype to a particular genetic sequence or sequences. It is now possible to isolate a specific region of a genome, produce a virtually unlimited number of copies, and determine the sequence of its nucleotides over night. This led to the discovery of genetic markers (discussed previously) which then enabled the application of statistical strategies to greatly refine the search for genes responsible for a particular disease. The ability to copy a gene is of no less significance. For example once a candidate gene has been identified multiple copies of the gene can now be produced to create a library. These copies could then be inserted into the DNA of animal models to allow further dissection of its biological function, i.e. testing its response to various pharmacological treatments, or as shall be described in the following paragraphs, copies can be used as ‘probes’ to monitor the transient expression of its in vivo counterpart.

**Hybridization**

Before describing the experimental methods it is essential to understand the term, ‘hybridization’. When aqueous solution of DNA is heated at 100 degrees C, or exposed to very high pH (pH > 13), the complementary base pairs that normally hold the two strands of the double helix together are disrupted and the double helix rapidly dissociates into two single strands. This process is call DNA denaturation, and was thought to be irreversible. However it was discovered that complementary single strands of DNA readily reform double helices by a process called hybridization if they are kept for a prolonged period at 65^0 C. Similar hybridization reactions can occur between any two complementary single-stranded nucleic chains (DNA/DNA, RNA/RNA or RNA/DNA). These specific hybridization reactions are widely used to detect and characterize specific
nucleotide sequences in both RNA and DNA molecules. Single stranded DNA molecules used to detect complementary sequences are known as probes; these molecules can carry radioactive or chemical markers to facilitate the detection of a hybridized reaction. Hybridization reactions using DNA probes are so sensitive and selective that they can detect complementary sequences present at a concentration of one molecule per cell. It is thus possible to determine how many copies of any DNA sequence are present in a particular DNA sample. Alternatively, DNA probes can be used in hybridization reactions with RNA, rather than DNA so as to find out whether a cell is expressing a given gene. In this case a DNA probe that contains part of the genes sequence is hybridized with RNA purified from the cell in question to see whether the RNA includes molecules matching the probe DNA and, if so in what quantities. Hybridization is key to many experimental techniques and without it monitoring the expression of genes would not be possible.

**Gel electrophoresis**

Gel Electrophoresis is a technique which is able to separate and distinguish between RNA segments of different lengths. This is useful for identifying expressed genes when combined with hybridization. Gel Electrophoresis exploits the fact that RNA (and DNA) is negatively charged. By placing RNA in a length of micro-porous polyacrylamide gel and applying an electrical potential difference between its ends, RNA of varying lengths will migrate to the positive terminal at different rates, i.e. the smaller the RNA segment the more easily it is able to translate through the micro porous gel. Hence by applying the potential difference for a short time period, RNA placed at the same position in the gel will be separated according to its length. Specially designed polyacrylamide gels allow the separation of molecules that differ in length by as little as a single nucleotide. Figure 2.14 shows several examples of typical patterns obtained when Gel Electrophoresis is performed. Note: Since the RNA molecules which are used for this method are radio labeled, their positions can be obtained by autoradiography.
Combing this method with hybridization techniques allows separated RNA to be identified as a specific gene. Hence when the RNA of a particular cell at a particular moment in time is isolated (remember that if a gene is expressed a particular DNA sequence is transcribed into and RNA sequence) and ‘Electrophoresised’, if the RNA is then hybridized with known DNA probes which describe a certain gene, the expression of genes at that time point can be established. This is the basic principle of Northern blotting.
Figure 2.15. First the RNA’s which have been isolated from the cells in question are fractionated on the basis of their sizes into a series of bands by gel electrophoresis. Then to make the RNA molecules accessible to DNA probes, a replica of the pattern of RNA bands on the gel is made by transferring (‘blotting’) the fractionated RNA molecules onto a sheet of nitrocellulose paper. The paper is then incubated in a solution containing a radio-labeled DNA probe whose sequence describes a known gene. The RNA labels that hybridize to the labeled DNA probe on the paper are then located by detecting the bound probe by autoradiography or by chemical means, thereby revealing which genes are expressed and which are not.

**Polymerase chain reaction**

Selected DNA segments can now be cloned in a test tube, by a process known as polymerase chain reaction or PCR. PCR allows the DNA from a selected region of a genome to be amplified a billion fold. This capability facilitates the mass production of DNA which can be used to amplify the genes expressed from a particular cell, so boosting the expression signal, or it can be used to create extensive libraries of DNA probes. First of all the segment of DNA to be cloned is heated to separate the two strands (step 1 Figure 2.16). Knowledge of this DNA sequence is used to design two synthetic DNA oligonucleotides which are complementary to the ends of each of the separated strands (figure 2.16). After strand separation, cooling of the DNA in the presence of a large excess of the two primers allows them to hybridize to the complementary sequences in the two DNA strands (step 2 figure 2.16). This mixture is then incubated with DNA polymerase (The molecular machinery or enzyme which produces replicate DNA in vivo; isolated from bacterium) and the four nucleotide bases so that DNA is synthesized , starting from the two primers (step 3, figure 2.16). In essence the DNA polymerase and a source of free-floating individual nucleotides, complementary to the exposed base pairs of the DNA strand, are ‘stitched’ together. Nothing special is produced in this first cycle of DNA synthesis, the power of the PCR method is revealed only after repeated rounds of DNA synthesis. The newly synthesized fragments serve as templates which will be heat-separated for successive rounds (figure 2.17). A single cycle requires only about 5 minutes, and the entire procedure can be easily automated. PCR therefore makes possible
the “cell-free molecular cloning” of a DNA fragment in a few hours. This technique is now used routinely to clone DNA from genes of interest directly.

![Diagram of Polymerase Chain Reaction (PCR)](image)

Figure 2.16: First cycle of Polymerase Chain Reaction (PCR).

![Diagram of Repeated rounds of DNA synthesis in PCR](image)

Figure 2.17: Repeated rounds of DNA synthesis in PCR.

**DNA microarrays**

So far we have discussed techniques that can be used to monitor the expression of only a limited number of genes at a time. To test for the expression of multiple genes using northern blotting techniques the blotting and hybridization procedures would have to be repeated many times over. DNA microarrays have revolutionized the way in which gene expression is now analyzed, by allowing the RNA product of thousands of genes to be monitored at once. By examining the expression of so many genes simultaneously it is now possible to identify and study gene expression patterns that underlie cellular physiology, for example scientists can now see which genes are switched on (or off) as cells grow, divide or respond to hormones or to toxins. DNA microarrays are little more
than glass microscope slides studded with a large number of DNA fragments, each containing a nucleotide sequence that serves as a probe for a specific gene. The most dense arrays may contain tens of thousands of these fragments in an area smaller than a postage stamp. These arrays are generated from DNA probes which have been produced by PCR and then spotted onto the slide by a robot thus the exact sequence and position of every probe on the array is known. Any nucleotide fragment that hybridizes to a probe on the array can now be identified as the product of a specific gene simply by detecting the position to which it is bound (figure 2.18). To use DNA microarrays to monitor gene expression RNA from the cells being studied is extracted and converted to cDNA. The cDNA is then labeled with a fluorescent probe. The microarray is incubated with this labeled cDNA sample and hybridization is allowed to occur (figure 2.18). The array is then washed to remove cDNA that is not tightly bound, and the positions in the microarray to which labeled cDNA fragments have bound are identified by an automated scanning-laser microscope. The array positions are then matched to the particular gene whose sample DNA was spotted in this location. In figure 2.18 RNA has been collected from two different cell samples for a direct comparison of their relative levels of gene expression. These samples are labeled, one with a red fluorochrome, and the other with a green fluorochrome. Hence red spots in the hybridized array indicate that the gene in sample 1 is expressed at a higher level than the corresponding gene in sample 2. Green spots indicate that expression of the gene is higher in sample 2 than in 1. Yellow spots indicate that the genes are expressed in equal amounts in both samples while the dark spots indicate little or no expression in either sample. Figure 2.19 shows an example of an extensive microarray measurement. The array summarizes a very large set of measurements in which the RNA levels of 1800 select genes (arranged top to bottom) were determined for 142 different human tumors (arranged left to right) each from a different patient.
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DNA probes describing known genes are amplified using PCR and printed into a matrix on a glass slide.

RNA from sample 1, labeled with red fluorochrome

Hybridization

RNA from sample 2, labeled with green fluorochrome

Figure 2.18: Use of DNA microarrays to monitor the expression of thousands of genes simultaneously.

Each small red element indicated that the given gene in the tumor is transcribed at a level significantly higher than the average across all the cell lines. Each green element indicates a less than average expression level, and each black element indicates an expression level that is close to average across the different tumors. This analysis shows that each type of tumor has a characteristic gene expression pattern. This information can be used to type cancer cells of unknown origin by simply matching the gene expression profiles to those of known tumors.

Figure 2.19: Differences in RNA expression patterns among different types of human cancer cells.
2.3. Osteoporosis

Osteoporosis is a disease characterized by an excessive decrease in bone mass leading to an increased susceptibility to fracture and skeletal deformation. A committee of the World Health Organization has defined osteoporosis based on bone density. Using standardized bone density measurements of the total hip, "normal" bone is greater than 833 mg/cm\(^2\). "Osteopenia" is between 833 and 648 mg/cm\(^2\). Osteoporosis is lower than 648 mg/cm\(^2\), and "Severe (established) osteoporosis" is when there has been a fragility fracture (2). Whilst a decrease in bone density can reduce the overall strength of bone, it is not the only factor which contributes to increased susceptibility to fracture, the quality of bone is also important. Bone quality is determined not only by bone mass (as measured by bone density) but also by the micro-architecture of bone, the crystal size and shape, the brittleness, the connectivity of the trabecular network, the vitality of bone cells, the ability to repair micro-cracks and the structure of bone proteins (3).

A common misconception about this disease is that it is considered to only afflict females, but the prevalence in men also increases exponentially with age. The rise in hip fracture rate occurs about 10 years earlier in women than in men. By the age of 90, about 17% of males have had a hip fracture, compared to 32% of females. Additional to the obvious costs on health, Osteoporosis is a global problem and carries with it significant socio-economic costs. This is illustrated by the IOF audit report “Call to Action” published in 2001, which claims that osteoporosis costs national treasuries in the EU over 4.8 billion euro annually in hospital healthcare alone.

Established treatments of osteoporosis

Osteoporosis is more common than it should be. Although science has not yet found a way to completely prevent bone loss, society can help itself by following a healthy lifestyle. A healthy diet with a balanced vitamin and calcium intake, together with regular exercise and no smoking are among the many factors conducive to stronger bones. There are medications with clinical approval which have been shown to reduce the rate of bone loss, however the clinical professions are quick to emphasize that these medications will
not be as effective if there is inadequate calcium, exercise, or nutrition. The different pharmaceutical treatments are discussed in the following paragraphs.

**Estrogen**

The major physiological effect of estrogen is to block activation of the bone metabolic unit. Estrogen effects may be mediated in part by growth factors and interleukins. For example, interleukin 6 is a potent stimulator of bone resorption, and estrogen blocks the osteoblast's synthesis of interleukin 6. Estrogen may also antagonize the interleukin 6 receptors. Transgenic mice without interleukin 6 do not develop osteoporosis after oophorectomy. Osteoclast apoptosis also appears to be regulated by estrogens. With estrogen deficiency, the osteoclasts live longer and are therefore able to resorb more bone. In response to the increased bone resorption, there is increased bone formation and a high-turnover state develops which leads to bone loss.

Estrogens also retard the bone resorbing effects of PTH. Women who are given PTH injections develop hypercalcemia and increased bone resorption; when the women are treated with estrogen before the PTH injections the effect is muted. This effect could potentially be related to effects on interleukins.

Epidemiological studies have suggested that the maximum prevention of fractures occurs in women who started estrogen within 5 years of menopause. Within the first five years of menopause, substantial disruption of trabecular architecture can be seen. This has been demonstrated in human biopsies using micro-CT techniques. Once the trabeculae are broken, they do not reconnect again, and bone strength may not be gained even if bone density increases.

However there are recognized side effects. Estrogen replacement therapy can increase a woman's risk of developing cancer of the uterine lining (endometrial cancer). To eliminate this risk, physicians prescribe the hormone progestin in combination with estrogen (4). However studies have shown that a combination of estrogen and progestin can increase the risk of heart disease. This observation was not seen when estrogen was taken alone (5).
SERMs (Selective Estrogen Receptor Modulators)

SERMs are “designer” estrogen-related medications that activate the estrogen receptors, but have different effects on different tissues. There are two kinds of estrogen receptors, and after binding to receptors, the drug-receptor complex can have various conformations. Some of these will act like estrogen others will inhibit the actions of estrogen. Many screening studies of related compounds have been done to search for those which act like estrogen in the desirable ways (stabilize bone mass, improve lipid profile) but do not act like estrogen in undesirable ways (cause breast cancer, stimulate the endometrium). The effects on the cardiovascular system are still uncertain: when SERMs were first developed it was felt that estrogen had a beneficial action on the heart, now this is doubted and debated. Tamoxifen is a SERM that is used to treat breast cancer. It has been shown to have some beneficial effects on the bones, but it does stimulate the endometrium. Raloxifene is a newer SERM that has been approved for prevention and treatment of postmenopausal osteoporosis. Other SERMs are being studied but are not available.

Bisphosphonates

Bisphosphonates are excellent inhibitors of bone resorption, with their potency varying according to the structure of the side chains. Treatment with bisphosphonates reduces the steady-state level of resorption dependent upon the administered dose (6,7). Many different osteoporosis models have been investigated (8-13). Bisphosphonates are also effective in decreasing bone loss and increasing mineral density in postmenopausal osteoporosis (14-19) and corticosteroid-induced bone loss (20). Bisphosphonates improve the biomechanical properties of bone in both normal animals and models of osteoporosis (8,21-24), along with hormone replacement therapy, calcium and vitamin D supplementation have led to a significant improvement in the management of osteoporosis. It has also been demonstrated that, in humans, bisphosphonates inhibit tumor-induced bone resorption, correct hypercalcaemia, reduce pain, prevent the development of new osteolytic lesions, prevent fractures and, consequently, improve the quality of life for the patients (25-29).
PTH (Parathyroid Hormone)

The role of PTH in control of bone mass is still not really understood. PTH stimulates osteoblastic activity, especially on trabecular surfaces. It also stimulates osteoclastic activity. In some cases the anabolic effect predominates over the increased resorption and osteosclerosis results. The current theory is that intermittent spikes of PTH, such as given by daily injection, will cause more increase in bone formation than in bone resorption. This has been shown in rats.

PTH has different effects on the cortical and trabecular bone. Patients with either primary or secondary hyperparathyroidism have increased bone density of the spine, but decreased cortical bone mass. Iliac crest bone biopsies show increased trabecular bone volume and thin cortices. Several past studies of patients with osteoporosis treated with PTH showed increases at the spine but decreases at cortical sites. Total body calcium decreased about 2%. Those studies using a combination of PTH and estrogen in postmenopausal women have shown increased bone density at both the spine and the hip, and some studies of intermittent PTH alone also show increases in total body calcium. QCT images demonstrate increases in cancellous bone of the hip, and an overall increase in the amount of cortical bone but with increased porosity. The anabolic actions of PTH diminish with time; after a mean of 19 months, the bone forming surfaces from bone biopsies are slightly lower than those at baseline. Studies using 85-Strontium kinetics, however, suggest that other parts of the skeleton might still be forming bone even when the iliac crest has returned to baseline (30). Intermittent PTH suppresses apoptosis of the osteoblasts (31) which is one reason there is a high bone formation rate. On the other hand, continuous PTH will increase the apoptosis (32).

The future: targeting genes which define osteoporosis

Genes contain the sequence for proteins, which are the building block of our bodies and help to regulate the operation of every organ. Knowledge about the genes and their functions can lead to advances in understanding and treatment of disease, for example gene and protein expression can be regulated such that the desired phenotype is enhanced, moderated or even eliminated. In the case of osteoporosis the identification of
a gene which regulates BMD would represent a huge step in allowing science to control bone loss and potentially lead to a cure for osteoporosis.

The introduction of new technology to measure bone mass helped provide the first clues that osteoporosis is a heritable disease. BMD studies of mother daughter pairs, twins and large cohorts estimated the heritability of this trait to be between 50% and 70% (33). This finding led most investigators to conclude that the phenotypic variation in BMD was caused by the action of a limited number of genes with discrete effects. Although data from these studies failed to yield major genes that defined osteoporosis, such studies, combined with genome wide scanning of multigenerational families, inferred that the genetic influence on bone acquisition was both complex and polygenic. In addition to this complex multifactorial nature of genetic influences, two other factors further complicate the search for osteoporosis genes. 1) Whilst there maybe polygenic determinants which have a direct influence on BMD these determinants themselves are influenced by genes that do not have a direct effect on BMD, i.e. epistasis (33,34). 2) There are numerous environmental factors that may modulate expression of one or more genes (33), i.e. Nutrition, hormonal interactions, the mechanical environment and lifestyle factors. These same technologies also led to the realization that BMD is a complex trait and is not the only influential factor. Analysis of bones micro architecture has identified “Bone quality” as another influential component and is a term which incorporates additional factors such as; bone turnover rate, trabecular spacing and trabecular connectivity.

With so many aspects contributing to the strength of bone discovering the genes responsible presents a sizeable task and cannot be done by studying human biology alone. The study of genetics in humans is limited to some degree by the tremendous heterogeneity among population, as well as multiple genetic, heritable and environmental determinants of the target phenotype. If the challenge is to be realized a more controllable genetic model is required, which is why the mouse as become such an important tool. The biological similarities between man and mouse make this small animal the ideal experimental surrogate and via the field of comparative genomics this small animal greatly increases the likelihood that candidate genes and effective therapies will be found.
2.4. The mouse as a model

This chapter provides an overview about current efforts in mouse genetics. It is adapted from Silver 1995 (35)

Many features of human biology at the cell and molecular levels are shared across the spectrum of life on earth; our more advanced organism-based characteristics are shared in a more limited fashion with other species. At one extreme are a small number of human characteristics (brain functions and behavior) that are shared by no other species or only by primates. But at a step below there is a whole set of characteristics, which are shared only with mammals. In this context, the importance of mice in genetic studies was first recognized in the biomedical fields of immunology and cancer research, for which a mammalian model was essential. Although it has been obvious that many other aspects of human biology and development should be amenable to mouse models, until recently, the tools just did not exist to allow for a genetic dissection of these systems.

The movement of mouse genetics to the forefront of modern biomedical research was catalyzed by the recombinant DNA revolution, which began 30 years ago. With the ability to isolate cloned copies of genes and to compare DNA sequences from different organisms came the realization that mice and humans as well as all other placental mammals are even more similar genetically than they were thought to be previously. An astounding finding has been that all human genes have counterparts in the mouse genome which can almost always be recognized by cross-species hybridization. Thus, the cloning of a human gene leads directly to the cloning of a mouse homolog which can be used for genetic, molecular, and biochemical studies that can then be extrapolated back to an understanding of the function of the human gene. Although the haploid chromosome number associated with different mammalian species varies tremendously, the haploid content of mammalian DNA remains constant at approximately three billion base pairs. It is not only the size of the genome that has remained constant among mammals; the underlying genomic organization has also remained the same as well. Large genomic segments (on average, 10-20 million base pairs) have been conserved virtually intact between mice, humans, and other mammals as well. In fact, the available data suggest that a rough replica of the human genome could be built by simply breaking the mouse
genome into 130-170 pieces and pasting them back together again in a new order (36,37). Although all mammals are remarkably similar in their overall body plan, there are some differences in the details of both development and metabolism, and occasionally these differences can prevent the extrapolation of mouse data to humans and vice versa (38). Nevertheless, the mouse has proven itself over and over again as being the model experimental animal par excellence for studies of nearly all aspects of human genetics. Besides the strong homology in the genome, the mouse is among mammals ideally suited for genetic analysis for several other reasons. First it is one of the smallest mammals known, second it has a short generation time, in the order of 10 weeks from being born to giving birth. Third, females breed prolifically in the laboratory with an average of 5-10 pups per litter. Fourth, an often forgotten advantage is the fact that fathers do not harm their young and that laboratory-bred strains are relatively docile and easy to handle. Finally, investigators are even able to control the time of pregnancies.

**Manipulation of the mouse genome and micro-analysis**

The close correspondence discovered between the genomes of mice and humans would not have been sufficient to drive researchers into mouse genetics without the simultaneous development, during the last decade, of increasingly more sophisticated tools to study and manipulate the embryonic genome. Today, genetic material from any source (natural, synthetic or a combination of the two) can be injected directly into the nuclei of fertilized eggs; two or more cleavage-stage embryos can be teased apart into component cells and put back together again in new "chimeric" combinations; nuclei can be switched back and forth among different embryonic cytoplasma; embryonic cells can be placed into tissue culture, where targeted manipulation of individual genes can be accomplished before these cells are returned to the embryo proper. Genetically altered live animals can be obtained subsequent to all of these procedures, and these animals can transmit their altered genetic material to their offspring. Progress has also been made at the level of molecular analysis within the developing embryo. With the polymerase chain reaction (PCR) protocol, DNA and RNA sequences from single cells can be characterized and enhanced versions of the somewhat older techniques of in situ hybridization and immuno-staining allow investigators to follow the patterns of individual gene expression
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through the four dimensions of space and time. Finally, with the automation and simplification of molecular assays that has occurred over the last several years, it has become possible to determine chromosomal map positions to a very high degree of resolution. Genetic studies of this type are relying increasingly on extremely polymorphic microsatellite loci to produce anchored linkage maps, and large insert cloning vectors, to move from the observation of a phenotype to a map of the loci that cause the phenotype, to clones of the loci themselves. All of these techniques provide the scientific community with the ability to search for answers to the many questions posed. This will invariably lead to more questions, but the potential is there to elucidate the mechanisms of many diseases and realize effective treatments.

The mouse and osteoporosis

Rodent models for testing hypotheses to skeletal disorders are not new. In fact this is how many of the established treatments came to market. The overiectomised rat is a well established tool and was used to test how estrogen deprivation affects the bone remodeling unit. At the forefront of technology today is the mouse model. Numerous mouse models exist, each of which attempt either identify or evaluate candidate genes associated with osteoporosis. A summary of the different model types is given in the proceeding paragraphs.

Transgenic, mutant and knockout mice

Knockout mice carry specific gene deletions while transgenic carry gene additions and mutant mice carry a spontaneous mutation at a particular genetic locus. These mouse models are used to assess the role of known, single genes and how they may regulate a specific phenotype. For example, targeted overexpression of IGF-I in transgenic mice using the osteocalcin promoter is characterized by a marked increase in both cortical and trabecular bone density at 6 weeks of age (39). Similarly, mice globally lacking expression of the $Cbfa1$ gene are characterized by the absence of osteoblast differentiation, failure to mineralize bone and lethality at birth (40). Spontaneous mutants such as the osteopetrosis mouse, which lacks a functional gene and its product, fail to exhibit differentiated osteoclasts which are required for normal bone resorption. Such
mouse models are extremely informative with respect to understanding single gene action in bone biology.

**Inbred strains of mice and quantitative trait loci**

Inbred strains of mice combined with the power of breeding strategies are used to identify the gene or the combination of genes responsible for a particular phenotype. This method is being used to uncover genes which regulate BMD.

Inbred strains are developed by repeated matings between siblings for at least 20 consecutive generations, which results in nearly 100% homozygosity at all alleles across the mouse genome, i.e. all mice have identical alleles thus an identical genetic makeup. Many different inbred strains exist, each strain with differing sets of phenotypic characteristics. One such difference is the wide variation in BMD among inbred strains. By crossing these strains in accordance with various breeding strategies (so producing hybrid strains) regions of the genome responsible for phenotypic variation (Quantitative trait loci) can be determined via statistical analysis methods. These statistical methods (QTL analysis) correlate the phenotypic variation with genotypic variation across the hybrid population. For example, by crossing two inbred strains of mice results in hybrid

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**Figure 2.20:** Schematic representation of crossing two inbred strains to produce an F2 generation exhibiting independent segregation and random assortment of unlinked genes. The histogram shows the variation of a specific phenotype across the F2 population (Bone mineral content in this case).
F1 mice (Termed the first filial generation) these are completely heterozygous and genetically identical with each other at all genetic loci. Intercrossing F1 progeny results in F2 mice in which there is independent segregation and random assortment of unlinked genes, which leads to a certain phenotype being distributed among the population at different degrees (figure 2.20). Bouxsein et al used this approach to map certain chromosomal regions which could be responsible for vertebral trabecular bone volume in mice (133). Inbred strains when crossed can provide an invaluable tool for locating and enumerating QTL’s, moreover the gene-gene interaction in the F2 population resulting in phenotypic values that are greater or less than the progenitor strain phenotype can offer critical insight into the complex genetic influence on a specific phenotype (41). However the initial QTL’s may reside in chromosomal regions up to 40 centimorgans (cM = recombination distances between specific markers), areas of the chromosome with thousands of potential candidate genes. Fine mapping and congenic construction (see next section) define narrower regions of the chromosome and allow for positional cloning and gene sequencing to take place.

The determination of QTL’s associated with a phenotype is made possible through the use of molecular markers. Molecular markers are essentially identifiable physical locations on a chromosome (e.g. gene) whose inheritance can be monitored (SNP’s for example, as discussed previously). Markers can be expressed regions of DNA (genes) or some segment of DNA with no known coding function but whose pattern of inheritance can be determined. These markers define a DNA map, i.e. a multitude of reference point which can be used to help identify QTL’s. Via this molecular profiling and statistical analysis the association between a phenotype and a marker genotype can be tested. For most species, an adequate coverage of the genome can be achieved with approx. 100–150 marker loci evenly spaced along the chromosomes. Once a marker density of approx. one marker/20 cM is reached, then it becomes more profitable to increase the number of progenies rather than the number of markers to increase the accuracy of QTL detection (42). For the mouse there are 8000 genotypic markers that are polymorphic across all strains.

At its simplest, QTL analysis relies on one-way ANOVA testing to assess whether the phenotypic means of the possible genotype classes at a specific chromosomal position
are significantly different. If a significant difference is found, then it can be concluded that a QTL is probably linked to the chromosomal position under investigation. A major limitation of ANOVA is that it does not provide information on the distance of the QTL from the associated marker; furthermore, it may not be possible to ascertain whether the detected effect is due to a minor QTL tightly linked to the marker, or to a major, but more distant, QTL. These limitations can be overcome through the use of statistical approaches based on information of multiple markers which provide greater accuracy for QTL mapping (42-47). In this case, a frequently used output-statistic to describe the results is the LOD (logarithm of the odds ratio) score; the LOD value at a particular chromosome position is computed as $\log_{10}$ of the ratio between the chance of a real QTL being present given the effect measured at that position divided by the chance of having a similar effect with no QTL being present. The most likely position of the QTL is thus at the peak of the LOD profile. The graphical output can also provide us with a confidence interval around the QTL peak, thus delineating the range of the most likely QTL position moving away from each side of the peak. To avoid declaring ‘false-positive’ QTLs (i.e. declaring the presence of a QTL when the QTL is absent), the threshold value of the LOD score should be set reasonably high (usually $>2.0$). Other diverse and more statistical approached can be employed to optimize the search for QTL’s (48-50).

QTL analysis, while lifting the ‘statistical fog’ surrounding conventional quantitative genetics (49), provides a powerful magnifying lens for deciphering the chromosome regions regulating complex traits. In this context, the introduction of QTL analysis in quantitative genetics can be compared with the introduction of the optical microscope in cell biology

**Congenic strains**

Once a QTL has been identified, its effect maybe tested via the generation of congenic strains. Individual or multiple linked loci from a donor strain are placed on the genetic background of a recipient strain (51). The strategy is based on repetitive backcrossing to the recipient (figure 2.21). The backcross system of congenic strain creation is straightforward in both concept and calculation. The first cross is always an outcross between the recipient inbred partner and an animal that carries the donor allele. The
donor animals need not be inbred or homozygous at the locus of interest, but the other partner must be both. The second generation cross and all those that follow to complete the protocol are backcrosses to the recipient inbred strain. At each generation, only those offspring who have received the donor allele at the differential locus are selected for the next round of backcrossing. With each generation the homozygosity of the recipient background increases. The residual heterozygosity resides at the region of interest carrying the donor alleles. Hence, the genetic background of the chromosomal region of interest has been replaced by that of the recipient. Congenic strains are therefore useful for confirming the existence of a QTL and for testing the quantitative effect of individual QTL’s. For example, if there is a strong QTL on Chromosome 1 from C3H inbred mice as found by F2 analysis, congenic strain mice in which Chromosome 1 QTL is now placed on a B6 background, allows the investigator to test the single effect of this genetic locus on a low density background. Thus one moves from breeding strategies and QTL analysis to individual congenic construction in order to define the locus of interest, as well as to test precisely how that locus could affect the phenotype. Two groups have reported generation of congenics using their most promising QTL’s for either whole body BMD or femoral BMD (52,53). The power of this strategy is illustrated by some very recent studies (54), providing further evidence that congenics can offer insight into mechanisms whereby peak bone mass is acquired. This type of strategy has also been successful in identifying genes associated with obesity, atherosclerosis, epilepsy and cancer susceptibility in the mouse.

![Figure 2.21: The making of congenic mouse strains.](image-url)
Other strategies use recombinant inbred mice, which have been successfully used to help refine QTL locations for bone density genes (55) however this area shall not be discussed. The previous paragraphs describing the use of mice and breeding strategies are intended, only, to give the reader a basic insight into the power of the mouse as a tool for exploring human diseases. It is quite obvious that despite major efforts no genes defining osteoporosis have been cloned. Still, inbred, recombinant inbred and congenic strains, as model systems, offer a wealth of information related to the acquisition and maintenance of peak bone mass.

### 2.5. Mechanical load induced bone adaptation

Mechanical loading is perhaps the most important single physiological/environmental factor regulating bone mass and shape. Although the basic form and development of bone are genetically encoded, their final mass and architecture are governed by adaptive mechanisms sensitive to the mechanical environment. Mechanical signals are transmitted to bone mainly by muscle contractions generating strains in the bone matrix (56). Loss of bone (osteoporosis) and muscle strength (sarcopenia) develop together with increasing age (57); characteristic to osteoporosis is the failure of structural adaptation by bones to the mechanical environment, which results in increased incidence of fractures in response to physiological loads or minimal trauma. Although age-related bone loss cannot be ascribed entirely to sarcopenia (57), a growing number of studies in humans report that resistance training is an effective means of preserving and increasing the mass of both muscle and bone at all ages (58-60). Likewise, a handful of studies in experimental animals have demonstrated a mechanical load-induced stimulation of bone formation.

Anatomically, the crucial structural component of all major skeletal load-bearing sites, namely proximal femur, vertebrae and distal radius, is trabecular bone. During growth increasingly vigorous mechanical usage increases global bone deposits by enhancing longitudinal growth through the addition of new spongiosa and new cortex, in addition to stimulating cortical modeling drifts of increased cortical cross-sectional area. However, in the adult organism, in which modeling drifts are usually ineffective and cortical bone turnover is relatively low, the effects of vigorous mechanical usage are targeted mainly to the spongiosa and endosteal cortical surfaces where losses and marrow
cavity expansion are retarded (61). Furthermore, significant gains in trabecular bone mass have been reported in exercising healthy humans (62). By contrast, decreased mechanical usage results in increasing numbers of basal multicellular units (BMU) and high bone turnover, with a clear shift from a balance between bone resorption and formation towards increased resorption and decreased formation (63).

These observations in humans have been repeatedly supported by experimental work in laboratory animals, thus confirming Frost’s Mechanostat theory (61). This theory defines four mechanical usage windows, with thresholds defined by minimum effective strains (MES): (i) trivial (subphysiological) loads which result in a negative, high trabecular bone turnover; (ii) physiological loads responsible for normal, balanced turnover; (iii) overload, which induces positively balanced turnover; and (iv) pathological excessive loading, or failure loads, which result in microfractures and in addition to enhanced lamellar bone formation produce woven bone, apparently as part of the fracture healing process (61,64). The effect of trivial loads has been confirmed in models employing immobilization, by methods such as sciatic denervation (65), limb fixation (66), hypogravity (67-69) and tail suspension (70,71). Decreased loading also occurs in joint injuries (72), and the effect on bone in the joint region results in decreased bone volume (73,74), primarily through architectural adaptation (75). The effect of overloading has been studied using a wide variety of approaches. For example, increased bone formation indices have been reported in animals forced into excessive exercising regimens (76). A 6-fold increase has been reported in woven spongiosa formed in vitro in intermittently pressurized hydraulic bone chambers (77). Woven bone is also produced on trabeculae in response to extraordinary loading conditions (78-80). Lower load magnitudes produce increases in trabecular lamellar bone (80-83). Mechanical loading also reduced ovariectomy induced loss of metaphyseal spongiosa (66); at least in rats, the respective signaling and anabolic effects generated by overloading are parathyroid hormone and estrogen dependent (84-87). Furthermore, trabecular anabolic bone adaptation specific to can be affected by specific load profiles such as high frequency, low magnitude vibration (83), and is reflected both in bone architecture (88) and mechanical strength (89). This is also shown to be the case for cortical bone. One study investigated cortical bone adaptation in C57BL/6 mice in response to a variety of loading
frequencies (1, 5, 10, 20, 30 Hz). It was found that cortical bone adaptation to mechanical loading increased with increasing loading frequency for 1-10 Hz peaking at 10 Hz (90). Another study introduced the idea of cellular desensitization during prolonged loading bouts, hypothesizing that bone cells desensitize rapidly to mechanical stimulation it was shown in a rat ulna model that mechanical loading is more effective if the loads are applied in discrete bouts, separated by recovery periods as opposed to prolonged loading periods (91).

A variety of in vitro models have been proposed to study the cellular and molecular mechanisms involved in the anabolic effect of loading. However, the relevance of these models to the in vivo situation is equivocal due mainly to the inability to definitively identify mechano-sensitive cells (e.g., osteocytes, osteoblasts, lining cells) and the absence of pathways by which loads applied to the cortical envelope are transferred to trabecular bone cells (e.g., cell and cell attachment molecule deformation, fluid flow (92)). Still, a few studies have assigned a mechanosensitive role to osteocytic, periosteal and endosteal cells by demonstrating changes in signalling molecules such as integrins and the glutamate receptor, tanacin-C, and RoBo-1 (93-96). However, these studies were carried out in cortical bone only and their relevance to trabecular bone cells remains to be investigated.

Studies addressing load-induced cellular and molecular mechanisms in trabecular bone are rather few. Compared to cortical bone, the spongiosa is enclosed in the cortical envelope and is substantially less accessible to cell isolation techniques, particularly in small laboratory animals such as rats and mice. Similar considerations also apply to the assessment of mechanical load induced trabecular deformation. Of particular relevance to the presently proposed project is the structural modeling of trabecular deformation generated by force applied to the vertebral cortex using micro-tomographic imaging (µCT) (97) and work emanating from the Chambers group. The latter investigators have devised a rat model in which the body of the eighth caudal vertebra is subjected to controlled, atraumatic cyclic compression administered via pins introduced into the bodies of the seventh and ninth vertebrae (85,98). Using electron microscopy and in situ hybridization they have recently demonstrated activation of trabecular lining cells and an early (1 h) increase in c-fos and IGF-I expression in trabecular osteocytes. Apparently,
the c-fos response is associated with Ca\(^{2+}\) signaling pathways and integrin binding (99). A late (72 h) increase in transcripts for collagen type I and osteocalcin was observed in trabecular osteoblasts/lining cells (100-102). While contributing important information, by and large these data remain incomplete inasmuch as it is very unlikely that increases in c-fos and IGF-I transcripts comprise the entire inter- and intracellular signaling cascade evoked by overloading. Nevertheless, these and a few other studies (94,102-104) assigned for the first time an experimentally supported physiologic role for osteocytes and lining cells, which the present project proposes to further substantiate and define in mice (105). The mouse is the only mammalian species with a well defined genome currently accessible to routine manipulations and is highly homologous when compared to the human genome. The availability of the proposed model for the study of cortical and trabecular bone adaptation will therefore provide, for the first time, unique opportunities to apply a range of transgenic technologies, not to mention breeding and QTL strategies, to study the molecular mechanisms involved in the response of both cortical and trabecular bone cells to mechanical loading.
References


Background


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3. Development of the mechanical loading device

The work presented here is aimed at developing a device which is able to load the fifth caudal vertebrae (C5) in vivo at a frequency of 10Hz for 3000 cycles, 3 times a week for a period of 4 weeks with amplitudes which will not induce structural failure of the proposed loading configuration. This intensive loading regime has been arrived at by considering other similar studies in which appreciable load induced anabolic effects have been observed in vivo in C57BL/6 mice (1,2).

3.1. Pinning of caudal vertebrae

![Diagram](image)

Figure 3.1: (a) Ideal axial compression of the target vertebra. (b) Additional bending caused by off-centre pins.

The success of the loading configuration is dependent on the mechanical stability of the pinned vertebra. The pin to bone interface will be subject to varying loads and fatigued by frequencies in excess of 1Hz. Hence a pin type must be identified which is small enough to be inserted into the vertebrae and which is strong enough to resist buckling and failure due to fatigue. Pin cross section and material will be critical factors which decide the mechanical limits of the system, i.e. the maximum force and the maximum number of cycles before joint failure. For example whilst the pin must be small it cannot be too small such that it induces failure in the bone via high localized stresses at the pin-bone interface or too small that the pin itself buckles. The pins must also pass through the longitudinal axis of the vertebra and lie on the same plane if ideal cyclic, axial
compression of the target vertebra is to be achieved. Deviation from ideal axial loading will only act to increase stresses so threatening the mechanical stability of the joint (figure 3.1). The significance of the pinning procedure is emphasized when considering the overall goal of the project, it is of vital importance that the pin-bone interface is designed such that it does not impose mechanical limits which lie below the mechanical threshold required to induce bone formation in the target caudal vertebra.

Additional to structural failure via mechanical means is the threat of pin loosening. Guo et al (3) reported occasional progressive loosening of the stainless steel pins used in the rat model due to bone resorption around the pin implant. Considering all of the discussed factors, it is clear the first challenge of this project is to identify a suitable pin type which promotes osteointegration, and to design a jig which facilitates accurate, reproducible pin insertion.

Caudal vertebrae, C4 and C6 shall be the bodies which will be pinned. The preliminary study which investigated the trabecular content of the C57Bl/6 inbred mouse strain, identified the fourth caudal vertebra (C4) has having the most trabecular bone, second to which was C5. However given that C4 is the first proximal vertebra (i.e. the first to protrude the body main body of the mouse) it cannot be the vertebra to which loading is applied. Therefore C4 and C6 must be pinned and the fifth caudal vertebra (C5) taken as the target for cyclical loading.

### 3.1.1. Design of Pinning device

![Figure 3.2](image)

Figure 3.2: (a) 3D schematic of the developed pinning device. (b) Wire frame schematic of the pinning device showing the pin delivery channels. (c) Fluoroscopic image of the pinning device in use.
To surgically implant stainless steel pins into the fourth and sixth caudal vertebrae a special pinning device, compatible with x-ray fluoroscopy was developed. The device makes use of a V-clamp to simultaneously secure the tail and centre the longitudinal axis (cranial-caudal axis) of the caudal vertebrae such that it is perpendicular to two pin channels (figure 3.2). The V-clamp is formed from two blocks of material, an upper clamp made from bronze and a lower clamp made from Perspex to facilitate fluoroscopic imaging. These two components are connected by two location-pins which enable the clamps to be pressed together to secure the tail. The stainless steel pins are loaded into two pin channels in the upper-clamp on top of which is placed a bronze plug. The pins are then inserted into the tail by gently pushing the plug with both thumbs and holding the lower clamp with the fingers. Whilst the longitudinal axis of the caudal vertebra is automatically centred by employing a V-clamp the vertebrae which are to be pinned (C4 & C6) are located using a digital mobile C-arm (OEC MiniView 6800, GE Medical Systems) which provides a fluoroscopic image of the vertebral units beneath the skin of the tail (figure 3.2c). Correct alignment is then achieved by verifying the location of the C4 and C6 relative to two steel markers indicating the trajectory of the pins to be inserted. The device pictured in figure 3.2 has been sized and manufactured specifically for inserting pins simultaneously into the mid-points of caudal vertebrae C4 and C6 belonging to 12 week old C57BL/6 mice. However the insertion point along each individual caudal vertebra can be selected by loading only one pin into the most proximally located pin channel and pinning each vertebra individually. Pinning in this way enabled investigation into the effect of pin location on the mechanical stability of the proposed loading configuration, which will be discussed later. Early prototypes used only Perspex so that the loaded pins themselves could be used to verify their alignment relative to the C4 and C6, however after repetitive use the pin channels became worn which acted to increase the geometric tolerances between the pins and the pin channels often resulting in skewed and inaccurate pin location. To avoid this bronze was used (a more durable material which would greatly prolong the life of the pinning device) and radio-opaque markers were integrated into the lower Perspex clamp (figure 3.3a). Additional to this design revision the depths of the pin channels were also increased.
Development of the mechanical loading device

which acted to reduce the amount of ‘play’ and conical indents where added to the lower surface of the plug, two factors which further increased the accuracy of pin location.

In-vitro pinning tests show that the finalized pinning device yields highly accurate and reproducible results. Figure 3.3b shows 3D images of ‘pinned’ caudal vertebrae scanned at 12µm resolution using desktop micro-computer tomography (Scanco, Bassserdorf, Switzerland). These vertebrae where purposefully pinned at a proximal location along the longitudinal axis of the tail and have had their pins removed before scanning.

![Figure 3.3: (a) Three different generations of the pinning device. (b) 3D micro-tomographic images of 0.4mm diameter pin holes in caudal vertebrae C4 and C6.](image)

3.1.2. Characterization of a robust loading configuration

Using the pinning device to insert stainless steel pins into tail segments, a series of mechanical test were performed to assess the mechanical performance of several different loading configurations. The purpose of this was to maximize the mechanical limits of the proposed loading configuration such that acute loading regimes could be imposed with the reduced likelihood that structural failure would occur. Realization of the most robust design would increase the probability that varying degrees of bone adaptation will be achieved in actual in-vivo studies.

The modified aspects of the loading configuration include, (1) pin diameter, (2) the position of the pin along the longitudinal axis of caudal vertebrae C4 and C6 and (3)
method of constraint. Point (1) is of critical importance as dimensions must be selected such that the bending stresses induced by the loading device do not exceed the yield strength of the pin itself. The pin diameter will also influence failure at the pin-bone interface as its diameter will be inversely related to the local stress concentration induced in bone, i.e. the smaller the pin diameter the higher the stresses in bone local to the pin-bone interface, increasing the probability that the pin will slice through bone. Point (2) investigates the structural support offered by the vertebra at different locations. If pinned at the centre of the vertebrae then the force applied to the pin is transferred directly to two supporting cortical sites, whilst these cortical sites may have sufficient load bearing capacity, the force maybe more evenly distributed if the pins where positioned at the relevant extremities i.e. positioning the C4 pin at the distal end and the C6 pin at the proximal end such that the distance between the two pins is minimized. This would mean both cortical and trabecular components could share the load which could increase the force at which failure occurs. Furthermore investigation of the pin position will also address the issue of additional bending moments which could be potentially detrimental to the loading configuration. Even if the pins are accurately located on the longitudinal axis of the bone, perfect axial compression of the target vertebra will still be difficult to achieve owing to the non-uniform geometry at the ends of the vertebrae. In the compression state, both C4 and C6 will articulate to some degree around the target vertebra, this articulation will not only act to reduce the direct force component applied to the target vertebra but it will also redefine the loading environment in such a way where failure of the system could occur more easily. The effect of this articulation will be more pronounced the further away the pins are from the ends of the target vertebra. This effect could also be minimized by positioning the pins at the distal end of C4 and the proximal end of C6. Finally point (3) investigates the additional strength which may be gained by partially confining the loading configuration to constrain movement in all directions other than in the cranial-caudal axis.

To quantify the strength, identify the failure mechanisms and characterize the most robust loading configuration 24 tail segments consisting of caudal vertebrae C4, C5 and C6 were excised from 12 week old C57BL/6 and divided into 8 groups (n = 3). The C4 and C6 caudal vertebrae of all groups were pinned using stainless steel insect pins
Development of the mechanical loading device

(Fine science Tools, GmbH) using the newly developed pinning device. The factors which differentiated the groups were (1) pin diameter, (2) pin location and (3) method of constraint (figure 3.4a & b). The test matrix clearly defining the different groups is shown in figure 3.4c. Once pinned the tail segments were fitted using their respective attachments and compressed using a mechanical testing device with a cross head speed of 1 mm/s (Model 1456, Zwick GmbH & co. KG, Ulm, Germany). Upon failure of the different loading configurations mechanical testing was immediately terminated. The tail segments were then transferred to a desktop micro-computer tomography unit (μCT 40, Scanco, Bassserdorf, Switzerland) and scanned at 12µm resolution to characterize associated failure mechanisms.

The failure mechanisms for each variation of the loading configuration were highly reproducible. Figure 3.5 provides a quick visual summary of the common failure mechanisms. Figures 3.5a and 3.5b are scout views obtained from centrally-pinned
unconfined and confined samples, post-failure, using desktop micro-computer tomography. They clearly show that in the unconfined case, where significant articulation around the ends of the target vertebra occurs, failure is initiated at the pin-bone interface in C6 which acts to split the vertebra apart along the longitudinal axis. In the confined case, where the degree of articulation is significantly reduced failure is initiated in C6 and C4, however rather than splitting the vertebrae the pin simply slices through the cortical shell. This is shown in figure 3.5c also. Figure 3.5d shows the typical failure modality for those samples which were eccentrically pinned, instead of slicing through the cortical shell and splitting the end plate, the pin’s close proximity to the endplate means that the endplate is simply cracked. The force displacement curve associated with each of these failure mechanisms shows that the confined case yields significantly stronger loading configurations for each pin diameter when compared to the unconfined case and that pinning with a pin of diameter 0.5mm yields the strongest configuration \( F_{\text{confined, 0.5mm}} = 44.3 \pm 3.5 \text{ N} \). There were no significant differences between the eccentrically and centrally pinned loading cases when constrained inferring that the location of the pin is not as important as initially believed to be and does not redefine the loading environment such that drastically different failure thresholds occur. It seems that the cortical shell alone has similar loading bearing capacity to the cortical plus trabecular structure which is present at the relevant extremities of the caudal vertebra. These data clearly show that if the probability of achieving load induced bone formation in the target caudal vertebra is to be maximized, a constrained loading configuration should be employed.

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**Figure 3.5:** (a) Scout view of the observed failure mechanism for and unconfined centrally pinned tail segment. (b) Scout view of the observed failure mechanism for and confined centrally pinned tail segment. (c) 3D tomographic image of C6 vertebra which was centrally pinned and loaded with constraining attachments. (d) 3D tomographic image of C6 vertebra which was eccentrically pinned and loaded with constraining attachments.
3.1.3. In vivo trials/ Osteointegration

One of the problems concerning the rat model which employed a similar loading configuration (3) was pin-loosening. The reasons for this have not been documented, but it is feasible that pin-loosening is caused by either bone resorption around the stainless steel implant, damage local to the pin-bone interface induced by mechanical fatigue or the potentially destabilizing forces which the pin is subjected to during normal cage activity of the rat. To avoid this we propose to use a pin type which would promote osteointegration of the pin itself, thereby reducing the probability that the pin would loosen and fall out of place. Surface modified Titanium pins (Ti) and Hydroxyapatite coated stainless steel pins (HA) were identified as those most likely to induce the desired effect as they are materials which have been successfully used in other implant studies (4,5). It should be noted that the selection of titanium pins would in no way invalidate the previously presented mechanical tests which characterized an optimal loading configuration using stainless steel pins. Bending theory shows that the stresses induced in titanium pins of the same diameter and for the same load cases lie far below the yield point as specified by the manufacturer.

To quantify the degree of osteointegration an in vivo pilot study was performed. 15 C57BL/6 female mice were divided into 3 groups of 5: A Sham group consisting of mice pinned with ordinary stainless steel pins, 0.5mm in diameter (Fine science tools), a Titanium-pinned group consisting of mice pinned with surface etched/sand blasted titanium alloy K-wires (Synthes) and finally a hydroxyapatite pinned group consisting of mice pinned with hydroxyapatite coated stainless steel pins 0.5mm in diameter (CAM implants BV). All pins types were surgically inserted into the fourth and sixth caudal vertebrae using the specially developed pinning device and anesthesia to render the mice unconscious. Following surgery the three groups of mice were housed in separate cages for a period of 2.5 weeks after which the mice were sacrificed using CO₂ inhalation. The pinned vertebrae were then carefully harvested from the tails and transferred directly to ethanol. To quantify the extent of osteointegration the pinned vertebrae were scanned using micro-computed tomography (Scanco AG, Basserdorf, Switzerland). The x-ray tube was operated at 70 kVp and 114 µA and the long axis of the pins was positioned perpendicular to the direction of x-ray propagation to minimize beam hardening effects.
Once scanned all samples were subjected to simple mechanical push tests. Mechanical testing was performed using a mechanical testing device (Model 1456, Zwick GmbH & co. KG, Ulm, Germany) in conjunction with a jig which aligned the implanted pins in the z-axis. Whilst applying a cross head speed of 0.5mm/s, the pins were pushed out and their associated force displacement curves recorded to identify the force at which failure of the pin-bone interface occurred.

Following 2.5 weeks of post surgical recovery, it was apparent that some of the titanium and stainless steel control pins had moved from their original position following insertion, however this was not the case for the hydroxyapatite coated pins. Image data retrieved from the scanning of the implanted pins proved to be inconclusive with respect to visualization of osteointegration. Severe artifacts caused by the high absorbtivity of the metallic pins made it impossible to segment the µCT images and distinguish between bone and implant material local to the pin-bone interface. This was not surprising in the case of the pins consisting of stainless steel but scans of titanium were expected to provide informative images. The mechanical tests however showed that the stainless steel pins coated with hydroxyapatite required significantly more force to push out than both the uncoated stainless steel pins and the surface modified titanium pins (figure 3.6). The yield force for the Hydroxyapatite coated pins was 5.8N +/- 1.16N compared to 2.05N +/- 1.68N for titanium and 1.81N +/- 0.699N for uncoated stainless steel. It is surprising to note that there is no significant difference between titanium and uncoated stainless steel. Other studies (4) have shown titanium to be highly biocompatible and induce appreciable amounts of osteointegration however the evidence presented here seems to indicate otherwise.

![Figure 3.6](image-url)
The fact that only the titanium and stainless steel control pins moved throughout the 2.5 weeks of post surgical recovery, provided early indications that the hydroxyapatite coated pins may have promoted sufficient degrees of osteointegration enabling the pin to remain fixed despite all of the potentially destabilizing forces which the pin is subjected to during normal cage activity of the mouse. The data gained from the mechanical tests support these early observations, since the force required to push out the HA coated pins was on average nearly 3 times that required to push out both uncoated stainless steel and titanium pins. However it is possible that osteointegration may not be responsible for this outcome. The high degree of surface roughness on the hydroxyapatite coated pins could mean that friction is responsible for the relatively high yield force. This possibility highlights a weakness of the test protocol which should have included additional groups of animals on which mechanical tests were performed shortly after pin insertion. Surprisingly the response of the surface modified titanium pins to mechanical testing displayed no significant difference to the response of the stainless steel control pins which we know are incapable of inducing osteointegration. This could be attributed to the length of the recovery time which may have not been long enough for the bone remodeling process to take effect and establish a sufficient, stabilizing degree of osteointegration. It was encouraging to see that none of the pins had fallen out of the tail however this also could be a question of time. Whilst these data neither fully confirm or dispute the fact that osteointegration is responsible for the relatively secure, stable fixation of the hydroxyapatite coated pins in the mouse vertebrae, it is clear that this pin type is the pin-type which will most likely maintain the structural integrity of the loading configuration throughout mechanical loading following a period of 2.5 weeks post surgical recovery.
3.2. **Hardware: Caudal Vertebrae Axial compression Device (CVAD)**

![Diagram of dual axis control system]

Figure 3.7: Schematic of the dual axis control system used to deliver a precisely controlled force to the fifth caudal vertebra of C57BL/6 mice.

The proposed Caudal Vertebra Axial compression Device (CVAD) is a dual axis, force-controlled, closed loop feedback device using a PID based control algorithm (Figure 3.7). The programs defining the exact loading protocols are coded using LabView 7.0 (National instruments) and executed by a servo control board (NI-7344, National Instruments) with an embedded real-time operating system, which drives two linear actuators (LA25-42-000A, Bei–Kimco Magnetics) via a multi-axis Servo Motor Drive (NI MID-7654, National Instruments). Each actuator is fitted in series with a 10N-load cell (13/2443 -16 TRANSMETRA haltec GmbH) providing the feedback signal which closes the control loop. The feedback signal is amplified using a small inline amplifier (IAM-15-10-50, ENTRAN sensors) and relayed back to an ADC input channel on the servo control board. Both the load cell and amplifier are excited by a small 15V power unit (PS-30 ENTRAN sensors). In addition an optical encoder (Mercury 2000, MicroE Systems) captures displacement information which is acquired by the same servo control board.
Development of the mechanical loading device

Axial, cyclic compression of the fifth caudal vertebra is achieved by transmitting the applied force via two metallic pins inserted into adjacent vertebrae (C4 and C6) (Figure 3.8b). By clamping the proximal most pin and securely connecting the distal most pin to the sliding structure driven by the linear actuator the target vertebra can be cyclically compressed (Figure 3.8a). The casing of the linear actuator is securely mounted onto a stainless steel base plate whilst the free-moving inner-core is guided by a linear bearing, giving the device two degrees of freedom (positive and negative displacement). To facilitate operation of the encoder a small optical strip is attached to the side of the housing containing the linear bearing. Screw holes on the top surface of the housing provide anchorage points for a bracket which aligns the load cell with the loading axis. The other side of the load cell is connected to an Applicator which secures the distal-most pin in the mouse tail whilst the proximal-most pin is securely fixed by a screw-clamp attached to the stainless steel base plate (Figure 3.9). To secure the tail in position prior to loading the segment of the mouse tail containing the distal-most pin is threaded through the screw cap and the Applicator until the pin sits in a horizontal slot cut into the front face of the Applicator. Once located the screw cap is rotated thereby securing the pin in-between the inner face of the screw cap and the Applicator. The clamp which secures the proximal most pin is designed to constrain the tail segment such that near perfect axial compression of the fifth caudal vertebra is achieved (Figure 3.9c). Note that the Applicator and clamp have been designed to constrain the mouse tail in an identical way to that which was identified as the most robust loading configuration (see section 1.1.2-
1.1.4). During fixation of the pins and throughout the operation of the device the mouse is placed on a custom made table where the nose of the mouse is placed into the nozzle of and anesthetizing system (Figure 3.8a). The anesthetizing system (Provet AG) is constituted of an oxygen supply which is mixed with vaporized isoflurane and delivered directly to the mouse via the inner-tube of a compound plastic tube (a small tube running through the centre of a larger outer tube). Excesses of the oxygen-isoflurane gas mixture, local to the point of inhalation are pumped via the outer tube to a gas filter so as not to endanger the unconscious mouse.

Figure 3.9: (a) 3D CAD drawing of the CVAD. (b) Exploded view of the components which secure the stainless steel pins during mechanical loading. (c) Side view of the clamping arrangement showing how the tail is constrained to achieve near perfect axial compression.
3.3. **Software: FlexMotion and LabView**

The CVAD is controlled by the NI-7344 servo control board (National Instruments), inserted into the PCI slot of a standard desktop computer. It is programmed with LabView, using a host of functions in the FlexMotion library. Conventionally FlexMotion software and hardware provide functionality and power for integrated motion systems, however they can be easily adapted to provide force control. Since the FlexMotion system controls electrical output signals according to electrical feedback signals, regardless of how these signals are converted or transduced, replacement of the encoder with a load cell will transform a motion control system into a force control system. The control system is defined via two separate software environments. The first,
LabView, is used to define various attributes of the loading protocol such as peak force, frequency etc, it is also here where the required sequence of events are described and executed. The second is MAX (Measurement and Automation Explorer). MAX verifies that a variety of different plug-ins, examples of which include data acquisition boards and the FlexMotion controller, are installed correctly and are communicating with the host computer as well as providing the user with an interactive environment for configuring, testing and trouble shooting. It is here where most of the fundamental parameters are defined to setup the device such that device is ready for general use, i.e. configuration of input and output channel addresses and PID values. However some of the parameters configured here can be redefined and overwritten in the main LabView program. The Graphical user interface developed for the control of the CVAD and the associated operating sequence is shown in figure 3.10. The GUI is split into two columns and shows a duplicate set of panels and charts for each of the two axes. The display is organized such that data specific the Axis 1 is shown in the first column and data specific to Axis 2 is shown in the second. Before the internal workings and the program architecture are explained in sufficient detail the basic sequence of events which constitute the loading protocol shall be discussed, making it easier to follow the proceeding technical descriptions. The following paragraphs describe the sequence of operations involved in successfully applying a precisely defined mechanical signal to the fifth caudal vertebrae of two mice.

Step 1
Prior to execution and placement of both mice into the system the user defines the characteristics of the two force signals (Maximum force, minimum force, Number of cycles) which will be transmitted at 10 Hz to the fifth caudal vertebrae both mice (figure 3.10, label A). LabView uses this information to construct a mechanical command signal defined by a discrete set of points which it will then write to two buffers on the NI-7344 controller. For simplicity the input and output units of force are Newtons, within the program ‘counts’ are used. Conversions to and from these units are done via two simple conversion factors specific to each load cell.
To ensure that a precise mechanical signal is transmitted both forces are zeroed before the mice are clamped into position. This is done by pressing the initialize button which acquires the magnitude of the voltage from each of the two ‘unloaded’ load cells for a short period of time, computes the average and sets both resultant voltages to represent zero force. To verify that the load cells are active, the raw voltage signals are displayed as small graphical charts at the top of the screen (figure 3.10, label B).

Step 3
The mice are placed into the system as described previously. It is likely that following attachment the tails will be in a state of mild tension or compression, therefore once in position a second set of graphical displays provides the user with a visual indication of the forces which are now applied to both mouse tails (figure 3.4, label C). Again to ensure the transmission of a precise mechanical load both forces must be set to zero. This is achieved by modifying the position of each loading axis via the manual adjustment screw (figure 3.8a) allowing the user to relieve the tails state of compression or tension.

Step 4
Mechanical loading of the fifth caudal vertebrae is initiated by pressing the button marked ‘Start Loading’. Here the two set of points describing the mechanical command signal for each axis, which were written to two onboard buffers, are executed whilst the feedback signals from the load cells are acquired and displayed for each axis during loading. The number of points belonging to each force signal which have been executed is also displayed, informing the user of the progress of the loading device (figure 3.10, label D).

Step 5
As a quality control measure at the end of a loading bout the accuracy of each transmitted mechanical signal is quantified and displayed by analyzing the recorded feedback signals from each load cell. This is done using a specially developed algorithm which isolates each minima and maxima of the recorded waveforms, and plots them against the number of cycles. By this method the user can visually assess the accuracy of each mechanical
signal (figure 3.10, label F). Averaged numerical values are displayed in cell below theses charts (figure 3.10, label H). As an additional feature, the system also records the history of each optical encoder, and processes both displacement signals in the same way (figure 3.10, label G), permitting the calculation of the variation of the apparent stiffness for each loaded mouse over time (figure 3.10, label I).

Step 6
Following the post processing of the load cell and encoder signals the user is then prompted to save the numerical summary in a text file format. After which LabView then clears the buffers on the FlexMotion controller of all data and re-initializes in preparation for subsequent bouts of mechanical loading.

3.3.1. Program design

An overview of the program architecture is given by figure 3.11. The mechanical command signal constructed by LabView is composed of two parts, a linear component and a sinusoidal component. The linear command signal, defines a straight line trajectory from 0N to the defined maximum force at a rate of (1N/s). This slow ‘ramp’ up to the peak value has been implemented to preserve the structural integrity of the loading configuration. The sudden pulse which results from immediately applying a high frequency signal from a 0N start point could potentially damage the pin-bone interface, using this method the device is allowed to gently compress the pinned tail segment to

Figure 3.11: Overview of the different modules which constitute the main LabView Program.
remove ‘slack’ from the system. The second part is composed of a cosine curve. Again using the maximum and minimum force values LabView constructs one period of cosine wave with 10 discrete points, the reason for which shall become clear. This single period is then repeated for the defined number of cycles. The two components are added together to give a complete finalized command signal. Note that minimum value of the high frequency component is not 0N but 1N, this is to avoid potential problems of system instability. Once LabView has created and array of points which define the complete mechanical command signal they are written to two buffers, one for each axis, these points are then executed by a process known as contouring, whereby the FlexMotion controller uses a cubic spline algorithm to smoothly interpolate between the discrete points. The controller can only execute each point at a maximum of 10 ms intervals hence the frequency is determined by the number of points which constitute one period, i.e. for a waveform to have a frequency of 10Hz the period must be described by 10 discrete points. Hence the fewer points there are to describe a single period the more the signal will deviate from a sinusoidal form. This is a limitation of the system and will prevent higher frequencies being used. This is less of a limitation when defining the rate at which the linear component of the command signal is executed. To achieve a steady linear trajectory which increases at the rate of 1N/s to the maximum defined force the number of points must be equal to the maximum force multiplied by 100 (the number of points which can be executed in one second).

An additional limitation is buffer size. Any number of buffers can be defined however their sum is unable to exceed 16000 points. The size of the array which describes a single command signal is significantly greater than the specified limit, requiring around 30,000 points. Hence 60,000 points must be executed in total with only 8000 points allocated for each buffer. This problem is solved by monitoring the status of the buffer and over-writing the linear component of each array once it has been executed with sinusoidal waveforms which fit perfectly with the sinusoidal waveforms belonging to the original signals. Because the buffer is configured to ‘wrap around’, once all the points in the buffer have been executed the controller will simply repeat the points in each buffer providing a continuous command signal. This will continue until a specified condition is met, in this case until the total number of points which have been executed is
equal to 30,000. Once this condition is met loading will then cease and the buffers are cleared in preparation for the next loading bout. It should be noted that operations which write or read from the buffer are computationally intensive and cause a momentary pause in execution. At anytime during operation of the system if the user defined-maximum force is exceeded by 1N a soft limit is triggered and the system is shut down, this is to protect the structural integrity of the mouse tail thereby ensuring that no subjects are lost from a particular loading group during a study. Once execution has ceased the history of the recorded feedback signals are analyzed using the specially developed MML algorithm which locates all of the maxima and minima associated with the force and displacement feedback signals. This information is used to provide a quality control measure such that the user can verify if the input forces are being successfully controlled to.

3.3.2. MML Algorithm (Maxima and Minima Location Algorithm)

To ensure that the CVAD is functioning correctly and accurately it is important to implement quality control of the transmitted mechanical signal. To do this a simple wave analysis algorithm was developed to analyze the recorded history of all feedback signals.
Development of the mechanical loading device

(Load Cell and Optical Encoder). The algorithm essentially registers all of the turning points belonging to each waveform, and plots them on the screen, making it easy for the user to quickly assess the accuracy of the actual transmitted mechanical signal. It simultaneously displays the mean maxima and minima for the first and last 50 cycles of each entire loading signal (figure 3.12).

The input to the algorithm is a recorded feedback signal which is a 2-D array of numerical values. The first column contains the index value or the integer value (i) giving the rank position of a particular point which constitutes the signal, typically ranging from 1 – 30000. The second column contains the actual corresponding signal value (v). The algorithm is applied to a single signal at a time and is configured to locate either minima or maxima. It first separates the recorded linear component of the signal from the sinusoidal component and then employs a forward and backward difference method acting on the actual signal values (v) to create a new 2-D array composed of the original index value and the suspected maxima or minima. This is done by assessing the sign of the difference between a suspected minima or maxima value (v) and subtracting from it the neighboring points (v+1) and (v-1).

Identification of maxima and minima in this way is relatively straight forward if the feedback signal is ‘clean’ however at a frequency of 10 Hz a noise component means that 2-4 maxima or minima maybe detected at a single turning point thus an extra step is required which is able to discern the true minima or maxima from the accompanying noise. To do this we must make use of the associated index value (i). The clusters of minima or maxima at a particular turning point indicative of noise are characterized by index values (i) which are close together. For true minima or maxima at a frequency of 10Hz the difference between consecutive indices is normally greater than 10. The ‘false’ minima or maxima can therefore be filtered out in a second step which assesses the magnitude of the difference between the array index values associated with each potential maxima or minima. Five forward differences are assessed between each index value (i) and (i+1), (i+2), (i+3), (i+4) and (i+5). For all those values where the absolute difference is less than 10 the maximum or minimum signal value (v, v+1, v+2, v+3, v+4, v+5) belonging to this subgroup is selected as the true maximum or minimum.
Before the development and implementation of this simple algorithm more obvious solutions were considered. Scanning through the array using a window with a size equal to the number of points in one period and taking the maximum or minimum value from this subset was implemented, however because one period was not always described by the same number of points the window would ‘drift’ during the analysis of 3000 cycles and report drastically false minima and maxima. Another convenient solution would have been to analyze the feedback signal in real-time or by triggering the analysis of one period just after it had been completed, however there is no way of accessing the clock onboard the FlexMotion controller to trigger real-time event analysis. The LabView clock is the only way to relay time information however this can be affected by other process running simultaneously on the host computer which is not advised. Another solution would have been to incorporate a DAQ board into the system, where the clock is accessible and use this to acquire the feedback signal. However this would have further complicated the device rendering it more expensive and less user-friendly.

3.4. Performance of the mechanical loading device

To ensure that each axis of the CVAD performed correctly and accurately, the load cells were first calibrated by applying a series of known weights and acquiring the voltage output using both a Voltmeter (FLUKE multimeter 23) and the appropriate 12 bit ADC feedback channel on the Flex motion controller. The Flex motion controller converts the input voltage signal into a digital value which is assigned a count value between 0 and 4096, where 0 counts = 0 V and 4096 counts = 5V (this range can be redefined in MAX).

To verify that the system of digital units used by the flex motion controller and LabView accurately represented the acquired voltage signal, the acquired voltage value was back calculated using the recorded count value using the following equation:

\[
\text{Voltage} = \text{Counts} \left( \frac{5}{4096} \right)
\]

Once the accuracy of the Flex motion acquisition system was confirmed, the linear coefficients relating counts to force were calculate for each axis and used in the main
LabView program such that the end user worked only with Newtons. The dynamic performance was assessed by a series of tests using pinned tail segments from 12 week old C57BL/6 mice. In the first set of tests 2 tails, excised from mice sacrificed using C02, were fitted into both axes and subjected to loading regimes consisting of 3000 cycles performed with amplitudes of 4N and 8N, for frequencies of 1Hz and 10Hz. Note that the oscillatory component of the loading regime was preceded by a linearly component, whereby the force applied to the target vertebra was ramped up to the maximum force at a rate of 1Ns⁻¹, this was to avoid sudden impulses which have been shown to damage the pinned vertebrae. Furthermore, applied force oscillated between the maximum defined force and a minimum of 1N, this ever-present load was required to maintain dynamic stability of the control system. Optimal Proportional, differential and integral control constants (Kp, Kd and Ki) were chosen for each axis such that the force output was accurately and stably defined. The accuracy of each axis was determined by using the MML algorithm to identify the values of the 3000 maxima and the 3000 minima present in the feedback signal and comparing the average of these values with those defined by the user. To assess reproducibility of the device each test was repeated 3 times. Throughout testing the frequency of the feedback signal was cross referenced by performing a Fast Fourier Transform analysis on the signal as acquired by a multifunctional DAQ card (National instruments, NI-6036E).

3.4.1. Performance data

![Graphs showing force vs. output voltage for two axes](image_url)

The graphs illustrate the linear relationship between force and output voltage for each axis. The equations provided, $\text{Force} = 0.0044 \times \text{Counts}$ for Axis 1, and $\text{Force} = 0.0055 \times \text{Counts}$ for Axis 2, indicate the sensitivity of the load cell to the applied force.
Loading both load cells with a series of known weights produced almost identical linear responses when measuring the voltage outputs with both a voltmeter and the FlexMotion control board (Figure 3.13 a & b) confirming that all of the components involved were functioning accurately thereby enabling the establishment of two distinct conversion factors which describe the linear relationship between force and counts. Note that the intercept which is present in figure 3.13 is not accounted for in the equations describing the conversions, this is because the force is ‘zeroed’ by the main LabView program before loading is executed. Extremely high accuracy was demonstrated when performing the tests at a frequency of 1Hz: When using the following PID value: KP1 = 0, Kd1 = 0, Ki1 = 1, KP2 = 0, Kd2 = 0, Ki2 = 2, the differences between the target values and actual recorded values were less than 0.01N furthermore the standard deviations associated with maximum and minimum values were less than 0.005 N. This however was not the case when loading at a 10Hz, using the same PID constants. Figures 3.14 (a) and (b) show the maxima and minima obtained when applying the MML algorithm following the repeat loading a mouse tail at 10Hz, in-vitro on axis 1 at two discrete force levels (4N and 8N). Results clearly show that the maxima and minima achieved throughout the 3000 cycles for both a single loading regime and repeated loading regimes are highly reproducible with standard deviations being less than 0.06 N. However both the minimum and maximum target values (minimum: 1N, maximum: 4N and 8N) are not sufficiently attained. In the case of both axes the recorded maxima are less than the target peak-values and the recorded minima are greater than the target minimum-value: for a target peak-value of 4N, the differences are around 0.25N and 0.3N respectively. These differences increase for a target-peak value of 8N: approximately 0.6N and 0.7N respectively. Hence the outcome of the control system when operating at 10Hz is such that the user defined force profile is marginally ‘compressed’, and the extent of this ‘compression’ is increased for higher input forces. To improve the accuracy of the feedback signal at 10Hz the PID values were re-adjusted, however even the smallest increase in either the integral gain (Ki) or the proportional constant (Kp) for either axis resulted in system instability. Fourier analysis of the all feedback signals show that the desired frequencies were accurately achieved: 1.01 Hz +/- 0.003 and 9.99 Hz +/- 0.02.
3.4.2. Feed-Forward strategy

To improve the accuracy of each loading axis a second set of tests were necessary to assess a feed-forward strategy. The tests described above performed at frequencies of 10Hz showed that adjustment of the PID settings alone could not achieve sufficient accuracy. The recorded maxima and minima were fractionally smaller and greater, than the desired maxima and minima respectively i.e. the outcome of the control system when operating at 10Hz is such that the user defined force profile is marginally ‘compressed’. It was noted that the extent of this compression, relative to the user-defined waveform increased as the desired peak force increased, (figure 3.14a & b). For a desired waveform with 4N maxima and 1N minima, the outcome would exhibit maxima reduced by 0.32N (mean) and minima increased by 0.34N, the magnitude of these changes increased for a desired waveform with 8N maxima and 1N minima (0.54N and 0.67N, respectively) . To compensate for this it was necessary to adopt a ‘feed-forward approach’ and ‘stretch’ the command signal by fractionally increasing the user defined maximum force ($UDf_{max}$) and fractionally decreasing the user defined minimum force ($UDf_{min}$). Pre-tests showed that control of the ‘stretching’ was not as simple as increasing both the $UDf_{max}$ and $UDf_{min}$ by equal factors and that an interdependency existed between both inputs: adjusting the $UDf_{max}$ value whilst fixing the $UDf_{min}$ value would change the output min value, the inverse of this statement was also true. The accuracy of this control system is therefore
determined, in addition to the PID settings, by adjusting the input values, whereby only
the correct combination of the adjusted inputs will result in the desired output waveform.
To determine the correct combination of ‘adjusted’ input values, such that any desired
waveform could be accurately achieved, i.e. waveforms with peak values of 2N, 4N, 6N
and 8N all with minimum values of 1N, tests were performed to identify the nature of the
relationship between ‘adjusted’ input values (AI\text{max} and AI\text{min}) provided by the user and
the averages of the observed output maxima and minima (OT\text{max} and OT\text{min}). Precise
characterization of this relationship would then allow the necessary compensation
mechanisms to be included in the background program architecture thereby establishing
an apparent 1:1 relationship between input and output.

Multiple tests were performed on each axis whereby two B6 mouse tails were
loaded for 300 cycles at a frequency of 10Hz for multiple combinations of AI\text{max} and
AI\text{min} values. A 9 X 8 test matrix was implemented using the following adjusted input
values: AI\text{max} = 4N, 4.5N, 5N, 5.5N, 6N, 7N, 8N, 9N, 10N, and AI\text{min} = -2N, -1.5N, -1N,
-0.5N, -0.25N, 0N, 0.5N, 1N. For each combination of input values the 300 output
maxima and minima were analyzed using the MML algorithm to provide a mean output
maxima (OT\text{max}) and mean output minima (OT\text{min}). The resultant 3-D data sets were then
used to identify pairs of values which gave mean output maxima of 4N and 6N along
with mean output minima equal to 1N. Two linear equations were then fitted to these
‘ideal’ pairs to determine the ideal pair of inputs for an output wave with 8N maxima and
1N minima (for each axis). Once characterized the accuracy of this feed forward strategy
was then assessed by using the MML algorithm over 3000 cycles to measure the accuracy
of output wave forms when loading multiple tail samples of C57BL/6 mice at different
ages (n_{12 weeks} = 3, n_{19 weeks} = 3) where the desired output maxima were 4N, 6N, and 8N
(for each axis).

3.4.3. Performance data using the feed forward strategy

Figure 3.15a shows part of a surface plot which describes a linear relationship between
AI\text{max}, AI\text{min} and OT\text{min}. Figure 3.15b shows a similar linear relationship between AI\text{max},
AI\text{min} and OT\text{max}. There are clearly multiple combinations of input values (shown by the
dotted lines for the case OT\text{min} = 1N and OT\text{max} = 4N) which satisfy one of the two
requirements of the output waveform i.e. either the output waveform has 1N minima or that the output waveform has 4N maxima. However there is only one combination were both conditions are met and it is this combination of input values which must be used if, in this case, Axis 1 is to be optimally tuned to deliver a sinusoidally varying force with 4N maxima and 1N minima. It is interesting to note that that the output minima are strongly influenced by the AI_{min} value and less effected by variation of the AI_{max} value. Similar behavior is also observed when considering the OT_{max} value. The AI_{max} value can be seen to exert a much stronger influence than the AI_{min} value. The ideal combination of AI_{max} and AI_{min} values was also determined for an output waveform with 6N maxima and 1N minima. These values can be seen in figure 3.15c where they are plotted along side the results of the 4N case. The straight lines fitted between the sets of ideal AI_{max} and AI_{min} values describes the relationship between the ideal system input and the optimal system output. When these two linear relationships were used to predict the input values
required to output a waveform having 8N maxima and 1N minima a highly accurate waveform was achieved. When loading a 12 week old tail, in vitro, on Axis 1 an average maximum of 7.99N +/- 0.03 N was achieved along with an average minimum of 1.02 N +/- 0.06N (as measured by the MML algorithm). Table 3.1 further demonstrates the accuracy and reproducibility of both CVAD axes when loading a number of different C57BL/6 mouse tails at 3 different force levels using the force input values as determined by the different linear relationships established for both axes.

<table>
<thead>
<tr>
<th>Peak Force</th>
<th>Mouse</th>
<th>AXIS 1</th>
<th>12 Weeks</th>
<th>19 Weeks</th>
<th>AXIS 2</th>
<th>12 Weeks</th>
<th>19 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>4N</td>
<td>A</td>
<td>3.98 +/- 0.04</td>
<td>5.98 +/- 0.05</td>
<td>1.02 +/- 0.01</td>
<td>1.02 +/- 0.01</td>
<td>3.99 +/- 0.06</td>
<td>1.04 +/- 0.03</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.97 +/- 0.06</td>
<td>5.99 +/- 0.01</td>
<td>1.02 +/- 0.02</td>
<td>0.98 +/- 0.07</td>
<td>4.03 +/- 0.08</td>
<td>0.98 +/- 0.04</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.89 +/- 0.04</td>
<td>3.95 +/- 0.08</td>
<td>1.0 +/- 0.03</td>
<td>1.0 +/- 0.01</td>
<td>4.02 +/- 0.08</td>
<td>1.06 +/- 0.01</td>
</tr>
</tbody>
</table>

| 6N         | A     | 5.98 +/- 0.05 | 6.07 +/- 0.08 | 1.02 +/- 0.01 | 1.05 +/- 0.01 | 5.90 +/- 0.03 | 1.02 +/- 0.01 | 6.01 +/- 0.08 |
|            | B     | 5.99 +/- 0.01 | 6.01 +/- 0.05 | 0.99 +/- 0.07 | 0.98 +/- 0.04 | 5.89 +/- 0.07 | 1.01 +/- 0.08 | 6.03 +/- 0.09 |
|            | C     | 6.02 +/- 0.09 | 5.99 +/- 0.07 | 1.05 +/- 0.06 | 1.1 +/- 0.04 | 6.09 +/- 0.07 | 1.06 +/- 0.01 | 5.97 +/- 0.05 |

| 8N         | A     | 7.98 +/- 0.02 | 8.05 +/- 0.06 | 0.96 +/- 0.05 | 0.94 +/- 0.03 | 8.00 +/- 0.08 | 1.09 +/- 0.01 | 7.94 +/- 0.02 |
|            | B     | 7.99 +/- 0.03 | 8.04 +/- 0.01 | 1.02 +/- 0.06 | 1.05 +/- 0.06 | 7.99 +/- 0.01 | 0.93 +/- 0.01 | 8.02 +/- 0.09 |
|            | C     | 8.01 +/- 0.05 | 7.96 +/- 0.04 | 1.08 +/- 0.03 | 1.09 +/- 0.01 | 3.98 +/- 0.06 | 1.1 +/- 0.01 | 7.92 +/- 0.04 |

Table 3.1: Output maxima and minima (italics) obtained using the MML algorithm when loading C57BL/6 mouse tails in-vitro for 3000 cycles on both axes 1 & 2 using the feed-forward strategy.
3.5. **Validation of the mechanical loading device**

This section is based on the following paper:


### 3.5.1. Abstract

To facilitate the investigation of bone formation, *in vivo*, in response to mechanical loading a Caudal Vertebra Axial compression Device (CVAD) has been developed to deliver precise mechanical loads to the fifth caudal vertebra (C5) of the C57BL/6 female mouse via two stainless steel pins inserted into the adjacent vertebrae (C4 and C6). A combined experimental and computational approach was then used to quantify the micro-mechanical strain induced in trabecular and cortical components following the application of static and dynamic loading regimes using the CVAD. Cortical bone strains were recorded using micro-strain gages attached to dorsal and ventral sites of the C5 vertebrae (n = 4). Peak forces of 1.5N, 2N, 4N, 6N and 8N were applied according to static and dynamic loading regimes (1Hz and 10Hz sinusoids). Regression analysis showed all peak strain measurements to vary linearly with applied peak forces ($R^2 > 0.95$). For an applied peak force of 8N the average peak cortical strains for all vertebrae were $900 \pm 111\mu\varepsilon$, $975 \pm 119\mu\varepsilon$, $751 \pm 220\mu\varepsilon$, and $793 \pm 142\mu\varepsilon$. In all cases dorsal and ventral strain gage responses were shown to be significantly different (6%-10%, $P < 0.001$), indicating the presence of an additional bending component whilst under compression. FFT analysis of output signals from the load cell and strain gages showed that loading frequencies of both 1Hz and 10Hz were successfully transferred to the target vertebra. Finite element models based on micro-computed tomography (12µm) were constructed for all C5 vertebrae. Both theoretical and experimental cortical strains correlated extremely well ($R^2 > 0.96$) for a Young’s modulus of 14.8 GPa, thus validating
the FE model. The model predicted average trabecular strains of $618 \pm 398\mu\varepsilon$, $559 \pm 350$, $528 \pm 330\mu\varepsilon$, $576 \pm 376\mu\varepsilon$ for an applied force of 8N, which were shown to be significantly less than predicted cortical strains ($P < 0.05$). In this study we have successfully applied mechanical loads to the C5 vertebrae of C57BL/6 mice, demonstrating the potential of this model to be used for in vivo loading studies aimed at stimulating both trabecular and cortical bone adaptation. We have also established a computational tool able to determine the 3D stress strain environment which when used in parallel with the quantification of both load induced bone morphology and the associated cellular responses will better facilitate the elucidation of the mechano-sensitive pathways involved in bone formation.

### 3.5.2. Introduction

In addition to the genetic background of the mammalian species, mechanical loading is perhaps one of the most important factors regulating bone mass and shape. Although the basic form and development of bone are genetically encoded, their final mass and architecture are governed by adaptive mechanisms sensitive to the mechanical environment. Several studies have shown load induced bone formation to be a function of the energy transferred to bone, showing load amplitude, frequency, duration and recovery time to be critical factors in determining the level of the anabolic response (1,6-8). A quantitative and qualitative understanding of how the mechanical environment governs bone adaptation, combined with knowledge of the associated cellular responses would provide opportunities to mimic or augment the response of bone to mechanical stimulation by pharmacological agents and may lead to novel strategies in the management of diseases such as osteoporosis. Currently little is known about the cellular mechanisms governing the adaptation of cortical bone and possibly more importantly trabecular bone, a significant structural component which has been shown to have a more enduring sensitivity to mechanical stimulation in mature human adults than that of cortical bone (9,10). Several studies have established mouse models for the study of cortical bone adaptation and associated biochemical pathways in response to mechanical loading using C57BL/6 and C3H/Hej inbred strains (11-13). However the whole cascade of biochemical responses remains poorly understood and specific genes or combinations
of genes have yet to be discovered, moreover the focus in these studies has been on cortical bone and not trabecular bone. The only model currently available for the elucidation of molecular mechanisms involved in load induced, trabecular and cortical bone formation is a model using rat caudal vertebrae (14,15). Here the eighth caudal vertebra (C8) in a rat tail was cyclically compressed via pins inserted into the adjacent vertebra (C87 and C9) at a frequency of 1Hz. Studies using this model have been hampered by technical hurdles such as the unavailability of bone cell isolates used to investigate global gene expression and identify the set of mechanical load regulated genes. Furthermore the principal limitation imposed by the rat model is its current inaccessibility to potential genetic manipulations. The goal of this study is to adapt the loading configuration used in this rat model for small inbred strains of mice, and to determine the micro-mechanical stress/strain environment resulting from the application of dynamic loading regimes (< 10Hz) in both cortical and trabecular components. Ultimately correlation of the micro-mechanical environment with associated structural adaptation and cellular responses will permit simulation of the mechano-biological processes involved in load induced bone morphology, contributing to a better understanding of how bone adapts to its mechanical environment. The objectives of this study can therefore be broken down into 2 specific aims: 1) Design a new force controlled loading device, which can accurately apply dynamic loading profiles at high frequencies to small murine caudal vertebrae. 2) Using a combined experimental and computational approach validate the in vivo mouse model for mechanically stimulated bone adaptation by establishing a quantitative relationship between the load applied and the mechanical strains subsequently induced in the target caudal vertebra.

3.5.3. Materials and method

A force controlled, Caudal Vertebra Axial Compression Device (CVAD) using a PID based control algorithm has been designed to apply a precisely controlled, cyclical, compressive load to the C5 vertebra in the C57BL/6 mouse tail at frequencies of up to 10Hz via two stainless steel pins (0.5mm in diameter, Fine Science Tools, Heidelberg, Germany) surgically inserted into C4 and C6 vertebrae (Figure 3.16). The programs defining the exact loading protocols are coded using LabView 7.0 (National Instruments,
Austin, Texas) and executed by a servo control board (NI-7344, National Instruments) with an embedded real-time operating system. Following amplification by a signal amplifier (MID-7654 National Instruments), the servo board outputs a signal to a linear electro-magnetic actuator (LA25-42-000A, PBA Systems Pte Ltd, Singapore). Compression of C5 is achieved by using the actuator to drive a shaft, mounted on linear bearings, connected to the distal pin. The proximal pin is clamped such that ideal uniaxial compression of the target vertebra is encouraged. The control loop is closed by a load cell (13/2443 -16 TRANSMETRA haltec GmbH, Neuhausen, Switzerland), fitted directly in series with the axis of compression and upstream of the target vertebra.

Figure 3.16: (a) Overview of the control system for the CVAD. (b) Photo showing the loading axis belonging to the CVAD. (c) Fluoroscopic image of a mouse, graphically edited to show the location of the stainless steel pins. To achieve compression of a C5 vertebra the mechanical signal is applied to the distal pin (left) whilst the proximal pin (right) is clamped.

To investigate how the force applied by the CVAD is transferred to the fifth caudal vertebra and to accurately quantify the 3-D stress/strain environment in the C5 vertebra a combined experimental and computational approach was employed whereby cortical strains of C5 vertebrae were measured in-vitro to yield data which was then used...
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to validate the theoretical strains calculated by finite element (FE) models of the same caudal vertebrae: Pairs of micro-strain gages (EA-06-015DJ-120, Vishay Micro-Measurements, Malvern, PA) were attached to 4 C5 caudal vertebrae (V1, V2, V3, and V4) belonging to pinned tail segments excised from 12-week-old C57BL/6 female mice (Harlan Ltd, Horst, The Netherlands) immediately after sacrifice. Two small incisions were made local to both dorsal and ventral aspects of the tail segments close to the midpoints of the C5 vertebrae. The small exposed areas of bone were cleaned off soft tissue and two micro-strain gages attached such that strain in the axial direction (the proximal-distal axis) was measured (Figure 3.17a and 3.17b). The strain gages were excited and their signals amplified and conditioned using a strain gage conditioning module (SCC-SG01, National Instruments). The strain gage output signals were acquired using an E-series multifunctional DAQ card (National Instruments, NI-6036E). The tail segments were then placed in the CVAD and loaded. To investigate the effect of the visco-elastic interconnecting vertebral discs (16) the tails were loaded according to three different loading regimes (Figure 3.17c). The pinned tail segments were first subjected to static loading regimes whereby the force applied by the CVAD was linearly increased at a rate of 1 Ns\(^{-1}\) and held constant at seven different peak forces (1N, 1.5N, 2N, 2.5N, 4N, 6N and 8N) for a period of 50 seconds. In between each loading step the tail was allowed a period of recovery to allow the visco-elastic matrix of the intervertebral disc to ‘exude back’ to a normal, pre-load state. The tails were then subjected to the first dynamic loading regime whereby the force was ramped up to the peak force (linearly increasing at a rate of 1Ns\(^{-1}\)), followed by a 1 Hz sinusoidal dynamic waveform for the same peak forces as described previously. Again in between each loading step the tail was allowed a period of recovery. Finally corresponding dynamic waveforms with a frequency of 10 Hz were applied in the same manner. The resultant output signals from the strain gages and the load cell were then processed to determine frequency and all maxima and minima. Once tested the C5 vertebrae were then harvested and scanned using micro-computed tomography (\(\mu\)CT) at 12 \(\mu\)m nominal resolution (\(\mu\)CT 40, Scanco Medical AG, Bassersdorf, Switzerland). Settings for the scanning parameters and subsequent image treatment are described elsewhere (P. Rüegsegger, B. Koller and R. Müller. A microtomographic system for the nondestructive evaluation of bone architecture. Calcif.
Figure 3.17: a) Experimental setup showing a mouse tail placed in the CVAD with strain gages attached, b) A graphically edited fluoroscopic image of a mouse tail showing the position of the two strain gages, c) Transverse cut through the C5 caudal vertebra showing dorsal and ventral regions of gage placement. d) The three different loading regimes applied to each tail.

Tiss. Int., 58:24–29, 1996). The resulting 3D images of the vertebra were converted into 3D FE models by mapping each bone voxel to an eight-node brick element, yielding models consisting of approximately 1,800,000 million elements. To model the intervertebral discs additional voxels were added to the proximal and distal ends of the vertebra such that the disc’s geometry was approximated (16). A initial value of 19 GPa was assigned as the young modulus to those voxels representing bone (17) , whilst a much softer value of 2.48 MPa was given to the voxels describing the intervertebral disc (16) both materials had a Poisson’s ratio of 0.3. All models were aligned in the z-axis, defined by the centre coordinates for mid-cortical sections according to the second moment of area. To mimic the loading condition applied in-vitro, the surface of the distal-most intervertebral disc was fixed in the axial direction while allowing free lateral movement. A compressive axial force of 8N was applied to the surface of the proximal most intervertebral disc (Figure 3.18). Because the FE model was linear elastic, the resulting strains and stresses were scaled accordingly to allow the necessary comparisons with experimental data. The models were solved using an element-by-element method (S.
K. Boyd, R. Müller and R. F. Zernicke. Mechanical and architectural bone adaptation in early-stage experimental osteoarthritis. J. Bone Miner. Res., 17:687-694, 2002.\(^{(18,19)}\) running on a super computer (CSCS, Manno, Switzerland) consisting of 8 IBM Regatta p690 SMPs for a total of 256 Power4 CPUs. Bone tissue strains were extracted from the cortical shell, local to the mid-section at dorsal and ventral sites previously occupied by the strain gages, and compared to the recorded experimental strains. The model was then used to determine the range of mechanical strains specific to the trabecular network.

**Figure 3.18**: Diagram showing the implemented FE model. Thresholded \(\mu\)CT images of the C5’s were aligned in the Z-axis, additional voxels were created to model the intervertebral discs. Load was applied to the distal end surface whilst the proximal end was fixed in the z-direction allowing free lateral movement.

**Statistical analysis**

Linear regression was performed to characterize the relationship between the peak strains acquired throughout the duration of loading and the force applied for both dorsal and ventral strain gages. To assess the level of symmetry that was achieved during loading ANCOVA was then used to compare the dorsal and ventral strain gage responses. The influence of the interconnecting visco-elastic vertebral discs during static and dynamic loading cases was investigated by performing FFT analysis on the strain-gage output signals. Finally to assess the validity of the FE model experimental and theoretical data sets were correlated using linear regression analysis. For all statistical analyses, the GNU statistical package R (Version 2.5.1, http://www.r-project.org) was used.
3.5.4. Results

Analysis of the recorded force feedback signals for each loading bout and for each tail segment demonstrates the high accuracy of the CVAD, showing deviations from the range of peak forces to be less than 2%, the same was also true of all the acquired static and dynamic strain signals for both ventral and dorsal gages. Linear regression, correlating average peak strains with the maximum forces applied, showed highly linear responses for all ventral and dorsal gages attached to all 4 vertebra (R$^2 > 0.95$). Figure 3.19 shows typical strain-force response curves. The average peak strains measured by dorsal and ventral gages at a peak force of 8N for static and dynamic loading formats are as follows: $V_1$ (Lat: $932.90 \pm 23.23\mu\varepsilon$, Med: $866.77 \pm 163.58\mu\varepsilon$), $V_2$ (Lat: $1056.26 \pm 67.71\mu\varepsilon$, Med: $894.76 \pm 104.72\mu\varepsilon$), $V_3$ (Lat: $625.65 \pm 79.25\mu\varepsilon$, Med: $877.17 \pm 260.10\mu\varepsilon$) and $V_4$ (Lat: $918.05 \pm 46.75\mu\varepsilon$, Med: $668.06 \pm 40.45\mu\varepsilon$). It should be noted that the standard deviations presented here are derived mainly from the variation between repeat measures using the different loading formats. The peak strain measurements reported within individual trials vary less than 2% and therefore make little contribution, hence two observations can be made from these data: Firstly, these values show that there are errors associated with repeat measurements. Comparison of the slopes specific to each trial using Ancova show the differences...
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between repeat responses to be highly significant (P < 0.001). Secondly the differences between dorsal and ventral responses indicate the presence of a bending component whilst under compression. These differences are also confirmed to be significant when using Ancova to compare the averaged dorsal and ventral responses for the different loading formats (P < 0.001). Furthermore such differences are positive (relative to the dorsal gage responses), comparison of the average dorsal and ventral gage responses for each loading format at a peak force of 8N show that the differences relative to the dorsal strain gages are 125µε, 162µε, 317µε, and 249µε for V₁, V₂, V₃ and V₄, respectively, indicating a bending component with a bias towards the dorsal side. There was no evidence that the interconnecting visco-elastic vertebral discs affected the frequency transmitted to the target vertebra. FFT analysis of the dynamic signals showed that for dynamic force signals with frequencies of 1Hz and 10Hz the resultant strain signals were 1.01 ± 0.05 Hz and 10.02 ± 0.04 Hz, values which were derived by taking the average frequencies of all tests for both ventral and dorsal strain gages.

![Figure 3.20: a) Typical force-strain response curve for dorsal and ventral cortical sites as determined by finite element analysis. The different slopes (P < 0.001) indicate bending of the target vertebrae whilst under compression. b) Strain color map showing the variation in axial strain at the cortical mid-section, showing bending to occur with a bias towards the ventral direction despite alignment in the z-axis.](image)

The force-strain response as calculated by FE at the cortical sites corresponding to the position of the strain gages exhibited, not surprisingly, a similar linear response (Figure 3.20a). Using 19 GPa for the young’s modulus of bone the average cortical strains for a peak force of 8N were: V₁ (Lat: 963.00 ± 344.11µε, Med: 732.80 ± 218.42µε), V₂ (Lat:
706.73 ± 106.91µε, Med: 605.41 ± 114.12µε), V₃ (Lat: 578.82 ± 103.06µε, Med: 558.74 ± 127.81µε) and V₄ (Lat: 672.23 ± 200.58µε, Med: 664.37 ± 110.00µε).

It should be noted here that the reported standard deviations are due to the variation of strains across all of the finite elements constituting the dorsal and ventral cortical surfaces previously occupied by the strain gages. Comparing these values with those obtained experimentally (quoted in the previous paragraph) clearly shows the FEA model to under predict the experimental strains by an average of 27.4 ± 27.6 % (P < 0.05). Furthermore comparison of dorsal and ventral sights at a peak force of 8N revealed significant differences of -88.83µε, -105.39µε, -20µε, -7.86µε (P < 0.001) relative to the dorsal site for V₁, V₂, V₃ and V₄, respectively, indicating a similar bending components whilst under compression but this time with a bias in the opposite direction to that described by the experimental results (Figure 3.20b). Re-adjustment of young’s modulus to 14.8 GPa, yielded an optimal fit when plotting ventral and dorsal experimental data points against the re-scaled FE data (R² > 0.96, Figure 3.21a). Analysis of the trabecular components using the re-adjusted FE model show the average peak axial strains (compressive) to be significantly less than the cortical component but with an increased range of variation,
V₁: 617.71 ± 398.31 µε, V₂: 559.03 ± 350.43, V₃: 528.03 ± 330.0 µε, V₄: 575.73 ± 376.38 µε, Figure 3.21b.

3.5.5. Discussion

Here we have successfully shown that we are able to surgically insert stainless steel pins into the small C4 and C6 caudal vertebrae of 12-week-old, C57BL/6 female mice. We have also shown that we are able to apply precise dynamic loading regimes at frequencies up to 10 Hz using a specially developed loading device. Furthermore we have shown, using micro-strain gages, that the force applied is successfully transferred to the target vertebra and that its frequency remains undiminished regardless of the interconnecting visco-elastic vertebral discs. The experimental strains acquired from both dorsal and ventral cortical sites were also demonstrated to correlate well with axial strains determined by FE analysis for corresponding regions ($R^2 < 0.96$) using a young modulus of 14.8 GPa. This value of Young modulus is lower than the range of values (19GPa – 30 GPa) published in several studies (17,20,21) for C57BL/6 bone at other anatomical locations, raising doubt over the accuracy of the theoretically determined strain magnitudes. The values of Young’s modulus determined here will have been affected by several sources of error. Firstly the small dimensions of the mouse vertebrae make precise, reproducible placement of the micro-strain gages difficult. Secondly the incorporation of a bending component, as shown by the differences between the dorsal and ventral responses, may have acted to reduce or elevate the experimental strains. Depending on the position of the neutral axis the observed bending may have yielded a more rigid or more compliant material when compared to the FE models. Normally one would expect the axis of bending to lie between the dorsally and ventrally positioned gages. In this case any additional increase in compressive strain caused on one side would then be compensated for by the reduction in strain caused by the tensile component on the opposite side. However given the lack of symmetry relative to the position of the gages (Figure 3.2b) coupled with their variable placement we are unable to make any assumptions regarding the neutral-axis of bending, thus we cannot neglect the possibility of bending modes whereby both gages sense the additional compressive or tensile components. This may also explain why the sets of experimental and theoretical...
data points become more divergent from the linear prediction (Figure 3.6b), the greater the force the greater the effect of the imposed bending component. Where FE is concerned, the alignment and boundary conditions are more consistent than those produced experimentally, however bending was still present, the average difference between dorsal and ventral cortical sites was shown to be -55.53µε (E = 19GPa) and -70.97µε (E = 13 GPa) as opposed to the difference for the average experimental values (213.25µε), thus a better approximation of ideal uniaxial loading is achieved. The convex geometry of the vertebral extremities makes it extremely difficult if not impossible to achieve perfect axial loading experimentally. Both pins must lie in the same plane and pass directly though the z-axis of the tail, furthermore the long-axis of both pins must be perpendicular to the long axis of the tail. The design of the clamp which ‘confines’ the tail is an attempt to minimize this effect, however the soft tissue surrounding the vertebrae and its blood supply represent obvious design limitations. Positioning of the pins closer to the articulating extremities to minimize the degree of articulation was considered, however preliminarily mechanical tests showed this arrangement to significantly reduce the strength of the loading configuration. Errors associated with image processing will have also contributed to the disagreement between practice and theory, beam hardening effects (22) which add bone mass to the µCT images and loss of structural porosity after thresholding will all act to increase the apparent stiffness of the finite element simulation.

The magnitude of the experimental errors incurred in this study are similar if not smaller than those evident in other studies which attempt to determine the mechanical strain on small murine bones under more controlled circumstances (23,24). Furthermore the data presented here gives a much more complete picture than that given in a similar study. Guo et al (14) performed similar measurements on rat tail vertebrae and compared their averaged results to FE models of half of a caudal vertebra (assuming symmetry about the mid-sagittal plane). The large standard deviations resulting from averaging the responses of the two gages (< 150%) contradicts their symmetrical assumptions, indicating the presence of a similar bending effect yielding an incomplete and inconsistent correlation of practice and theory. In this study we have been consistent in our comparisons and achieved an excellent correlation between theoretical and
experimental results and whilst this may raise doubt over the true value of Young’s modulus for bone it is clear that this computational approach can be used to investigate how relative changes in the micro-mechanical environment influence the biochemical cascade of cellular responses which lead to load induced bone formation.

In this study we have shown that we can adapt the loading configuration used in rats for small mice, demonstrating the potential of this model to be used in in-vivo loading studies of load induced trabecular and cortical bone adaptation. We have also established a computational tool able to determine the 3D mechanical strain environment which when used in parallel with the quantification of load induced bone morphology and the associated cellular responses will enable simulation and a better understanding of the mechano-biological processes involved in load induced bone adaptation.

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3.6. Fatigue testing

To mechanically stimulate groups of mice such that varying degrees of bone formation are achieved it has been proposed to load groups of mice for 3000 cycles every other day for a total of 4 weeks with an amplitude that will not induce structural failure of the proposed loading configuration. Pseudo static mechanical testing of the mice vertebrae has shown that the proposed loading configuration fails at a force of 44.3 ± 3.5 N. However when repetitively loading at frequency of 10Hz for 3000 cycles the failure force will be drastically reduced.

To investigate the most acute loading regime the fourth and fifth caudal vertebrae of tails excised from 12 week old C57BL/6 mice (n = 3) were pinned with stainless steel insect pins (0.5mm diameter) and loaded in-vitro using the CVAD. An acute loading regime (frequency: 10Hz, 3000 cycles) with an amplitude of 8N was applied. To test the feasibility of 12 separate loading bouts and to trouble shoot any problems associated with repetitive use of the CVAD this was repeated each day for 14 days. After the 14 days or immediately after any external signs of structural failure, i.e. a significant reduction of the
distance between the two pins in a loaded state, the stainless steel pins were removed and
the vertebrae were scanned at 12µm resolution using desktop micro-computer
tomography (µCT 40, Scanco, Basserdorf, Switzerland) and the structural integrity of all
three vertebral bodies assessed visually. In between loading the mouse tails were stored
in a phosphate buffer solution and refrigerated.

Throughout the 14 days of mechanical loading no external signs of structural
failure were evident. However µCT images showed signs of structural damage for two of
the tails (figure 3.22), where two out of three sets of C4’s and C6’s had cracks
propagating from the hole at the pin-bone interface. It appears that the cracks occurring in
C4 propagate in a distal-proximal direction and those in C6 in the inverse direction which
is consistent with the direction of the reaction force at each pin-bone interface. None of
the target vertebrae showed any sign of structural damage.

Figure 3.22: (a) µCT image of a pinned vertebra (with pin removed) showing no structural damage. (b)
µCT image of a damaged C4 caudal vertebra following 42000 loading cycles. (c) µCT image of a damaged
C6 caudal vertebra following 42000 loading cycle.

The results confirm that structural damage of the proposed loading configuration does
occur however this is not at the expense of the mechanical integrity of the system as a
whole. Despite the cracks the pins remain in the correct position and the applied force is
still transferred to the target vertebra, hence the loading configuration could survive an
acute loading regime consisting of 12 sets of 3000 cycles at a frequency of 10Hz with
amplitude of 8N. Furthermore the resistance of the loading configuration to structural
damage should be dramatically improved in vivo as living bone is able to regenerate and
repair itself in between loading bouts, it is therefore possible that such cracks may not
occur and that the system maybe capable of handling even higher forces. However
because we are dealing with a sensitive biological system if such cracks do occur an auto-repair response could be provoked which could further complicate the biochemical pathway associated with the anabolic response of the target vertebra to mechanical stimulation, ultimately leading to false results. To identify the optimum acute loading regime in which no cracks were present would be difficult. Owing to the fact that we are blind to the events which occur inside the tail a large test matrix would be required where each sample would have to be scanned and inspected for crack initiation. This would be a different matter if we wanted to detect structural failure of the proposed loading configuration. In this case the MML algorithm could be modified to detect sudden changes in the displacement feedback signal which will occur as the pin-bone interface yields. Additionally for true optimal values to be found tests would have to be performed in vivo, the logistical problems in organizing such tests (application to the ethics committee cost of mice etc) would drastically increase the time and resources required. It would be less costly in the long run to employ a trial and error approach and underestimate the most acute loading regime, performing a second iteration if necessary with the benefit of experience.
Chapter 3

References


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Chapter 4
Load induced changes in trabecular and cortical bone
4. Load induced changes in trabecular and cortical bone

This chapter presents the results of in vivo studies in which the developed CVAD is deployed to mechanically stimulate the C5 vertebrae of two inbred mouse strains. The aims of these two studies were to: 1) Characterize the effect of pinning on the morphometry of the target vertebrae following surgery and throughout the defined loading period in both B6 and C3H mice and 2) Investigate the differences in mechano-sensitivity between B6 and C3H mice.

4.1. The C57BL/6 mouse model

In order to facilitate the investigation of the biochemical pathways involved in both cortical and trabecular bone formation a Caudal Vertebrae Axial compression Device (CVAD) has been developed to mechanically stimulate the fifth caudal vertebrae (C5) of C57BL/6 (B6) female mice, via two hydroxyapatite coated stainless, steal pins inserted into caudal vertebrae C4 and C6. Here we report the effect of surgical intervention and mechanical loading on both trabecular and cortical bone adaptation in C57BL/6 (B6) mice. Stainless steel pins (coated with Hydroxyapatite) were inserted into the C4 and C6 of 50, 12 week old, female B6 mice. Following surgery, 80 mice (including the 50 pinned mice) were randomly divided into 8 groups: 4 control groups and 3 loading groups such that the effect of pinning and mechanical stimulation on the morphometry of C5 vertebrae could be accurately characterized. It was intended to subject the 3 loading groups to an acute loading regime (3000 cycles, 10 Hz, 3 times a week for 4 weeks) for 3 different load amplitudes (2N, 4N and 8N), however during the 3 weeks of post surgical recovery 36% of the pinned mice exhibited a severe inflammatory response local to the pinned vertebrae, prompting cancellation of the 8N groups and a redistribution of the mice such that the remaining groups had sufficient numbers for statistical significance. Those mice exhibiting a severe inflammatory response were assigned to special control groups for analysis (Literature suggests the inflammatory response could have been induced by particle wear debris emanating from the implanted pins). At 15 weeks of age loading commenced. Upon sacrifice C5 vertebrae were scanned using micro-computed tomography (µCT) at 6 µm voxel size. Analysis of µCT images showed pinning to have a
Load induced changes in trabecular and cortical bone

negative effect on bone morphometry, comparison of the unpinned age matched control group with the pinned control group showed global, BV/TV and BV to be reduced by 5% and 6% (P < 0.05) at the end of loading. A dramatic osteolytic effect in C5 was shown to be associated with the severe inflammatory response, comparison of the ‘make-shift’, severely inflamed group with the unpinned control group showed global trabecular BV/TV to diminish by 20% (P < 0.01). There was a progressive inflammatory response in the loaded groups, prompting speculation that any mechanically-induced anabolic effect could have been diminished by the presence of inflammatory associated catabolic mechanisms. Despite this, comparison of µCT data for the 4N loading group and the pinned, unloaded, age-matched group detected several positive anabolic responses: A significant 10% increase in Primary Spongiosa BV (P < 0.05) along with significant regional increases in both cortical and trabecular BV (maximum increase = 5%, P < 0.01, assessed using Repeated measures Anova). In this first in vivo trial we have successfully demonstrated that we can surgically insert hydroxyapatite coated pins into the C4 and C6 vertebrae of C57BL/6 mice, whilst inducing and acceptable reduction in bone quality (ignoring the severely inflamed groups), we have also shown that we can accurately apply a mechanical load to the C5 vertebrae of for a sustained period of time and stimulate a minimal degree of bone formation. Given that we were unable to load at higher magnitudes and given that the inflammatory response could most likely be eliminated by eliminating the generation of implant wear debris the results presented here provide a clear indication of the model’s potential for the study of both trabecular and cortical bone formation.

**Introduction**

In addition to the genetic background of the mammalian species mechanical loading is perhaps one of the most important factors which regulate bone mass and shape. Although the basic form and development of bone are genetically encoded, their final mass and architecture are governed by adaptive mechanisms sensitive to the mechanical environment. An understanding of the biological pathways by which mechanical forces regulate the structure of bone qualitatively and quantitatively would provide opportunities to mimic or augment the response of bone to mechanical stimulation by pharmacological
agents and may lead to novel strategies in the management of osteoporosis. However little is known about the cellular mechanisms responsible for both cortical and trabecular bone adaptation, the only model currently available for the elucidation of molecular mechanisms involved in load induced, trabecular and cortical bone formation is a model using rat caudal vertebrae (1). Studies using this model have been hampered by technical hurdles such as the unavailability of bone cell isolates used to investigate global gene expression and identify the set of mechanical load regulated genes. Furthermore the principal limitation imposed by the rat model is its current inaccessibility to potential genetic manipulations. To overcome this several studies have established mouse models for the study of cortical bone adaptation and associated biochemical pathways in response to mechanical loading using C57BL/6 and C3H/HeJ inbred strains (2-4). However specific genes and or combinations of genes have yet to be discovered, moreover the focus in these studies has been on cortical bone and not trabecular bone, a significant structural component which has been shown to have a more enduring sensitivity to mechanical stimulation in mature human adults than that of cortical bone (5,6). The objective of this study was to establish a fully functioning mouse model for the study of both cortical and trabecular bone adaptation using the newly developed Caudal Vertebra Axial compression Device (CVAD). The CVAD is able to apply a uniaxial, cyclical, compressive force to the fifth caudal vertebrae (C5) of C57BL/6 female mice via hydroxyapatite coated pins (0.5mm diameter) inserted into the adjacent caudal vertebrae (C4, C6). Using the CVAD we applied a cyclic mechanical load at magnitudes which are capable of inducing both cortical and trabecular strains conducive to bone formation, for 3000 cycles at 10 Hz, 3 times a week for 4 weeks. The loading protocol was guided by considering numerous studies which show bone formation to be a function of the mechanical energy transferred to bone (7-9). To fully characterize this in vivo model, the study has been designed to assess the effect of both surgical intervention and the natural ageing process on bone adaptation, in addition to mechanical stimulation. Owing to the well characterized genome of the mouse which is accessible to manipulations by transgenic and knockout technologies the successful establishment of an inbred mouse model for the study of load-induced anabolic activity in both cortical and trabecular bone would better facilitate molecular approaches aimed at the identification of mechano-
sensitive, bone-forming genes. Furthermore, replacement of the C57Bl/6 mouse with a complementary inbred strain such as the C3H/Hej may provide opportunities for the application of QTL based strategies, further enhancing the probability that mechano-sensitive genes implicated in bone formation will be found.

4.1.1. Materials and methods

Mechanical loading apparatus

Figure 4.1: (a) Overview of the control system for the CVAD. (b) Photo showing the loading axis belonging to the CVAD. (c) Fluoroscopic image of a mouse, graphically edited to show the location of the Hydroxyapatite coated, stainless steel pins. To achieve compression of a C5 vertebra the mechanical signal is applied to the distal most pin whilst the proximal most pin is clamped.

A closed-loop feedback device complete with a graphical user-interface has been designed to apply a precisely controlled, cyclical, compressive load to the C5 vertebra in the C57BL/6 mouse tail at a frequency of 10Hz via two hydroxyapatite coated pins surgically inserted into C4 and C6 vertebrae (figure 4.1c). The device is controlled via LabView 7.0 (National Instruments) installed on a desktop computer which communicates with a servo control board (NI-7344 National instruments). Following amplification by a signal amplifier (MID-7654 National instruments), the servo board outputs a signal to a linear electro-magnetic actuator (LA25-42-000A, Bei–Kimco
Magnetics). Compression of C5 is achieved by using the actuator to drive a shaft, mounted on linear bearings, connected to the distal-most Hydroxyapatite coated pin. The proximal-most pin is clamped such that the only positive and negative translations along the axis of compression are permitted. The control system is closed by a load cell (13/2443 -16 TRANSMETRA haltec GmbH) (figure 4.1a & b). As a quality control measure the feedback signal from the load cell was recorded and all force maxima and minima determined. Surgically insertion of the stainless steel, hydroxyapatite coated pins was performed using a special pinning device, compatible with x-ray fluoroscopy. The device makes use of a V-clamp to simultaneously secure and automatically locate the cranial-caudal axis of the mouse tail. The coated pins are loaded into channels integral to the V-clamp and are manually pushed through the centers of the vertebrae, perpendicular to the cranial-caudal axis. A digital mobile C-arm (OEC MiniView 6800, GE Medical Systems) was used to locate C4 and C6.

**Experimental design**

![Experimental design diagram](image-url)

Eighty, 9 week old C57Bl/6 female mice (Harlan Ltd) were housed in a husbandry unit in groups of 5 and given 3 weeks to acclimatize to their new environment. At 12 weeks of age (T0) the mice were divided into 8 groups of 10: Group 1 (T0_{BASE}) served as the
baseline control group, the mice in this group were sacrificed at 12 weeks of age, providing a benchmark C5 which was used to assess the contribution of normal growth to the morphometry of C5 vertebrae. Groups 2 and 3 (T₃_PIN, T₃_AMC) formed the pinned the un-pinned control groups. The mice belonging to the pinned control group had stainless steel insect pins (0.5mm diameter, Fine Science Tools) coated with hydroxyapatite (Cam Implants BV) surgically inserted into their C4 and C6 vertebrae at T0. Both groups were sacrificed at 15 weeks of age (T3) just before the start of loading. These groups were designed to assess the effect of growth and the pinning procedure on C5 morphometry prior to the commencement of loading and following 3 weeks of post surgical recovery. Groups 4, 5, and 6 (T₇₂N, T₇₄N, T₇₈N) were pinned in the same way at T0 and constituted the loading groups. Sinusoidally varying forces (3000 cycles, 10Hz) were applied to the C5 of mice in these groups with amplitudes of 2N, 4N and 8N respectively. This was repeated 3 times a week for duration of 4 weeks. At 18 weeks of age (T7), following 4 weeks of loading all mice were sacrificed. To help quantify the amount of bone formation attributable to mechanical loading alone and to further assess the effect of pinning groups 7 and 8 (T₇_PIN, T₇_AMC) formed the unloaded, pinned and unloaded non-pinned control groups which were also sacrificed at T7. All mice were sacrificed using CO₂ inhalation. Upon sacrifice of the mice the C5’s were harvested and immediately fixed in formalin for 48 hours, after which they were transferred to saline containing 70 % ethanol. Figure 4.2 provides a Schematic representation of the experimental design. Throughout pin insertion and loading the mice were anesthetized using an oxygen-isoflurane mixture (Provet Medical AG). To monitor the health of the mice, each mouse was weighed prior to loading and after each loading bout. The accuracy of the CVAD was assessed by analyzing the recorded force feedback signals for each loading bout and for each mouse to determine each peak and minimum force value present in the applied dynamic signals.

Quantification of bone adaptation

Bone morphometry was assessed using micro-computed tomography with 5 times frame averaging (6µm voxel size, 50 kVp, 160µA, Scanco Medical AG Switzerland) to obtain 3D, digital images of all C5 vertebrae. Morphometric parameters were determined using a direct 3D approach (10). Bone Volume (BV_{Wh}), Tissue Volume (TV_{Wh}) and Bone
Volume Density (BV/TVWb) were determined globally and locally for the whole bone (figure 4.3a). Local analysis was performed by subdividing the whole bone into 19 overlapping regions: 10 distinct regions, each having a height equal to 10% of the caudal vertebrae’s total height and 9 additional overlapping regions, spanning the midpoints of the 10 distinct regions (figure 4.3a).

![Figure 4.3: (a) Digital image of a whole C5 vertebra showing cortical, trabecular components, including the primary spongiosa, subdivided into 19 overlapping regions (1Wb-19Wb). (b) Subdivided Internal volume of interest enclosing primary spongiosa (Regions Sp1, Sp2) and trabecular bone (Regions 1 Tb -15 Tb). (c) Subdivided cortical shell. The 15 cortical regions (1Cℓ -15 Cℓ) match those which define the 15 trabecular regions.](image)

The spongiosa and cortical components of the vertebrae were analyzed in a similar way. An internal volume of interest comprised of both primary and secondary spongiosa (trabecular bone) was subdivided into 19 overlapping regions, (figure 4.3b). The first and last regions (Sp1, Sp2) contained primary spongiosa local to both proximal and distal growth plates. The transition of primary spongiosa into secondary spongiosa was captured in regions Tr1 and Tr2. The remaining regions (1Tb -15 Tb) enclosed only trabecular bone. Bone Volume Density (BV/TVsp), Bone Volume (BVsp) and Tissue Volume (TVsp) were calculated for the sum of the two proximal and distal volumes containing primary spongiosa (Sp1 & Sp2). Trabecular Bone Volume Density (BV/TV Tb), Trabecular Bone Volume (BV Tb), Trabecular Tissue Volume (TV Tb), Trabecular Thickness (Tb.Th) and Trabecular Number (Tb.N) were determined locally for regions 1Tb -15 Tb and globally (for a single volume described by regions 1Tb -15 Tb). The cortical shell was subdivided such that the overlapping regions matched the 15 overlapping regions defining the trabecular component of each caudal vertebra (figure 4.3c). Cortical
Bone Volume Density (BV/TV(_Ct)), Cortical Bone Volume (BV(_Ct)) and Cortical Tissue Volume (TV(_Ct)), Cortical Thickness (Ct.Th) and Marrow Volume (MV) were also determined globally and locally.

### Statistical analysis

To investigate the effect of time, pinning and mechanical loading on global bone morphometric parameters Anova was used to contrast the relevant groups at different time points and load levels. Where significance was found pairwise t-tests with a bonferroni correction were used to determine the significance of the differences. To investigate the regional effects of time, pinning and mechanical loading Repeated Measures Anova was used. Again bonferroni corrections were applied where necessary.

#### 4.1.2. Results

Following the surgical insertion of the hydroxyapatite coated pins into C4 and C6 vertebrae and 1 week prior to loading it was noticed that 22 of the 60 pinned subjects (36 %) exhibited varying degrees of inflammation local to the site of pinning. To quantify the degree of inflammation all mice were visually inspected and assigned with a degree of inflammation, degree 1: no inflammation, degree 2: moderate inflammation and degree 3, severe inflammation. Out of the 22 which exhibited signs of inflammation, 15 were

Figure 4.4: (a) Schematic representation of experimental design detailing the re-organization of groups following the severe inflammatory response of some mouse tails to pin insertion. (b) Chart detailing the progression of the inflammatory response throughout the 4 weeks of loading. The number of mice and the severity of swelling are shown for groups T7_2N, T7_4N, T7_PIN and T7_INF before and after loading.

Following the surgical insertion of the hydroxyapatite coated pins into C4 and C6 vertebrae and 1 week prior to loading it was noticed that 22 of the 60 pinned subjects (36 %) exhibited varying degrees of inflammation local to the site of pinning. To quantify the degree of inflammation all mice were visually inspected and assigned with a degree of inflammation, degree 1: no inflammation, degree 2: moderate inflammation and degree 3, severe inflammation. Out of the 22 which exhibited signs of inflammation, 15 were
severely inflamed such that they could not be fitted into the CVAD whilst the remaining 7 suffered only a moderate degree of inflammation. The reduced numbers of healthy subjects prompted immediate reorganization of the experiment. To facilitate the redistribution of mice such that the remaining groups had sufficient ‘healthy’ numbers for statistical significance the T7*N group was eliminated. The 15 animals exhibiting severe inflammation were divided into two non-loaded control groups: T3*INF, n = 10 and T7*INF, n = 5. The unaffected animals and those exhibiting only moderate inflammation were then reorganized such that the control groups and loaded groups possessed the following ratio’s of healthy and moderately swollen mice: T3*PIN (2/9), T7*2N (2/9), T7*4N (2/9), T7*PIN (1/8). A schematic of the revised experimental design is shown in figure 4.4a. Throughout the 4 weeks of loading regular visual inspection and scoring of the tails revealed that, in some cases, inflammation became progressively worse (figure 4.4b). In the T7*2N group the number of moderately inflamed tails increased by 1, whereas in the T7*4N group, all tails became inflamed to some degree: 4 mouse tails displayed moderate tail inflammation, 3 mice exhibited severe swelling, in 2 cases the tails were so severely inflamed that the mice had to be eliminated from the study. In the case of the non loaded control group, T7*PIN, the number of moderately swollen tails had been judged to have increased from 1 to 5. In the case of T7*INF, in which the tails which were scored as severely inflamed, there were no signs of improvement throughout the 4 weeks of loading. To assess the impact of swelling the effects of sustained, severe swelling on bone morphometry were measured by comparing bone morphometric indices specific to groups T3*INF and T7*INF with the indices of groups T3*AMC, T7*AMC, T3*PIN, and T7*PIN. The results of this are included in the analysis of the effect of age and pinning on bone morphometry and are presented in the section titled ‘Assessment of the anabolic response of C5 to ageing, pinning and prolonged inflammation’. The outcome of the progressive inflammation observed during the 4 weeks of loading was investigated by correlating an inflammation score (INFscore) with several bone morphometric indices for groups T7*2N, T7*4N and T7*PIN. The INFscore takes into account the progression of inflammation and provides a measure of the overall severity for the entire 4 weeks during loading: At the end of each loading bout scores of 0, 2, and 4 were assigned to each mouse exhibiting no inflammation, moderate inflammation and severe inflammation, respectively. Following
the 4 weeks of loading (12 bouts of loading) the individual scores were summed and expressed as a percentage of the most severe score possible (12 x 4). Correlations with bone morphometric indices were then checked for by plotting BV/TV, BV and TV for all regions of analysis, against the INF-score. The effects of progressive inflammation are included in the mechano-sensitivity analysis of bone and are presented in the section titled: ‘Assessment of the anabolic response of C5 to Mechanical stimulation and progressive inflammation’.

Despite the unexpected inflammatory response of some of the mice, no mice were lost due to death and the average weights of the mice throughout loading appeared not to decrease suggesting that the general health of each mouse remained satisfactory throughout the experiment. In addition none of the stainless steel, hydroxyapatite coated pins showed any visible signs of loosening, neither in the T7PIN group nor the loaded groups (T72N, T74N) inferring that the structural integrity of the pinned vertebrae remained intact for 36000 cycles. Furthermore analysis of the recorded force feedback signals for each loading bout and for each mouse demonstrates the high accuracy at which the CVAD controls to the user defined force. The average amplitudes applied during the entire experiment were 2.022N +/- 0.013 N and 4.0314 +/- 0.0199N for the T72N and T74N groups respectively.

Assessment of the anabolic response to ageing, pinning and prolonged inflammation

Whole bone

The baseline control group (T0BASE), show the average values of BV/TVWh, BVWh and TVWh to be, 50.5%, 3.88mm³ and 7.68mm³, respectively for a C5 vertebra at 12 weeks of age. The age-matched controls showed Global BV/TVWh to increase by 9% for the entire 7 week period (T0-T7), figure 4.5a. This is accompanied by a 12% global increase in BVWh (P < 0.001) and a non significant 2.5% increase in global TVWh, these translate to absolute increases of 0.5mm³ and 0.24mm³, respectively. The pinned control groups described similar transient behavior (4.5a) such that at T7 global BV/TVWh and global BVWh are 5% (P = 0.12) and 4.5% (0.29mm³, P = 0.07) less, respectively when compared to the age-matched control. There is no significant difference between pinned and age-
matched groups at T7 in global TV\textsubscript{Wh} (P > 0.5). Regional analysis comparing BV/TV\textsubscript{Wh}, BV\textsubscript{Wh} and TV\textsubscript{Wh} for pinned and age-matched controls at T7, reveals significant group-region interaction P-values (P\textsubscript{int} < 0.01). Visual inspection of regional variation for both BV/TV\textsubscript{Wh} and BV\textsubscript{Wh} at T7 shows that differences due to pinning occur primarily in proximal and distal regions (figure 4.5b). The differences observed here are not indicative of bone loss. They indicate a retardation of growth, as regional comparisons between T7\textsubscript{PIN} and T0\textsubscript{BASE} show regional BV to be higher.

![Figure 4.5: (a) Variation of global BV/TV\textsubscript{Wh} as measured by age-matched, pinned and inflamed control groups. (b) Regional variation of BV\textsubscript{Wh} as measured by age-matched, pinned and inflamed control groups at T7. (c) Regional variation of TV\textsubscript{Wh} as measured by age-matched, pinned and inflamed control groups at T7.](image)

Negative differences of up to 9\% (0.05mm\textsuperscript{3}) for BV\textsubscript{Wh} are reported when comparing T7\textsubscript{PIN} and T7\textsubscript{AMC}, whilst differences at central regions are almost undetectable (< 0.008mm\textsuperscript{3}). Inflammation is shown to have a negative impact, clearly demonstrated by a progressive 8\% decrease in global BV/TV\textsubscript{Wh} (P = 0.11, figure 4.5a). This is shown to be a result of a marginal non significant 1.2\% (0.05mm\textsuperscript{3}) increase in global BV\textsubscript{Wh} with time (P > 0.5) and a dramatic significant 11.3\% (0.87mm\textsuperscript{3}, P < 0.001) increase in global TV\textsubscript{Wh}. At T7 global TV\textsubscript{Wh} is shown to be 10\% (0.63mm\textsuperscript{3}) higher than that for the age-matched control group (P < 0.01) whilst BV\textsubscript{Wh} is shown to be 10\% (0.45mm\textsuperscript{3}, P < 0.05) less.
Highly significant group-region interaction terms are detected for BV/TV<sub>Wh</sub> and BV<sub>Wh</sub> when comparing T7<sub>INF</sub> with T7<sub>AMC</sub> (P<sub>int</sub> < 0.001) figure 4.5b. These differences however are not attributable to a retardation of growth but actual bone loss, as is revealed when comparing T7<sub>INF</sub> with T0<sub>BASE</sub> at a local level. A maximum negative difference of 22% (0.1mm<sup>3</sup>) is reported for regional BV<sub>Wh</sub> when comparing T7<sub>INF</sub> and T7<sub>AMC</sub>. TV<sub>Wh</sub> appears to be consistently 8% (0.07mm<sup>3</sup>) greater than that for the age-matched control group across all regions (P<sub>int</sub> > 0.5, figure 4.5c).

**Trabecular bone**

At T0 the average values for C5 trabecular parameters are as follows: BV/TV<sub>Tb</sub> = 19.24%, BV<sub>Tb</sub> = 0.63mm<sup>3</sup>, TV<sub>Tb</sub> = 3.26mm<sup>3</sup>, Tb.Th = 67µm, Tb.N = 2.95mm<sup>-1</sup>. Throughout the entire 7 week period the age-matched control groups show a non-significant 5% increase in global BV/TV<sub>Tb</sub> (figure 4.6a). This is accompanied by a non-significant 5% (0.03mm<sup>3</sup>) increase in global BV<sub>Tb</sub> (P > 0.5), and a non-significant 3.3% (0.11mm<sup>3</sup>) decrease in global TV<sub>Tb</sub> (P = 0.38). Global Tb.Th is shown to increase significantly by 18.3% (12µm, P <0.01), whilst Tb.N decreases by 2.6% (0.08mm<sup>-1</sup>, P = 0.2). The pinned control groups describe a similar global transient trend to that described by the age-matched controls although reduced in magnitude (figure 4.6a). At T7 global BV/TV<sub>Tb</sub> and BV<sub>Tb</sub> are shown to be 13% (P =0.14) and 9% (0.059mm<sup>3</sup>, P = 0.25) less when compared to T7<sub>AMC</sub>. Global TV<sub>Tb</sub>, Tb.N are shown to similar (P > 0.5), whilst global Tb.Th is shown to be significantly 8% less (7µm, P < 0.05). Regional analysis shows that non-significant differences exist for both BV/TV<sub>Tb</sub> and BV<sub>Tb</sub> of up to 8% (P<sub>int</sub> = 0.24) and 9.8% (0.016mm<sup>3</sup>, P<sub>int</sub> = 0.27), respectively when comparing T7<sub>PIN</sub> with T7<sub>AMC</sub>. Regional reductions in Tb.Th are inferred of up to 9.7% (&lt; 7.6µm, P<sub>int</sub> = 0.2). The differences in BV/TV<sub>Tb</sub> and BV<sub>Tb</sub> are more pronounced in proximal and distal regions where there is more trabecular bone (figure 4.6b). In the central regions were few trabeculae exist, the differences in BV<sub>Tb</sub> are almost undetectable (&lt; 0.0016mm<sup>3</sup>). No significant regional differences exist for both TV<sub>Tb</sub> and Tb.N (P<sub>int</sub> > 0.5). The subtle negative effect of pinning can be seen in figure 4.7 which compares caudal vertebrae from T7<sub>PIN</sub> and T7<sub>AMC</sub> groups with median BV/TV<sub>Wh</sub> values.
Figure 4.6: (a) Variation of global BV/TV\textsubscript{Tb} as measured by age-matched, pinned and inflamed control group. (b) Regional variation of BV\textsubscript{Tb} at T7 for control groups T7\textsubscript{AMC}, T7\textsubscript{PIN} and T7\textsubscript{INF}. (c) Regional variation of TV\textsubscript{Tb}T7 for control groups T7\textsubscript{AMC}, T7\textsubscript{PIN} and T7\textsubscript{INF}.

Analysis of the inflamed groups shows global BV\textsubscript{Tb} and Tb.N to diminish significantly by 24% (0.11mm\textsuperscript{3}) and 22% (0.66mm\textsuperscript{-1}) respectively, (P < 0.01) between T0 and T7. A non-significant 6% (4µm) reduction in global Tb.Th is reported (P > 0.5) together with a surprising significant 16.4% increase in global TV\textsubscript{Tb} (0.53mm\textsuperscript{3}, P < 0.01). Combined these changes result in a significant 34% reduction in global BV/TV\textsubscript{Tb} (P < 0.01, figure 4.6a). Not surprisingly highly significant global differences exist when comparing the inflamed group with the age-matched control at T7 for all morphometric indices. Global BV\textsubscript{Tb}, Tb.Th, Tb.N are 27% (0.18mm\textsuperscript{3}), 18% (16µm), and 17% (0.58mm\textsuperscript{-1}) less, respectively (P < 0.001), whilst global TV\textsubscript{Tb} is 20% (0.64mm\textsuperscript{3}, P < 0.001) higher, subsequently BV/TV\textsubscript{Tb} is significantly reduced by 39% (P < 0.01). This is reflected in regional analysis were highly significant group-interaction terms are detected when comparing T7\textsubscript{AMC} and T7\textsubscript{INF}. Regional BV\textsubscript{Tb} (figure 4.6b), and Tb.N are shown to be up to 26% (0.04mm\textsuperscript{3}), and 21.5% (0.92mm\textsuperscript{-1}) less, respectively (P\textsubscript{int} < 0.0001). Tb.Th is shown to be up to 21.4% (16.5µm) lower for all regions (P\textsubscript{int} = 0.3). This is accompanied by a volumetric expansion shown by the consistent increase in TV\textsubscript{Tb} (≈ 14%, 0.07mm\textsuperscript{3}) across all regions (P\textsubscript{int} < 0.05, figure 4.6c) resulting in significant regional losses in
Load induced changes in trabecular and cortical bone

BV/TV of up to 34% (P < 0.001). The dramatic reduction of bone quality is clearly shown in figure 4.7. The loss in bone mass is shown to increase progressively towards proximal and distal regions.

At T0, typical values for global cortical parameters are shown to be: BV/TV = 33%, BVCl = 1.93mm³, TVCl = 5.76mm³, Ct.Th = 0.118mm, MV = 3.83mm³. The age-matched control groups show a significant 12% increase throughout the entire 7 week period (P < 0.001, figure 4.8a) in global cortical BV/TVCl accompanied by significant 15% (0.3mm³) and 12% (0.015mm) increases (P < 0.001) in global BVCl and Ct.Th. Global TVCl shows a non-significant 3% (0.17mm³) increase (P =0.22) whilst a 3% (0.13mm³) reduction in MV (P = 0.19) is reported. The pinned control groups describe a similar global trend to that described by the age-matched control such that at T7 there are no significant differences between the pinned control group and the age-matched control group for all global cortical parameters. Global BV/TVCl (figure 4.8a) and BVCl are shown to be 5% (P = 0.21) and 6% (0.14mm³, P = 0.23) less than the corresponding parameters for T7AMC. Global TVCl, Ct.Th and MV are shown to be similar (P > 0.5). Regional analyses at T7, comparing pinned and age-matched controls reveal a significant group-region interaction term for BVCl (Pint < 0.001). Inspection of the regional variation of BVCl for T7AMC and
T7_{PIN}, (figure 4.8b) show BV_{Ct} to be up to 8% (0.026mm^3) less for the pinned controls at proximal and distal-most cortical regions. Little variation in BV_{Ct} occurs at central cortical-regions (< 0.005mm^3). Non-significant regional differences are reported for BV/TV_{Ct}, Ct.Th, TV and MV (P_{int} > 0.5).

Figure 4.8: (a) Variation of global BV/TV_{Ct} as measured by age-matched, pinned and inflamed control group. (b) Regional variation of BV_{Ct} at T7 for age-matched, pinned and inflamed control groups. (c) Regional variation of MV at T7 for age-matched, pinned and inflamed control groups.

Again these regional differences show the growth of cortical bone to be retarded, cortical bone mass is shown not to disappear when comparing T0_{BASE} and T7_{PIN} regionally. Global comparison of T0_{BASE} and T7_{INF} shows that global TV_{Ct} and MV increase significantly by 11% (0.68mm^3, P < 0.01) and 17% (0.63mm^3, P < 0.01) respectively, whilst BV_{Ct} and Ct.Th increase non-significantly by 3%(0.056mm^3, P > 0.5) and 0.67% (0.0008mm, P > 0.5). This results in a non-significant 7.7% global decrease in BV/TV_{Ct} (P= 0.37), figure 4.8a. Global comparisons of T7_{INF} with T7_{AMC} show BV_{Ct} and Ct.Th to be 17% (P < 0.01) and 10.7% (0.014mm, P < 0.01) less. Global TV_{Ct} and MV are shown to be 8.6% (0.514mm^3, P < 0.01) and 20.4% (0.76mm^3, P < 0.001) higher respectively, resulting in global BV/TV_{Ct} being 10% less (P < 0.01). Regional analysis at T7 comparing T7_{INF} to T7_{AMC} shows BV_{Ct} and Ct.Th to be up to 16%( 0.05mm^3, P_{int} <
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0.0001) and 13% (0.01mm, P_{int}< 0.05) less (figure 4.8b). As was the case for pinned controls these regional differences demonstrate a retardation of growth, no osteolytic effects are observed. TV\textsubscript{Ct} (P_{int} > 0.5) and MV (P_{int} < 0.001) are shown to be approximately 6.5% (0.06mm\textsuperscript{3}, P > 0.5) and 17% (0.11mm\textsuperscript{3}, P < 0.001) consistently higher over the 15 cortical regions, (figure 4.8c) reflecting the volumetric expansion seen for trabecular regions (figure 4.6c). Consequently regional BV/TV\textsubscript{Ct} is shown to be up to 21% (P_{int} < 0.12) lower.

Primary spongiosa
T\textsubscript{0\_BASE} shows average values of BV/TV\textsubscript{Sp}, BV\textsubscript{Sp}, and TV\textsubscript{Sp} to be 59.7%, 0.57mm\textsuperscript{3}, and 0.95mm\textsuperscript{3} respectively. Comparison of T\textsubscript{0\_BASE} and T\textsubscript{7\_AMC} show global BV/TV\textsubscript{Sp} to increase by 11% (P < 0.001), which corresponds to a 15% (0.087mm\textsuperscript{3}) increase in global BV\textsubscript{Sp} (P < 0.001) and 2.5% (0.034mm\textsuperscript{3}) increase in global TV\textsubscript{Sp} (P < 0.001). As was the case for both cortical and trabecular components the pinned controls describe a similar transient response but again reduced in magnitude. Comparison of T\textsubscript{7\_AMC} and T\textsubscript{7\_PIN} shows global BV/TV\textsubscript{Sp}, BV\textsubscript{Sp} and TV\textsubscript{Sp} to be 4.6% (P = 0.24), 9.5% (0.062mm\textsuperscript{3}, P < 0.05) and 4% (0.049mm\textsuperscript{3}, P = 0.27) less, respectively for T\textsubscript{7\_PIN}. The inflamed controls show a non significant 3% loss in global BV/TV\textsubscript{Sp} (P > 0.5) throughout the 7 week period. This is accompanied by a non-significant 9.8% (0.055mm\textsuperscript{3}, P < 0.14) increase in global BV\textsubscript{Sp} and a dramatic 12.5% (0.12mm\textsuperscript{3}) significant increase in global TV\textsubscript{Sp} (P < 0.001). When compared to T\textsubscript{7\_AMC}, the T\textsubscript{7\_INF} group shows BV/TV\textsubscript{Sp} and BV\textsubscript{Sp} to be 12% (P < 0.01), and 4.8% (0.031mm\textsuperscript{3}, P > 0.5) less, respectively. Global TV\textsubscript{Sp} is shown to be 8.7% higher (0.086mm\textsuperscript{3}, P < 0.05).

Assessment of the anabolic response to mechanical stimulation
The INF\textsubscript{score} correlates extremely poorly with all morphometric indices (BV/TV\textsubscript{Wh}, Tb, ct, sp, BV\textsubscript{Wh}, Tb, ct, sp and TV\textsubscript{Wh}, Tb, ct, sp) for both global and regional analysis (R\textsuperscript{2} < 0.1) suggesting that the progressive inflammation that was observed did not influence bone morphometry.
Comparison of the 0N (T7\text{PIN}), 2N and 4N loading groups for global BV/TV$_{Wh}$ show no significant differences (P = 0.3). Global BV$_{Wh}$ is shown to increase non-significantly by 5% (0.2mm$^3$, P = 0.19) when comparing 0N and 4N groups, similarly a 3.7% (0.29mm$^3$) increase in global TV$_{Wh}$ is reported (P = 0.27). Regional analysis shows there to be no significant difference for BV/TV$_{Wh}$ when comparing 0N and 4N loading groups (P\text{\text{\text{int}}} > 0.5), there are however marginal significant regional increases in BV$_{Wh}$ (figure 4.9a) and TV$_{Wh}$ (P\text{\text{\text{int}}} < 0.01) of up to 6.3% (0.04mm$^3$, P\text{\text{\text{int}}} < 0.01) and 5.8% (0.06mm$^3$, P\text{\text{\text{int}}} < 0.01), respectively. Anova comparison of the loading groups for global trabecular BV/TV$_{Tb}$ again reveals no significant differences (P > 0.5). Both global BV$_{Tb}$ and TV$_{Tb}$ are shown to increase non-significantly by 3% (P > 0.5) when comparing 0N and 4N loading groups. Global Tb.Th shows a non-significant 3% increase (P > 0.5), whilst Tb.N gives a non significant 3% decrease (P > 0.4). Regional analysis of BV/TV$_{Tb}$ yields non-significant differences, however regional comparisons of 0N and 4N loading groups for both BV$_{Tb}$ and TV$_{Tb}$ reveal a highly significant group-region interaction terms, describing increases of up to 10.4% (0.009mm$^3$, P\text{\text{\text{int}}} < 0.001) and 5.65% (0.026mm$^3$, P\text{\text{\text{int}}} < 0.001), respectively.
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(figure 4.9b). No significant group-region interaction terms were evident for Tb.Th and Tb.N. Furthermore comparisons of mean regional changes show that regional changes in Tb.Th, Tb.N or TV_{Tb} do not always correspond to regional increases in BV_{Tb}.

Global cortical analysis reveals a non significant increase in global BV/TV_{Ct} less than 1% (P > 0.5) when comparing 0N and 4N loading groups. Global BV_{Ct} and TV_{Ct}, Ct.Th and MV are all shown to increase by less than 3% (P > 0.5). Highly significant regional differences are however detected. The group-region interaction term associated with regional comparisons of 0N and 4N loading groups show BV_{Ct}, Ct.Th and MV to be significantly different (P < 0.001). BV_{Ct} is shown to increase in cortical regions 1_{Ct-9}_{Ct} (figure 4.9c), where the maximum reported increase is 6.14% (0.0164mm^3, P int < 0.001). Again comparisons of mean regional changes show that regional changes in Ct.Th, TV_{Ct} and MV do not always correspond to mean regional increases in BV_{Ct}.

Analysis of the Primary spongiosa shows a 2%, non-significant increase in global BV/TV_{Sp} (P > 0.5) when comparing unloaded and the group loaded at 4N. However both global BV_{Sp} and TV_{Sp} are both shown to increase significantly by 10.9% (0.064mm^3, P < 0.05) and 9.2% (0.085mm^3, P < 0.05) for the same comparison.

4.1.3. Discussion

This study has shown, ageing, pinning, an inflammatory response associated with pinning and mechanical stimulation to influence the bone morphology of the C5 caudal vertebra. Analysis of the age-matched controls demonstrates the continued growth of the caudal vertebra. Global analysis suggests that growth is targeted predominantly towards primary spongiosa and cortical bone, where significant 11% and 12% increases are reported in global BV/TV_{Sp} and BV/TV_{Ct} respectively as apposed to a non significant 5% increase in BV/TV_{Tb}. The significant increases in TV_{Wh} and TV_{Ct} combined with the non significant decreases in MV and global TV_{Tb} suggest that the newly formed bone is deposited on both endostial and periosstal cortical sights, in addition to the formation of bone at both proximal and distal primary spongiosa. Direct comparison of the pinned control groups with the age-matched controls shows that the insertion of hydroxyapatite coated pins into C4 and C6 do not have a significant effect on global morphometric parameters. However regional analysis does infer that there is a significant local retardation in growth. When
comparing age-matched and pinned control groups at T7 differences in $BV_{Ct}$ between corresponding regions progressively increase towards proximal and distal regions (figure 4.8b), the same trend is also observed for trabecular regions (figure 4.6b) however significant group-region interactions are not detected. The combined effect of these differences is shown to produce significant regional differences for whole bone (figure 4.5b). These data indicate that the pinning procedure interrupts the normal growth process of the target vertebra, however it is clear from the images (figure 4.7) that the observed retardation in growth does not significantly affect structural integrity of the C5 caudal vertebrae.

Whilst the pinned controls demonstrate acceptable differences this is not the case for the groups associated with the severe inflammatory response. At the end of 7 weeks prolonged inflammation has been shown to severely affect trabecular bone, resulting in a dramatic 22% decrease in global $BV_{Tb}$, attributable to 25% loss in Tb.N. The effect on global cortical bone is less dramatic, no change in global $BV_{Ct}$ is observed throughout the time course of the experiment suggesting development of the cortical bone is in someway inhibited, resulting in significant global and regional differences when compared to both pinned and age-matched control groups at T7 (figure 4.8b). Furthermore the observed significant global and regional increases in $TV_{Tb}$ and $MV$ combined with the significant global and non-significant regional increases in $TV_{Wh}$ and $TV_{Ct}$ show structural expansion to be another effect of the inflammatory response. Coupled with the losses in $BV$, the observed structural expansion results in global and regional reductions for $BV/TV$ for all components (figure 4.5a, 4.6a, and 4.8a). These factors are of particular concern when considering the progressive inflammation observed in the loaded groups, it is therefore feasible that any anabolic effect resulting from mechanical stimulation could have been diminished by the seemingly catabolic mechanisms associated with an inflammatory response. The poor correlation of the $INF_{score}$ with global and regional morphometric parameters suggest otherwise, indicating that inflammation has to be present for a sustained period of time before any osteolytic effects are seen. However without proper characterization the presence of a counter-acting catabolic effect cannot be neglected. One possible cause of the inflammatory response could be wear debris from the Hydroxyapatite coating. Literature shows that wear debris from prosthetic implants
Load induced changes in trabecular and cortical bone

can induce a pro-inflammatory response and osteolytic mechanisms (11,12). The inflammatory response and the associated osteolytic effect seen in C5 could therefore be eliminated by using pins which are not susceptible to the generation of wear debris. Uncoated stainless steel pins would be the obvious option however there is a strong possibility that these pins would loosen as no osteointegration local to the pin-bone interface would be induced (the sole purpose of the hydroxyapatite coating). Another potential solution would be to pharmacologically treat the inflammation. However the introduction of external biological agents could unnecessarily complicate the elucidation of the biochemical pathways responsible for bone formation.

When comparing the groups loaded at 0N (T7\text{PIN}) and 4N, no significant global increases in BV/TV\text{Wh}, Tb, Ct, or BV\text{Wh}, Tb, Ct, were detected by \(\mu\)CT, however regional analyses of \(\mu\)CT data did show significant local increases in BV\text{Wh}, Tb, Ct, when comparing T7\text{PIN} with T7\text{4N} (figure 4.9). Corresponding changes in other structural parameters (Tb.N, Tb.Th, Ct.Th, TV\text{Ct} and MV) were not present indicating that the magnitudes of the local increases in BV (< 5%) were insufficient to yield significant changes in other geometric parameters. A significant 10% increase in both BV\text{Sp} and TV\text{Sp} was also detected (P < 0.05) for primary spongiosa. These data offer clear proof of load-induced anabolic activity in response to mechanical stimulation despite the possible presence of catabolic mechanisms.

In this first in vivo trial we have successfully demonstrated that we can surgically insert hydroxyapatite coated pins into the C4 and C6 vertebrae of C57BL/6 mice, whilst inducing an acceptable reduction in bone quality in a high proportion of mice, we have also shown that we can accurately apply a mechanical load to the C5 vertebrae of for a sustained period of time. We have also induced detectable magnitudes of bone formation at both cortical and trabecular sites thereby demonstrating the model’s potential. By increasing the applied load and taking measures to reduce the inflammatory response, it should be possible in future studies to increase the amount of mechanically induced bone formation, such that the associated biochemical responses are sufficiently amplified to reveal the underlying mechanisms of bone formation.
4.2. The C3H/Hej mouse model

A Caudal Vertebrae Axial Compression Device (CVAD) was used to mechanically stimulate the fifth caudal vertebrae (C5) of C3H/Hej female mice via two stainless steal pins inserted into the C4 and C6 caudal vertebrae. The aim of this study was to show that the surgical and intensive loading protocol, already applied to B6 mice could be successfully applied to C3H mice thereby establishing a second mouse model which can be used in future studies to compare the mechnano-sensitivity of both cortical and trabecular bone in two different strains of mice. This could ultimately facilitate the identification of mechnano-sensitive genes in both cortical and trabecular bone via a Quantitative trait loci (QTL) approach. Stainless steel pins were inserted into the C4 and C6 of 50, 12 week old, female C3H mice, the extremities of which were morphed such that they could not be pulled out. Following surgery, 70 mice (including the 40 pinned mice) were randomly divided into 7 groups: 4 control groups and 2 loading groups such that the effect of pinning and mechanical stimulation on the morphometry of C5 vertebrae could be accurately characterized. Mice belonging to the 3 loading groups were subjected to an acute loading regime (3000 cycles, 10 Hz, 3 times a week for 4 weeks) for 3 different load amplitudes (2N and 4N). Upon sacrifice C5 vertebrae were scanned using micro-computed tomography (µCT) at 6 µm voxel size. Throughout the duration of the experiment no severe inflammation occurred. Analysis of µCT images showed pinning to have an effect on bone morphometry, relative to the morphology associated with the natural ageing process. Comparison of the pinned control group with the unpinned age matched control group at the end of the experiment showed whole global BV and TV to be increased by 4.5% and 6.5% respectively ( P = 0.07, P < 0.05). Isolation of the cortical component alone showed these global increases to be 7% and 7.7% (P < 0.05). Both global and regional analysis of whole, cortical and trabecular bone morphometric parameters show that the C3H female mice did not respond to mechanical stimulation. Percentage increases for global and regional BV at cortical and trabecular sites were less than 2% (P > 0.2) when comparing groups loaded at 0N and 4N. In this second in vivo trial we have successfully demonstrated that we can surgically insert stainless steel pins into the C4 and C6 vertebrae of C3H/Hej mice, whilst inducing acceptable changes in C5 morphology. We have also shown that we can accurately apply
a mechanical load to the C5 vertebrae of for a sustained period of time without inducing structural failure. These data supports the view that C3H are less responsive to mechanical loading than their B6 counterpart.

4.2.1. Introduction

Mechanical stimulation is one of the most important factors regulating the development and structural maintenance of bone (13-16). Studies have shown that increased mechanical stress on bone tissue changes bone morphology such the there is an increase in bone mass and biomechanical strength. Conversely it has been shown that a lack of mechanical stress results in bone loss (17-23). Recent studies in humans have shown the anabolic response to a given mechanical load to be highly variable (24-27). A similar variation has also been observed in inbred strains of mice, in particular C57BL/6 (B6) and C3H/Hej (C3H) (18,28) suggesting that skeletal mechano-sensitivity is genetically determined. Successful identification of these mechano-sensitive genes would lead to and increased understanding of the biochemical pathways involved in bone formation which in turn could lead to the development of novel strategies for the management of osteoporosis.

To further investigate the genetic regulation of mechanical loading several studies have applied the Quantitative Trait Loci strategy (QTL). In this approach, two inbred mouse strains exhibiting a phenotypic difference of interest are crossed, and any genetic loci which co-segregate with the phenotype are identified. C57BL/6 (B6) and C3H/Hej (C3H), mice which demonstrate numerous contrasting phenotypes (29-32) including mechano-sensitivity, are therefore ideal candidates. Kesavan et al (33) used these two inbred mouse strains and has located chromosomal regions containing genetic loci which could regulate the adaptive response of cortical bone to mechanical loading. However so far no single gene has been identified that influences the skeletal response to mechanical loading, furthermore this and other studies (2,4,18,34) have focused their efforts on the cortical component of bone. There is little information concerning trabecular bone which is perhaps the most important component of bone when considering that the loss of trabeculae in the vertebra during ageing is a primary contributor to the development of Osteoporosis. In addition trabecular bone has been shown to have a more enduring
sensitivity to mechanical stimulation in mature human adults than that of cortical bone (5,6).

The purpose of this study is to establish a second mouse model for the study of both cortical and trabecular bone adaptation in response to mechanical loading using the C3H inbred strain. We have previously demonstrated that we can successfully insert stainless steel pins into the 4th and 6th caudal vertebrae of B6 female mice at 12 weeks of age without significantly altering bone mass and bone micro-architecture. We have also demonstrated that we can apply an accurate acute mechanical loading regime using a specially developed loading device (CVAD) without inducing structural failure, furthermore we have successfully induced a positive, albeit minimal, anabolic response in both cortical and trabecular sites. Here we intend to demonstrate that the same protocol can be used for C3H mice. This will enable us to test the hypotheses that C3H mice are less mechano-sensitive to mechanical stimulation when compared with B6 mice in future studies, using loads in excess of 4N. This could ultimately facilitate a QTL approach targeted at the identification of mechano-sensitive genes regulating both cortical and more importantly trabecular bone adaptation in response to mechanical loading.

4.2.2. Materials and Methods

Mechanical loading apparatus

To increase the experimental throughput of mice the Caudal Vertebrae Axial Compression Device (CVAD) was modified to include a second identical loading axis (figure 4.10a). The additional loading axis, comprised of a linear actuator (LA25-42-000A, Bei-Kimco Magnetics) connected in series with a load cell (13/2443 – 16 Transmettra Haltec GmbH), was connected to the second axis of a 4-axis servo control board (NI-7344 national instruments) via a 4 axis signal amplifier (MID-7654 National instruments). Compression of the fifth caudal vertebra was achieved by connecting two morphed, stainless steel pins (diameter: 0.5mm, Fine Science Tools), inserted into C4 and C6 vertebrae, to the CVAD (figure 4.10b). To surgically implant the stainless steel pins into C4 and C6 vertebrae a special pinning device, compatible with x-ray fluoroscopy, was developed. The device makes use of a V-clamp to simultaneously secure and
automatically locate the cranial-caudal axis of the mouse tail. A digital mobile C-arm (OEC MiniView 6800, GE Medical Systems) is then used to locate C4 and C6. The pins which are loaded into channels integral to the V-clamp are manually pushed through the centre of the vertebrae, perpendicular to the cranial-caudal axis. To ensure that the pins remain in the caudal vertebrae throughout the entire duration of the experiment the ends of the pins are bent using a specially developed mechanical press. Throughout this procedure the mice were anesthetized using an oxygen isofluorane mixture.

Figure 4.10: (a) Overview of the dual axis Caudal Vertebra Axial Compression Device (CVAD). (b) Fluoroscopic image of a mouse, graphically edited to show the location and form of the stainless steel pins once they have been surgically inserted. The mechanical signal is applied to the distal most pin whilst the proximal-most pin is clamped.

**Experimental design**

To fully quantify the effects of ageing, pinning and mechanical loading in the C3H/HeJ inbred strain a study similar to that reported for B6 mice was performed. 70, 9 week old C3H/HeJ female mice (Harlan Ltd) were housed in a husbandry unit in groups of 5 and given 3 weeks to acclimatize to their new environment. At 12 weeks of age (T0) the mice were divided into 7 groups of 10: Group 1 (T0_BASE) served as the baseline control group, the mice in this group were sacrificed at 12 weeks of age, providing a benchmark C5 which was used to assess the contribution of normal growth to the morphometry of C5 vertebrae. Groups 2 and 3 (T3_PIN, T3_AMC) formed the pinned the un-pinned control groups. The mice belonging to the pinned control group had stainless steel insect pins (0.5mm diameter, Fine Science Tools) surgically inserted into their C4 and C6 vertebrae
at T0. Both groups were sacrificed at 14 weeks of age (T3) just before the start of loading.

These groups were designed to assess the effect of growth and the pinning procedure on C5 morphometry prior to the commencement of loading and following 3 weeks of post-surgical recovery. Groups 4, 5, and 6 (T7_0N, T7_2N, T7_4N) were pinned in the same way at T0 and constituted the loading groups. Following the application of a 1N pre-load, sinusoidally varying forces (3000 cycles, 10Hz) with amplitudes of 2N and 4N were applied to the C5 of mice in T7_2N and T7_4N groups respectively. T7_0N served as the sham group and was fitted into the device for the same amount of time but received not loading. This was repeated 3 times a week for duration of 4 weeks. At 18 weeks old (T7), following 4 weeks of loading all mice were sacrificed. To help quantify the amount of bone formation attributable to mechanical loading alone and to further assess the effect of pinning groups 7 and 8 (T7_PIN, T7_AMC) formed the unloaded, pinned and unloaded non-pinned control groups which were also sacrificed at T7. All mice were sacrificed using CO2 inhalation. Upon sacrifice of the mice the C5’s were harvested and immediately fixed in formalin for 48 hours, after which they were transferred to saline containing 70% ethanol. Figure 4.11 provides a schematic representation of the experimental design.
Throughout pin insertion and loading the mice were anesthetized using an oxygen-isoflurane mixture (Provet Medical AG). To monitor the health of the mice, each mouse was weighed prior to loading and after each loading bout. The accuracy of the CVAD was assessed by analyzing the recorded force feedback signals for each loading bout and for each mouse to determine each peak and minimum force value present in the applied dynamic signals.

**Quantification of bone adaptation**

Figure 4.12: (a) Digital image of a whole C5 vertebra showing cortical, trabecular components, including the primary spongiosa, subdivided into 19 overlapping regions (1\textsubscript{Wh}-19\textsubscript{Wh}). (b) Subdivided Internal volume of interest enclosing primary spongiosa ( Regions Sp\textsubscript{1}, Sp\textsubscript{2}) and trabecular bone (Regions 1\textsubscript{Tb} –15\textsubscript{Tb}). (c) Subdivided cortical shell. The 15 cortical regions (1\textsubscript{Ct} –15\textsubscript{Ct}) match those which define the 15 trabecular regions.

Bone morphometry was assessed using micro-computed tomography with 5 times frame averaging (6µm voxel size, 50 kVp, 160µA, Scanco Medical AG Switzerland) to obtain 3D, digital images of all C5 vertebrae. Morphometric parameters were determined using a direct 3D approach (10). Bone Volume (BV\textsubscript{Wh}), Tissue Volume (TV\textsubscript{Wh}) and Bone Volume Density (BV/TV\textsubscript{Wh}) were determined globally and locally for the whole bone (figure 4.12a). Local analysis was performed by subdividing the whole bone into 19 overlapping regions: 10 distinct regions, each having a height equal to 10% of the caudal vertebrae’s total height and 9 additional overlapping regions, spanning the midpoints of the 10 distinct regions (figure 4.12a). The spongiosa and cortical components of the vertebrae were analyzed in a similar way. An internal volume of interest comprised of both primary and secondary spongiosa was subdivided into 19 overlapping regions, (figure 4.12b). The first and last regions (Sp\textsubscript{1}, Sp\textsubscript{2}) contained primary spongiosa local to
both proximal and distal growth plates. The transition of primary spongiosa into secondary spongiosa was captured in regions Tr\(_1\) and Tr\(_2\). The remaining regions (1\(_{Tb}\) - 15\(_{Tb}\)) enclosed only trabecular bone. Bone Volume Density (BV/TV\(_{Sp}\)), Bone Volume (BV\(_{Sp}\)) and Tissue Volume (TV\(_{Sp}\)) were calculated for the sum of the two proximal and distal volumes containing primary spongiosa (Sp\(_1\) & Sp\(_2\)). Trabecular Bone Volume Density (BV/TV\(_{Tb}\)), Trabecular Bone Volume (BV\(_{Tb}\)), Trabecular Tissue Volume (TV\(_{Tb}\)), Trabecular Thickness (Tb.Th) and Trabecular Number (Tb.N) were determined locally for regions 1\(_{Tb}\) -15\(_{Tb}\) and globally (for a single volume described by regions 1\(_{Tb}\) -15\(_{Tb}\)). The cortical shell was subdivided such that the overlapping regions matched the 15 overlapping regions defining the trabecular component of each caudal vertebra (figure 4.12c). Cortical Bone Volume Density (BV/TV\(_{Ct}\)), Cortical Bone Volume (BV\(_{Ct}\)) and Cortical Tissue Volume (TV\(_{Ct}\)), Cortical Thickness (Ct.Th) and Marrow Volume (MV) were also determined globally and locally.

**Statistical analysis**

To investigate the effect of time, pinning and mechanical loading on global bone morphometric parameters Anova was used to contrast the relevant groups at different time points and load levels. Where significance was found pair wise t-tests with bonferroni correction were used to determine the significance of the differences. To investigate the regional effects of time, pinning and mechanical loading Repeated Measures Anova was used. Again bonferroni corrections were applied where necessary.

**4.2.3. Results**

Following the insertion of the stainless steal pins there were no signs of severe inflammation, there were signs of moderate inflammation, however this was shown not to correlate in any way with bone morphometric indices. Throughout the loading experiment no mice were lost due to death, and average weights remained constant suggesting the maintenance of good general health. Owing to their morphed extremities, the stainless steel pins remained fixed in all C4 and C6 caudal vertebrae, indicating that no structural damage was caused at the pin-bone interface following intensive fatigue loading. Analysis of the force history specific to both loading axes showed that force was
transferred to the loaded subjects with high accuracy. The average amplitudes applied during the entire experiment were 2.03N +/- 0.05N, 2.04N +/- 0.02N and 4.05N +/- 0.03N, 4.08N +/- 0.04N for Axes 1 and 2 respectively.

**Assessment of the anabolic response to ageing and pinning**

**Whole bone**

Figure 4.13: (a) Variation of global BV/TV\(_{Wh}\) as measured by age-matched and pinned control groups. (b) Regional variation of BV\(_{Wh}\) as measured by age-matched and pinned control groups at T7. (c) Regional variation of TV\(_{Wh}\) as measured by age-matched and pinned control groups at T7.

T0\(_{BASE}\) shows the average values of BV/TV\(_{Wh}\), BV\(_{Wh}\) and TV\(_{Wh}\) to be 63\%, 4.87mm\(^3\), and 7.73mm\(^3\), respectively. The age-matched controls show development of the C5 caudal vertebrae to continue throughout the entire 7 week period, Global BV\(_{Wh}\) and TV\(_{Wh}\) are shown to increase by 8.8\% (0.43mm\(^3\), P < 0.05) and 5.8\% (0.45mm\(^3\), P = 0.2), yielding a significant 3\% significant increase in global BV/TV\(_{Wh}\) (P < 0.05), figure 4.13a. In contrast the pinned controls show significant 13\% (0.67mm\(^3\), P < 0.001) and 12.7\% (0.99mm\(^3\), P < 0.001) increases in both global BV\(_{Wh}\) and TV\(_{Wh}\), resulting in non-significant increase in global BV/TV\(_{Wh}\) (figure 4.13a) of less than 1\% (P > 0.5).
Comparison of the pinned and age-matched controls at T7, reveals global $BV_{Wh}$ and global $TV_{Wh}$ to be 4.5% (0.24mm$^3$, $P = 0.07$) and 6.5% higher (0.54mm$^3$, $P < 0.05$) higher respectively for the pinned control group. Subsequently global $BV/TV_{Wh}$ is 2% higher ($P = 0.08$) for the age-matched controls. Significant group-region interaction $P$-values ($P_{int}$) were detected when comparing corresponding regions for both pinned and age-matched controls at T7. $BV_{Wh}$ and $TV_{Wh}$ were shown to be up to 10% (0.05mm$^3$) and 8% (0.07mm$^3$) higher in the case of pinned controls ($P_{int} < 0.001$, figure 4.13b & c), resulting in significantly higher regional values for $BV/TV_{Wh}$ in favour of the age-matched control group ($< 3\%$, $P_{int} < 0.001$).

**Trabecular bone**

![Figure 4.14](image)

Figure 4.14: (a) Mean regional variation of $BV_{Tb}$ for age-matched ($T7_{AMC}$) and pinned ($T7_{PIN}$) control groups. (b) Mean regional variation of $Tb.Th$ for $T7_{AMC}$ and $T7_{PIN}$. (c) Mean regional variation of $Tb.N$ for $T7_{AMC}$ and $T7_{PIN}$. (d) Global variation of $BV/TV_{Tb}$ as described by $T_{0.BASE}$ and the age-matched and pinned controls at T3 and T7.

$T_{0.BASE}$ shows the average trabecular parameters to be the following: $BV/TV_{Tb} = 12.3\%$, $BV_{Tb} = 0.28mm^3$, $TV_{Tb} = 2.27mm^3$, $Tb.Th = 0.087mm$, $Tb.N = 2.1mm^{-1}$. Visual and regional analysis show little if any bone mass to exist in the central trabecular regions.
resulting in negligible, almost undetectable differences between pinned and age-matched controls of BV/TV<sub>Tb</sub> and BV<sub>Tb</sub> (< 0.0002mm<sup>3</sup>, figure 4.14a). Inconsistencies in the data arise when these values are cross referenced with structural parameters. In regions 6<sub>Tb</sub>-9<sub>Tb</sub> there are disproportionate increases in Tb.Th and Tb.N (Figure 4.14b & c). The inaccurate quantification of Tb.N and Tb.Th is attributable to masking artifacts i.e. the inclusion of a few spatially distributed cortical voxels into the trabeculae VOI such that regional BV/TV<sub>Tb</sub> is less than 0.01%, furthermore because there is almost no bone mass, the means and standard errors of structural indices are strongly affected by the one or two samples which enclose these artifacts or those few voxels which describe legitimate trabecular bone.

Figure 4.15: Transverse µCT slices showing typical bone structure in proximal, central and distal regions of C5 vertebrae for groups T0<sub>BASE</sub>, T7<sub>PIN</sub> and T7<sub>AMC</sub>. The images are taken from C5 vertebrae with median values for BV/TV<sub>WH</sub>.

For this reason subsequent global and regional calculations will exclude regions 6<sub>Tb</sub>-9<sub>Tb</sub>. During the entire 7 week period the age-matched controls describe a significant 12.8% increase in global BV/TV<sub>Tb</sub> (P < 0.05), resulting from a significant 17% (0.05mm<sup>3</sup>) increase in global BV<sub>Tb</sub> (P < 0.01) and a non-significant 3.7% (0.08mm<sup>3</sup>) increase in global TV<sub>Tb</sub> (P > 0.5). These increases are accompanied by a 9% (8µm) significant increase in Tb.Th (P < 0.01) and a non-significant 5% (1.04mm<sup>-1</sup>) decrease in Tb.N (P >
0.44). The pinned control groups demonstrate a non-significant increase in global BV/TV\textsubscript{Tb} of less than 2% with time (P > 0.5, figure 4.15d) this is accompanied by significant 13.4% (0.037mm\textsuperscript{3}) and 11.2% (0.26mm\textsuperscript{3}) increases in global BV\textsubscript{Tb} and global TV\textsubscript{Tb}, respectively (P < 0.05). Global Tb.Th is shown to increase significantly by 7.4% (6.5µm, P < 0.05) whilst Tb.N demonstrates a non significant 6.7% decrease (0.14mm\textsuperscript{-1}, P > 0.5). When compared to the age-match control group at T7 global BV/TV\textsubscript{Tb} and BV\textsubscript{Tb} for the pinned group is on average 9.8% (P < 0.01) and 3% (0.009mm\textsuperscript{3}) less (P > 0.4). Global TV\textsubscript{Tb} on the other hand is 7.5% (0.18mm\textsuperscript{3}) significantly higher (P < 0.05), Global Tb.Th is 1.8% (1.7µm) less (P > 0.1), whilst the magnitude of the reduction in Tb.N is shown to be 1.8% less (0.03mm\textsuperscript{-1}, P > 0.1). Regional analysis at comparing T7\_PIN and T7\_AMC shows BV\textsubscript{Tb} to be up to 1% (0.008mm\textsuperscript{3}) significantly less in some regions (P\textsubscript{int} < 0.01) for T7\_PIN, conversely TV\textsubscript{Tb} is shown to be consistently higher, by up to 9.6% (0.033mm\textsuperscript{3}, P\textsubscript{int} > 0.12). Consequently regional BV/TV\textsubscript{Tb} is shown to be up to 15% less (P\textsubscript{int} < 0.01). No significant group-region interaction terms are reported for Tb.Th and Tb.N. Despite the reported significant variations in trabecular structure the magnitudes of the changes are such that no differences between pinned and age-matched controls can be detected visually (figure 4.15)

**Cortical bone**

At T0 the average values of the cortical parameters are as follows: BV/TV\textsubscript{Ct} = 50.6%, BV\textsubscript{Ct} = 2.77mm\textsuperscript{3}, TV\textsubscript{Ct} = 5.46mm\textsuperscript{3}, Ct.Th = 0.18mm and MV = 2.69mm\textsuperscript{3}. The age-matched controls describe an almost significant 8.5% (0.23mm\textsuperscript{3}) increase (P = 0.06) throughout the entire 7 week period for global BV\textsubscript{Ct} and a non-significant 8% (0.33mm\textsuperscript{3}) increase (P > 0.1) in global TV\textsubscript{Ct}, resulting in a non-significant 2.3% increase in global BV/TV\textsubscript{Ct}, figure 4.16a. The increase seen in global BV\textsubscript{Ct} is reflected by a 4.3% (7.6µm) increase in global Ct.Th (P =0.17) whilst global MV is shown to increase non-significantly by 3.7% (0.1mm\textsuperscript{3}, P > 0.5). The pinned controls demonstrate significant 16.7% (0.46mm\textsuperscript{3}, P < 0.001) and 14.3% (0.79mm\textsuperscript{3}, P < 0.001) increases in both global BV\textsubscript{Ct} and TV\textsubscript{Ct}, resulting in a similar non-significant 2% increase in global BV/TV\textsubscript{Ct}, figure 4.16a. Global Ct.Th and MV are shown to increase significantly by 7.8% (14µm, P < 0.01) and 12% (0.32mm\textsuperscript{3}, P < 0.01). When global parameters for both age-matched and
pinned controls are compared at T7, no difference is shown to exist for BV/TV\(_{\text{Ct}}\) (figure 4.16a). There are however significant differences for all other global parameters. Global BV\(_{\text{Ct}}\), TV\(_{\text{Ct}}\), Ct.Th and MV are shown to be 7% (0.22mm\(^3\)), 7.7% (0.45mm\(^3\)), 3.3% (6.2\(\mu\)m) and 8% (0.22mm\(^3\)) higher for the pinned control group at T7 with respect to the age-matched control group at T7 (P < 0.05).

Regional analysis detects significant regional differences between pinned and age-matched controls at T7 for all cortical parameters apart from BV/TV\(_{\text{Ct}}\) (Pint > 0.5). Mean regional values of BV\(_{\text{Ct}}\) are shown to be consistently higher than the age-matched control group by up to 10% (0.045mm\(^3\), P\(_{\text{int}}\) < 0.001, figure 4.17b). Similarly TV\(_{\text{Ct}}\), Ct.Th and MV (figure 4.16c) are all shown to be up to 10% (0.09mm\(^3\)), 5% (12.7\(\mu\)m) and 10% (0.048mm\(^3\)) higher than the corresponding regions belonging to the age-matched control group (P\(_{\text{int}}\) < 0.01).
Primary spongiosa

At T0, the average values of the Primary Spongiosa parameters are as follows: BV/TV\textsubscript{Sp} = 77\%, BV\textsubscript{Sp} = 0.69mm\textsuperscript{3}, TV\textsubscript{Sp} = 0.88mm\textsuperscript{3}. During the 7 week period the age-matched controls show a significant 4\% increase in BV/TV\textsubscript{Sp} (P < 0.01), explained by a significant 11.7\% (0.08mm\textsuperscript{3}) increase in BV\textsubscript{Sp} (P < 0.05) and a non-significant 7\% (0.06mm\textsuperscript{3}) increase in TV\textsubscript{Sp} (P > 0.1). The pinned control demonstrate a non-significant 1.5\% increase in global BV/TV\textsubscript{Sp} (P > 0.1) and significant 15\% (0.11mm\textsuperscript{3}) increases in BV\textsubscript{Sp} and TV\textsubscript{Sp} respectively (P < 0.01) such that when T7\textsubscript{PN} is compared to T7\textsubscript{AMC}, BV\textsubscript{Sp} and TV\textsubscript{Sp} are 3\% (0.02mm\textsuperscript{3}, P > 0.1) and 5.5\% (0.05mm\textsuperscript{3}, P = 0.056) greater. BV/TV\textsubscript{Sp} however is shown to be 3\% greater for the age-matched control group (P < 0.01).

Assessment of the anabolic response to mechanical stimulation

No significant global changes were detected for any parameter when comparing loading groups T7\textsubscript{0N}, T7\textsubscript{2N} and T7\textsubscript{4N} for whole bone analysis. Global BV/TV\textsubscript{Wh} was shown to decrease by 0.86\% when comparing T7\textsubscript{0N} and T7\textsubscript{4N} (P > 0.5), whilst global BV\textsubscript{Wh} and TV\textsubscript{Wh} were shown to increase non-significantly by 1\% (0.05mm\textsuperscript{3}, P > 0.5) and 2\% (0.16mm\textsuperscript{3}, P > 0.5) respectively. Furthermore corresponding regional analysis showed no significant regional differences for both BV\textsubscript{Wh} (figure 4.17a) and TV\textsubscript{Wh} (P\textsubscript{int} > 0.2). The same holds true for trabecular global and regional analyses. Global BV/TV\textsubscript{Tb} was shown to decrease by 1.63\% (P > 0.5) whilst global BV\textsubscript{Tb}, TV\textsubscript{Tb}, Tb.Th and Tb.N were shown to increase non-significantly by 0.4\%, 2\%, 1.2\% and 0.3\% respectively (P > 0.5). Similarly no significant regional changes were detected for any of the morphological parameters (P\textsubscript{int} > 0.5, figure 4.17b). Global BV/TV\textsubscript{Ct} is shown to decrease non-significantly by 0.6\% (P > 0.5), whilst BV\textsubscript{Ct}, TV\textsubscript{Ct} and MV are shown to increase non-significantly by 1.4\%, 1.9\% and 2.5\%, respectively (P > 0.4). Global C.Th is shown to decrease by 0.14\% (P > 0.5). Regional analysis again fails to report any significant group-region interaction terms for all cortical parameters (figure 4.17c). The lack of response to mechanical stimulation is confirmed by the absence of any significant change in the primary spongiosa. BV/TV\textsubscript{Sp}, BV\textsubscript{Sp} and TV\textsubscript{Sp} are all shown to change by
4.2.4. Discussion

Here we have shown that between the ages of 12 weeks and 19 weeks there is a continued addition of bone mass to the C5 vertebra, both in the subjects which have not had their C4 and C6 vertebrae pinned and those which have. The age-matched controls have shown that throughout the 7 week period cortical bone, trabecular bone and primary spongiosa bone mass increase significantly by 7% (P = 0.06), 16.5% (P < 0.01) and 10% (P < 0.05), reflecting absolute increases of 0.23mm$^3$, 0.05mm$^3$ and 0.08mm$^3$, respectively. Surprisingly the pinned controls showed greater increases in Global BV for both cortical (figure 4.16b), and primary spongiosa components. Throughout the entire 7 week period cortical and primary spongiosa bone masses for the pinned controls increased significantly by 16.7% (P < 0.01) and 14.9% (P < 0.01) which translate to absolute increases of 0.46 mm$^3$ and 0.1 mm$^3$. Consequently at T7 the pinned controls have 7% (P < 0.05) and 3% (P > 0.1) more cortical and primary spongiosa bone mass compared to age-matched controls. Owing to similar significant changes in global TV$_{Ct}$, $s_p$ and MV the addition of bone mass is not reflected by higher global bone density at T7.
for pinned controls. Significant increases for pinned controls with time in global Ct.Th of 7.8% (0.013 mm$^3$), TV$_{Ct}$: 14.3% (0.79 mm$^3$), TV$_{Sp}$: 13% (0.12 mm$^3$) and MV: 12% (0.32 mm$^3$), $P < 0.01$, infer a distribution of bone such that the cortex and the primary spongiosa expand. This is reaffirmed by regional analysis which shows global BV$_{Ct}$, Ct.Th, TV$_{Ct}$ and MV to be consistently higher than corresponding regions at T7 in the age-matched controls by up to 10% (0.04 mm$^3$), 5% (0.013 mm$^3$), 8% (0.084 mm$^3$) and 9.3% (0.048 mm$^3$) respectively ($P_{int} < 0.01$). The observed pinning-anabolic effect did not transfer to the trabecular component of bone, where no significant global changes in BV$_{Tb}$ were reported; moreover small significant regional losses in BV$_{Tb}$ of up to 6% (0.002 mm$^3$) were evident. Given that we are interrupting normal biological processes by implanting pins into C4 and C6 caudal vertebra we would expect to see reduction in bone mass for all components, as was observed in the B6 model. Here we have observed an effect which initiates an altogether different remodeling process which results in C5 vertebrae with increased masses of cortical bone a primary spongiosa. The underlying mechanisms of this anabolic response to pinning cannot be satisfactorily explained from the data presented here, however it is clear that the surgical insertion of stainless steel pins into C4 and C6 vertebrae does not significantly effect the morphology of C5 such that we cannot use it as a model for the study of cortical and trabecular bone adaptation. Figure 4.15, shows that the changes induced by pinning are difficult to distinguish visually.

No mechano-sensitive effect was detected when mechanically stimulating the C5 vertebrae. No significant global or regional percentage increases in BV for either the cortical or trabecular components of bone were present when comparing the T7$_{0N}$ group with either of the two loaded groups, T7$_{2N}$ and T7$_{4N}$. These results provide early indications that the C3H mouse strain is less mechano-sensitive than its B6 counterpart, however comparisons of their geometries shows that C3H have thicker cortical cross sections and trabeculae, which may result in lower micro-structural strains for the same applied load. Hence before we can confirm these two strains as ideal candidates for a QTL analysis aimed at elucidating the genes responsible for mechano-sensitivity or lack there of, further studies are required to investigate the anabolic response at elevated loads whilst accounting for their geometric differences.
Load induced changes in trabecular and cortical bone

To summarize, in this second in vivo trial we have successfully demonstrated that we can surgically insert stainless steel pins into the C4 and C6 vertebrae of C3H/Hej mice, whilst inducing acceptable changes in C5 morphology and without inducing a severe inflammatory response. We have also shown that we can accurately apply a mechanical load to the C5 vertebrae of for a sustained period of time without inducing structural failure. The fact that we have failed to stimulate bone formation supports existing evidence that C3H mice lack mechano-sensitivity when compared to B6 mice. Furthermore we have demonstrated that by using stainless steel pins with no coating we have avoided the damaging osteolytic effect as seen with in the B6 study. Even though the genetic backgrounds for the two mice are different this result is encouraging, hence by using non-coated stainless steel pins in future studies we have successfully established two mouse models which can be used to determine the relative mechano-sensitivities of both C3H and B6 inbred mouse strains.

4.3. Comparative loading study

Most in vivo studies addressing the skeletal responses of mice to mechanical loading have targeted cortical bone. To investigate trabecular bone responses also we have developed a caudal vertebral axial compression device (CVAD) that transmits mechanical loads to compress the fifth caudal vertebra via stainless steel pins inserted into the forth and sixth caudal vertebral bodies. Here we used the CVAD in C57BL/6 and C3H/Hej mice to investigate whether the effect of regular bouts of mechanical stimulation on bone remodeling and bone mass were dependent on dose and genotype. A combined micro-computed tomographic and dynamic histomorphometric analysis carried out at the end of a 4-week loading regimen for load amplitudes of 0N, 2N, 4N and 8N revealed that an amplitude of 8N stimulated significant increases in trabecular and cortical bone mass mainly in the C57BL/6 strain. Both biological strains exhibited substantial increases in bone formation rates and decreases in osteoblast number. These findings demonstrate that the effect of loading on the structural and functional parameters of bone is dose- and genotype dependent. The caudal vertebral loading model established here is proposed for further studies addressing the molecular processes involved in the skeletal responses to mechanical stimuli.
4.3.1. Introduction

Mechanical loading, is perhaps the most important environmental determinant of bone mass and functional integrity. It is well established that insufficient mechanical stimuli, as in the case of prolonged bed rest and hypogravity, lead to massive bone loss. Conversely it has also been demonstrated that mechanical overloading results in enhanced bone formation and a net gain in bone mass (35-38). These stimuli affect both cortical and trabecular bone in particular trabecular bone which has been shown to have a more enduring sensitivity to mechanical stimulation in human adults (5,6).

Osteoporosis, the most prevalent degenerative disease in western societies, has been partly attributed to a reduction in muscle mass and function and consequently decreased mechanical usage of the skeleton (39). Hence, it is anticipated that an understanding of the processes involved in the skeletal response to mechanical forces could lead to the identification of molecular targets for the development of anti-osteoporotic therapies.

The availability of in vivo models for load regulated bone adaptation is key to understanding the underlying biochemical mechanisms. To study cortical bone several studies have established in vivo mouse models which demonstrate significant increases in bone formation in response to dynamic load regimens at tibial sites (28,40,41). These models have been used to investigate the biochemical pathways associated with load-induced cortical bone adaptation using C57BL/6 and C3H/Hej inbred strains (2-4). These strains are of particular interest as they exhibit a number of contrasting phenotypes specific to both cortical and trabecular bone, including the mechano-sensitivity of cortical bone (18,28-30). Furthermore breeding strategies have been employed together with quantitative trait loci (QTL) analysis in an effort to refine the search for the chromosomal regions responsible for the complementary phenotypes (32,33). However specific genes and or combinations of genes which regulate load induced bone adaptation have yet to be discovered. Compared to cortical bone, trabecular bone has been studied less extensively. One of the few models currently available to assess load induced changes in trabecular bone uses a mechanical device to apply an axial compressive force to the 8th caudal vertebrae of a rat via K-wires inserted into the two, adjacent caudal vertebrae (1,42). However, studies employing this model have been hampered by the current
inaccessibility to genetic manipulations, which are available mainly in mice. Two studies have reported load induced trabecular bone adaptation in mice. De Souza et al (43) mechanically stimulated mice tibia using dynamic loads with magnitudes between 5 and 13 N and demonstrated a significant increase in trabecular bone volume density when pooling all the loaded groups together. Using a similar approach Fritton et al (44) showed trabecular bone volume density and trabecular thickness to increase significantly by 15 % and 12 % when applying a single 6 week long loading regimen. Here we present an alternative in vivo model for the study of both cortical and trabecular bone adaptation. Using an approach similar to the rat vertebra model we hypothesize that the response of murine cortical and trabecular bone to mechanical loading is both dose and genotype dependent. The objectives of this study are therefore twofold: 1) To show that the caudal vertebrae of B6 and C3H mice can be mechanically stimulated in a similar manner to that performed in rats (1) and 2) To compare the skeletal responsiveness to mechanical stimulation in both B6 and C3H mouse strains.

4.3.2. Materials and methods

Mechanical loading apparatus

To mechanically load murine vertebrae a dual-axis closed-loop feedback device was used. This device has been developed to apply a precisely controlled cyclical, compressive load to the fifth caudal vertebrae (C5) of mice at a frequency of 10Hz via stainless steel pins surgically inserted into the fourth (C4) and sixth caudal (C6) vertebrae (Figure 4.18). For more information refer to the previous publication which details the validation of the device (45) As a quality control, the feedback signal from the load cell was recorded and all force maxima and minima determined. Surgical insertion of the stainless steel pins was performed using a special pinning device, compatible with X-ray fluoroscopy. The device makes use of a V-clamp to simultaneously secure and automatically locate the cranio-caudal axis of the mouse tail. The pins are loaded into channels integral to the V-clamp and are manually pushed through the centers of the vertebrae, perpendicular to the cranial-caudal axis. A digital mobile C-arm (OEC
MiniView 6800, GE Medical Systems, Glattbrugg, Switzerland was used to locate C4 and C6 for pinning.

Experimental Design

Female mice, 40 B6 and 40 C3H, 9 weeks of age (Harlan Ltd.) were housed in a husbandry unit in groups of 5 and given 3 weeks to acclimatize to their new environment. Then, all mice had stainless steel insect pins (0.5 mm diameter, Fine Science Tools, Heidelberg, Germany) surgically inserted into their C4 and C6 vertebrae and given 3 weeks to recover before loading commenced. For loading, the mice were divided into 8 loading groups (n = 10): B60N/2N/4N/8N and C3H0N/2N/4N/8N. B60N and C3H0N formed the sham groups whilst the remaining groups were submitted to an intensive loading regime whereby sinusoidally varying forces (3000 cycles, 10Hz) were applied to all C5’s with amplitudes corresponding to the group subscript. This was repeated 3 times a week for a duration of 4 weeks. To vitally label newly formed bone 100 ml of the fluorochrome calcein (Sigma, 15mg/Kg) was administered intraperitonally 4 days and 1 day prior to sacrifice. To protect the structural integrity of the pinned vertebrae, sudden impulses were avoided by linearly increasing the force at a rate of 1 Ns⁻¹ until the peak force was attained at which point the dynamic signal was applied. At 19 weeks of age, following 4
weeks of loading all mice were sacrificed using CO2 inhalation. Upon sacrifice of the mice the C5’s were harvested and immediately fixed in formalin for 48 hours, after which they were transferred to saline containing 70% ethanol. Throughout pin insertion and loading the mice were anesthetized using an oxygen-isoflurane mixture (Provet Medical AG, Lyssach, Switzerland). To monitor the health of the mice, each mouse was weighed prior to loading and after each loading bout. The accuracy of the CVAD was assessed by analyzing the recorded force feedback signals for each loading bout and for each mouse to determine each peak and minimum force value present in the applied dynamic signals. All animal procedures were approved by the local animal ethics committee (Kantonales Veterinäramt Zürich, Zürich, Switzerland).

**Quantification of bone adaptation**

Bone morphometry was assessed using micro-computed tomography (micro-CT) with 5 times frame averaging (6 µm voxel size, 50 kVp, 160µA, Scanco Medical AG, Basserdorf, Switzerland) to obtain 3D, digital images of all C5 vertebrae. Using a direct 3D approach (10) morphometric parameters specific to trabecular and cortical bone were determined for a global volume these included: Trabecular bone volume density (BV/TV\(T_b\)), trabecular bone volume (BV\(T_b\)), trabecular tissue volume (TV\(T_b\)), trabecular thickness (Tb.Th), trabecular number (Tb.N), cortical bone volume density (BV/TV\(C_t\)), cortical bone volume (BV\(C_t\)), cortical tissue volume (TV\(C_t\)), cortical thickness (Ct.Th) and marrow volume (MV). Global trabecular and cortical volumes were defined by adapting an algorithm developed earlier (46) to automatically isolate the outer volume, cortical bone and an internal volume comprised of spongiosa, both primary and secondary (trabecular bone). Primary spongiosa was then excluded from analysis by discarding distal and proximal sub-volumes to ensure only trabecular bone remained (Figure 4.19A). For B6 mice each of these two regions measured 10% of the total height of the total, isolated volume. Owing to the presence of more distal primary spongiosa in C3H mice the discarded distal and proximal regions measured 10% and 15% respectively. The global cortical region of analysis was similarly defined such that it was comprised of the cortical shell enclosing the global trabecular volume. Where global analysis failed to detect a significant load induced anabolic effect local analyses were performed. This was
executed by subdividing both cortical and trabecular global volumes into overlapping regions each having a height equal to 10% of the total height of the volume originally isolated (containing both primary and secondary spongiosa). For B6 mice this resulted in 15 overlapping regions (Figures 4.19B & C).

Similarly for C3H mice this resulted in 14 overlapping regions. Histomorphometric indices for trabecular and cortical regions were quantified using histological techniques for trabecular (Tb), periosteal-cortical (Pe) and endosteal-cortical (En) surfaces. Both single labelled (sLS) and doubled labelled (dLS) surfaces were measured the sum of which was used to determine the mineralized perimeter of bone: Md.Pm<sub>Tb/Pe/En</sub> (a surrogate of osteoblast number). Mineral appositional rate (MAR<sub>Tb/Pe/En</sub>; a surrogate of osteoblast activity) was also obtained by dividing the average distance between the double labels on the bone surfaces by the number of days between injections. In addition bone formation rates (BFR<sub>Tb/Pe/En</sub>), were determined by multiplying the sum of sLS and dLS by MAR. To permit inter-strain comparison Md.Pm and BFR were normalized by total bone surface perimeter (Pm) to give Md.Pm/Pm and BFR/Pm. All indices were determined from 3 single histological slices taken from the mid-sagittal plane of each double labelled vertebra according to the standards in histomorphometry (47). Tartrate Resistant Acid Phosphatase (TRAP) staining was also performed on adjacent sections to measure the number of osteoclasts present per unit of trabecular perimeter (N.Oc/Pm<sub>Tb</sub>).
Statistical analysis

A Students T-test was used to compare the base line morphological parameters specific to both biological strains. To investigate the effect of mechanical loading on each strain, ANOVA was used to compare global CT-measured morphometric parameters and dynamic histomorphometric parameters for each loading group. Where significance was detected post-hoc pair-wise T-tests with bonferroni corrections were used to determine the significance of the differences relative to the 0N loading groups. In the cases where global micro-CT analysis failed to detect a load induced anabolic effect repeated measures anova with the necessary Bonferroni corrections was used to compare the different regions and assess the local effects of loading specific to each mouse strain, yielding a group-region interaction p-value ($P_{int}$). To compare the relative mechano-sensitivity of the two biological strains, two-way ANOVA was used to contrast their dose responses as measured by both micro-CT and histomorphometry. Owing to the difference in bone size between the two strains the micro-CT measured dose responses were first normalized using their respective 0N loading groups. Again where significance was detected post-hoc pair-wise T-tests with Bonferroni corrections were used to determine the significance of inter-strain differences at different loads. Regional effects were not compared between mouse strains. For all statistical analyses, the GNU statistical package R (Version 2.5.1, http://www.r-project.org) was used. Results were considered statistically significant for adjusted P-values lower than 0.05.

4.3.3. Results

Throughout the duration of the experiment no mice were lost due to death and the average weights of the mice throughout loading did not decrease, suggesting that the general health of each mouse remained satisfactory. Analysis of the recorded force feedback signals for each loading bout and for each mouse demonstrates the high accuracy at which the CVAD controls the user defined force. The average force amplitudes applied during the entire experiment were $2.02N \pm 0.01 N$ and $4.03 \pm 0.02N$ and $8.06 \pm 0.07N$. indulge changes in trabecular and cortical bone
Table 4.1: Mean absolute values for global morphological parameters specific to trabecular and cortical components for B6 and C3H loading groups

<table>
<thead>
<tr>
<th>Group</th>
<th>BV/TV (%)</th>
<th>BV (mm$^3$)</th>
<th>TV (mm$^3$)</th>
<th>Tb.Th (µm)</th>
<th>Tb.N (mm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B60N</td>
<td>20.9 ± 0.62$^a$</td>
<td>0.61 ± 0.02$^a$</td>
<td>2.96 ± 0.07$^a$</td>
<td>76.7 ± 2.21$^a$</td>
<td>2.95 ± 0.03$^a$</td>
</tr>
<tr>
<td>B62N</td>
<td>20.5 ± 0.83</td>
<td>0.63 ± 0.03</td>
<td>3.11 ± 0.05</td>
<td>78.5 ± 2.19</td>
<td>2.85 ± 0.05</td>
</tr>
<tr>
<td>B64N</td>
<td>22.5 ± 0.43</td>
<td>0.66 ± 0.01</td>
<td>2.95 ± 0.06</td>
<td>78.7 ± 1.39</td>
<td>3.05 ± 0.08</td>
</tr>
<tr>
<td>B68N</td>
<td>26.3 ± 0.73$^b$</td>
<td>0.80 ± 0.04$^b$</td>
<td>3.02 ± 0.08</td>
<td>93.5 ± 1.63$^b$</td>
<td>3.03 ± 0.08</td>
</tr>
<tr>
<td>C3H0N</td>
<td>11.9 ± 0.26</td>
<td>0.29 ± 0.008</td>
<td>2.48 ± 0.05</td>
<td>91.5 ± 1.39</td>
<td>1.99 ± 0.06</td>
</tr>
<tr>
<td>C3H2N</td>
<td>12.4 ± 0.41</td>
<td>0.31 ± 0.01</td>
<td>2.46 ± 0.03</td>
<td>92.5 ± 1.60</td>
<td>1.96 ± 0.04</td>
</tr>
<tr>
<td>C3H4N</td>
<td>11.7 ± 0.40</td>
<td>0.29 ± 0.01</td>
<td>2.53 ± 0.06</td>
<td>92.7 ± 1.49</td>
<td>2.00 ± 0.07</td>
</tr>
<tr>
<td>C3H8N</td>
<td>13.5 ± 0.64$^c$</td>
<td>0.33 ± 0.01</td>
<td>2.48 ± 0.06</td>
<td>98.7 ± 3.42</td>
<td>1.99 ± 0.09</td>
</tr>
</tbody>
</table>

Almost significant relative to 0N loading group of same biological strain (P < 0.06).

Significant relative to C3H0N (P < 0.001).

Significant relative to 0N loading group of same biological strain (P < 0.001).

Trabecular bone

The mean baseline global parameters for trabecular bone as measured by the B60N and C3H0N loading groups are shown in Table 4.1. Compared to the C3H strain these data show that in the absence of mechanical loading mean global trabecular bone volume and trabecular number are 110% (0.32 mm$^3$) and 48% (0.96 mm$^{-1}$) higher for the B6 strain respectively (P < 0.0001) whilst mean global trabecular thickness is 16% (14.9 µm) lower. These data can be considered to be representative of normal B6 and C3H populations as previous pilot studies have shown pinning of C4 and C6 caudal vertebrae to have a negligible effect on the typical morphology of C5 vertebrae for both trabecular and cortical bone.

When comparing the micro-CT data sets for all B6 loading groups Anova detected highly significant changes in global BV/TV$^{T_b}$, BV$^{T_b}$ and Tb.Th (P < 0.001). No significant differences were detected for global TV$^{T_b}$ and Tb.N (P > 0.1). Comparison of B68N and B60N groups shows global BV/TV$^{T_b}$ and BV$^{T_b}$ to increase by 26% (Table 4.1, Figure 4.20A) and 29% (0.18 mm$^3$) respectively (P < 0.001). No significant changes were observed in global TV$^{T_b}$. Analysis of the global structural indices show the increase in bone mass to correspond to a 22% increase (16.9µm, P < 0.001) in Tb.Th and a non-significant 3% increase (0.09 mm$^{-1}$, P > 0.5) in Tb.N. Regional comparison of BV/TV$^{T_b}$, BV$^{T_b}$ and Tb.Th for the B60N and B68N loading groups confirms the reported, global
anabolic effect. Furthermore significant local increases of up to 14.56% (0.69 mm$^{-1}$) are observed for Tb.N (Figure 4.20B), $P_{\text{int}} < 0.01$. No significant global differences were detected when comparing B6$_{2N}$ and B6$_{4N}$ groups with the 0N loading group. Significant local increases in $\text{BV}_{\text{Tb}}$, of up to 26% (0.025 mm$^3$), were detected when comparing B6$_{4N}$ and B6$_{0N}$ ($P_{\text{int}} < 0.05$). No other significant local increases were observed. The effect of mechanical stimulation on the trabecular component of bone can be clearly seen (Figure 4.21).

Figure 4.20: (A) Percentage increase in global $\text{BV}/\text{TV}_{\text{Tb}}$ for B6 and C3H mice loaded at 0N, 2N, 4N and 8N. $^a$ Significantly different 0N loading group of same biological strain ($P < 0.01$). $^b$ Significant inter-strain difference ($P < 0.01$). (B) Mean regional variation of Tb.N (absolute values) as measured by B6$_{0N}$ and B6$_{8N}$ loading groups, standard errors shown. (C) Mean regional variation of $\text{BV}/\text{TV}_{\text{Tb}}$ (absolute values) as measured by C3H$_{0N}$ and C3H$_{8N}$ loading groups. All Error bars show the standard error.
Figure 4.21: Visual comparison of loaded caudal vertebrae from both B6 and C3H mice. The grey-scale images show transverse sections from the uppermost slice of region 3_Tb. Samples with median BV/TV_Tb from each loading groups are displayed.

When comparing the histological data sets for all B6 loading groups Anova detected significant differences for mineralizing perimeter (Md.Pm/Pm_Tb) and bone formation rate (BFR/Pm_Tb). Md.Pm/Pm_Tb was found to increase significantly by 72% (21.2 mm/mm x10^{-2}) for the group loaded at 2N, by 64% (18.7 mm/mm x10^{-2}) when loaded at 4N and by 89% (26.1 mm/mm x10^{-2}) when loaded at 8N, (P < 0.05; Figure 4.22A). A similar but non-significant trend was reported for MAR_Tb. Increases of 36% (0.55 µm/day) and 29% (0.45 µm/day) were observed for loads of 2N and 4N respectively (P > 0.2), whilst an almost significant 56% (0.86 µm/day) was reported for a load of 8N (P = 0.051; Figure 4.22B). Significant increases in bone formation rate of 54% (109 µm^3/µm^2/day x10^{-2}) and 82% (163 µm^3/µm^2/day x10^{-2}) were reported for B6_2N and B6_8N groups only (P < 0.01), with a near significant increase of 43% (86.5 µm^3/µm^2/day) being shown for B6_4N (P = 0.06). The Osteoclast number per perimeter was shown to significantly decrease for groups loaded at 2N and 8N by 71% (1.57 mm^{-1}) and 52% (1.15 mm^{-1}) respectively (P < 0.05) with non-significant reduction of 35% (0.77 mm^{-1}) being reported for 4N (P > 0.2).
Load induced changes in trabecular and cortical bone

Figure 4.22: Bar charts showing the change of Md.Pm/Pm, MAR and BFR/Pm with load for B6and C3H mice at trabecular (A) periosteal-cortical (B) and endosteal-cortical (C) sites. a Significantly different to the 0N loading group of same strain (P < 0.05). b Significantly different to C3H strain. c Almost significantly different to 0N loading group of same biological strain (P < 0.06). Error bars show standard deviation.

When comparing all loading groups for the C3H strain Anova detected significant differences for global BV/TV_Tb (P < 0.05). Comparison of the C3H8N and C3H0N shows global BV/TV_Tb to be increased, almost significantly, by 14.22% (P = 0.055; Figure 4.20A) owing to a 13.5% (0.039 mm^3, P < 0.2) increase in global BV_Tb and a non significant decrease in global TV_Tb (Table 4.1). Global Tb.Th and Tb.N were shown to increase non-significantly by 7.62% (7 µm) and 2.49% (0.058 mm\(^{-1}\)) respectively. Local
analysis revealed significant group-region interaction terms for BV/TV\(_{Tb}\) and BV\(_{Tb}\) when comparing C3H\(_{8N}\) and C3H\(_{0N}\). BV/TV\(_{Tb}\) was shown to increase by up to 31\% (P\textsubscript{int} < 0.01; Figure 4.20C) whilst BV\(_{Tb}\) was shown to increase by up to 32\% (0.026 mm\(^3\), P\textsubscript{int} < 0.01). The maximum reported increases in Tb.Th and Tb.N were 14.7\% (11.7 µm) and 4.87\% (0.11 mm\(^{-1}\)) respectively, (P\textsubscript{int} > 0.5). Comparison of the other loading groups with the C3H\(_{0N}\) group yielded no significant global or regional differences. Analysis of the histomorphometric indices shows that a significant 48\% (27.7 mm/mm x10\(^{-2}\)) increase in Md.Pm/Pm\(_{Tb}\) was found for the C3H\(_{8N}\) group only (P < 0.05; Figure 4.22A). Significant 15\% (0.19µm/day) and 41\% (0.50µm/day) increases in MAR\(_{Tb}\) are observed in C3H\(_{4N}\) and C3H\(_{8N}\) loading groups respectively (P < 0.05; Figure 4.22b). This however, only translates to a significant 110\% (0.78 µm\(^3\)/µm\(^2\)/day x10\(^{-2}\)) increase in BFR\(_{Tb}\) for C3H\(_{8N}\) (P < 0.01; Figure 4.22C). Significant changes in osteoclast number were also observed. For loads of 2N, 4N and 8N osteoclast number decreased significantly by 59\% (2.59 mm\(^{-1}\)), 73\% (3.21 mm\(^{-1}\)) and 77\% (3.40 mm\(^{-1}\)), respectively (P < 0.01).

Inter-strain comparison of the trabecular global components shows that global percentage increases in BV/TV\(_{Tb}\), BV\(_{Tb}\), are significantly higher for the B6 strain when loaded at 4N and 8N. The percentage increase in global BV/TV\(_{Tb}\) is shown to be 575\% and 296\% higher for the B6 strain for loads of 4N and 8N respectively (P < 0.05; Figure 4.20A), likewise global percentage increases in BV\(_{Tb}\) are 1998\% (0.05 mm\(^3\)) and 322\% (0.14 mm\(^3\)) for loads of 4N and 8N respectively. Global percentage increases in Tb.Th are significantly higher for the B6 strain (394\%, 10.1 µm, P < 0.05) for a load of 8N only. No significant differences are reported for global percentage increases in Tb.N and TV\(_{Tb}\). Furthermore no significant differences occur in any parameter for an applied load of 2N. Md.Pm/Pm\(_{Tb}\) is shown to be consistently higher for the C3H strain, significantly so for loads of 2N and 8N. Conversely MAR\(_{Tb}\) is shown to be consistently higher for the B6 strain, however this is only shown to be statistically significant for a load of 2N (Figure 4.22B). No significant differences exist between strains when comparing BFR/Pm\(_{Tb}\) (Figure 4.22C).
Load induced changes in trabecular and cortical bone

Cortical bone

Figure 4.23: (A) Percentage increase in global $\text{BV}_{\text{Ct}}$ for B6 and C3H mice loaded at 0N, 2N, 4N and 8N, standard errors shown. (B) Mean regional variation of $\text{BV}_{\text{Ct}}$ (absolute values) as measured by B6$_{0N}$ and B6$_{8N}$ loading groups. $^b$ Significantly different to C3H strain ($P < 0.05$). $^c$ Almost significantly different to 0N loading group of same biological strain ($P < 0.06$). Standard errors are shown.

Table 4.1 shows that unloaded C3H C5 vertebrae possess significantly more cortical bone mass resulting in a thicker cortical shell and larger cross sectional area. When comparing global cortical parameters for all B6 loading groups anova detected a significant difference for $\text{BV}_{\text{Ct}}$ only ($P < 0.05$). Comparison of the B6$_{8N}$ group with the B6$_{0N}$ group showed global $\text{BV}_{\text{Ct}}$ to be 11% (0.22 mm$^3$) higher ($P = 0.058$; Figure 4.23A). Global Ct.Th was shown to increase by 5.44% (7 μm, $P > 0.2$), TV$_{\text{Ct}}$ by 5.9% (0.32 mm$^3$, $P > 0.2$) and MV by 3.01% (0.11 mm$^3$, $P > 0.2$). Localized anabolic effects were detected however. Regional analysis yielded significant group-region interaction terms for both $\text{BV}/\text{TV}_{\text{Ct}}$ and $\text{BV}_{\text{Ct}}$ when comparing B6$_{8N}$ and B6$_{0N}$ ($P_{\text{int}} < 0.001$). $\text{BV}/\text{TV}_{\text{Ct}}$ and $\text{BV}_{\text{Ct}}$ are shown to increase by up to 11.2% and 17.6% (0.04 mm$^3$; Figure 4.23B) when loaded at 8N. This corresponded to non-significant regional increases of up to 9% (11 μm) for Ct.Th, 8.3% (0.05 mm$^3$) for TV$_{\text{Ct}}$ and 7.9% (0.022 mm$^3$) for MV, ($P_{\text{int}} > 0.5$). No significant differences were detected when comparing both global and local parameters for the other loading groups. Histology shows no significant differences to exist between loading groups for Md.Pm/Pm at periostial cortical sites (Figure 4.22A). The mineralized perimeter is however much greater at endostial cortical sites, where a significant 57% (28.4 mm/mm x10$^{-2}$) increase is reported for the B6$_{8N}$ loading group ($P < 0.01$). Owing to
the absence of double calcein labels at periostial-cortical sites in the B6\textsubscript{0N} group MAR\textsubscript{Pe} and BFR\textsubscript{Pe} are shown to be greater for all other loading groups, however none of the reported increases are significantly different to B6\textsubscript{0N} (Figures 4.22B & C). No Significant differences were detected for Mineral Apposition Rate at endostial cortical sites. Bone formation Rate is however shown to be significantly higher for a load of 8N at endostial cortical sites by 123\% \left(0.86 \ \mu m^3 / \mu m^2 / day \times 10^{-2}\right); Figure 4.22C).

When comparing loading groups for the C3H strain anova found no significant differences for any global or local parameter, as measured by micro-CT (Figure 4.23A). Analysis of the histomorphometric indices shows the mineralized perimeter to increase at periostial-cortical sites with increasing load (Figure 4.22A) however the only significant increase is that reported by C3H\textsubscript{8N} (233\%, 37.56 mm/mm \times 10^{-2}, P< 0.05). No significant increases with load are reported in mineralized perimeter at endostial-cortical sites. Comparison of C3H\textsubscript{0N} and C3H\textsubscript{8N}, shows mineral apposition rate at periostial-cortical sites to increase almost significantly by 65\% \left(0.81 \ \mu m/day, P = 0.06\right), which translates into a significant 371\% \left(0.84 \ \mu m^3 / \mu m^2 / day \times 10^{-2}\right) increase in BFR\textsubscript{Pe} (P < 0.05). Both MAR and BFR at endostial-cortical sites show significant 34\% \left(0.44 \ \mu m^2/day\right) and 76.8\% \left(0.57 \ \mu m^3 / \mu m^2 / day \times 10^{-2}\right) increases for an applied load of 8N respectively, significant values are also reported for a load of 2N.

Inter-strain comparison shows that no significant global differences exist for BV/TV\textsubscript{Ct}, however the percentage increase in global BV reported in the B6 strain is significantly different to that of the C3H strain (Figure 4.23A). Whilst micro-CT shows actual 3D adaptation to be greater for the B6 mice, histomorphometry shows activity to be significantly higher in the C3H strain for periostial cortical surfaces. Md.Pm/Pm\textsubscript{Pe}, MAR\textsubscript{Pe} and BFR/Pm\textsubscript{Pe} are all greater for the C3H strain, significantly so in all cases apart from MAR when loaded at 4N (Figures 4.22A, B & C). No significant inter-strain differences exist for any of the histological parameters at Endosteal-cortical sites.

4.3.4. Discussion

In this study we hypothesized that load induced cortical and trabecular bone adaptation is both dose and genotype dependent. The significant global increase in trabecular BV/TV for a load of 8N combined with the significant local increases in trabecular BV/TV for a
Load induced changes in trabecular and cortical bone

load of 4N confirms a dose dependency for trabecular bone in B6 mice. Analysis of the other structural parameters for the 8N group infers that the global increase in bone volume density can be attributed to both the thickening of existing trabeculae and the formation of new trabeculae, demonstrating that a fully anabolic model has been established. At a local level, for a load amplitude of 4N, no significant changes in any structural parameter accompanied the regional increases detected in bone mass, i.e. trabecular thickness. This could be explained by the magnitude of change being smaller than the 6 micron resolution of the imaging system. Furthermore the absence of any significant three dimensional changes when loading at 2N suggests that the osteogenic threshold for B6 trabecular bone lies somewhere between 2N and 4N. Trabecular dose dependency was not inferred by histomorphometry. Whilst the elevated values of Md.Pm/Pm, MAR and the significant increase BFR concur with micro-CT data for 8N, the relative increases reported by the 2N and 4N loading groups do not reflect the dose dependency as described by micro-CT. All dynamic indices for 2N and 4N are similar, yet only regional significant changes were detected in 3-dimensions for the 4N loading group. Rather than the osteogenic threshold being set somewhere between 2N and 4N, these data may allude to a lag in the remodelling process for a load of 2N, thus if loading were to continue similar 3-dimensional geometric changes would start to occur in the 2N loading group. This observation hints at a mechanism which is dependent on the cumulative amount of mechanical energy transferred to the bone matrix i.e. fatigue induced micro-cracks (48,49). However, caution must be exercised when attempting to interpret histological data in this manner. Histomorphometry represents the bone forming activity during the last three days of loading from a limited volume (3 x 5 µm thick slices), reported dynamic indices my therefore not represent the temporal pattern of bone formation responsible for the increases in bone mass as detected by micro-CT, this maybe further confounded by the two dimensional nature of histology. Despite being less pronounced, cortical bone formation is also shown to be dose dependent by micro-CT in the B6 strain. Although not significant, global BV was shown to linearly/exponentially increase with load. A load induced anabolic effect was confirmed at a local level for a load of 8N, where significant group-region interaction terms were reported for both BV/TV_Ct and BV_Ct. Non-significant regional increases in Ct.Th followed a similar pattern.
to those described by BV\textsubscript{Ct}. The increases in both B6 BV\textsubscript{Ct} and Ct.Th, coupled with the non-significant increases in both MV and TV\textsubscript{Ct} makes it difficult to draw any conclusions regarding the modality of remodelling at cortical sites. Rather than just simply depositing new bone on either or both of the cortical surfaces (endosteal and periosteal) it appears that a subtle thickening of the cortical shell is accompanied by both internal and external volumetric expansion. From an engineering perspective this would clearly accommodate the optimal support of increased axial loads. Such a modality would require some kind of radial shift where greater periosteal bone formation rates are accompanied by resorption at endostial sites. This mechanism is not supported by histomorphometry which, for a load of 8N, shows a greater significant increase in bone formation at endosteal cortical sites compared to a smaller and non significant increase at periosteal sites. However, this could also be explained by the temporal and spatial limitations of histomorphometry which were previously addressed. The precise characterization of load induced morphology would therefore require more detailed studies which are longitudinal in nature and which employ three dimensional, in vivo imaging techniques. The cortical dose response reported by micro-CT is to some degree reflected by the trends observed in Md.Pm/Pm and BFR at periosteal sites. This is not the case however at endosteal sites, which again could be explained by the limitations of histomorphometry.

Despite the incongruence between histomorphometry and micro-CT, micro-CT has demonstrated a clear dose response in both trabecular and cortical compartments of bone. The trabecular dose response achieved here is more pronounced than the response reported in two studies employing the tibia loading model (43,44). Souza et al showed that by loading the tibias of B6 mice (female, 8 weeks old) on alternate days for two weeks using a dynamic loading regime (5-13N, 2Hz with 10s rest periods between cycles, 40 cycles/day) induced a 37% increase in trabecular BV/TV when pooling all the loading groups together. This increase was not accompanied by increases in trabecular number or trabecular thickness. It is of particular interest to note that when Souza applied the same loading regime to 12 week old and 20 week old B6 mice trabecular bone mass was shown to significantly decrease. In view of the significant trabecular anabolic activity observed here in 15 week old mice and despite the obvious differences between the loading regimens these data suggest that trabecular mechano-sensitivity maybe
Load induced changes in trabecular and cortical bone
determined by anatomical location. Furthermore, when comparing our study with the study performed by Fritton et al (44) we see that load magnitude maybe the most important component of an anabolic mechanical signal for stimulating trabecular bone: Fritton et al reported similar percentage increase in tibial trabecular BV/TV for two groups of female B6 mice (10 weeks of age) loaded for 2 weeks and 6 weeks using the same dynamic mechanical signal (Peak force: 3N, Frequency: stride frequency, number of cycles per bout: 1200 cycles 5 days/week).

No dose response was observed in trabecular bone for the C3H strain however, a near significant 14.22% increase in global trabecular BV/TV was reported when loading at 8N. This was shown to be significant at a local level, where increases of up to 31% and 32% in BV/TV_{Tb} and BV_{Tb} were observed. Such changes were insufficient to induce significant increases in Tb.Th and Tb.N. Trabecular histomorphometry supports the anabolic effect reported by micro-CT measurements for a load of 8N. No significant increases in global or regional cortical bone mass were reported by micro-CT for any load. However, histology showed that for a load of 8N there was a significant increase in the number of osteoblasts recruited to periostial-cortical surfaces, furthermore there was a near significant increase in periosteal mineral apposition rate and a significant increase in periosteal bone formation rate. This was accompanied by significant increases in both MAR and BFR at endosteal-cortical sites. These data suggest that if loading in the C3H strain was continued beyond 4 weeks, enough bone mass maybe deposited to yield significant three dimensional changes in cortical bone. The comparative lack of bone formation in the C3H strain as shown by micro-CT suggests that mechano-sensitivity is genotype dependent, furthermore comparison of B6 and C3H histomorphometry appears to suggest that the response of C3H mice lag that of B6 mice: osteoblast recruitment, osteoblast activity and bone formation rates at periostal cortical sites are shown to be higher for the C3H strain whilst dynamic indices are similar at trabecular and endostal-cortical sites. This apparent temporal lag again alludes to the possible role of fatigue based mechanisms for load induced bone remodelling which is a viable assumption given that C3H cortical and trabecular bone is shown to be both thicker and stiffer at vertebral sites (29,50). This latter point highlights a limitation of the study and brings into questions the dependency of mechano-sensitivity on genotype. It is possible that for
equivalent axial loads the micro strains induced in C3H strains are lower than those induced in B6 vertebrae, hence the lack of response observed in the C3H strain could be explained by a reduced mechanical stimulus rather than genetic differences. Conversely, the same load must be transferred through fewer trabeculae. This may affect the way cortical and trabecular bone share the load, which could act to amplify the mechanical strains present on both cortical and trabecular components. Despite the questions raised by the differences in geometry and material properties the case for genetic dependency is supported by several loading studies, albeit at different anatomical locations, which have shown a smaller mechano-sensitive effect in C3H mice despite higher levels of microstrain. Kesevan et al (4) demonstrated that B6 mice were more receptive to mechanical loading compared to C3H mice when loading their tibias using a 4 point bending modality even though higher mechanical strains were induced in C3H mice. B6 cortical thickness was shown to increase by 28% compared to <10 % in C3H mice, when loaded for 6 days/week over a period of 2 weeks (Peak force: 9N, frequency: 2Hz). A similar effect was shown by Robling et al (28) when loading B6 and C3H ulna. Cortical bone in C3H mice as shown to have a higher osteogenic threshold (2392 $\mu$ε) compared to that for B6 (1769 $\mu$ε), furthermore once the osteogeneic threshold was exceeded cortical bone formation per unit increase in mechanical strain was significantly less for C3H mice.

In performing this study we have demonstrated that we can surgically insert stainless steel pins into the C4 and C6 vertebrae of both B6 and C3H mice, we have also shown that we can accurately apply dynamic mechanical loads to their C5 vertebrae for a sustained period of time, whilst inducing a significant dose response in the cortical and trabecular bone of B6 mice. We have also provided strong evidence that the mechano-sensitivities for both cortical and trabecular bone are genetically determined. The availability of this approach provides an alternative to the tibia loading model thus affording opportunities to explore site specific mechano-sensitivity in both cortical and trabecular bone. The vertebra loading model established here is proposed for further studies addressing the molecular processes involved in the skeletal responses to mechanical stimuli.
References


Chapter 5
Prediction of load induced cortical and trabecular bone adaptation
5. Prediction of load induced cortical and trabecular bone adaptation

Here we investigate the spatial relationship between load induced bone adaptation and mechanical strain for both cortical and trabecular compartments. In a previous study we demonstrated appreciable 25.9% and 11% increases in both trabecular and cortical bone volume density, respectively when subjecting the fifth caudal vertebrae (C5) of C57/BL6 (B6) mice (12 weeks of age) to an acute loading regime (Amplitude of 8N, 3000 cycles, 10 Hz, 3 times a week for 4 weeks). We have also established a validated Finite Element (FE) model of the C5 vertebra (12 weeks of age) using micro-computer tomography (µCT), which characterizes, in 3D, the micro-mechanical strains present in both cortical and trabecular compartments due to the applied dynamic loads. To investigate the relationship between load-induced bone adaptation and mechanical strains, in vivo and in silico data sets were compared. Using data from the previous cross-sectional study we divided cortical and trabecular compartments into 15 sub-regions and determined, for each region, a bone formation parameter ∆BV/BS (a cross-sectional measure of the bone volume added to cortical and trabecular surfaces following the described loading regime). Linear regression was then used to correlate mean regional values of ∆BV/BS with mean values of mechanical strains derived from the FE models which were similarly regionalized. The mechanical parameters investigated were Strain Energy Density (SED) the orthogonal strains (εx, εy, εz) and shear strains (εxy, εyz, εzx). Furthermore, to investigate how the load induced bone was deposited mean regional values of ∆BV/BS were correlated with mean regional percentage increases in structural indices specific to both cortical and trabecular compartments. For cortical regions regression analysis showed mean regional values of ∆BV/BS to correlate extremely well with Strain Energy Density (SED), the percentage increase in Tissue Volume (TVCt), and Marrow Volume (MVCt), R² = 0.82, 0.81 and 0.64 respectively (P < 0.01). For trabecular regions ∆BV/BS significantly correlated with percentage increase in trabecular thickness (Tb.Th) and Trabecular Number (Tb.N), R² = 0.79 and 0.26, respectively (P < 0.05). However no significant correlation was observed with any mechanical parameter. These results hint at
the presence of two distinct physiological mechanisms underlying load regulated bone adaptation in both cortical and trabecular compartments.

5.1.1. Introduction

Osteoporosis is a disease characterized by an excessive decrease in bone mass, which can lead to skeletal fragility and an increased susceptibility to fractures, dramatically reducing a person’s quality of life and in some cases leading to morbidity. Osteoporosis has been partly attributed to a reduction in muscle mass and muscle function resulting in a decrease in mechanical usage of the skeleton (1). Conversely it has also been demonstrated that mechanical overloading results in enhanced bone formation and a net gain in cortical and cancellous bone mass (2-4). Numerous studies have demonstrated load induced bone formation to be a function of the energy transferred to bone, showing load amplitude, frequency, duration and recovery time to be critical factors in determining the level of the anabolic response (5-8). However the exact spatial relationship between the micromechanical environment and consequent bone adaptation is poorly understood. A quantitative understanding of how the mechanical environment regulates bone adaptation could lead to an improved understanding of bone formation and bone quality maintenance as well as an ability to predict micro-architectural changes in bone geometry. This may ultimately reveal novel approaches for the treatment or prevention of age related fractures and the successful management of diseases such as osteoporosis.

Several computational models (9-12) have attempted to provide theoretical frameworks which explain load induced adaptations. These models employ absolute values of mechanical strains or strain gradients as their primary inputs, operating on the assumptions that bone formation is driven either by one of two underlying physiological mechanisms: 1) Micro damage interrupting communication between osteocytes, which is hypothesized to be a function of absolute strain magnitudes (13-15) or 2) Shear stresses imposed by fluid flow through canaliculi, hypothesized to be a function of the mechanical strain gradient (16,17). Whilst these models are able to mimic bone adaptation when qualitatively compared to in vivo bone adaptation many lack quantitative, in vivo validation. A few studies have attempted to address this issue. One Study using the rat
fore-limb compression model has successfully correlated axial increases in bone mass with axial mechanical strains using Finite element analysis (FE). However periosteal bone formation failed to correlate with mechanical strains in the plane transverse to the direction of loading (15). In another study significant correlations between mechanical strains and 2D bone formation indices were found to exist in loaded trabecular bone, using the rat-tail model. Guo et al (18) used FE to quantify a rat tail model for the study of trabecular bone adaptation. By scanning vertebrae using micro-CT at 34 µm resolution and converting the resulting images to 3D FE models they were able to establish significant correlations between trabecular bone mechanical strains and bone formation, as measured by histology (19), $R^2 < 0.40$. Despite these attempts the three dimensional relationship between the micro-mechanical environment and bone adaptation in both cortical and trabecular compartments remains largely undefined. Recently we have developed a model similar to the rat-tail model using the mouse. By employing a specially developed loading device to dynamically compress the 5th caudal vertebrae (C5) of C57BL/6 (B6) mice (20) we were able to show, using µCT, appreciable three dimensional increases in both cortical and trabecular compartments of bone. Furthermore we have also established a fully validated finite element (FE) model of the loaded C5 vertebrae in B6 mice; it is capable of characterizing the micro-mechanical environment resulting from applied dynamic loading regimes (21). Here, by combining these two studies, we propose to further investigate the relationship between the micro-mechanical environment and subsequent bone adaptation, this time in both cortical and trabecular compartments.

5.1.2. Materials and methods

Experimental Design

In the previous study (20) the C5 vertebrae of 4 groups (n =10) of C57BL/6 (B6) female mice, 12 weeks of age, were subjected to an intensive loading regime consisting of 3000 cycles, delivered at 10Hz, 3 times a week for 4 weeks at amplitudes of 0N, 2N 4N and 8N. To investigate the relationship between the micromechanical environment induced by the applied loads and subsequent bone formation, in-vivo data was extracted from the
aforementioned study and compared to in-silico data sets derived from FE models of C5 vertebrae: To quantify load induced bone formation data specific to the 0N (B6\(0N\)) and 8N (B6\(8N\)) loading groups were used only. Owing to a lack of significant anabolic activity data specific to the 2N and 4N loading groups were neglected. Mechanical strain distributions were determined from finite element models of C5 vertebrae belonging to pinned mice sacrificed at the beginning of the dynamic loading regime (B6\(T0\)), 4 weeks prior to the end of loading.

**Quantification of bone adaptation**

![Digital image of a whole B6 vertebra (C5) showing cortical and trabecular compartments which are subdivided into 15 overlapping regions (1–15).](image)

Bone morphometry was assessed using micro-computed tomography with 5 times frame averaging (6\(\mu m\) voxel size, 50 kVp, 160\(\mu A\), Scanco Medical AG Switzerland). Using a direct 3D approach (22) morphometric parameters specific to trabecular and cortical bone were determined for regional volumes of interest, these included: Trabecular Bone Surface (BS\(Tb\)), Trabecular Bone Volume (BV\(Tb\)), Trabecular Thickness (Tb.Th), Trabecular Number (Tb.N), Cortical Bone Surface (BS\(Ct\)), Cortical Bone Volume (BV\(Ct\)), Cortical Tissue Volume (TV\(Ct\)), Cortical Marrow Volume (MV), Cortical Thickness (Ct.Th) and cortical cross-sectional area (T.Ar\(Ct\)). Regional trabecular volumes were defined by adapting an algorithm developed by Kohler et al (23) to isolate an internal volume comprised of spongiosa, both primary and secondary. The regions were then created by subdividing this volume into overlapping regions each having a height equal to 10% of the total height of the volume originally isolated. This resulted in 19
overlapping regions: 10 distinct regions and 9 additional overlapping regions, spanning the midpoints of the 10 distinct regions (figure 5.1). Primary spongiosa was then excluded from analysis by discarding the first two and last two sets of overlapping regions, leaving 15 regions of interest containing trabecular bone only. The cortical regions of analysis were comprised of the cortical shell enclosing the trabecular regions of interest (figure 5.1). It should be noted here that the heights of the vertebrae belonging to B6T0 were not significantly different to the more mature groups B60N and B68N. To quantify bone formation in 3-dimensions from the cross sectional data available a new parameter (ΔBV/BS) was calculated. It was determined by calculating, for each region, the difference in mean bone volume between the groups loaded at 8N and 0N. The regional ΔBV’s were then normalized by the mean regional values for bone surface area (BS) specific to the B6T0 group. Hence this parameter can be considered as measuring the amount of new bone which is deposited, due to a load of 8N, onto the bone surface available at the start of loading.

**Finite element model**

To determine the initial micro-mechanical environment induced by the applied loads, μCT images of the vertebra belonging to the B6T0 group were converted into 3D FE models by mapping each bone voxel to an eight-node brick element. To model the intervertebral discs additional voxels were added to the proximal and distal ends of the vertebra such that the disc’s geometry was approximated (24) yielding models consisting of approximately 1,800,000 elements. Following previous validation of this FE model (21) an initial value of 14.8 GPa was assigned as the Young’s Modulus to those voxels.
representing bone (25), and 2.48 MPa was given to the voxels describing the intervertebral disc (24). Both materials had a Poisson’s ratio of 0.3. All models were aligned in the z-axis, defined by the centre coordinates for mid-cortical sections according to the first moment of area. The z-axis corresponded to the proximal-distal direction of the tail, the x-axis corresponded to the medial-lateral direction, whilst the y-axis was defined by the ventral-dorsal direction. To mimic the loading conditions applied in vivo, the surface of the distal intervertebral disc was fixed in the axial direction while allowing free lateral movement. The compressive axial force was applied to the surface of the proximal intervertebral disc (Figure 5.2). The models were solved using an element-by-element method (26,27) running on a super computer (CSCS, Manno, Switzerland) consisting of 8 IBM Regatta p690 SMPs for a total of 256 Power4 CPUs. Bone tissue strains were extracted from the same trabecular and cortical regions described before (Figure 5.1). The mechanical parameters analyzed were: Strain Energy Density (SED), orthogonal strains ($e_x$, $e_y$ and $e_z$) and Shear Strains ($e_{xy}$, $e_{yz}$, $e_{zx}$). To visualize the spatial distribution of mechanical strains and the resulting deformations Paraview (Kitware Inc. New York) was used.

**Statistical analysis**

To investigate where load induced bone was deposited linear regression was used to correlate mean regional values of $\Delta$BV/BS with mean regional percentage increases in morphometric parameters for both trabecular and cortical bone (calculated with respect to the 0N loading group). Similarly to investigate the relationship between the micromechanical environment and load induced bone adaptation, linear regression was used to test for correlations between the mean regional bone formation parameter $\Delta$BV/BS and mean regional values for the different mechanical parameters analyzed. For all statistical analyses, the GNU statistical package R (Version 2.5.1, http://www.r-project.org) was used.
5.1.3. Results

Bone formation

Trabecular bone formation activity appeared to be highest in the proximal and distal regions where an average of 0.011mm ± 0.0018 and 0.015mm ± 0.0024 of bone was deposited (Figure 5.3). At regions 9 and 10 a distinct lack of anabolic activity is apparent. Linear regression analysis shows that trabecular ΔBV/BS correlates significantly with the percentage increase observed in trabecular thickness, $R^2 = 0.79$ ($P < 0.001$, figure 5.4a). A significant correlation is also shown to exist between trabecular ΔBV/BS and the percentage increase in Tb.N, $R^2 = 0.46$ ($P < 0.05$, figure 5.4b), however visual inspection of the distribution of points reveals that strength is added to the correlation primarily by the high increases observed in regions 1 and 15. Analysis of cortical ΔBV/BS showed regional bone formation profile to be almost parabolic in form (figure 5.3), having a peak value of 0.016mm ± 0.0052 at region 7. Linear regression showed significant correlations to exist between ΔBV/BS and the percentage increase in both $TV_{Ct}$ and $MV_{Ct}$ ($R^2 = 0.81$ and 0.64 respectively, figure 5.4c & d), inferring that the addition of new bone contributes to a structural expansion of the cortical shell in the plane transverse to the direction of loading. It would appear that this volumetric expansion is accompanied by subtle increases in cortical thickness and cross-sectional area. However percentage increases in these two parameters do not correlate particularly well with ΔBV/BS (figure 5.4e & f). In the central cortical regions, where external and internal volumetric expansion appears to be the greatest (regions 6-9), the corresponding changes in cortical thickness and cross sectional area appear to be the smallest. Conversely at distal and
proximal regions where volumetric increases are the smallest, increases in cortical thickness and cross-sectional area appear to be the greatest.

Figure 5.4: Correlation of mean, regional ∆BV/BS with mean, regional percentage increases in Tb.Th (a), Tb.N (b), TV_Ct (c), MV_Ct (d) and Ct.Th (e) and T.Arc_Ct (f) as measured by the 8N and 0N loading groups. The numbers shown next to the plotted points identify the region.
Finite element model

Examination of the FE models showed that the distributions of mechanical strains span a greater range for trabecular bone than for cortical bone (Figure 5.5, Table 5.1). Analysis of the mean strains determined by the FE model show that the trabecular network is predominantly in a state of compression where regional mean, axial compressive strains reach $841.64 \pm 111.62$ and $709.04 \pm 103.88$ in both proximal and distal regions (regions 3 and 14, figure 5.6a). Note the standard deviations presented here describe the variation of the mean mechanical parameter across the analyzed group of C5 vertebrae ($n = 10$) and not the variation of mechanical strains across all of the finite elements constituting the defined regions. Minimum mean trabecular compressive strains occur local to the centre of the vertebrae where there are fewer trabeculae ($366.65 \pm 199.46$, region 7). Inspection of the orthogonal strains $e_x$ and $e_y$ shows that the trabecular network is in a state of tension in the plane transverse to the direction of loading. This is confirmed by visualization of the deformed micro-structure (figure 5.7). Maximum values of $e_x$ and $e_y$ occur in proximal and distal regions (regions 2 and 14). The small deviations of shear strain $e_{xy}$ from zero indicates that there is little shearing of the trabecular network in the plane transverse to the direction of loading. Shearing is shown to be present however in central regions (5-10) in planes parallel to the axial direction of loading, as demonstrated by the regional variation of $e_{yz}$ and $e_{zx}$. Figure 5.6b shows the same strain components for cortical bone regions. Compressive axial strain magnitudes corresponding to the direction of loading are superior to the magnitudes of both the tensile and shear strain magnitudes. Maximum mean compressive strains are shown to occur local to the centre of the vertebrae ($884.63 \pm 180.57$, region 7). All cortical regions are subject to similar levels of tension ($\approx 200\mu$ε) in the x-y plane, whilst mean shear strain values in all three orthogonal planes are close to $0\mu$ε. Comparison of the cortical and trabecular axial strains shows that higher axial strains occur in trabecular bone for proximal and distal regions whilst cortical axial strains in central regions greatly supersede corresponding trabecular strains. The same is true of the regional variation in mean strain energy density (SED), figure 5.8.
Table 5.1. The average standard deviation across all regions for each mechanical parameter.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>µ(σ) Trab</th>
<th>µ(σ) Cort</th>
</tr>
</thead>
<tbody>
<tr>
<td>εx (µε)</td>
<td>159 ± 29</td>
<td>102 ± 29</td>
</tr>
<tr>
<td>εy (µε)</td>
<td>158 ± 24</td>
<td>109 ± 38</td>
</tr>
<tr>
<td>εz (µε)</td>
<td>359 ± 35</td>
<td>234 ± 72</td>
</tr>
<tr>
<td>εxy (µε)</td>
<td>223 ± 61</td>
<td>153 ± 40</td>
</tr>
<tr>
<td>εyz (µε)</td>
<td>440 ± 87</td>
<td>228 ± 33</td>
</tr>
<tr>
<td>εzx (µε)</td>
<td>422 ± 81</td>
<td>236 ± 32</td>
</tr>
<tr>
<td>SED (Pa)</td>
<td>5084 ± 1155</td>
<td>2676 ± 728</td>
</tr>
</tbody>
</table>

Secondary standard deviation shown describe the variation of µ(σ) across the group of C5 vertebrae analyzed (n = 10)

Figure 5.5: Histogram showing the typical distribution of trabecular and cortical axial strains from one region (data shown is from region 13 of one C5 vertebrae).

**Relationship between bone formation and the micro-mechanical environment**

Linear regression applied to the data sets describing mean regional bone formation in cortical bone and the corresponding mean mechanical parameters as determined by the FE model, show highly significant correlations to exist between ΔBV/BS, εx, εy, εz and SED (R² = 0.78, 0.56, 0.89 and 0.82 respectively, P < 0.001, figure 5.9). A weak, but significant, correlation was found to exist for εyz (R² = 0.31, P < 0.05), no significant correlations were found to exist for either εxy or εzx. Trabecular mechanical parameters εx, εy, εz and SED correlated poorly with ΔBV/BS, (R² < 0.1, P > 0.1). The only significant correlation was found between ΔBV/BS and εyz (R² = 0.31, P < 0.05). A qualitative comparison of figures 5.3 & 5.8 quickly confirms the quality of cortical and trabecular correlations with SED. It is clear to see that the regional profile of cortical
ΔBV/BS follows a similar pattern to that described for cortical SED whilst the two corresponding profiles for trabecular bone exhibit little similarity.

Figure 5.6: Regional variation of mean mechanical strains ($e_x$, $e_y$, $e_z$, $e_{xy}$, $e_{yz}$, $e_{zx}$) for both Trabecular (a) and cortical (b) bone. Standard deviations are shown and describe the variation of the mean mechanical parameter across the group B6T0.
Figure 5.7: (a) Visualization of a finite element simulation from one loaded vertebra. Axial strains in the y-z plane from one slice are shown. (b) Visualization of the positive axial strains which can occur in horizontally positioned trabeculae connected to the inner cortical shell at central cortical regions.

Figure 5.8: Regional Variation of the mean SED for both trabecular and cortical compartments. Standard deviations are shown and describe the variation of the mean value of SED across the group B6Tb.
Chapter 5

5.1.4. Discussion

The regional numerical data obtained from the FE simulations shows that compressive strains in the axial direction dominate in both trabecular and cortical structures, inducing a state of tension in the transverse plane. Moreover, it appears that axial load is unevenly shared by both cortical and trabecular components. When referring to figures 5.6a, 5.6b and 5.8, the mean regional axial strains and strain energy densities are greater in the proximal and distal regions for trabecular bone compared to cortical bone, whilst at the centre the same mechanical parameters are greater for cortical bone. Visual analysis of the FE results confirms that in proximal and distal regions axial loads are transferred primarily through trabeculae. More specifically it can be seen that load is transferred directly to vertically orientated trabeculae which lie beneath the intervertebral discs (figure 5.7a). Visual analysis of the mechanical strains in trabeculae at central regions (figure 5.7b) on the other hand shows that the bias towards cortical loading at central
regions is somewhat misrepresentative. It can be seen that both positive and negative strains are present in horizontally and vertically position trabeculae respectively. The inclusion of these positive strains in averaged numerical results therefore reduces the perceived contribution of trabeculae, however it appears that axial load is shared evenly (at central regions) between all structural members which are aligned with the direction of loading. Nevertheless there are cases where trabeculae passing through the midsection are disconnected and thus structurally redundant.

When analyzing the relationship between the regional micro-mechanical strains induced by a load of 8N and subsequent $\Delta$BV/BS linear regression shows that cortical bone formation is linearly related to the mean regional value of SED (figure 5.9, $R^2 = 0.82$). This provides evidence that beyond a certain threshold, somewhere between 4N and 8N, bone formation becomes directly proportional to magnitude of the mechanical strains sensed by the cortical bone matrix. In other words, FE is able to predict the quantity of the bone deposited along the axis of the cortical shell due to a load amplitude of 8N. Analyses of the structural changes which accompany the axial deposition of new cortical bone reveal that newly formed bone is not just simply deposited onto all available surfaces. It appears that newly formed bone is somehow distributed to induce radial expansion of the cortical shell. This was alluded to in the previous study (20), and is confirmed here by the strong significant correlations between $\Delta$BV/BS and the observed percentage increases in both $TV_{Ct}$ and $MV_{Ct}$. Furthermore detailed inspection of the associated percentage increases in $Ct$.Th and $T.Ar_{Ct}$ (figure 5.4) show that volumetric changes and the accompanying changes in cross-sectional area and cortical thickness are not consistent over the length of the cortical shell. It appears that the large volumetric changes which occur at central regions (6-10) are accompanied by relatively smaller increases in cortical thickness and cross-sectional area, compared to distal and proximal regions where smaller volumetric changes are accompanied by larger increases in cortical thickness and cross-sectional area. Given the hour glass shape of the C5 vertebrae and the fact that both cortical thickness and cross sectional area increase towards the centre it seems that bone is being deposited such that the C5 vertebrae is morphing towards a more cylindrical form with homogeneous wall thickness. From an engineering perspective this clearly accommodates the optimal support of increased loads. From this
we can conclude that the deposition of bone on periosteal surfaces is being accompanied by resorption at endosteal sites, such that cortical bone ‘drifts’ in a radial direction. It would have therefore been interesting to have analyzed the distribution of mechanical strains in the plane perpendicular to the direction of loading, particularly at periosteal and endosteal cortical surfaces. However, given the design of this study we are unable to distinguish between the quantities of bone added to periosteal surfaces and removed from endosteal surfaces. It should be noted that significant endosteal bone formation rates as measured by histomorphometry in the previous study (20) contradict the ‘drift’ hypothesis. However, such measurements may not be representative of the overall 3D dynamic changes. Furthermore, histomorphometry represents a small period of time towards the end of the loading period. The results presented here partly agree with data presented by Kotha et al (15) who showed, using the rat forelimb compression model, that the pattern of cortical bone formation in the longitudinal direction was highly correlated to the predicted peak compressive strains at seven cross-sections ($R^2 = 0.89$, $p < 0.05$). This led them to hypothesize that fatigue induced damage was the mechanism underlying load induced bone formation. Interestingly, no evidence of a cortical drift was presented here. This is most likely attributed to the presence of substantial bending modalities, which in turn can be attributed to the different loading configuration used. Kotha et al did attempt to correlate periosteal bone formation with circumferential mechanical strains in the plane perpendicular to the direction of loading however mechanical strains were unable to explain the in-plane pattern of bone formation ($e.g.$: $R^2 = 0.21$, SED: $R^2 = 0.29$ $p < 0.01$). These results contrast those presented by Gross et al (28) who reported that the circumferential gradient in strain correlated moderately well ($R^2 = 0.36$) with the pattern of periosteal bone formation in the turkey ulna, while SED did not correlate with bone formation ($R^2 = 0.01$). In their model, the three combined gradients in axial strain explained 60% of the variation in bone formation, leading the authors to suggest the importance of fluid flow in the long term adaptive response to mechanical loading as apposed to fatigue induced micro-damage.

In the trabecular bone compartment the significant correlations between $\Delta BV/BS$ and both the percentage increases in trabecular thickness (Tb.Th) and trabecular number (Tb.N) suggest that new bone is both added to existing trabeculae and appears in the form
of new trabeculae. Inspection of the linear correlation between bone formation and the percentage increase in Tb.N (figure 5.4b) shows that strength is added to the correlation by the two outermost proximal and trabecular regions, however rather than being two random outliers previous analysis (20) suggests that the formation of new trabeculae is limited to just proximal and distal regions. Surprisingly linear regression analysis shows that FE is unable to predict trabecular bone formation as no significant correlations exist between any mean regional mechanical parameter and trabecular $\Delta BV/BS$ (figure 5.9, $R^2 = 0.08$). The trabecular dose response observed in the previous study clearly shows that trabecular bone formation is linked to the mechanical environment, however based on the data presented here the nature of the relationship between the mechanical environment and trabecular bone formation cannot be properly characterized. These data disagree with the study performed by Kim et al who showed significant correlations to exist between mechanical strains and trabecular bone formation during the first two weeks of loading (19), $R^2 < 0.40$. However bone formation was quantified in 2D using histological techniques and may not be representative of the spatial addition of load induced bone formation in 3D.

The polarized nature of the correlations specific to both cortical and trabecular bone, in this study, could represent an indication that their adaptations are governed by different physiological mechanisms. However, even with such strong and weak correlations, before drawing any conclusions we must be aware of the lack of specificity of our approach. The correlations presented here are based on average values which could be concealing more convincing relationships at a local level. Further inspection of the data was therefore performed to investigate the influence of potential statistical artifacts. First, we investigated whether the poor correlation in trabecular bone could be explained by the greater variation of mechanical strains in the trabecular network (Table 5.1, figures 5.5, 5.7b & c) masking any potential relationship i.e. the inclusion of tensile strains in horizontally positioned trabeculae. A rudimentary iterative thresholding strategy was therefore implemented whereby the mean mechanical parameter was recalculated following the exclusion of strains below several threshold values. This approach failed to yield improved correlations. We then assessed whether the variations of values calculated for $\Delta BV/BS$ were masking any potential correlations. The standard
deviations of both BV and BS within the individual groups are relatively small (< 10%). Nevertheless, owing to the cross sectional nature of the study and the normalization of data large variations in the parameter ΔBV/BS are introduced (> 50%, figure 5.3). However, comparison of the trabecular standard deviations with those determined for cortical regions, where strong correlations are observed, shows the variation of ΔBV/BS to be no greater. To further investigate statistical sensitivity the regions of analysis were reduced in number and increased in size. This approach produced no improvements. Strong correlations were maintained for cortical bone, whilst correlations for trabecular bone remained poor. Finally it was also hypothesized that another source of error could be the small nominal values of both BV and BS in central trabecular regions. Here the mean value of ΔBV/BS would be particularly sensitive to small perturbations in either BV or BS. However exclusion of the central regions, even in the cases were fewer regions were defined, failed to improve the value of R².

Another factor contributing to the poor correlation observed in trabecular bone could be the suitability of the assumptions upon which the FE models are based. The assumed homogeneity of cortical bone may be sufficiently descriptive owing to the alignment of the cortical shell with the axial direction of loading (despite being anisotropic). The orientation of the trabecular network however is more varied by comparison, and given its heterogeneous nature (29) may not be suitably represented by a single elastic constant. Accounting for this could therefore significantly modify the regional mechanical strain profile seen in figure 5.8 and therefore alter the correlation between trabecular bone formation and the micro-mechanical environment. Another aspect which has not been modelled and which could change the strain profile is a non-linear material response. Our model simulates the conditions at the start of fatigue loading. It has been shown in other studies (30) that the accumulation of micro-damage resulting from in vivo fatigue loading led to a 70% acute loss of whole-bone stiffness. Hence material properties of both cortical and trabecular bone are time and load dependent. Furthermore any relationship between the micromechanical environment and subsequent trabecular bone formation may not be evident owing to the period of time which the attempted correlations bridge, i.e. the micromechanical environment at time zero maybe responsible for morphological changes which manifest after only one week,
these changes will in turn alter the micromechanical environment (depending on mineralization rates etc) which will then result in an entirely different pattern of bone formation, hence the time points we are comparing maybe disconnected. Finally the value of ∆BV/BS most likely includes bone resorption at areas where there is disuse thus more local analyses are required which correlate mechanical strains with both regions of bone resorption and bone formation.

In performing this study we have supported the view that load induced cortical bone formation is driven by strain magnitude. Moreover, in identifying a cortical drift we have shown that bone adapts by different mechanisms in the absence of significant bending component. We have also, for the first time, presented evidence which suggests that the physiology underlying load regulated bone adaptation may be different in trabecular bone than it is in cortical bone. If confirmed this may have significant implications for strategies aimed at augmenting bone strength to treat or prevent degenerative disorders such as osteoporosis. Whilst many questions remained unanswered the study performed here demonstrates the potential of this model to uncover the physiological mechanisms responsible for both cortical and trabecular, load induced bone adaptation. The implementation of longitudinal studies, using this mouse model, combined with improved FE models would permit point to point correlation of the micromechanical environment and bone formation. It would also be necessary to extend the investigation to include other physiological relevant mechanical parameters amongst which are strain gradients, principle strains and dilatational strains (31-33). These improvements would allow us to gain a better understanding of load regulated bone adaptation, especially if such studies were compared to the results of a second loading modality, using the same animal model, with a bias towards structural bending.
Chapter 5

References


6. Synthesis

Osteoporosis is a disease characterized by an excessive decrease in bone mass leading to an increased susceptibility to skeletal fracture and deformation, symptoms which can have a dramatic, negative impact on the quality of a person’s life and which in more extreme cases can lead to death. A common misconception about this disease is that it is considered to only afflict females but the prevalence in men also increases exponentially with age. The rise in hip fracture rate occurs about 10 years earlier in women than in men. By the age of 90, about 17% of males have had a hip fracture, compared to 32% of females. Additional to the obvious costs on health, osteoporosis is a global problem and carries with it significant socio-economic costs. This is illustrated by the IOF audit report “Call to Action” published in 2001, which claims that osteoporosis costs national treasuries in the EU over 4.8 billion Euro annually in hospital healthcare alone. Clinically approved strategies aimed at treating the disease employ hormonal based medications which disrupt the bone remodeling process via provocation of bone forming cells or the inhibition of bone resorbing cells. All however have limited effects and in some cases there are negative consequences. Medical research is now attempting to target genes which define osteoporosis using the mouse as a model system for human diseases. Owing to the recent deciphering of the mouse genome and the high homology that exists between the human and mouse genomes, inbred strains of mice represent ideal models for genetic studies. Using the mouse to identify genes implicated in the bone remodeling process could therefore lead to advances in understanding which may enable the precise regulation of genes and proteins responsible for particular bone phenotypes i.e. bone mineral density or bone strength. One interesting phenotype under investigation is the response of bone to mechanical loading or its ‘Mechano-sensitivity’. Mechanical loading is the most important physiological/environmental factor regulating bone mass and shape. It has been demonstrated in humans that cyclic overloading enhances bone mass in both cortical and trabecular components. An understanding of the biological pathways (from gene expression to protein function) governing load stimulated bone formation could therefore provide opportunities to mimic or augment bone mechano-sensitivity using pharmacological agents thereby leading to the development of novel strategies in the management of osteoporosis.
The goal of this thesis was to provide the foundations for genetic studies aimed at elucidating the biochemical pathways involved in the mechano-sensitive response of two components of bone; cortical bone and trabecular bone. Furthermore we also sought to investigate the quantitative relationship between the micro-mechanical environment and subsequent bone morphology so as to better understand the drivers of load induced bone formation. To achieve these goals a stepwise engineering approach was employed to adapt a loading modality already established in a rat model for small inbred strains of mice whereby a single caudal vertebra could be dynamically compressed via two pins inserted into adjacent vertebrae.

Chapter 3 describes much of the design work and experimentation which was carried out (in vitro) to establish the correct methodology for both pin insertion and the application of dynamic load profiles similar to those which had been shown to produce a load induced anabolic effect in other animal models. Firstly a simple surgical tool was designed to facilitate the insertion of stainless steel pins into caudal vertebrae C4 and C6 adjacent to the target vertebra (C5) in an accurate and highly reproducible manner. To maximize the probability that loads capable of inducing an anabolic response could be transferred to both trabecular and cortical components of bone in the eventual in vivo trials, mechanical tests were performed to characterize the most robust configuration of the proposed loading modality. Structural damage for high, static compressive loads was shown to be minimized by a partially constrained loading configuration which prevented movements of the tail in the planes perpendicular to the direction of loading using stainless steel insect pins with a diameter of 0.5mm. Furthermore it was shown that coating the pins with hydroxyapatite would make it much more difficult for the pins to be displaced by the mice during normal cage activity when they were not being loaded, this was likely due to the promotion of osteointegration at the pin-bone interface. However as shall be discussed later the use of hydroxyapatite coated pins was discontinued owing to an associated undesirable inflammatory response. Secondly to facilitate the application of ‘anabolic’ dynamic loading regimes, Chapter 3 presents the design of a dual axis loading device, complete with quality control software. The device makes use of two linear motors and a servo-control board to execute dynamic, user-defined load profiles. Deployment of the device in vitro demonstrated that it was possible to submit the target vertebrae to dynamic sinusoidal load profiles with amplitudes of 8N and frequencies of 10Hz for 14 X 3000 cycles without compromising the structural integrity of the proposed loading configuration. Moreover the errors incurred in its dynamic
performance were less than 1%. Thus before any in vivo trails were attempted a system capable of delivering dynamic profiles conducive to bone formation was successfully established.

To complete the validation of the loading device and confirm that the energy provided by the system was successfully transferred to the target vertebra via the interconnecting viscoelastic vertebral discs the final section in chapter 3 presents the results of experiments designed to determine the dynamic characteristics of the resultant micro-strains sensed by the target vertebrae. This also provided data which was used to validate static FE models of the entire target vertebrae such that micro-structural stresses and strains could be determined and used to investigate the relationship between the micro-mechanical environment and any subsequent morphology. This combined experimental and computational approach was executed by pinning tail segments from B6 mice, attaching micro-strain gauges to the lateral and medial cortical sites of C5 vertebrae and loading them in vitro using the developed loading device. μCT scans of the target vertebrae where then taken and used to produce FE meshes by converting the 3D image voxels directly to 8-node brick elements. Tests showed that the frequency of the applied dynamic load remained undiminished and that a direct linear relationship existed between the magnitude of the applied load and the magnitude of the cortical strains sensed by the micro-strain gauges, thereby validating the linear elastic assumption of the FE model. Furthermore by using a young modulus of 14.8Gpa for bone a 1:1 relationship was established ($R^2 = 0.84$) between the cortical strains determined experimentally and those determined by the FE model. Thus in addition to validating the loading device a computational tool capable of characterizing the micro-mechanical environment within loaded caudal vertebrae was successfully established. There are however several limitations. Firstly experimental data showed that compression of the target vertebrae using the proposed loading configuration was less than ideal. Owing to the soft tissue around the tail additional bending components were introduced which were not modeled. Secondly it was assumed that both cortical and trabecular bone are homogeneous isotropic materials with the same material constants, whilst this maybe a feasible assumption further experimental validation of the structural deformations resulting from the applied loads are required, in particular those of the trabecular network.

Chapter 4 presents the results of in vivo trials designed to asses the effects of pinning on the development of the target vertebra and the effects of mechanical stimulation. Two inbred strains of mice with different genetic backgrounds suspected of having contrasting responses to
mechanical loading were used, a trait which could be ultimately exploited using comparative genomic techniques to assist in the identification of genes responsible for mechano-sensitivity. Here it was intended to load groups of mice at 3 different magnitudes of 2N, 4N and 8N, for 3'000 cycles, 3 times a week for 4 weeks at a frequency of 10Hz. However in the very first trial using the B6 strain differing degrees of inflammation occurred local to the insertion sites of hydroxyapatite coated pins. In the more severe cases some of the tails could not be fitted into the loading device. To ensure sufficient numbers for statistical significance this prompted reorganization of the group structures such that the 8N loading group was cancelled. The severely inflamed cases were then allocated to special control groups whereby the effects of severe inflammation on the normal development of the target vertebrae could be quantified. Analysis of these groups using µCT showed severe, established inflammation to stop cortical growth and initiate catabolic mechanisms in trabecular bone. In the other groups where inflammation was negligible or moderate and progressive a rudimentary classification of the degree of inflammation failed to correlate with changes in trabecular and cortical bone density suggesting a certain amount of time was required before catabolic mechanisms took effect. Following further investigation, in vitro scans showed hydroxyapatite particles to be widely dispersed in the soft tissue surrounding the tail, this combined with literature studies identified the hydroxyapatite coating as the likely reason for the auto-inflammatory response. Ignoring the severe cases of inflammation pinning was shown to marginally retard the growth of both cortical and trabecular bone. When comparing the pinned unloaded control group with the age-matched control group at the end of the study bone volume density (BV/TV) for the entire caudal vertebrae was shown to be reduced by 5%. Despite these negative influences mechanical loading at a magnitude of 4N was shown to stimulate marginal significant increases when compared to the unloaded-pinned control group. By dividing both cortical and trabecular regions into sub-volumes of interest we were able to detect significant local increases in bone volume (BV) of up to 5% (P < 0.05) for both trabecular and cortical components. Furthermore a significant 10% (P < 0.05) increase in BV for bone local to both proximal and distal growth plates (primary spongiosa) was detected thereby underlining the potential of the model. In a second identical trial using C3H mice the use of hydroxyapatite coated pins was discarded in favour of plain stainless steel pins which were morphed at the ends to prevent them from being removed from the tail. This had the effect of eliminating the severe inflammatory response which was observed in the first trial, suggesting the
reaction to hydroxyapatite coating was a major cause of the negative inflammatory response. Analysis of the pinned groups showed that pinning did not retard the normal development of both cortical and trabecular components, furthermore mechanical stimulation was shown to have no effect suggesting that C3H mice possessed a complimentary mechano-sensitive phenotype. Even though we failed to establish an appreciable dose response in the B6 mouse strain these first in vivo trials showed that surgical intervention to facilitate the use of the proposed loading modality induced acceptable changes in the normal development of C5 vertebrae, moreover confirmation of minimal but significant load induced changes demonstrated the potential of this model.

Chapter 4 section 4.3 presents the optimization of the model. Using stainless steel pins with no hydroxyapatite coating B6 and C3H mice were loaded using the same protocol for amplitudes of 0N, 2N, 4N and 8N. It was shown in this second round of trials that bone mass was significantly augmented for a load of 8N in both cortical and trabecular components of B6 target vertebrae when compared to the 0N group. At trabecular sites µCT measurements showed bone volume density (BV/TV) to increase by 25.9% (P < 0.05) this was accompanied by a significant global increase of 21.9% (P < 0.001) in trabecular thickness (Tb.Th). Furthermore when dividing the trabecular content into sub volumes of interest local significant increases of up to 14.6% (P < 0.001) were observed for trabecular number (Tb.N) suggesting that a fully anabolic model had been achieved whereby mechanical loading stimulates the thickening of existing trabeculae and even their formation at distal and proximal regions. At cortical sites a near significant 11% (P =0.06) global increase in bone volume (BV) was reported for a load of 8N together with significant local changes of up to 17.6% (P < 0.001). The mechanisms for cortical adaptation were less apparent however. The data suggests that mechanical loading at 8N initiates a remodeling process whereby the cortical shell expands whilst maintaining a certain cortical thickness. Dynamic histomorphometry, as measured by two calcein injections performed 4 days and 1 day prior to the end of loading, supports the µCT results, however, it was interesting to see that 2N and 4N loading groups displayed similar significant increases in histological indices inferring that sustained loading would also result in the significant augmentation of bone mass. This might allude to fatigue damage as the primary driver for bone formation. For C3H mice when loaded at 8N trabecular bone volume density (BV/TV) increased by 14.2 (P < 0.05) as measured by µCT however this was not accompanied by significant global or regional changes in trabecular thickness or trabecular number. Histology however did show significant increases in
the recruitment of osteoblasts, osteoblast activity and bone formation rates at trabecular and
cortical surfaces for a load of 8N. In terms of absolute and percentage increases the results from
the two mouse strains indicate that for the defined loading regime B6 mice are more mechano-
sensitive than C3H mice. Whilst this study has clearly shown that bone formation can be
regulated by mechanical loading, the mechanisms by which this manifests (i.e. thickening and
formation of new trabeculae, cortical expansion) are based only on the qualitative comparisons of
individual structural parameters. The cross sectional nature of this study therefore represents a
limitation. The implementation of a longitudinal study using in vivo imaging techniques would
enable a much more informative view of how bone adapts to mechanical loading. Furthermore
whilst the presented data suggests that the mechano-sensitive response of bone is genetically
determined, we have not accounted for the geometric differences between the two biological
strains a factor which could significantly modify the mechanical strain fields present in the target
vertebrae for a specific load.

Chapter 5 investigated the relationship between the micro-mechanical environment
induced in the micro-architecture of the target vertebra by the applied loads and subsequent
morphology. Upon dividing the vertebrae into sub-volumes or finite regions relationships were
searched for between mean regional mechanical parameters and the regional percentage increases
in bone attributable to a specific load. More specifically linear regression was used to correlate
strain energy density and the principle strains, as computed by FE models of vertebrae before
loading commenced, with $\Delta BV_L/BS_{0N}$. This parameter quantifies bone formation in 3D from
cross sectional data sets. It was determined by calculating the difference in mean bone volume
between a loaded group (subscript ‘L’) and the group loaded at 0N, for each region. This yielded
a mean $\Delta BV_L$ for each region and an associated error. The mean regional differences in $\Delta BV_L$
were then normalized by the mean regional values for bone surface area specific to the sham
group ($BS_{0N}$). Hence this parameter can be considered as measuring the amount of new bone
volume added, for a specific load, to the bone surface initially available in a particular region.
Results showed that regional cortical bone formation ($\Delta BV_{8N}/BS_{0N}$) correlated highly with mean
regional values of SED ($R^2 = 0.81$), suggesting that bone formation is directly proportional to the
magnitude of the mechanical strain sensed by the bone cells implicated in the remodeling
process. This however was not the case in trabecular bone where no correlation existed ($R^2 = -
0.072$). In addition to assessing correlations between mean regional mechanical parameters
\( \Delta BV_{8N}/BS_{0N} \) was correlated with the mean regional percentage changes in trabecular thickness, trabecular number, marrow volume, cortical thickness etc. This reaffirmed the observations that trabecular load induced bone formation was explained by both the thickening of existing trabeculae and the formation of new trabeculae. Furthermore correlations for cortical bone supported the idea that the structural expansion of the cortical shell accommodated increased loads.

The work presented in Chapter 5 has for the first time simultaneously assessed the relationship, in 3D, between the micro-mechanical environment and bone formation in both cortical and trabecular compartments of bone. The relative strengths of the linear correlations between mechanical strain and bone formation, observed in both compartments, challenges current theory related to load induced bone formation. Their polarized nature suggests different mechanisms drive cortical and trabecular bone formation. However, these data could also be explained by the cross sectional nature of the study which led to relatively high variations in both the computed mechanical parameters and the values of bone formation, both of which could be sources of significant error. The assumptions on which the FE models are based must also be questioned. It was assumed that both cortical and trabecular bone are homogeneous isotropic materials. Whilst this assumption is more suited to cortical bone, given its consistent alignment with the axial compressive force, the anisotropic properties of trabecular bone may need to be included to better simulate the mechanical behavior of trabeculae which display a greater variation of spatial orientation. Further work would therefore be required to improve the existing FE model. This would include improved definition of the anisotropic constants for trabecular bone in particular, which would in turn require further validation steps. Instead of relying on comparisons with micro-strain gages attached to the cortical shell (chapter3), step-wise image guided analysis of trabecular deformations could be implemented using image registration techniques to determine actual micro-structural strains.

In conclusion, the work of this thesis has provided an in vivo model which will facilitate future studies aimed at investigating the biochemical pathways involved in bone formation. Firstly, the appreciable dose response observed in the B6 mouse could be used to investigate the role of genes which are already known to be implicated in the bone remodeling process. Isolation of mRNA from osteocytes in both cortical and trabecular bone from different time points combined with PCR and micro DNA analysis could reveal much about the importance of these
genes thereby refining the search for the biochemical mechanisms which control load induced bone formation. Secondly, comparative genomic techniques could be used to compare the B6 and C3H genomes to find the chromosomal regions responsible for the allegedly differing degrees of mechano-sensitivity. Thirdly, other biological strains could be loaded to test the effect of specific gene additions or gene deletions which may have been identified by the first and second strategies to confirm their influence or further investigate the effect of the gene(s) or combination of genes. Finally molecular imaging techniques could be used in longitudinal loading studies to provide further insight into the role of certain genes and proteins. In addition to enabling further exploration, this thesis also questions current theory regarding load-induced bone formation. Strain energy density is assumed to be directly proportional to bone formation and is predominantly used as the primary input variable for many computer models simulating load induced bone adaptation for both cortical and trabecular bone. No studies have shown, in 3D, that strain energy density correlates well with bone formation in the cortical compartment and not with that in the trabecular compartment for the same bone unit. These findings therefore merit further investigation. The implementation of longitudinal studies using the latest in vivo imaging techniques would enable real-time changes to be monitored. When combined with ‘individualized’ FE models this approach could reveal much more about the relationship between micro-structural strains and stresses and load induced bone morphology in both cortical and trabecular bone. To summarize, this thesis has provided a genetically accessible model and a platform which when developed will enable us to gain a better understanding of the mechano-biology for both trabecular and cortical compartments of bone.

Finally, much of this discussion has focused on trabecular and cortical bone, appreciable dose responses were also detected in the primary spongiosa local to both growth plates. Considering the target vertebrae as and enclosed organ it would make sense to extend any further investigation to include these components as it is feasible that a synergy exists between the different components of bone.
Curriculum Vitae

Duncan James Webster

Born 20th July 1976, Doncaster, South Yorkshire, United Kingdom

2003 – 2008 PhD Thesis: A Combined Experimental and Computational Model for Genetic Control of Micro Structural Bone Adaptation, under the supervision of Prof. Dr. Ralph Müller at the Institute for Biomechanics, ETH Zurich.


2000-2001 Ove Arup and Partners: The Advanced Technology Group UK & USA: Mechanical Stress Engineer (First class honors achieved)


1994 -1999 University of Bath, United Kingdom: Masters of Mechanical Engineering with French.

1992-1994 The Hayfield School, United Kingdom: A-Levels, Mathematics (A), Physics (A), Design and Technology (A)