Institute of Ageing and Chronic Disease

Centre for Integrated Research into Musculoskeletal Ageing

Establishment of a functional musculoskeletal test system in mice to determine the effects of possible interventions on age-related musculoskeletal dysfunction

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Abstract

The age-related loss of skeletal muscle mass and function is a key contributor to physical frailty in older individuals. In addition to this, the increased development of bone and joint disorders such as osteoporosis and osteoarthritis with ageing present a complex problem. Current understanding of the mechanisms responsible for age-related musculoskeletal deterioration is poor.

The overall aims of this study were to integrate methods of measuring and visualising age-related changes in musculoskeletal tissues in a mouse model of ageing and to test a range of differing potential interventions known to protect individual tissues during ageing on the whole musculoskeletal system. Data demonstrated that C57Bl6 mice are a reliable model to study ageing, particularly in muscle and bone (Muscle mass reduced by ~40% and trabecular bone halved between 3 and 28 months of age); however these mice did not demonstrate evidence of any gross changes in the hind limb joints or tendons. C57Bl/6 mice were supplemented dietary sodium nitrate for either a short (18-24 months old) or longer (9 to 24 months old) to determine whether supplementation at key stages of development of musculoskeletal tissue dysfunction would result in preservation of muscle or bone without the need for more invasive treatment. No significant differences were reported in the long term treatment, however short-term treatments of mice with nitrate resulted in a significant decrease in muscle mass and function and this was associated with changes in trabecular bone disputing our initial hypothesis. HSP10 overexpression in muscles of mice has been shown to preserve muscle force generation and cross-sectional area in old mice previously. As proof of principle, the direct effect of such preservation of muscle on bone was tested. Preservation of muscle was evident as in the previous study; however, little effect of this was seen on bone disputing the dominant role the muscle has over bone tissue.

A cohort of wild mice was then used to challenge the widely used Bl/6 mouse model and demonstrated substantial spontaneous deterioration of joint tissues as well as increased loss of muscle and bone tissue compared to the Bl/6 mice. These results indicated a possible altered ageing process in the lab bred C57Bl/6 mouse which may impact the research using them.

Doctor of Philosophy Declaration

I hereby declare that this dissertation is a record of work carried out in the Institute of Ageing and Chronic Disease at the University of Liverpool during the period from October 2013 to October 2016.

The dissertation is original in content except where otherwise indicated.

March 2017

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(Gareth Anthony Nye)

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List of abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
ADRs	Adverse Drug Reactions
Akt-mTOR	Protein kinase B- mechanistic target of rapamycin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BDNF	Brain derived neurotropic factor
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenic protein
BMU	Bone multicellular units
c-met	Hepatocyte growth factor receptor
CSA	Cross-sectional area
DEXA	Dual-energy X-ray absorptiometry
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factors
H_2O_2	Hydrogen peroxide
H&E	Haematoxylin and eosin
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HSF1	Heat Shock Factor 1
HSP	Heat shock protein
IGF1	Insulin-like growth factor 1

IL	Interleukin
IP	Intraperitoneal
IU/day	International units/day
KC	Chemokine (C-X-C motif) ligand 1
KO	Knockout
Lbx1	Ladybird Homeobox 1
Lo	Muscle optimal length
MCP-1	Monocyte chemoattractant protein 1
Mef2	Myocyte enhancer factor 2
MTORC1	Mammalian target of rapamycin complex 1
Myf5	Myogenic factor 5
N	Newton
NFkB	Nuclear factor κ-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
OCN	Osteocalcin
OVX	Ovariectomy
Pax3	Paired box 3
PBS	Phosphate-buffered saline
PGC1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Po	Maximum isometric tetanic force
P/O ratio	Phosphate/Oxygen Ratio
PTH	Parathyroid hormone
PTHr	Parathyroid hormone receptor
PTH1-34	Teriparatide
RANK	Receptor activator of nuclear factor kappa-B

RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SEM	Standard error of the mean
SOD1	Superoxide dismutase 1
TGFβ	Transforming growth factor beta
TNFα	Tumour necrosis factor
Trap5b	Tartrate-resistant acid phosphatase 5b
VEGF	Vascular endothelial growth factor

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Chapter 1

Introduction

1.1 Ageing: the role of musculoskeletal decline

We currently live in an increasing ageing population both within the UK and in other developed countries. Figures from before 2000, based on the current UK population of that time estimated that the number of people over 65 would reach 13 million by 2030 (Statistics, 2013). In 2010, the UK parliament reported that the UK population over the age of 65 was currently at 10 million people and further estimated that by 2030, over 15 million people would be in this age group.

The UK-based charity, Age UK, published more recent findings (June 2016) suggesting that 11.4 million people in the UK are currently over the age of 65 (Statistics, 2015), with 23.2 million people over the age of 50 – this equates to roughly a third of the population (Statistics, 2015). There are now more people over the age of 60 than under the age of 18 (Statistics, 2015) but despite the growing aged population, 11% of older people would describe their life as very poor, poor or neither good nor poor (Voice, 2008). On mean, women today will be in poor health for around the last ten years of life, with men having seven years of poor health (Statistics, 2015). Thus, it is clear that research based interventions should aim to not solely extend lifespan, but to increase health span (Kirkland and Peterson, 2009) keeping people healthier for longer and reducing the time spend in poor health (Figure 1.1).
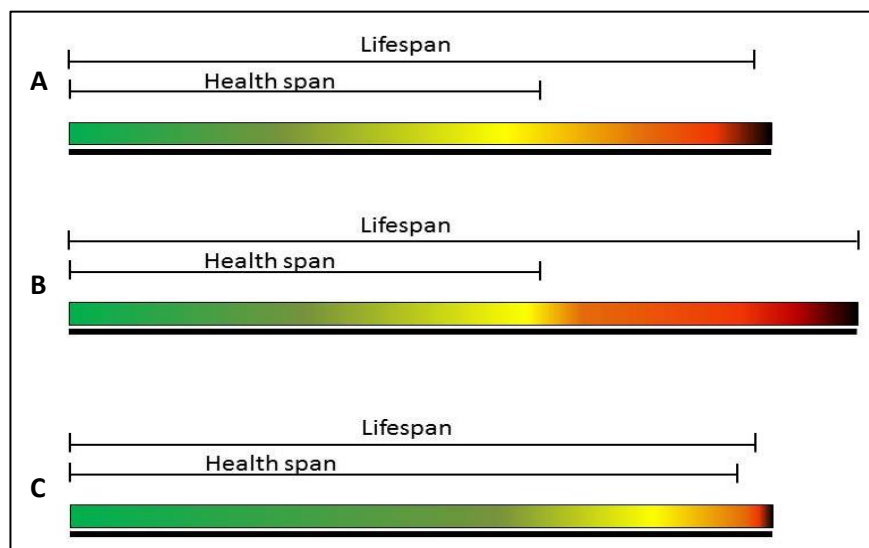
Fig 1.1 Differing approaches to treating increasing age**.** A shows the normal lifespan of an average person. B shows the effect of lifespan extension only. C shows the extension of health span only. Redrawn from Kirkland and Peterson (2009)

There are a number of age-related conditions that can cause a reduction in health span (Kirkland and Peterson, 2009, Burch et al., 2014). These include the presence of cancers (Statistics, 2015, Ahmad et al., 2015), cardiovascular and respiratory diseases (Statistics, 2015), cognitive disorders (Fratiglioni et al., 1997), metabolic disorders such as diabetes (Kirkman et al., 2012) and musculoskeletal disorders (Degens and Alway, 2003, Kelsey, 1989). Classically, basic research has focused on one of two approaches – to slow ageing or to prevent death. This has often been reduced to specific prevention or treatment of one of the age-related conditions (Figure 1.2).
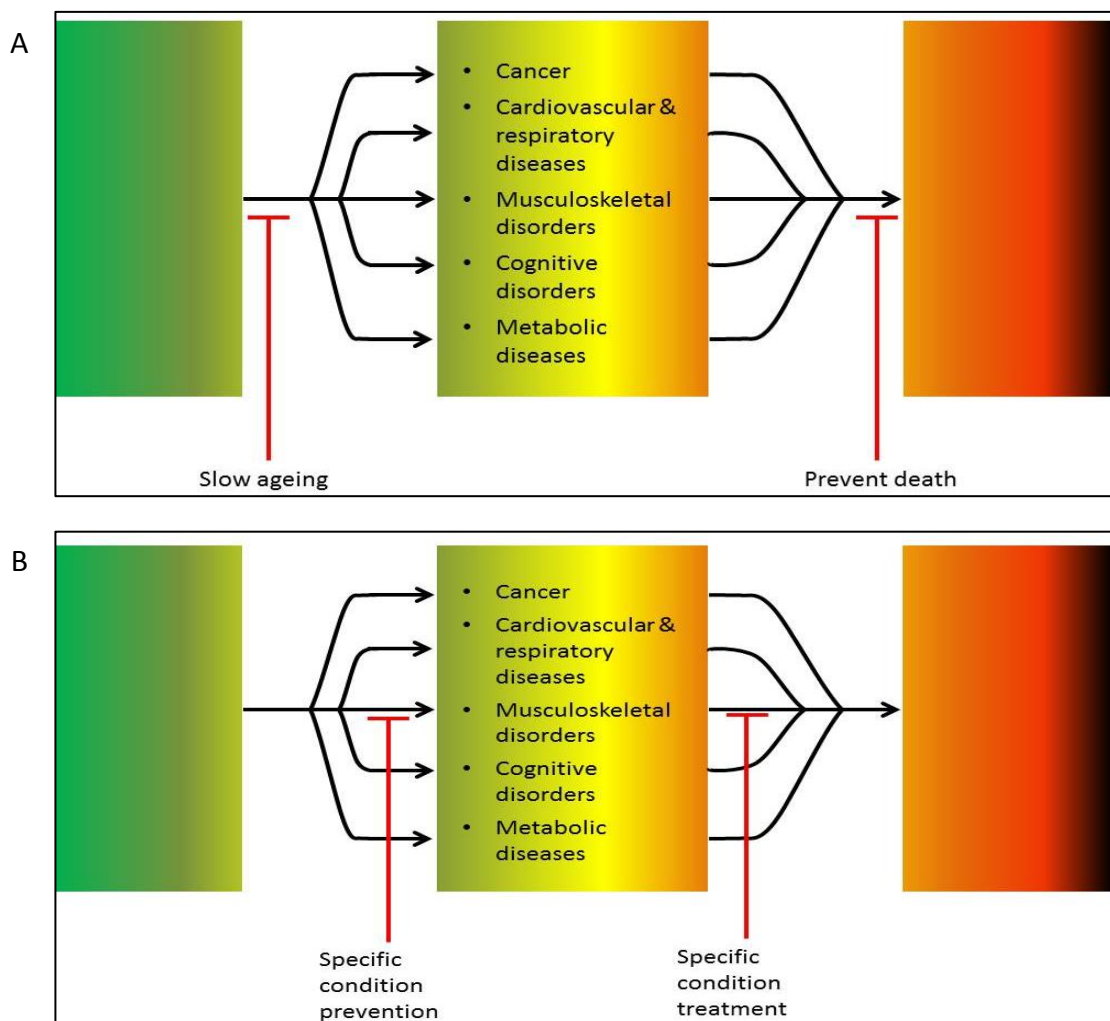
Fig 1.2 Differing approaches to treating age-related conditions. A shows how ageing can be slowed to prevent the onset of age-related conditions as much as possible, or preventing death by treating the age-related diseases and prolonging life. B shows a more realistic example where one specific disease is either prevented or treated allowing other age-related conditions to develop.

Although there have been successes with using specific approaches, it undervalues the level of connectivity of a majority of the age-related conditions. Removing cancer as an exception, the remaining conditions can be considered closely connected e.g. decline in musculoskeletal function leads to both an increased risk of falls (Montero-Fernandez and Serra-Rexach, 2013) (compounded by decreases in cognitive function (Rosano et al., 2006)) and decreases in daily activity (Hamrick et al., 2006, Roubenoff and Hughes, 2000). Both can feedback to further reduce musculoskeletal function (Perkin et al., 2015, Wijlhuizen et al., 2007). The decrease in daily activity can lead to increased mass (Wang et al., 2008), reducing activity further, and can lead to metabolic disorders (Colditz et al., 1995, Wisse, 2004) which can lead to cardiovascular and respiratory disorders (Kannel et al., 1996, Aaron et al., 2004). All three of these effects can further reduce musculoskeletal function (Moseng et al., 2014) (Figure 1.3).

This may well go some way to explain the rapid decline in health in later life – declines in one tissue can lead to knock-on effects which all feedback onto each other.
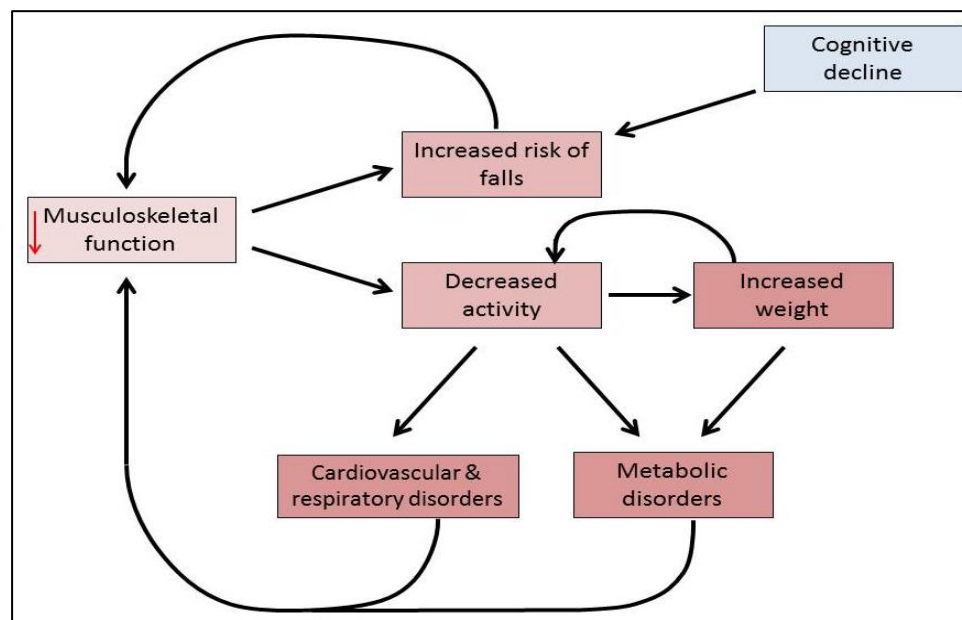
Fig 1.3 Example of placing musculoskeletal decline as the centre of age-related health decline**.** The decrease in musculoskeletal function can lead to increasing falls and decreasing activity which both feedback directly and indirectly causing further declines. These effects are known to influence other age-related conditions and co-morbidities. (Martini et al., 2012)

Based on the above, although not as life threatening when compared to other age-related conditions, decline in musculoskeletal function can be highly influential in coordinating the general decline we see in later life. Musculoskeletal disorders are currently the leading causes of disability in the aged population (Statistics, 2015). The UK Global Burden of Disease study placed musculoskeletal conditions as the largest single cause of years lived with disability, this increases in the older population (Murray et al., 2013).

Musculoskeletal disorders consist of reduced quality or function of one or a combination of bones, muscles, joints or tendons and ligaments. These states can be brought about through reduction or dysfunction in tissues specifically (e.g. reduction in bone mass leading to osteoporosis and increased fracture risk) or through a reduction in multiple tissues causing limited mobility, function and overall frailty. Conditions can broadly be placed into three categories consisting of conditions causing pain, conditions which increase fragility and conditions brought about by increases in inflammation.

Approximately a third of people aged over 65 will fall at least once per year (3 million people) (Statistics, 2015), compounded by high levels of osteoporosis (Bilezikian et al., 2008), costing the NHS upwards of £6 million a day (based on hip fractures alone) (Statistics, 2013). Of the 70,000 osteoporotic hip fractures each year, about 30% of patients will die within a year due to causes related to the fracture (Statistics, 2015). Compounding this, 1 in 2 women and 1 in 5 men over the age of 50 will have an osteoporosis induced fracture, between 50-70% of women will have an osteoporotic fracture (Statistics, 2013). This shows that with the ageing population growing at a faster rate than predicted, certain age-related illnesses are not being successfully managed. Lifespan is unfortunately outpacing health span.

On mean an older person loses between 2-4% of muscle mass (Lexell, 1995) and 1% of bone mass every year of life (Pouilles et al., 1996). Despite this, there is no clear understanding of this process. Thus, musculoskeletal ageing is placed in the centre of the cause of a large proportion of age-related morbidity and death. In order to fully research a beneficial solution, first a fundamental knowledge of musculoskeletal ageing is needed, not from a selective, single tissue approach, but

from a multi-tissue multidisciplinary approach and from this we can begin to successfully test interventions with an aim of improving the whole musculoskeletal system.

There is a clear need to both improve musculoskeletal function in older people and prevent the decline for as long as possible if not indefinitely. There is strong evidence to place this as the most important step in improving the quality of later life.

1.2 Structure and function of musculoskeletal tissue

1.2.1 Skeletal muscle structure and function

Skeletal muscle as an organ has evolved to provide us with wide ranging and hugely beneficial functions including coordinated movement such a running/walking and for posture maintenance. Being highly adaptable with both a robust and plastic nature, it has allowed for many steps in evolution to commence. The muscle bulk itself is a bundle of differing tissues including muscle, nerve, blood vessels and connective tissue. Muscles connect to the skeleton via tendons (Figure 1.4) (MacIntosh, 2006).

The main function of skeletal muscle is that of locomotion. Muscle also aids in maintaining body temperature and is a major source and store of dietary protein. Muscle makes up 40-50% of total body mass on mean and stores 50% of total protein within the body, (MacIntosh, 2006).

Skeletal muscles develop early in the embryo from somites (Buckingham et al., 2003). Early muscles require a large number of external signals and factors before they commit to forming a skeletal muscle cell with the expression of Pax3, c-met, Lbx1, MyoD, Myf5, Myogenin, Mox2, Six and Mef2 (Buckingham et al., 2003). In humans the first muscle fibres start to form at around embryonic day 11-14 (primary fibres) with further fibre formation (secondary fibres) and innervation occurring before embryonic day 16. Primary fibres will tend to develop into slow glycolytic fibres, secondary fibres tending to form fast twitch fibres (Buckingham et al., 2003).
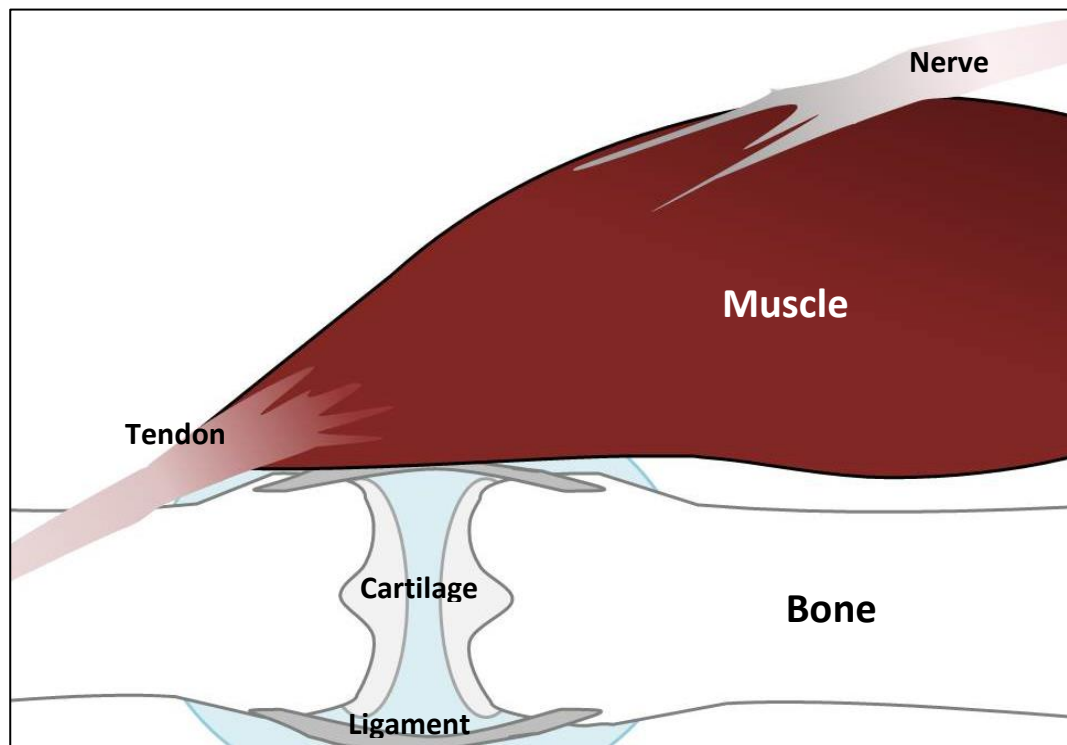
Fig 1.4 The musculoskeletal tissues are in close proximity**.** The arrangement of musculoskeletal tissues is as above. Innervated muscles surround bone and are attached via tendons. Bones are joined via ligaments forming joints with the protective cartilage present at the ends of each bone (Martini et al., 2012).

The fully formed skeletal muscle has a consistent architecture despite large plasticity. Muscles contain long bundles of muscle fibres formed initially from myoblasts. Each fibre has a plasma membrane called the sarcolemma. Each fibre is in turn made up of up to 1000 myofibrils of roughly 1-2um in diameter which are made up of proteins including actin and myosin providing the contractile properties (MacIntosh, 2006).

The actin and myosin filaments are highly organised into structures termed sarcomeres (Huxley and Hanson, 1954). These units give the striated appearance visible in skeletal muscles. Dark bands and light bands are the cause of this striation. Dark bands termed A bands are caused by the overlapping actin and myosin filament and are divided by the M line (a dark line) whilst lighter bands called I bands are divided by the Z disk (filament anchoring site) and are composed of non-overlapping filaments. Sarcomeres are the area between two Z disks (MacIntosh, 2006).

The structure of these filaments allows the maximum force to be produced with the least amount of energy input. The contraction is first initiated upon receipt of

neural signals from the neuromuscular junction (MacIntosh, 2006). This causes the opening and activation of voltage gated Na/K channels along the fibre which cause large influxes of Na and a net positive charge along the fibre, propagating an action potential (Sandow, 1952a, Sandow, 1952b, Huxley, 1957).

The action potential spreads down specialised sections of the fibres called transverse tubules (T tubules) causing release of Ca from the sarcoplasmic reticulum. These free ions bind to troponin located on the actin filament. This causes allosteric modification of the related protein tropomyosin, freeing up myosin binding sites on the actin protein. Myosin heads then readily bind to actin filaments forming cross bridges. Hydrolysis of ATP bound to the myosin head by myosin ATPases allows movement of myosin heads along the actin filament, a process known as the power stroke (Sandow, 1952a, Sandow, 1952b, Huxley, 1957).

This process will continue in a cyclical nature along the actin filament (approximately 10nm movements per power stroke) as long as ATP is present and can be hydrolysed. A sliding motion occurs and as the length of actin and myosin filaments remain constant, the muscle fibre as a whole contracts (Sandow, 1952a, Sandow, 1952b, Huxley, 1957).

Fibres within a muscle can be divided according to how each performs under stimulation and their biochemical characteristics once fully developed. Type I fibres are also termed slow twitch. Type II fibres can be classed as fast twitch (MacIntosh, 2006). Type I fibres have larger numbers of mitochondria and contract slowly compared to type II. These do however have a larger capacity for aerobic metabolism and a higher fatigue resistance (MacIntosh, 2006).

Type II fibres can be further subdivided. Type IIA fibres are relatively fatigue resistant with intermediate levels of glycolytic activity. Type IIB fibres contain smaller numbers of mitochondria leading to a limited capacity for aerobic respiration but a high capacity for anaerobic metabolism. Muscles with a larger proportion of these fibres are ideal for fine rapid movements (MacIntosh, 2006).

Muscle is clearly still of much interest as it is hugely plastic. Muscle can hypertrophy after exercise but more importantly to our ageing population, there is

atrophy in muscle mass and decline in force generation through age known as sarcopenia which severely limits mobility. The cause of this is yet unknown although several hypotheses have been proposed (Rennie et al., 2004, Rennie, 2009).

1.2.2 Bone structure and function

Bone is a highly specialised tissue which forms the skeleton with cartilage (Figure 1.4). Like muscles, bone is pliable. Bones throughout life are formed, removed, modelled and remodelled numerous times (Bilezikian et al., 2008). Bones are needed to produce locomotion in tandem with the muscle. Bones are also a major source and store of calcium within the body (Smith and Wordsworth, 2005). Despite being in close proximity to muscles, the developmental pathway of bone is relatively distinct from muscle.

Early bones are formed by mesenchymal stem cells which migrate from the neural crest and condense into appropriate clusters (Olsen et al., 2000). These cells first differentiate into chondrocytes at approximately embryonic day 11 and begin to produce cartilage templates for bone (Smith and Wordsworth, 2005). Although not fully understood, Transforming Growth Factor beta (TGFβ), Bone Morphogenic Protein (BMPs) and Fibroblast Growth Factors (FGFs) along with Sox9 are essential for this process to occur (Smith, Smith and Wordsworth, 2005).

As the cartilage templates in the embryo increase in size, chondrocytes within the centre start to dehydrate, enlarge and calcify via production of alkaline phosphatase. This may be due to parathyroid hormone related peptide signalling (Chung et al., 1998). The increase in cell volume drives bone growth (Smith and Wordsworth, 2005). At this stage blood vessels form and invade the cartilage templates, forming the periosteum which contains osteoprogenitor cells later becoming osteoblasts. These cells form a bone collar to support the newly forming bone. These cells continue to enter the bone using the calcified inner matrix as scaffolds to produce trabecular bone via secretion of osteoid. Osteoclasts formed from the macrophage cell line enter via blood vessels to break down bone from the centre to create the bone marrow cavity. This process is continually active and allows for further bone growth and maintenance until after puberty (Smith and Wordsworth, 2005).

The final bone structure (in long bones) consists of a thick, dense outer layer of bone named the cortical bone, with the trabecular bone forming struts within this casing (Figure 1.5) (Smith and Wordsworth, 2005).

Between 80-90% of the volume of cortical bone is calcified, under 25% of the volume of trabecular bone is calcified. This is due to the presence of other tissues such as blood vessels, fat and other connective tissues within the trabecular bone compartment. Although both compartments contain osteogenic cells, the trabecular bone compartment is the site of most cell activity and interaction with other tissues (Smith and Wordsworth, 2005).
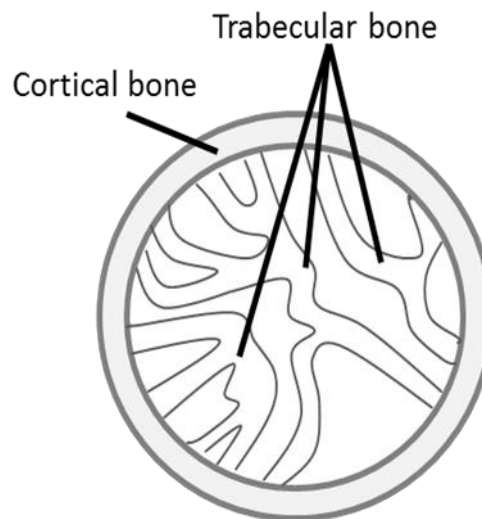
Fig 1.5 Schematic displaying the difference in position of cortical and trabecular bone.

Once formed, bone undergoes remodelling events to counteract stresses and strains. Osteoblasts and osteoclasts are the cells primarily involved in this growth and remodelling of bone tissue. Their effects are controlled through multiple pathways involving both mechanical and chemical stimuli in a similar fashion to muscle cells.

Osteoblasts work simultaneously with each other to synthesize collagen, osteocalcin and osteopontin as well as hydroxyapatite to produce the bone matrix. It is this matrix which provides both large compressive strength and elastic deformation (Smith and Wordsworth, 2005).

Osteoclasts are also present within bone and function to breakdown the bone matrix into component parts to release minerals such as calcium. This is achieved by releasing large amounts of hydrogen ions to acidify and dissolve the mineralised bone matrix (Smith and Wordsworth, 2005).

Between these two classes of cells, bone is resorbed and formed to allow for modelling events. The cells involved in these events form bone multicellular units (BMUs) and comprise of osteoclasts cutting and absorbing into bone with osteoblasts following with new bone matrix, blood vessels and nerves (Smith and Wordsworth, 2005). This process can occur on both types of bone and it is estimated that approximately 20% of the bone surface is undergoing remodelling processes, with millions of BMUs occurring at any one time in a process that takes between 2-8 months (Smith and Wordsworth, 2005).

A third class of bone cell is also present, the osteocyte. Although this is the most abundant cell located in bone (Bilezikian et al., 2008), both function and regulation are poorly understood. Osteocytes are formed from osteoblasts that become encased in the bone matrix they themselves are producing (Smith and Wordsworth, 2005). These cells remain viable and highly functional, with a half-life of approximately 25 years.

Osteocytes stay within space inside the matrix named lacunae with long protrusions called processes being found within canaliculi (Smith and Wordsworth, 2005). The processes are important in nutrient exchange as well as in mechanosensory mechanisms (Noble, 2008). Although the nature of these mechanisms has not yet been elucidated, it is thought that nitric oxide may play a role (Klein-Nulend et al., 2014). There are also theories suggesting the flow of fluid surrounding the osteocyte may be the method of mechanosensing (Verbruggen et al., 2014). It is proposed the osteocytes die via apoptosis (Figure 1.6) (Smith and Wordsworth, 2005).
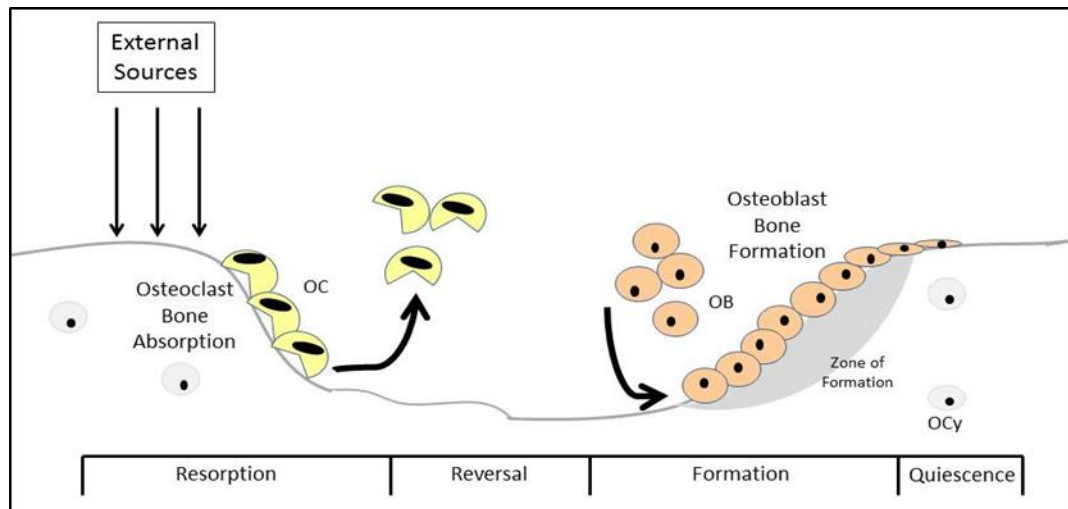
Fig 1.6 Schematic displaying function of bone cells during normal bone maintenance. External sources such as mechanical forces or hormonal responses trigger osteoclasts (OC) to break down bone. Following this, osteoblasts (OB) lay down new bone. During this bone laying, some osteoblasts are encased and become osteocytes (OCy).

1.2.3 Tendon structure and function

Tendons connect muscles to bone to facilitate locomotion and transmit forces (Figure 1.4). Although there are subtypes of tendons, the most useful are the energy storing tendons, such as the Achilles tendon in humans, allowing the attached muscles to generate greater force (Thorpe et al., 2012).

Tendons are made up of parallel arrangements of collagen (mainly type 1 collagen) interspersed with elastin, proteoglycans and other inorganic components such as copper and calcium (Lin et al., 2004). Collagen molecules are arranged in fibrils, these in turn arrange to form fascicles (Caldini et al., 1990). These fascicles are joined together in bundles by an endotendineum and groups of fascicles are bound by the epitenon (Caldini et al., 1990). Proteoglycans found along the length of the fibrils aid the tendon by providing resistance to compressive stress complementing the resistance of collagen fibrils to tensile stress (Raspanti et al., 2002). The turnover of tendon fibres is controlled by tenocytes, the fibroblast-like cell found within tendons.

1.2.4 Joint structure and function

Joints are the location where two or more bones meet and allow movement and provide some mechanical support during locomotion (Figure 1.4). Joints are

classified depending on how the bones are arranged to each other. In most cases the bones are not directly connected to one another and are surrounded by a synovial cavity and facilitated by an articular capsule and associated ligaments. Articular cartilage is located on both ends of the bones to facilitate free movement of the joint as well as essential shock absorbance whilst moving (Martini et al., 2012).

1.3 Pathophysiology of ageing in musculoskeletal tissues

Ageing of certain musculoskeletal tissues in humans can occur as early as thirty to forty years of age and this occurs at a comparable time in the lifespan throughout a large proportion of species.

Musculoskeletal ageing is a complex and highly intertwined process. It involves the loss of tissue and function in muscle, bone, tendons and ligaments as well as articular cartilage. This has effects on the level of physical activity (Hamrick et al., 2006). Reduced activity correlates with an increase in fat deposition which can further influence the ageing process (Wang et al., 2008). Not only is there a general loss of function with age, there are also distinct features that can be regarded as disease.

1.3.1 Loss of muscle mass and function with age

By the time a person reaches their 80s, they will have lost approximately 50% of their muscle mass (Faulkner et al., 2007). As muscle mass represents 60% of total body mass, this loss is clearly detrimental to each individual through reductions in locomotion, strength, co-ordination as well as more metabolic processes. It is to be noted that the more muscle mass a person has developed in earlier life, the longer a person can remain fully mobile (Pollock et al., 1997, Steen, 1988).

Despite this, there is still not a current clinical definition of sarcopenia, due in part to the lack of standardised measurements for muscle function. Although muscle mass measurements are well defined, mass does not always correlate with function.

Most studies use muscle mass, muscle strength or a functional measure of muscle capability through physical exercise as a marker, either alone or in any number of

combinations. More recently a common use of grip strength, walking speed and muscle mass measurements have been used to determine if a patient has sarcopenia. A European working group produced guidelines to this end consisting of patients being aged 65 or over, displaying gait speed of less than 0.8m/s and have low muscle mass, with low grip strength being another deciding factor. Other co morbidities related to sarcopenia should also be factored in however such as cancer, heart disease, renal failure and strokes. From this definition we see incidences varying widely from population groups, incidence also varying dramatically when taking into account other recognised definitions of sarcopenia (Woo et al.).

The consensus states however that loss of muscle mass is due to loss of individual fibres and eventual atrophy and weakening of remaining fibres, in particular type II fibres (Larsson, 1978) (Coggan et al., 1992). This leaves a larger proportion of type I fibres in muscles of older people. What compounds this muscle loss is the replacement of muscle with fat and connective tissues which disrupts muscle function (Taaffe et al., 2009). This loss of muscle mass and individual fibre loss has a direct impact on force production and overall strength which can also influence associated musculoskeletal tissues (Thom et al., 2007). There also appears to be a greater susceptibility of muscle to contraction-induced damage in the remaining fibres and an inability to repair the damage once it occurs (Close et al., 2005). Although the cause of this muscle fibre loss is still unknown, there are related events that may be involved such as inflammation, disease, genetics or as discussed next, the innervation of muscle.

Nerve input to muscle motor units is an essential part of muscle contraction. It is the release of excitatory neurotransmitter from the nerve that potentiates the influx of calcium that lead to the muscle contraction. Denervation of these motor units or poor structure and transmission through neuromuscular junctions is evident with increasing age leading to a large decrease in force production (Finkelstein et al., 1993). There are currently no known mechanisms identified as responsible for this deterioration. Animal models have provided some theories as to the cause of neuronal dysfunction. One of the most interesting studies has used transgenic mice with SOD1 removed at the whole body level as well as specifically in muscle or nerve, or re-introduced into the nerve in the whole body knockout

mouse (Jang et al., 2010). Whole body deletion of SOD1 leads to premature loss of muscle mass mimicking that of age-related sarcopenia (Jang et al., 2010). It has been shown that reintroducing SOD1 back into the nerves only is sufficient to restore function in both nerves and muscle (Sakellariou et al., 2014b). This would strongly suggest that age-related increases in reactive oxygen and nitrogen species occur in the nerves and that this potentially is the initiating factor in the loss of innervation. There are also decreases in anabolic signals and hormones such as IGF1 and conversely there are increases in catabolic signals which both combine to cause decreases in muscle mass (Barton-Davis et al., 1999). IGF1 is produced in response to growth hormones and is a key driver of muscle growth in particular. One way in which it is thought to achieve this is by improving muscle satellite cells' ability to proliferate (Barton-Davis et al., 1999). However in environments with high levels of inflammatory markers, this ability is reduced and may, at least in part, be the cause of the age-related reduction in IGF1 sensitivity (Barbieri et al., 2003).

1.3.2 Age-related changes in tendons

Age-related changes in tendons and ligaments centre on a change in collagen production and maintenance leading to a reduction of elasticity (Narici and Maganaris, 2006). Tendon cell function also changes with age with dramatic reductions in cell number (possibly due to increasing extracellular matrix) although the vast majority of this reduction in cell numbers has been proposed to occur earlier in life (between 2-9 months of age in the rat) (Nagy et al., 1969). However other studies show no changes with age (Thorpe et al., 2016). Studies have shown a decrease in tendon tissue turnover after late teens in humans (Heinemeier et al., 2013), (although there have been reports of small increases in tendon cross-sectional area (Magnusson et al., 2003)) and a likely decrease in strength (Connizzo et al., 2013). Inconsistent studies mean that no firm conclusion can be made. Although these are not major influential changes, together with a loss of force generation by the muscles, this may lead to a compounded effect on locomotion. This change in movement can further impact on balance and can increase the risk of falls within older people. The lack of human studies in this field mean no firm conclusions can be made on the age that changes occur within the tendon (Magnusson et al., 2003, Couppe et al., 2009).

1.3.3 Loss of bone mass and structure with age

Bones lose strength as a result of loss of bone tissue, particularly around the trabecular bone which normally functions to provide strength and support, a condition known as osteoporosis. Thus, there is an increased risk of fractures with increasing age. 1 in 5 men over the age of 50 will have an osteoporosis induced fracture; between 50-70% of women will have an osteoporotic fracture (Statistics, 2015). Osteoporosis was first recorded by Cooper in 1829 (Cooper, 1829) and a full description of the disorder given by Cooke in 1955 (Cooke, 1955). Despite this, the cause of the disorder is not yet fully understood. However, the close links between muscle and bone may suggest that age-related loss of muscle force and therefore loss of mechanosensation is a key mechanism in the age-related loss of bone. As with the muscle, bone also becomes less responsive to anabolic signals such as IGF1 and vitamin D and bones are affected by increases in inflammatory factors (Redlich and Smolen, 2012) which serve to reduce osteoblast numbers and increase osteoclast activity eventually shifting the balance of bone formation/resorption into a highly resorptive state (Redlich and Smolen, 2012). Trabecular bone appears to be more at risk of this change. The effect on each bone is unique, although it can be broadly split into two categories – bone thinning and bone erosion/deletion (Fig 1.7) (Smith and Wordsworth, 2005). The mechanical need for each trabecular bone may be the determining factor (Smith). The higher the bone mass present at earlier life, the later a person will display symptoms of osteoporosis suggesting that the rate of bone loss is similar for all individuals.

1.3.4 Age-related changes in cartilage

Along with age-related changes in bone tissue, articular cartilage also diminishes and with it there is a large reduction in shock absorption within the joints. This breakdown exposes bone surfaces to each other and can lead to painful joints (Li et al., 2013). Inflammatory markers and cytokine changes may also be associated with joint conditions such as osteoarthritis which can also damage the underlying bone (Sokolove and Lepus, 2013).
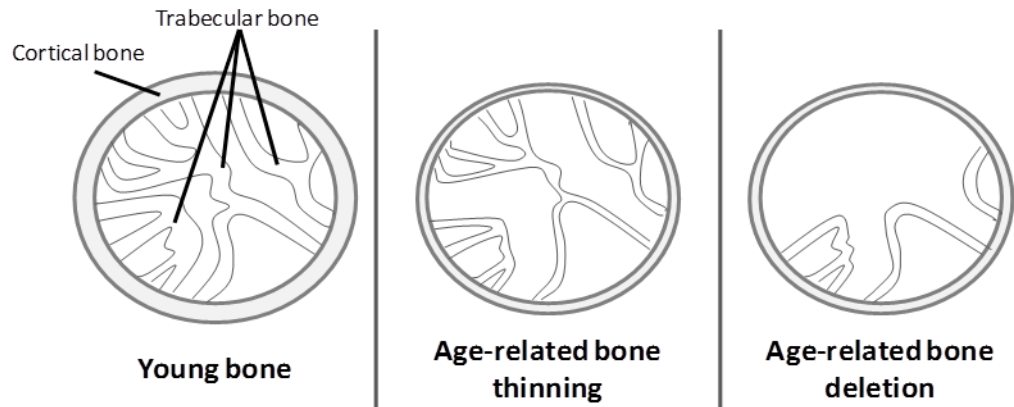
Fig 1.7 Differential age-related loss of trabecular bone showing both loss of bone through thinning and loss through deletion of trabecular bone. Also displayed is age-related thinning of cortical bone (Smith and Wordsworth, 2005).

1.3.5 Other age-related changes that affect musculoskeletal tissues

Although the changes outlined above are the most influential concerning musculoskeletal tissues, there are other age-related changes that can impact these tissues and contribute to the detrimental changes. Fat tissue is known to produce numerous factors including cytokines that can alter the inflammatory environment of nearby tissues (Bucci et al., 2013). During increasing age, fat deposition is increased in the body and can integrate into both muscle and bone tissues (Bucci et al., 2013). These fat depositions can then produce local detrimental effects compounding the already negative environment.

Elevated pro-inflammatory cytokines and increases in reactive oxygen species systemically can also influence musculoskeletal tissues and have been widely discussed in the literature and will be further discussed later. Age-related changes in hormones are also prevalent and particularly influential in bone health and have been widely discussed elsewhere (Riggs, 2000, Kamel et al., 2002). Furthermore, reduction in neural signalling and reduction in small blood vessels also occur through age and have additional effects for all musculoskeletal tissues (Faulkner et al., 2007).

It is clear that ageing of musculoskeletal tissues is a complex and intertwined process. There are still many gaps in our knowledge that can only be filled with basic, clinical and translational approaches to research.

1.4 Muscle-bone cross-talk

Muscle bone cross-talk has been long proposed since the late 1800s (Wolff, 1988). The interactions between muscle and bone through life and the changes related to ageing display a close link between the two tissues above a physical association due to proximity.

Some links are clear. As discussed previously, both tissues originate in close association from somites during early development and both reach their peak mass through shared environmental and physical stimuli, albeit at potentially different times. For example, increases in both muscle and bone mass are tightly correlated during bouts of exercise, conversely both tissues atrophy during times of disuse at similar rates (Wolff, 1988, Rubin, 1984). Even prior to birth, muscle contractions are needed for bone development (Bren-Mattison et al., 2011). Without this, bone develops without being mechanically suitable for movement. In knockout mouse models were skeletal muscle does not develop (MyoD$^{-/-}$, Myf5$^{-/-}$) foetuses are born with deformed bones lacking mineralisation (Gomez et al., 2007).

Whether changes are brought about by one tissue or both is still contentious. Many studies suggest muscle has a dominant role over bone in synchronicity of the two tissues (Bren-Mattison et al., 2011, Jang et al., 2010).

Regardless of possible synchronicity, both tissues share a number of signalling pathways in development and throughout life. An example of this is growth hormone and IGF1 which both cause proliferation and differentiation of osteoblasts and myoblasts therefore increasing mass in both tissues (Alzghoul et al., 2004, Banu et al., 2003). Wnt signalling is also important in development of both tissues (Rudnicki and Williams, 2015). These signalling molecules however are by no means specific signalling molecules to muscle and bone.

There are more specific examples of a close link between muscle and bone. The gross structures of long bones within the body are often unique and show various asymmetries through differential bone deposition (Sharir et al., 2011, Auerbach and Ruff, 2006). This has been described to be due to site-specific mechanical stress and strain laid on the bone from the attached muscle (Vickerton et al.,

2014). This works in an attempt to counteract the weakest parts of the bone to gain overall strength and stability (Vickerton et al., 2014). The forces applied to bone from muscle far outweigh the environmental forces in order to generate movement. Strains applied to bone above 3000 microstrain (A strain expressed in terms of parts per million) will typically cause bone formation, strains under 500 microstrain cause bone resorption (Rubin and Lanyon, 1985, Burger and Klein-Nulend, 1999). It is theorised that the mechanotransduction occurs in the osteocyte in response to changes in fluid movement along the bone (Burger and Klein-Nulend, 1999). Tail suspension models in mice have been used to develop a model of inactivity separate from disrupting the nerves, which may have a separate effect on the bones (Roland et al., 2005, Amblard et al., 2003). Although muscles were still able to contract in this model, the removal of mass bearing activities can lower the force generated by muscles. Within 24 hours there were signs of repression of actin and myosin and after 10 days this model had showed reduction in both muscle and bone mass (Roland et al., 2005). These effects are reversed when the tail suspension has ceased (Roland et al., 2005). Only load bearing exercise is capable of affecting the skeleton – swimming has been shown to have no effect on bone mineral density even with improvements of shoulder, back and grip strength (Courteix et al., 1998, Emslander et al., 1998).

Experiments have shown that there is more detail to the cross-talk of muscle and bone then a simple application of forces. Muscle paralysis can decrease bone mass even when the muscle is loaded (Warden et al., 2013). This would suggest another level of interaction. More subtle links can also be found and often become more prominent during ageing. During sarcopenia a number of pathways all act to reduce the overall muscle mass. These include Akt-mTOR signalling, inflammatory cytokines, NFkB, myostatin, increases in reactive oxygen species and more (Meng and Yu, 2010). As mentioned above there are still uncertainties as to the cause of sarcopenia and sarcopenia is often accompanied by osteoporosis. Many of the pathways that impact on muscles ability to maintain mass also impact osteoblasts and the potential to increase bone mass e.g. oxidative stress (Bai et al., 2004).

Although there is substantial evidence for a close relationship between both muscle and bone, a growing theory is that further communication occurs between

these tissues through release of signalling molecules, creating the possibility of an endocrine loop that directly concerns muscle and bone. This theory arose through the study of bone fracture healing rates. The rate is accelerated when surrounded by fully functioning muscle. In mouse studies the introduction of muscle coverings (Harry et al., 2008) or minced muscle (Zacks and Sheff, 1982) are beneficial to fracture healing and this beneficial effect can be blocked by small pore filters. This would suggest other external signals could be converted within the muscle to induce further signals influencing bone biology (Schnyder and Handschin, 2015). These external signals are termed myokines.

One such molecule released from muscle is irisin which has been recently shown to have a possible role in this bone-muscle cross-talk. Researchers showed that irisin injected systemically can elicit an anabolic response in the cortical aspect of bone only (Colaianni et al., 2015). This was through increased activation of osteoblasts (Colaianni et al., 2015). There is currently no evidence that this molecule is present within humans and as with many of the reported myokines, is the subject of much controversy.

It should however be noted that most data published thus far on muscle bone crosstalk is purely correlative and hypothetical. Bone has a surrounding membrane that physically separates bone and muscle tissue called the periosteum (Allen et al., 2004). There are some doubts as to whether even the smallest myokines could cross this bone membrane and diffuse into the cellular levels to affect a response (Hamrick et al., 2010). The periosteum has been described as being completely sealed in some papers or semi permeable using hydraulic mechanisms to facilitate movement (Qin et al., 2003, Gardinier et al., 2010), a hypothesis which may have been partially confirmed with irisin (Colaianni et al., 2015). Recent studies have shown that the periosteum will act as a semi-permeable sieve for any molecules under 40kDa (Lai et al., 2014). This study also showed that penetration times for potential myokines range from 10 seconds to 6 hours and this would potentially allow for myokines to penetrate and reach bone targets (Lai et al., 2014). The range of charges and structures of each myokine is an influencing factor on time of transport. This also gets further complicated with the addition of fat and collagen deposits with increasing age (Fasshauer and Bluher, 2015).

1.4.1 Muscle as a source of chemical signals

Muscle is a secretory organ capable of releasing a variety of compounds. These factors, termed myokines, may act in an autocrine, paracrine or even endocrine manner to facilitate changes in the body (Schnyder and Handschin, 2015). Studies suggest that muscle itself has the ability to produce several hundred different factors.

The first protein found to be secreted by muscle was myostatin (Allen et al., 2008). This protein has many wide ranging functions (Elliott et al., 2012). Myostatin is principally concerned with inhibiting muscle growth and differentiation (Elliott et al., 2012). It is also involved in metabolism and maintaining adipose tissue mass (Allen et al., 2011). Myostatin is suppressed by aerobic and strength exercise (Allen et al., 2011, Hittel et al., 2010). It is therefore clear to see that during age, myostatin may play a greater role, as less activity would mean possible increases in myostatin levels further preventing muscle maintenance.

The first myokine found to be secreted into the bloodstream after muscle stimulation was the cytokine interleukin 6 (IL6) (Pedersen and Febbraio, 2008). Levels of IL6 in the plasma can rise up to 100 fold in response to exercise and increases proportional to the level of exercise (Febbraio and Pedersen, 2005). This occurred without any evidence of muscle damage, indicating that IL6 may have a role in signalling separate to the IL6 mediated pathways in macrophages (Scheller et al., 2011).

The function of IL6 is not certain and there is compelling evidence suggesting both positive and negative effects. The theorised beneficial role for IL6 signalling was to mediate metabolism within the muscle to improve muscle mass (Serrano et al., 2008). IL6 release was greater when muscular levels of glycogen are low; glucose intake during exercise reduces IL6 release (Febbraio et al., 2003). There may also be a role for IL6 in anti-inflammatory responses related with exercise, in particular reducing TNFα levels (Starkie et al., 2003). However chronic release of IL6 directly induces muscle loss (Scheller et al., 2011). Of interest to bone health, IL6 released from mechanically stimulated myotubes in culture can improve

osteoclastogenesis and therefore increase bone resorption (Juffer et al., 2014). IL7 has a similar double edged role in bone formation (Lee and Surh, 2005).

Some other notable myokines are also produced by muscle, often in relation to exercise. Brain derived neurotropic factor (BDNF) is produced within the muscle however this has not been shown to be released into circulation (Pedersen, 2011). BDNF has roles in the oxidation of fat tissue and also has positive effects on neurological tissue to improve innervation (Pedersen, 2011). IL15 overexpression has also been shown to affect fat tissues when released into the circulation (Pedersen, 2011); there is also an increase in bone mass in IL15 overexpression transgenic mouse models (Quinn et al., 2009). Decorin is a leucine rich proteoglycan secreted by muscle during contraction (Kanzleiter et al., 2014). Decorin is involved in muscle hypertrophy and actively reduces atrophy pathways in the muscle by binding to and blocking myostatin (Kanzleiter et al., 2014). Other proteins include FGF2, FGF21, IGF1, IL4, calprotectin and irisin (which has been described above) (Pedersen, 2011). Whether these proteins are released in sufficient amounts to affect other tissues remains unclear.

1.4.2 Bone as a source of chemical signals

Classically bone has been seen as an inert, structural organ within the body. More recent advances however have shown the bone as a contributor to whole body physiology through secretion of proteins into the blood stream which in turn play a role in metabolism. The key cell to this appears to be the osteocyte (Dallas et al., 2013). There are some in vitro studies suggesting that osteocyte like cells will express factors such as IL6, VEGF (Juffer et al., 2012, Kaji, 2013) and IGF1 (Sheng et al., 2013) that are known to influence muscle mass.

Osteocalcin, in particular undercarboxylated-osteocalcin (OCN), is an osteoblast-derived hormone shown to increase muscle sensitivity to insulin and can increase mitochondrial number in mice (Lee et al., 2007, Ferron et al., 2012). OCN knockout mouse models show reduced muscle mass and reduced running ability indicating an important role for this hormone (Brotto and Abreu, 2012). In knockout models, the bone defects appear before the muscle loss, indicating that at least in this model, OCN may play an important role in maintaining muscle

mass (Tagliaferri et al., 2015). This suggests disruption of the bone can feedback to muscle.

1.5 Current treatment options to maintain musculoskeletal structure and function in older people

Despite the increase in the aged population and the work undertaken in this field of research, the current treatment options for combating age-related musculoskeletal conditions are few and often ineffective. What is detrimental to work in this field is that older people can have decreased ability to process drugs, thus a normal dose of medicine can build-up in the body leading to an adverse reaction. Adverse Drug Reactions (ADRs) account for 6.5 per cent of hospital admissions for older people (Pirmohamed et al., 2004).

Treating sarcopenia currently appears to be a combination of improved diet and resistance training to increase muscle mass with some improvements seen although the longer-term effects of such treatments are unclear (Waters et al., 2010). A major problem with treating sarcopenia is that there is currently no clinical definition of sarcopenia (Santilli et al., 2014).

A common age-related joint problem is osteoarthritis. Treatment options here are similar to those seen in sarcopenia with gentle exercise and improvements in diet being the major treatments (Messier et al., 2013). Above these lifestyle interventions the NHS recommend a steady increase in pain management from paracetamol to codeine depending on length of disease state and level of pain (NHS, 2017). When painkillers are no longer effective enough, intra-articular corticosteroid injections are advised however a patient will only be given up to three injections per year. The only definitive treatment option is joint replacement (Fortin et al., 2002).

Tendon problems can be wide ranging from acute tears from excessive force, or inflammatory conditions can develop from overuse. The outcome of the repair depends highly on the location of the injury. According to the NHS, tendon injuries are treated in a similar method to joints with rest and pain management until surgery becomes a viable option (NHS, 2017). There has been very little work on finding successful treatments to aid tendonopathies.

According the NHS, treatment of osteoporosis mainly consists of drugs called bisphosphonates which are thought to reduce the rate of bone break down within the body (NHS, 2017). These drugs are given as either tablet or injection and improvements often not seen until between 6 and 12 months after commencing treatment. The possible length of treatment is up to 5 years (Diab and Watts, 2013). This treatment does however have side effects including irritation of the oesophagus, difficulty swallowing, stomach pain and in rare instances osteonecrosis of the jaw (Kennel and Drake, 2009). Further treatments include strontium ranelate which is known to cause nausea and diarrhoea (Cianferotti et al., 2013); selective oestrogen receptor modulators which can cause menopausal symptoms and can rarely cause deep vein thrombosis (Diez-Perez, 2006), parathyroid hormone which can be prescribed in special cases where other treatments are not showing improvement, and an improvement of diet to include more calcium and vitamin D. More detail on each treatment plus treatment options that are not currently used in the UK will now be described.

1.5.1 Current bone anabolic agents

1.5.1.1 Parathyroid hormone

Parathyroid hormone (PTH) is a naturally occurring hormone found within the blood of reptiles, birds, mammals and fish. This hormone was first recorded in experiments carried out in the early 1970s. It is produced and secreted by the parathyroid glands chief cells as a polypeptide 84 amino acids in length, 9.4 kDa in mass (Jin et al., 2000). PTH has a half-life in serum of approximately 4 minutes (Bieglmayer et al., 2002). The normal concentrations of PTH range from 8-51pg/ml in humans (Jin et al., 2000).

PTH is primarily released in response to low serum levels of calcium recorded by calcium sensitive receptors in the parathyroid gland. Small decreases in magnesium and increases in phosphate are also triggers for PTH release (Poole and Reeve, 2005).

PTH acts through two types of receptor to facilitate different outcomes. PTH receptor 1 is found in high concentrations in the bones and kidneys. PTH receptor

2 is found within the central nervous system, testes, placenta and pancreas (Martini et al., 2012).

In bone, PTH causes the release of calcium from reservoirs contained within the bone (Poole and Reeve, 2005). This is achieved through augmentation of the normal bone turnover processes. PTH binds to osteoblast cells (functioning to lay new bone) which in turn increases the release of Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL). RANKL binds to RANK on premature osteoclast cells (functioning to resorb bone), causing fusion and maturation of the premature osteoclasts, in turn increasing bone resorption (Poole and Reeve, 2005). Short-term PTH exposure will however activate osteoblasts more than osteoclasts leading to a net increase in bone mass (Poole and Reeve, 2005).

In the kidneys, the action of PTH binding to PTHr1 enhances the reabsorption of the minerals calcium and magnesium from the distal tubules of the thick ascending limb increasing the levels of calcium in the circulation (Smith).

The only current anabolic therapy for osteoporosis is a recombinant form of PTH named Teriparatide (PTH1-34) (Eli Lilly and Company, Indiana, USA). Teriparatide has been FDA approved since 2002 for use in osteoporotic men and high risk postmenopausal women and is commonly administered via daily intramuscular injection. Teriparatide has some adverse effects including nausea, headaches and some limb pain. There is also a slight risk of osteosarcoma (1 in 100,000) (Rizzoli and Reginster, 2011).

Despite being used clinically for 12 years, there is still much debate surrounding the method of administration of Teriparatide, the dose and whether combination therapies are more beneficial. The number of publications on the effect of PTH particularly in mice (through varying experimental designs) is still escalating.

Since 2000, a large amount of research has been undertaken to understand both the mode of action for PTH, and the best method of administering the drug to maximise the outcome for patients. Focusing on mouse studies in particular there has been little progress, due in part to differing experimental designs. However, some interesting findings have been reported.

Between 2000 and 2002 when Teriparatide was first introduced, only a small number of papers were published detailing the effect PTH has in "wild type"/normal mouse. In 2002, Iida-Klein et al. described the site-specific action of PTH in C57BL/6J mice (Iida-Klein et al., 2002). Here the authors used 10 week old female C57BL/6J mice and subjected them to a subcutaneous dose of 40ug/kg human PTH (1-34), for 5 days a week for both 3 and 7 weeks. This study showed an anabolic effect of PTH on bone within 2 weeks of treatment with increased bone mineral density (BMD) displayed in the femur and tibia. Using histomorphometric analysis of the tibia, clear increases in trabecular number, area and width were shown with increases in osteocalcin, tartate-resistant acid phosphatase and RANKL. The lumbar spine however only showed significant increases in these measures at the 7 week time point. This study clearly shows a site-specific effect of PTH (Iida-Klein et al., 2002).

The study of Mohan et al. (2002) compared the actions of PTH analogues to improve the increases in bone mass. This study compared the effects of treatment of PTH (1-34) with PTH (1-31) and PTH (2-34) in mice via daily subcutaneous administration of the PTH analogues at 80ug/kg for 15 days (Mohan et al., 2000). This was then followed up with a 22 week study using only PTH (1-34) and PTH (1-31). This work showed that PTH (1-34) was by far the best analogue for increasing bone mass, however, with each type exhibiting differing effects on the bones studied (Mohan et al., 2000).

A key paper by Masiukiewicz (2000) showed that the cytokine interleukin (IL)-6 is an important mediator in PTH action (Masiukiewicz et al., 2000). This group show that IL6 promotes osteoclastogenesis (formation of new osteoclasts) which in turn leads to bone resorption. When oestrogen-deprived female mice were given PTH infused for 5 days, IL6 was significantly increased with a greater increase in bone reabsorption markers (Masiukiewicz et al., 2000). Treatment with oestrogen prevented this increased response (Masiukiewicz et al., 2000). This combination study showed that although PTH is a useful drug, its action can be affected by a large number of factors.

The second of these studies by Samuels et al (2001) used 17beta-estradiol (E2) in combination with PTH (Samuels et al., 2001). Ten week old female mice were

given 10µg/kg of PTH and 40µg/kg E2 and showed that in combination, mice displayed a greater increase in bone mineral density of the femur when measured with Dual-energy X-ray absorptiometry (DEXA) scan with increased cortical and periosteal bone formation (Samuels et al., 2001).

A more recent study (Feng et al., 2015) showed an increase in PTH effectiveness (80µg/kg per day) combined with high calcium levels (2% of diet) (Feng et al., 2015). This study showed significant increases in bone mineral density with increased osteoblast activity and viability time (Feng et al., 2015). However, mice used in this study were 4 weeks of age, so this study has not shown whether this action is enough to maintain bone mass or repair age-related declines in bone.

Taking these latter studies together, we see that although PTH (1-34) is a highly effective drug for increasing bone mass, female hormones are needed to improve the effect.

Other studies have caused confusion within the field due in part to varied experimental designs and outcome measures. Some similarities can be drawn however. The majority of papers published since 2001 have used female mice, usually under the age of five months. These studies all showed an improvement in bone quality and mass but the relevance in terms of osteoporosis is unclear.

Only two papers published since 2000 have used old mice in a study of PTH action. Knopp et al (2005) studied the effect of daily subcutaneous injections of 95ng/kg PTH for 4 weeks in C57BL/6 mice aged 3 and 18 months (Knopp et al., 2005). This study showed that through this regime, the action of PTH was more pronounced in the old mouse, with particular effects in the spine (Knopp et al., 2005). Jilka et al (2010) showed similar increases in the spine as well as the femur (Jilka et al., 2010). One further finding was that PTH appeared to reduce cellular reactive oxygen species generation in the bones of old mice. Here it was hypothesised that PTH had a greater action in older mice due to the higher oxidative stress found within tissues (Jilka et al., 2010) which is also seen in many tissues of aged mammals and different species.

The effects PTH appear to have in younger mice cannot be fully extrapolated to an older mouse due, in part, to the severe age-related changes these mice undergo

in all tissues. There should be care when choosing animal models to study with a greater use of old animals.

Despite the large volume of research in the wider PTH field, less significant progress has been made in terms of mouse studies. It is clear however that attempting to study an ageing phenomenon in younger mice does not provide relevant answers. This may also explain why PTH is currently not widely used as a treatment option and rarely prescribed in the UK.

Recently, interest in PTH has been revitalised following work detailing the use of PTH in tendon repair. Lee et al. (2015) showed significant increases in tendon extracellular matrix deposition combined with increased fibre organisation and tensile strength of the repairs with daily subcutaneous injections of 40ug/kg (Lee et al., 2015). However the mice used where aged between 6 and 8 weeks of age and so more studies are required to confirm these effects in old age.

1.5.1.2 Bisphosphonates

Bisphosphonates are a further class of osteoporosis drugs which are a family of compounds sharing two phosphonate groups. Bisphosphonates can be further divided into two classes, non-nitrogen containing and nitrogen-containing bisphosphonates. Bisphosphonates work to encourage osteoclast apoptosis, slowing the loss of bone. These drugs achieve this effect by mimicking the structure of pyrophosphate, an important enzyme in cells, and target bone by preferentially binding to calcium (van Beek et al., 2003). The bisphosphonate is ingested by osteoclasts during bone turnover (van Beek et al., 2003). Non-nitrogen containing bisphosphonates are metabolised by the osteoclasts and competes with ATP, eventually reducing the cells' ability to respire, leading to apoptosis (Frith et al., 1997). Nitrogen-containing bisphosphates bind and block farnesyl diphosphate synthase, an enzyme in the HMG-CoA reductase pathway (van Beek et al., 2003). This in turn disrupts protein trafficking within the cell. These drugs may also disrupt lipid modification of important cell signalling proteins (van Beek et al., 2003).

Bisphosphonates were first used in the 1960s for bone disorders, however it was not until the 1990s when the first commercial drug was released for use (Shinkai

and Ohta, 1996). There are currently two bisphosphonate drugs widely used for the treatment of osteoporosis, alendronate (Merck, Massachusetts, USA) and risedronate (Warner Chilcott, New Jersey, USA). Zoledronate (Novartis, Basal, Switzerland) has also been in use from 2007. In recent literature, alendronate has been the most studied, however the majority of studies in mice have been with a combination of drugs at varying concentrations and differential experimental designs.

These studies provide a valuable insight into how best to use bisphosphonates for the treatment of osteoporosis. When alendronate is given in combination with PTH, an increase in bone mineral density (Samadfam et al., 2007), an enhancement of the beneficial effects of PTH alone (Yamane et al., 2009) and evidence that alendronate is site-specific with synergistic effects with PTH in the spine but additive effects in the femur is seen (Johnston et al., 2007).

There is some conflicting evidence. Alendronate appears to blunt remodelling of the trabecular bone when combined with PTH (Yamane et al., 2009) but was shown to improve trabecular bone mineral density when combined with vitamin K2 (Sasaki et al., 2010).

Risedronate has been less studied. As with alendronate, risedronate has only been examined in combination with other drugs or treatments. Prior treatment with vitamin K2 significantly improved the action of risedronate, particularly in the femur (Matsumoto et al., 2009). Risedronate is capable of preventing and reversing the deleterious effects on bone of both glucocorticoids (Balooch et al., 2007) and lipopolysaccharides (LPS) (Kwak et al., 2009).

Since 2000, little research has been published looking in depth at the actions of bisphosphonates in mice. Publications show large variations in experimental design and the relevance is diminished by using younger mice. Although the drug is effective and combination therapies appear to increase these benefits, until larger more detailed studies have been undertaken in aged animals, there cannot be direct correlations between these findings and the potential for the treatment of the human disease.

1.5.1.3 Sclerostin

Sclerostin is a protein consisting of 213 amino acids and secreted by osteocytes found within the bone matrix (Winkler et al., 2003). The function of Sclerostin has only recently been alluded to; it appears to bind to the low-density lipoprotein receptor related proteins 5 and 6 (Li et al., 2005), members of a family of transmembrane receptors.

Sclerostin works to inhibit the Wnt signalling pathway and thus decrease bone formation by osteoblasts in a negative feedback loop (Fig 1.8) (Winkler et al., 2003). The production is inhibited by the actions of parathyroid hormone, loading and some cytokines and the production is increased via calcitonin (Bellido et al., 2005).
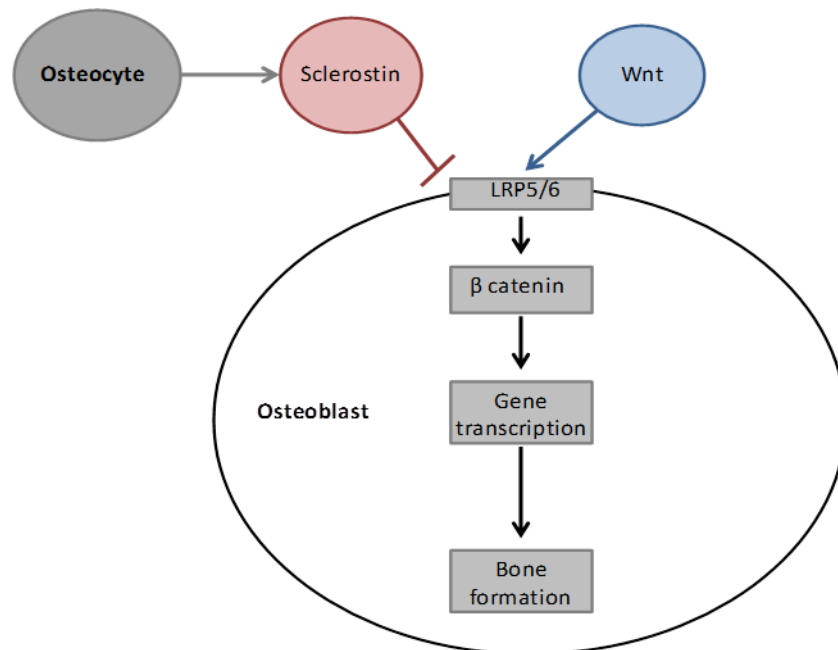
Fig 1.8. Action of Sclerostin to prevent bone formation in the osteoblast.

Antibodies to neutralise the presence and effect of Sclerostin are currently being developed for use in osteoporosis and associated bone disorders. Although not expected to reach the market until at least 2017, pre-clinical trials are ongoing with an anti-Sclerostin antibody (Romosozumab, Amgen) and have shown positive results.

Research in mice using inhibitors of Sclerostin has only become prominent in the last 10 years. Despite this, many papers clearly show one such inhibitor,

Romosozumab, can be a very powerful drug in reversing the effects of osteoporosis (Graeff et al., 2015).

Some of the earliest studies involving Sclerostin used mouse knockout (KO) models. Two papers using the KO models (Li et al., 2008, Lin et al., 2009) showed that whole body knock out Sclerostin increased bone mineral density and bone volume with increased bone formation rate. These models were also resistant to mechanical unloading-induced bone loss.

Further work using antibodies to reduce Sclerostin have been increasing in popularity and form the basis for ongoing clinical trials. These studies clearly show increase in bone strength and volume. There were also improvements in trabecular bone strength and mass. There was a higher force and maximum load to failure with some extra benefits in protecting the articular cartilage and increased fracture repair. Roschger, et al. 2014 found however that in a Col1a1 mouse model, anti-Sclerostin has a reduced effect in more severe disease states (Roschger et al., 2014).

Although the majority of this work has focused on differing mouse models, the benefits with anti-Sclerostin antibody therapies are clearly defined. As with other studies, differing experimental designs make drawing firm conclusions difficult however the improvements in bone structure and function seen in short time frames are promising. It is hoped that future work in mice can complement the ongoing human trials to produce a highly beneficial drug treatment.

1.5.1.4 Non-Pharmacological Interventions

Bone is mainly built upon a bone matrix consisting of both organic and inorganic materials. Considering the process of bone formation, resorption and remodelling, the diet provides a large amount of essential vitamins and minerals necessary to facilitate this.

The bone mineral content or inorganic bone matrix is formed around a mineral form of calcium named hydroxyapatite. Hydroxyapatite consists of calcium and phosphate mineral ions. The organic portion of bone matrix is primarily made up of type 1 collagen. Vitamin C has an important role in the synthesis of collagen through its action on the collagen precursor procollagen.

The need for correct collagen formation and functional inorganic matrix is made clear when looking at disease states. Both osteogenesis imperfecta and chondrodysplasias are conditions affecting the skeleton caused by mutations in collagens (Smith and Wordsworth, 2005).

Bone remodelling is a continuous process throughout life. Approximately 10% of the adult skeleton is remodelling every year. One of the main triggers for this bone remodelling is calcium homeostasis. At times of low calcium in the diet, calcium is released from the bones through PTH action described previously, when calcium is high in the bloodstream bone formation occurs to hold calcium in the mineral form to lay new bone matrix (Smith and Wordsworth, 2005).

Another vitamin needed from correct bone structure and function is vitamin D (Lips and van Schoor, 2011). Vitamin D has a major role in the intestines to allow efficient calcium absorption by the intestines. Although vitamin D can be synthesised through sunlight exposure, in countries such as Britain, Russia and other Northern European countries, exposure is generally not sufficient and skeletal conditions such as rickets or osteomalacia can occur. Vitamin D can be found within the diet and further supplementation or fortification has been used in the past to improve dietary levels (Lips and van Schoor, 2011).

Early in 2000, Kodama, et al (2005) published a study detailing the effect calcium-free diets have on the skeleton of two wild-type mouse strains (C3H/HeJ and C57Bl/6) (Kodama et al., 2000). Over a course of 2 weeks, femur mass decreased along with rate of bone formation. This effect was reversed when calcium was reintroduced to the diet (Kodama et al., 2000). Kasukawa, et al (2003) using IGF1 knockout mouse models, showed that with a low calcium diet for two weeks, that loss of IGF1 exacerbates the effect of calcium depletion (Kasukawa et al., 2003). It was also shown from this work that PTH levels were increased and vitamin D concentrations were reduced suggesting a deficiency (Kasukawa et al., 2003). This work was followed up by Niziolek, et al (2009) who used rapamycin (a downstream IGF1 inhibitor) for 6 weeks (Niziolek et al., 2009). This group showed marked increases in Trap5b (a bone resorption marker) and suppression of the anabolic effects of PTH (Niziolek et al., 2009).

IGF1 is known to cause hypertrophy in a large number of cell types including those found in bone (Sheng et al., 2014). A number of sources claimed that large amounts of IGF1 could be found in deer antler extracts associated with its fast growth. Antler has been fed to 2 month old female SAMP8 mice (Chen et al., 2007) in order to test the effect of increased IGF1. The extract was introduced into the diet of ovarectomised mice for 15 months and showed opposite effects to the IGF1 KO studies (Kasukawa et al., 2003). Chen et al, 2007 showed that the decreases in bone density and calcium contents were maintained with antler extract and that PTH levels were moderated.

The effect of increases in the protein content of the diet on bone tissue has also been studied. Increased collagen levels in diet given to mice following ovariectomy were shown to reduce bone loss and improve cortical thickness (Guillerminet et al., 2012). Given prior to ovariectomy, increased collagen was shown to prevent bone loss altogether (Rouy et al., 2014). When diets were replaced with soy protein and given to mice there was reduced cortical and trabecular bone thickness reduced bone volume and reduced rate of bone formation (Rouy et al., 2014). These studies demonstrate the need for an appropriate level of protein in the diet to maintain bone mass.

Due to the components of bone, plant and vegetable extracts have been examined for beneficial effects on bone. The benefit of using plants and vegetables is that this would allow for a non-pharmaceutical treatment option. Polycan, a beta glycan polysaccharide extracted from yeast, suppressed the ovariectomy associated decreases in bone mineral content, density, and bone thickness and decrease numbers of osteoclasts after 4 weeks of 125mg/kg (Shin et al., 2007). The detrimental effects of low calcium diets can be reversed with high levels of sap from the Acer trees (known to contain extremely high levels of calcium) when given for 7 weeks (Lee et al., 2008). Dried plum extract was used at 25% of normal diet for 6 months in both 6 and 18 month old mice (Halloran et al., 2010). This study showed a 50% increase in bone volume and increase in total bone. Less concentrated extract resulted in the same effect in the younger group (Halloran et al., 2010).

Vitamins and minerals play an important role in bone quality and strength. Vitamin D in particular is necessary in the absorption of calcium into the body. A study in 4 month old mice given 18pmol/day of vitamin D for 6 weeks showed an increase in bone volume and quality and increases in circulating bone formation markers indicating that added vitamin D can facilitate the formation of new bone (Duque et al., 2005). Vitamin K2 has also been shown to improve bone mass and strength, in particular, when given in combination with other known bone anabolic agents (Matsumoto et al., 2009, Sasaki et al., 2010). Another vitamin, vitamin B12 may, in early life, aid maintenance of bone mass and in the growth and development of bone (Roman-Garcia et al., 2014). Nutrients such as magnesium and selenium are also required for bone formation. Two studies using low levels or none at all in the diet led to decreases in osteoblast numbers, loss of trabecular bone and bone volume as well as increases in bone resorption markers (Rude et al., 2003, Cao et al., 2012).

It is evident that maintaining a healthy and balanced diet throughout life can at least partially counteract the negative ageing effects of a poor diet on bone and could improve bone mass in older people. This may be an attractive alternative to drug interventions, or complementary therapies to modulate and enhance drug effects. The converse is also evident, poor diets can have a greater detrimental effect then that of ageing alone.

1.5.1.5 Nitrates

Following on from the recent interest in dietary interventions for use in improving bone health (along with other tissues); dietary nitrates have shown potential due to the recent increase in popularity within athletes and sports nutritionists.

The active molecule of interest is nitric oxide which is now known to be achieved through stepwise reductions from nitrate. This appears to be facilitated through commensal bacteria in the oral cavity of mammals. The common effect of conversion to NO is to modulate blood flow but it has been shown that the body can store and recycle the nitrate-nitric oxide products.

The most common source of nitrates is from the diet with leafy green vegetables being particularly high in nitrates (van Velzen et al., 2008). The nitrate is actively

taken up into the blood via the salivary glands whereby it is concentrated 10-20 times within the saliva. Approximately 25% of nitrates are taken up this way. Whilst remaining in the oral cavity, anaerobic bacteria containing nitrate reductase enzymes reduce approximately 20% of nitrate to nitrite (Doel et al., 2005). These bacteria use the nitrate to produce ATP in the absence of oxygen forming nitrite as a by-product (Doel et al., 2005). The level of nitrite found in saliva is also increased (Lundberg and Govoni, 2004).

This nitrite is converted to NO through the acidic conditions in the stomach (Benjamin et al., 1994). Much of the nitrite found here can pass into the circulation without being converted (Lundberg and Govoni, 2004). Once within the circulation, there are numerous tissues that can convert nitrite into the active NO molecule.

Early studies using NO for bone treatment were promising. In 2001, a review was published summarising the effects of NO on bone cells (van't Hof and Ralston, 2001). This review detailed a biphasic effect of NO, low concentrations caused induction of bone resorption, and high concentrations inhibited bone resorption. A similar effect was seen with bone formation (van't Hof and Ralston, 2001). Small amounts of NO produced constitutively by osteoblasts may act as autocrine stimulators of growth within osteoblast cells (Ralston et al., 1994). When using animals with a subtype of nitric oxide synthase (eNOS) knocked out, major defects in bone formation and osteoblast activity were found (Armour et al., 2001). This group also demonstrated the formation of NO during mechanical stress of both osteoblasts and osteocytes (van't Hof and Ralston, 2001). This process may be active in increasing bone formation in times of increased mechanical stress.

Studies *in vivo* in animals examining the role of NO on bones have been promising, but complement the work showing a biphasic response to NO. The major NO donor used is nitroglycerin, an organic nitrate, used primarily for heart related conditions. It has been shown that nitroglycerin prevented ovariectomy (OVX) and corticoid induced bone loss above and beyond the effect of oestrogen (Wimalawansa, 2000). Nitroglycerin has also been shown to have beneficial effects on both trabecular and cortical bone when given in combination with more

recognised therapies (Wimalawansa, 1995). Furthermore, one daily application of nitroglycerin was sufficient to induce these effects, cumulative effects were not evident.

Orally administered nitrates produce headaches as their side effects. These however can often be tolerated by the patient. The need for only a small dose removes the effect of lowering blood pressure which may have detrimental effects in older people with underlying heart conditions.

It is therefore plausible to suggest that increases in dietary nitrates may lead to increases in NO reaching the skeleton and having beneficial effects in older people and individuals diagnosed with osteoporosis.

1.5.2 Effect of bone anabolic agents on skeletal muscle

There have been very few studies focusing on the action of bone anabolic agents and their effect on skeletal muscle. Considering how closely interlinked both tissues appear to be in both mechanical and biochemical pathways, any action on one tissue could influence the other either directly or indirectly.

1.5.2.1 Parathyroid hormone

Despite parathyroid hormone being used extensively for treating bone disorders, its relationship with muscle has not been fully studied. Early studies from the 1980 are focused on increases in PTH levels in uremia and hyperparathyroidism. Here, three studies showed that high levels of PTH reduced the ability of muscle to metabolise protein, amino acids and nucleotides sufficiently (Garber, 1983). Further studies outlined a diminished ability of muscles to produce and utilise energy sufficiently (Baczynski et al., 1985). This was hypothesised to be the reason for muscle dysfunction within these conditions. However a paper published in 1987 disagreed with this hypothesis suggesting PTH had no effect on muscle with respect to glucose uptake, protein synthesis or degradation or amino acid release (Wassner and Li, 1987). Thompson et al (1996) showed that PTH had no effect on oxidative ATP capacity despite altering phosphate levels within the muscle (Thompson et al., 1996). Visser et al (2003) published data from the longitudinal ageing study of Amsterdam showing that the serum levels of PTH rise with age and may be influential in the progression of sarcopenia in

association with reduced vitamin D (Visser et al., 2003). Although noting many weaknesses with the study, this paper suggested a possible link between PTH and muscle. Citing earlier papers where changes in energy production and protein metabolism were evident, the authors also suggested the role for PTH in maintaining calcium levels could influence the disruption of muscle structure and function (Visser et al., 2003). These authors also hypothesised that due to close links between PTH and IL6; there may be secondary effects on muscle because of the detrimental effect IL6 may have muscle (Visser et al., 2003).

Komrakova et al (2010) measured muscle mass, fibre size and mitochondrial activity in the gastrocnemius muscle of 3 month old normal and ovarectomised rats during PTH treatment for fracture healing (Komrakova et al., 2010). This study found no significant changes in muscle measures during this period although later study by the same author did show the potential for increased vascularisation in muscle following PTH treatment (Komrakova et al., 2010).

1.5.2.2 Bisphosphonates

There have been very few studies examining the action of bisphosphonate drugs on muscle tissue. Some of the first patient reports indicated an early onset of bone, joint and/or muscle pain after bisphosphonate use, suggesting that the drugs appear to be influencing other nearby tissues. This was again reported in 2007 and was noted to be a dose-dependent effect, however no further insight into the causes of the pain was given (Bock et al., 2007). Widrick, et al (2007) published a study monitoring the effects of endurance exercise and alendronate treatment in ovarectomised mice (Widrick et al., 2007). This study showed that exercise increased muscle mass in soleus and extensor digitorum longus muscles as well as increasing tetanic and twitch forces (Widrick et al., 2007) but that the bisphosphonate treatment had no influence on this (Widrick et al., 2007). The most recent study published in 2015 showed data on female human patients using bisphosphonate drugs for at least 3 years (Uchiyama et al., 2015). This group measured age, BMI, BMD and muscle cross-sectional area of the muscle surrounding the thigh. A significant reduction in muscle cross-sectional area in the group of long-term bisphosphonate users was shown and this was uncoupled to

increases in bone mineral density indicating that bisphosphonate action was only on the bone and did not improve muscle mass (Uchiyama et al., 2015).

Overall, bisphosphonate drugs appear to be detrimental to the muscle tissue, or at best have no effect. However more robust studies are required before any firm conclusion can be made.

1.5.2.3 Vitamin D.

Studies have demonstrated that Vitamin D has the potential to of improve both muscle and bone structure and function. Several studies have recently been published showing the beneficial effects of vitamin D on muscle, the positive effects on bone have already been outlined above. Park et al in 2014 demonstrated a clear association between vitamin D levels and sarcopenia in women (Park et al., 2014). This group showed a 1.46 fold increased risk of sarcopenia with a reduction in vitamin D by 10ng/ml in women (Park et al., 2014). Cangussu et al (2015) showed data from a double-blind, placebo-controlled clinical trial in postmenopausal women receiving oral doses of 1000IU/day of vitamin D3 demonstrating a 25.3% increase in muscle strength after 9 months and controlled muscle mass loss when compared to the placebo group (Cangussu et al., 2015). A further multicentre, randomized, controlled, double-blind, 2 parallel-group trial among 380 patients with sarcopenia (Bauer et al., 2015) showed similar trends, using a vitamin D and leucine-enriched whey protein nutritional supplement twice daily for 13 weeks, the authors showed increased muscle mass, and muscle function in the leg (Bauer et al., 2015).

Thus it appears that modifications to dietary intake are a potential for musculoskeletal improvement in older age. It also highlights how small changes to diet may influence ageing without the need for drug intervention.

1.5.2.4 Nitrates

There is strong body of evidence for beneficial effects of nitrates on skeletal muscle especially in relation to improved exercise outcomes. Larsen et al. (2011) showed improvement in oxidative phosphorylation efficiency, improved mitochondrial P/O ratio and a reduction in oxygen cost during exercise in individuals treated with daily doses of 0.1mmol/kg of sodium nitrate (Larsen et

al., 2011). This was followed up by Ferguson et al. (2013) with data showing that supplementation with 0.1mmol/kg/day of nitrate improved vascular control and in predominately fast twitch muscle, elevated skeletal muscle oxygen delivery during exercise (Ferguson et al., 2013a). As well as direct influences on muscle, indirect effects can also improve muscle function such as the effect of nitrate on blood vessels leading to vasodilation, which in turn promotes better oxygen delivery to muscles and organs (Ferguson et al., 2013a, Ferguson et al., 2013b), particularly where bloody supply is reduced, as is proposed in old age.

It is clear that despite the large amount of research being carried out in this field, many conflicting views, methods and outcomes have cast doubt on what may be valuable results in the treatment of musculoskeletal dysfunction in old age. If a robust study could be undertaken to accurately determine the effects that nitrate has on muscle, there is a potential for increased dietary nitrates as an intervention to improve structure and function of multiple musculoskeletal tissues.

1.5.3 Potential muscle anabolic agents

The lack of consensus, and the fact that sarcopenia is such a multifactorial disease, has had a direct impact on the development of potential interventions and thus is significantly under represented when compared to bone anabolic therapies.

1.5.3.1 Dietary Protein

The muscle within our body is reliant on a large source of protein from the diet. Dietary protein is needed for the correct maintenance of muscle mass through the use of amino acids to trigger muscle anabolic pathways (Martini et al., 2012). There is sufficient opinion to state that the levels of protein recommended through adulthood are not enough to maintain muscle mass in the elderly. This contributes to the loss of muscle mass in old age (Landi et al., 2016).

The most effective method of increasing protein within the diet of older people is still highly debated. One key paper showed that giving older people 80% of the daily protein needed in one meal was more effective over a two week time period then evenly distributing the protein over four meals (Arnal et al., 1999). However giving this amount of protein in one meal has been shown to be difficult to

maintain over large time periods and so older people are recommended to eat approximately 25-35g of protein at every meal daily (Bauer et al., 2013).

Often, increased dietary protein is given in combination with an increase in physical activity. It has been shown that resistance exercise in particular is the most beneficial form of exercise as long as the protein levels are high. There is however some debate over when is the best time to ingest this protein. Protein intake following activity has been shown to stimulate protein synthesis, when given at the same time, there is the production of an anabolic environment throughout the body (Landi et al., 2016). The highest level of muscle protein synthesis is observed 60 minutes following activity, so ingesting protein at this time would appear to be the most beneficial (Landi et al., 2016).

1.5.3.2 Heat shock proteins

An intervention that has been extensively researched in muscle is the potential benefit of increased cellular content of heat shock proteins (HSPs). HSPs are molecular chaperones and are a cellular defensive mechanism in response to stresses such as pathological increases in reactive oxygen species (Velichko et al., 2013). HSPs protect cellular proteins from damage. HSPs in muscle have been studied due to evidence of significant reductions in the ability of cells of old mammals to produce these proteins (Vasilaki et al., 2003, Vasilaki et al., 2006a).

Transgenic animal studies have been used in this area to demonstrate the effects of lifelong over-expression of HSPs on muscle function. HSP70 overexpression protected against age-related decreases in muscle force generation and allowed for improved muscle regeneration in old mice (McArdle et al., 2004). HSP10 also preserved force generation and the cross-sectional area of the muscles in old mice (Kayani et al., 2010). Both displayed a reduced level of oxidative damage hypothesising this is the mechanism responsible for the age-related benefit. There are however very few drug interventions to induce a similar effect to the transgenic models. Treatment of mice with 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG) resulted in increased HSP70 levels in the muscles of both adult and old mice but did not result in increased muscle mass or function in the old mice but did improve muscle regeneration (Kayani et al., 2008a). A further HSP70 inducer, arimoclomol, has been shown to slow the

progression of the muscle wasting disease amyotrophic lateral sclerosis (Kalmar et al., 2008).

1.5.3.3 Antioxidant drugs

One of the major hypothesises concerning the cause of musculoskeletal ageing involves the altered production or inadequate removal of reactive oxygen species within tissues (Szczesny et al., 2010). SS31 has been used in animal studies (Min et al., 2011). SS31 is a potent antioxidant that locates to the mitochondria and can significantly attenuate the production of the reactive oxygen species, H_2O_2, which is known to be elevated in old age (Min et al., 2011). It has been shown to reverse age-related depletion of ATP synthesis, improving muscle function and provides an attenuation of H_2O_2 production preventing damage to the muscle proteins (Siegel et al., 2013).

1.5.3.4 Myostatin

Animal studies have focused on myostatin – a negative regulator of muscle mass. Inhibition of myostatin leads to a dramatic increase in muscle mass and muscle force generation however this does not improve specific muscle force production (Gentry et al., 2011). Treatment of mice with bimagrumab (an antibody that targets a receptor ActRII which myostatin binds to inhibiting myostatin) resulted in increased muscle mass with attenuation of muscle weakness (Lach-Trifilieff et al., 2014).

1.5.3.6 Effects of muscle anabolics on the skeleton

Despite evidence that increases in muscle mass and muscle force production can have direct beneficial effects on the underlying bone, there have been few studies investigating the effect of muscle anabolic drugs on bone. Studies have often presented opposing views.

Inhibition of activin A has been looked at in a similar vein to myostatin to induce muscle hypertrophy (Yaden et al., 2014). Studies have shown an increase in bone volume in mice whilst using this inhibitor. In post-menopausal women, treatment with this drug resulted in increases in bone formation and bone density (Yaden et al., 2014). Inhibiting activin via soluble activin receptor IIB fusion proteins results

in improved muscle mass even compared to myostatin studies. It has also been shown to directly improve trabecular bone throughout the skeleton (Lotinun et al., 2012).

1.5.4 Treatment options known to improve both bone and muscle tissue

1.5.4.1 Exercise

One of the most common potential therapies aimed at improving mass and function of musculoskeletal tissues is regular weight-bearing and resistance exercise (Tang et al., 2006). The benefits of these include increased muscle mass, improved balance, reduced falls and improvement to bone mass. Exercise may achieve these improvements through targeting the MTORC1 pathways to increase muscle protein synthesis. The optimal amounts and form of exercise may also reduce the loss of key type II muscle fibres that are reduced during age, increasing the area of those that remain. Aerobic exercise such as swimming or running is known to improve cardiovascular function, increasing heart rate, lung capacity and blood flow (Frankel et al., 2006). It has been shown that this type of exercise can lead to increases in muscle fibre cross-sectional area, mitochondrial volume and muscle enzyme activity (Short et al., 2004).

Resistance exercise has the largest effect on muscle tissue and can increase muscle mass and improve muscle function including increased muscle force generation (Sipila and Suominen, 1995). Improvements can be seen with just one session of resistance training per week (Taaffe et al., 1999). The key to improving muscle mass and function in older people appears to be through prolonged resistance exercise. 2-3 sessions a week of resistance exercise improved physical function, gait speed, balance and muscle strength in older people (Latham et al., 2003).

However, prolonged exercise regardless of type does not prevent the onset of sarcopenia. In a group of veteran athletes of either endurance or power activities, there was still evidence of similar rates of muscle loss with increased age (Drey et al., 2016). These veteran athletes do however have the benefit of increased muscle mass compared with community dwelling older people which may allow for the continuation of muscle function until a later point in life (Drey et al., 2016).

Another benefit of exercise on muscle in particular is the adaptation, with improvement of antioxidant defences via upregulation of relevant enzymes (Kayani et al., 2008b) and decreased inflammatory cytokines (Marzetti et al., 2008, Nicklas et al., 2008). Exercise has also been shown to reduce inflammatory cytokines which are detrimental throughout the body (Greiwe et al., 2001, Petersen and Pedersen, 2005). Exercise also clearly increases mitochondria number via activation of PGC1a and by doing so potentially increases the muscles functionality.

Studies into the effect of exercise on osteoporosis are a subject of contention. Although many studies are published, very few follow similar experimental designs and protocols, making comparisons difficult.

Despite this, regular exercise is proposed as the most a beneficial approach for osteoporosis (Berard et al., 1997, Gomez-Cabello et al., 2012). The type of exercise that is potentially beneficial differs slightly to what is reported for muscle and suggests a programme of high load non mass bearing exercise should be used in the elderly.

Positive effects of exercise on muscle and bone are not permanent and differ between old and young populations, however regular exercise appears to be important. After 6 months of exercise cessation, positive effects in muscle and bone are lost (Taaffe and Marcus, 1997, Taylor et al., 2004).

There are currently no exercise types in particular that result in universal benefits for all musculoskeletal tissues however resistance exercise is clearly beneficial for muscles and this may induce improvements in the other tissues. There is a need for increased exercise to be introduced prior to reaching old age to ensure people are reaching their peak muscle and bone mass before the declines are seen.

1.5.4.2 Antioxidant therapies

One of several hypothesises proposed as a mechanism of musculoskeletal ageing involves the aberrant production of or inadequate removal of reactive oxygen species within tissues (Szczesny et al., 2010).

The function of reactive oxygen species (ROS) has been studied over a considerable time period and a large amount of research has been undertaken to characterise the potential sources of ROS. The overarching assumption was that ROS were detrimental to cells and tissues and that cumulative damage by ROS to lipids in cellular membranes, DNA, proteins and other sub cellular structures lead to cellular damage and eventually death (Vasilaki et al., 2006b).

In the tissues of older people and animals including muscle tissues, oxidation of integral proteins such as myosin is present, preventing adequate functionality. There is also evidence of increased levels of mitochondrial DNA damage and decreased numbers of functional mitochondria with age (Vasilaki et al., 2006b, Vasilaki et al., 2006a). Damaged mitochondria are proposed to produce excessive ROS which in turn feedback to cause further damage (Miquel et al., 1980, Baines et al., 2014). However, recent work in animal models have shown no correlation between oxidative damage and life span, potentially arguing against the hypothesis that oxidative damage is the direct cause of ageing, although it may be a compounding factor (Gems and Doonan, 2009, Perez et al., 2009).

With further understanding of the role of ROS as essential signalling molecules, the theory has been altered to incorporate recent interest in defective signalling involving these reactive oxygen species (Jackson, 2016). Signalling by ROS is essential for a number of processes, including increased expression of protective proteins within cells, (Jackson, 2016). In young adult mammals, increased ROS leads to an increase in cellular protective proteins. However in older mammals this response does not occur. It is proposed that there is already a chronic increased level of ROS within the tissues of older species and a chronic activation of the regulatory, protective pathways (Jackson, 2016). This in turn leads to a lack of further increases in ROS when additional stimuli are present, leading to a lack of adaptations to acute stresses (Vasilaki et al., 2006b). In addition to this, the chronic increased production of ROS continues to damage lipids, proteins and DNA within the cell (Vasilaki et al., 2006b). Thus, researchers have focused on modifying the chronic increase in ROS generation in tissues of older mammals in an attempt to reduce the inability to produce ROS in response to acute stimuli and a number of drug targets are being developed. SS31 is proposed to be a potent antioxidant that locates to the mitochondria (Cho et al., 2007) although whether it

is the action of SS31 as an antioxidant that provides protection is controversial. It has been shown to reverse age-related depletion of ATP synthesis and improve muscle function. Another drug, MitoQ has been developed however long-term supplementation did not produce beneficial effects in old mice, failing to improve muscle function in old age compared with placebo treated mice (Sakellariou et al., 2016).

1.5.4.3 Heat shock proteins

HSPs are molecular chaperones found within the cell within various subcellular compartments. The main function of HSPs during normal cellular conditions is maintenance and control of protein folding, transportation and maturation of nascent proteins. Heat shock proteins are rapidly synthesized when cells are exposed to a wide variety of stresses including inflammation, hypoxia in an attempt to maintain this control on protein formation and to avoid denaturing or misfolding (Vasilaki et al., 2003).

Increasing HSP content of cells is an attractive proposition, since evidence shows a clear decline in the amount and functionality of heat shock proteins with age. Currently being studied are the effects of HSP10 and HSP70 as mentioned previously in relation to preserving muscle (Kayani et al., 2008a, Sasaki et al., 2010).

In bone, less work has been done in this area due in part to the complexity of studying the skeleton in detail compared to the musculature and the impossibility of studying oxidant levels in vivo in bone. It has been shown that free radicals and reactive oxygen species are a driver of osteoclastogenesis and are essential in bone resorption (Srinivasan et al., 2010). It is therefore reasonable to suggest increases in antioxidants will reduce the production of osteoclasts and prevent the resorption of bone. One study has correlated that decreases in bone mineral density follows increases in oxidative stress (Smietana et al., 2010). Further papers stress the possibility for associations but often fail to reach significant data.

Despite this, Pycnogenol has been used in animal studies in an attempt to reduce the reactive oxygen species in bone and has produced significant benefits in a model of ovariectomy (Huang et al., 2015).

1.5.4.4 Anti-inflammatory agents

Age-related increase in inflammatory cytokines is a condition in a large proportion of older animals and people. Whether this is a consequence of ageing of tissues or caused by the presence of pathologies is debatable (Bartlett et al., 2012). Inflamm-ageing as it has been termed has been linked to decline in muscle mass and function and to the decline in bone mass and function as well as joint problems and other age-related conditions (Franceschi et al., 2000).

Age-related inflammation includes the chronic increased release and circulation of cytokines such as TNFα and IL6 which are both highly correlated with declines in muscle function (Franceschi et al., 2000). More recently, evidence that muscles can express and secrete cytokines (myokines) has led to the theory that muscle may play some role in the increase of inflammatory cytokines in old age (Nielsen and Pedersen, 2008).

TNFα has a direct effect on muscle fibres resulting in reduction in total muscle protein as it initiates protein degradation (Li et al., 1998, Li et al., 2000). Further studies with hospitalised old patients have shown significant declines in grip strength with increasing incidence of inflammatory mediators with further studies supporting this link (Norman et al., 2014).

It is proposed that use of non-steroidal anti-inflammatory drugs may reduce the detrimental effects of increased inflammatory cytokines with age however very few studies have been carried out examining the role of anti-inflammatory drugs for muscle and bone (Munoz-Ortego et al., 2014). A study in Crohn's disease has suggested that anti TNFα drug named infliximab is a potential intervention to reduce the loss of muscle mass caused by increases in inflammation. TNFα inhibitors have also shown promise in treating osteoporosis as osteoclasts (bone removing cells) are highly dependent on TNFα to function (Allali et al., 2003). Studies in patients with Ankylosing Spondylitis display promising improvement in bone mass and strength when treated with such drugs (Kang et al., 2014).

Studies have also focused on the potential use of dietary anti-inflammatory compounds to modify musculoskeletal function. Resveratrol is a naturally occurring substance found in plants which has recently been shown to have anti-

inflammatory effects. Recent studies show beneficial effects of this agent on both muscle and bone however there is no evidence that treatment will prevent the loss of muscle mass (Jackson et al., 2011). In bone the effects are clearer, displaying improved bone in rodent models of post-menopausal osteoporosis as well as improved responses to age-related bone loss when given prior to bone loss (Durbin et al., 2014, Pearson et al., 2008).

1.6 Hypothesis

The main hypothesis of this study is that with increasing age in mice, there is a decline in the mass and function of all musculoskeletal tissues. The secondary hypothesis is that this decline could be prevented through a series of interventions including increased lifelong activity, a whole body over-expression of Heat Shock Protein 10 (HSP10) or an increase in dietary nitrates.

1.7 Aims

This aims of this study were as follows:

- To integrate methods of visualising and measuring age-related changes in musculoskeletal tissues.
- To identify changes in musculoskeletal tissues throughout the life span of C57BL/6 mice.
- To determine the effects of increased sodium nitrate intervention, both short-term and longer-term, on changes seen in the musculoskeletal system of C57Bl/6 mice with age.
- To examine the effect of lifelong overexpression of HSP10, known to preserve muscle CSA on other tissues from the musculoskeletal system.
- To compare data from C57Bl/6 mice with changes in musculoskeletal tissues of male and female wild mice which demonstrate a significant increase in activity

The use of an integrated approach to measuring and visualising age-related change in musculoskeletal tissues is primarily found in Chapter 3 but is addressed throughout the thesis. Chapter 3 looks to complete aim 2 and will identify changes in musculoskeletal tissues of the C57Bl/6 mouse through age. We will then move into providing these mice with a non-invasive dietary intervention of sodium nitrate in Chapter 4. Chapter 5 will address how overexpressing an antioxidant protein in the C57Bl/6 mice through life will affect ageing of musculoskeletal tissues. Chapter 6 will address whether C57Bl/6 mice are the ideal model to study musculoskeletal ageing in mice and whether lab based mice are artificially ageing compared to wild mice.

Chapter 2

Experimental Methods

2.1 Chemicals and reagents

Unless stated otherwise, all chemicals and reagents used in this study were obtained from Sigma chemical company, Dorset, UK

2.2 Mouse husbandry

All experiments were undertaken in accordance with UK home office guidelines under the UK Animals (scientific Procedures) Act 1986 and with ethical approval from the University of Liverpool Animal Welfare Ethical Reviews Body (AWERB).

2.2.1 C57 Bl6 mice

For the ageing study presented in Chapter 3, a total of 42 C57BL/6 mice were used (6 mice per group) at a range of ages with power calculations based on previous studies. Mice were culled at 3, 6, 9, 12, 18, 24 and 28 months of age. These ages were based on literature and previous experiments by the McArdle group. C57Bl/6 mice were purchased from Charles River (Lyon, France) and was delivered 1 month prior to the required age except for the 28 month cohort which were delivered at 24 months of age and allowed to age within the Biomedical Services Unit at the University of Liverpool. Mice were fed a CRM (P) rodent diet with ad libitum access to food and were maintained under barrier conditions in microisolator cages on a 12-h dark/light cycle. A detailed breakdown of mouse chow is included in Figure 2.1. Twenty eight months was deemed a suitable end point based on survival data provided by Jackson laboratories, Maine, USA (Fig 2.1)

2.2.2 Wild mice

Mice used in the wild mouse study of Chapter 4 were obtained from an ongoing behavioural study led by Professor Jane Hurst at the Leahurst campus at the University of Liverpool.

Male and female mice where captive bred *Mus musculus domesticus* derived from ancestors captured from five differing populations around the North West of England, UK. Ancestor mice were captured within 20 miles of each other except

for two, which were captured 80 miles away. This was to introduce genetic variance.

Calculated Analysis

Proximate Analysis

NUTRIENTS		Total	Supp (9)
Moisture (1)	%	10.00	
Crude Oil	%	3.36	
Crude Protein	%	18.35	
Crude Fibre	%	4.23	
Ash	%	6.27	
Nitrogen Free Extract	%	57.39	

Digestibility Co-Efficients (7)

		Total	Supp (9)
Digestible Crude Oil	%	3.05	
Digestible Crude Protein	%	16.44	

Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)

		Total	Supp (9)
Total Dietary Fibre	%	15.06	
Pectin	%	1.40	
Hemicellulose	%	8.85	
Cellulose	%	3.89	
Lignin	%	1.40	
Starch	%	42.37	
Sugar	%	3.90	

Energy (5)

		Total	Supp (9)
Gross Energy	MJ/kg	15.01	
Digestible Energy (15)	MJ/kg	12.27	
Metabolisable Energy (15)	MJ/kg	11.19	
Atwater Fuel Energy (AFE) (8)	MJ/kg	13.93	
AFE from Oil	%	9.08	
AFE from Protein	%	22.03	
AFE from Carbohydrate	%	68.90	

Fatty Acids

		Total	Supp (9)
Saturated Fatty Acids			
C12:0 Lauric	%	0.03	
C14:0 Myristic	%	0.14	
C16:0 Palmitic	%	0.33	
C18:0 Stearic	%	0.06	
Monounsaturated Fatty Acids			
C14:1 Myristoleic	%	0.02	
C16:1 Palmitoleic	%	0.10	
C18:1 Oleic	%	0.87	
Polyunsaturated Fatty Acids			
C18:2(ω6) Linoleic	%	0.96	
C18:3(ω3) Linolenic	%	0.11	
C20:4(ω6) Arachidonic	%	0.11	
C22:5(ω3) Clupanodonic	%		

Amino Acids

		Total	Supp (9)
Arginine	%	1.19	
Lysine (6)	%	1.04	0.17
Methionine	%	0.28	0.02
Cystine	%	0.29	
Tryptophan	%	0.22	
Histidine	%	0.46	
Threonine	%	0.69	
Isoleucine	%	0.77	
Leucine	%	1.46	
Phenylalanine	%	0.96	
Valine	%	0.91	
Tyrosine	%	0.69	
Taurine	%		
Glycine	%	1.55	
Aspartic Acid	%	1.00	

NUTRIENTS		Total	Supp (9)
Glutamic Acid	%	3.72	
Proline	%	1.34	
Serine	%	0.78	
Hydroxyproline	%		
Hydroxylysine	%		
Alanine	%	0.21	

Macro Minerals

		Total	Supp (9)
Calcium	%	0.83	0.72
Total Phosphorus	%	0.64	0.19
Phytate Phosphorus	%	0.23	
Available Phosphorus	%	0.41	0.19
Sodium	%	0.27	0.22
Chloride	%	0.40	0.35
Potassium	%	0.69	
Magnesium	%	0.22	0.01

Micro Minerals

		Total	Supp (9)
Iron	mg/kg	130.65	60.21
Copper	mg/kg	16.42	6.90
Manganese	mg/kg	91.05	44.90
Zinc	mg/kg	86.59	52.86
Cobalt	µg/kg	494.92	420.30
Iodine	µg/kg	390.43	310.17
Selenium	µg/kg	265.49	100.34
Fluorine	mg/kg	9.63	

Vitamins

		Total	Supp (9)
β-Carotene (2)	mg/kg	1.28	
Retinol (2)	µg/kg	5218.35	4500.38
Vitamin A (2)	iu/kg	17376.38	15001.26
Cholecalciferol (3)	µg/kg	76.94	75.00
Vitamin D (3)	iu/kg	3077.42	3000.00
α-Tocopherol (4)	mg/kg	93.03	72.81
Vitamin E (4)	iu/kg	102.81	80.09
Vitamin B$_1$ (Thiamine)	mg/kg	15.84	9.83
Vitamin B$_2$ (Riboflavin)	mg/kg	13.28	11.76
Vitamin B$_6$ (Pyridoxine)	mg/kg	17.65	13.74
Vitamin B$_{12}$ (Cyanocobalamine)	µg/kg	78.17	75.00
Vitamin C (Ascorbic Acid)	mg/kg	1.80	
Vitamin K (Menadione)	mg/kg	185.05	180.00
Folic Acid (Vitamin B$_9$)	mg/kg	4.30	2.94
Nicotinic Acid (Vitamin PP) (6)	mg/kg	78.92	27.65
Pantothenic Acid (Vitamin B$_{3/5}$)	mg/kg	25.24	11.56
Choline (Vitamin B$_{4/7}$)	mg/kg	899.51	75.63
Inositol	mg/kg	2253.88	12.78
Biotin (Vitamin H) (6)	µg/kg	488.74	230.85

Notes
1. All values are calculated using a moisture basis of 10%. Typical moisture levels will range between 9.5 - 11.5%.
2. a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene.
   b. Retinol includes the Retinol equivalents of β-Carotene.
   c. 0.48 µg Retinol ≡ 1 µg β-carotene ≡ 1.6 iu Vitamin A activity
   d. 1 µg Retinol ≡ 3.33* iu Vitamin A activity
   e. 1 iu Vitamin A ≡ 0.3 µg Retinol ≡ 0.6 µg β-carotene
   f. The standard analysis for Vitamin A does not detect β-carotene
3. 1 µg Cholecalciferol (D$_3$) ≡ 40.0 iu Vitamin D
4. 1 mg all-rac-α-tocopherol ≡ 1.1 iu Vitamin E activity
   1 mg all-rac-α-tocopherol acetate ≡ 1.0 iu Vitamin E activity
5. 1 MJ ≡ 239.23 Kcalories ≡ 239.23 Calories ≡ 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy ≡ Atwater Fuel Energy ≡ ((CO%/100)*9000)+ ((CP%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
15. Calculated.

Fig 2.1 Detailed breakdown of CRM (P) rodent diet provided by Special Diets Services.

Each male mouse was individually housed in a 48 cm × 11.5 cm × 12 cm cage for the duration of the experiment (M3, North Kent Plastic Cages Ltd., UK), females were housed in 45 x 28 x 13cm cages (MB1, North Kent Plastics, UK) in single-sex small family groups (2-4 animals) with Corn Cob Absorb 10/14 substrate and paper-wool nest material, with ad libitum access to food (LabDiet 5002) and water.
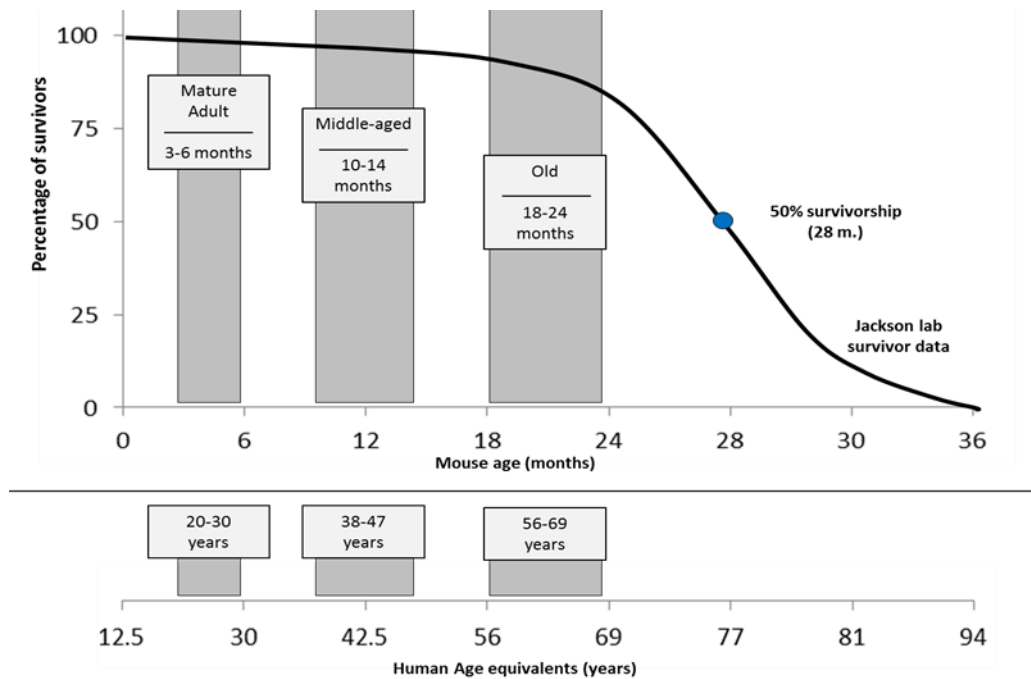
Fig 2.2 C57Bl/6 survival data showing human age comparisons. Adapted from *The Mouse in Biomedical Research 2nd Edition*. Fox JG, et al, (2007).

2.2.3 HSP10 overexpressor mice

Male HSP10 overexpressor mice studied in Chapter 5 have been previously described (Kayani et al., 2010). Mice were originally produced to overexpress both HSP60 and HSP10. Mice were generated via chimeric transgenes containing of human gene variants of HSP10 and HSP60 which were inserted into pCAGGS vectors. The pCAGGS construct should allow both genes to be under the control of the human cytomegalovirus (CMV) intermediate early (hCMV-IE) enhancer and chicken β-actin promoter and intron 1. The CAGGS-HSP10 fragment was cut from the Bluescript II KS vector using Xho1/Not1 digestion to produce a 3.3kb fragment. The CAGGS-HSP60 fragment was cut from the BlueII KS vector using Sal 1/Not1 digestion to produce a 4.8kb fragment. Both fragments were purified

and used for microinjection into mouse oocytes. Previous western blot analysis demonstrated that only HSP10 content was significantly elevated but HSP60 was not overexpressed in skeletal muscle or other tissues of these transgenic mice at 6 months old. HSP10 levels remained elevated until at least 24 months old (Kayani et al., 2010). The mice from this study are therefore referred to as HSP10 overexpressor mice. Mice were housed within the Biomedical Services Unit at the University of Liverpool. Mice were fed a CRM (P) rodent diet and were maintained under barrier conditions in microisolator cages on a 12-h dark/light cycle.

2.2.4 Mice used for sodium nitrate intervention

Two groups of male C57Bl/6 mice used for the sodium nitrate intervention were purchased at 7 and 16 months of age from Charles River and allowed to age within the Biomedical Services Unit at the University of Liverpool. Mice were fed a CRM (P) rodent diet and were maintained under barrier conditions in microisolator cages on a 12-h dark/light cycle.

The long-term nitrate study was started when the male mice reached 9 months of age and involved 20 mice split into two groups of 10 mice. The control group was given 250ml standard drinking water changed weekly. The treatment group was given 1mM sodium nitrate (21mg/250ml) in the drinking water which was also changed weekly. This was repeated until the mice reached 24 months of age. The short-term study was started when mice were 18 months of age. For the short-term study, 16 mice were split into two groups of 8 and treated as above. The study ended when the mice reached 24 months of age.

2.3 Sample collection and preparation

Following completion of all procedures, mice were killed by cervical dislocation. Muscles from both hindlimbs and a range of organs were dissected and mass were recorded. Following this, tissues were snap-frozen in liquid nitrogen and stored at -80°C.

For tendon analysis, the *Achilles* tendon and *posterior tibialis* tendon with associated muscle tissue were left attached to the foot prior to dissection and were

snap-frozen in liquid nitrogen and stored at -80°C. Tendons mass and function was only assessed for the C57Bl/6 ageing study presented in Chapter 3.

Hindlimbs had all remaining muscle tissue removed from the bone. Bones were then stored in Formalin solution for 24 hours. Following this, hindlimbs were transferred to 70% ethanol for long-term storage.

2.4 Microscopy and imaging

Microscopy and imaging of samples used in this study has been detailed in Sakellariou et. al (2016). Anterior tibialis muscles were cryosectioned at -20°C through the mid-belly with a thickness of 12µm and sections were stained with fluorescent immunohistochemical staining. Sections were rinsed in phosphate buffered saline and permeabilized in 0.2% Triton X-100 for 5 minutes. Extracellular matrix was identified through fluorescein labelled wheat germ agglutinin (Vector Laboratories, Burlingame, California). Nuclei were identified with 4,6-diamidino-2-phenylindole (DAPI, 1µg/ml). Cross sections from 6 muscles/age group were examined by blinded observers to allow total number of fibre and individual fibre CSA to be measured. Image J software was used to quantify fibre CSA.

2.5 *In situ* muscle contractions induced by electrical stimulation

Mice subjected to in situ muscle force measurements were anesthetised with intraperitoneal (IP) injections of ketamine hydrochloride (66 mg/kg) and medatomidine hydrochloride (0.55 mg/kg), with supplemental injections provided to maintain an adequate level of anaesthesia throughout the procedure.

The contractile properties of *extensor digitorum longus* (EDL) muscles were measured in situ as previously described (McArdle et al., 2004). To assess the maximum isometric tetanic force (Po) of the EDL muscle, the distal tendon from anesthetised mice was secured to the lever arm of a servomotor (Aurora Scientific, Ontario, Canada). The knee of the hindlimb was fixed, the peroneal nerve was exposed, and platinum wire electrodes were placed across the nerve. Muscle optimal length (Lo) was determined using a series of 1 Hz stimulations and the muscle was set at the length that generated the maximal force. For determination of Po, EDL muscles were electrically stimulated to contract at Lo

and optimal stimulation voltage (8–10 V) at 2min intervals for 300ms with 0.2ms pulse width. Po was assessed by increasing the frequency of stimulation from 10 to 50 Hz and subsequently in 50-Hz increments to a maximum of 300Hz. Po was identified when the maximum force reached a plateau despite increasing stimulation frequency.
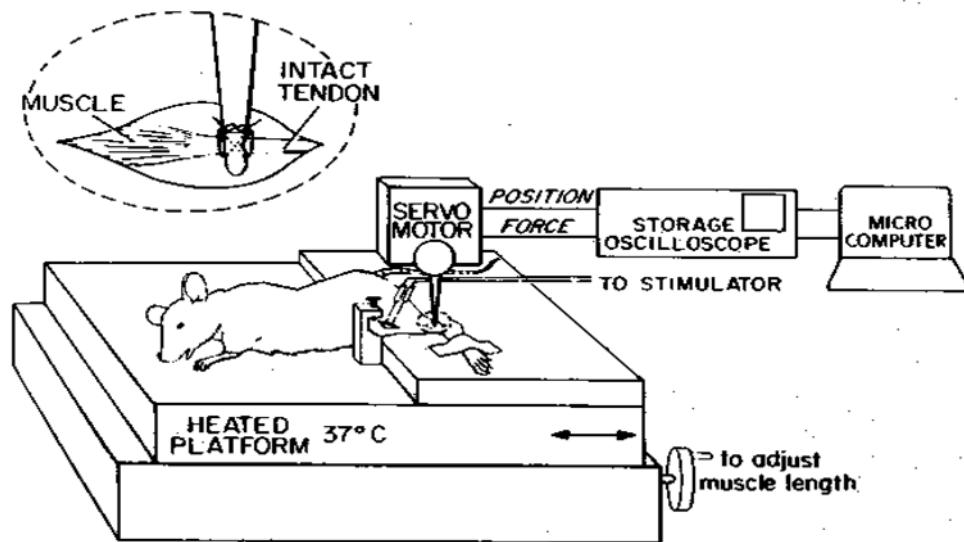
Fig 2.3 Schematic displaying arrangement for in situ muscle force measurements (Taken from Brooks & Faulkner)

2.6 Tendon stretching through application of force

2.6.1 Preparation of the *posterior tibialis* tendon sample

Intact mouse feet were removed with the *posterior tibialis* muscle attached via the tendon and stored at -80°C until use. Prior to testing, each specimen was removed from the freezer and allowed to defrost for 1hr before excising the tibialis posterior tendon from the intact foot and removing any excess tissue leaving only the tendon (Fig 2.3).

Fig 2.4 Representative mouse foot sample with Achilles and *posterior tibialis* tendon visible.

Samples were placed on glass slides along with three drops of PBS to maintain tissue hydration. Images were taken of each tendon. Five diameter measurements were taken from each image at approximately 100 μm intervals using a Nikon Eclipse Ci microscope equipped with a 10x/0.30 lens and a Nikon DS-Fi2 camera. Means of the five measurements were used to calculate the cross-sectional area of the tendon.

2.6.2 Mechanical testing of the *posterior tibialis* tendon

All tensile tests were performed on an Instron 3366 material testing machine (Instron, Massachusetts, USA) equipped with a 10N load cell in conjunction with Bluehill materials testing software (Instron, Massachusetts, USA) (Fig 2.4). Samples were secured into custom clamps using a cyanoacrylate adhesive (superglue). Specimens were allowed to hydrate in phosphate-buffered saline (PBS) for 15 minutes prior to the start of the test.

Specimens were secured into the clamps using a 2 mm spacer to ensure consistent gauge length. Prior to loading into the uniaxial tensile testing machine, the grip-to-grip distance was measured using a digital Vernier micrometre. In instances where glue overran, the glue-to-grip distance was measured for strain calculations. Each specimen was loaded into the custom clamps initially under slight compression and then each specimen was elongated until a load of 0.01 N was achieved, so that a reference point could be established for repeatable testing.
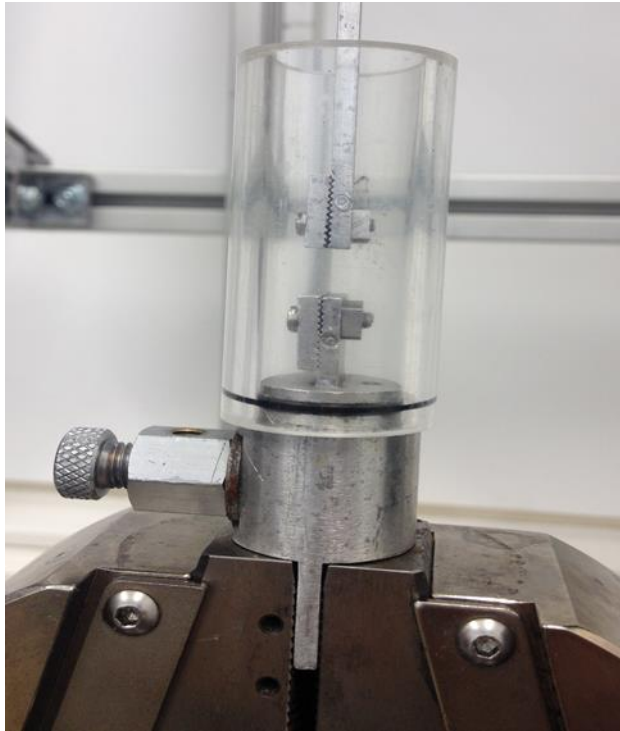
Fig 2.5 Instron 3366 material testing machine

Each specimen was subjected to 6 loading cycles to a peak load of 1.0 N and the results from the last cycle were used to determine the stress-strain behaviour of the specimen.

The selected strain rate was chosen as it has been previously used on mouse achilles tendons (Ansorge et al., 2011). Specimens were subjected to a 6 minute recovery period succeeding each cycle. Due to a large percent of specimens slipping out of the clamps at larger loads, failure properties were not reported.

The load-elongation parameters were obtained, so the stress and strain could be calculated. The stress, σ, was calculated by dividing the applied load, F, by the mean cross-sectional area (πr^2), A, and the strain, ε, was calculated by dividing the elongation, ΔL, by the original length, L.

In addition, the tangent modulus, a measure of material stiffness, was calculated from the gradient of the corresponding tangent line of the stress-strain curve (i.e. $d\sigma/d\varepsilon$).

2.7 Histological analysis of mouse knee joint

2.7.1 Preparation of knee joints

Knee joints were stored as hindlimbs in 70% ethanol. Prior to sectioning, hindlimbs were decalcified in EDTA solution at room temperature in a non-agitated environment for 4 weeks. Following decalcification knee joints were embedded in paraffin in a coronal orientation with the back of the joint facing downwards. Sections were cut on a HM355S microtome (Thermo Fisher Scientific, Massachusetts, USA) at 6 micron thickness throughout the joint.

2.7.2 Haematoxylin and eosin (H&E) staining

Sections were placed in Harris' Haematoxylin for 3-5 min, rinsed in distilled H_2O and counterstained in eosin for 30 sec. Sections were then dehydrated in 3x Absolute alcohol and cleaned in xylene. Cover slips were mounted onto microscope slides using DPX mountant. Sections were analysed with the use of an inverted microscope (Zeiss Axiovert 200M microscope (Oberkochen, Germany)).

2.7.3 Safranin-O staining

Sections were placed in Weigert's Iron Haematoxylin for 5 minutes and rinsed in distilled H_2O. Sections were then differentiated in 1% acid-alcohol solution for 2 seconds and rinsed. Following this, sections were placed in 0.02% Fast Green solution for 1 minute, 1% acetic acid for 30 seconds, 1% Safranin O for 30 minutes and then briefly rinsed in 95% ethanol. Sections were then dehydrated in 3x Absolute alcohol and cleaned in xylene. Cover slips were mounted onto microscope slides using DPX mountant.

2.7.4 Histological scoring of the mouse joint

Histological scoring was undertaken by experts at Leahurst campus, University of Liverpool as previously described (Glasson et al., 2010). Femoral and tibial cartilage was scored for lesions on a scale between 0-6, formation of osteophytes were also assessed based on a scoring scale of 0-3 (Glasson et al., 2010). Samples were scored blindly and performed twice to assess inter observer variability at

Leahurst campus. Normality test (Kolmoigorov-Smirnov test) was performed on data. This indicated the n number was too small to allow for wild male and female to be separated based on sex in Chapter 4. Therefore wild female and male results were pooled together.

2.8 MicroCT scanning of mouse hindlimb bones

2.8.1 Preparation of hindlimbs for MicroCt scanning

Both hind limbs with knee joint intact were dissected from each mouse. Muscle tissue was removed and bones stored in Formalin for 24hrs. Bones then washed with PBS and placed in 70% ethanol.

2.8.2 MicroCT scanning

Both the tibia and femurs bones of each hindlimb were scanned using a μCT system across the knee joint (Skyscan 1272 X-Ray microtomograph, Bruker Corporation, Kontich, Belgium) to evaluate trabecular architecture and cortical bone measures (Fig 2.5).

Fig 2.6 Representative X-Ray image of mouse hindlimb showing general area of bones scanned for analysis.

For trabecular scanning, high-resolution scans with an isotropic voxel size of 4.3 μm were acquired (60 kV, 0.5 mm aluminium filter, 0.3° rotation angle). The isotropic voxel size was changed to 10 μm for cortical analysis.

2.8.3 Analysis of mouse hindlimb bones following MicroCT scan

The scans were reconstructed using NRecon software (Bruker, Massachusetts, USA). For analysis, a 1 mm section of the metaphysis was taken for the measurement of trabecular bone, using the base of the growth plate as a standard reference point. A 500 μm section of the mid-shaft was taken for the analysis of cortical bone, using the base of the growth plate as a standard reference point. CTAn software (Bruker, Massachusetts, USA) was used to analyse the appropriate parameters previously outlined (Bouxsein *et al*. 2010) as shown in Table 2.1.
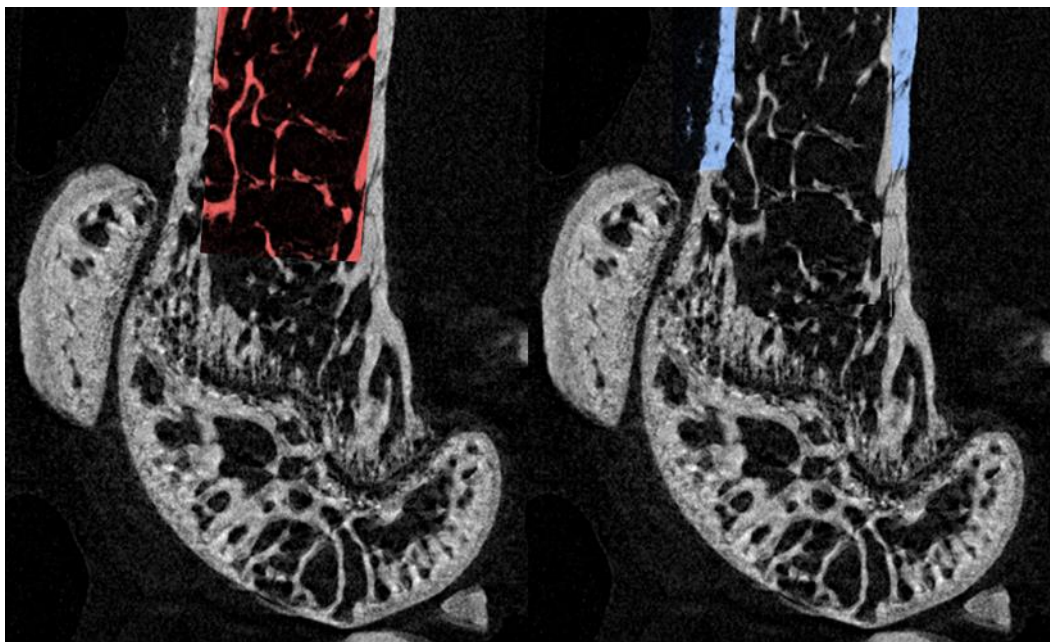
Fig 2.7 Representative MicroCT images of the femur highlighting different regions for analysis – Red area represents the trabecular bone area analysed, blue area represents the cortical region.

Tissue volume	
TV	Volume of the entire region of interest
mm^3	
Bone volume	
BV	Volume of the bone present in the region of interest
mm^3	
Percent bone volume	
BV/TV	Ratio of the segmented bone volume to the total volume of the region of interest
%	
Bone surface density	
BS/TV	Ratio of the segmented bone surface to the total volume of the region of interest
1/mm	
Mean total crossectional bone area	
B.Ar	cross-sectional area inside the periosteal envelope
mm^2	
Cortical bone thickness	
Cb.Th	Average cortical thickness
mm	
Trabecular thickness	
Tb.Th	Mean thickness of trabeculae, assessed using direct 3D methods
mm	
Trabecular number	
Tb.N	Measure of the average number of trabeculae per unit length
1/mm	

Table 2.1 Table of commonly used bone measures within this study with units and description.

2.9 Statistical analyses

Data was tested for normality and equal variance using SigmaPlot (v11.0) (Systat Software, Inc., London, UK). Direct comparison between two sets of data was made by Student's *t*-test. For multiple comparisons, data was analysed for statistical significance by one-way ANOVA incorporating pairwise comparisons. Data are presented as mean ±SEM. Values $P<0.05$ were considered significant.

Chapter 3

Characterising age-related changes in
musculoskeletal tissues of C57Bl/6 mice

3.1 Introduction

Signs of musculoskeletal ageing such as a loss of muscle mass and force, loss of bone mass or loss of joint functionality do not commonly present until the 4th decade of life in humans. Once the decline in these tissues appears, it often takes a further forty years to develop into a full pathology (Faulkner et al., 2007). This slow time course of changes presents a challenge to studies examining the effects of ageing on musculoskeletal tissues in humans and has resulted in a lack of robust studies into the pathology and pathophysiology of musculoskeletal ageing, despite a large number of studies focusing on individual tissue pathologies.

Animal models are commonly used to address this issue. Animals are used to study the effects of age-related conditions in a reduced time scale due to their shorter lifespan. Despite the quadruple locomotion used by mice, multiple musculoskeletal tissues display many similarities between mice and humans. Since the sequencing of the C57Bl/6 mouse genome, it has been shown that 99% of mouse genes have human homologues (Mouse Genome Sequencing et al., 2002). There are also many examples of mouse studies involving alterations of genes/proteins to potentially accelerate or slow the ageing process. In muscle, this includes a lack of antioxidant enzymes (Sakellariou et al., 2014a), deletion of maintenance proteins such as heat shock proteins (Kim et al., 2006) or a reduction of calorie intake (Sohal and Forster, 2014). In bone, many studies involve the removal of mouse ovaries in order to replicate the effects of post-menopausal osteoporosis in women (Bouxsein et al., 2005). These ovarectomised mice are commonly studied but experiments are often undertaken in relatively young mice and so the results cannot always be deemed translatable to ageing. Although there has been some cataloguing of changes in bone tissue throughout life in mice which allow us to base further research on, they are not comprehensive (Willinghamm et al., 2010).

Description of the ageing process through life in a robust mouse model would allow for the discovery of potential interventions to prevent or delay any decline. However, despite the large amount of work in this area, very little work has been done which details 'normal' lifelong effects of age of muscle and bone tissues in animals. Less common are age-related studies of cartilage and tendon tissues as

well as changes in extracellular factors which may drive such changes such as cytokines and/or hormone levels.

3.1.1 Fundamental changes in musculoskeletal tissues through life

With increasing age, muscle mass and muscle function is reduced resulting in a pathology termed sarcopenia. In humans, the decline of muscle mass starts at the age of ~40 and decline occurs leading to approximately 40-50% loss of muscle mass by the time a person is in their 80s (Faulkner et al., 2007).

The cause of this decline in muscle mass and function is yet to be fully elucidated. As discussed in Chapter 1

Tendons and ligaments are also subject to age-related changes. These changes centre on altered collagen production and maintenance leading to a reduction of elasticity (Narici and Maganaris, 2006). Although not a major influential change, taken in combination with the reduced ability of the muscles to generate force, this can compound the loss of muscle force and ultimately leads to a change in gait and locomotion.

Osteoporosis is the term describing the loss of bone mass and strength. This is not solely an age-related condition with both menopause and certain drugs (such as glucocorticoids) causing loss of bone; however it is highly prevalent in older populations. With this, there is an increased risk of fractures with increasing age.

Despite this, the cause of the disorder is not yet fully understood, however the close links between age-related changes in muscle and bone may suggest that loss of muscle force generation and therefore loss of mechanosensation is a key factor. Whatever the cause, the balance between bone formation and resorption is altered in favour of resorption which causes a net loss of bone tissue. This is summarised in Section 1.3.4.

Along with changes in bone tissue, articular cartilage also diminishes with age and with it there is a large reduction in shock absorption capability within the joints (Li et al., 2013). Changes in inflammatory markers and cytokine levels may also lead to joint conditions such as osteoarthritis which can affect the underlying bone (Sokolove and Lepus, 2013).

3.1.2 Interplay between musculoskeletal tissues

Throughout life, there are close interactions and relationships between all tissues of the musculoskeletal system. The changes in the way that these tissues interact in later life are theorised to be a key factor in overall decline in musculoskeletal tissues as a whole.

The interactions between muscle and bone described previously through life suggest a close link between the two tissues.

It is suggested that differential mechanical events and other external signals could be converted within the muscle to induce further signals influencing bone biology (Schnyder and Handschin, 2015) and possibly within bone to effect muscle in a feedback loop mechanism.

There are also close links between bone tissue and cartilage. As articular cartilage is removed with age there is a large reduction in the ability of bones to absorb shock within joints. This breakdown exposes bone surfaces to each other and can lead to painful joints (Li et al., 2013).

3.2 Aims

This aims of this chapter were as follows:

- To integrate methods of visualising, measuring and quantifying age-related changes in musculoskeletal tissues in C57Bl/6 mice.
- To identify changes in musculoskeletal tissues throughout the life span of C57BL/6 mice with particular focus on muscle, bone, tendon and cartilage.
- To determine interactions between the musculoskeletal tissues studied.

Hypothesis – as mice age, there is a detrimental loss of mass and function in muscle, bone, tendons and in the joint and that there is a close association between the tissues that can influence the ageing process.

3.3 Methods

3.3.1 Mice

All experiments were undertaken in accordance with UK home office guidelines under the UK Animals (scientific Procedures) Act 1986 and with ethical approval from the University of Liverpool Animal Welfare and Ethical Review Body (AWERB).

A total of 42 male C57BL/6 mice were used with 6 mice per group. The number of mice per group was determined using power calculations based on previous studies. Exclusion of mice with overt pathology at 28 months old resulted in the reduction of the group to n=4. Mice were culled at 3, 6, 9, 12, 18, 24 and 28 months of age. These ages were chosen based on the current published literature.

C57Bl/6 mice purchased from Charles River (Lyon, France) were used for this study and delivered to the Biomedical Services Unit at the University of Liverpool at least 1 month prior to the required age.

3.3.2 Tissue collection and preparation

All tissues were removed post-mortem as described in Section 2.3. Briefly, all muscles and organs were removed and snap frozen in liquid nitrogen prior to storage at -80°C. Feet with both *achilles* and *posterior tibialis* tendon attached were stored at -80°C. Hindlimbs, with the remaining muscle tissue removed were stored in formalin solution for 24 hours before being transferred into 70% ethanol.

3.3.3 Muscle analysis

Anterior tibialis, EDL, *soleus* and *gastrocnemius* muscles from both hindlimbs were dissected and weighed. Following this, muscles were snap frozen in liquid nitrogen and stored at -80°C.

3.3.4 Bone analysis

Detailed analysis of bones is described in Section 2.8. Briefly, both tibia and femurs of each hindlimb were scanned using a μCT system (Skyscan 1272 X-Ray microtomograph, Bruker Corporation, Kontich, Belgium) at high-resolution with an isotropic voxel size of 5 μm for trabecular bone (60 kV, 0.5 mm aluminium

filter, 0.7° rotation angle). The isotropic voxel size was changed to 10 μm for cortical analysis. Two images were taken at each rotation angle. The scans were reconstructed using NRecon software (Bruker, Massachusetts, USA). CTAn software (Bruker, Massachusetts, USA) was used to analyse the appropriate parameters (Bouxsein *et al*. 2010).

3.3.5 Tendon analysis

Tendon analysis was described in detail in Section 2.6. Briefly, images were taken of each tendon specimen, using a Nikon Eclipse Ci microscope equipped with a 10x/0.30 lens and a Nikon DS-Fi2 camera (Nikon, Tokyo, Japan).

Tensile tests were performed on an Instron 3366 material testing machine (Instron, Massachusetts, USA) equipped with a 10N load cell in conjunction with Bluehill materials testing software (Instron, Massachusetts, USA). Samples were secured into custom clamps using a cyanoacrylate adhesive (superglue).

Each specimen was subjected to 6 loading cycles to a peak load of 1.0 N and the results from the last cycle were used to determine the stress-strain behaviour of the specimen.

3.3.6 Joint analysis

Joint analysis has been described in detail Section 2.7. Briefly, one hindlimb from each mouse was transferred into a decalcification solution (EDTA, pH 7) and left at room temperature for 4 weeks without agitation. Samples were then embedded coronally in paraffin. Sections were cut using a HM355S microtome (Thermo Fisher Scientific, Massachusetts, USA). The cutting was carried out until the condyles could be seen. Sections were stained for Haematoxylin & Eosin and Safranin-O.

Joints from two age groups (6 and 24 month old) of mice were initially analysed since the process is a laborious process from dissection to analysis. As no significant differences were seen between these two age groups, no further age groups were analysed.

3.3.7 Statistics

Data was tested for normality and equal variance using SigmaPlot (v11.0) (Systat Software, Inc., London, UK). Direct comparison between two sets of data was made by Student's *t*-test. For multiple comparisons, data was analysed for statistical significance by one-way ANOVA incorporating pairwise comparisons. Data are presented as mean ±SEM. Values $P<0.05$ were considered significant.

3.4 Results

3.4.1 Measurement of age-related changes in body mass
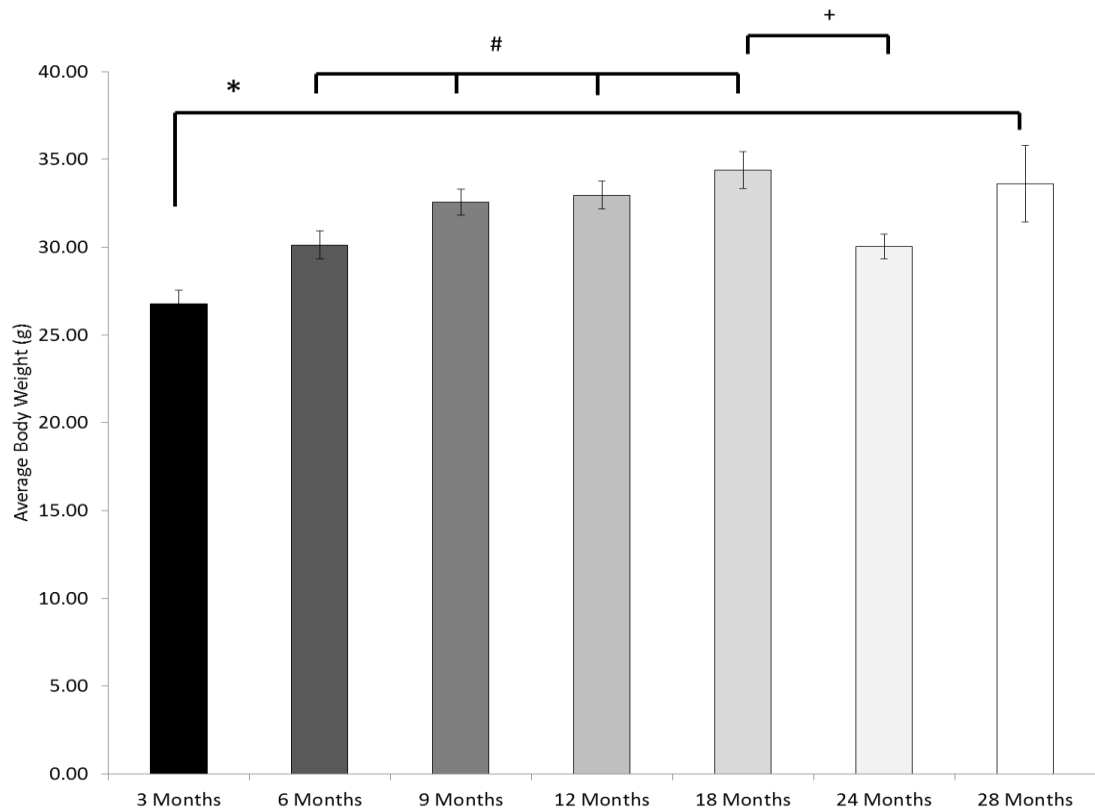
Fig 3.1 Mean body mass of male C57Bl/6 mice at different ages (Data presented as mean +/- SEM) (n=6 except n=4 in 28 month old group). * represents a significant difference compared with mice at 3 months old, # represents a significant difference compared with mice at 6 months old, + represents a significant difference compared with mice at 18 months old.

Mean body mass of C57Bl/6 mice at different ages are presented in Figure 3.2. There was a steady increase in mean body mass up to nine months old compared with body mass of mice at 3 and 6 months. Body mass did not change significantly from 9 months of age in the mouse until 24 months old when there was a significant decrease in body mass compared with body mass at 18 months old..

3.4.2 Age-related changes in organ mass

		3 Months	6 Months	9 Months	12 Months	18 Months	24 Months	28 Months
Heart	Average	147	197 **a**	179 **a**	240 **a**	236 **a,b**	205 **a**	198 **a**
	SEM	5	13	13	31	13	9	35
Lungs	Average	156	208 **a**	222 **a**	240 **a**	231 **a**	228 **a**	269 **a,b**
	SEM	10	12	11	11	10	13	15
Liver	Average	1382	1580 **a**	1632 **a**	1758 **a**	1888 **a**	1212 **e**	5441 **e**
	SEM	51	76	94	131	40	128	1138
Kidneys	Average	334	402 **a**	452 **a**	496 **a,b**	525 **a,b,c**	450 **a,d**	461 **a,b,d**
	SEM	13	13	14	24	26	35	22
Spleen	Average	68	71	100 **a,b**	100 **a,b**	129 **a,b**	66 **d**	281 **e**
	SEM	2	5	9	7	21	11	137
Brain	Average	254	388 **a**	386 **a**	386 **a**	344 **a**	383 **a**	369 **a**
	SEM	25	31	18	25	46	23	37

Table 3.1 Mean organ mass (mg) from mice at different ages. a represents a significant difference in values compared with mice at 3 months old, b represents a significant difference compared with 6 months old, c represents a significant difference compared with 12 months old, d represents and significant difference compared with 18 months old and e represents a significant value compared to all preceding ages (n=6 per group except n=4 in 28 month old group). Data presented as mean +/- SEM.

		3 Months	6 Months	9 Months	12 Months	18 Months	24 Months	28 Months
Heart OW/BW	Average	5.5	6.5 **a**	5.5 **b**	7.2 **a**	6.9 **a**	5.0 **b,c,d**	6.1
	SEM	0.2	0.3	0.3	0.8	0.3	0.6	1.4
Lungs OW/BW	Average	5.8	6.9 **a**	6.9 **a**	7.3 **a**	6.8 **a,c**	6.7	8.0 **a,b,d**
	SEM	0.3	0.3	0.3	0.3	0.1	1.4	0.5
Liver OW/BW	Average	51.6	52.3	50.1	53.3	55.0	39.1 **a,b,d**	156.9 **e**
	SEM	1.0	1.5	2.3	3.4	1.5	9.8	26.1
Spleen OW/BW	Average	2.6	2.3	3.1 **a,b**	3.0 **a,b**	3.8 **a,b,c**	5.6	7.6 **a,b,c,d**
	SEM	0.1	0.1	0.3	0.2	0.1	3.0	3.2

Table 3.2 Mean organ mass as a proportion of body mass (mg/g). a represents a significant difference in values compared with mice at 3 months old, b represents a significant difference compared with 6 months old, c represents a significant difference compared with 12 months old, d represents and significant difference compared with 18 months old and e represents a significant difference compared to all preceding ages (n=6 per group except n=4 in 28 month group). Data presented as mean +/- SEM.

Table 3.1 shows that heart mass significantly increased at 6 months old compared with mice at 3 months old and this was maintained until 28 months old. When expressed as a proportion of body mass, heart mass was again significantly increased by 6 months old but significantly reduced at 24 months of age compared with mice at 6, 12 and 18 months old. Lung tissue shows a significant increase in mass in mice at 6 month old but this remained consistent in mass until 28 months of age where a further increase was seen. The increase at 28 months old remained

significant when expressed as a proportion of body mass. Liver mass showed significant increases at 6 months of age, with a small but significant reduction in mass at 24 months of age. At 28 months there was a >3 fold significant increase in liver mass, however the large standard error would indicate that this was variable. When taken as a proportion of body mass liver mass remained consistent in the mouse until 24 months old when a reduction was seen. Again liver mass in mice at 28 months old was significantly increased. The spleen mass significantly increased at 9 months of age. As with the liver, at 24 months a reduction in spleen mass was seen which was followed by a large increase in mice at 28 months old. When taken as a proportion of body mass there was a gradual age-related increase in spleen mass. Kidneys showed an age-related increase in mass from 9 months until 18 months of age at which point the mass was reduced. Brain reached a peak mass in mice at 6 months old and remained consistent throughout the life span studied.

3.4.3 Age-related changes in muscle mass

		3 Months	6 Months	9 Months	12 Months	18 Months	24 Months	28 Months
AT	Average	63	56	64	58	57	45 e	38 e,f
	SEM	5	4	7	4	5	4	1
EDL	Average	11	12	13	12	12	12	6 e
	SEM	1	1	1	2	3	2	1
Sol	Average	9	0	0	11	11	10	**7 e**
	SEM	1	1	1	2	1	1	1
Gas	Average	174	175	184	160 a	159 a	138 a	105 e
	SEM	8	7	13	5	6	12	5

Table 3.3 Mean muscle mass (mg) at different ages from four hindlimb muscles. a represents a significant difference in values compared with mice at 3 months old, d represents and significant difference compared with 18 months old, e represents a significant difference compared with all preceding ages, f represents significant difference compared with 24 months old (n=6 per group except n=4 in 28 month old group). Data presented as mean +/- SEM.

		3 Months	6 Months	9 Months	12 Months	18 Months	24 Months	28 Months
AT MW/BW	Average	2.3	1.9 a	1.9 a	1.7 a,b	1.7 a,b	1.7 a,b	1.2 e
	SEM	0.1	0.0	0.2	0.1	0.1	0.1	0.2
EDL MW/BW	Average	0.4	0.4	0.4	0.4	0.4	0.4	0.2 e
	SEM	0.1	0.0	0.0	0.1	0.1	0.0	0.0
Sol MW/BW	Average	0.3	0.3 a	0.3 b	0.3 b	0.3 b	0.4	0.2 e
	SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gas MW/BW	Average	6.5	5.8 a	5.6 a	4.9 a,b	4.6 a,b	4.9 a,b	3.2 e
	SEM	0.2	0.1	0.2	0.2	0.1	0.2	0.3

Table 3.4 Table displaying mean muscle mass as a proportion of body mass (mg/g). a represents a significant difference in values compared with mice at 3 months old, b represents a significant difference compared with 6 months old and e represents a significant difference compared with all preceding ages (n=6 per group except n=4 in 28 month old group). Data presented as mean +/- SEM

Table 3.3 shows data for mean hindlimb muscle mass at different ages in C57BL/6 mice. There was no significant change in *anterior tibialis* muscle mass of mice until 24 months old and mass was further declined in mice at 28 months of age. There was no significant change in EDL mass until mice were 28 months of age. *Soleus* muscles significantly increased in mass in mice at 6 months old when compared with 3 months old; there was a significant decline in *soleus* mass in mice at 28 months of age. For the *gastrocnemius* muscle, there was a small but significant decrease in muscle mass at 12 months old, which then further declined at 28 months of age.

Interestingly, when presented as a proportion of body mass (Table 3.4), *anterior tibialis* muscle showed an apparent decline in mass from 6 months of age with the

largest decline seen in mice at 28 months old. *Gastrocnemius* muscle showed a significant decline from 6 months of age in the mouse with the largest decline in mass at 28 months of age.

3.4.4 Analysis of age-related changes in *anterior tibialis* fibre number and CSA
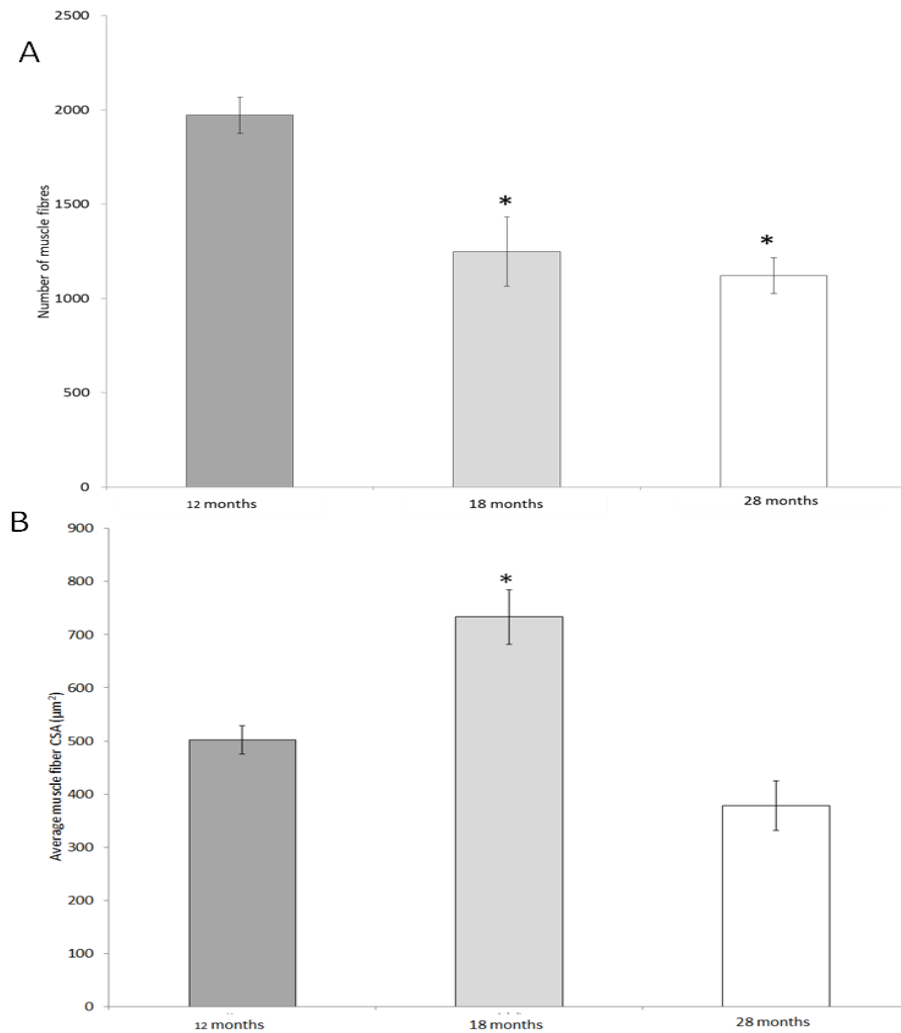
Fig 3.2 Mean fibre number and cross-sectional area in *anterior tibialis* muscles of C57Bl/6 mice at 3 ages. * represents a significant difference compared with mice at 12 months old (Data presented as mean +/- SEM) (n=6)

Fibre number data and CSA measurements are extremely time consuming and so muscle of mice at the ages 12, 18 and 28 months were chosen for further analysis based on changes seen in muscle mass data (Table 3.3) at time points when muscle mass appeared relatively stable, through to the age where the largest decline in mass was seen. There was a significant decline in the number of fibres in the *anterior tibialis* muscle at 18 months old compared with mice aged 12 months. No further decline was seen in fibre number of muscles of mice at 28 months old. Mean CSA increased in the anterior tibialis of mice between 12 and 18 months old but was reduced in mice at 28 months of age compared with 18 months of age.

3.4.5 Age-related changes in the *posterior tibialis* tendon
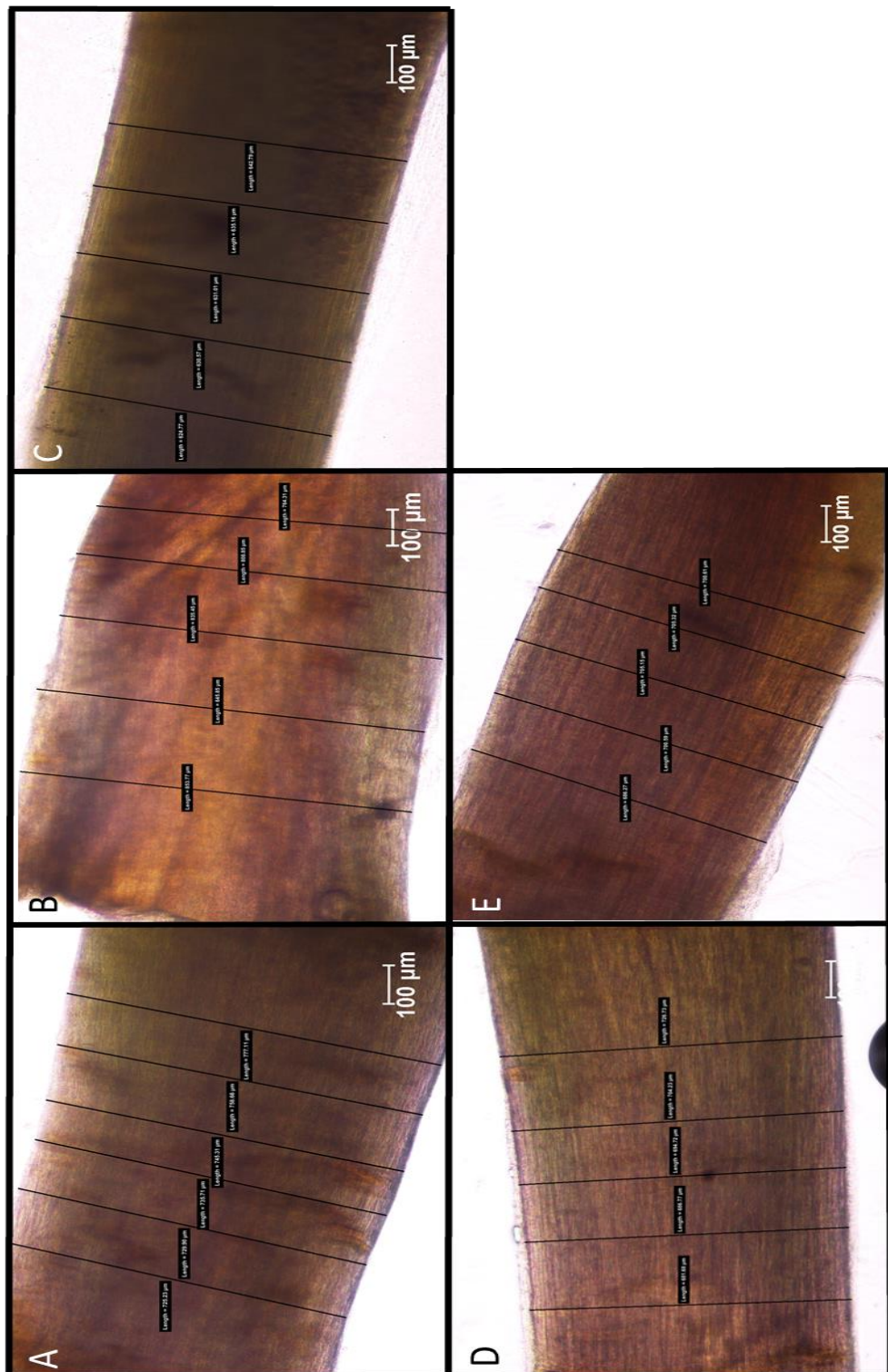
Fig 3.3 Microscopic images of *posterior tibialis* tendon at 10x magnification. Displayed images are taken from mice at 6(A), 9(B), 12(C), 18(D) and 24(E) months of age.
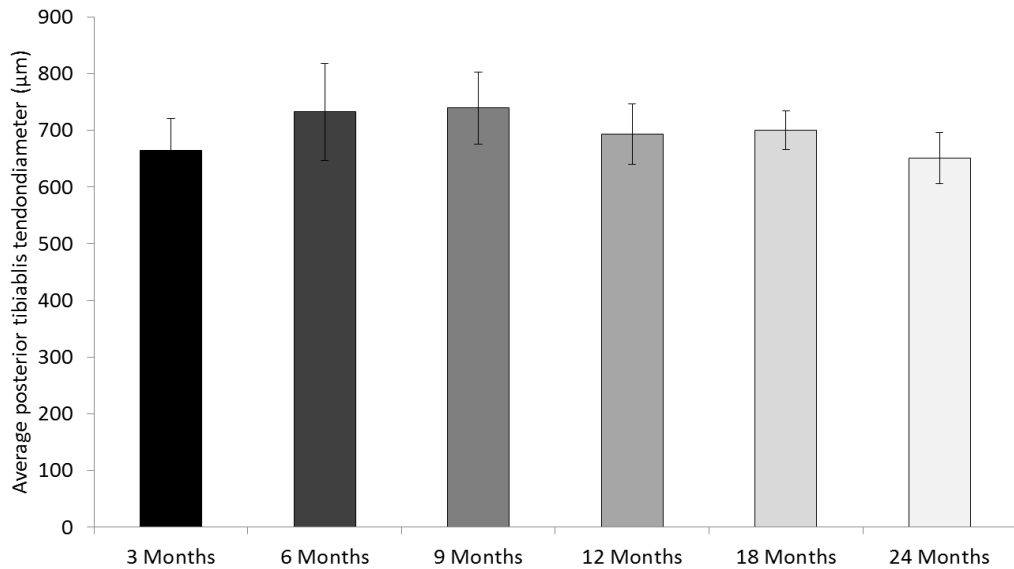
Fig 3.4 Mean diameter of *posterior tibialis* tendon of mice at different ages. (Data presented as mean +/- SEM) (n=6)

There were no significant differences in mean diameter of the *posterior tibialis* tendon at any age.
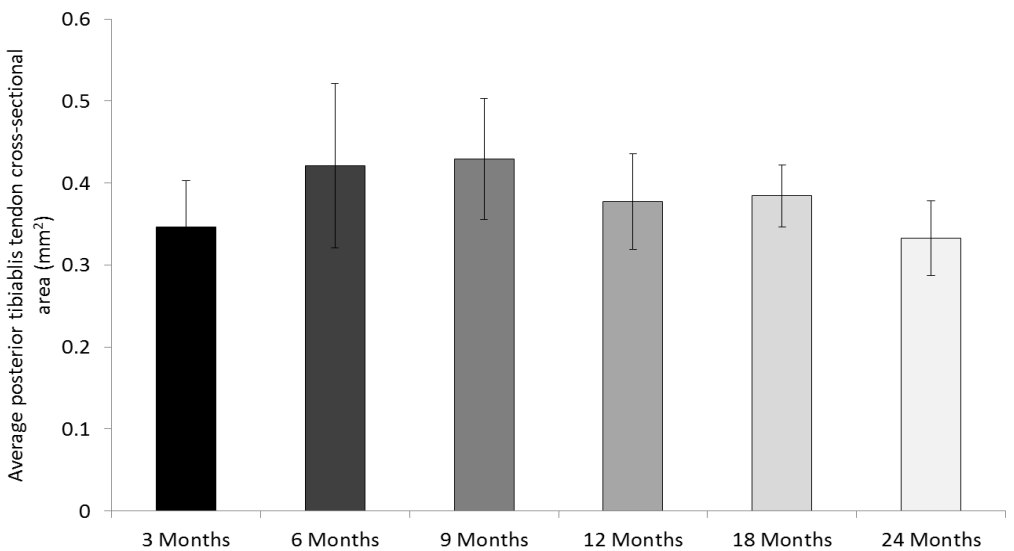
Fig 3.5 Mean cross-sectional area of *posterior tibialis* tendon in C57BL/6 mice at different ages. (Data presented as mean +/- SEM) (n=6).

There were no significant differences in mean cross-sectional area of the *posterior tibialis* tendon at any age.
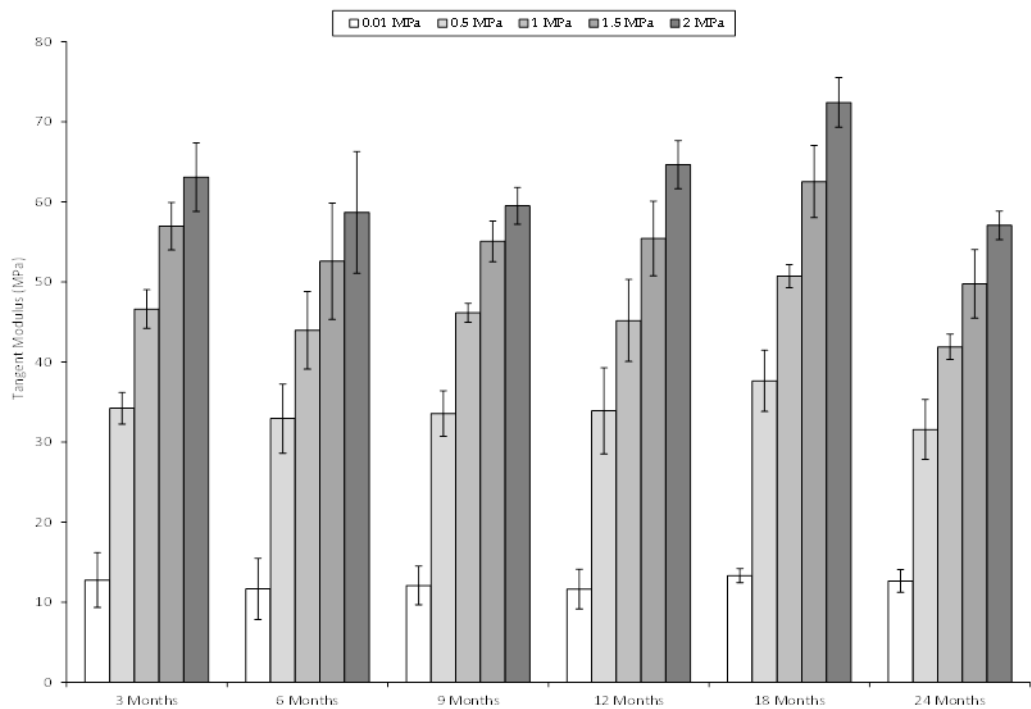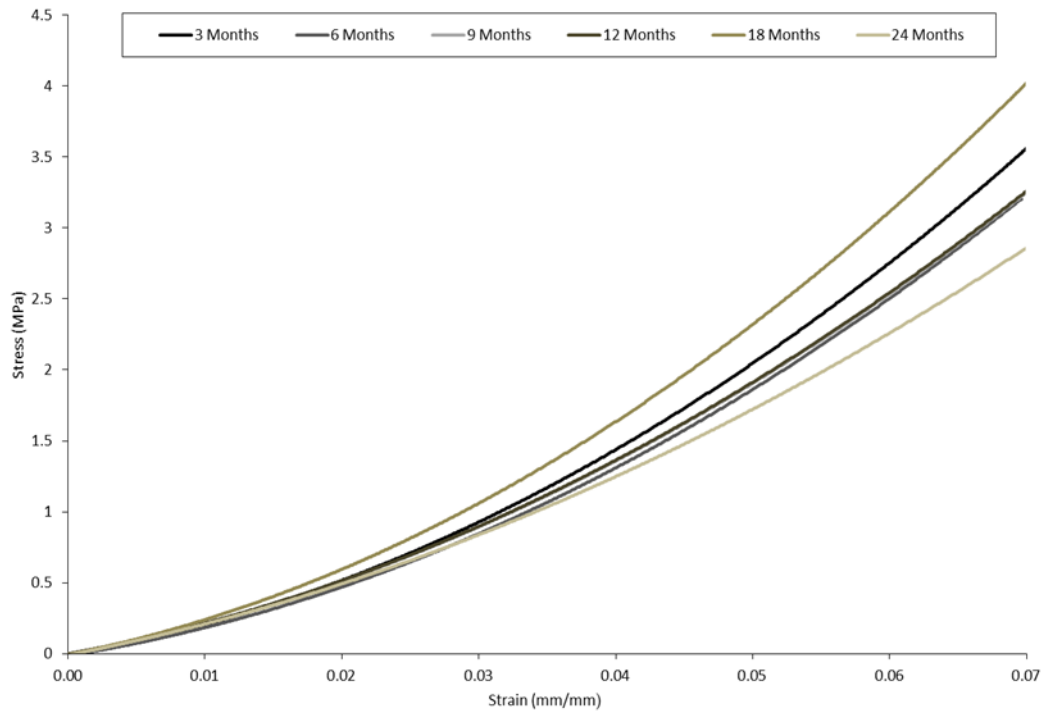
Fig 3.6 Age-related changes in stress/strain relationship of *posterior tibialis* tendons (Data presented as mean +/- SEM) (n=6). A presented as stress over strain, B is presents as tangent modulus (MPa).

No significant differences were seen in stress/strain curves at any age in the *posterior tibialis* tendon. The tendon of older mice appears to be unable to process higher stress/strains (based on the tangent modulus).

3.4.6 Analysis of joint and ligament structure in 6 and 24 month old C57Bl/6 mice
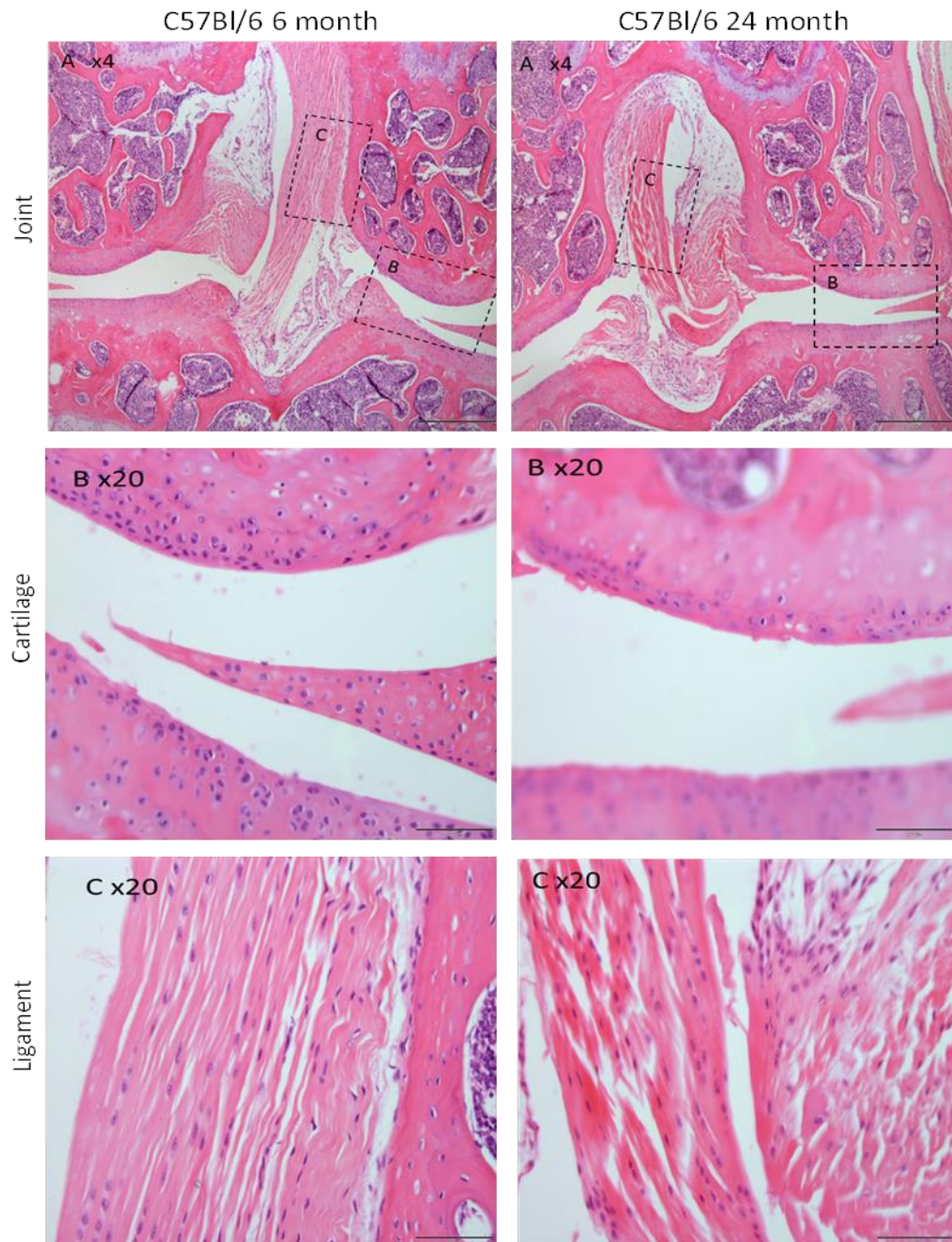
Fig 3.7 Representative histological sections of the knee joint in the C57Bl/6 mice at 6 and 24 months of age. Sections were stained with H&E. A was magnified x4 to show the whole joint, B (cartilage region) and C (cruciate ligament region) were magnified x20 to show detailed structures.

There was a visible reduction in the total number of cell present in both the cartilage and ligament with increasing age. The ligament showed a visual reduction in size with increased age and also appeared to have fibril disorganisation. The meniscus also showed some reduction in size.
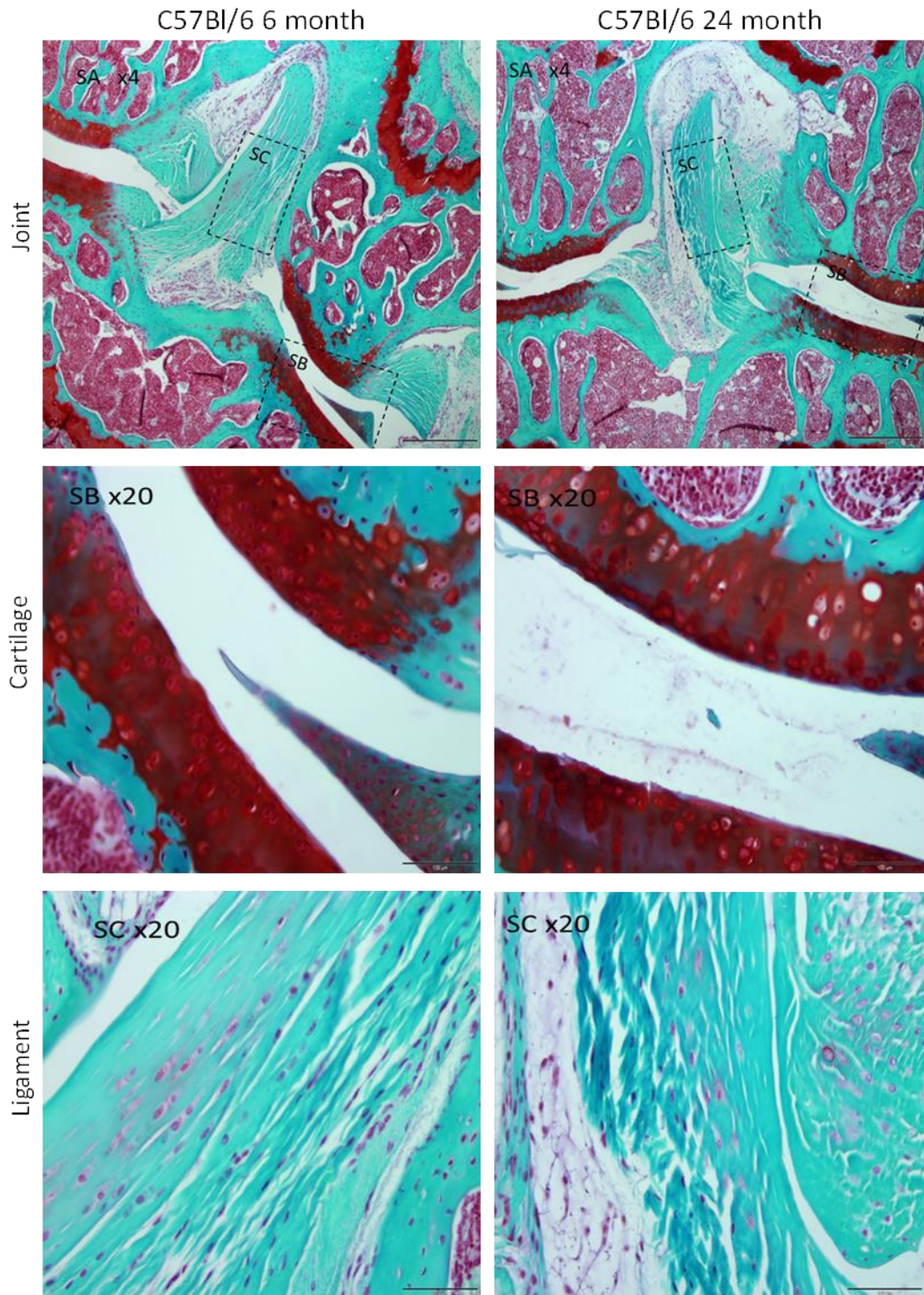
Fig 3.8 Representative histological sections of the knee joint in the C57Bl/6 mouse at 6 and 24 months of age. Images SA-SC were stained with Safranin-O to show proteoglycans (red). SA was magnified x4 to show the whole joint, SB (cartilage region) and SC (cruciate ligament region) were magnified x20 to show detailed structures.

There did not appear to be any major loss of proteoglycan content in the cartilage and there were no signs of cartilage damage or lesions.

3.4.7 Visualisation of age-related changes in hindlimb bones
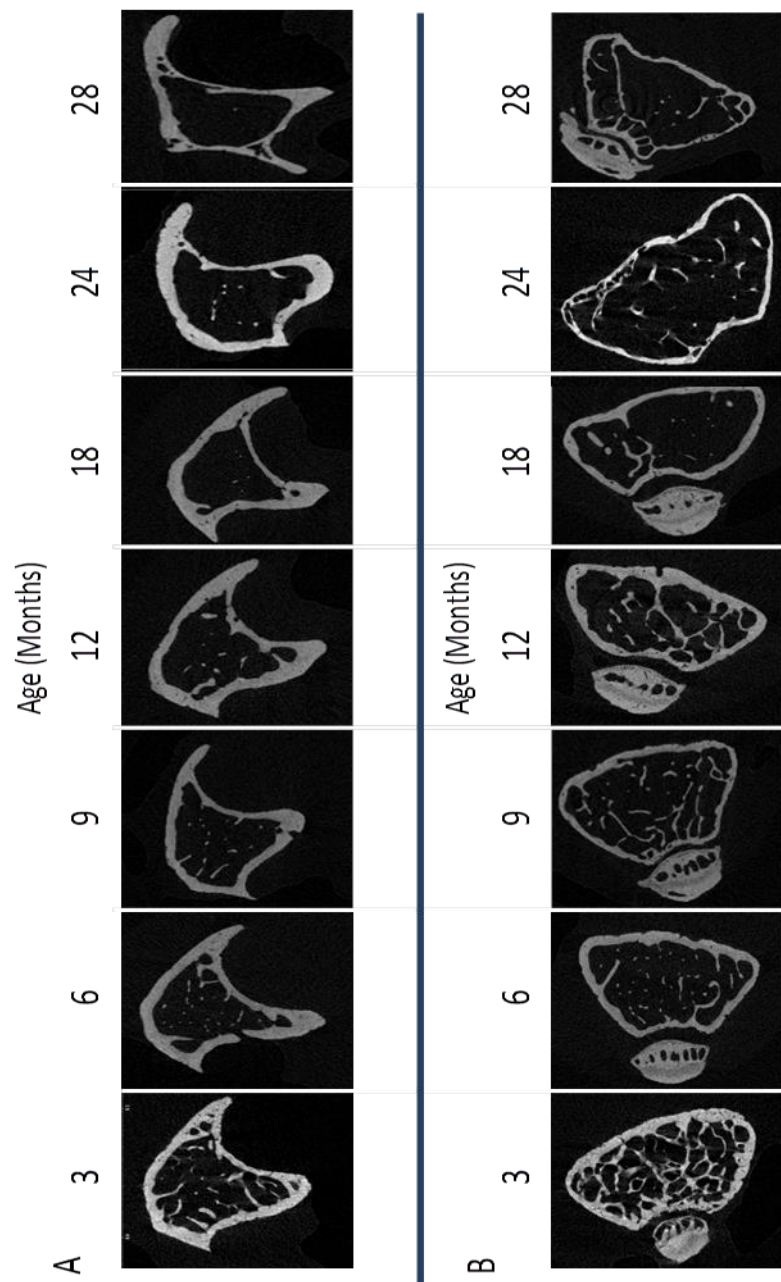
Fig 3.9 Representative MicroCT images of the tibia (A) and femur (B) in the C57Bl/6 mouse at different ages.

Representative images are presented from both the femur and tibia of the mouse at different ages (Figure 3.9). Images were chosen based on mean cortical and trabecular bone values. Images clearly show both the cortical and trabecular bone regions in both tibia and femur. A change in 2D shape of both bone sites was seen. Loss of trabecular bone was also seen with increasing age.
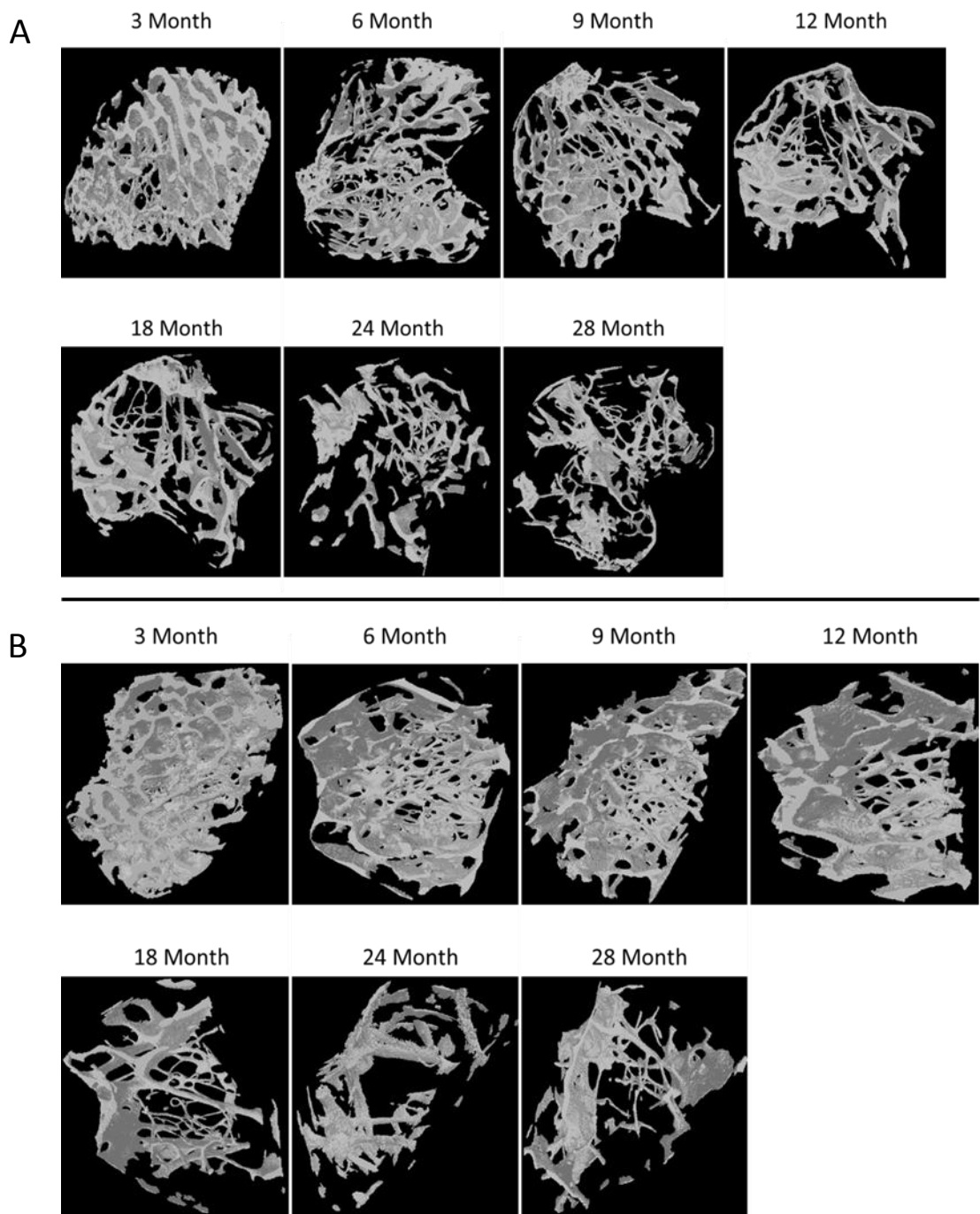
Fig 3.10 Representative reconstructed 3D scans of trabecular bone of tibia (A) and femur (B) of mice at different ages.

When images were reconstructed, loss of trabecular bone was clearly evident in both bones analysed (Figure 3.10). The trabecular bone mass appeared less connected with increasing age. Smaller trabecular bones appeared to be lost first with the larger trabeculae becoming thinner with increasing age.

3.4.8 Age-related changes in cortical and trabecular bone of tibia and femur bones
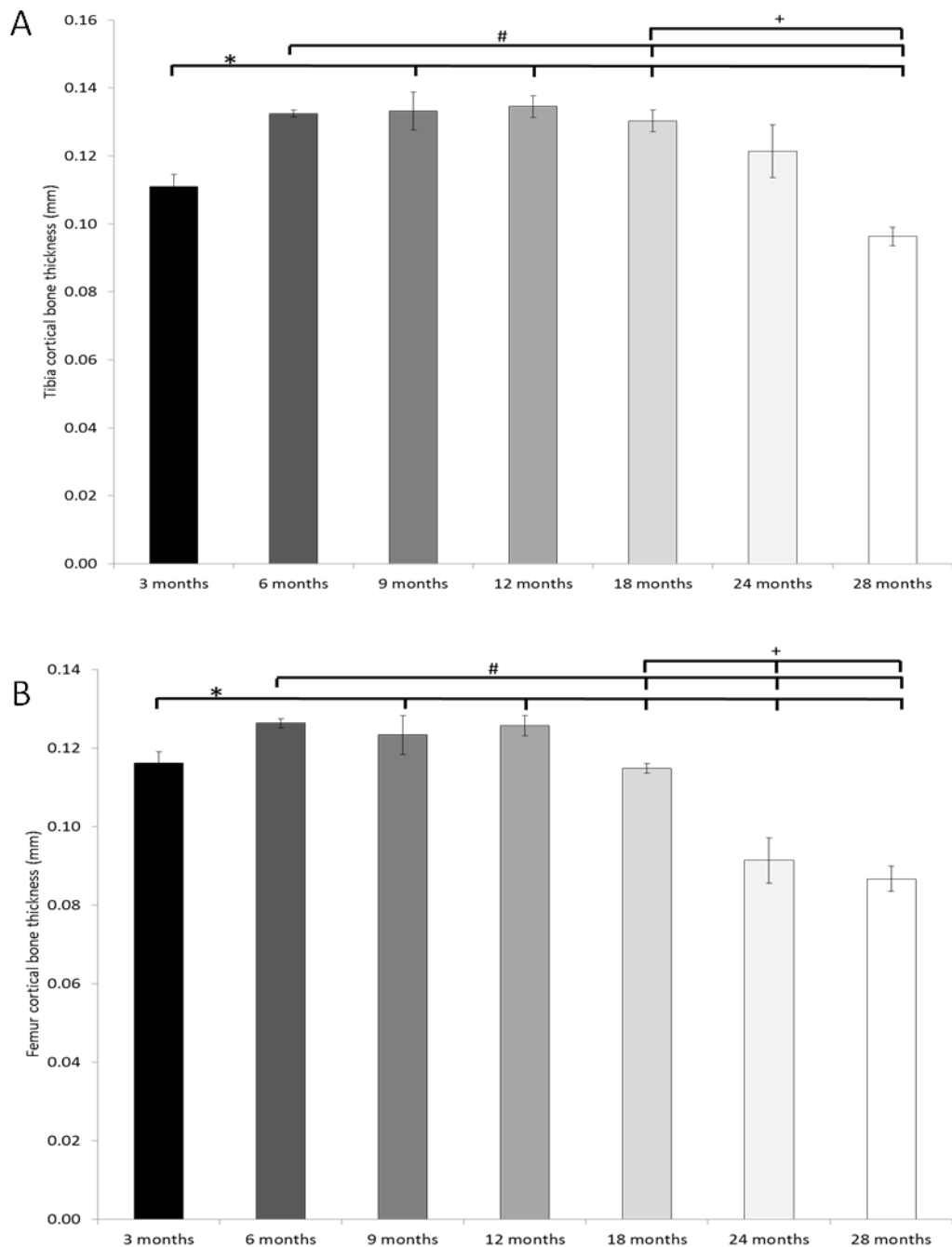
Fig 3.11 Mean cortical bone thickness of tibia (A) and femur (B) in mice at different ages (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with mice at 3 months old, # p <0.05 compared with mice at 6 months old, + p <0.05 compared with mice at 18 months old.

The tibia showed a significant increase in cortical bone thickness at 6 months of age. This remained at a constant level until 18 months of age, after which levels decreased to values similar to those seen at three months old. There was a further

significant decrease in tibia cortical bone thickness at 28 months of age which was significantly reduced compared to values at 3, 6 and 18 months old.

Figure 3.11 B shows the cortical thickness of the femur. There was a significant increase in thickness between 3 and 6 months of age. This remained at a similar value for up to 12 months of age in the mouse. At 18 months of age in the mouse, femur cortical thickness was significantly reduced compared with values at 6 months old and the decline continued at both 24 and 28 months of age.
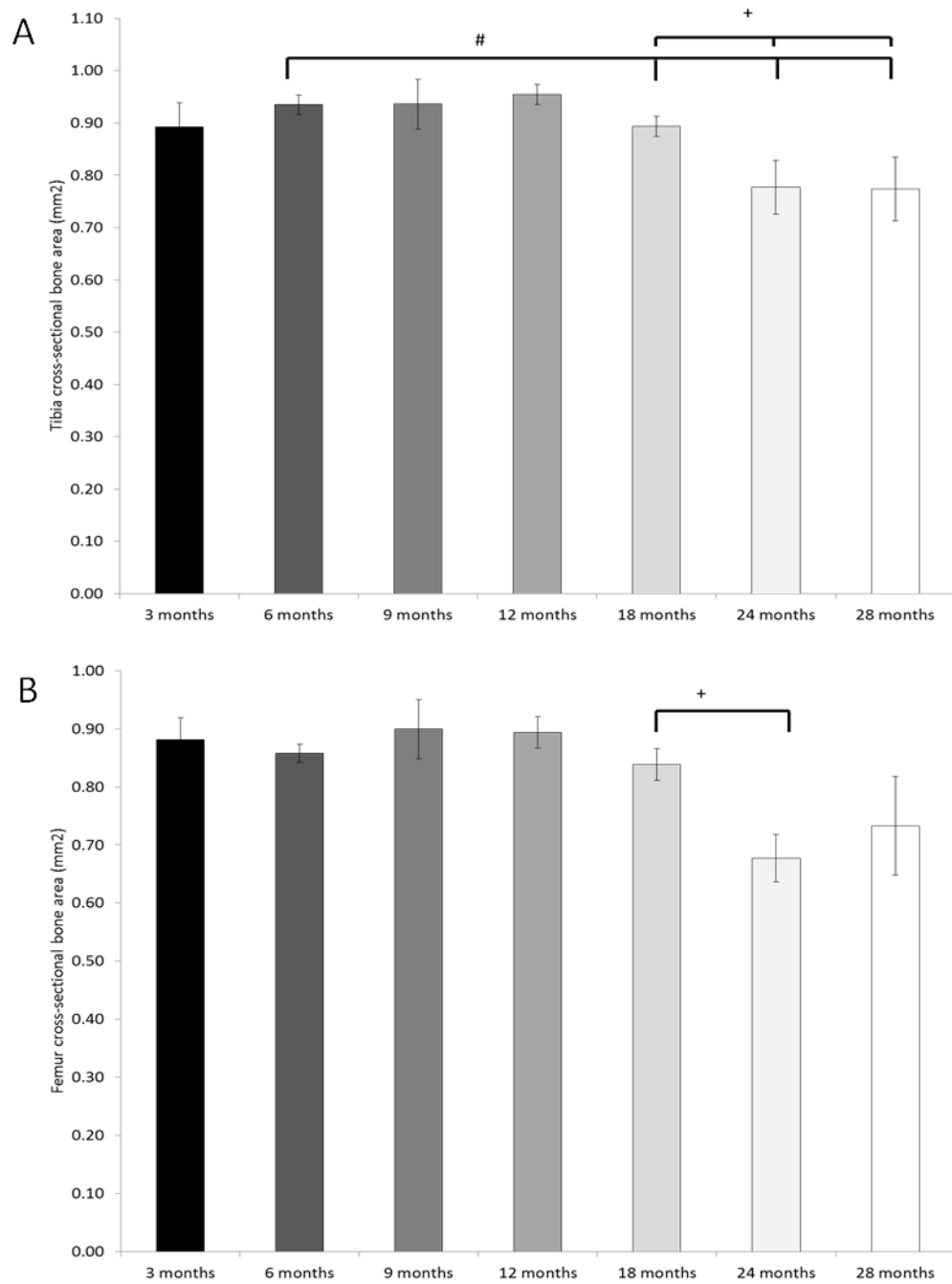
Fig 3.12 Mean cross-sectional area of tibia (A) and femur (B) in mice at different ages (Data presented as mean +/- SEM) (n=6) # p <0.05 compared with mice aged 6 months old, + p <0.05 compared with data for mice aged 18 months old.

There was a significant reduction in cross-sectional area of both the tibia and femur from 18-24 months of age in the mouse which remained evident at 28 months of age, and was significant in the tibia.
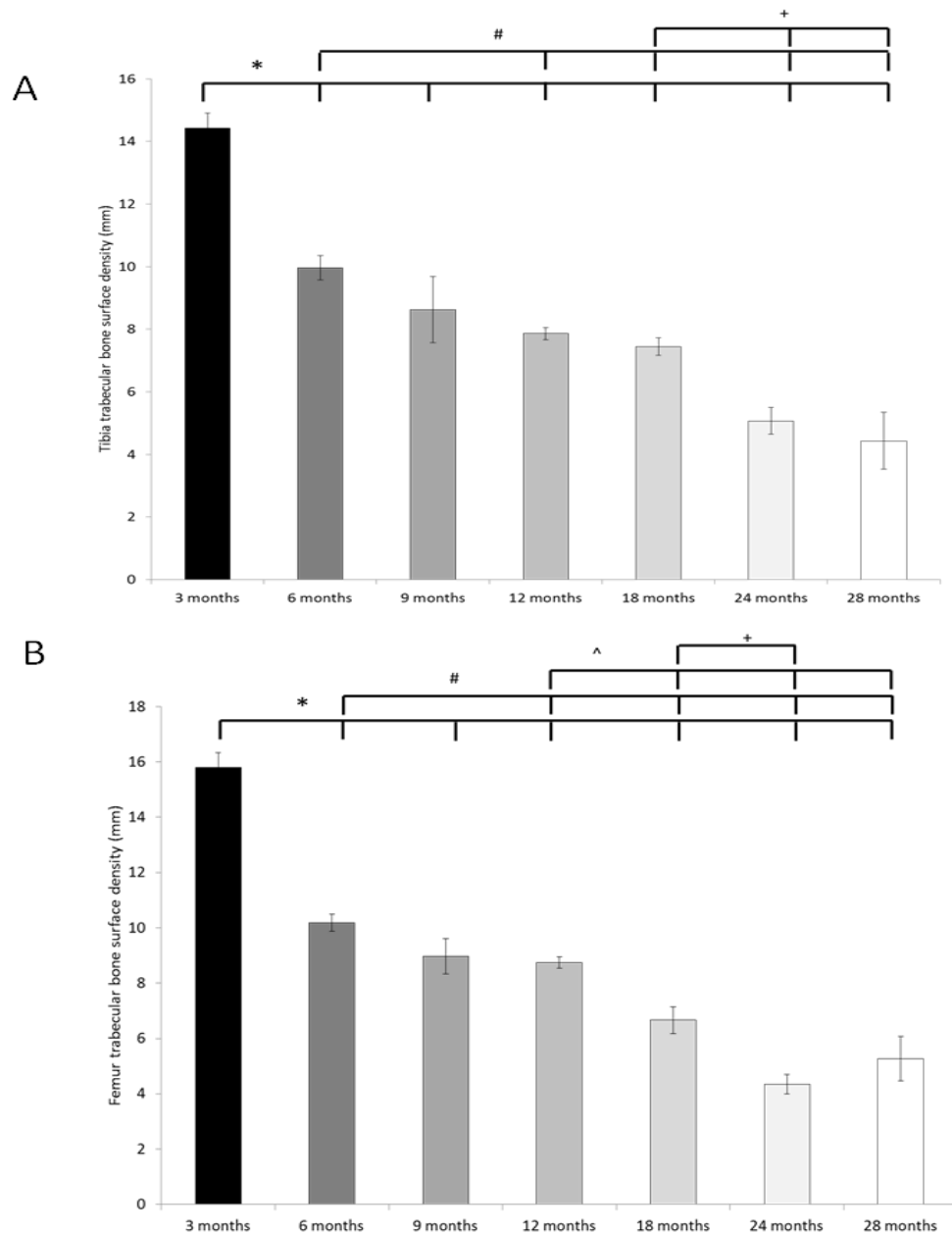
Fig 3.13 Mean trabecular bone surface density of tibia (A) and femur (B) at different ages (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with mice aged 3 months old, # p <0.05 compared with mice aged 6 months old, ^ p <0.05 compared with mice aged 12 months old and + p <0.05 compared with mice aged 18 months old.

There was a dramatic age-related loss of bone surface density in both the tibia and femur throughout life. In particular, there was a 31% decrease in the tibia and a 35% decrease in the femur surface density in mice at 6 months old compared with 3 month old mice. This was followed by a more gradual decrease in bone surface density in mice after 12 months of age which continued for up to 24 months old.
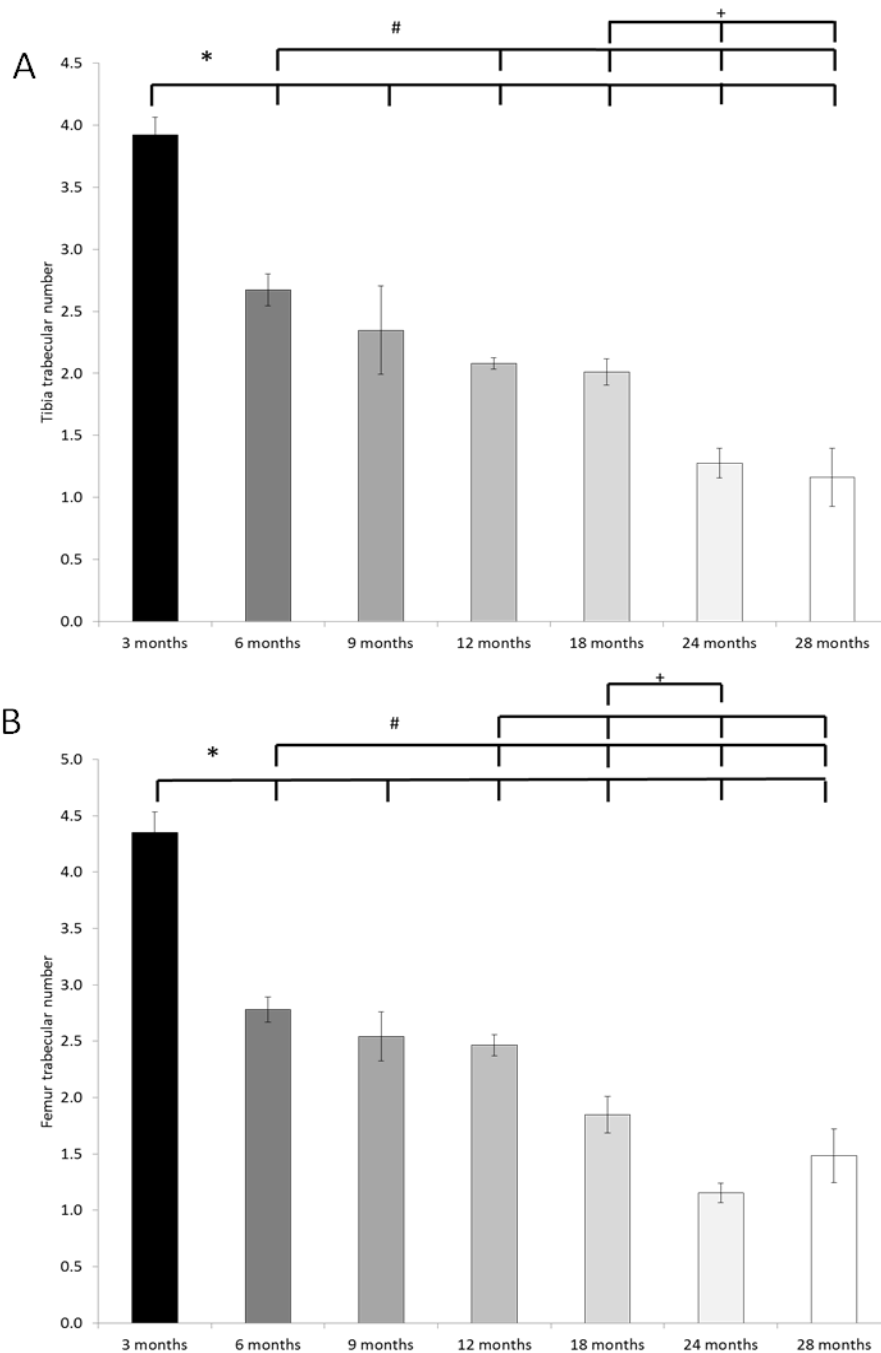
Fig 3.14 Mean number of trabecular bone/mm^2 present in the tibia and femur in mice at different ages (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with mice aged 3 months old, # p <0.05 compared with mice aged 6 months old, ^ p <0.05 compared with mice aged 12 months old and + p <0.05 compared with mice aged 18 months old.

There was a large reduction in the trabecular number present in both the tibia and femur in mice between 3 and 6 months of age which equated to approximately 30-35% loss of trabeculae. A further decrease in trabecular number was seen in mice at 12 months old which was further significantly reduced at 24 months old.

A	C57 Bl6	Tibia trabecular number	%Change
	6 Months	2.51	21.73
	12 Months	2.07	SEM ±2.68
	12 Months	2.07	36.93
	24 Months	1.56	SEM ± 5.10

B	C57 Bl6	Femur trabecular number	%Change
	6 Months	2.47	11.04
	12 Months	2.35	SEM ±3.01
	12 Months	2.35	52.46
	24 Months	1.09	SEM ± 5.33

C	C57 Bl6	Tibia trabecular number	%Change
	6 Months	2.51	50.48
	12 Months	1.56	SEM ±4.72
		Femur trabecular number	
	12 Months	2.47	57.92
	24 Months	1.09	SEM ± 4.29

Table 3.5 (A) shows the percentage change at chosen age points of trabecular number in the tibia trabecular bone. (B) shows the percentage change at chosen age points of trabecular number in the femur trabecular bone. (C) shows total percentage loss of trabecular bone in the tibia and femur between 6 and 24 months in the mouse. (Data presented as mean +/- SEM)

The percentage of trabecular bone lost over time appeared to differ between bone sites. The tibia appeared to lose more trabecular bone between 6 and 12 months compared with the femur but lost less between 12 and 24 months. At both sites more bone loss occurred between 12 and 24 months of age compared with 6-12 months.

In general, both bone has lost >50% of trabecular bone by 24 months old compared with 6 months old.

<table>
<tr><td rowspan="2">A</td></tr>
</table>

C57 Bl6	Gastrocnemius weight	Femur cortical bone thickness	Pearson correlation
6 Months	0.17	0.13	
12 Months	0.16	0.13	0.92
24 Months	0.14	0.09	

C57 Bl6	Anterior tibialis weight	Tibia cortical bone thickness	Pearson correlation
6 Months	0.06	0.13	
12 Months	0.06	0.13	1.00
24 Months	0.05	0.12	

B

C57 Bl6	Gastrocnemius weight	Femur trabecular number	Pearson correlation
6 Months	0.17	2.47	
12 Months	0.16	2.35	0.95
24 Months	0.14	1.09	

C57 Bl6	Anterior tibialis weight	Tibia trabecular number	Pearson correlation
6 Months	0.06	2.51	
12 Months	0.06	2.07	0.84
24 Months	0.05	1.56	

Table 3.6 Pearson correlation calculations comparing the cortical thickness (A) and trabecular number (B) of the tibia and femur and their attached muscles mass.

There was a total positive correlation when the relationship between *anterior tibialis* mass and tibia cortical thickness was compared. There was a strong positive correlation between *gastrocnemius* mass and femur cortical thickness.

There were strong correlations between loss of muscle mass and loss of trabecular bone at both sites however the correlation was stronger for the gastrocnemius and femur. This would indicate a close relationship between the mass of the muscle and the characteristics of the underlying bone.
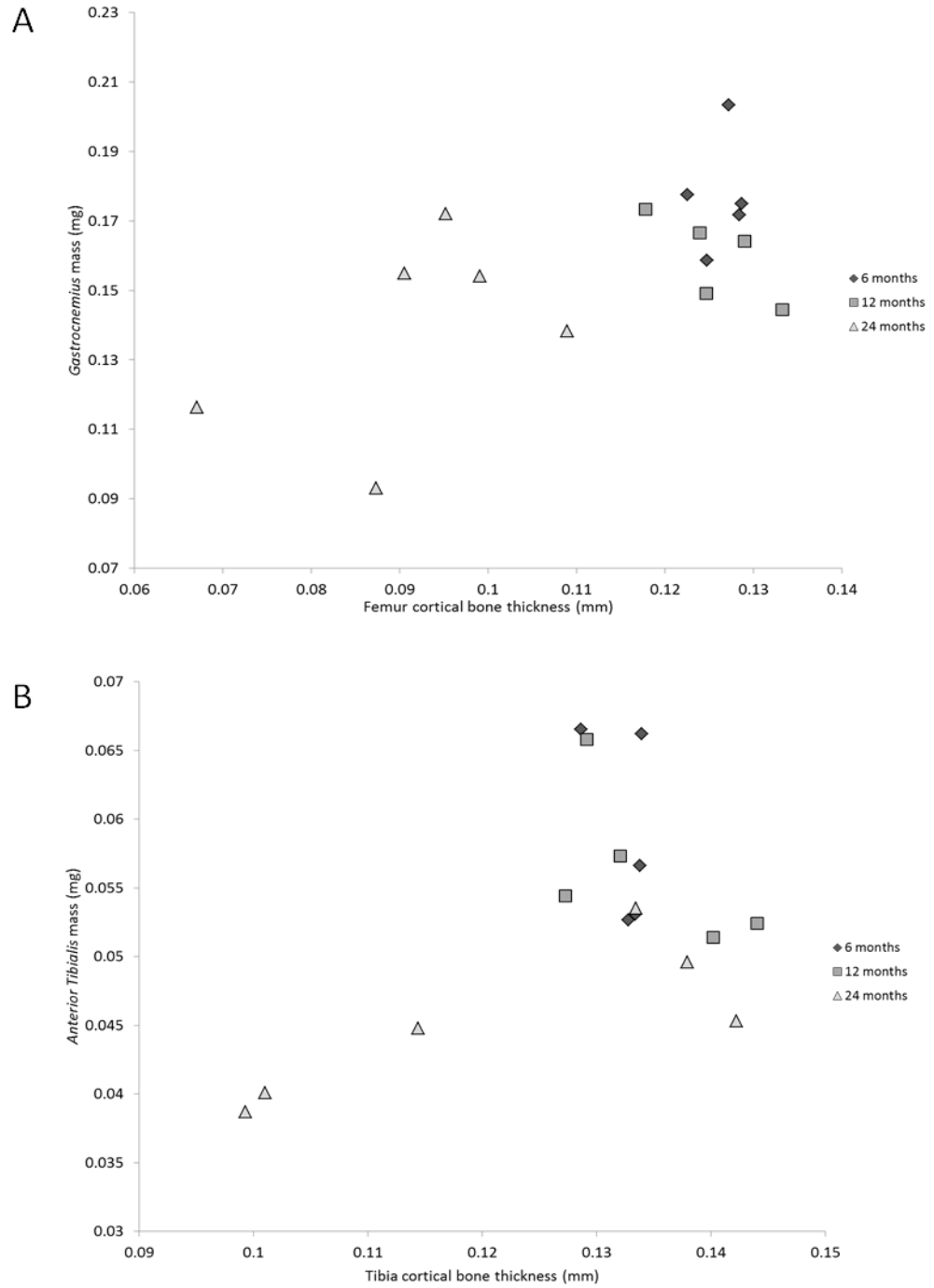
Fig 3.15 Correlations showing muscle mass against the cortical bone thickness of the underlying bone. A shows *gastrocnemius* and femur, B shows *anterior tibialis* and tibia
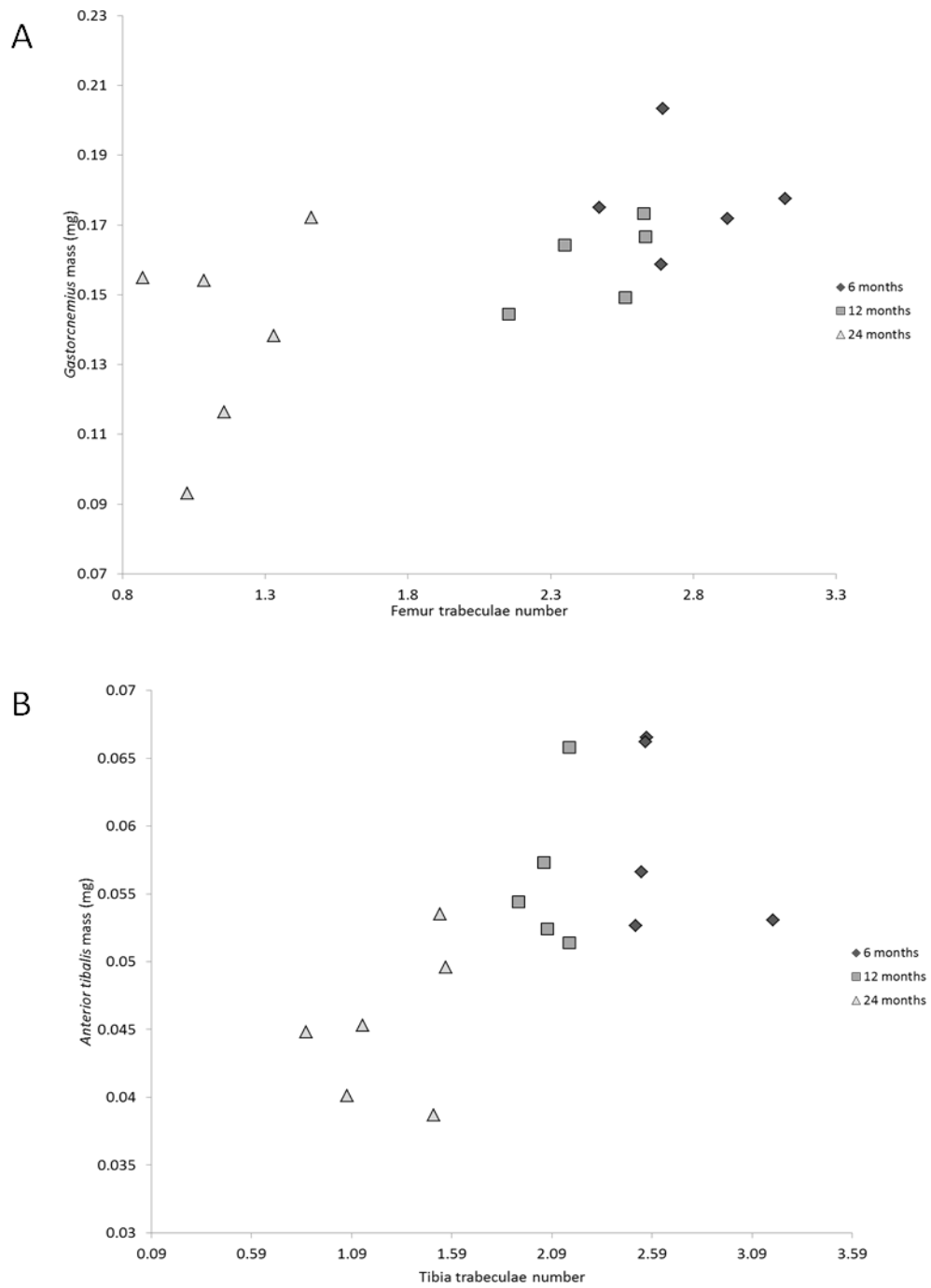
Fig 3.16 Correlations showing muscle mass against the trabecular bone number of the underlying bone. A shows *gastrocnemius* and femur, B shows *anterior tibialis* and tibia
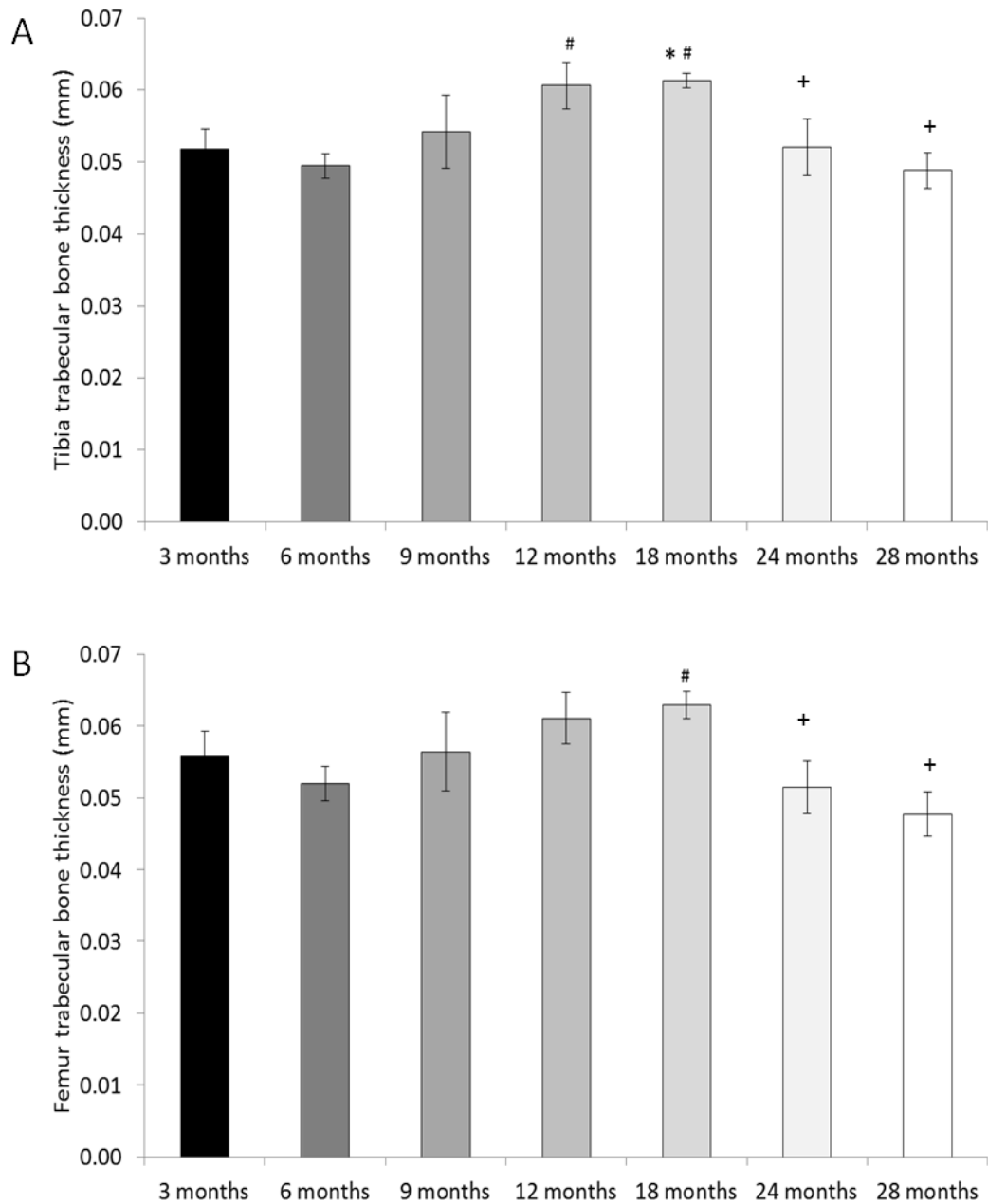
Fig 3.17 Changes in trabecular bone thickness in the tibia and femur of mice at different ages. (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with mice aged 3 months old, # p <0.05 compared with mice aged 6 months old, + p <0.05 compared with mice aged 18 months old.

Increases in trabecular thickness were seen in both bones of the mouse at 12-18 months of age. However, a significant decrease in trabecular bone thickness was seen at 24 months of age compared with values at 18 months old. This reduction remained evident in both the tibia and femur in mice at 28 months old.
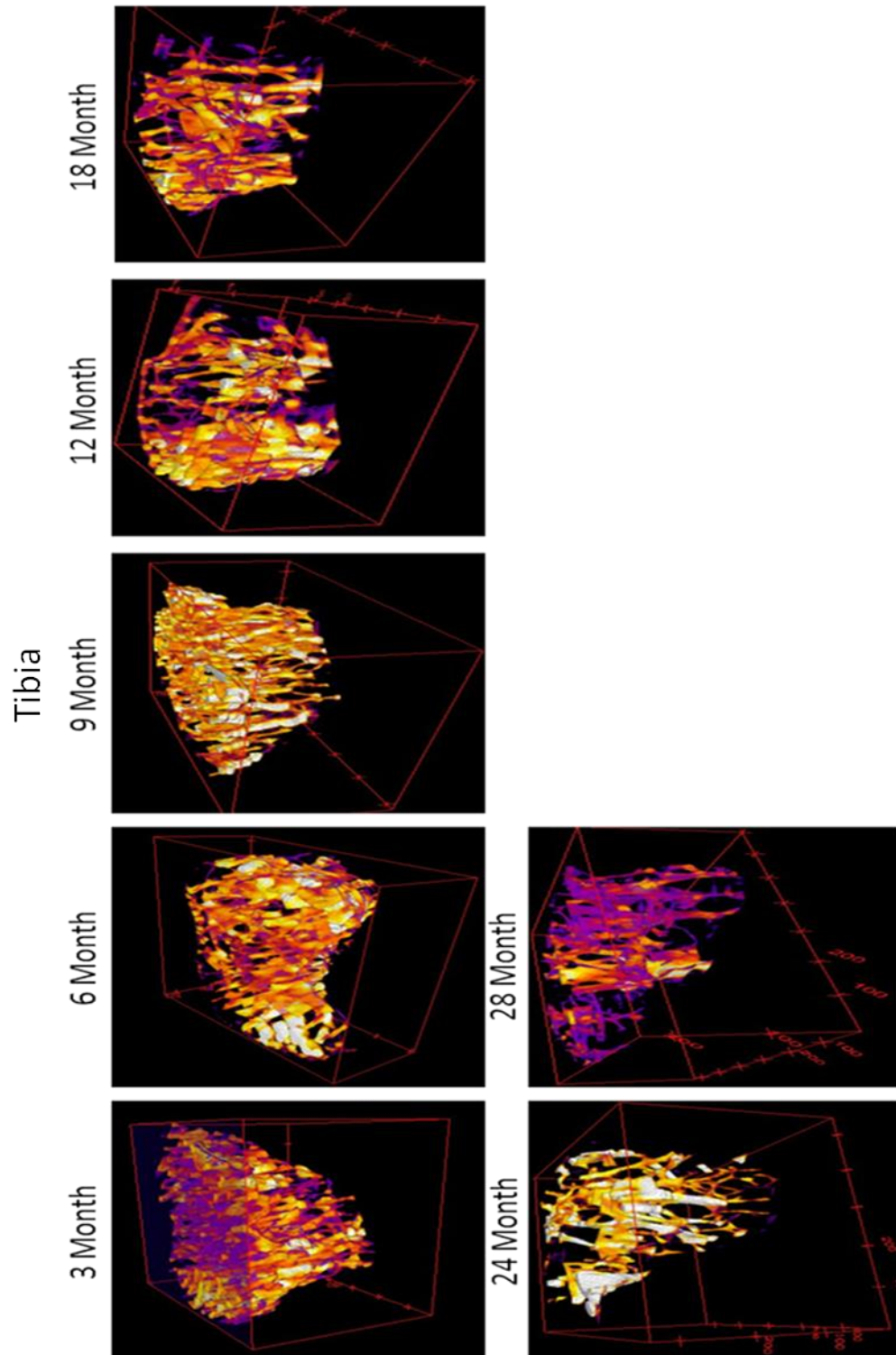
Fig 3.18 3D representation of trabecular bone thickness through age in the tibia. White colours represent the thicker areas, purple colours representing the thinner areas. Scale is specific to each individual bone based of mean and SEM as such, no key is present.

There were visual decreases in trabecular thicknesses in the tibia between 3 and 28 months of age. The loss of trabecular bone was also evident.
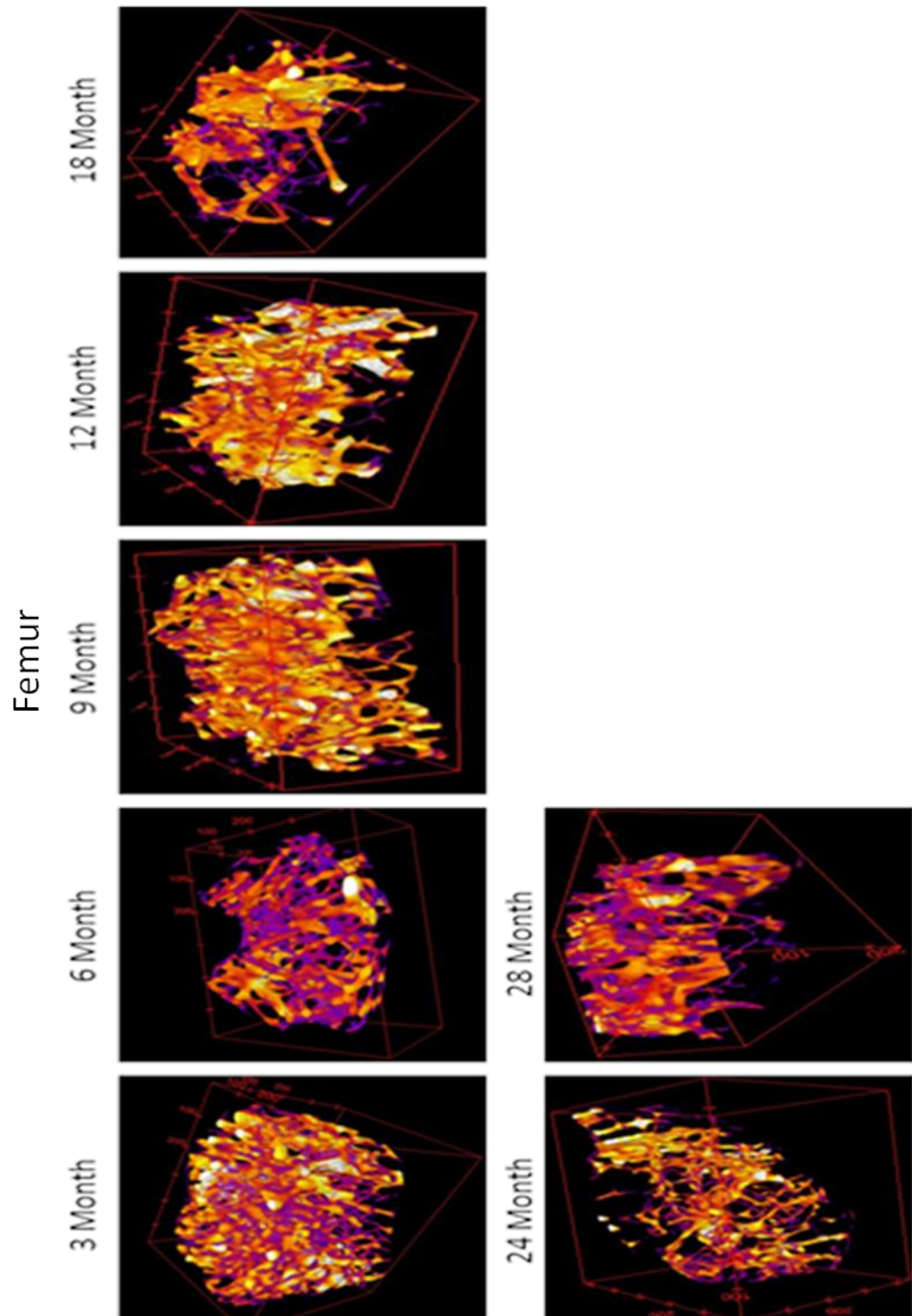
Fig 3.19 3D representation of trabecular bone thickness through age in femur. White colours represent the thicker areas, purple colours representing the thinner areas. Scale is specific to each individual bone based of mean and SEM as such, no key is present.

There were visual decreases in trabecular thicknesses in the femur between 3 and 28 months of age. The loss of trabecular bone was also evident
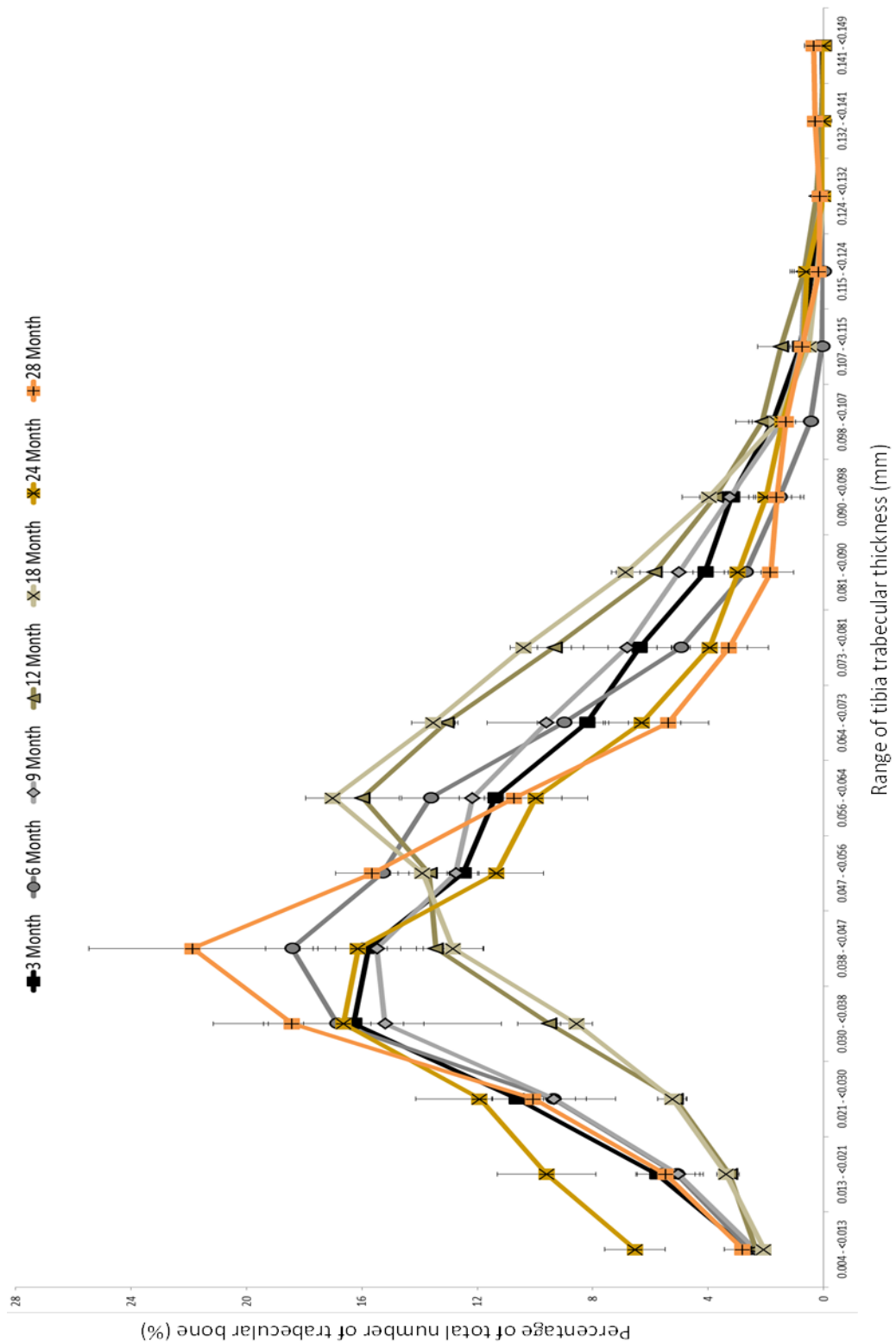
Fig 3.20 Combined range of mean trabecular thickness in the tibia for each age point in the mouse. (Data presented as mean +/- SEM)

Figure 3.19 shows the range of trabecular thicknesses in the mouse tibia at different ages. Data show an apparent shift towards thicker trabecular up to 18 months old, followed by a reduction at 24 and 28 months old.
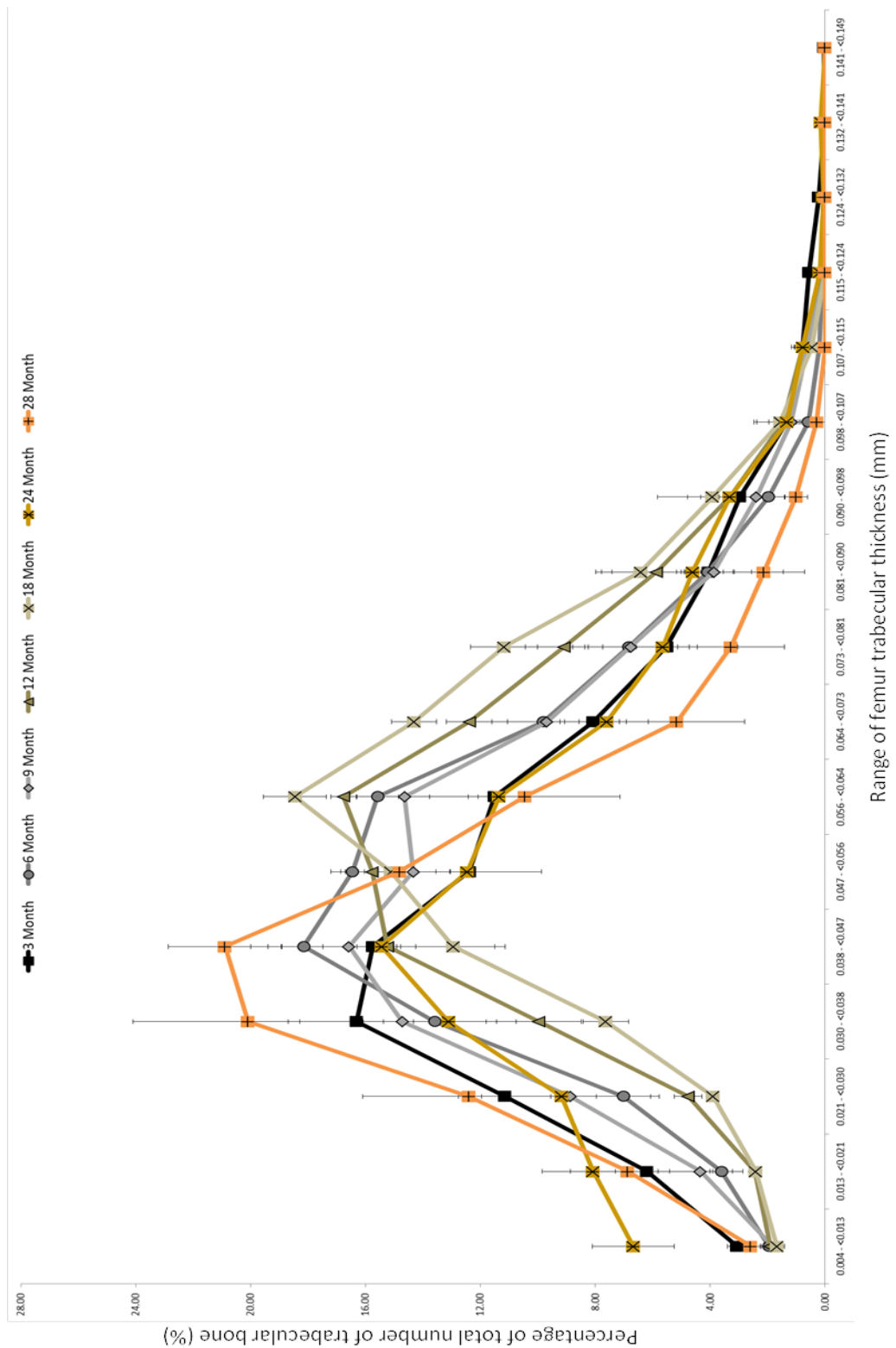
Fig 3.21 Combined range of mean trabecular thickness for each age point in the femur. (Data presented as mean +/- SEM)

Figure 3.20 shows the range of trabecular thicknesses in the mouse femur at different ages. Data show an apparent shift towards thicker trabecular up to 18 months old, followed by a reduction at 24 and 28 months old.

3.5 Discussion

3.5.1 C57Bl/6 mice as a model of ageing

A mouse model was chosen for this study to produce successful and reproducible methods of measuring age-related changes in musculoskeletal tissues. By using mice, the aims could be achieved in an appreciable timescale and provide fully characterised data sets that could be used to examine potential effects of possible future interventions.

C57Bl/6 mice are a well-recognised strain of mice and was chosen as it has been widely used in laboratory settings; often these mice have been used to produce transgenic mice with altered genetics. Despite this, little work has focused on lifelong changes in the musculoskeletal system of these mice.

From the data presented, it is apparent that C57Bl/6 mice are a good model of musculoskeletal ageing, particularly for muscle and bone ageing. The data were correlative to changes seen in human studies (Faulkner et al., 2007).

3.5.2 Age-related changes in the C57BL/6 mouse

3.5.2.1 Changes in body mass

Body mass increased with increasing age up to 9 months of age with no further significant increases in body mass between 9 and 18 months (Figure 3.2). There was a decrease in body mass of mice seen at 24 months of age. Despite this decrease, body mass at 24 months of age was still significantly increased compared with 3 months old. Body mass increased again at 28 months of age.

The early increases in body mass can be attributed to increases in muscle tissue, organ tissue and a previously observed but not recorded deposition of fat tissue which all increase with age (Table 3.1, 3.3). The lack of any further increases in mass during 9 to 18 months of age in the mouse reflects the data showing relative stability of tissue mass during the time period. The decrease in mass of mice at 24 months of age reflects concurrent loss of muscle mass particularly evident in larger muscles such as the *anterior tibialis* and *gastrocnemius* (Table 3.3). The following increase in body mass at 28 months was theorised to be an artefact of this study. In the 28 month group, there was an increased presence of liver

tumours which was not evident in the 24 month old mice (Table 3.1, Figure 3.24). This excess growth of the liver was noted and seems to have resulted in an increase in overall body mass. Removing mean liver mass from mean body mass resulted in the body mass remaining steady after 12 months of age, suggesting that the mass of the liver is a major factor in the age-related increase seen in body mass (Figure 3.23). Thus, care needs to be taken when expressing tissue mass relative to body mass and it may be more accurate to express tissue mass relative to body mass minus liver mass, or at least be aware of potential effects of increased liver mass on data.
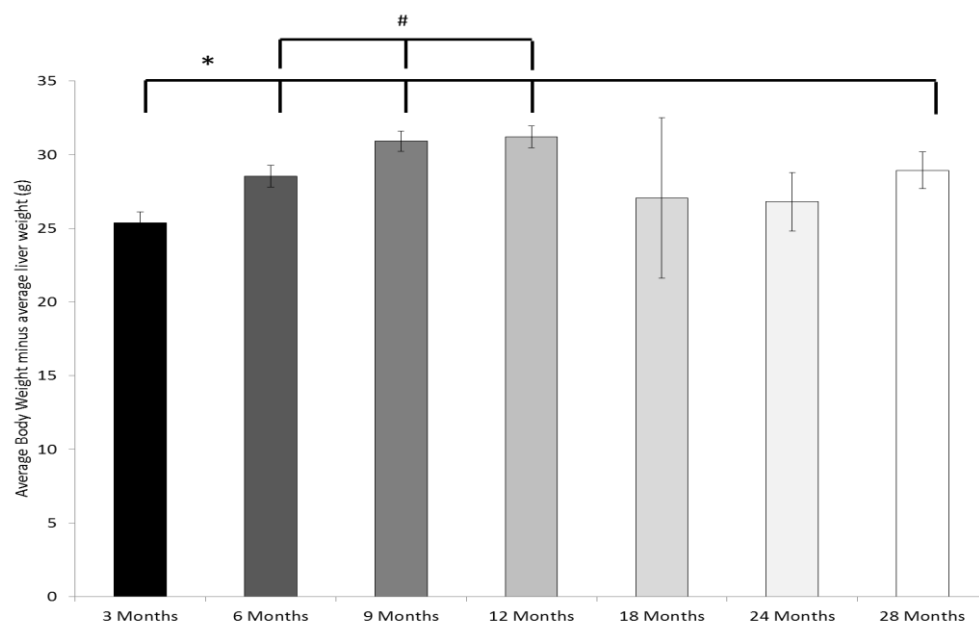
Fig 3.22 Mean body mass of C57Bl/6 mice minus liver mass at different ages (Data presented as mean +/- SEM, n=6) * $p < 0.05$ compared with mice aged 3 months old, # $p < 0.05$ compared with mice aged 6 months old.

3.5.2.2 Age-related changes to organ mass

The mass of all measured organs are shown in Table 3.1. In general in a similar manner to body mass, the mass of all tissues reached peak values between 12-18 months old. The heart did not reach peak mass until 12 months of age, after 18 months this began to decline again however by 28 months the mass was still significantly higher than values recorded at 3 months of age in the mouse. Lung mass increased until mice were 12 months old at which point mass remained constant until a further increase at 28 months of age. The liver appeared to gradually increase in mass until 18 months old at which point mass was reduced.

However, at 28 months of age in mice this mass was increased. This was an artefact of increased presence of liver tumours (Figure 3.24). Considering the increased risk of tumours in the liver, and the influence liver mass has on the body mass, it would suggest that 28 months may not be a suitable age to use.

Fig 3.23 An example of a liver tumour taken from a 28 month old C57Bl/6 mouse.

The combined kidney mass also gradually increased in mass with age until 18 months old, at which point mass starts to decline. The brain of the mice reached its peak mass at 6 months and did not decline until the mice were 28 months old. The mass of the spleen continued to increase throughout life reaching a peak at 28 months old. The spleen is an organ that is known to have roles in active immune responses. Increases in spleen size can be due to infection or changes to the regulation of the immune system which may potentially suggest a modified inflammatory environment within the mice at older ages (Zhu et al., 2014).

By expressing organ mass as a proportion of body mass, the heart mass remained relatively constant. Lungs still reached a peak at 12 months. Liver mass remained constant throughout life until 24 months where they appeared to decrease in mass until 28 months of age. There was a clear age-related increase in the size of the spleen throughout life in the mouse indicating increased inflammatory or diseased states with increased age (Table 3.2).

Changes in organ mass through life in this study are similar to published data (Lessard-Beaudoin et al., 2015) with the exception of liver mass. In the current study, this has been shown to be due to increased tumours in the 28 months cohort. This would suggest that the mice in this study (Lessard-Beaudoin et al., 2015) do not present with tumours or that data may have been (correctly) removed for mice with showing liver tumours, although this is not explicitly stated.

Interestingly, this increase in liver mass with increased incidence of liver tumours has been reported previously in a mouse model of accelerated ageing, the SOD1$^{-/-}$ mouse (Jang et al., 2010), suggesting that modified ROS signalling plays a role in the increase of tumour incidence.

3.5.2.3 Changes in hindlimb muscle mass

Four muscles of the hindlimbs were used to measure changes in muscle mass. These were chosen due to ease of dissection with clear tendon demarcation.

Overall declines in muscle mass were seen with increasing age consistently throughout the 4 measured muscles (Table 3.3). In the larger *anterior tibialis* and *gastrocnemius* muscles, a decline was seen from an earlier age when compared with the smaller *soleus* and EDL muscles. This may be due in part to the larger changes in mass being easier to measure. The gastrocnemius loses approximately 13% of mass (21mg) between 18 and 24 months old whereas the 8% loss by the soleus does not reach significance as this equates to a loss of only 1mg. Clearly a difference of 21mg is easier to measure and will therefore influence the data analysis.

 Focusing on *gastrocnemius* muscle, there was a significant decline in *gastrocnemius* muscle mass by 12 months old. In the *anterior tibialis* this was seen at 24 months of age. Both muscles saw a further, large decline in mass in mice between 24 and 28 months.

When expressed as a proportion of body mass, all muscles lost mass through age and again the largest decreases were seen in mice aged 24 and 28 months although, for reasons stated earlier, data for 28 month old mice need to be treated with caution. The mass of the EDL and *soleus* remained consistent in these mice until 28 months of age however both *gastrocnemius* and *anterior tibialis* muscles

displayed significant declines in mass from 6 months of age with a further decrease at 12 months old (Table 3.4).

When removing the liver mass from the total body mass, no major changes in the pattern of muscle loss were seen and only minimal changes in terms of muscle mass to body mass value. This suggests that although changes in liver mass at 28 months old does influence body mass, this does not significantly influence the muscle mass (Table 3.9).

		3 Months	6 Months	9 Months	12 Months	18 Months	24 Months	28 Months
AT MW/BW	Average	2.4	2.0 a	2.0 a	1.8 a,b	1.7 a,b	1.7 a,b	1.4 e
	SEM	0.1	0.0	0.2	0.1	0.1	0.1	0.2
EDL MW/BW	Average	0.4	0.4	0.4	0.4	0.4	0.4	0.2 e
	SEM	0.1	0.0	0.0	0.1	0.1	0.0	0.0
Sol MW/BW	Average	0.3	0.4 a	0.3 b	0.3 b	0.3 b	0.4	0.2 e
	SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gas MW/BW	Average	6.9	6.2 a	5.9 a	5.1 a,b	4.9 a,b	5.1 a,b	3.7 e
	SEM	0.2	0.1	0.2	0.2	0.1	0.2	0.3

Table 3.7 Mean muscle mass as a proportion of body mass following removal of liver mass (Data presented as mean +/- SEM).

These data are similar to other studies examining muscle loss in mice and add to the current information on life-long loss of muscle mass in this species (Hamrick et al., 2006, Graber et al., 2015, Brooks and Faulkner, 1988, Larsson and Ansved, 1995, Brooks and Faulkner, 1991).

The presence of significant liver tumours in mice at 28 months old is also likely to play a significant role in the muscle loss seen in mice at this age. There are well characterised cachectic effects of tumours on skeletal muscle and so additional caution is needed when using this age of mice as a model of ageing.

When studying muscle changes in more detail in the *anterior tibialis* muscle, changes in the total muscle fibre number were seen despite no significant change in overall muscle mass. In mice aged between 12 and 18 months, a significant reduction in the number of muscle fibres was seen. This was offset by significant increases in individual fibre cross-sectional area which appears to have resulted in the muscle mass remaining constant at the 18 month time point. In 24 month old mice, muscle mass was significantly lower compared with earlier time points and this seems to be caused by atrophy of the remaining muscle fibres with no further loss of fibres evident at this age (Figure 3.3).

This would suggest that although muscle mass may remain steady until 18 months old in C57Bl/6 mice, underlying changes to the muscle structure and function were occurring prior to this, in agreement with other reported studies (Graber et al., 2015).

The causes of the muscle fibre loss are still unclear. This is proposed to be due to changes in innervation. In particular the neuromuscular junction that controls fibre contraction is known to deteriorate with age. If a muscle fibre is dennervated, it undergoes atrophy and is eventually is lost. If this occurs over a long period of time, surrounding fibres may adapt to increasing demand and there is a hypertrophy in these fibres in an attempt to maintain the overall need of the muscle to produce force. Once the muscle reaches a critical threshold where the muscle fibre loss continues, the remaining fibres cannot adapt any further and a loss of the ability of muscles to produce sufficient force will then be seen. This will manifest as muscle weakness in the older person. As discussed in Chapter 1, there are a number of additional factors which may play a role in this later stage fibre atrophy, including the need for adequate dietary protein intake.

3.5.2.4 Age-related changes in tendon characteristics

The *posterior tibialis* tendon was chosen to study due to the ease at which it could be reproducibly dissected and analysed and ease of measurement of a wide range of tendon properties (Figure 3.4). However, analysis of mouse tendons is particularly challenging given their small size.

There were no statistically significant changes in mean tendon diameter (Figure 3.5) or total cross-sectional area (Figure 3.6). There are currently no studies that have determined the cross-sectional area of the posterior tibialis tendon to directly compare these data to but it is possible that, due to the small size of the tendon, any changes were too small to record, in a similar manner to the mass of the smaller hindlimb muscles such as the *soleus* and EDL muscle discussed previously.

Due to the small size of the tendons, it is also possible that stress-strain measurements may have been influenced directly by the grips needing to attach to the tendon. Despite this, a decrease in the tangent modulus with increasing age

was seen when higher forces were applied (Figure 3.7). This would suggest an inability of tendons in old mice to tolerate larger forces applied from muscle. This may have arisen due to reduction in the forces applied by the muscle on the tendon *in vivo* at older ages, as mean muscle mass and force generation was reduced with increasing age. The age-related changes in mechanical properties of tendons described here may reflect morphological and biochemical changes previously reported in mice (Shadwick, 1990) which include increased collagen content, increased fibril diameter, reduction of fibril crimp angle and decreased water content (Elliot, 1965). These data also support the study by Dunkman (2013) (Dunkman et al., 2013) who reported that tendons of 18 month old C57Bl/6 mice showed less stress in response to strain and were more viscous, so more likely to lose energy from applied forces, ultimately leading to a functional deficit (Dunkman et al., 2013).

3.5.2.5 Age-related changes in the knee joint of C57Bl/6 mice

Using histological sectioning, the joint was visualised and the major structures identified for characterisation and comparison (Figure 3.8). No major structural changes in terms of bone shape were seen at the joint and there were no signs of osteophyte structures growing which are signs of osteoarthritis (Gelse et al., 2003). There were also no clear signs of cartilage loss which is common with human ageing (Figures 3.8, 3.9).

There were some structural changes in the soft tissues such as the ligament which appeared to lose fibril organisation and visually appeared to be reduced in diameter. The meniscus also became smaller with age (Figures 3.8, 3.9). There was a clear loss of cells within the cartilage and ligaments with age indicating a reduced cellular activity and therefore reduced tissue maintenance (Figure 3.8, 3.9). These may cause restricted movement within the hindlimb.

Although the lack of deterioration of cartilage in the joint was unexpected, this has been reported in other studies especially with the C57Bl/6 mice which rarely show loss of cartilage with increasing age on the patellar/femur joint or medial views of the tibia femur joint (van der Kraan et al., 2001). This would suggest that C57Bl/6 mice do not naturally develop osteoarthritis and that joints remain in a stable state from the age of 6 months until at least 24 months of age. This could

possibly be due to the quadrupedal movement of mice and so equal distribution of mass between four joints rather than two as with humans. Although models of osteoarthritis have been established within the mouse through disrupting the joint via disruption of the medial meniscus (DMM) (Culley et al., 2015) or through gene knockouts to provide some pathophysiology, the lack of evidence of this event occurring naturally would suggest that wild type laboratory mice are not suitable models for studying the development and treatment of osteoarthritis. The DMM model in particular is a process aiming to mimic meniscus injury which is presumed to be a predisposing factor for osteoarthritis (Culley et al., 2015).

3.5.2.6 Age-related changes in hindlimb bones of C57Bl/6 mice

Using a combination of original microCT images and 3D renderings of the internal skeletal compartments, age-related changes in bones could be clearly seen (Figures 3.10, 3.11).

MicroCT images provide contextual imagery to see changes at comparable positions in each bone with both cortical and trabecular bone compartments being visible at different ages in the mouse. Images also allowed for the shape of each bone to be determined, which can often be lost from other measurements such as mean bone cross-sectional area. Via 3D rendering, visualisation of the trabecular bone in 3D space can also be achieved. This allows for the age-related changes in bones to be identified more clearly (Figure 3.11).

Measurements were taken on the two main bones found within the hindlimb, representing differential effects of mass bearing as well as the attachment of *anterior tibialis* and *gastrocnemius* muscles to these bone sites were shown.

Measurements taken from the tibia and femur are shown in Figures 3.12-3.22 and representative images are shown in Figures 3.10 and 3.11. In cortical bone, the thickness of the measured bone peaked at 6 months old. This remained relatively steady until 18-24 months old, following which was a reduction in values. There was a further decrease between 24 and 28 months of age (Figure 3.12). This initial increase was not as marked when examining mean bone cross-sectional area, CSA remained relatively constant until 18-24 months at which point values decreased and remained low through to 28 months old, particularly in the tibia (Figure 3.13).

This would suggest that the small increases in cortical bone thickness in mice up to 18 months old contributes at least in part to the small increases seen in CSA at these ages, but that cortical bone thickening occurs primarily on the inner surface reducing the centre volume of the bone rather than increasing the CSA significantly (Figure 3.10). The decrease in cortical thickness at 24-28 months old is however reflected in a reduced CSA at this age in both bones, suggesting that changes in cortical thickness contribute significantly to the decrease in bone CSA.

Changes in trabecular bone measures can be seen in Figure 3.14-3.19 and this is visualised in 3D in Figure 3.11. The percentage of bone in the measured volume was reduced throughout life with the large reductions seen in mice at two distinct time points, between 3 and 6 months as well as at 12 months old when compared which continued to decline up to 24 months old (Table 3.8). This was associated with a reduction in the number of individual trabecular bones present. Again large reductions were seen in mice between 3 and 6 months and 18 to 24 months of age. This also led to reductions in the connectivity of the trabecular bone as a whole (Table 3.5).

Despite these changes, individual trabecular bone thickness was not significantly changed until later in life (24 months old) (Figure 3.16). When comparing the range of trabecular thicknesses with the same value in mice at 3 months of age, differential ranges were seen with increasing age. There was a gradual shift towards a higher level of thicker trabecular bone until 18 months old. This was due to smaller trabecular bones increasing in size and potentially the removal of some smaller trabecular bones occurring. Eventually the larger trabecular bones also became thinner. This would suggest that despite an age-related loss of trabecular bone within the tibia and femur, remaining trabecular bone is remodelled and increased in thickness until approximately 18 months of age (Figure 3.14, 3.15).

What was evident is that the tibia displayed a larger number of trabecular bone present at three months when compared with the femur. However in mice by 28 months of age, the femur has retained more individual trabecular bone.

Interestingly, when comparing the number of trabecular bone present through age with the mean mass of the attached muscle (femur and *gastrocnemius* muscle,

tibia and *anterior tibialis* muscle) using Pearson correlations, a close age-related relationship between the two separate tissues was seen. This relationship was particularly striking when examined at 6, 12 and 24 months of age which represents time points at which significant changes were seen (Figure 3.13).

This close relationship would suggest the mass of the muscle attached to the bone, and therefore the potential force that the said muscle can apply to the bone, may directly influence the amount of cortical and trabecular bone present. To further support this hypothesis, change in trabecular number was converted to a percentage. In mice between 6 and 24 months of age there was roughly 55% of trabecular bone lost at both bone sites. When this change in trabecular bone was divided further to show changes in mice between 6 and 12 months (where muscle mass was stable) and 12 to 24 months of age (where total muscle mass and individual muscle fibre numbers were significantly reduced), proportionally more bone was lost at this age when compared with between 6 and 12 months old. An early loss of bone tissue between 3 and 6 months in mice (whilst muscle is still growing and producing mechanical stimulus) was also seen, which appeared to be due to remodelling events to allow for optimal force handling. This loss was slowed whilst muscle reached a peak mass and continues in tandem with loss of muscle fibres.

These finding together would therefore suggest a close relationship between the two tissues which is potentially dependent on mechanical influences from the muscle to bone. This theory has been established through computer modelling and is an increasing popular technique to monitor the effects of muscle force on underlying bone (Vickerton et al., 2014). Although other factors discussed in Chapter 1 such as paracrine and endocrine responses may also be having a role in this process.

There have been few publications reporting age-related changes in the tibia and femur of the mouse. One such paper (Halloran et al., 2002) showed that tibia trabecular bone volume decreased continuously from 1.5 months of age until the end of study at 24 months old. Data presented in the current study agrees with a lifelong loss of tibia trabecular bone volume and this remained evident at 28 months of age. Data presented here however also showed a steep decrease in tibia

trabecular bone volume from 3 to 6 months of age, and no further loss until after 12 months of age which differs to data represented by (Halloran et al., 2002)). Although Halloran et al., (2002) reported a decrease in the number of trabecular bone in the tibia; these authors only recorded a 37% loss between 5.5 months and 24 months compared to 52% measured in this study. Halloran et al., (2002) also noted no significant losses of trabecular bone between 12 and 24 months, a finding that was not seen in the current study (Halloran et al., 2002). A further discrepancy was seen in connectivity density values with Halloran et al., (2002) reporting constant values from 12 months of age following an increase prior to this, whereas in the current study no significant changes between 6 and 24 months of age were recorded.

Differing patterns of changes in the cortical bone of the tibia are also seen when comparing the two studies. In the current study, an increase in cortical bone thickness between 3 and 6 months of age was seen; there were no further significant changes until values decline again from 18 months onwards. Halloran et al. (2002) described a decrease in cortical bone thickness following a peak at 6 months of age (Halloran et al., 2002).

The differences reported in the Halloran et al., (2002) study and the current work may be due to differing microCT scanners and the bones being scanned at a much higher resolution (18μm compared to 4.5 μm). Another issue with the Halloran (2002) study was that only the tibia was studied and having measured both tibia and femur in the current study, each site aged marginally differently from each other. Although time points earlier than 3 months may have been beneficial to study based on Halloran et al., (2002), high levels of remodelling seem to be occurring at these ages. This may have changed the outcome when looking at the entire life course, but may be beneficial to record in the future.

Another study detailing age-related changes in the bone of C57Bl/6 mice was published by Glatt et al (Glatt et al., 2007). This study again mirrored the results presented in this study but Glatt et al. (2007) focused on early life more than later life. Again Glatt et al. (2007) described lifelong decreases in trabecular bone volume and trabecular number although values differed slightly from the presented values in this study (Glatt et al., 2007). This may be due to the scanning

techniques. Glatt et al. (2007) reported 12μm resolution which was improved compared to Halloran et al (2002) but was still higher then used in this study (Glatt et al., 2007). The Glatt paper did however show the benefits of recording two sexes and two different bone sites which our study did not do and would be beneficial to do so in the future.

Based on the evidence presented, it is reasonable to suggest that the major influencing factor in the age-related loss of bone mass is the changes in the muscle mass and force, after initial remodelling events seen at the age of 6 months. This change may due to a reduction in muscle force causing the mechanosensitive osteocytes within the bone to signal to the osteoclasts that the bone is not needed and the minerals held within the bone would be better used elsewhere (Wolff, 1988, Bren-Mattison et al., 2011). This would lead to reductions in both cortical and trabecular bone thickness and if broken down enough, can lead to the trabecular bone numbers decreasing.

Another theory is that external influences such as changes in hormones, changes in cytokines and/or changes in external signals from the muscle (myokines) may be triggering the resorption of bone either through activation of osteoclasts or blocking the action of osteoblasts.

It is most likely that a combination of these possible mechanisms is responsible but it is clear that the key to maintaining bone mass into later life may be through keeping the muscle healthy.

3.6 Conclusions

The data presented here have fully characterised the major changes in the musculoskeletal tissues throughout life with particular focus on ageing within the male C57Bl/6 mouse.

- Body mass increased from 3 months old and peaked at 9 months old until a significant loss was seen at 24 months old. The influence of liver mass on body mass suggests that care needs to be taken when expressing tissue mass relative to body mass.

- Organs reach peak mass between 6 and 9 months of age and do not show major declines in mass in later life. Exceptions are organs influenced by the presence of tumours (Liver) or potential signs of infection (Spleen).

- All hindlimb muscles examined showed a significant loss in mass between 24 and 28 months of age with the larger muscles, losing more muscle mass at an earlier age. The changes in muscle fibre number and size earlier in life appears to be the initial point of decline in muscle function with some evidence of compensatory hypertrophy (Sakellariou et al., 2014a).

- Ageing of musculoskeletal tissues is closely linked, particularly between muscle and bone. Bone modelling events occurring early on in life are potentially controlled in part by increasing muscle mass and force.

- There were no major changes in tendon tissue with age. This may be due to the method used to measure function, the tendon chosen or may be that tendons are resistant to age-related changes in contrast to those evident in muscle and bone.

- There appeared to be no major signs of naturally occurring osteoarthritis in the C57Bl/6 mouse. As previously discussed this could be due to the fundamental difference in movement between mice and humans.

In summary, this study has integrated methods of measuring and quantifying age-related changes in musculoskeletal tissues in C57Bl/6 mice and has identified the time points where major changes in structures are seen. This model of musculoskeletal ageing in C57Bl/6 is a powerful model to study age-related changes in muscle mass and bone although may not be as useful in measuring changes in the joint or tendon with age.

Taking these values and the methods developed for measuring and images musculoskeletal tissues, we feel confident in applying these to experimental procedures involving interventions to prevent of delay the age-related changes in muscle and bone in this mouse model.

Having looked at how the C57Bl/6 mouse ages under uninterrupted environments, we will look to prevent these detrimental changes seen with age. To fully translate any therapy into future human work, we will look first to alter the diet to promote improvements in age related changes found in musculoskeletal tissues, providing a less invasive form of treatment.

Chapter 4

Age-related changes in musculoskeletal tissues of C57Bl6 mice following short and long-term treatment of mice with sodium nitrate

4.1 Introduction

4.1.1 Current dietary interventions aimed at improving age-related loss of musculoskeletal mass and function

The transition from middle age to elderly is usually associated with a decrease in body mass, partly through loss of appetite (Morley, 1997). From the age of 20 to the age of 80, total dietary intake decreases up to 1200 kcal in men and 800 kcal in women per day which can be further compounded by other factors such as hospitalisation (Trumbo et al., 2002). In addition, advanced age often leads to changes in food preference and a switch to sweeter foods, ultimately missing dietary targets for protein, vitamin and mineral intake. Age UK (June 2015) estimates 1.3 million people aged over 65 suffer from malnutrition.

As we shall see, although improving diet in aged populations has had positive effects in modulating some of the age-related decline in tissues (particularly muscle), these results have often been in combination with other interventions such as exercise and so far, few dietary interventions alone have resulted in significant improvement musculoskeletal tissues structure and function in humans.

A well characterised dietary intervention aimed at improving muscle function is increased protein intake. As discussed previously in Section 1, muscle relies on a large supply of protein from the diet in order to function correctly. As we age, there is evidence of reduced protein intake (Kerstetter et al., 2003) and it is very rare to find older individuals who consume the highest recommended protein values (Trumbo et al., 2002). There are a number of studies showing that increased total protein intake or increasing the intake of essential amino acids can improve some measures of muscle quality in older people (Farsijani et al., 2016). Despite the popularity of increasing protein in the diet, very few studies have been conducted to determine the effects modified dietary protein intake on bone mass and function – a weakness shared with many dietary studies.

One dietary intervention has recently been proposed to potentially be beneficial to both muscle and bone tissues. This is increased dietary nitrate.

4.1.2 Nitrate effects within the body

Nitrate and nitrite were originally deemed unnecessary residues from the food chain that may carry a potential carcinogen risk or as the inert oxidative end products of nitric oxide metabolism (Gilchrist et al., 2010). This approach has changed since ~2000 and it is now known that these compounds are capable of eliciting physiological effects through the conversion to nitric oxide and other active nitrogen oxides (Lundberg et al., 2008). Nitrates are therefore thought of as dietary sources of nitric oxide, complementing the cellular nitric oxide synthase pathways. This had led to research into the possible function of nitrate and nitrite supplementation within the body (Lundberg et al., 2008).

The most abundant source of nitrates in the diet is green vegetables (van Velzen et al., 2008). Nitrate is actively taken up into the blood via the salivary glands where it is concentrated 10-20 times within the saliva (van Velzen et al., 2008). Approximately 25% of nitrates are taken up this way. It has been reported that a portion of vegetables high in nitrate such as spinach contain more nitrate than is formed endogenously over the course of a day (van Velzen et al., 2008).

Production of nitrite from nitrate appears to be facilitated through commensal bacteria in the oral cavity of mammals. Anaerobic bacteria in the oral cavity contain nitrate reductase enzymes which reduce approximately 20% of nitrate to nitrite (Doel et al., 2005). These bacteria assimilates nitrate for use in the production of energy in the absence of oxygen, forming nitrite as a by-product (Doel et al., 2005, Moreno-Vivian et al., 1999) so the level of nitrite found in saliva is increased (Lundberg and Govoni, 2004).

Nitrite is converted to nitric oxide (NO) through the acidic conditions in the stomach (Benjamin et al., 1994). Much of the nitrite found here can pass into the systemic circulation without being further converted (Lundberg and Govoni, 2004). Once within the circulation, there are numerous tissues that can convert nitrite into the active NO molecule. These include both haemoglobin and myoglobin amongst other compounds (Lundberg and Govoni, 2004). The well characterised function of NO is to modulate blood flow in times of hypoxia. In hypoxic conditions, nitric oxide synthase enzymes perform poorly. It has been

shown that the body can actually store and recycle the nitrate-nitric oxide products (Lundberg and Govoni, 2004).

Both nitrate and nitrite are known to modulate oxidative stress, mitochondrial function and have been shown to reduce oxygen consumption during exercise - a property that has led to a much interest in sports science research (Larsen et al., 2007).

4.1.3 Effects of nitrate supplementation on muscle mass and function

The effects of nitrate supplementation on muscle especially during exercise have been relatively widely studied. Larsen et al (2007) showed that supplementation of people with 0.1 mmol/kg/day of sodium nitrate was capable of reducing the amount of oxygen needed by humans to perform cycling exercises (Larsen et al., 2007). This was followed by another report showing a 16% improvement in tolerable duration of high intensity exercise. Improvements have been shown in walking, running, cycling, and rowing exercises, with positive results in both acute and long-term supplementation (Affourtit et al., 2015).

In muscle, it has been stated that nitrate supplementation alters the rate of oxidative ATP synthesis (reducing the amount of oxygen needed) and lowers the oxygen and ATP cost of contractile force production (Whitfield et al., 2016, Kemp, 2016). Within cell culture models of muscle, treatment with nitrate can lead to mitochondrial biogenesis, increasing cellular respiration (Bailey et al., 2009).

The effects of nitrate supplementation however appear to be specific to fast twitch fibres of muscles which may be due to a lower oxygen levels within the microvasculature of these fibres during force production (Hernandez et al., 2012). This furthers the evidence seen of the benefit of nitrates particularly during hypoxic conditions.

Positive effects of supplementation appear to be somewhat blunted in well trained athletes especially those trained in endurance exercise and there is a possible differential effect in ageing populations (Bailey et al., 2009).

Older people have limitations in oxygen delivery to tissues including muscle and reduced skeletal muscle blood flow, both of which are improved in younger subjects during exercise with supplementation of nitrates (Bailey et al., 2009). The potential to improve or reverse this with nitrate supplementation is therefore of major importance.

4.1.4 Effects of nitrate supplementation on bone

Most studies examining the effect of nitrates on bone have examined the product of nitrates, nitric oxide. The effects of NO on bone cells (van't Hof and Ralston, 2001) was summarised detailing a biphasic effect of NO on bone, low concentrations causing induction of bone resorption and high concentrations inhibiting bone resorption. NO supplementation of mice had the same effect on bone formation (van't Hof and Ralston, 2001). In mice lacking a subtype of nitric oxide synthase (eNOS), major defects in bone formation and osteoblast activity were found (Armour et al., 2001). This is promising considering that the amount of nitric oxide gained from the diet can potentially outweigh that of the synthase enzymes.

The major NO donor used in animal studies is nitroglycerin, an organic nitrate, used in humans primarily for treatment of heart related conditions. Nitroglycerin treatments prevented ovarectomy- and corticoid-induced bone loss above and beyond the effect of treatment with oestrogen (Wimalawansa, 2000) and has been shown to have additional beneficial effects on both trabecular and cortical bone when given in combination with more recognised therapies (Wimalawansa, 1995). Furthermore, one daily dose of nitroglycerin was sufficient to exert these effects, cumulative effects were not evident.

What is less clear is the role that dietary nitrates may have on bone mass but given the body of research in both bone and muscle, it may be possible to modulate the age-related effects of these two tissues by this treatment.

4.2 Aims

This aims of this study were:

- To examine the effects of short-term treatment of mice (from 18-24 months old) with sodium nitrate on muscle and bone mass and function in C57Bl/6 mice.
- To examine the effects of long-term treatment of mice (from 9-24 months old) with sodium nitrate on muscle and bone mass and function in C57Bl/6 mice.

Hypothesis – the treatment of mice with nitrates prior to the development of deteriorations in both muscle and bone will result in protection against or delay of development of previously characterised deteriorations.

4.3 Methods

4.3.1. Mice

C57Bl/6 mice used for the sodium nitrate intervention studies were obtained at 7 and 16 months of age from Charles River (France) and aged within the Biomedical Services Unit at the University of Liverpool. Mice were fed a CRM (P) rodent diet and were maintained under barrier conditions in microisolator cages on a 12-h dark/light cycle. Body mass were recorded monthly.

The long-term study was started when one cohort of mice reached 9 months of age and involved 20 mice split into two groups of 10 mice. The control group was given 250ml standard drinking water changed weekly. The treatment group was given 1mM sodium nitrate (21mg/250ml) in the drinking water which was also changed weekly. This was repeated until the mice reached 24 months of age. The short-term study began when mice reached 18 months of age. 16 mice were split into two groups of 8 and treated the same way as above. The study was concluded when the mice reached 24 months of age.

For analysis, the control mice from each intervention study have been pooled into one group (n=12) as no differences were noted between any indices measured in the muscle or bone of these two groups of mice.

Six control mice, 2 mice in the long-term treatment group and 2 mice in the short-term treatment group died prior to the end of the study.

4.3.2 Tissue collection and preparation

All tissues were removed post mortem as described in Section 2.2.

4.3.3 Muscle analysis

Extensor digitorum longus (EDL) muscle contractile properties were measured *in situ* as described in Chapter 2. Muscle optimal length (Lo) was determined using a series of 1 Hz stimulation and set at the length that generates the maximal force. To assess the maximum isometric tetanic force (Po) of the EDL muscle, EDL muscles were electrically stimulated to contract via nerve stimulation at Lo and optimal stimulation voltage (8–10 V) at 2min intervals for 300ms with 0.2ms pulse width.

Anterior tibialis (AT), *extensor digitorum longus* (EDL), *soleus* and *gastrocnemius* muscles from both hindlimbs where dissected and weighed. Following this, muscles were snap frozen in liquid nitrogen and stored for further analysis at -80°C

4.3.4 Bone analysis

Analysis of bones was described in Section 2.8.

4.3.5 Statistics

Data was tested for normality and equal variance using SigmaPlot (v11.0) (Systat Software, Inc., London, UK). Direct comparison between two sets of data was made by Student's *t*-test. For multiple comparisons, data was analysed for statistical significance by one-way ANOVA incorporating pairwise comparisons. Data are presented as mean ±SEM. Values $P<0.05$ were considered significant

4.4 Results

4.4.1 Changes in body mass following intervention with sodium nitrate
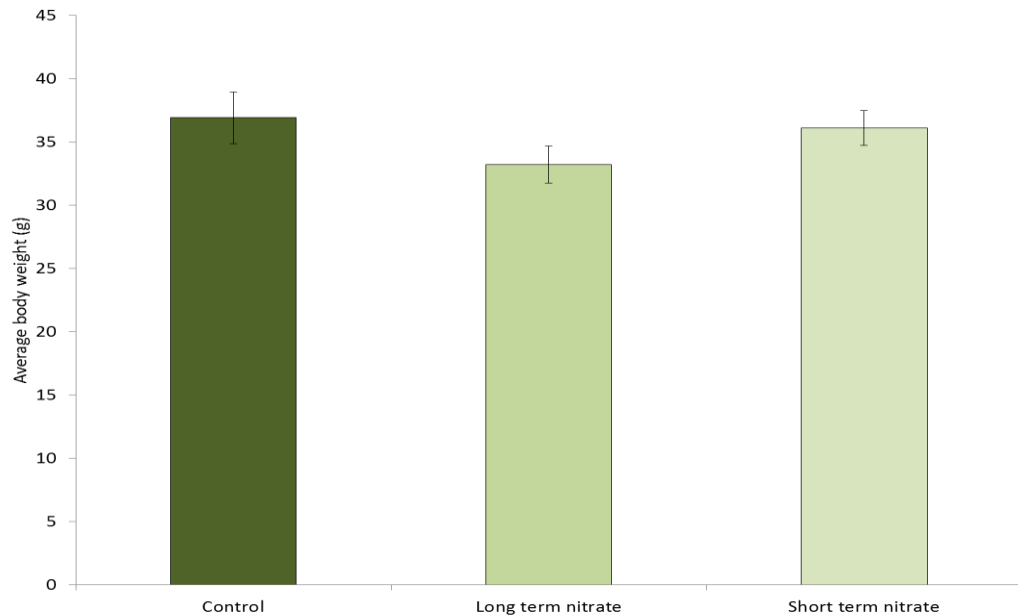
Fig 6.1 Mean body mass of 24 month old control male C57Bl/6 mice following long or short-term nitrate treatment (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated).

Figure 6.1 shows the mean body mass of mice at 24 months of age in C57Bl/6 mice following short or long-term nitrate intervention. There were no significant differences in body mass following either intervention compared with untreated control mice.
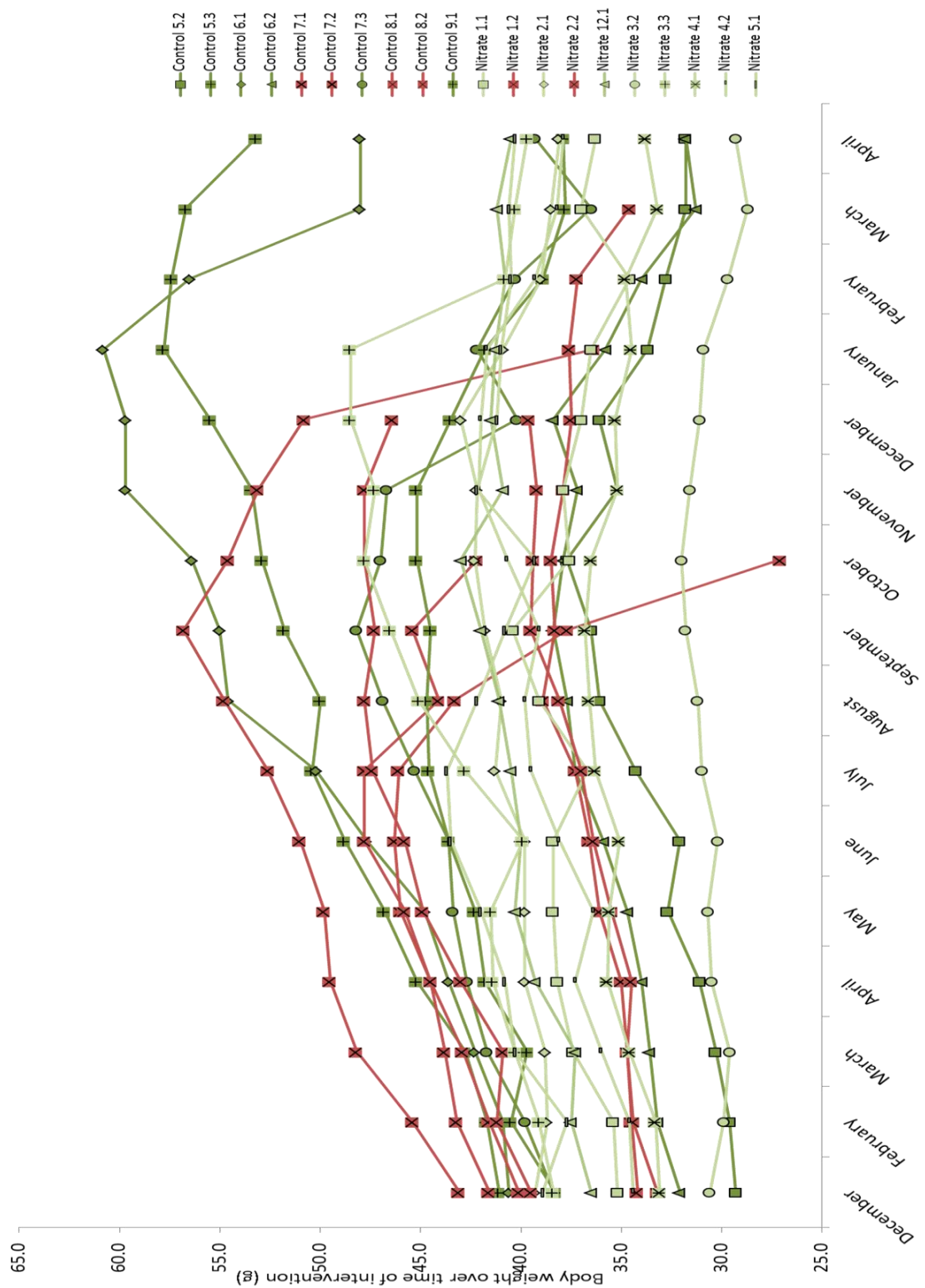
Fig 4.2 Mean body mass of C57Bl/6 mice with and without long-term nitrate intervention over the experimental span (n=7 for control animals, n=8 for treated). Red lines indicate animals that died prior to the end of experiment due to health conditions.

Mean body mass through the span of the experiment were presented. There were more deaths in the untreated group. Control group animals appeared to gain mass more often over the course of the experiment.
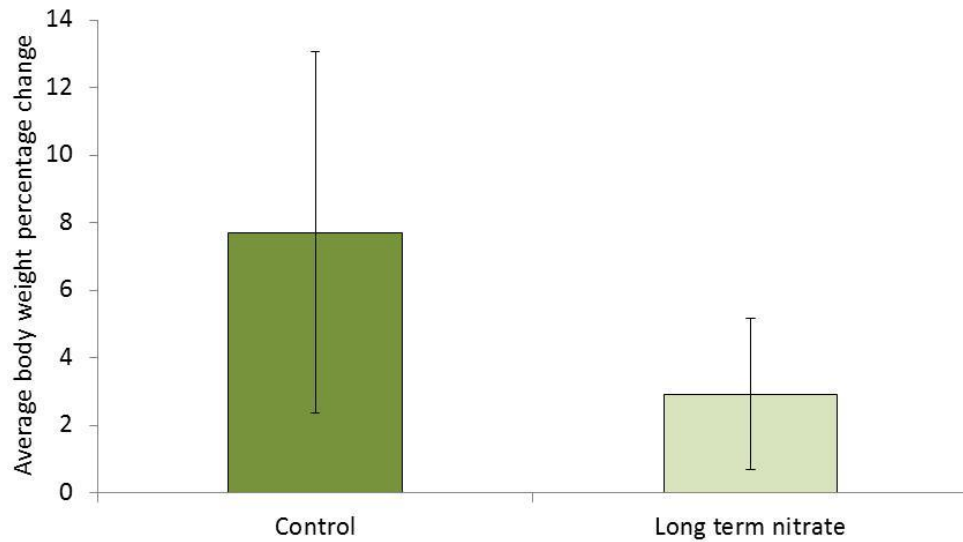
Fig 4.3 Mean change in body mass of C57Bl/6 mice with or without long-term nitrate intervention at 24 months of age displayed as a percentage of starting mass (Data presented as mean +/- SEM, n=7 for controls, n=8 for treated).

Mean body mass change over the intervention time was presented. There were no significant changes.
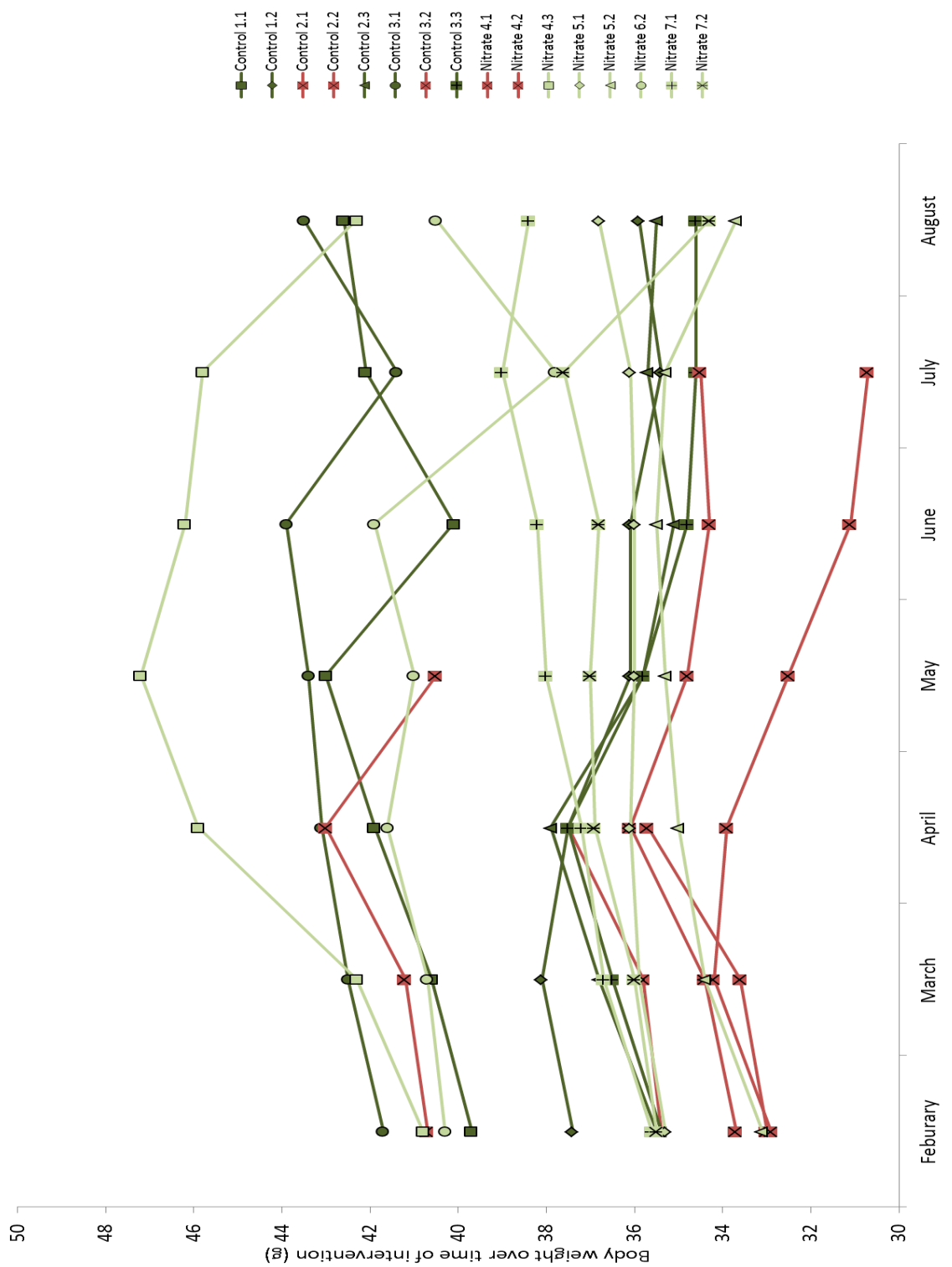
Fig 4.4 Mean body mass of C57Bl/6 mice with and without short-term nitrate intervention over the experimental span. (n=5 for control, n=6 for treated) Red lines indicate animals that died prior to the end of experiment due to health conditions.

Mean body mass through the span of the experiment were presented. There were more deaths in the untreated group.
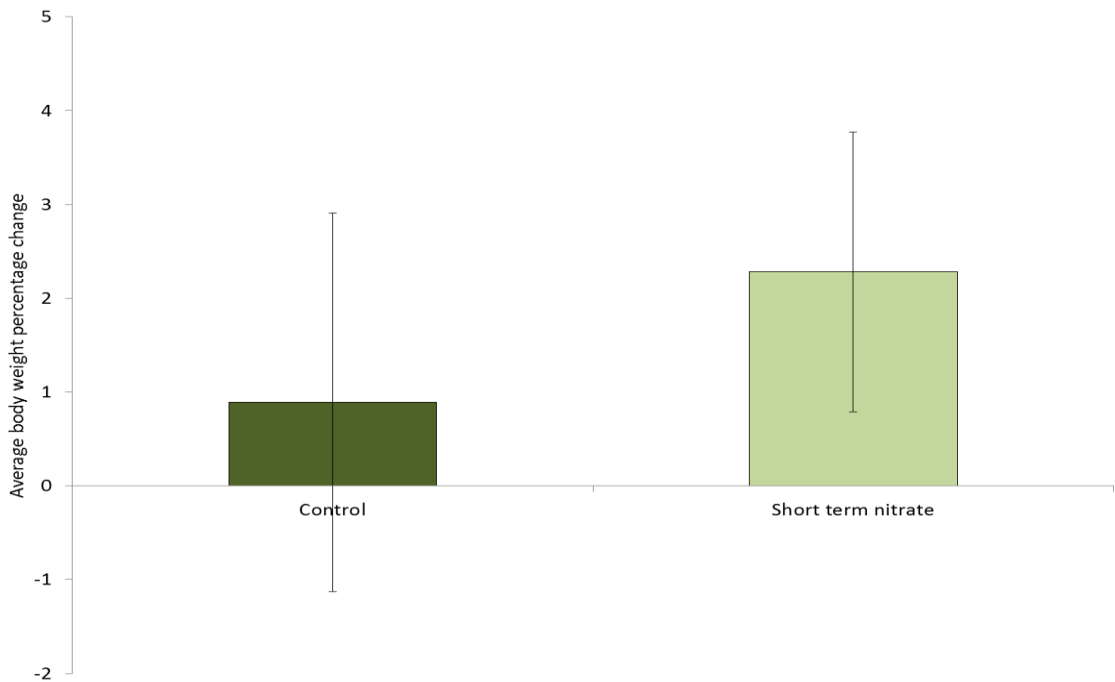
Fig 4.5 Mean change in body mass of C57Bl/6 mice with or without short-term nitrate intervention at 24 months of age displayed as a percentage (Data presented as mean +/- SEM, n=5 for controls, n=6 for treated).

Mean body mass change over the intervention time is shown in Figure 6.5. There were no significant differences in the change in body mass between the groups.

4.4.2 The effects of long and short-term nitrate intervention on organ mass in C57Bl/6 mice

		Control	Long term nitrate	Short term nitrate
Heart	Average	200	229 *	224 *
	SEM	7	13	13
Lungs	Average	258	277	331 *
	SEM	15	12	38
Liver	Average	1751	1562	2222
	SEM	103	76	576
Kidneys	Average	547	569	564
	SEM	26	25	40
Spleen	Average	89	138 *	110
	SEM	6	23	13

Table 4.1 Mean organ mass at 24 months of age with or without sodium nitrate intervention (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated group) * values were significantly changed compared with control data.

		Control	Long term nitrate	Short term nitrate
Heart/BW	Average	5.5	7.2 *	6.6 *
	SEM	0.2	0.4	0.4
Lungs/BW	Average	7.1	8.7 *	9.8 *
	SEM	0.4	0.4	1.1
Liver/BW	Average	48.2	48.9	65.8
	SEM	2.5	2.4	17.1
Kidneys/BW	Average	15.0	17.9 *	16.7
	SEM	0.6	0.8	1.2
Spleen/BW	Average	2.4	4.1 *	3.3 *
	SEM	0.1	0.7	0.4

Table 4.2 Mean organ mass as a proportion of body mass (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated group) * represents values significantly changed compared with control.

Treatment of mice with nitrate for either long or short-term resulted in increased heart mass compared with that of untreated control mice. Long-term nitrate intervention also caused an increase in spleen mass. Short-term intervention led to increased lung mass.

When taken as a proportion of body mass, heart, lungs, and spleen mass were increased following both short and long-term nitrate intervention compared with data from untreated control mice. Long-term intervention also increase kidney mass.

4.4.3 The effects of long and short-term nitrate intervention on muscle mass and force generation in C57Bl/6 mice

		Control	Long term nitrate	Short term nitrate
AT	Average	49	50	38 #
	SEM	2	3	8
EDL	Average	11	13 *	7 *#
	SEM	0	1	2
SOL	Average	9	10	7
	SEM	0	0	2
Gas	Average	143	147	114 #
	SEM	4	7	22

Table 4.3 Mean muscle mass in mice following short or long-term intervention from four hindlimb muscles. (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated group groups) * were significantly different compared with control; # values were significantly different compared with long-term treated.

		Control	Long term nitrate	Short term nitrate
AT/BW	Average	1.3	1.5 *	1.2 #
	SEM	0.1	0.1	0.1
EDL/BW	Average	0.3	0.4 *	0.2 * #
	SEM	0.0	0.0	0.0
SOL/BW	Average	0.3	0.3	0.2
	SEM	0.0	0.0	0.0
Gas/BW	Average	3.9	4.5 *	3.7 #
	SEM	0.1	0.2	0.3

Table 4.4 Mean muscle mass as a proportion of body mass following short or long-term intervention. (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated group) * were significantly different compared with control; # values were significantly different compared with long-term treated.

Following short-term intervention with sodium nitrate there was a general decrease in muscle mass which reached significance in the EDL muscle.

Following long-term intervention with sodium nitrate, some evidence of increased mass in the EDL muscle but when data was expressed as per unit of body mass, the muscle mass values for AT, EDL and gastrocnemius muscles were all significantly higher than those in the control group. This appears to be primarily due to small decrease (~10%) in body mass of the mice without a concurrent decrease in muscle mass.

Data also demonstrated a differential effect of short and long-term treatment with sodium nitrate whereby the small increases in muscle mass following long-term treatment was not evident following short-term treatment.
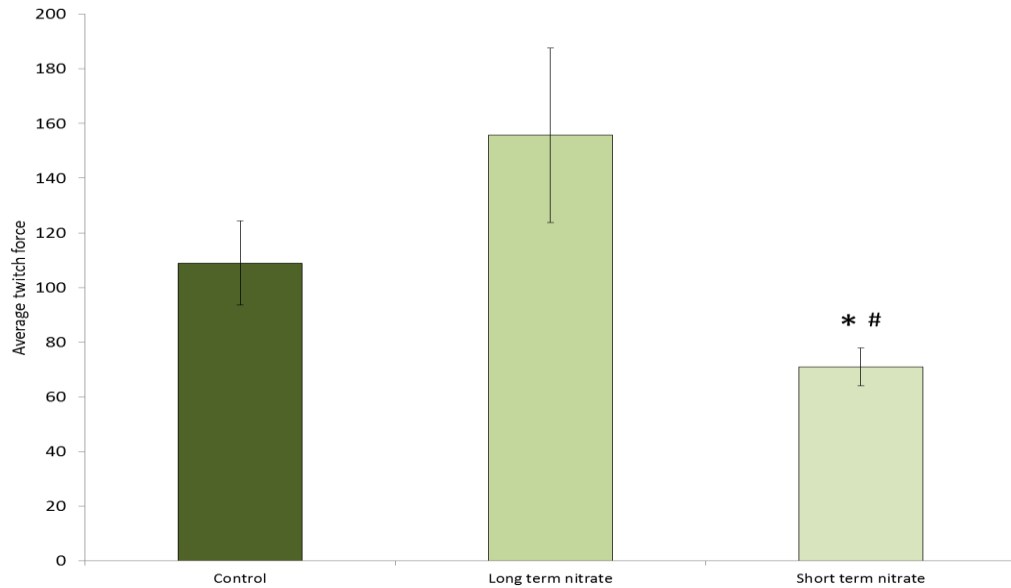
Fig 4.6 Mean twitch force of the EDL muscle of C57Bl/6 mice following long or short nitrate intervention (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated group, n=6 for short-term treated group), * represents significant difference compared with control group, # represents significant difference compared with long-term nitrate treated group.

There were no significant differences in mean twitch force between EDL muscles of the control group and muscles of the long-term nitrate intervention group. Short-term intervention resulted in a significant reduction in twitch force of the EDL compared with both control and long-term intervention groups.
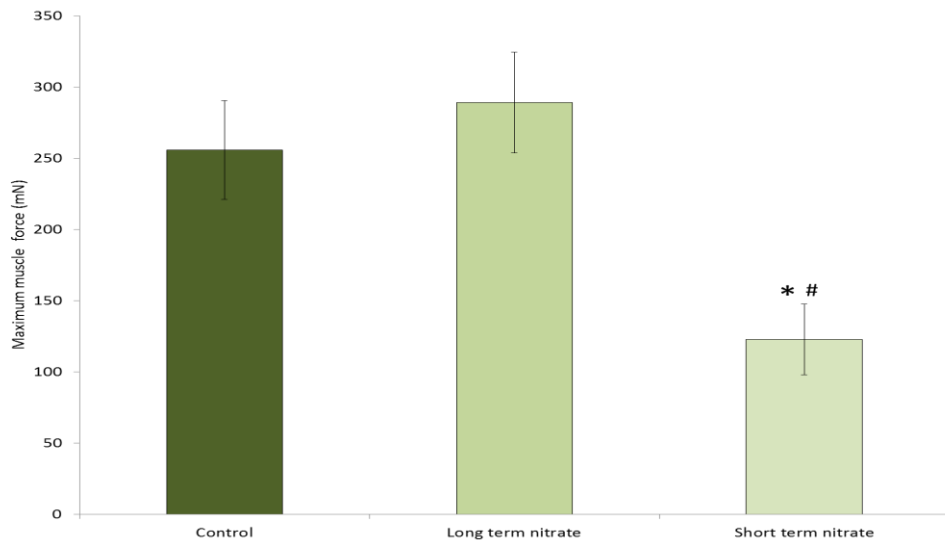
Fig 4.7 Mean maximum tetanic force of the EDL muscle of C57Bl/6 mice following long or short nitrate intervention (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated group), * represents significant change compared with control group, # represents significant change compared with long-term nitrate.

No significant differences in maximum tetanic force generation were seen between EDL muscles of control and long-term nitrate intervention groups. Short-term sodium nitrate intervention resulted in a significant reduction in maximum tetanic force of the EDL compared with both control and long-term intervention groups.
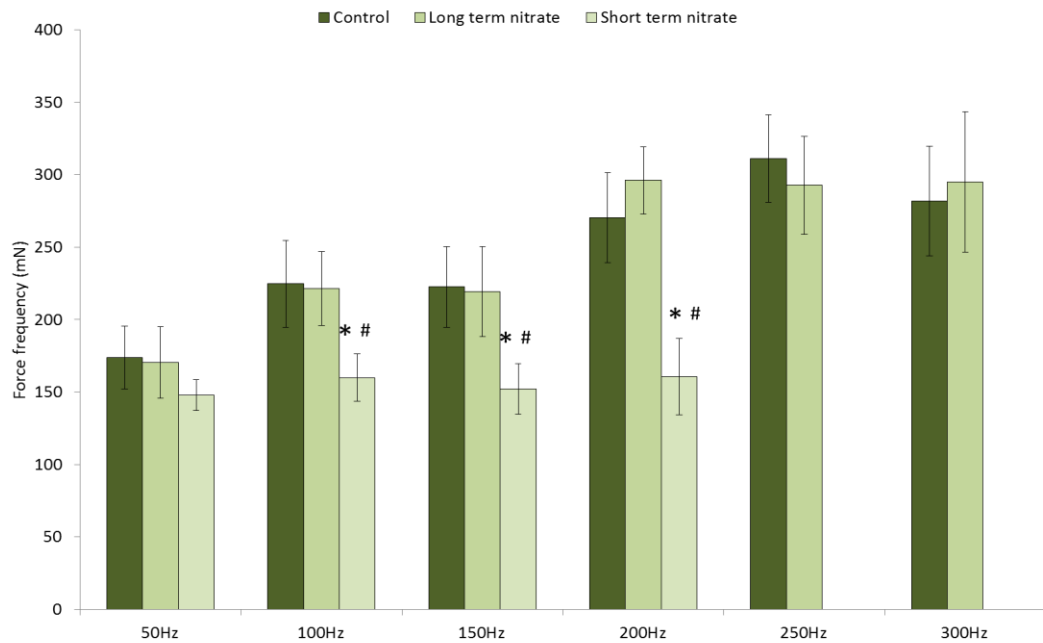
Fig 4.8 Mean force frequency of C57Bl/6 mice with or without long or short-term nitrate intervention at a range of stimulations (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated group), * represents significant change compared with control group, # represents significant change compared with long-term nitrate.

Mean force frequency data following intervention is presented in Figure 6.8. There were no significant differences between control and long-term treated group. Data for the short-term treated group show that the maximum forces were significantly reduced compared with both control and long-term treatment groups resulting in no further analysis taken after 200Hz.
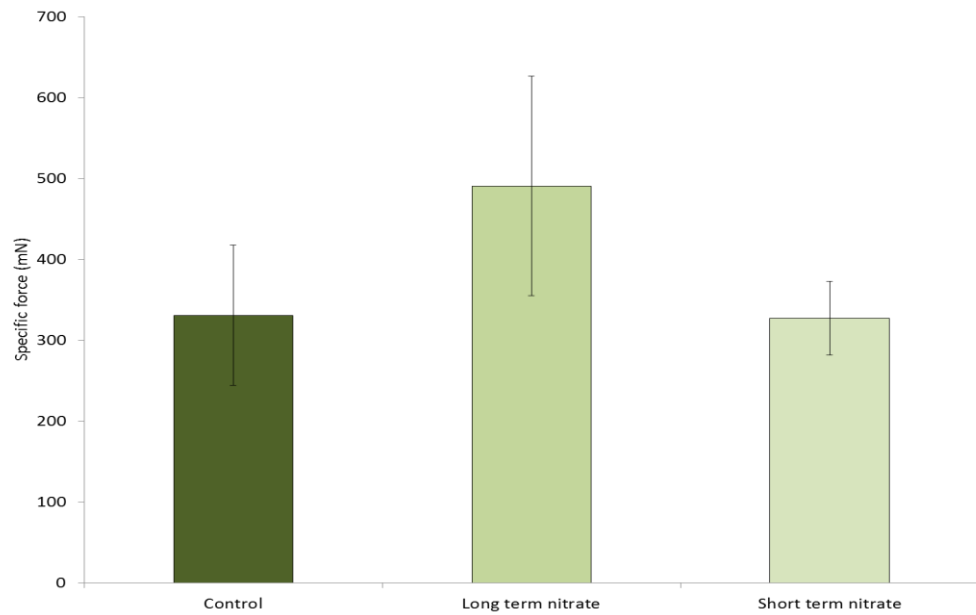
Fig 4.9 Mean specific force of the EDL muscle of C57Bl/6 mice following long or short nitrate intervention (Data presented as mean +/- SEM) (n=12 for combined controls, n=8 for long-term treated, n=6 for short-term treated group),

There were no significant differences in specific force generation by the EDL between control and either nitrate intervention group.

4.4.4 Visualisation of changes in the tibia and femur in C57Bl/6 mice following nitrate intervention
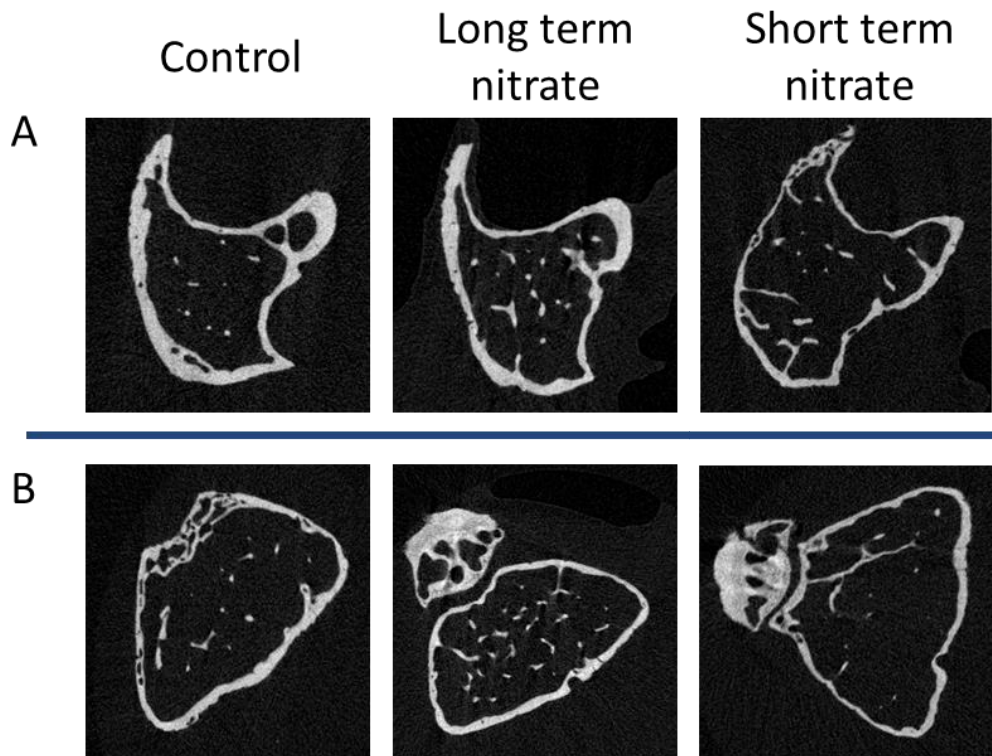
Fig 4.10 Representative MicroCT images of the tibia (A) and femur (B) at 24 months of age in C57Bl/6 mice with or without long-term nitrate intervention.

A representative image from each bone at 24 months of age has been selected based on mean bone values to visualise both the cortical and trabecular bone. There were no visual differences in bone appearance between the treated and untreated groups.
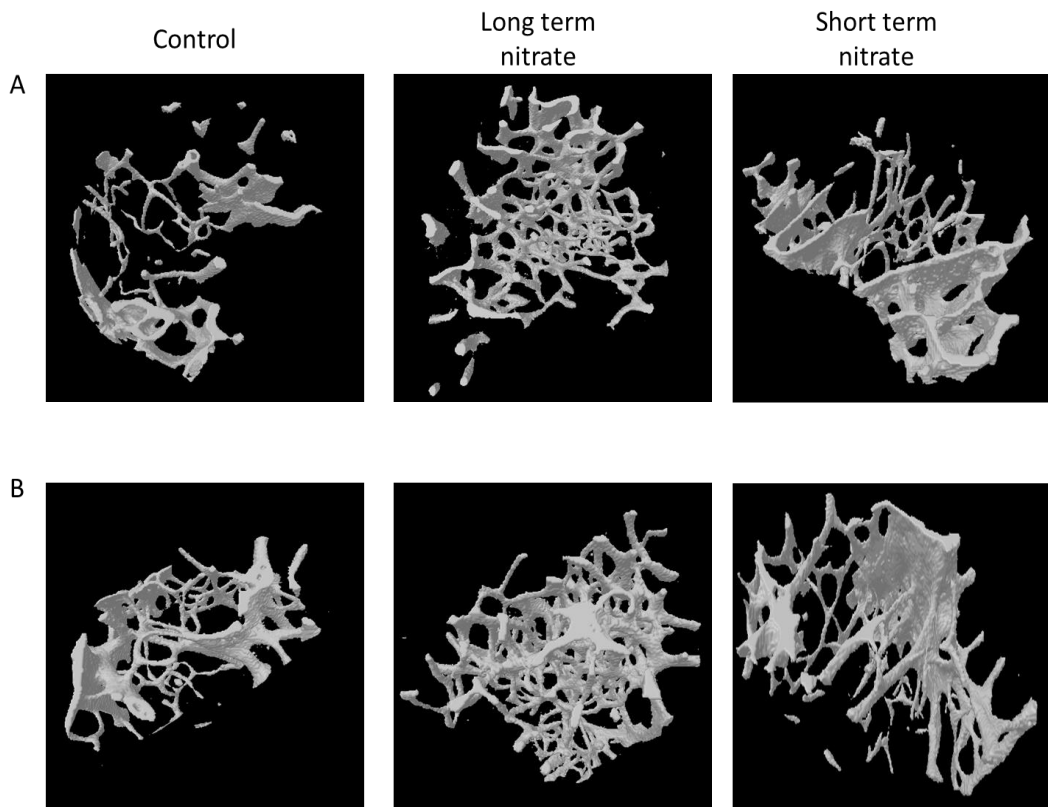
Fig 4.11 3D representative models of trabecular bone in tibia (A) and femur (B) at 24 months of age from both treated and untreated group.

No visual differences in appearance of bone between the treated and untreated group were seen.

4.4.5 Effects of long and short-term nitrate intervention on cortical and trabecular bone measures in C57Bl/6 mice
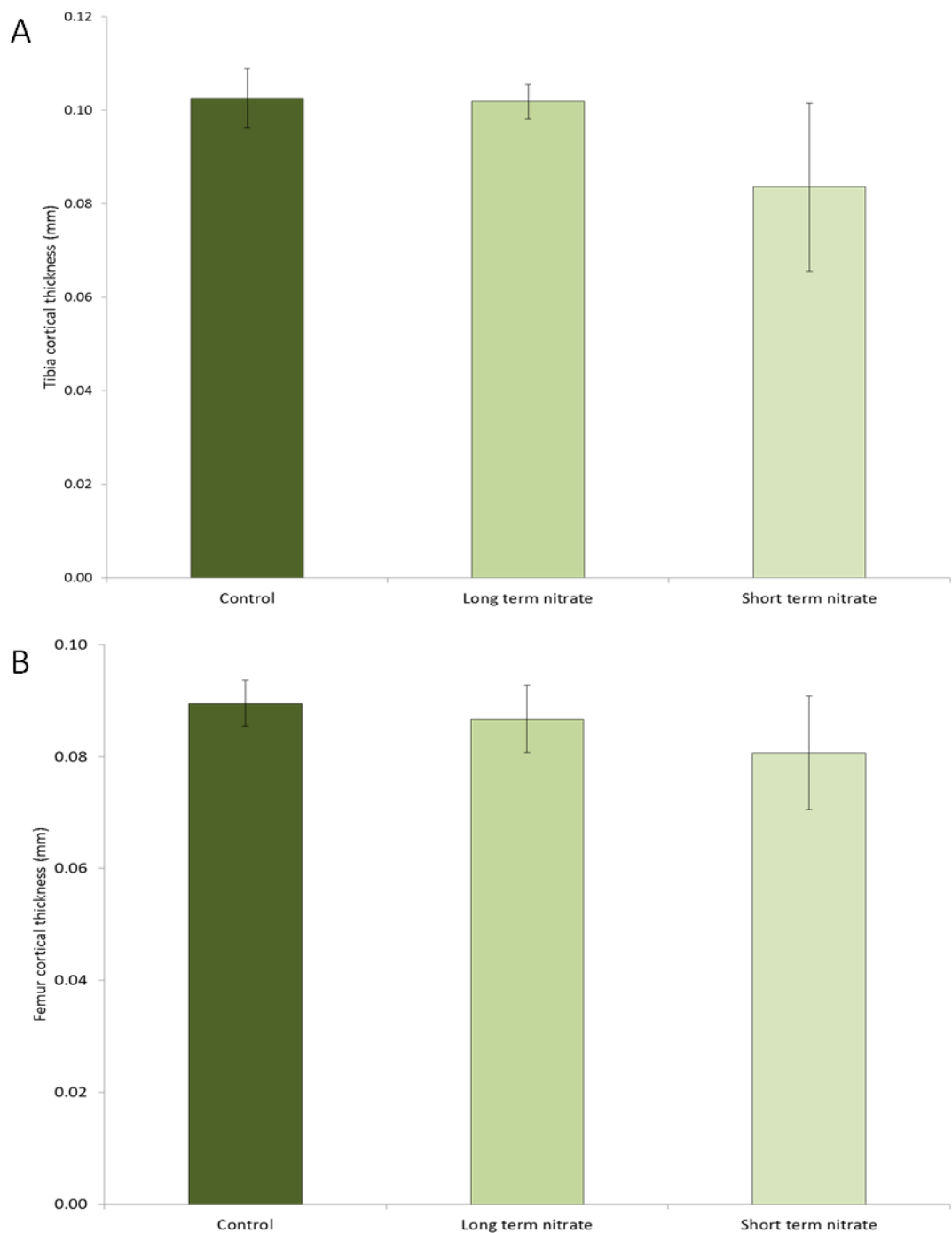
Fig 4.12 Mean cortical bone thickness of tibia (A) and femur (B) with or without long or short nitrate intervention (Data presented as mean +/- SEM, n=12 for controls, n=7 for long-term treatment group, n=6 for short-term treatment group)

No significant differences were seen in cortical thickness following short or long-term nitrate intervention in either bone site compared with control values.
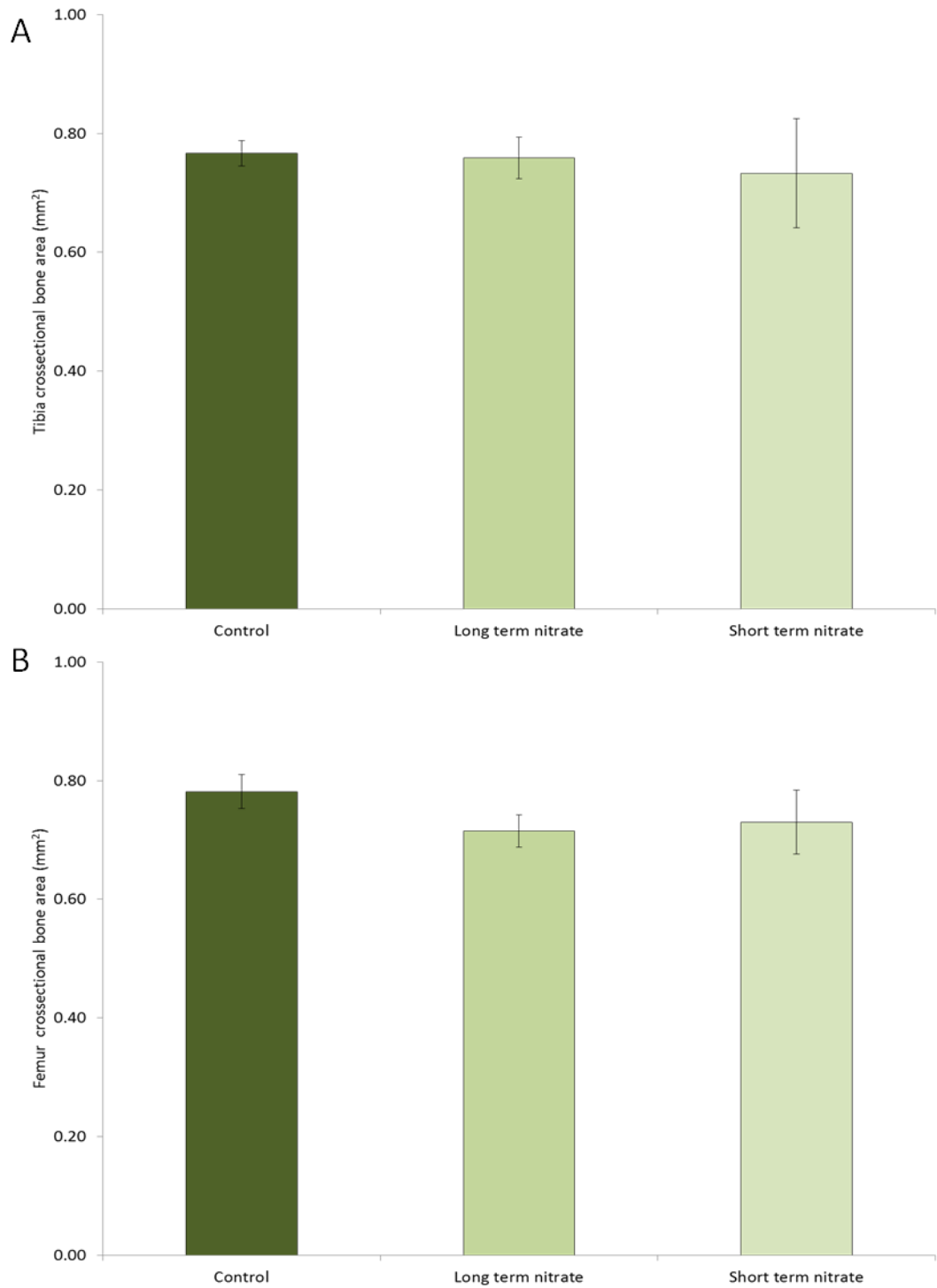
Fig 4.13 Mean cross sectional area values for tibia (A) and femur (B) with or without long or short nitrate intervention (Data presented as mean +/- SEM, n=12 for controls, n=7 for long-term treatment group, n=6 for short-term treatment group)

There were no significant differences in cross-sectional bone area following long or short-term nitrate intervention in either bone site compared with control values.
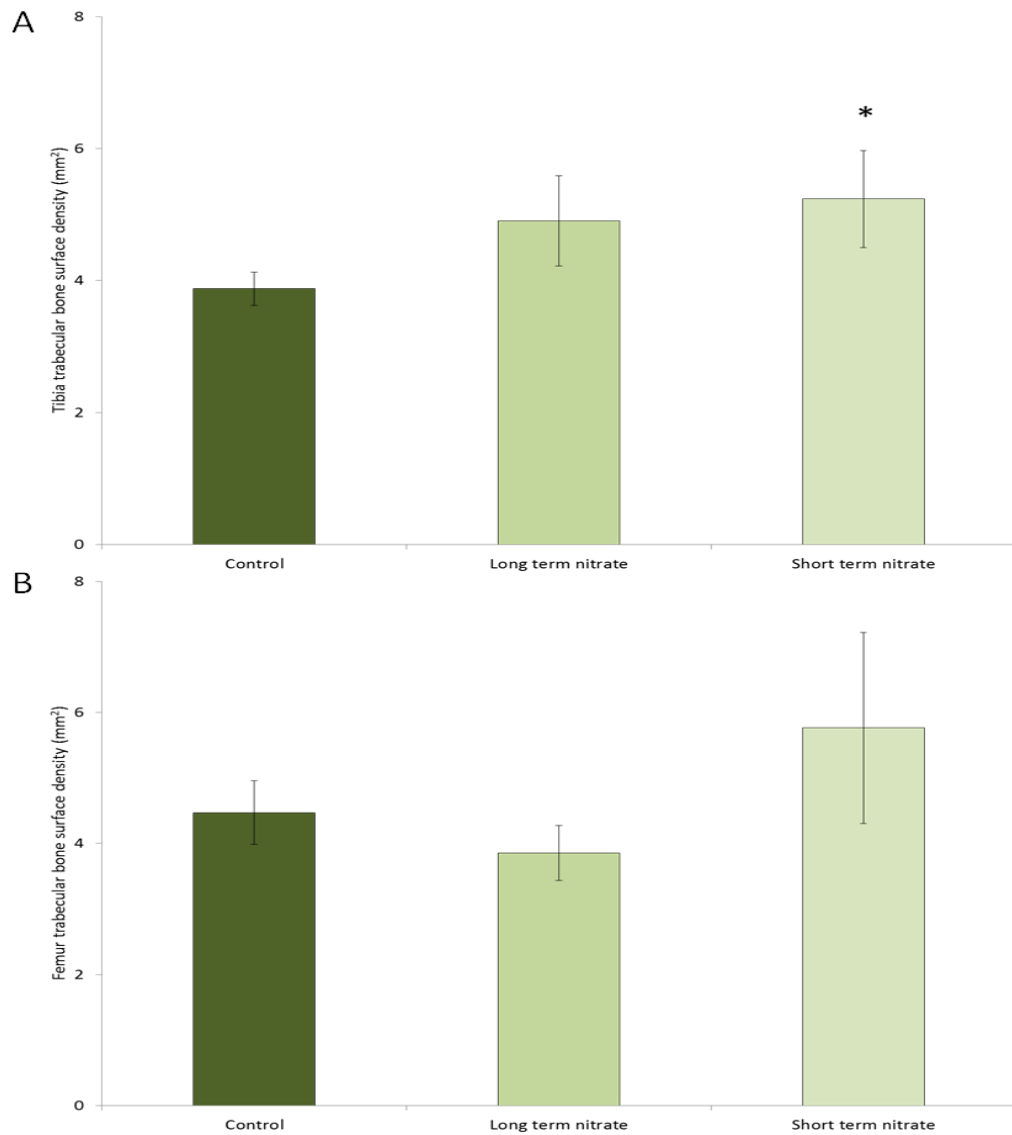
Fig 4.14 Mean trabecular bone surface density values for tibia (A) and femur (B) with or without long or short-term nitrate intervention (Data presented as mean +/- SEM, n=12 for controls, n=7 for long-term treatment group, n=6 for short-term treatment group). * represents significant differences compared with control group.

No significant differences were seen in trabecular bone surface density following long-term nitrate intervention in either bone studied compared with control values.

Tibia bone surface density was significantly increased following treatment with short-term treatment with nitrate compared with the tibia bone surface density of control untreated mice and a similar but non-significant trend was seen in the femur.
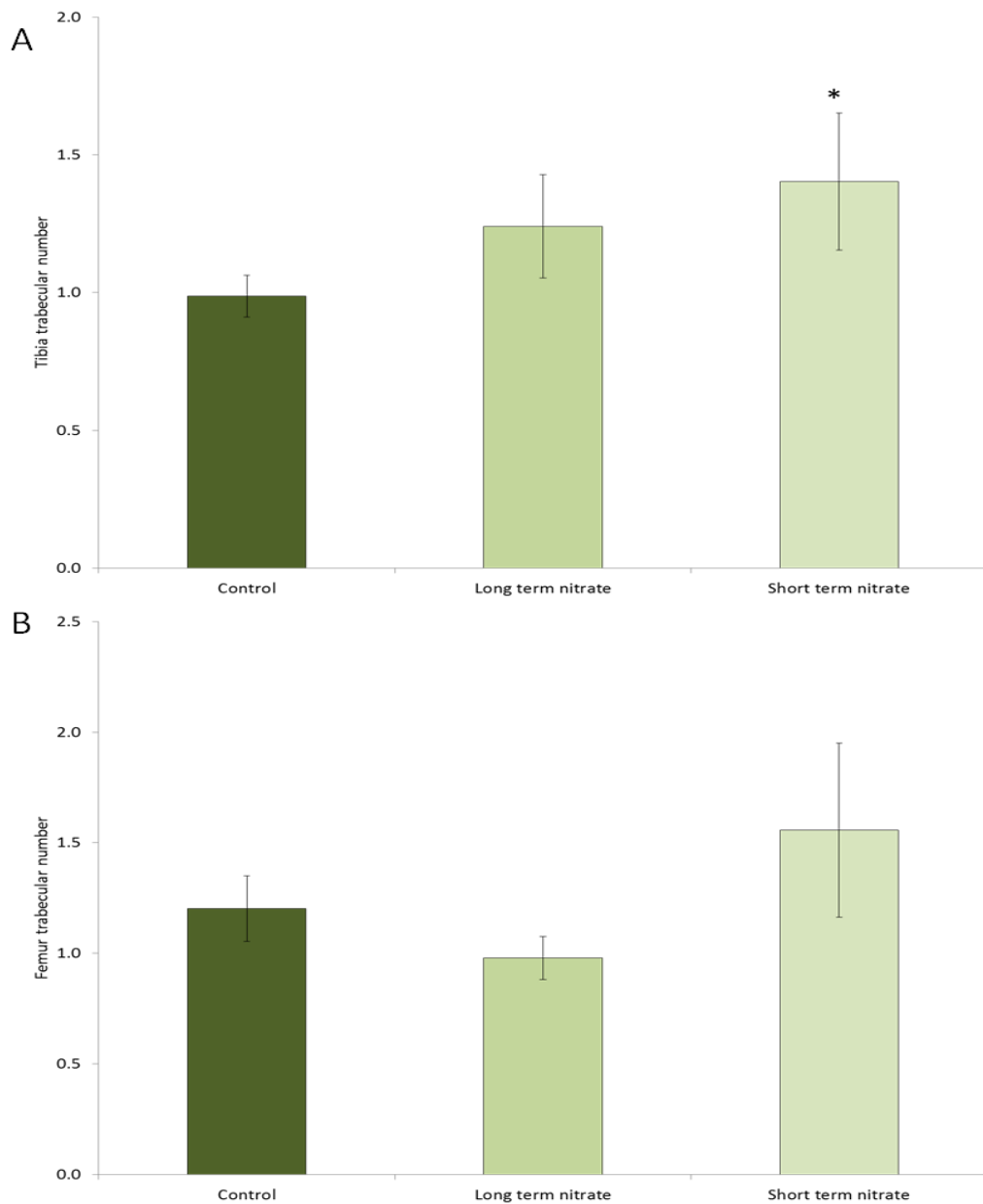
Fig 4.15 Mean number of trabecular bone per mm present in the tibia and femur with or without long or short-term nitrate intervention (Data presented as mean +/- SEM, n=12 for controls, n=7 for long-term treatment group, n=6 for short-term treatment group). * represents significant values compared with control.

No significant differences were seen in either the tibia or femur in the number of trabecular bone following long-term nitrate intervention compared with that of control mice.

There was a significant increase in trabecular number in the tibia following short-term treatment compared with that of untreated control mice. A similar pattern was seen in the femur but these data did not reach significance
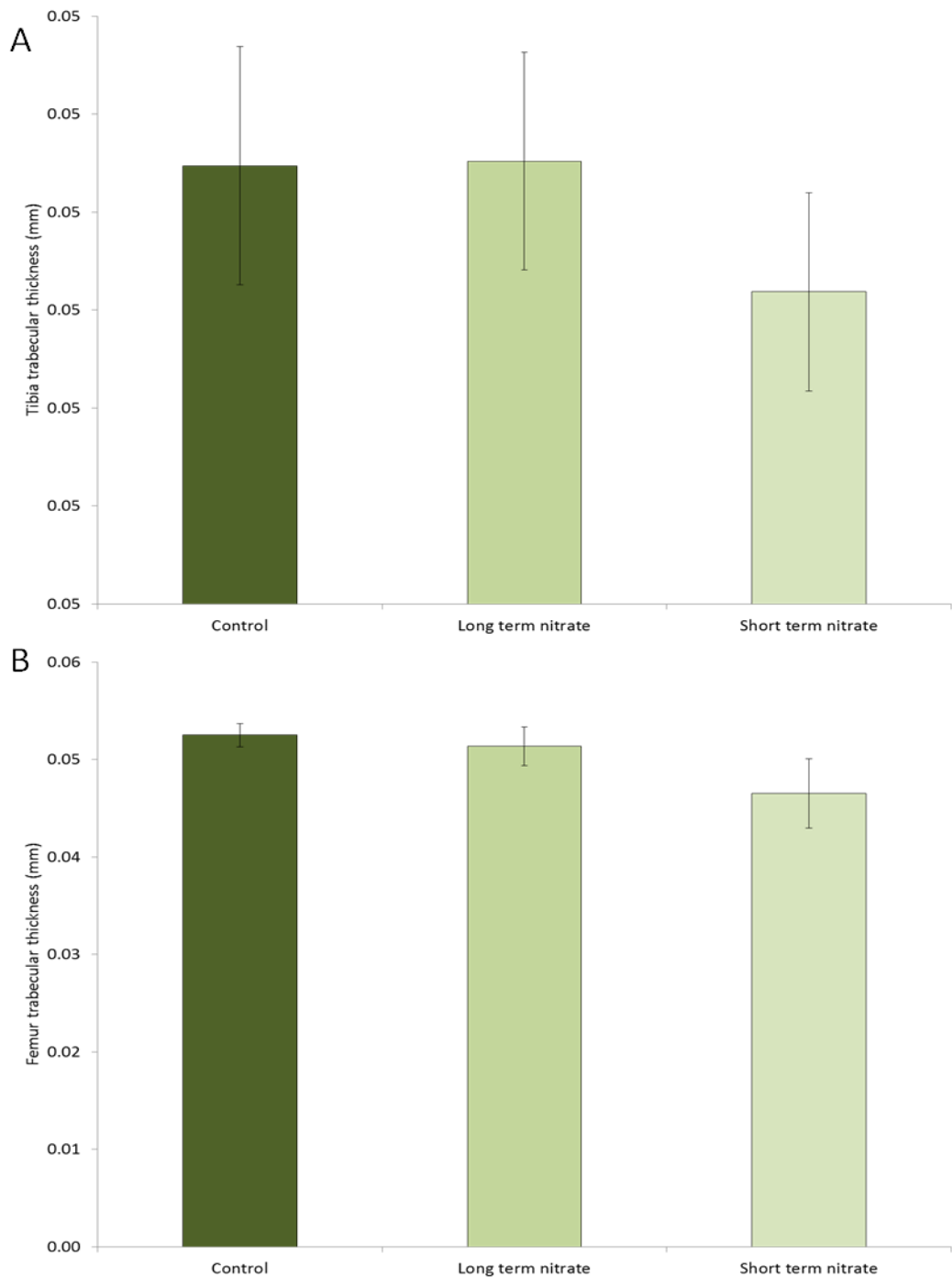
Fig 4.16 Changes in trabecular bone thickness at 24 months of age in the tibia and femur with or without long or short-term nitrate intervention (Data presented as mean +/- SEM, n=12 for controls, n=7 for long-term treatment group, n=6 for short-term treatment group).

No significant difference in trabecular bone thickness was recorded between treated and untreated groups at 24 months of age. There was a trend towards reduced thickness in the short-term nitrate group.
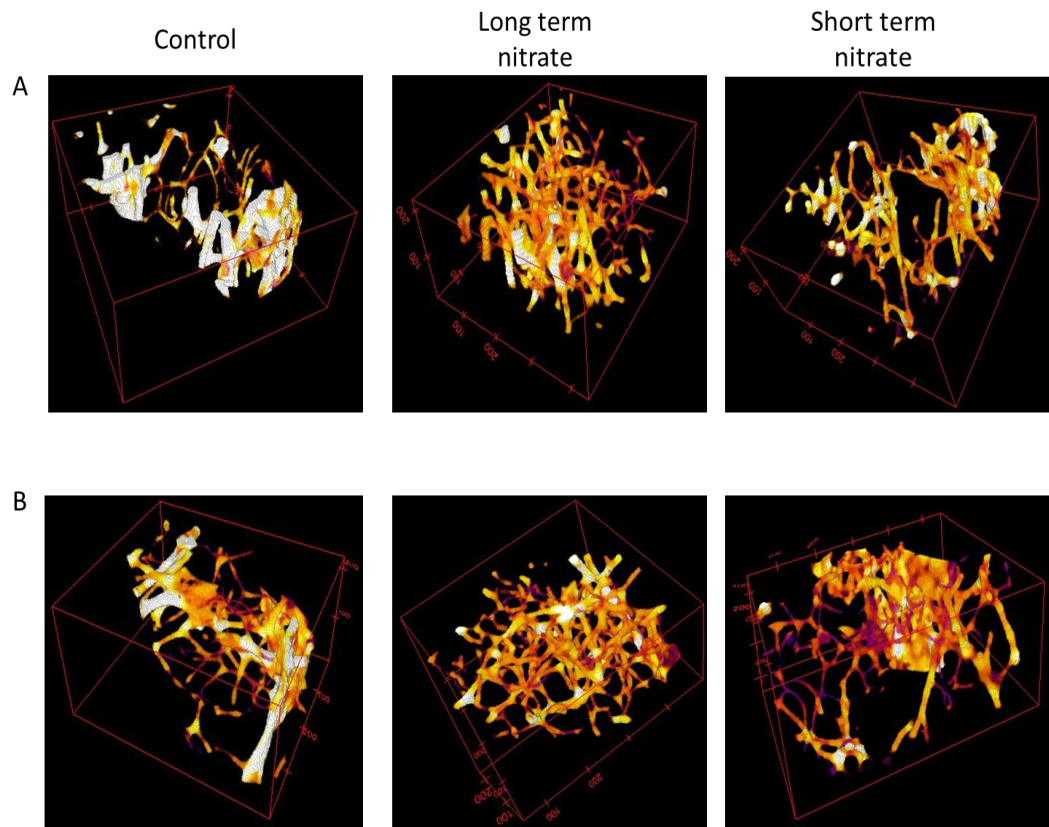
Fig 4.17 3D representation of trabecular bone thickness through age in the tibia and femur of untreated mice or mice treated with sodium nitrate. White colours represent the thickest points, purple colours representing the thinnest areas. As each colour chart is based on individual thicknesses, no key is available.

The changes in trabecular thickness properties are generally evident in 3D modelling.

4.5 Discussion

4.5.1 Effects of long-term sodium nitrate intervention on age-related changes in muscle and bone of C57Bl/6 mice

4.5.1.1 Effect of long-term treatment of mice with sodium nitrate on body mass and organ mass

Long-term treatment of mice with sodium nitrate had no effect on body mass.

This allows further analysis of tissue mass to be undertaken without any confounding factors of changes in body mass.

Long-term treatment of mice with nitrate resulted in a significant increase in the mass of both heart and spleen tissue. When expressed as a proportion of body mass, the long-term treatment also resulted in a significant increase in the lung mass compared with lung mass of the control untreated group.

The mechanisms responsible for these increases in tissue mass are unclear, although nitrates are known to induce changes in the vasculature and control blood flow (Pironti et al., 2016). The increase in heart mass could be due to dilation of blood vessels with the heart compensating by increasing mass and force to increase blood circulation. The increase in lung mass would indicate an increased maximum oxygen intake to match any increases in blood flow. The benefits of this to musculoskeletal tissue would potentially be to increase oxygenation of tissues and organs.

4.5.1.2 Effect of long-term treatment of mice with sodium nitrate on muscle mass and force generation

Following long-term nitrate intervention only the EDL muscle showed a small but significant increase in mass compared with the EDL mass of control untreated mice.

However, when expressed as a proportion of body mass, the mass of the anterior tibialis, EDL and gastrocnemius muscle were significantly increased compared with the control untreated group.

Interpretation of the data is difficult since these changes seem to be due to a small, non-significant reduction in body mass. Although this would suggest that long-term nitrate intervention has had a significant effect on muscle mass based on the control group in this study.

Due to the increase in heart and lung mass, it might be speculated that there would be an improvement in blood flow and oxygenation to the muscles although this was not measured in this study. This is one possible mechanism by which any increased muscle mass was seen as there is a better supply of nutrients.

This increase in muscle mass was not accompanied by any significant changes in twitch force, maximum force or specific force compared with the control group despite presenting a significantly increased EDL muscle mass compared with the control group suggesting little functional effects of long-term nitrate supplementation or muscle function in old mice.

These data contradict recently published studies discussed previously. However the majority of studies published involve human participants which may be one reason for the discrepancy. Results presented here also disagree with recently published study in comparable mice. Hernandez et al 2012 show improvement in muscle force generation in C57Bl/6 mice following an intervention of only seven days (Hernandez et al., 2012). The muscle force generation data was measured *ex vivo* compared with *in vivo* measurements in this study. This study provided the same nitrate concentration in drinking water, but was undertaken in adult mice. It maybe that muscles of old mice are resistant to the effect of nitrate in a similar manner to other anabolic agents (discussed within Chapter 1).

4.5.1.3 Effect of long-term treatment of mice with sodium nitrate on bone ageing

Treatment of mice with sodium nitrate for long-term had no effect on either the tibia or femur with respect to cortical bone thickness, bone area, trabecular bone surface density, trabecular number or trabecular thickness. This indicates that long-term nitrate intervention at this dose is not effective on bone structure or function.

If as previously suggested, muscle strength influences bone strength, then the lack of significant changes in muscle force generation may also be an influencing factor in the lack of changes to bone structure.

There do not appear to be any published studies on the effect of sodium nitrate intervention on muscle or bone in mammals. Nitroglycerin is the most widely used nitric oxide donor in animal studies and studies using nitroglycerin have been described as beneficial for bone (Wimalawansa, 2000). Although sodium nitrate is capable of increasing the levels of NO within the body, it may be that it is not able to elicit effects on bone at the concentration given in this study.

4.5.2 Effects of short-term treatment of mice with sodium nitrate on age-related changes in muscle and bone of C57Bl/6 mice

4.5.2.1 Changes to body mass

Mean body mass following short-term treatment of mice with sodium nitrate is shown in Figure 4.15. As with the long-term intervention no changes in mean body mass were seen at 24 months old.

Since no significant effects was seen in body mass following either treatment regime, body mass is not likely to be a factor to consider in further analysis of tissue mass. Similarly, no evidence of gross changes in liver mass were seen in these mice (in comparison with Table 3.1, suggesting that liver tumours are not a confounding factor in this study)

4.5.2.2 Changes in organ mass

In a similar manner to the long-term treatment, the mass of the heart and lungs were significantly greater following treatment. When expressing these data as a proportion of body mass, the heart, lungs and spleen were significantly increased again in a similar manner to that seen following long-term treatment.

This may suggest again that nitrate treatment resulted in increases in the circulatory system and thus increasing blood flow and oxygen transport throughout the body by increases in lung capacity and heart mass (Pironti et al., 2016).

Thus, it is clear that intervention with sodium nitrate result in improvements in heart and lung mass independently from the length of intervention or the age at which the intervention is started.

4.5.2.3 Changes to muscle mass and force

Although recorded muscle masses were generally smaller than the control values only the EDL muscle was significantly reduced compared with the control group. This becomes relevant when considering muscle force data as this muscle was used for force measurements.

When taken as a proportion of body mass, the EDL was still significantly smaller in the treated group compared with the control.

The twitch and maximum tetanic force generation values were significantly reduced in EDL muscles of mice treated using the short-term intervention compared with the control values. When force frequency was measured, there was a failure to respond to increases in frequency of stimulation and this became significant at 200Hz. However, specific force was not significantly reduced compared with the controls. This would suggest differences in twitch and maximum force can be completely explained by the smaller EDL muscle in this group.

The mechanisms by which short-term treatment of mice with sodium nitrate results in loss of muscle mass are unclear. One would speculate that it may be due to a potential increase in oxygen uptake from increased heart and lung function. The increase oxygen would lead to an increase in respiration. A by-product of respiration is reactive oxygen species production. Aged cells are unable to effectively deal with reactive oxygen species as well as reactive nitrogen species (which can be derived from NO the end product of nitrate intervention) and are therefore potentially detrimental to muscle of older mammals (Ansari and Mahmood, 2015, Ansari et al., 2015).

The differential effects of short and long-term treatment in mice with nitrate is also surprising but demonstrates the importance of determination of temporal effects of interventions, particularly in an ageing cohort.

4.5.2.4 Changes to bone ageing

Following short-term intervention with sodium nitrate no significant differences were seen compared with controls in either bone in cortical bone thickness, bone area or mean trabeculae bone thickness. In the tibia there was a significant increase in trabecular number and trabecular bone surface density. This trend was also seen in the femur although the data did not reach significance.

The mechanisms responsible for this effect are currently unknown as it was not seen following the long-term nitrate intervention. The change cannot be attributed to changes in muscle as loss of muscle mass and force is proposed to reduce the number of trabecular bone.

The changes in trabecular bone were associated with an increased percentage of smaller trabeculae suggesting that the trabecular bone is preserved by the treatment.

Thus unlike the longer-term treatment, the short-term nitrate intervention had a major effect on trabecular bone although the mechanisms by which this protection occurs are unclear.

4.6 Conclusions

- There appeared to be differential effects of treating mice with sodium nitrate from the age of 9 months or the age of 18 months.
- By treating mice from 18 months, the data suggest this was detrimental to muscle mass and force generation.
- Treating mice from 9 months old resulted in some prevention of muscle mass but no major effect on force generation when compared with control mice.
- Treating mice from 9 months old had no major effects on bone structure but treating mice from 18 months appeared to result in some preservation of trabecular bone. The mechanism by which sodium nitrate displays this temporal effect is unclear but is not related to any positive effects on muscle function.

- The extent of any effects of nitrate intervention may be related to the increased mass of the heart, lungs and thus changes in the circulatory system.

As the nitrate study did not prove conclusive in preventing the age related change we saw in Chapter 3, we must now look to a more invasive process. We will look to use the same mouse type but overexpress proteins known to counteract the increased presence of free radicals that may damage the cellular content.

Chapter 5

Overexpression of heat shock protein 10 (HSP10) in mice to prevent age-related decline in musculoskeletal tissues

5.1 Introduction

The previous chapter showed that introducing a general intervention to treat musculoskeletal ageing is not a feasible way of gaining mass and function in these tissues. Many hypothesize that loss of muscle mass and function results directly in detrimental changes seen in bone (Bren-Mattison et al., 2011, Jang et al., 2010, Wolff, 1988). There is evidence to suggest that by maintaining muscle mass and function bone tissues will be preserved (Burr, 1997).

One leading theory behind the reduction in muscle function is an inability to reverse and repair contraction induced muscle damage leading to reduced force production with advancing age (Faulkner et al., 1995).

Although the mechanisms for muscle losing the ability to repair itself with increasing age are not known, it has been observed that there are age-related loss in proteins responsible for controlling cellular stress and protecting the muscle fibres from long lasting damage such as heat shock proteins (HSPs) (Vasilaki et al., 2003).

Several mouse models exist whereby muscle mass and/or function can be maintained into old age compared with wild type mice. These include models from our own lab such as those overexpressing HSP10 (Kayani et al., 2010).

5.1.1 Heat shock proteins

Heat shock proteins (HSP) are found within all organisms and play integral roles in cellular defence against cellular stresses (De Maio, 1995). The function of HSPs within cells is primarily to chaperone the folding and unfolding of nascent or misfolded proteins. This is hugely beneficial particularly in times of cellular stress as cell content of HSPs can be rapidly up-regulated and so can preserve the functionality of integral cellular proteins. Heat shock proteins are classified according to their molecular mass and include HSP10, 60, 70, 90 (Cuervo and Wong, 2014, Georgopoulos and Welch, 1993).

One of the most widely recognised HSPs is HSP70. The main role of HSP70 is to assist in the folding of newly translated proteins from the ribosome. HSP70 also

has more specific functions such as in preventing cellular apoptosis by neutralising caspases found within the cell cytosol (Tavaria et al., 1996).

However, to facilitate the wide ranging functions HSPs have within cells, many HSPs are specifically located within sub-compartments of the cell. In particular, mitochondria have a number of HSPs including HSP10 and HSP60 (Cheng et al., 1990) (Deocaris et al., 2006). These two HSPs have been shown to work together as the HSP60/10 chaperonin complex which aids protein folding within mitochondrial matrix and prevents protein aggregation of denatured proteins (Cheng et al., 1990, Lin et al., 2001).

Studies have shown that HSP10 may play a role independent of HSP60 involving both classic chaperone function explained above, but also possible roles in cellular signalling (Czarnecka et al., 2006). Although HSP10 is mostly localised within mitochondrial matrix, increased concentration of HSP10 has been found within cell cytosol in certain situations (e.g. cancerous cells) (Czarnecka et al., 2006). How HSP10 is retained within the cytosol during times of cellular stress or damage is unknown.

5.1.2 Role of heat shock proteins in muscle tissues of adult and old mice

Older mice have an attenuated heat shock protein responses in muscles following various stresses (Vasilaki et al., 2002). In particular, it has been shown that, following a damaging exercise protocol, adult mice can repair muscle and regain maximum force production whereas older mice have a 44% decrease in force production at 28 days recovery following damage. This decrease did not occur in transgenic animals overexpressing some heat shock proteins (McArdle et al., 2004). This effect appears to be fibre specific and is more apparent in faster skeletal muscles such as *gastrocnemius* suggesting that the loss of muscle fibres with age reduces the ability of the whole muscle to raise heat shock protein defences in times of cellular stress. The inability to produce HSPs following stress would suggest a mechanism by which loss of muscle force production occurs with loss of proteostasis with increasing age.

5.1.3 Using heat shock proteins as a therapy for age-related diseases

Chaperonopathies are a series of diseases in which molecular heat shock proteins (chaperones) are an integral role in pathological disease states. Often this involves either an abnormally formed heat shock protein at normal cellular levels or reduced levels of the heat shock protein (Cappello et al., 2014).

Transgenic mice provide a reliable way of increasing the cellular levels of HSPs throughout the body. In particular, multiple mouse models have been developed to study the effects of up-regulating or overexpressing certain HSPs and measuring the beneficial or negative outcomes of this.

One model uses muscle specific over-expression of HSP10. Work in these mice has shown that HSP10 can prevent the age-related loss of force production and showed some benefit in preserving cross-sectional area in the EDL muscle (Kayani et al., 2010). The role of HSP10 in bone tissue has not been studied.

5.2 Aims

The aims of this study were as follows:-

- To examine the effect of muscle-specific overexpression of HSP10 on age-related changes in musculoskeletal tissues of mice compared with wild type mice

The hypothesis was that overexpression of HSP10 in muscles reportedly results in preservation of muscle mass and strength and that this preservation had a positive effect on bone structure in old mice.

5.3 Methods

5.3.1. Mice

HSP10 overexpressor mice have been previously described in Chapter 2.2.3 and Kayani et al 2010 (Kayani et al., 2010). Mice were generated via chimeric transgenes containing of human gene variants of HSP10 and HSP60 which were inserted into pCAGGS vectors. The pCAGGS construct allowed both genes to be under the control of the human cytomegalovirus (CMV) intermediate early (hCMV-IE) enhancer and chicken β-actin promoter and intron 1. The CAGGS-HSP10 fragment was cut from the Bluescript II KS vector using Xho1/Not1 digestion to produce a 3.3kb fragment. The CAGGS-HSP60 fragment was cut from the BlueII KS vector using Sal 1/Not1 digestion to produce a 4.8kb fragment. Both fragments were purified and used for microinjection into mouse oocytes. Mice were housed within the Biomedical Services Unit at the University of Liverpool. Mice were fed a CRM (P) rodent diet and were maintained under barrier conditions in microisolator cages on a 12-h dark/light cycle.

5.3.2 Tissue collection and preparation

All tissues were removed post mortem as described in Section 2.3.

5.3.3 Muscle analysis

Anterior tibialis, EDL, *soleus* and *gastrocnemius* muscles from both hindlimbs where dissected and weighed. Following this, muscles were snap frozen in liquid nitrogen and stored for further analysis at -80°C.

5.3.4 Bone analysis

Analysis of bones was described in Section 2.8.

5.3.5 Statistics

Data was tested for normality and equal variance using SigmaPlot (v11.0) (Systat Software, Inc., London, UK). Direct comparison between two sets of data was made by Student's *t*-test. For multiple comparisons, data was analysed for statistical significance by one-way ANOVA incorporating pairwise comparisons. Data are presented as mean ±SEM. Values $P<0.05$ were considered significant.

5.4 Results

5.4.1 Age-related changes in body mass in both C57Bl/6 and HSP10 overexpressor mice
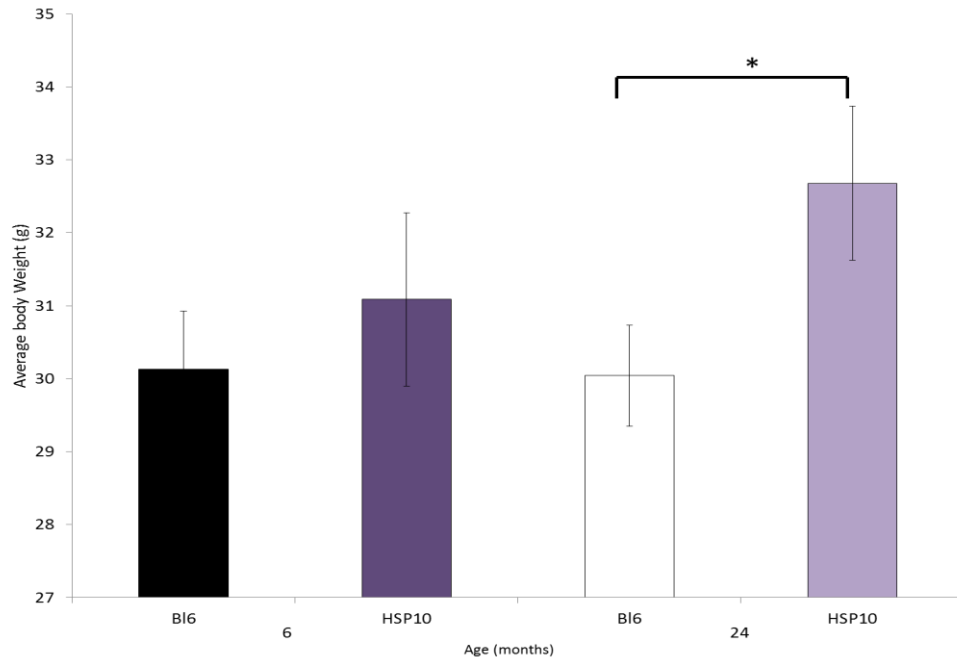
Fig 5.1 Mean body mass of male C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse.

Mean body mass at 6 and 24 months of age in C57Bl/6 mice and HSP10 overexpressor mice are shown in Figure 5.1. No significant differences were seen in body mass of C57Bl/6 or HSP10 overexpressor mice between 6 and 24 months of age. However, the 24 month HSP10 overexpressor mouse was significantly heavier compared to the C57Bl/6 mouse at 24 months of age.

5.4.2 Age-related changes in organ mass in both C57Bl/6 and HSP10 overexpressor mice

		Bl6 6	HSP10 6	Bl6 24	HSP10 24
Heart	Average	197	198	206	230
	SEM	13	8	9	8 * b
Lungs	Average	208	224	229	274
	SEM	12	**18**	13	21 *
Liver	Average	1580	1681	1212	2137
	SEM	76	**42**	128	153 * b
Kidneys	Average	403	439	450	610
	SEM	13	6	35	19 * b
Spleen	Average	71	94	67	109
	SEM	5	6	11	5 * b
Brain	Average	388	340	383	367
	SEM	31	25	23	45

Table 5.1 Mean organ mass (mg) at different ages. a represents significant differences compared with 6 month old Bl6 mice, b represents significant differences compared with 6 month old HSP10 overexpressor mice and * represents significant differences compared with age matched C57Bl/6 mouse data. Data presented as mean +/- SEM.

		Bl6 6	HSP10 6	Bl6 24	HSP10 24
Heart OW/BW	Average	6.5	6.4	5.0	7.2
	SEM	0.3	0.4	0.6 a	0.4 *
Lungs OW/BW	Average	6.9	7.2	6.7	8.6
	SEM	0.3	0.5	1.4	0.8
Liver OW/BW	Average	52.3	54.3	39.1	66.9
	SEM	1.5	1.0	9.8 a	6.6 * b
Spleen OW/BW	Average	2.3	3.0	5.6	3.4
	SEM	0.1	0.18 *	3.0	0.2

Table 5.2 Mean organ mass as a proportion of body mass at different ages. a represents significant differences compared with 6 month old Bl6 mice, b represents significant differences compared with 6 month old HSP10 overexpressor mice and * represents significant differences compared with age matched C57Bl/6 mouse data. Data presented as mean +/- SEM.

C57Bl/6 mice showed significant increases in liver mass at 24 months of age. When expressed as a proportion of body mass, heart and liver were significantly heavier at 24 compared with 6 months old.

HSP10 overexpressor mice had significantly increased heart, liver, kidneys and spleen mass at 24 months old compared with 6 months old. When expressed as a

proportion of body mass, only the liver was significantly heavier at 24 compared with 6 months of age.

The kidneys of the 6 month old HSP10 overexpressor mouse were significantly smaller than those of the C57Bl/6 mouse. The spleen was significantly heavier compared with that of the C57Bl/6 mouse. At 24 months of age, the lungs and spleen were significantly heavier and the kidneys were significantly smaller in the HSP10 overexpressor mouse compared with the C57Bl/6 mouse.

When expressed relative to body mass, the spleen of the 6 month old HSP10 overexpressor mouse was significantly heavier than that of the C57Bl/6 and the heart and liver of the 24 month old HSP10 mouse was significantly increased compared to C57Bl/6 mice at 24 months old.

5.4.3 Age-related changes in muscle mass in C57Bl/6 and HSP10 overexpressor mice
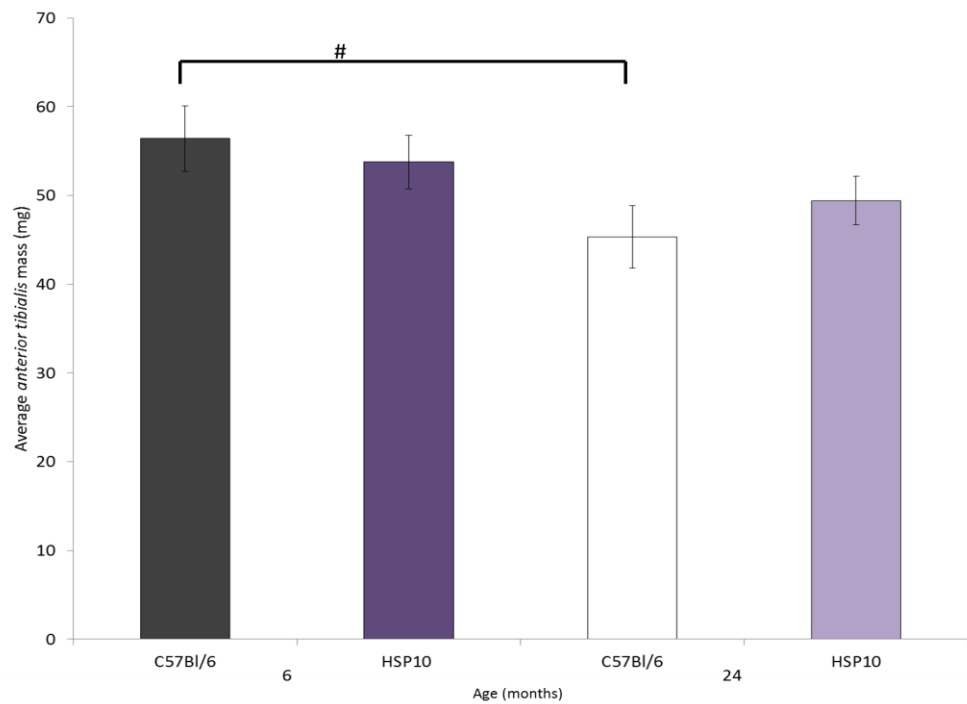
Fig 5.2a Mean *anterior tibialis* muscle mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). # represents significant difference compared with cohort matched 6 month old value.

There was a significant decrease in mass of the *anterior tibialis* muscle between 6 and 24 months of age in the C57Bl/6 mice. In contrast there was no significant change in anterior tibialis mass between 6 and 24 months of age in the HSP10 overexpressor mice.
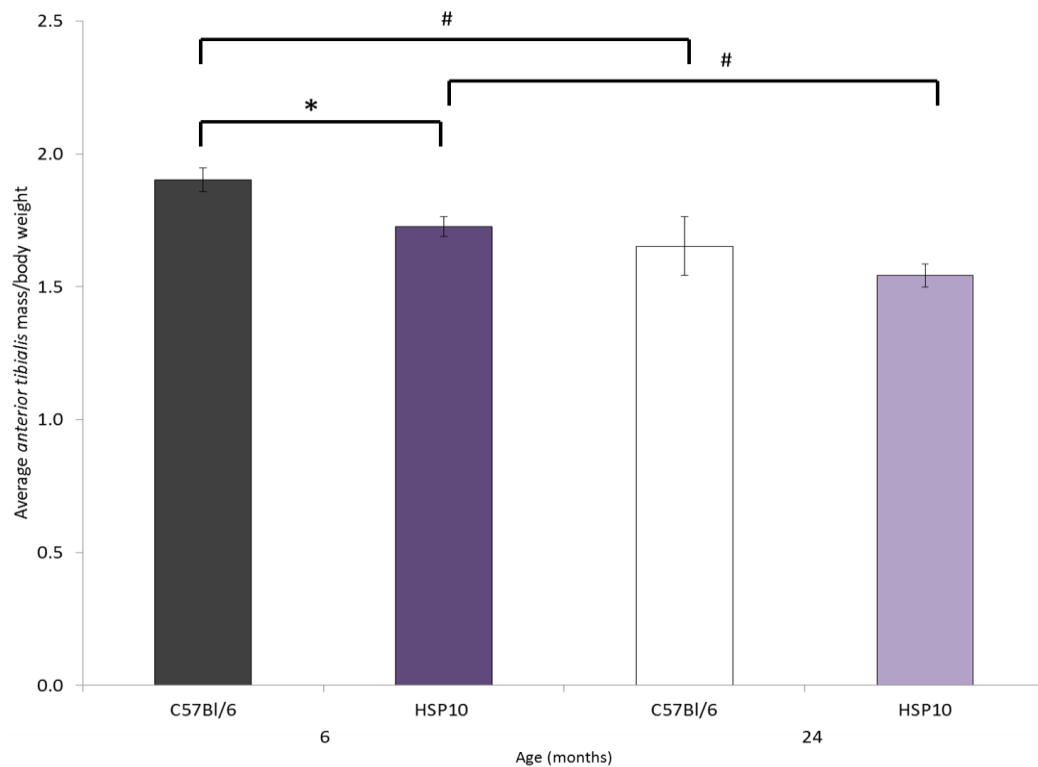
Fig 5.2b Mean *anterior tibialis* muscle mass as a proportion of body mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse, # represents significant difference compared with cohort matched 6 month old value.

There was a significant decrease between 6 and 24 months of age in the *anterior tibialis* muscle as a proportion of body mass in the C57Bl/6 mice.

There was a significant decrease between 6 and 24 months of age in the *anterior tibialis* muscle as a proportion of body mass in the HSP10 overexpressor mice.

The muscle weight value for HSP10 overexpressor mice at 6 months of age was therefore significantly lower when compared with age-matched C57Bl/6 mice.
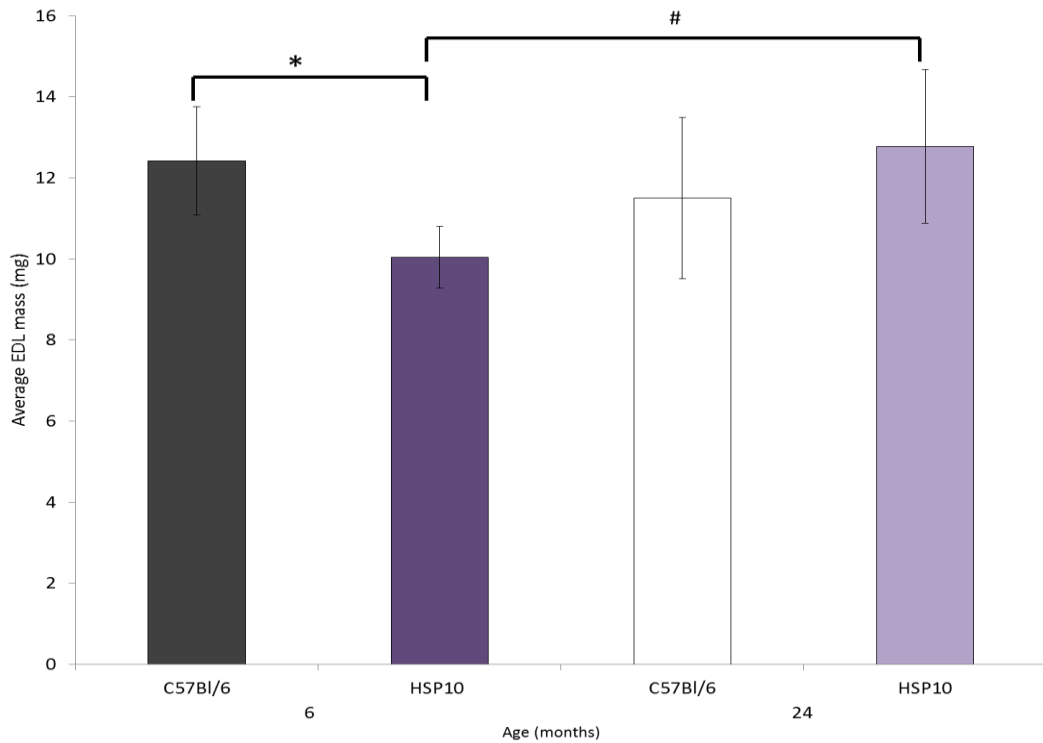
Fig 5.3a Mean EDL muscle mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse, # represents significant difference compared with cohort matched 6 month old value.

There was no significant change in EDL mass between 6 and 24 months of age in the C57Bl/6 mice. In contrast a small but significant increase between 6 and 24 months of age in the EDL muscle of the HSP10 overexpressor mice was seen.

At 6 months of age, the EDL mass from the HSP10 mice was significantly decreased compared to EDL mass from age matched C57Bl/6 mice.
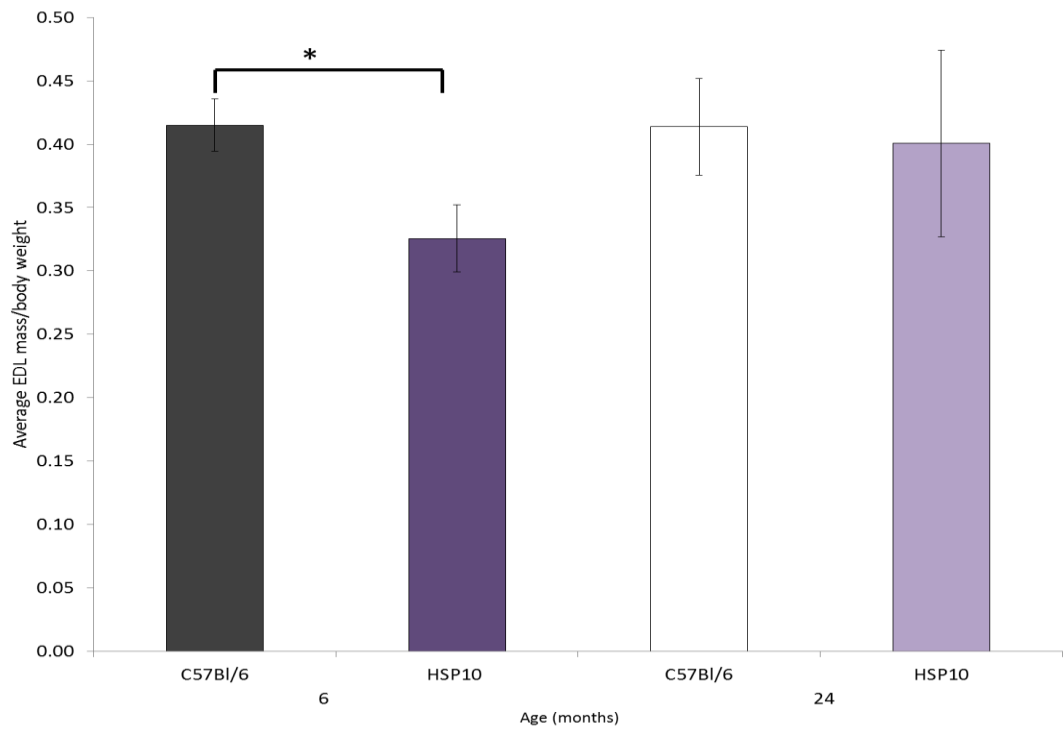
Fig 5.3b Mean EDL muscle mass as a proportion of body mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse.

There was no significant differences between 6 and 24 months of age in the EDL muscle as a proportion of body mass in the C57Bl/6 mice or in the HSP10 overexpressor mice.

The value for HSP10 overexpressor mice at 6 months of age was significantly lower when compared with age-matched C57Bl/6 mice.
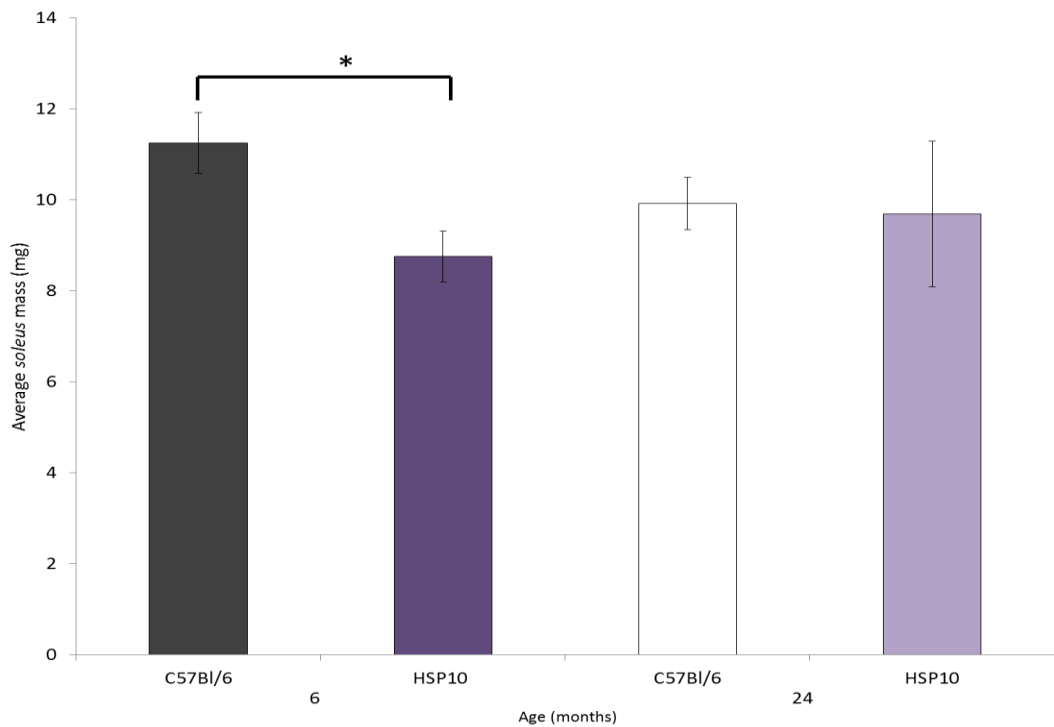
Fig 5.4a Mean *soleus* muscle mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse.

There was no significant change in *soleus* mass between 6 and 24 months of age in the C57Bl/6 mice or HSP10 overexpressor mice.

At 6 months of age, the *soleus* mass from the HSP10 mice was significantly decreased compared to soleus mass from age matched C57Bl/6 mice. There were no significant differences at 24 months of age.
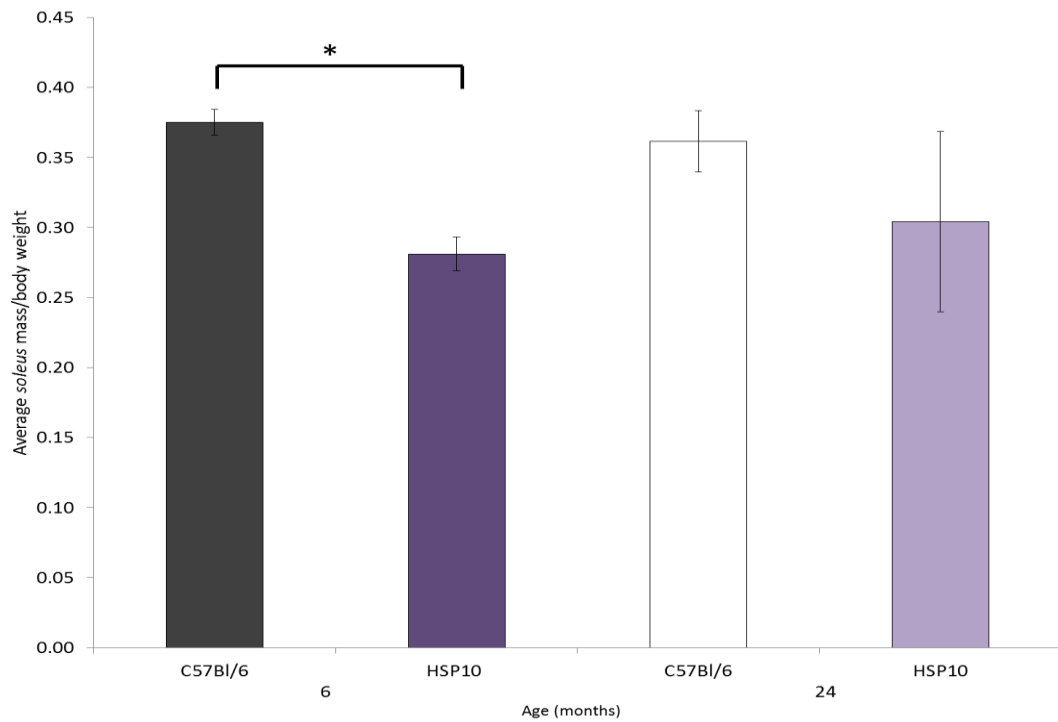
Fig 5.4b Mean *soleus* muscle mass as a proportion of body mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse.

There were no significant changes between 6 and 24 months of age in the *soleus* muscle as a proportion of body mass in the C57Bl/6 mice or HSP10 overexpressor mice.

The value for HSP10 overexpressor mice at 6 months of age was significantly lower when compared with age-matched C57Bl/6 mice.
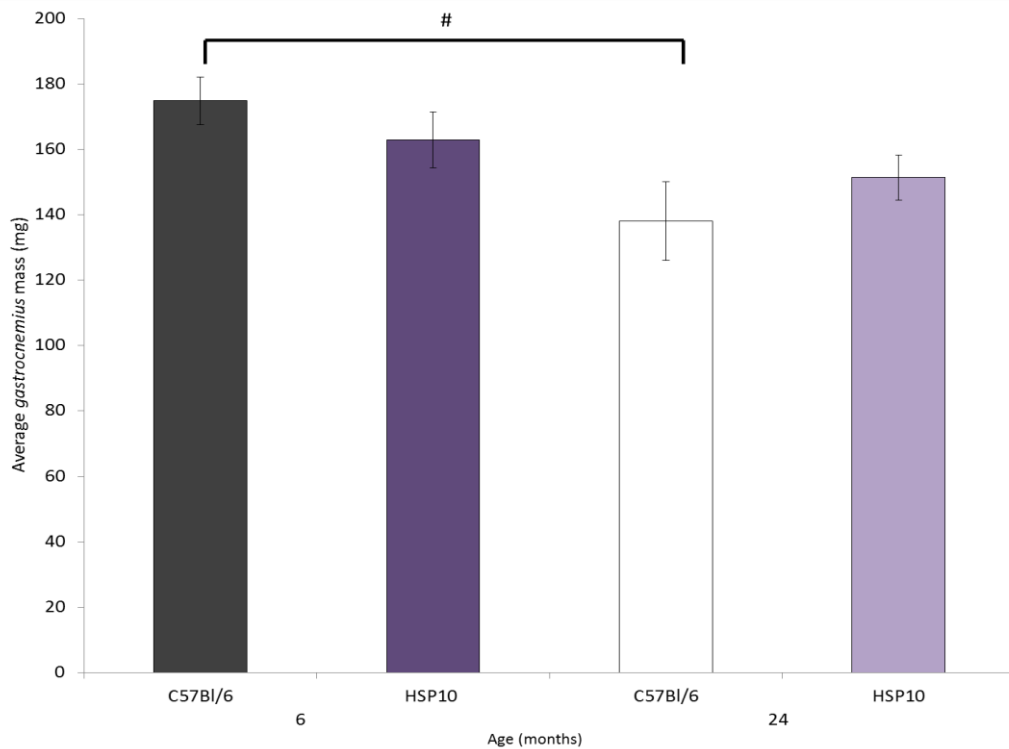
Fig 5.5a Mean *gastrocnemius* muscle mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). # represents significant difference compared with cohort matched 6 month old value.

There was a significant decrease in *gastrocnemius* muscle mass between 6 and 24 months of age in the C57Bl/6 mice

There was no significant change in *soleus* muscle of the HSP10 overexpressor mice. There were no significant differences between the two cohorts of mice at the same age.
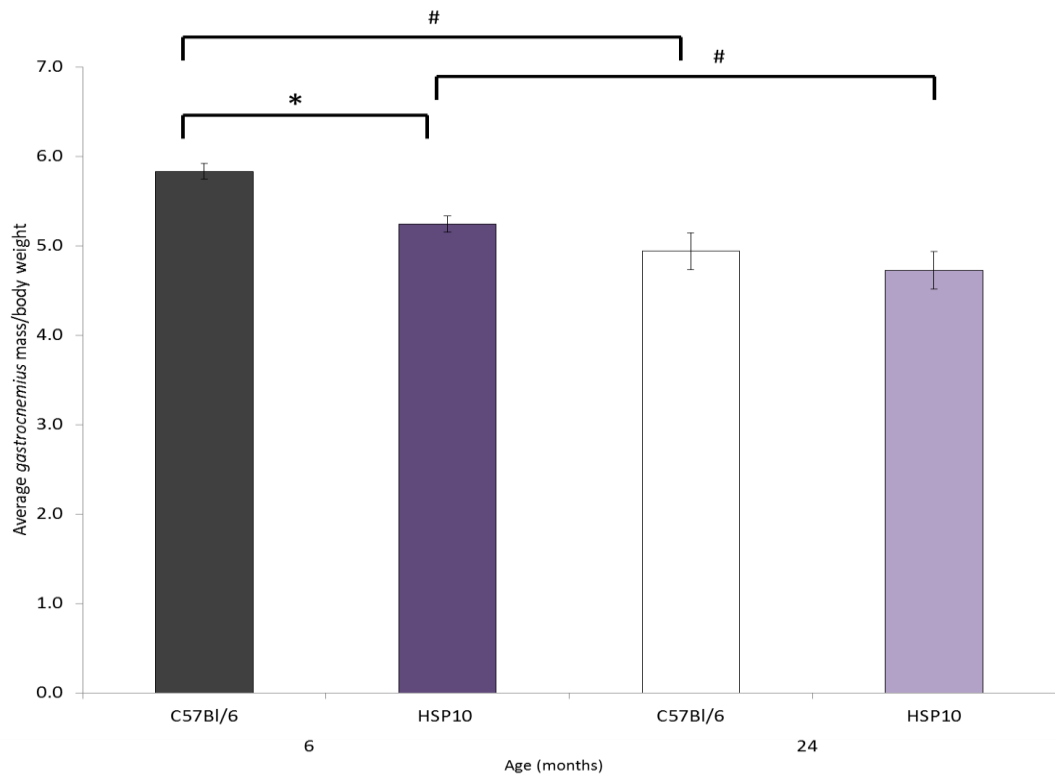
Fig 5.5b Mean *gastrocnemius* muscle mass as a proportion of body mass from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse, # represents significant difference compared with cohort matched 6 month old value.

There was a significant decrease between 6 and 24 months of age in the *gastrocnemius* muscle as a proportion of body mass in the C57Bl/6 mice.

There was a significant decrease between 6 and 24 months of age in the *gastrocnemius* muscle as a proportion of body mass in the HSP10 overexpressor mice.

The value for HSP10 overexpressor mice at 6 months of age was significantly lower when compared with age-matched C57Bl/6 mice.

5.4.4 Age-related changes in the tibia and femur in both C57Bl/6 and HSP10 overexpressor mice
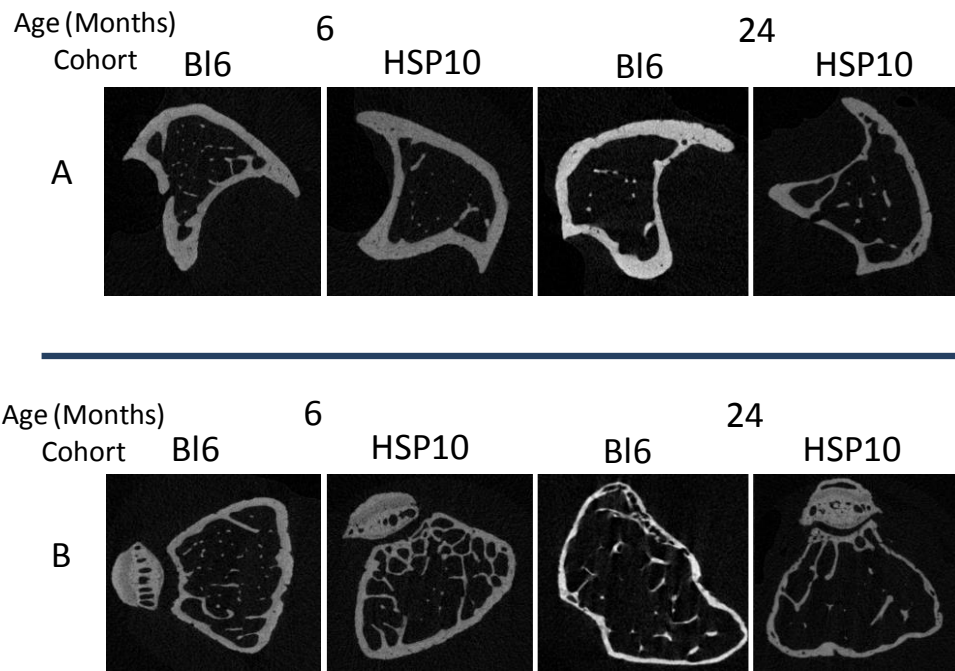
Fig 5.6 Representative MicroCT images of the tibia (A) and femur (B) at 6 and 24 months of age in the C57Bl/6 and HSP10 overexpressor mouse.

A representative image from each site at 6 and 24 months of age was selected based on mean bone values to visualise the cortical and trabecular bone. A change in 2D shape of both bone sites was seen at 24 compared with 6 months old with no gross differences visible between C57Bl/6 and HSP10 overexpressor mice at either age.
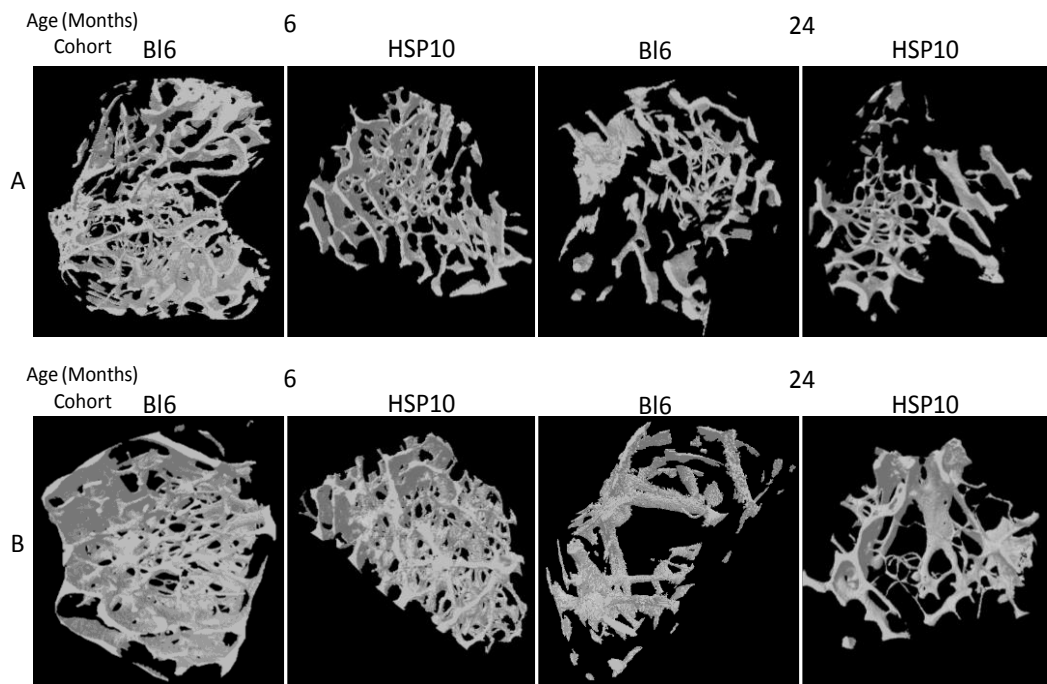
Fig 5.7 Representative 3D models of trabecular bone in tibia (A) and femur (B) at 6 and 24 months of age in C57Bl/6 and HSP10 overexpressor mice.

Loss of trabecular bone was seen at both bone sites. The trabecular bone mass became less connected in both strains of mice. Smaller trabecular bones appeared to be lost first with larger bones becoming thinner. There were no visual differences in trabecular bone between the two mouse strains.

5.4.5 Comparison of age-related changes cortical and trabecular bone measures in both C57Bl/6 and HSP10 overexpressor mice
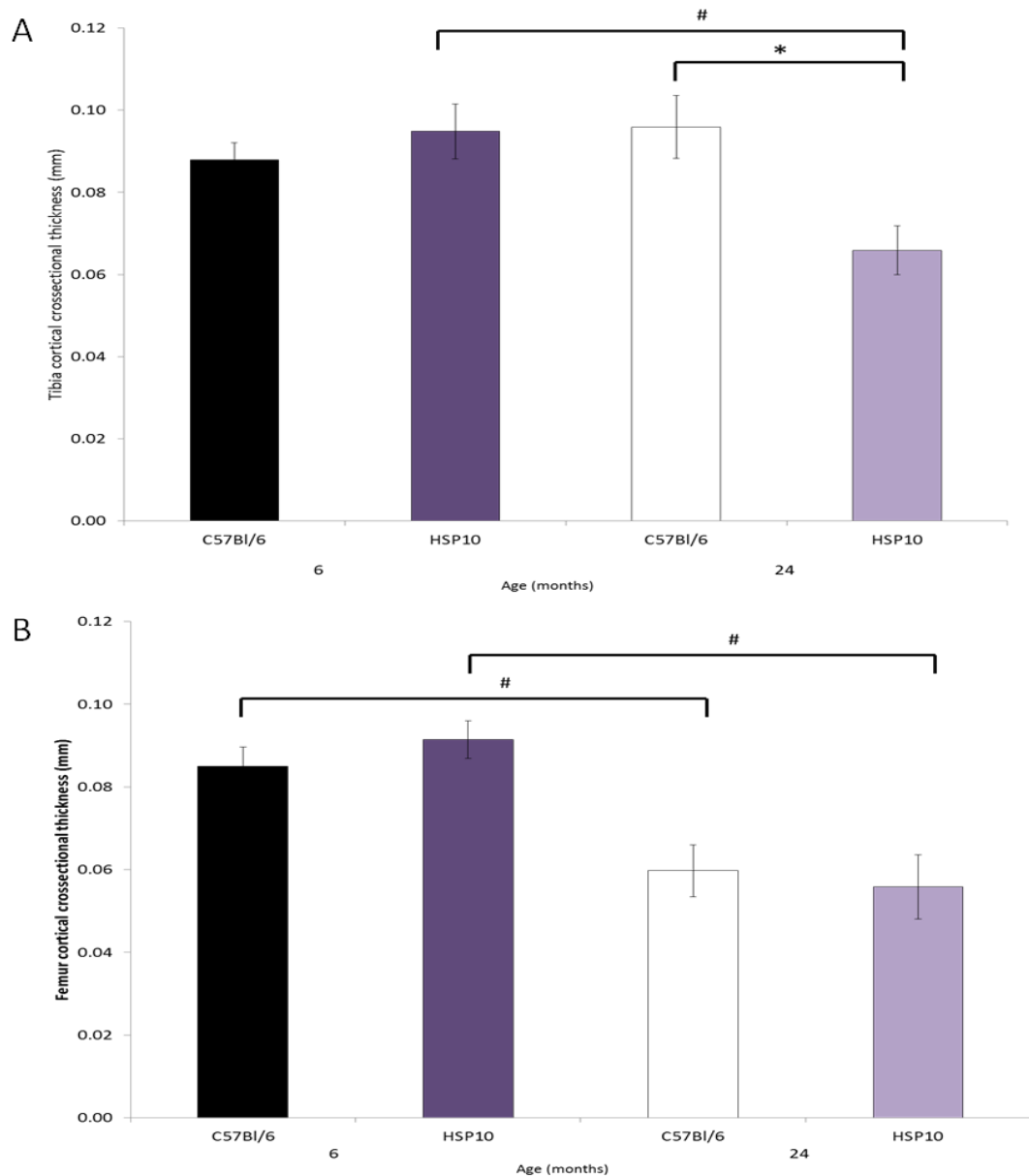
Fig 5.8 Mean cortical bone thickness values for tibia (A) and femur (B) from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse, # represents significant difference compared with cohort matched 6 month old value.

There was no significant change in the cortical bone thickness of the tibia between 6 and 24 months of age from the C57Bl/6 mice whereas there was a significant decrease in cortical bone thickness of the femur between at 24 months of age in the C57Bl/6 mice. This decrease in cortical thickness with age was evident in both the tibia and femur of HSP10 overexpressor mice.
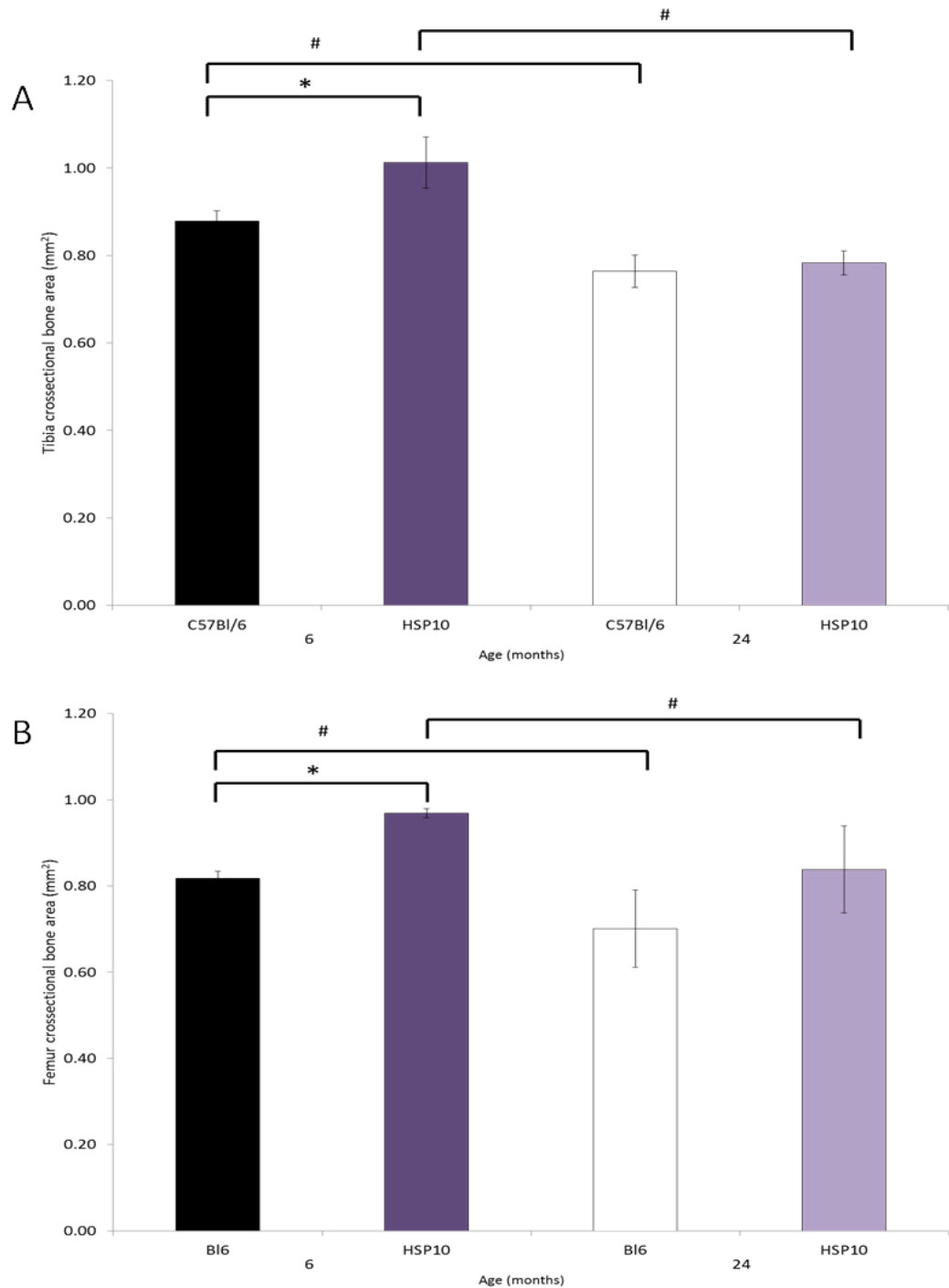
Fig 5.9 Mean cross sectional area values for tibia (A) and femur (B) of C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mice, # represents significant difference compared with strain matched 6 month old value.

Both HSP10 overexpressor mice and C57Bl/6 mice showed a decrease in area at 24 months of age at both bone sites when compared with 6 month values. The HSP10 overexpressor mice had a larger area of tibia and femur bone at 6 months old compared with age matched C57Bl/6 mice.

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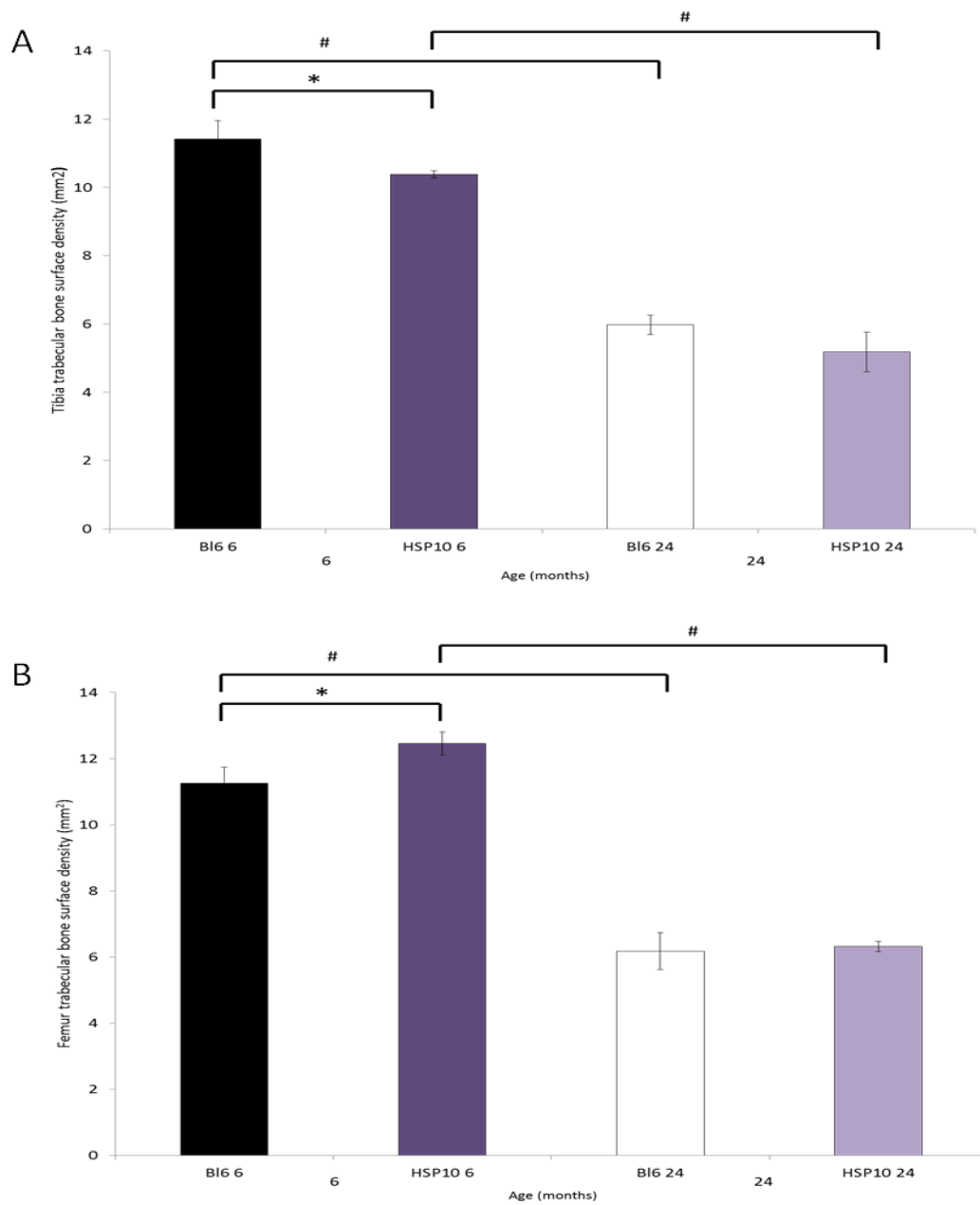
Fig 5.10 Mean bone surface density values for tibia (A) and femur (B) from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mice, # represents significant difference compared with strain matched 6 month old value.

At both bone sites both C57Bl/6 and HSP10 mice had significantly decreased bone surface density at 24 months of age compared with 6 month old strain matched mice. The HSP10 overexpressor mice showed a minor reduction in bone surface density values at 6 months old compared with age matched C57Bl/6 mice. In contrast, in the femur, HSP10 overexpressor mice showed a minor but significantly larger surface density at 6 months old compared with age-matched C57Bl/6 mice.
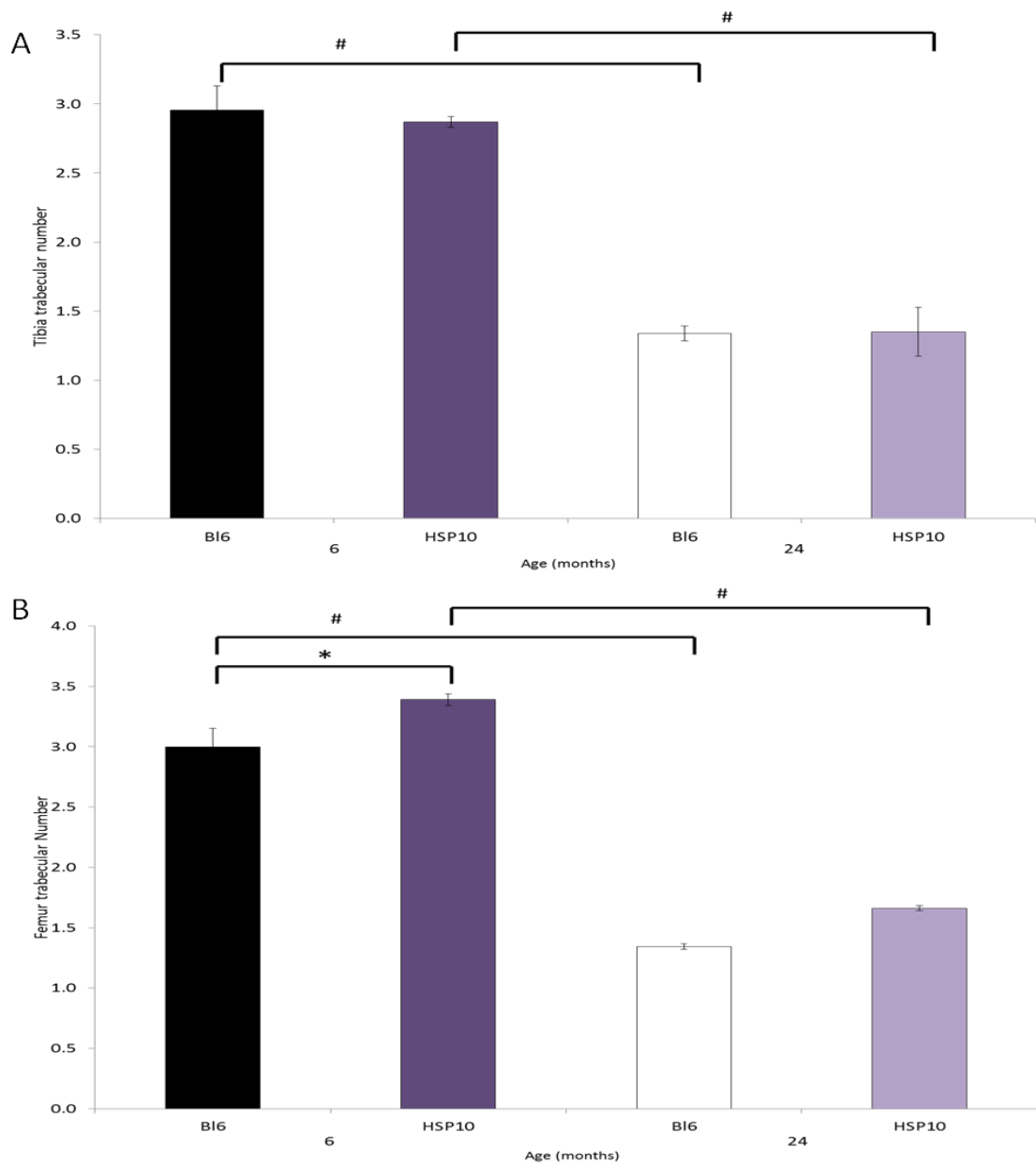
Fig 5.11 Mean number of trabeculae present in tibia (A) and femur (B) from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM, n=6). * represents significant difference compared with age matched C57Bl/6 mouse, # represents significant difference compared with cohort matched 6 month old value.

At both bone sites, C57Bl/6 and HSP10 mice showed significantly decreased numbers of trabeculae present at 24 months of age compared with 6 month old mice.HSP10 overexpressor mice showed a decrease in trabeculae numbers at 24 months of age in both bone sites when compared with 6 month old. In the femur, HSP10 overexpressor mice showed a minor but greater number of trabeculae per mm at 6 months old compared with C57Bl/6 mice at this age.
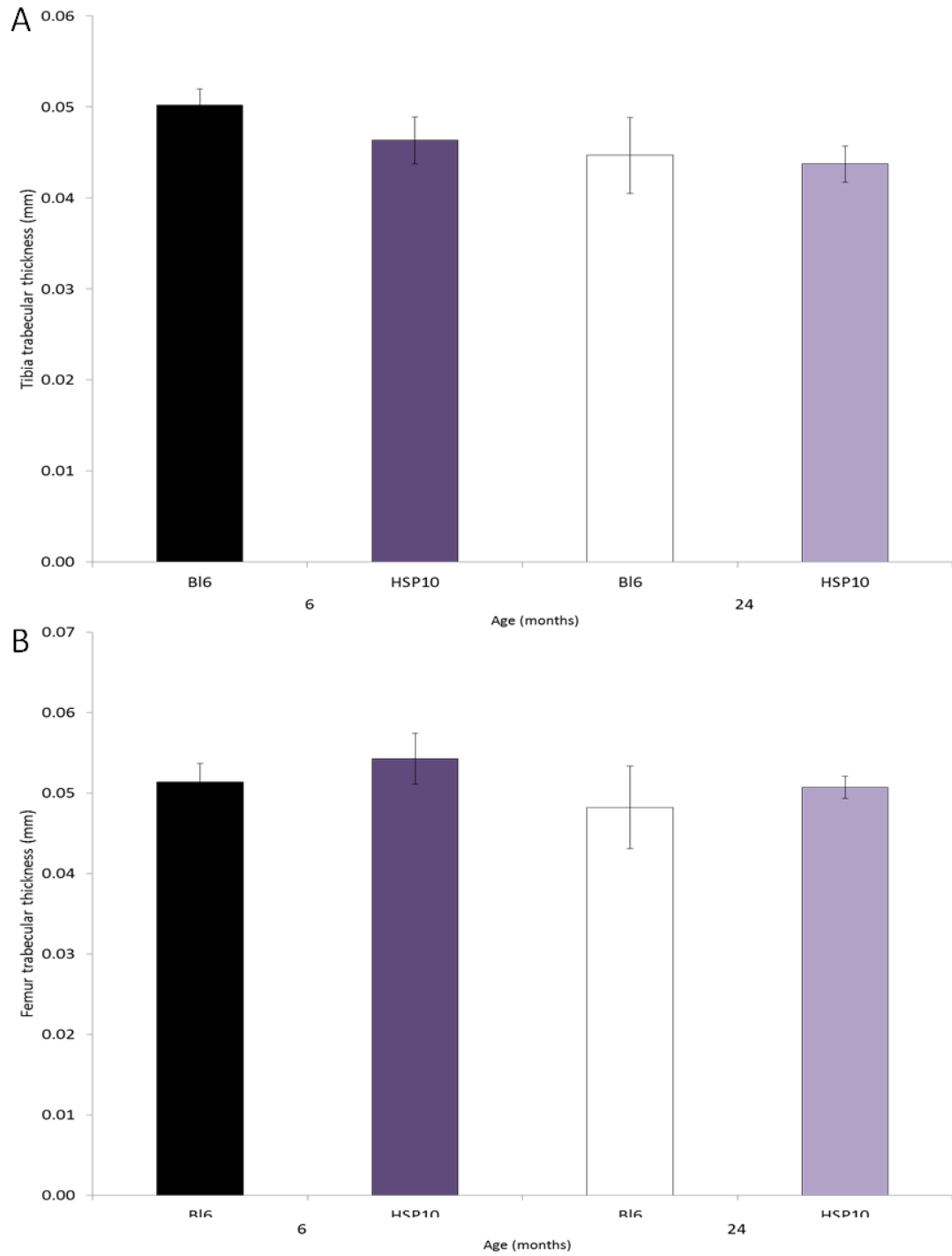
Fig 5.12 Changes in trabecular bone thickness in the tibia (A) and femur (B) from C57Bl/6 and HSP10 overexpressor mice at 6 and 24 months of age. (Data presented as mean +/- SEM)

No significant effect of age of HSP10 overexpression on trabecular thickness was seen.
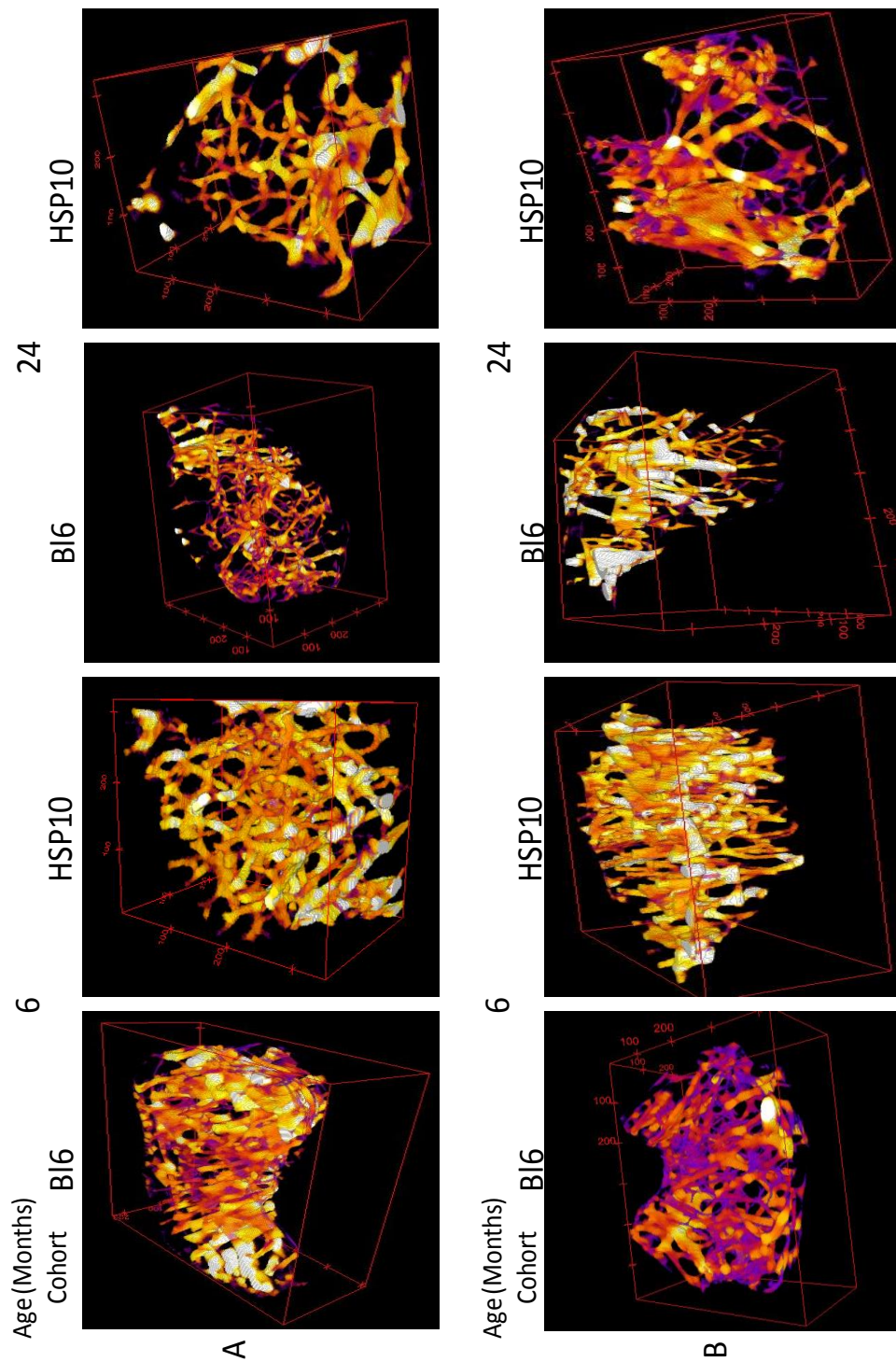
Fig 5.13 3D representation of trabecular bone thickness through age in the tibia and femur in C57Bl/6 and HSP10 overexpressor mice. White colours represent the thickest points, purple colours representing the thinnest areas.

Despite the lack of change in the mean values, clear decreases in trabecular thicknesses were seen between 6 and 24 months of age. The loss of trabecular bone was also evident.

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5.5 Discussion

5.5.1 Age-related changes in the HSP10 overexpressor mice and comparison to age matched C57Bl/6 mice

5.5.1.1 Age-related changes in hindlimb muscle mass

The mass of 4 major hindlimb muscles was measured at both 6 and 24 months of age in the HSP10 overexpressor mice and compared with data from the wild type C57Bl/6 mice. No significant changes were seen with age in any of the four muscles measured in the HSP10 overexpressor mice indicating that there appeared to be preservation of muscle mass in these mice with advancing age.

This is in contrast to data from the C57Bl/6 mice were significant decreases occurred in the mass of the *anterior tibialis* and *gastrocnemius* muscles although no significant changes in the EDL and *soleus* muscle were seen. These data would indicate that the over-expression of HSP10 preserves muscle mass as previously reported (Kayani et al., 2010).

However, when expressing the muscle mass as a proportion of body mass, significant changes in the muscle mass were seen. In the larger, *anterior tibialis* and *gastrocnemius* muscles significant decreases in mass with increased age were seen. No significant changes were seen in the EDL and *soleus* muscles.

Comparing these normalised values with those seen in the C57Bl/6 mice, the pattern of muscle loss was the same in both groups - the *anterior tibialis* and *gastrocnemius* muscle showed clear declines with age. When comparing the groups at each age, the muscles of the C57Bl/6 were significantly larger as a proportion of body mass at 6 months old in all four muscles measured. This differential effect was lost at 24 months old. This would therefore indicate that the total age-related loss of muscle was higher in the C57Bl/6 mice compared with the HSP10 overexpressor mice.

Data presented here are similar to those published in 2010 (Kayani et al., 2010). It must be noted however differential ages were used – our study used 6 month and 24 month old mice, the Kayani paper used mice aged 10-12 months and 26-28

months of age (Kayani et al., 2010). Where the two studies differ was that the new study measured more muscles than the 2010 paper.

In summary, although loss of muscle mass was still somewhat evident, there was clear preservation of the rate of loss with ageing in the muscles of HSP10 overexpressor mice in terms of overall muscle loss. These findings indicate an effect of HSP10 in preserving muscle with increasing age which agrees with earlier work (Kayani et al., 2010).

5.5.1.2 Age-related changes in the bones of the hindlimb

Based on raw micro CT scan images and the 3D rendering from these scans, there was a visible loss of trabecular bone in both tibia and femur through age. This was visibly comparable to the changes seen in similar images from the C57Bl/6 mice.

When focusing on measures of cortical bone, in the femur there was an age-related thinning of the cortical bone. There appeared to be a trend toward this also occurring in the tibia but the values were not significant. However both bones displayed a reduced cross sectional area with age indicating that the thinning of the cortical bone was leading to an overall smaller bone. This effect was more apparent on the tibia which showed differential ageing of the two hindlimb bones.

In C57Bl/6 mice similar trends were seen. The cortical thickness of the tibia did not show age-related thinning, but this was apparent in the femur. Although there were no significant differences across age, when comparing the 24 month age point in both groups, there was a significant reduction in cortical thickness in the tibia, but no changes in the femur. Again this would indicate that there were differential changes in the two hindlimb bones but HSP10 overexpression was not improving the age-related changes.

When looking at bone cross sectional area, the same pattern was seen in C57Bl/6 mice as in HSP10 overexpressor mice. There was an age-related reduction in cross sectional area in both bones measured. There were no further differences within age groups between the two mouse types indicating that this reduction in area was not improved by overexpression of HSP10.

It was interesting to note that despite the mice not losing muscle mass, the bones were still detrimentally affected. This would suggest that HSP10 was not preserving bone tissue unlike in the muscle. This could be explained through the role of HSPs being a lot lower in bone then other tissues such as muscle. There is currently no real understanding on the antioxidant levels of roles in bone cells due in part, to the difficulty in isolating bone cells for further analysis. It may be that increases in ROS may not play a role in the ageing of bone and in the disruption of bone cells. We then must turn to the role muscle has on controlling bone tissue. As muscle mass does not decline in this model, we cannot be certain of muscles dominant role. We can therefore only really base these results on the genetics of the mice and that bone is predisposed to breakdown as the mouse ages or that as the mice age and they take in fewer nutrients (reported previously) then the need for calcium is rerouted to maintaining the muscle and thus taken away from the bone. Further studies are needed to fully understand these points.

When looking at the changes in trabecular bone with increasing age in the HSP10 overexpressor mice, there was a reduction in trabecular bone number at both bone sites with associated reductions in bone surface density. This was mimicked in the C57Bl/6 mice with similar reductions. There were no significant differences between age groups except for a slightly higher trabecular number at 24 months old in the HSP10 overexpressor mice group.

Despite the loss of trabecular bone with age in both groups, no changes in mean thickness of the trabecular bone was seen. However when applying thickness to the 3D model, large variations were seen in the age groups at both sites. When looking at the mean ranges of trabecular thickness through in the tibia of the HSP10 overexpressor mouse, there was an age-related decrease in the thicker trabecular bone and a shift towards thinner trabecular bone. The change in the femur was focused more on the loss of thicker trabecular bone with no significant increases in thinner bone. This would suggest that the loss of trabeculae bone was a result of thinning of the bone causing the thinnest trabeculae to be removed.

When the ranges of trabecular thickness were compared between the HSP10 overexpressor and the C57Bl/6 mice there were no significant changes in either site at 6 months old. However, at 24 months there were significantly lower

numbers of thicker trabecular in the HSP10 overexpressor mice and a larger number of smaller trabecular bones. This would suggest that the trabecular bone thinning and loss was more apparent in the HSP10 overexpressor mouse then the C57Bl/6 mouse.

Taken together, although there were few significant changes between the C57Bl/6 and HSP10 overexpressor mouse, the bones of the HSP10 overexpressor mouse appeared to have been affected by age to a greater extent than the C57Bl/6 mouse. This could be a failed ability to adapt to the maintained muscle mass in the HSP10 mouse or that HSP10 overexpression was detrimental to the bone itself.

5.6 Conclusions

The potential for increasing heat shock proteins in aged tissues to elicit a beneficial effect has long been considered and many attempts have been undertaken to achieve this response.

- We can utilise overexpression of heat shock proteins in mice to show lifelong effects of heat shock protein increases which clearly could not be achieved in humans.
- From this study, it can be clearly seen that overexpression of heat shock protein 10 had beneficial outcomes in terms of muscle tissue through age. The larger muscles measured in this study were resistant to age-related loss of mass to 24 months old.
- There were no significant changes in the bone between the groups at 6 months, indicating overexpression of HSP10 in muscle and preservation of muscle did not affect bone loss. Thus by the age of 24 months old, the HSP10 overexpressor mice were showing similar losses to C57Bl/6 mice.

In summary, over-expression of HSP10 in muscle of mice appeared to rescue the age-related loss of muscle mass to some extent but this did not appear to confer any additional benefits on the underlying bone.

It has become apparent that work in the C57Bl/6 mouse is not providing positive outcomes in preventing the onset of age related musculoskeletal decline or recovering the damage in old age. Is this because the C57Bl/6 mouse is already

bred to be at its optimum and protected from negative stimuli? Is the C57Bl/6 mouse a true representation of mouse ageing?

Chapter 6

Characterising age-related changes in musculoskeletal tissues of male and female wild mice

6.1 Introduction

We have already show in the previous chapters how ageing occurs in the lab based strain of mice C57Bl/6 and how the chosen interventions only have very limited effects on reversing the detrimental changes we see with increased age. It is now sensible to investigate whether our original chose of model animal was not optimal and whether these lab based strains are already optimised for ageing well.

6.1.1 Using wild mice as a model of human ageing

Mouse models are commonly used to study the effects of age-related conditions primarily due to the shorter life span and the ability to manipulate gene expression in tissues. The use of model organisms to research ageing is not a new phenomenon and despite the quadruped locomotion used by mice, multiple studies have shown many similarities between mice and humans. Mouse models used tend to be genetically identical mice which provide the reliable information. This has the benefit of reproducibility and allows for integration of data across experiments (Threadgill et al., 2011). Since the sequencing of the C57Bl/6 mouse genome, it has been shown that 99% of mouse genes have human homologues (Boguski, 2002). Although this would indicate that the data can be translated into human work, there is a drive for other mouse models of ageing. Due to mouse lines being derived from brother-sister mating through generations, the mouse strains are inbred (Bailey, 1971). In essence the genetic pool of inbred laboratory strain mice has become smaller and smaller over time. When compared with the large variation in natural genetic polymorphisms displayed within the human population, the use of these laboratory mice is not optimal.

Alongside these findings, work comparing wild mice and laboratory mice concluded that there are a number of differences between the two groups including genetic diversity, less responsiveness to environmental cues, higher growth rates and more docile behaviour in the laboratory strain mice (Garland et al., 1995, Olsson and Sherwin, 2006). Studies have also shown that laboratory strain mice are generally larger in mass through the life span and display a lack of interest in voluntary activity – both of which would impact on musculoskeletal ageing in these rodents (Garland et al., 1995). However it must be noted that most

wild mice will adapt to a domesticated lifestyle within approximately 10 generations, which may impact on any findings (Nelson et al., 2013).

Studies have attempted to reintroduce genetic variability amongst common laboratory mouse strains. Miller et al (2009) published a paper detailing a programme coordinated by the National Institute on Ageing (Miller et al., 1999). This involved a four-way cross of commonly used laboratory mouse strains creating offspring with 25% of genes from each grandparent. These mice were therefore not genetically similar to each other, creating a new mouse strain (Miller et al., 1999). The Collaborative Cross Study further developed these initial experiments and mice were derived from 8 inbred strains through three generations of random intercrossing and at least 20 generations of brother-sister mating to produce new strains (Threadgill et al., 2011). These processes have allowed for mice to be developed with high genetic diversity and this is being further applied to future mouse lines. It has also removed genetic drifting seen in more common laboratory strains with apparently the same strain in multiple labs developing 'local' changes in genetics due to lack of local diversity. However both techniques used large amounts of time and expense to create genetically unique mice that are still reared in a laboratory environment.

6.1.2 Differential ageing between male and female mice

The main consequences of ageing in musculoskeletal tissues are a severe and progressive loss of muscle mass and force, a continued loss of bone mass and bone quality leading to osteoporosis, an increased risk of joint diseases such as arthritis and an increased risk of fractures due to increased falls.

Although these are ubiquitous between the sexes, the rate of development and presentation of each disease state can be widely different. One of the main causes of these differences is proposed to be the dimorphisms in size and body mass between males and females. Females on mean tend to be of smaller size and mass compared with male counterparts and it directly influences the mean size of muscles and bones (Hanrahan and Eisen, 1973). As muscle and bone mass is lost at generally similar rates in both sexes until menopause (at which females show an accelerated loss), females may develop the symptoms of sarcopenia and osteoporosis at an earlier time point then males. This leads to more time spent

with mobility problems and a higher risk of falls since female lifespan tends to be longer.

Three key papers show age-related changes in the muscles of female compared to male mice. Sheard and Anderson et al (2012) published data on age-related decline in muscle fibre number in male and female mice (Sheard and Anderson, 2012). In accordance with work presented in Chapter 3, Sheard and Anderson et al. (2012) showed that the leg muscles of the male mouse had significant muscle fibre loss. This study also showed that this occurred in leg muscles of female mice. Despite earlier work to the contrary (Rowe and Goldspink, 1969), the Anderson study could find no correlation with specific fibre typing and prevalence of fibre loss. Rowe (1969) did however find a sex related difference in the proportion of slow fibres in the soleus muscle, with female mice having a higher proportion of slow fibres compared with male mice. This study also showed a higher percentage of fibres lost in the EDL with increasing age in the female compared with the male mice.

Complementing this work, a further study by Moran et al (2005) showed that the force generating capacity of soleus muscles from aged female mice decreased by 20% (Moran et al., 2005). This decrease was not attributed to reduced muscle size or reduction in contractile protein levels in the soleus (Moran et al., 2005). Again, female mice displayed reduced values compared with the male counterparts. However a new finding was that the significant decrease in force generation occurred earlier in the female mice. The authors recorded the age of approximately 10-16 months of age for this decrease in force generation capacity in females compared with the males (This was not seen in the EDL muscle in this study) (Moran et al., 2005).

This age range is of significance in female mice as it is the age at which levels of ovarian hormones drop and the ovaries fail in mice (Cargill et al., 2003). This would imply that the decreases in force generation occur simultaneously with the onset of these hormonal changes. However whether this was coincidental or not was not investigated in this study.

The onset of the menopause has direct consequences with skeletal health and is a key differing point in the ageing of musculoskeletal tissues between the sexes.

Postmenopausal osteoporosis is the most common form of osteoporosis. The decline of ovarian hormones occurs in humans many years before the onset of the menopause (48-50 years) and in the first ten years of the menopause, humans on mean lose 1% of bone mass every year, which does not occur until later time points in males.

Although the mechanisms by which that loss of oestrogen affect bone turnover is not yet understood fully, it is known that all bone cells carry a receptor for oestrogen and it is hypothesised that the loss of these hormones, potentially in combination with an increase in serum cytokine levels leads to the loss of bone (Smith, 2005).

What has clouded the field is the use of mouse models of menopause. Often studies use younger mice which undergo ovariectomy to induce artificial oestrogen deficiency. One study by Glatt et al. (2007) examined the age-related changes in bone of both male and female mice without surgical or pharmaceutical intervention (Glatt et al., 2007). The authors showed that changes in trabecular bone occurred much earlier and to a greater extent in the female mice when compared with male. Female mice also showed a more rapid decline in bone volume during the adolescence phase and slowed down in adulthood (Glatt et al., 2007). This would mimic data of muscle loss previously described. Females in general in this study displayed lower values for each of the bone measurements and showed a higher rate of decline through age (Glatt et al., 2007).

6.1.3 Benefits of increased activity on age-related conditions

There is a large body of research investigating the effect of inactivity or reduced exercise levels has on ageing and musculoskeletal tissue decline. Physical inactivity is closely associated with admissions to hospital with chronic conditions (Bielemann et al., 2015). There is also a high correlation between increasing age and decreasing physical activity and this has a clear negative impact on skeletal muscle mass and function (Kortebein et al., 2007, Gianoudis et al., 2015) (Sardinha et al., 2015). This is also seen with loss of bone mass (Emaus et al., 2014, Dallanezi et al., 2016).

Resistance exercise has the largest effect on muscle tissue. It has been shown that resistance exercise can increase muscle mass and improve muscle function including increased muscle force generation (Sipila and Suominen, 1995). However, prolonged exercise regardless of type does not prevent the onset of sarcopenia. In a group of veteran athletes of either endurance or power activities, there was still evidence of muscle loss with increased age (Drey et al., 2016).

Regular exercise is proposed as the most beneficial approach for osteoporosis (Berard et al., 1997, Gomez-Cabello et al., 2012). The type of exercise that is potentially beneficial to bone differs slightly to what is reported for muscle and suggests a programme of high load non mass bearing exercise is optimal.

Exercise has also been shown to reduce serum levels of inflammatory cytokines which increase in old age and are also thought to be detrimental in later life (Greiwe et al., 2001, Petersen and Pedersen, 2005).

There are currently no exercise regimes in particular that show universal benefits for all musculoskeletal tissues however resistance exercise is clearly beneficial for muscles and this may induce improvements in the other tissues. Increased exercise prior to reaching old age may ensure individuals reach peak muscle and bone mass before the declines start as with the veteran athletes.

6.1.4 Wild mice for study of increased activity

Wild mice used in this study were obtained from Professor Jane Hurst (Green et al., 2015). Mice were captive bred *Mus musculus domesticus* derived from ancestors captured from five different populations in the northwest of England, UK. All ancestors were captured within 20 miles of each other except for 2, which were captured 80 miles away to ensure genetically differing strains (Green et al., 2015) (Ramm et al., 2015). The captive bred offspring were housed in standard cages with increased enrichment. As well as being the offspring of wild mice, they also displayed increased levels of activity and were more prone to climbing and jumping activities (Green et al., 2015, Ramm et al., 2015). This may be due to cage enrichment (Olsson and Sherwin, 2006) although C57Bl/6 mice do not show increased activity even with this enrichment.

In contrast to the C57Bl/6 mice ageing described in the previous chapter, here we aim to establish a model of ageing in both male and female mice from a genetically wild background. These mice also have the benefit of increased levels of activity.

6.2 Aims

The aims of this study were as follows;

- To investigate age-related changes in musculoskeletal tissues of wild mice compared with the C57Bl/6 laboratory strain of mice.
- To investigate differences in ageing between male and female wild mice.

The hypothesis of this study was that musculoskeletal tissues of wild mice demonstrate delayed ageing compared with tissues of C57Bl/6 mice due to the reported increased activity of the wild mice. In addition, female mice will demonstrate earlier onset of musculoskeletal dysfunction compared with male mice independent of the strain of mice.

6.3 Methods

6.3.1 Mice

All experiments were undertaken in accordance with UK home office guidelines under the UK Animals (scientific Procedures) Act 1986 and with ethical approval from the University of Liverpool Animal Welfare and Ethical Review Body.

A total of 38 mice were used. These were divided into groups based on sex and age. Final group numbers for males were – 6 mice aged 6 months, 6 mice aged 12 months and 4 mice aged 24/25 months. Final group numbers for female mice were – 4 mice aged 6 months, 6 mice aged 12 months, 6 mice aged 24/25 months and 6 mice aged 31 months. These age groups were chosen to match key time points identified in Chapter 3 although the age groups were somewhat limited due to the availability of the wild mice. Six months was chosen to record muscle masses prior to significant declines in mass occurring, 12 months to represent a mid-point in the life course, 24 months to represent a time of significant muscle and bone decline in C57Bl/6 mice. 31 month old female mice were included as additional time point as they remained in good health. They were included to examine whether these mice have prolonged preservation of musculoskeletal tissue compared to the C57Bl/6 laboratory strain.

Mice were kindly donated by Professor Jane Hurst (University of Liverpool, UK).

6.3.2 Tissue collection and preparation

All tissues were obtained post mortem as described in Section 2.3. Briefly, all muscles and organs were removed and snap frozen prior to storage at -80°C. Feet with both *achilles* and *posterior tibialis* tendon attached were stored at -80°C. Hindlimbs had remaining muscle tissue removed and were stored in formalin solution for 24 hours before transferred into 70% ethanol solution.

6.3.3 Muscle analysis

Anterior tibialis, *extensor digitorum longus*, *soleus* and *gastrocnemius* muscles from both hindlimbs where dissected and weighed. Following this, muscles were snap frozen in liquid nitrogen and stored for further analysis at -80°C.

6.3.4 Bone analysis

Analysis of bones was described in Section 2.8. Briefly, both tibia and femurs of each hindlimb were scanned using a μCT system (Skyscan 1272 X-Ray microtomograph, Bruker Corporation, Kontich, Belgium) at high-resolution with an isotropic voxel size of 5μm (60kV, 0.5mm aluminium filter, 0.7° rotation angle). The isotropic voxel size was changed to 10μm for cortical analysis. Two images were taken at each rotation angle. The scans were reconstructed using NRecon software (Bruker, Massachusetts, USA). CTAn software (Bruker, Massachusetts, USA) was used to analyse the appropriate parameters previously outlined (Bouxsein *et al*. 2010).

6.3.5 Analysis of hindlimb joint structure

Joint analysis has been described in Section 2.7. Briefly, one hindlimb from each mouse was transferred into a decalcification solution (EDTA, pH 7), left at room temperature for 4 weeks without agitation and then embedded in paraffin coronally.

Transverse sections were cut via HM355S microtome (Thermo Fisher Scientific, Massachusetts, USA). The cutting was done until the condyles were seen. Sections were stained for H&E and Safranin-O. Normality test indicated the n number was too small to allow for wild type male and female to be separated based on sex. As this was a prolonged process, 6 and 24 month time points were chosen for the analysis to match optimal stages shown by data gained in Chapter 3.

6.3.6 Statistics

Data was tested for normality and equal variance using SigmaPlot (v11.0) (Systat Software, Inc., London, UK). Direct comparison between two sets of data was made by Student's *t*-test. For multiple comparisons, data was analysed for statistical significance by one-way ANOVA incorporating pairwise comparisons. Data are presented as mean ±SEM. Values $P<0.05$ were considered significant.

6.4 Results

6.4.1 Measurement of age-related changes in body mass in both male and female wild mice
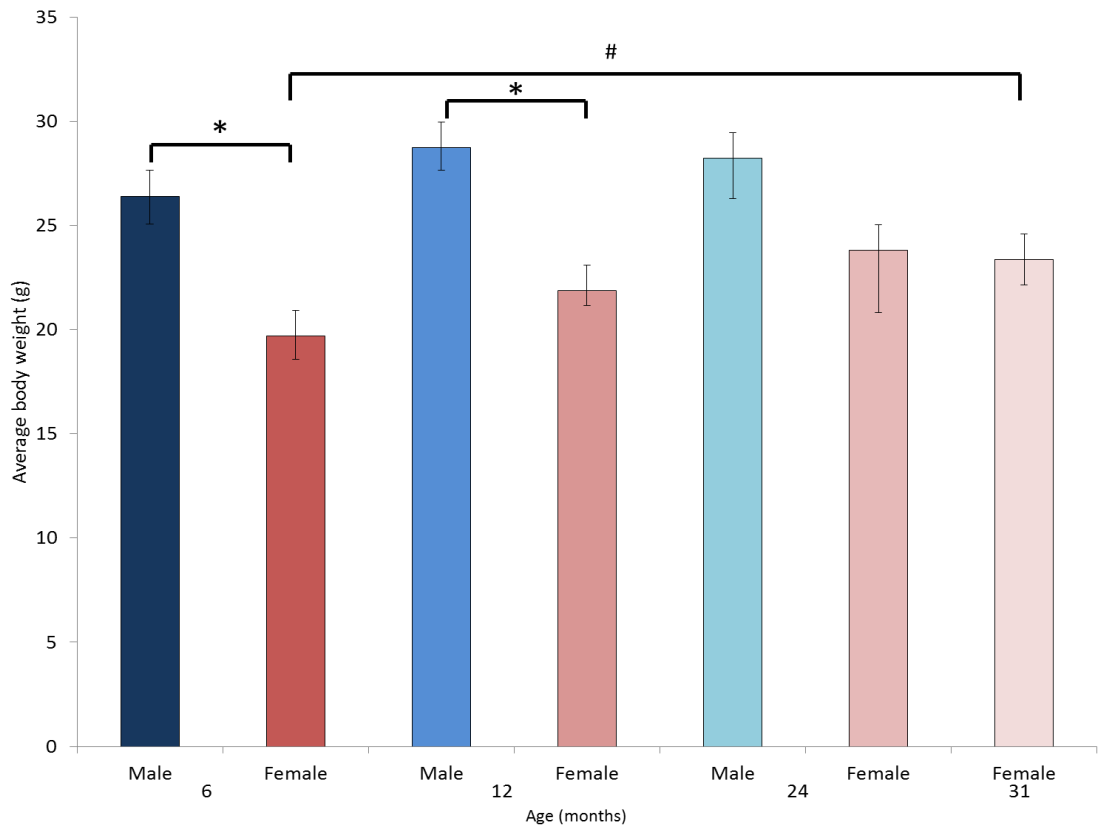
Fig 6.1 Mean body mass of male and female wild mice at different ages (Data presented as mean +/- SEM) (n=6). * represents significant differences compared with values for age matched male mice value, # represents significant differences compared with sex matched 6 month old mice value.

Mean body mass at different ages in both wild male and female mice are presented in Figure 4.1. Female mice were significantly smaller compared with males at both 6 and 12 month old. The body mass of male mice did not significantly change up to 24 months old. Female mice were significantly heavier at 31 months old compared to 6 months old.

6.4.2 Age-related changes in organ mass in both male and female wild mice

Age		6		12		24		31
Sex		Male	Female	Male	Female	Male	Female	Female
Heart	Average	127	79 *	143 a	121 * c	135	135 c,d	140 c,d
	SEM	5	11	7	4	15	6	9
Lungs	Average	141	83 *	176 a	146 * c	139 b	172 * c,d	153 c
	SEM	9	19	8	6	10	17	8
Liver	Average	909	730 *	1019	788 *	1116	1012 c,d	876 c
	SEM	75	51	80	44	231	162	64
Kidneys	Average	306	249 *	385 a	281 *	146 a,b	152 c,d	319 c,d,e
	SEM	8	19	6	12	19	9	11
Spleen	Average	37	27 *	46	57 * c	77 a,b	127 * c,d	77 c,d
	SEM	4	7	4	5	17	51	13
Brain	Average	228	163 *	304 a	348 c	237 b	345 * c	308 c
	SEM	26	23	10	35	37	39	23

Table 6.1 Mean organ mass in wild male and female mice at different ages. (Data presented as mean +/- SEM) (n=6 per group) * represents significant difference compared with age matched male mice. Letters depict a significant sex matched difference, (**a** - 6 month old male mice, **b** - 12 month old male mice, **c** - 6 month old female mice, **d** - 12 month old female mice and **e** - 24 month old female mice).

Age		6		12		24		31
Sex		Male	Female	Male	Female	Male	Female	Female
Heart OW/BW	Average	4.8	4.1 *	5.0	5.5 *,c	7.5 a,b	5.7 c	5.9 c,d
	SEM	0.2	0.9	0.2	0.2	1.9	0.2	0.2
Lungs OW/BW	Average	5.3	4.4	6.1 a	6.7 c	5.4 b	7.3 *	6.5
	SEM	0.2	1.3	0.2	0.3	0.3	0.7	0.3
Liver OW/BW	Average	34.3	37.3	35.3	36.0	34.4	41.7	37.2
	SEM	1.6	3.3	1.9	1.6	3.0	5.4	2.2
Spleen OW/BW	Average	1.4	1.5	1.6	2.6 *,c	19.0	5.2 d	3.33 c
	SEM	0.1	0.5	0.2	0.2	17.4	2.1	0.6

Table 6.2 Mean organ mass as a proportion of body mass. * represent significant difference compared with age matched male mice (Data presented as mean +/- SEM) (n=6 per group). Letters depict a significant sex matched difference, (**a** - 6 month old male mice, **b** - 12 month old male mice, **c** - 6 month old female mice, **d** - 12 month old female mice and **e** - 24 month old female mice).

The tissue mass of female mice were generally smaller than those of age-matched male mice although this difference was generally lost when data were expressed per unit body mass suggesting that body size was primarily responsible for the difference in these mass.

Male mice had significantly increased mass for the heart, lungs, kidneys and brain at 12 months old compared with mice at 6 months old. The mass of the lung, kidneys and brain were decreased in mass in male mice at 24 months old compared with those in 12 month old mice.

There was a significant increase in mass of most measured organs in female mice at 12 months old compared with the 6 month old mice and most tissues had generally increased further in mass at 24 months old.

When accounting for body mass by converting data to a proportion of body mass, most of the observed changes with age in male mice were lost, with the exception of the heart and spleen. In contrast, most tissues in female mice maintained the increases seen at 12 months compared with 6 months old.

Inclusion of the 31 month old female mice has demonstrated that tissue mass remained elevated compared with data for 6 month old mice, In general, reduced mass were seen in some tissue of mice at this age, although these did not reach significance.

6.4.3 Age-related changes in muscle mass of male and female wild mice

Age		6		12		24		31
Sex		Male	Female	Male	Female	Male	Female	Female
AT	Average	39	22 *	44 a	37 *c	34 b	32 * c,d	20 d,e
	SEM	2	2	1	1	4	2	1
EDL	Average	9	2 *	9	7 *c	7 a,b	7 c	6 c
	SEM	1	1	1	2	1	1	1
Sol	Average	6	1 *	0	6 c	6 b	8 *c	4 c,e
	SEM	0	0	1	3	1	1	1
Gas	Average	130	90 *	133	112 *c	108 a,b	111 c	96 d,e
	SEM	7	2	5	4	10	7	6

Table 6.3 Mean muscle mass (mg) of mice at different ages from four hindlimb muscles * represents significant difference compared with age matched male mice. Letters depict a significant sex matched difference, (**a** - 6 month old male mice, **b** - 12 month old male mice, **c** - 6 month old female mice, **d** - 12 month old female mice and **e** - 24 month old female mice). (n=6 per group, data presented as mean +/- SEM)

Age		6		12		24		31
Sex		Male	Female	Male	Female	Male	Female	Female
AT MW/BW	Average	1.5	1.1 *	1.5	1.7 c	1.2 a,b	1.4 c,d	1.1 d,e
	SEM	0.1	0.1	0.1	0.0	0.1	0.1	0.0
EDL MW/BW	Average	0.3	0.1 *	0.3	0.3 c	0.3 a,b	0.4 c	0.3 c
	SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sol MW/BW	Average	0.2	0.1 *	0.3 a	0.3c	0.2 b	0.4 c,d	0.2 c,d,e
	SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gas MW/BW	Average	5.0	4.6	4.6	5.1	3.9 a,b	4.9	4.1 c,d,e
	SEM	0.4	0.3	0.2	0.2	0.1	0.5	0.0

Table 6.4 Mean muscle mass as a proportion of body mass. * represents significant difference compared with age matched male mice. Letters depict a significant sex matched difference, (**a** - 6 month old male mice, **b** - 12 month old male mice, **c** - 6 month old female mice, **d** - 12 month old female mice and **e** - 24 month old female mice). (n=6 per group, data presented as mean +/- SEM). (n=6 per group, data presented as mean +/- SEM)

Muscles were generally smaller in 6 and 12 month old female mice compared with male mice although this difference was lost at 24 months old. This difference was lost at 12 months old when data were expressed per unit of body mass, suggesting that the smaller muscle size of female mice was a reflection of smaller body mass.

The *anterior tibialis* muscle and *soleus* of male mice reached peak mass at 12 months old. The mass of all male mice were significantly reduced in 24 month old mice compared with 12 month old mice.

In the female mice, all muscles reached peak mass at 12 months of age and the *anterior tibialis* muscle showed some evidence of reduced mass at 24 months

compared with 12 months old. No evidence of muscle loss seen in any muscle. At 31 months of age most muscles showed a significant reduced mass.

Expressing muscle mass as a proportion of body mass had little effect on a differences seen in absolute muscle mass. Thus, when expressed as a proportion of body mass of the anterior tibialis and gastrocnemius muscle in the male mice declined at 24 months old compared with the 12 month old mice. The mass of EDL muscle was significantly reduced at 24 months old compared with EDL mass at the 6 month old with no changes in the soleus muscle.

Similarly, when expressed as a proportion of body mass, anterior tibialis muscle of female mice showed evidence of reduction in mass at 24 months compared with 12 months old but no evidence of age-related reduction in mass, in a similar manner to absolute muscle mass.

6.4.4 Analysis of joint and ligament structure by histological sections of knee joints of male and female wild mice
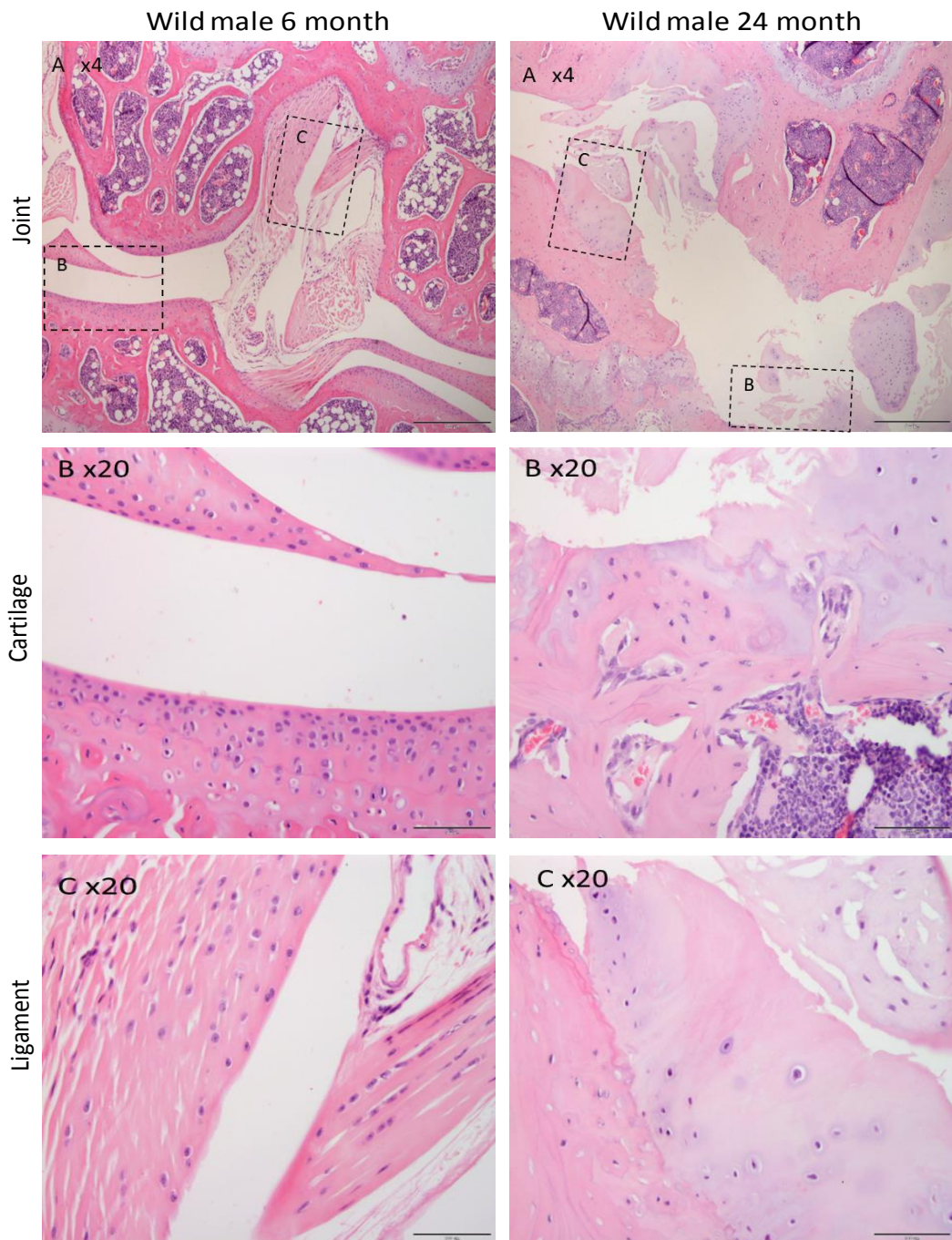
Fig 6.2 Representative histological sections of the knee joint in wild male mice at 6 and 24 months of age. Photographs A-C were stained with H&E. A was magnified x4 to show the whole joint, B (cartilage region) and C (cruciate ligament region) were magnified x20 to show detailed structures.

There was a visual change in the shape and structure of the joint in the male wild mice between 6 and 24 months of age. The meniscus and ligament were no longer recognisable at 24 months of age.
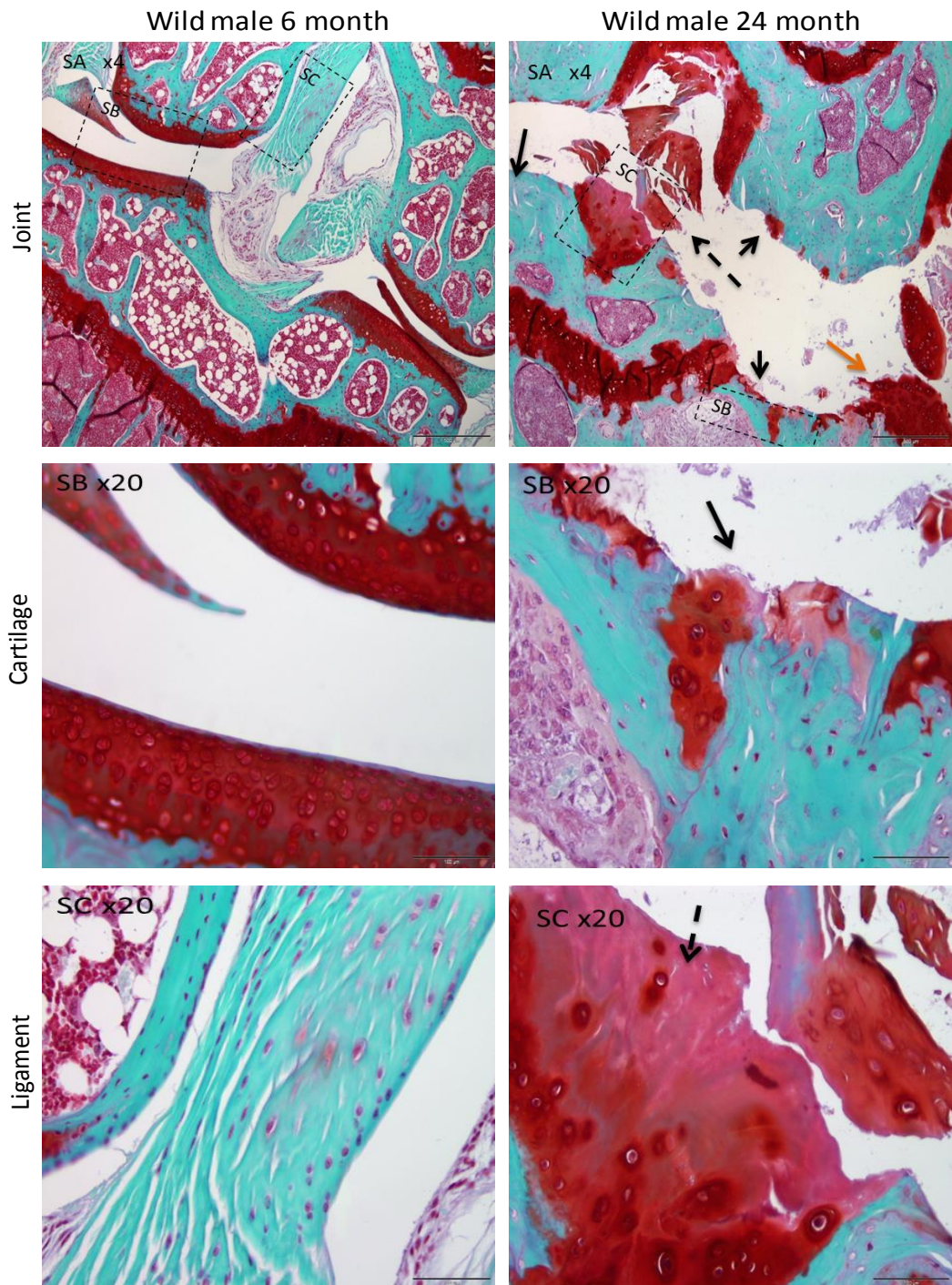
Fig 6.3 Representative histological sections of the knee joint in wild male mice at 6 and 24 months of age. Photographs SA-SC is stained with Safranin-O to show the proteoglycans (red). SA was magnified x4 to show the whole joint, SB (cartilage region) and SC (cruciate ligament region) were magnified x20 to show detailed structures. Erosion to the calcified cartilage (black arrow). Abnormal structure of cruciate ligaments with (dashed arrow). Osteophyte formation was also observed (orange arrow).

The ligament was stained for proteoglycan structures. Clear erosion of cartilage was evident between 6 and 24 months of age, with cartilage lesions and boney osteophyte formation.

Fig 6.4 Representative histological sections of the knee joint in wild female mice at 6 and 24 months of age. Photographs A-C were stained with H&E. A was magnified x4 to show the whole joint, B (cartilage region) and C (cruciate ligament region) were magnified x20 to show detailed structures.

There was a visual change in the shape and structure of the joint in the female wild mice between 6 and 24 months of age. The meniscus and ligament were no longer recognisable at 24 months of age.
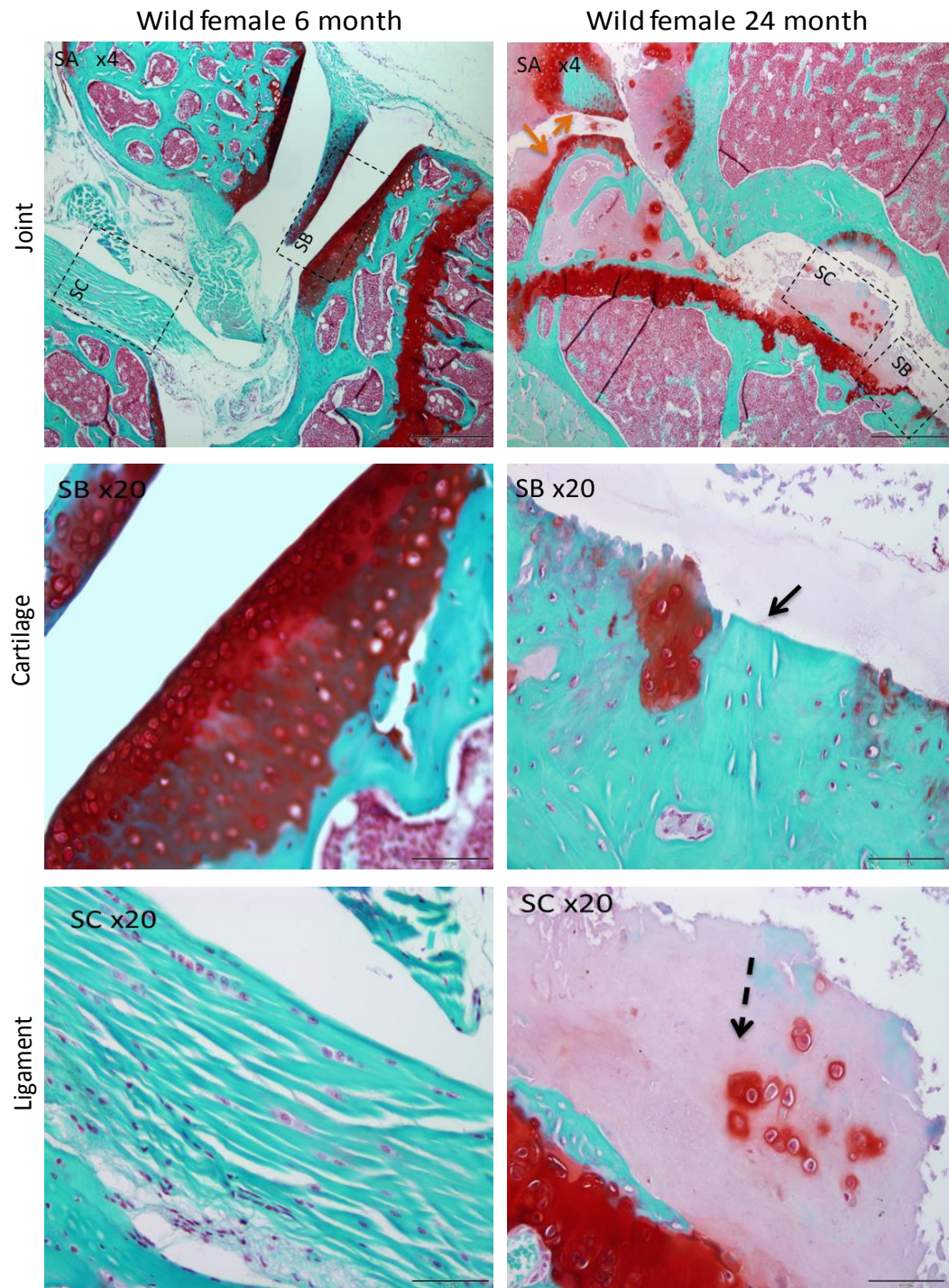
Fig 6.5 Representative histological sections of the knee joint in wild female mice at 6 and 24 months of age. SA-SC is stained with Safranin-O to show the proteoglycans (red). SA was magnified x4 to show the whole joint, SB (cartilage region) and SC (cruciate ligament region) were magnified x20 to show detailed structures. Erosion to the calcified cartilage (black arrow). Abnormal structure of cruciate ligaments with (dashed arrow). Osteophyte formation was also observed (orange arrow).

The ligament was stained for proteoglycan structures. There was a clear erosion of cartilage between 6 and 24 months of age, with cartilage lesions and bony osteophyte formation
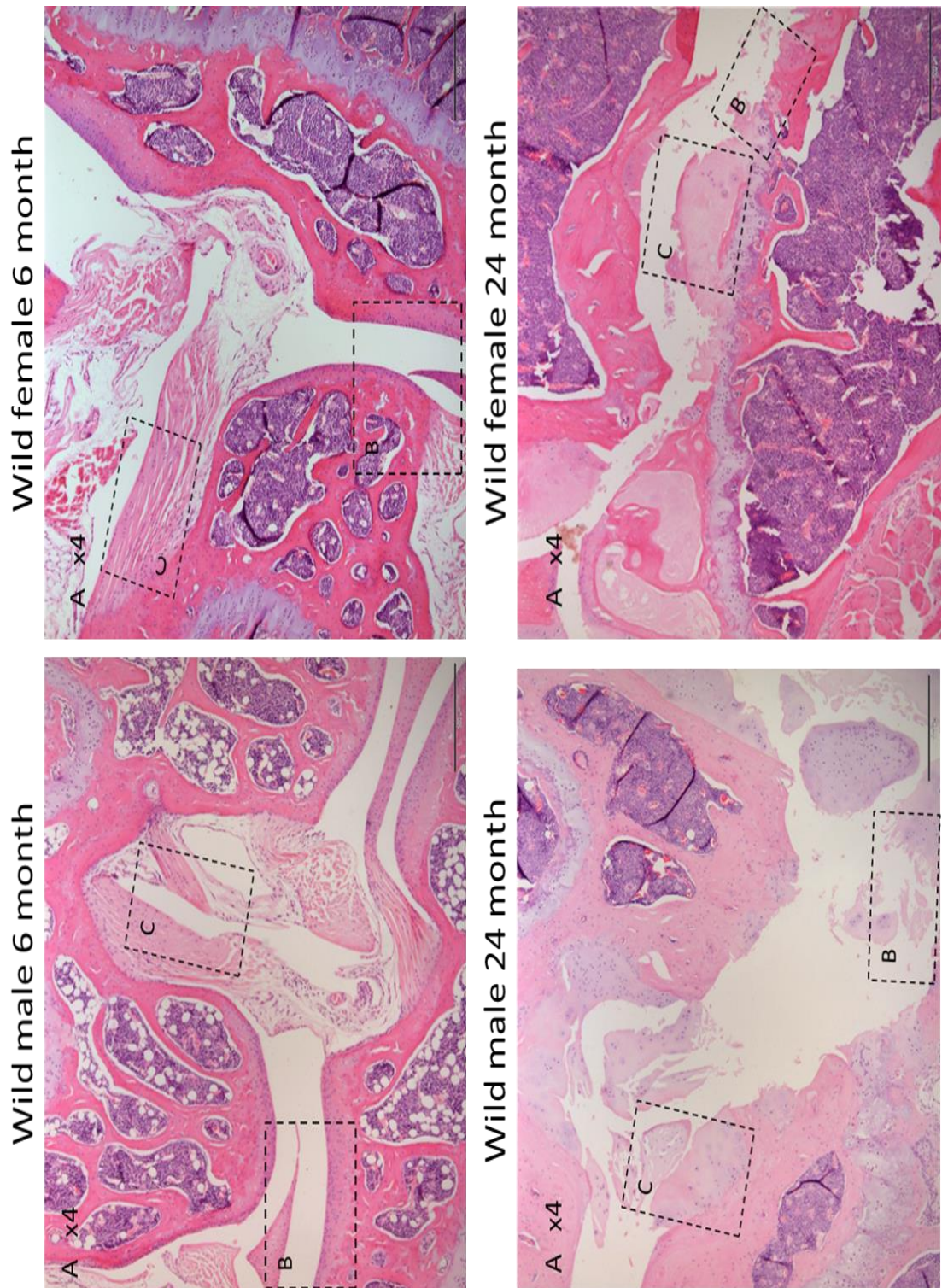
Fig 6.6 Representative histological sections of the knee joint in wild male and female mice at 6 and 24 months of age. Photographs were stained with H&E. and was magnified x4 to show the whole joint,

There were no gross differences between the male and female wild mice suggesting that the changes seen were not sex-dependent but were a naturally occurring condition in the wild mouse population.
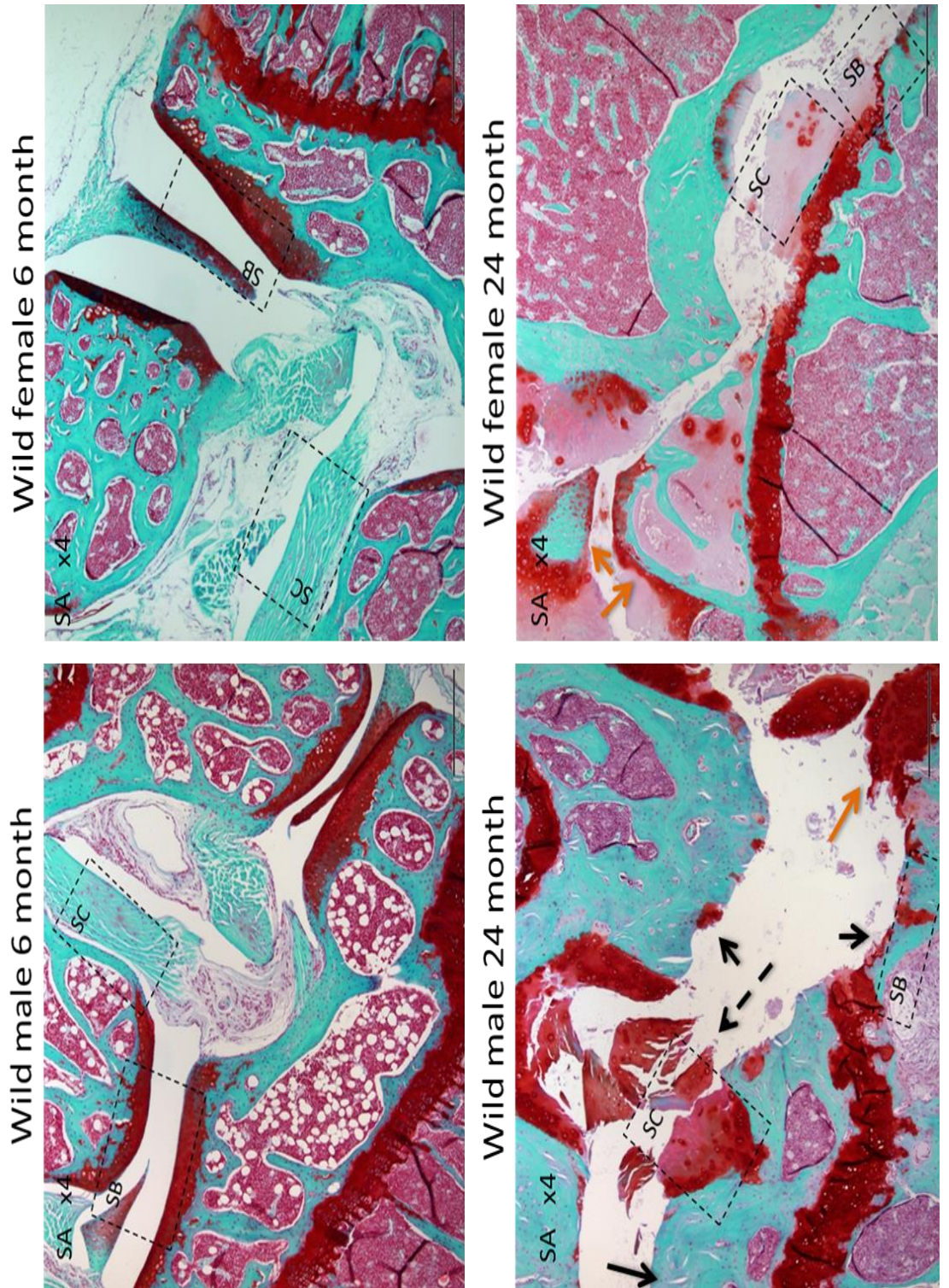
Fig 6.7 Representative histological sections of the knee joint in wild male and female mice at 6 and 24 months of age. Photographs were stained with Safranin-O to show the proteoglycans (red) and was magnified x4 to show the whole joint,

There were no gross differences between the male and female wild mice at any age suggesting that the changes displayed were not sex-dependent but were a naturally occurring condition in the wild mouse population.

6.4.5 Visualisation of age-related changes in the hindlimb bones in male and female wild mice
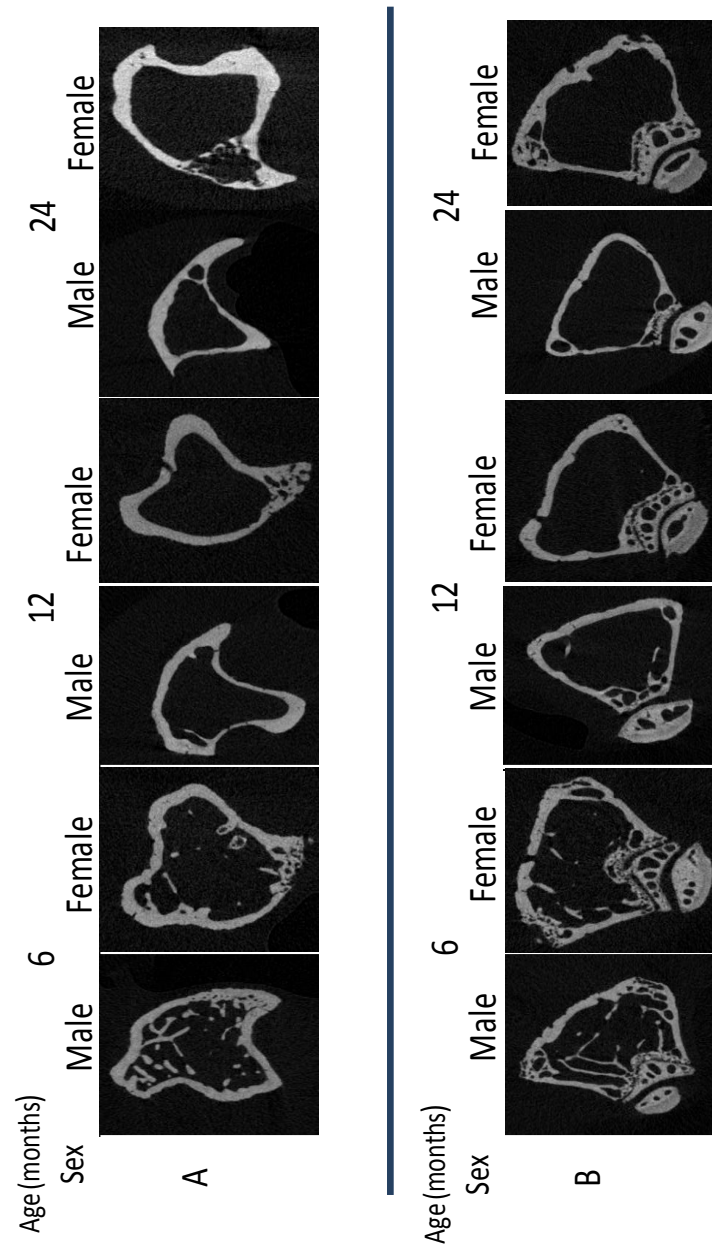
Fig 6.8 Representative microCT images of the tibia (A) and femur (B) in the wild male and female mice at different ages.

A representative image from each bone through the life course studied was selected based on mean bone values. In both sexes the cortical and trabecular bone was clearly evident. A change in 2D shape of both bones was shown with increasing age. Loss of trabecular bone was also seen at advancing age, with potential differential effects seen between male and female mice.
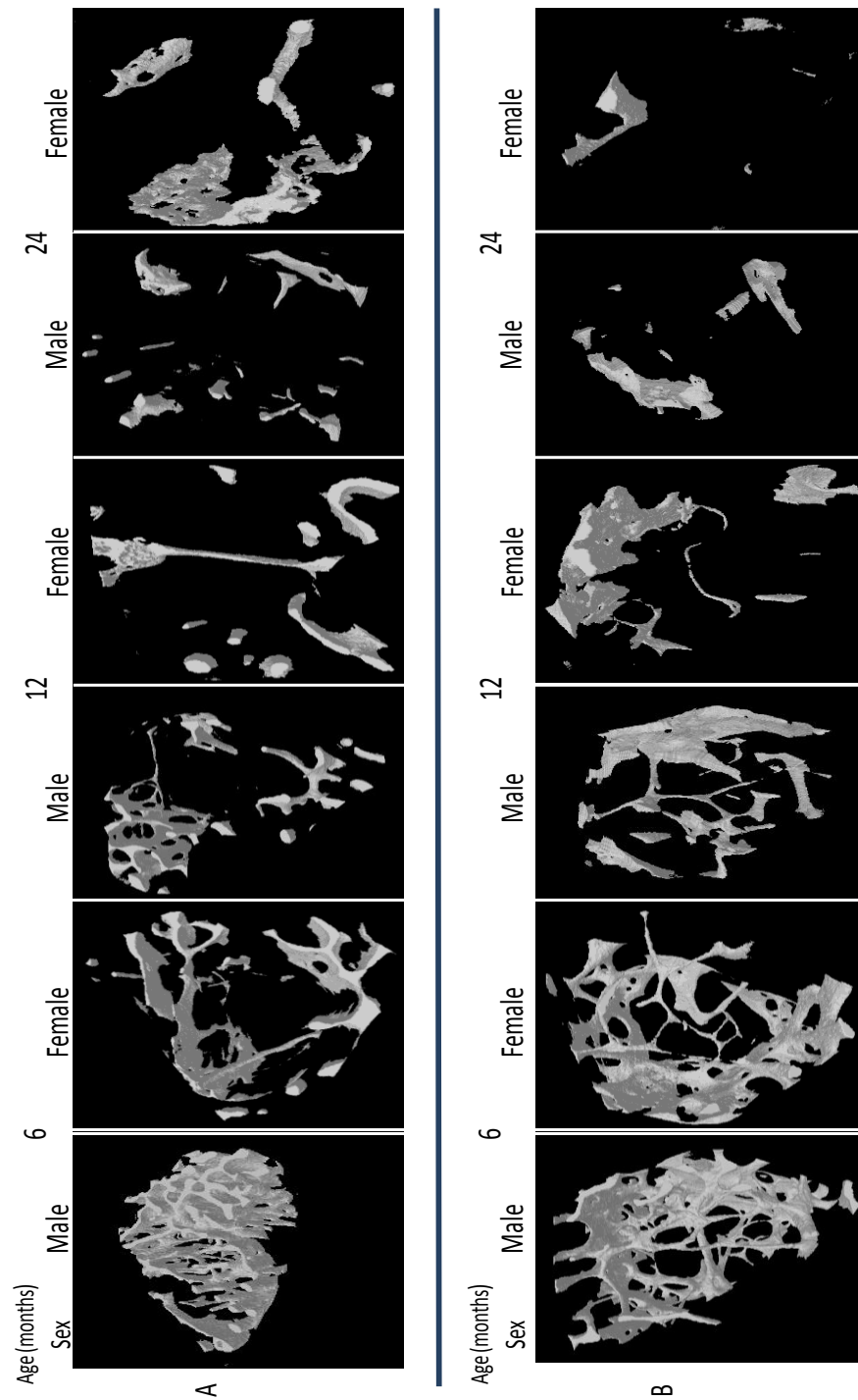
Fig 6.9 Representative 3D models of trabecular bone in tibia (A) and femur (B) through age in the wild male and female mouse.

Loss of trabecular bone was seen in both bones studied. The trabecular bone also became less connected. Smaller trabecular bones appeared to be lost first with larger bones becoming thinner. There were clear visual differences between the male and female mice.

6.4.6 Age-related changes in cortical and trabecular bone in hindlimb bones of male and female wild mice
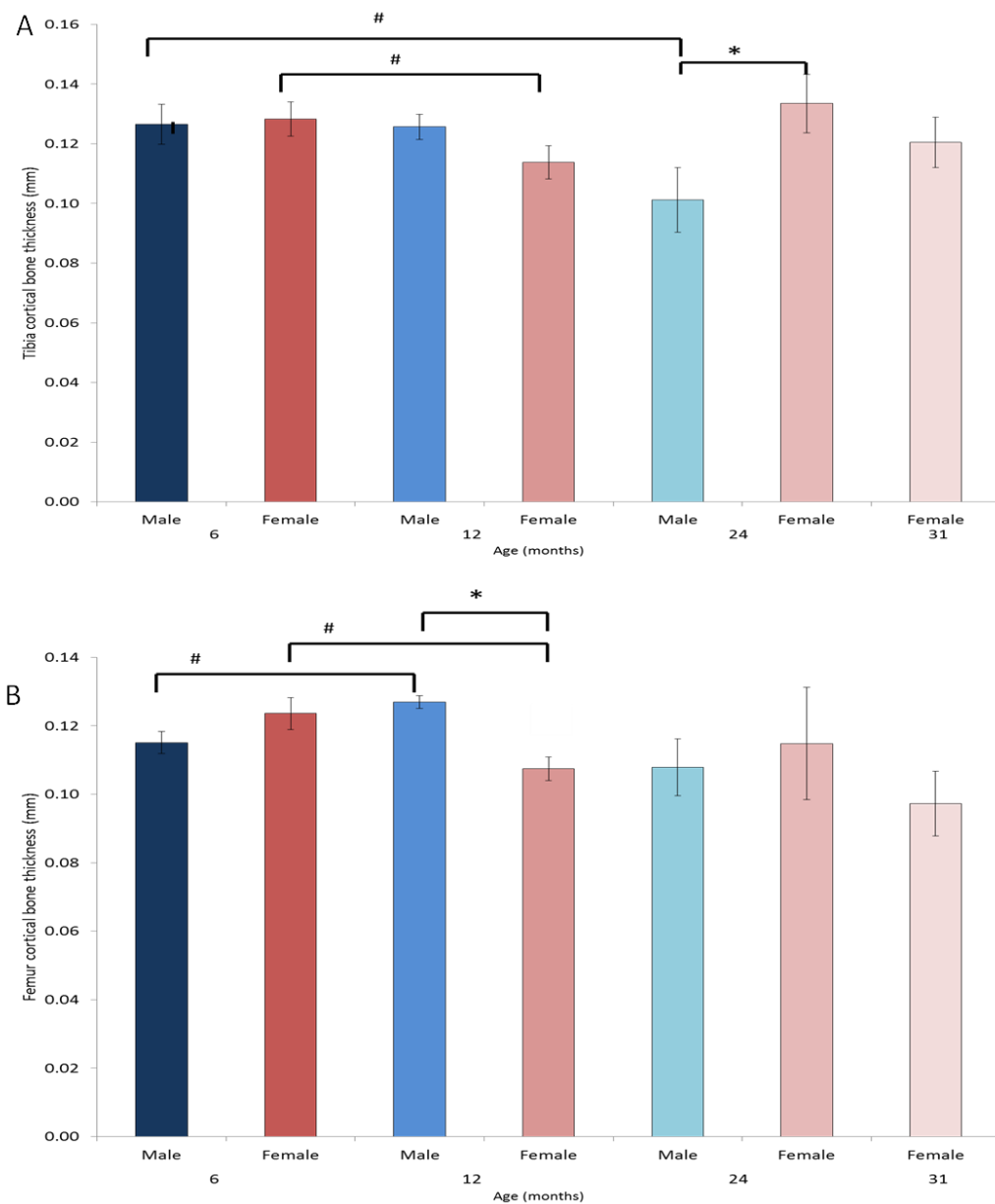
Fig 6.10 Mean cortical bone thickness of tibia (A) and femur (B) at different ages in wild male and female mice (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with values for age matched male mice, # p <0.05 compared with sex matched mice at 6 months old.

Data showed that cortical bone thickness generally decreased in tibia of male mice at 24 months old but this was not evident in the femur of the male mice. Female mice showed a small but significant decrease in tibia and femur cortical thickness at 12 months compared with 6 months old although the relevance of this is difficult to interpret as this was not maintained at 24 or 31 months old. No clear sex effects were seen.
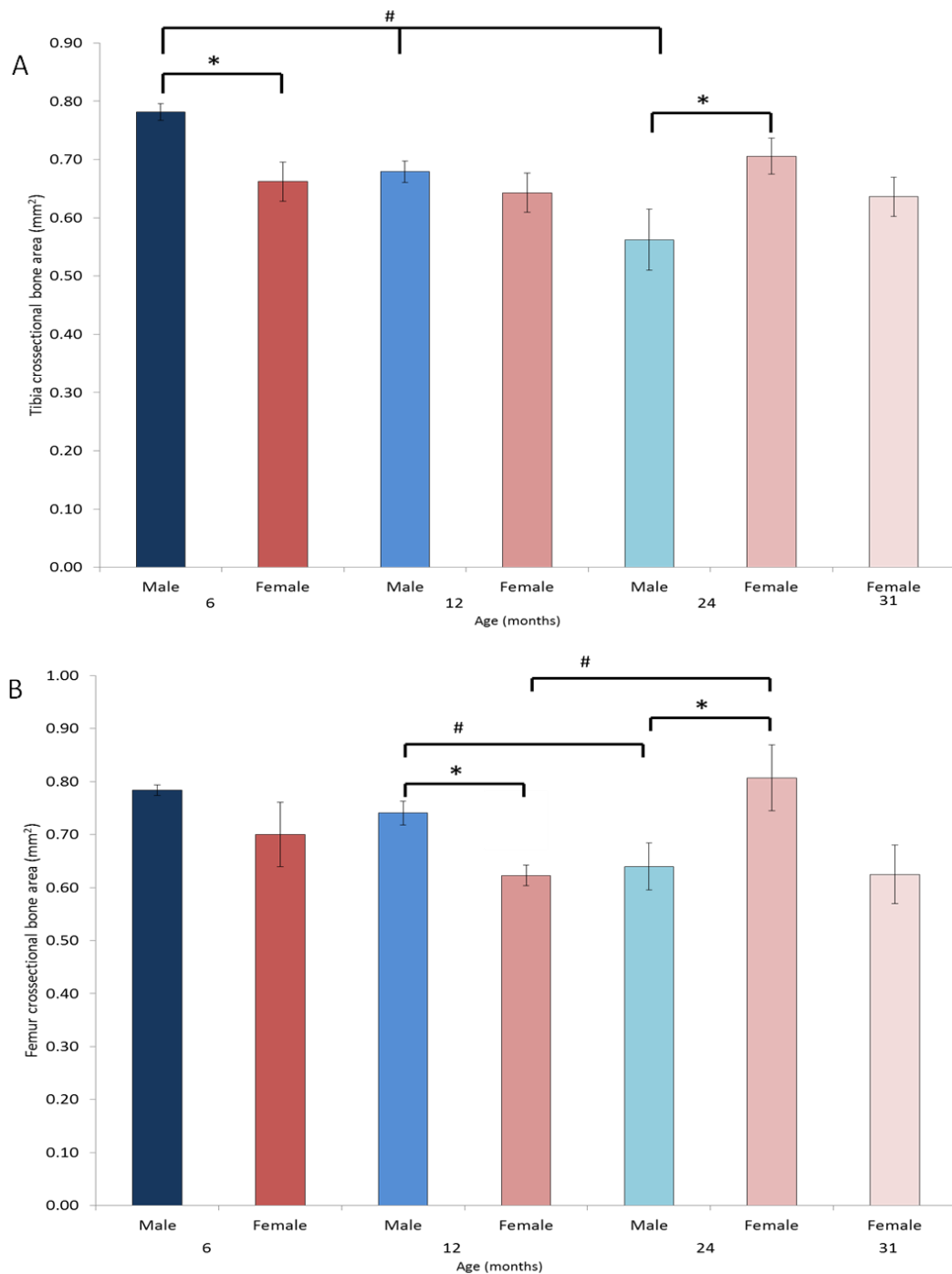
Fig 6.11 Mean bone cross-sectional area of tibia (A) and femur (B) at different ages in wild male and female mice (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with age matched data for male mice, # p <0.05 compared with data sex matched for mice at 6 months old.

Data show a minor but significant reduction in tibia in bone CSA in 6 month old female compared with male mice with a similar although non-significant reduction seen in the femur. Tibia and femur CSA was generally reduced with age in male mice but not in female mice, resulting in a higher CSA in both tibia and femur of female compared with male mice at 24 months old.
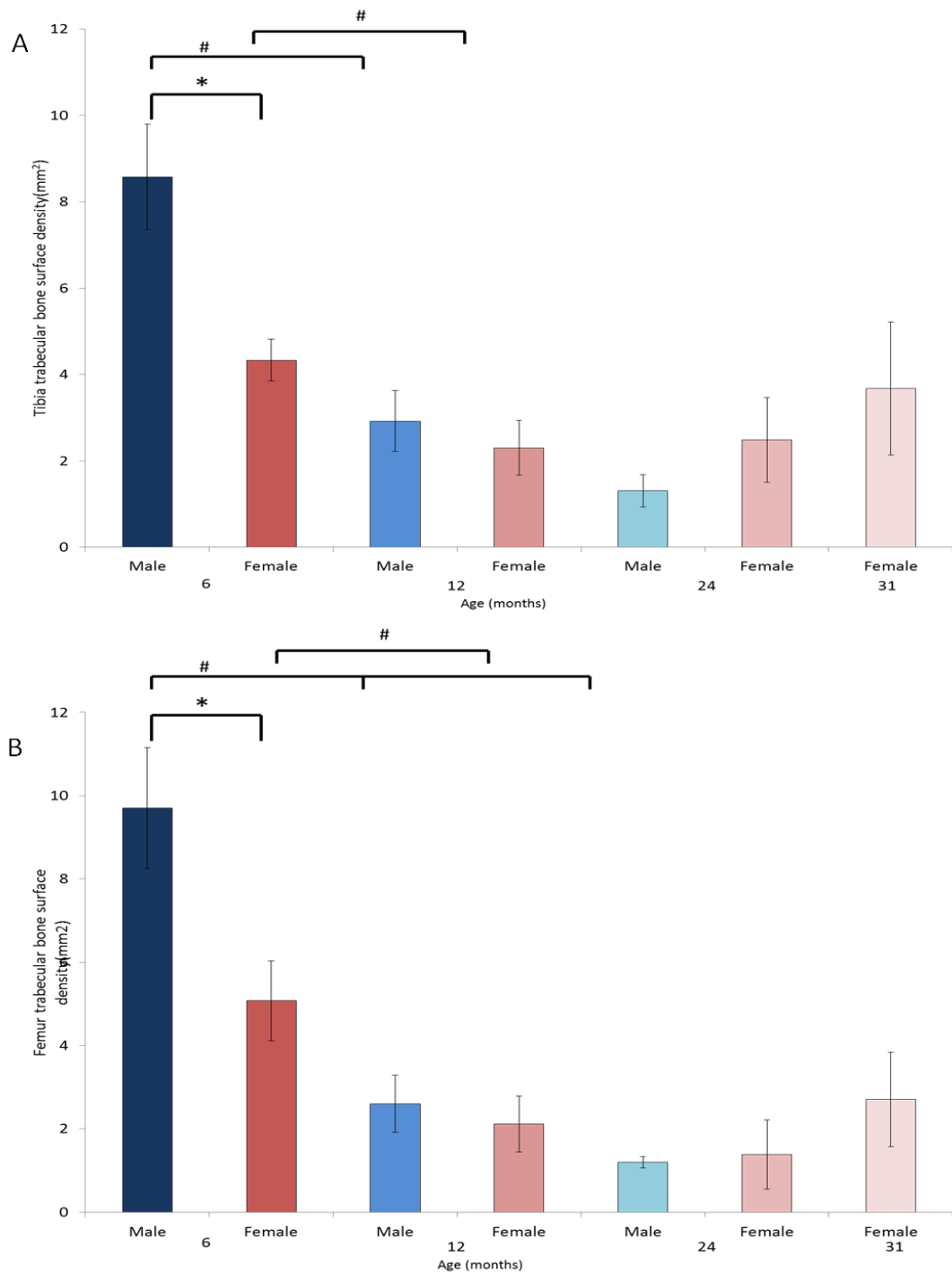
Fig 6.12 Mean trabecular bone surface density values for tibia (A) and femur (B) at different ages in wild male and female mice (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with data for age matched male mice, # p <0.05 compared with data for sex matched 6 month old mice.

Trabecular bone surface density of both the tibia and femur was significantly lower in 6 month old female compared with male mice. This was then dramatically reduced in male mice with increasing age and in female mice, resulting in loss of a high proportion of bone surface density by 24 months old in both male and female mice.

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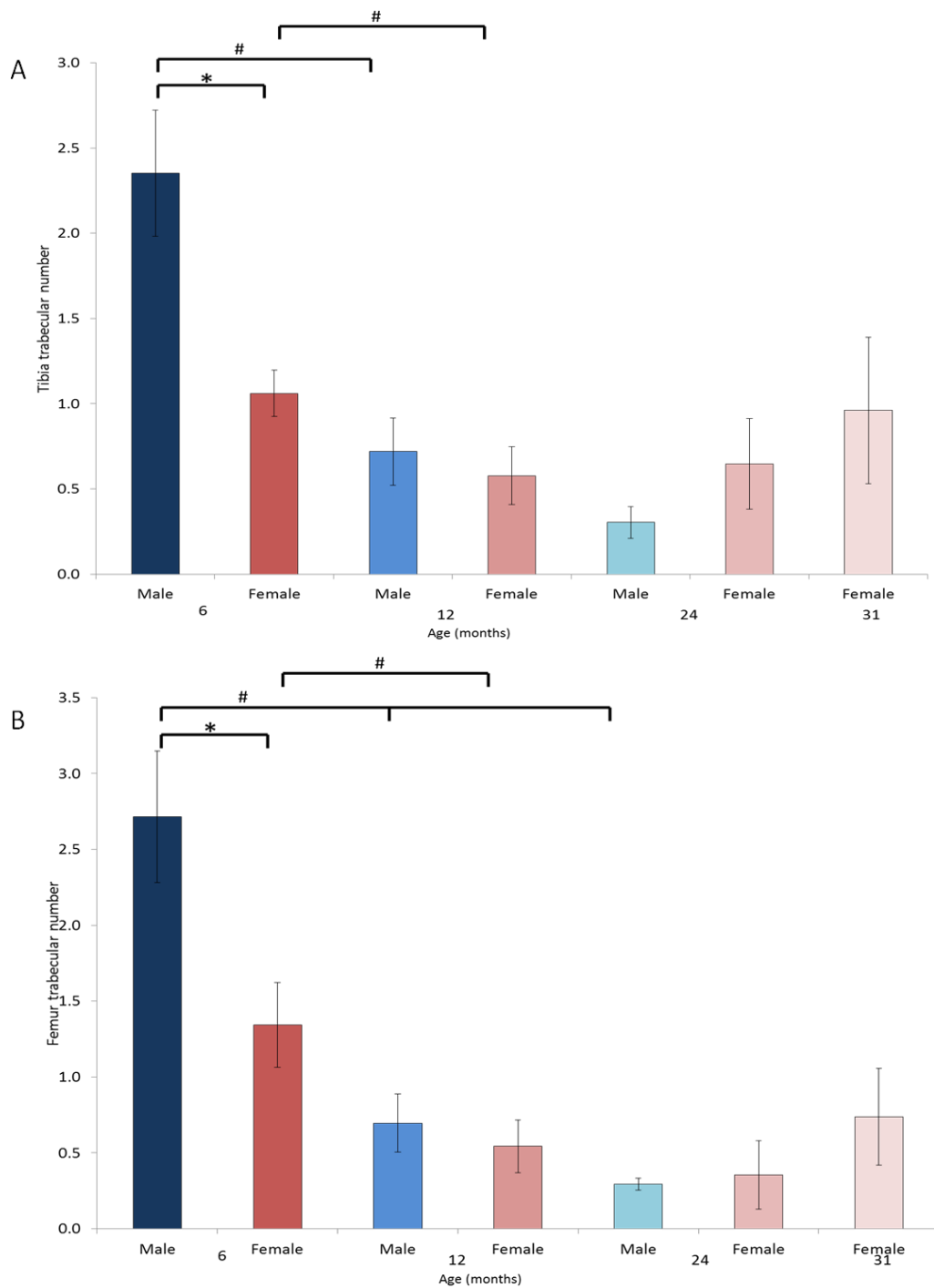
Fig 6.13 Mean trabecular number/mm values for tibia (A) and femur (B) at different ages in wild male and female mice (Data presented as mean +/- SEM) (n=6) * $p < 0.05$ compared with values for age matched male mice, # $p < 0.05$ compared with values for sex matched 6 month mice.

In a similar manner to trabecular bone density, trabecular bone number was significantly lower in female mice at 6 months old and both showed a dramatic and significant reduction by 12 months resulting in a dramatic loss of trabecular bone by 24 months old.
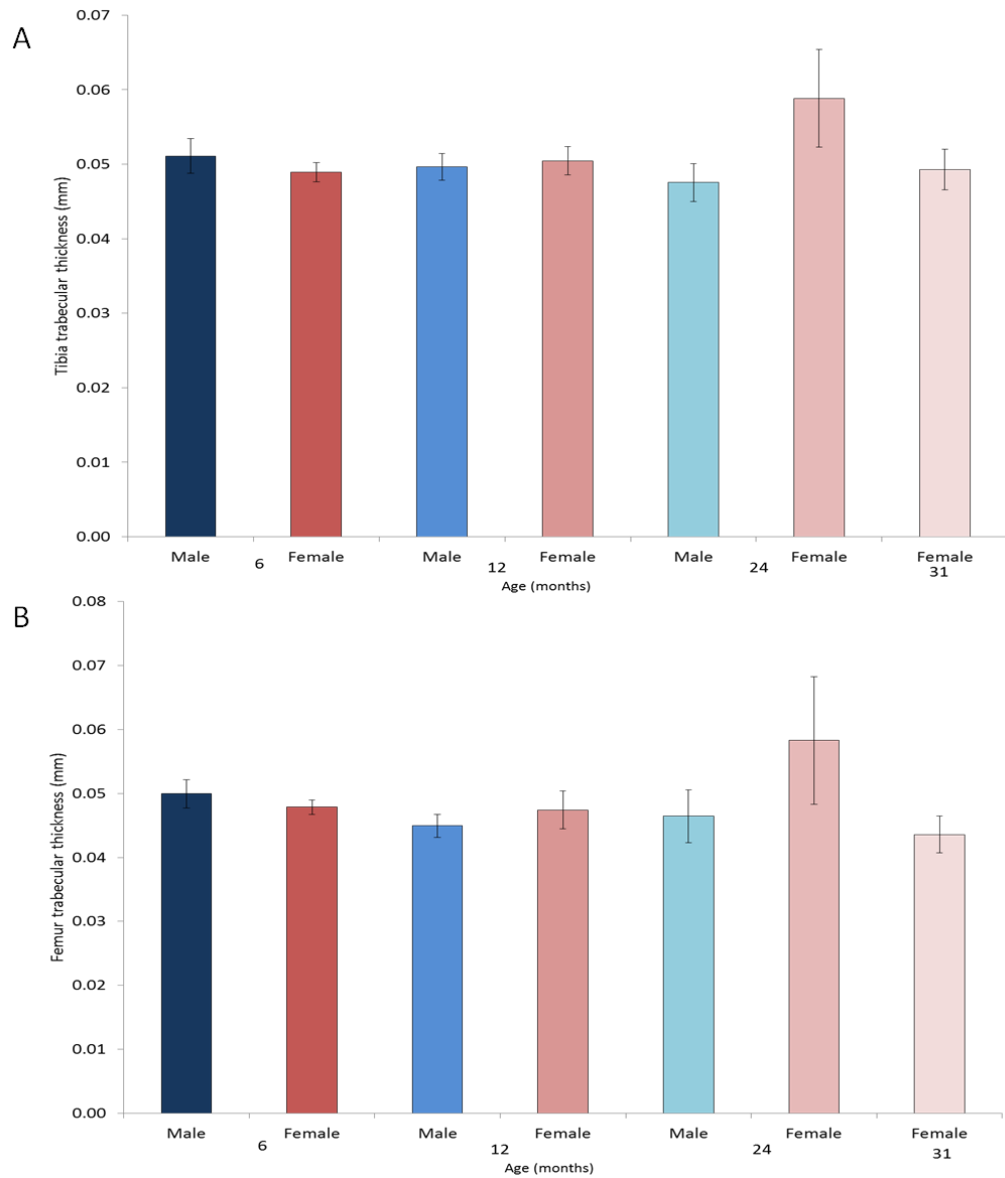
Fig 6.14 Changes in trabecular bone thickness at different ages in the tibia and femur in wild male and female mice (Data presented as mean +/- SEM) (n=6) * p <0.05 compared with male values.

There were no significant differences in trabecular bone thickness in either bone at any age in either male or female mice.
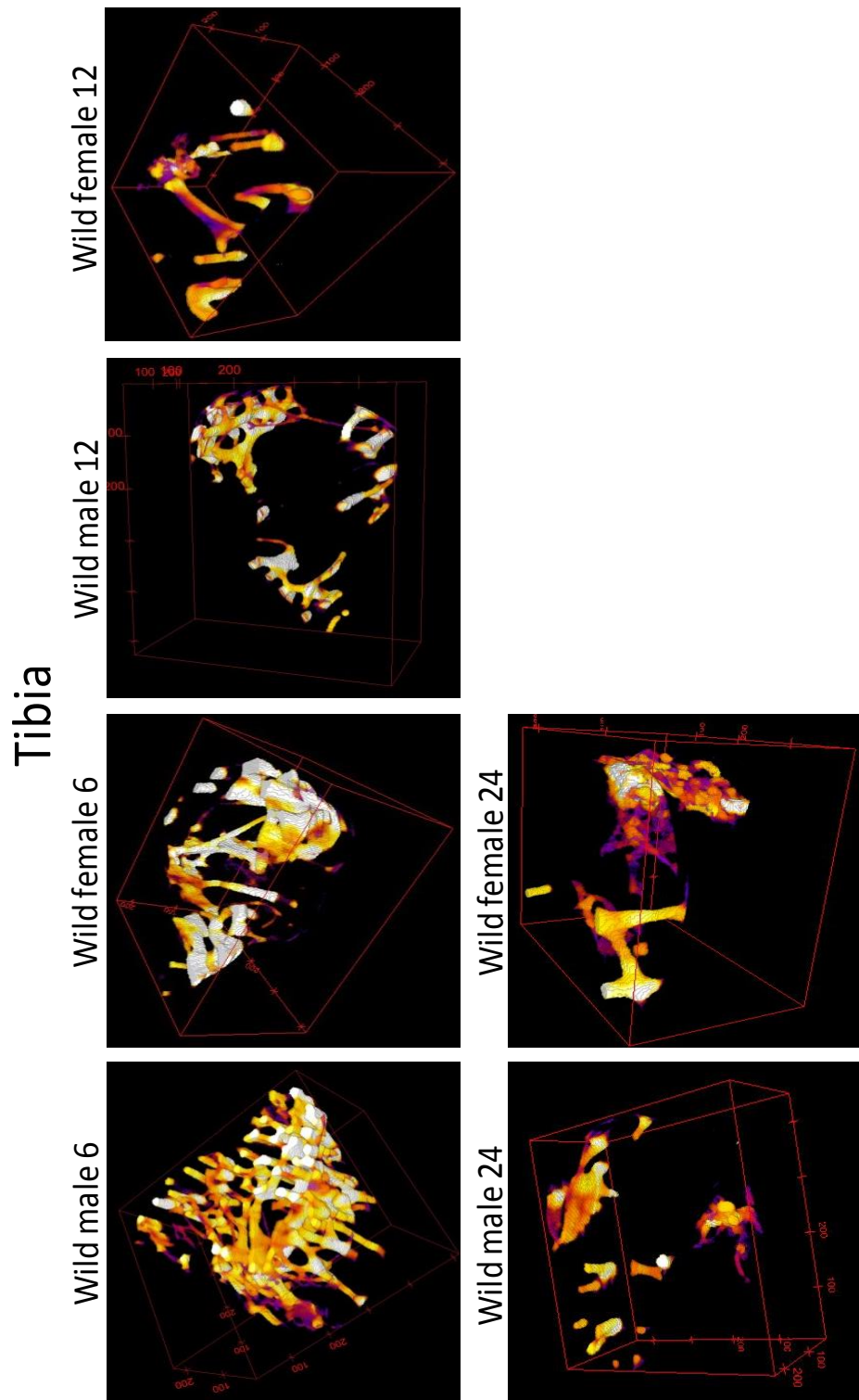
Fig 6.15 3D representation of trabecular bone thickness through age in the tibia. White colours represent the thicker areas, purple colours representing the thinner areas. Scale is specific to each individual bone based of mean and SEM as such, no key is present.

There were visual decreases in trabecular thicknesses in the mouse between 6 and 24 months of age and differences between the sexes. The loss of trabecular bone was also evident.
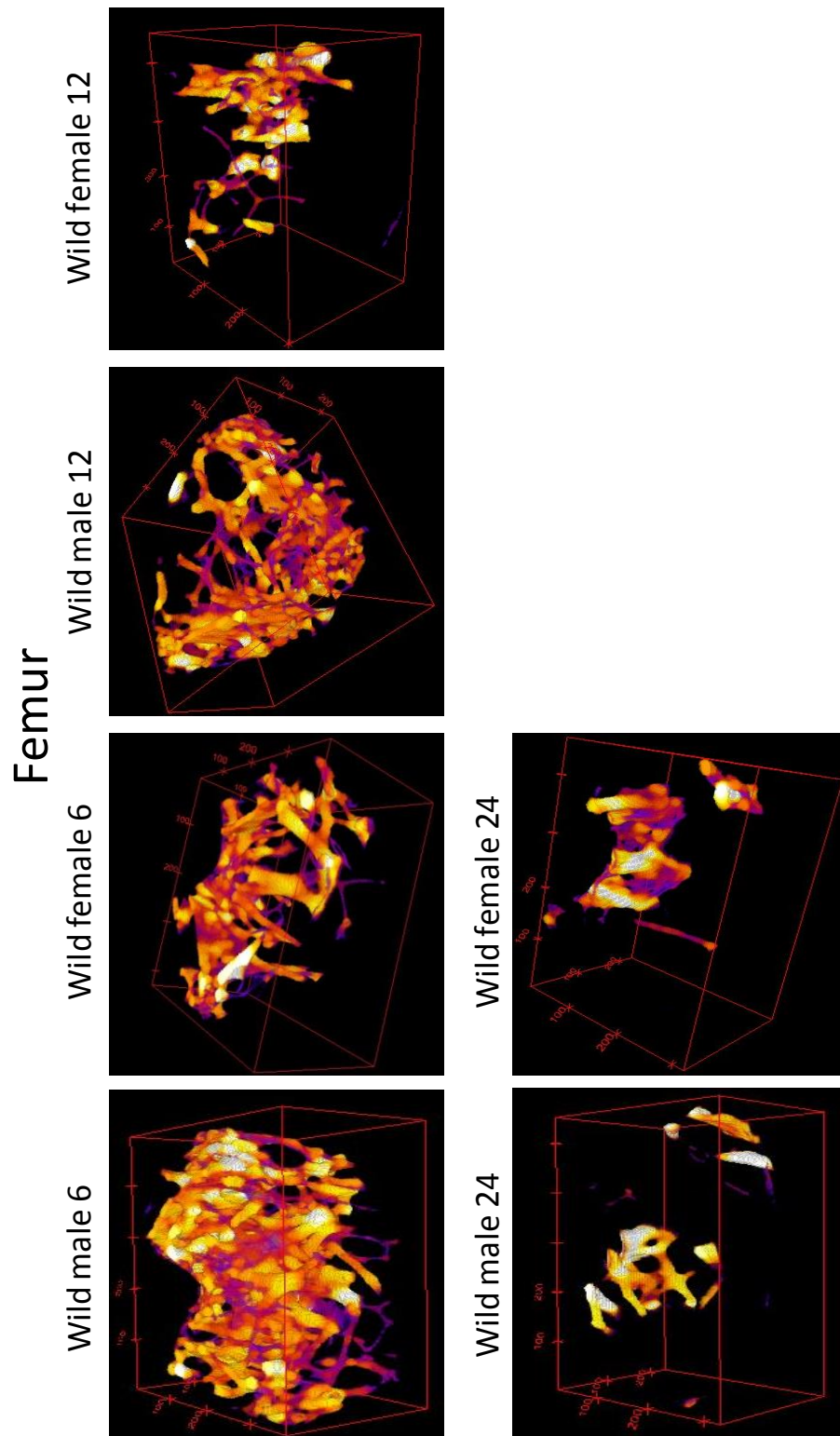
Fig 6.16 3D representation of trabecular bone thickness through age in the femur. White colours represent the thicker areas, purple colours representing the thinner areas. Scale is specific to each individual bone based of mean and SEM as such, no key is present.

There were visual decreases in trabecular thicknesses in the mouse between 6 and 24 months of age and differences between the sexes. The loss of trabecular bone was also evident.

6.4.7 Comparison of age-related changes in body mass in both male C57Bl/6 and male wild mice
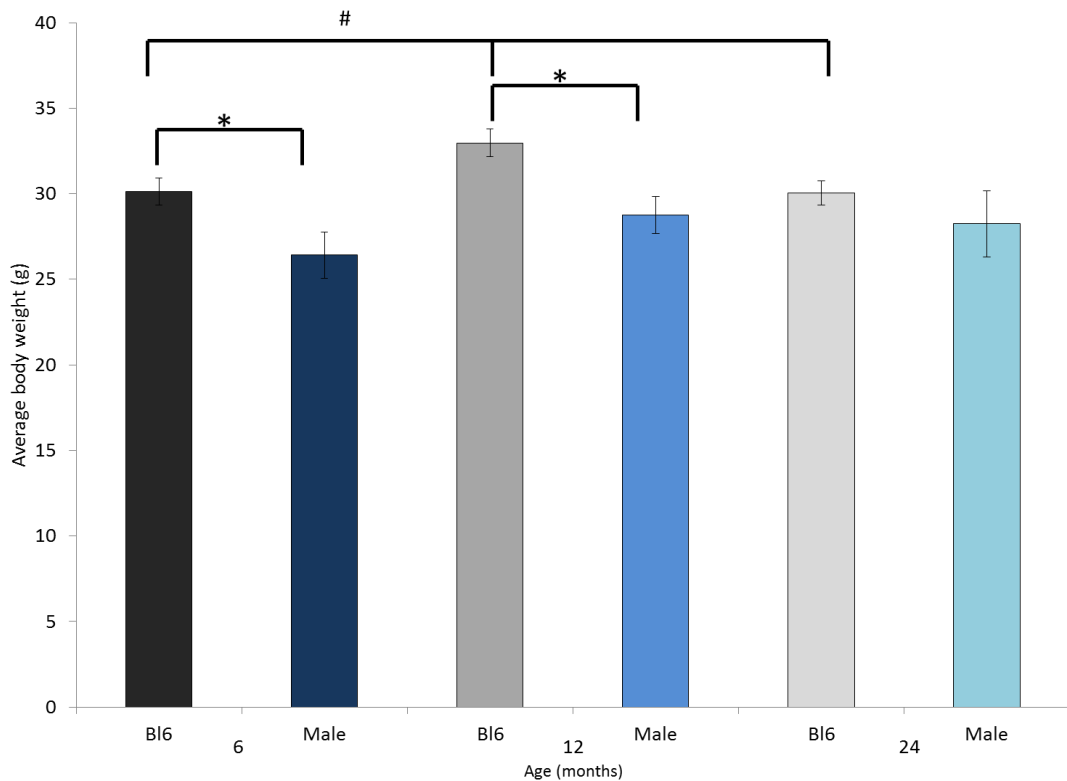
Fig 6.17 Mean body mass of male C57Bl/6 mice and male wild mice at different ages (Data presented as mean +/- SEM) (n=4/6* p <0.05 compared with value for age matched male C57Bl/6 mice.

The body mass of wild male mice were significantly reduced compared with C57Bl/6 males at both 6 and 12 month old, but whereas C57Bl/6 mice showed an age-related loss of body mass, this wasn't evident in wild mice, resulting in no significant difference in body mass between C57Bl/6 and wild mice at 24 months old.

6.4.8 Comparison of age-related changes in organ mass in both male C57Bl/6 and male wild mice

Age		6		12		24	
Cohort		Bl6	Wild	Bl6	Wild	Bl6	Wild
Heart	Average	197.2	127.2 *	240.2	142.6 * c	205.7	135.1 *
	SEM	13.4	5.5	30.8	6.5	8.8	15.0
Lungs	Average	208.3	140.9 *	240.9 a	176.2 * c	228.5	138.6 * d
	SEM	12.1	8.5	11.4	8.1	12.8	10.2
Liver	Average	1580.3	908.9 *	1758.4	1019.3 *	1212.1 a,b	1116.3
	SEM	76.2	75.1	130.9	79.7	127.7	231.3
Kidneys	Average	402.9	306.4 *	496.8 a	384.9 *,c	450.1	145.7 * c,d
	SEM	12.5	8.1	23.9	5.8	35.5	18.9
Spleen	Average	70.8	37.2 *	100.3 a	46.2 *	66.9 b	77.5 c,d
	SEM	5.4	4.5	7.4	4.1	10.6	16.5
Brain	Average	388.4	228.3 *	386.7	303.9 * c	383.1	237.4 * d
	SEM	31.4	26.5	25.4	9.6	22.7	36.9

Table 6.5 Mean organ mass at different ages. * represent significant differences compared with data from age matched C57Bl/6 mouse data (n=6 per group). Letters depict a significant breed matched difference, (**a** - 6 month old Bl/6 mice, **b** - 12 month old Bl/6 mice, **c** - 6 month old wild mice, **d** - 12 month old wild mice). Data presented as mean +/- SEM.

Age		6		12		24	
Cohort		Bl6	Wild	Bl6	Wild	Bl6	Wild
Heart OW/BW	Average	6.5	4.9 *	7.2	4.9 *	5.0 a,b	7.5 c,d
	SEM	0.3	0.2	0.8	0.2	0.6	1.9
Lungs OW/BW	Average	6.9	5.3 *	7.3	6.1 *,c	6.7	5.4 d
	SEM	0.3	0.2	0.3	0.2	1.4	0.3
Liver OW/BW	Average	52.3	34.3 *	53.3	35.3 *	39.1 a,b	34.4
	SEM	1.5	1.6	3.4	1.9	9.8	3.0
Spleen OW/BW	Average	2.3	1.4 *	3.0 a	1.6 *	5.6	19.0
	SEM	0.1	0.1	0.2	0.2	3.0	17.4

Table 6.6 Mean organ mass as a proportion of body mass. * represent significant differences compared with age matched C57Bl/6 mouse data (n=6 per group). Letters depict a significant breed matched difference, (**a** - 6 month old Bl/6 mice, **b** - 12 month old Bl/6 mice, **c** - 6 month old wild mice, **d** - 12 month old wild mice and **e** - 24 month old female mice). Data presented as mean +/- SEM.

The mass of tissues was significantly reduced in male wild mice at all ages compared with age matched male C57Bl/6 mice (Table 4.7) and this decrease was maintained when data was expressed per unit of body mass (Table 4.8).

6.4.9 Comparison of age-related changes in muscle mass in male C57Bl/6 and male wild mice

Age		6		12		24	
Cohort		Bl6	Wild	Bl6	Wild	Bl6	Wild
AT	Average	56.4	38.7 *	57.7	43.7 *c	45.3 a,b	34.1 * d
	SEM	3.7	1.8	3.6	0.8	3.5	4.1
EDL	Average	12.4	8.6 *	11.6	8.7	11.5	6.8 * c,d
	SEM	1.3	0.6	1.9	0.6	2.0	0.7
Sol	Average	11.2	6.1 *	10.6	8.2 c	9.9 a	6.4 * d
	SEM	0.7	0.3	1.5	1.3	0.6	0.9
Gas	Average	174.9	129.8 *	160.1 a	132.6 *	138.1 a,b	108.2 c,d
	SEM	7.3	7.3	5.2	5.3	12.0	10.4

Table 6.7 Mean muscle mass (mg) of mice from four hindlimb muscles at different ages. * represent significant differences compared with age matched C57Bl/6 mouse data (n=6 per group). Letters depict a significant breed matched difference, (**a** - 6 month old Bl/6 mice, **b** - 12 month old Bl/6 mice, **c** - 6 month old wild mice, **d** - 12 month old wild mice and **e** - 24 month old female mice).. Data presented as mean +/- SEM.

Age		6		12		24	
Cohort		Bl6	Wild	Bl6	Wild	Bl6	Wild
AT MW/BW	Average	1.9	1.5 *	1.7 a	1.5 *	1.7 a	1.2 * c,d
	SEM	0.0	0.1	0.1	0.1	0.1	0.1
EDL MW/BW	Average	0.4	0.3	0.4	0.3	0.4	0.2 * c,d
	SEM	0.0	0.0	0.1	0.0	0.0	0.0
Sol BW/MW	Average	0.4	0.2 *	0.3	0.3 c	0.4	0.2 *,d
	SEM	0.0	0.0	0.0	0.0	0.0	0.0
Gas BW/MW	Average	5.8	5.0	4.9 a	4.6	4.9 a	3.9 * c,d
	SEM	0.1	0.4	0.2	0.2	0.2	0.1

Table 6.8 Mean muscle mass as a proportion of body mass. * represent significant differences compared with age matched C57Bl/6 mouse data (n=6 per group). Letters depict a significant breed matched difference, (**a** - 6 month old Bl/6 mice, **b** - 12 month old Bl/6 mice, **c** - 6 month old wild mice, **d** - 12 month old wild mice and **e** - 24 month old female mice). Data presented as mean +/- SEM.

The mass of muscles was significantly reduced in male wild mice at 6 months compared with age matched male C57Bl/6 mice (Table 4.9). All muscles were significantly reduced by 24 months in both wild and C57Bl/6 mice.

When expressed as a proportion of body mass anterior tibialis and soleus muscles where still significantly smaller in the wild mice at 6 months. All muscles of both types where significantly smaller at 24 months compared with 6 month old values.

6.4.10 Comparison of age-related changes in joint and ligament structure in both male C57Bl/6 and male wild mice
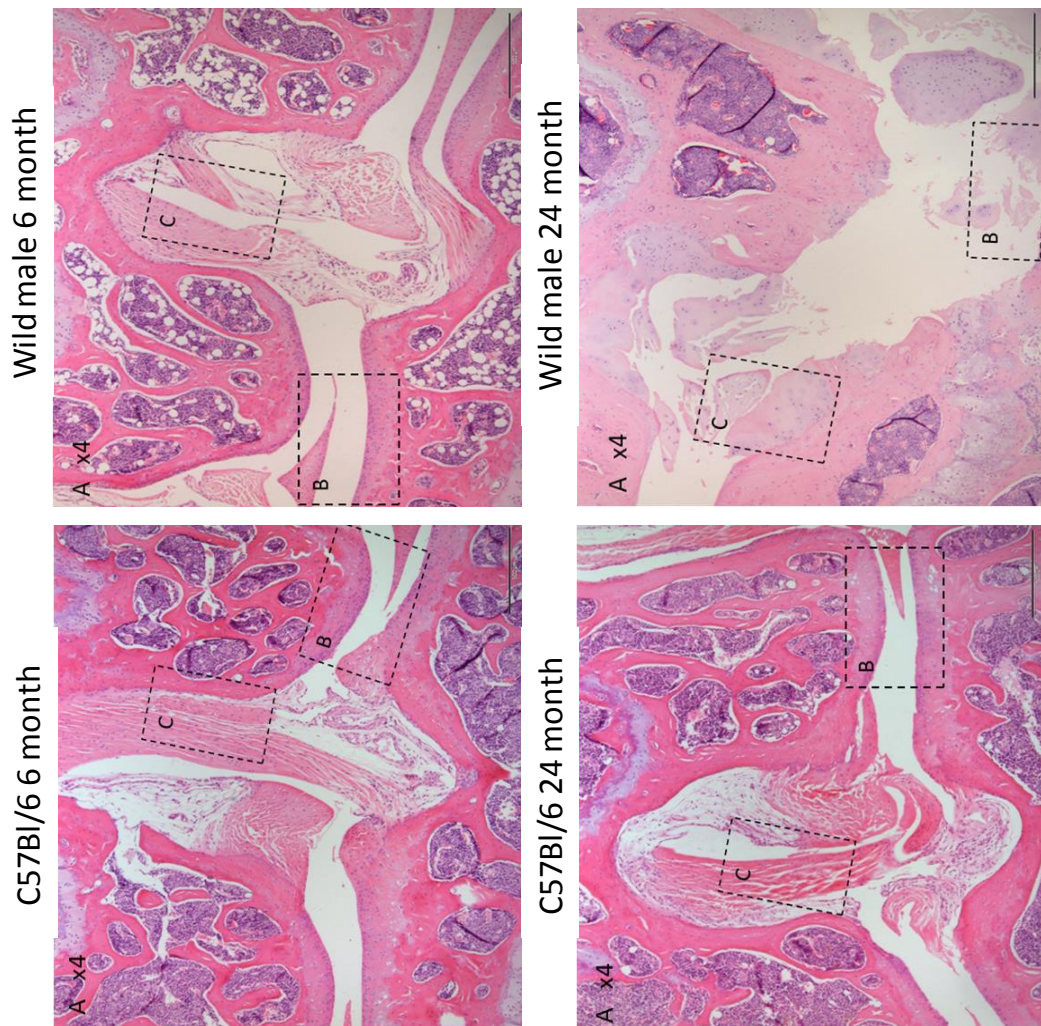
Fig 6.18 Representative histological sections of the knee joint of C57Bl/6 and wild male mice at 6 and 24 months of age. Photographs A-C were stained with H&E. A was magnified x4 to show the whole joint, B (cartilage region) and C (cruciate ligament region) were magnified x20 to show detailed structures.

There were no major visual differences in the structure of the joint or associated joint tissues between C57Bl/6 mice and the wild male mice at 6 months old. However significant differences were evident at 24 months old in wild mice including disruption of the ligament and meniscus. These changes were not evident in the C57Bl/6 mice at this age.
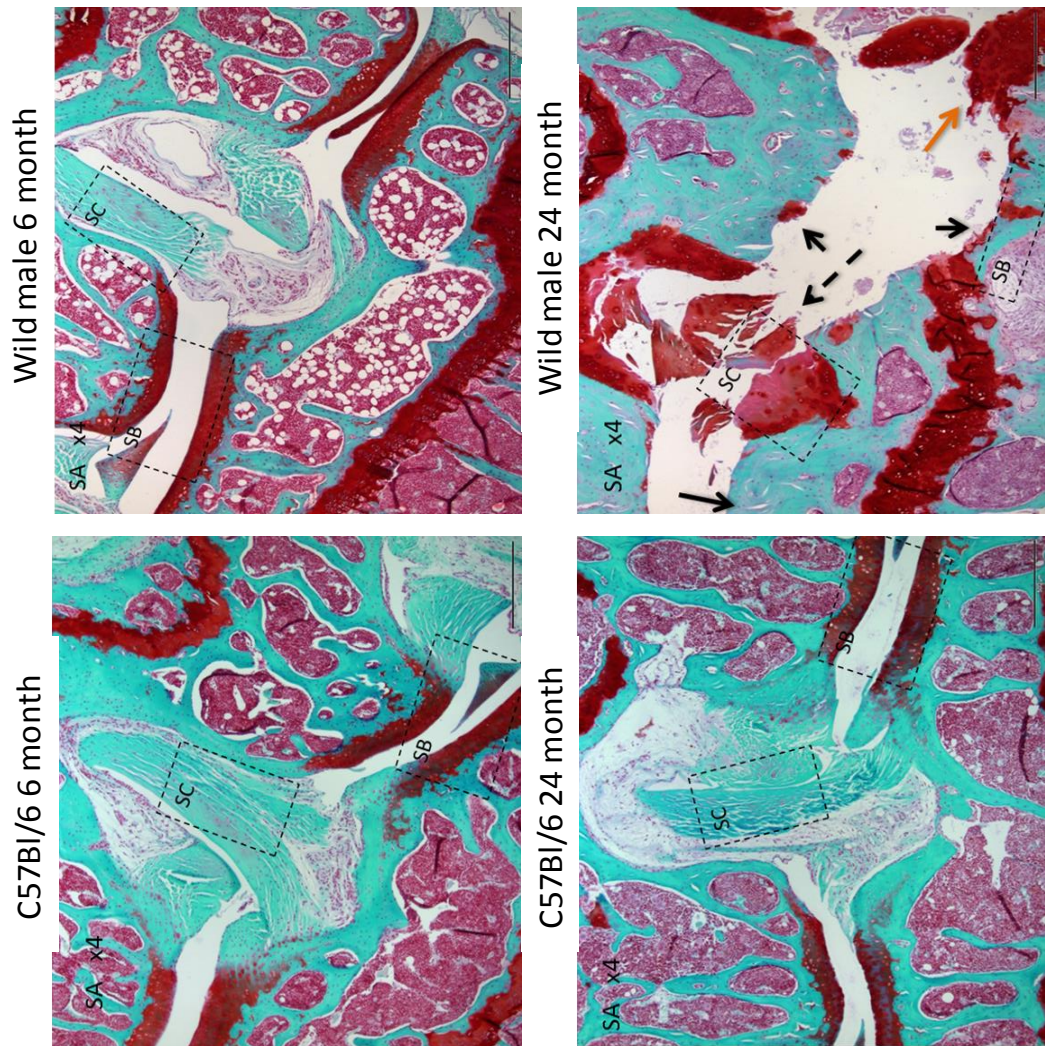
Fig 6.19 Representative histological sections of the knee joint in C57Bl/6 and wild male mice at 6 and 24 months of age. SA-SC is stained with Safranin-O to show the proteoglycans (red). SA was magnified x4 to show the whole joint, SB (cartilage region) and SC (cruciate ligament region) were magnified x20 to show detailed structures. Erosion to the calcified cartilage (black arrow). Abnormal structure of cruciate ligaments with (dashed arrow). Osteophyte formation was also observed (orange arrow).

There were no major visual differences in the structure of the joint or joint tissues between C57Bl/6 mice and the wild male mice at 6 months old. However significant differences were evident at 24 months old in wild mice including reduction of articular cartilage and increase in cartilage lesions and formation of osteophytes in wild male mice, none of which were seen in the C57Bl/6 mice.

6.4.11 Visualisation of age-related changes in the tibia and femur in both male C57Bl/6 and male wild mice
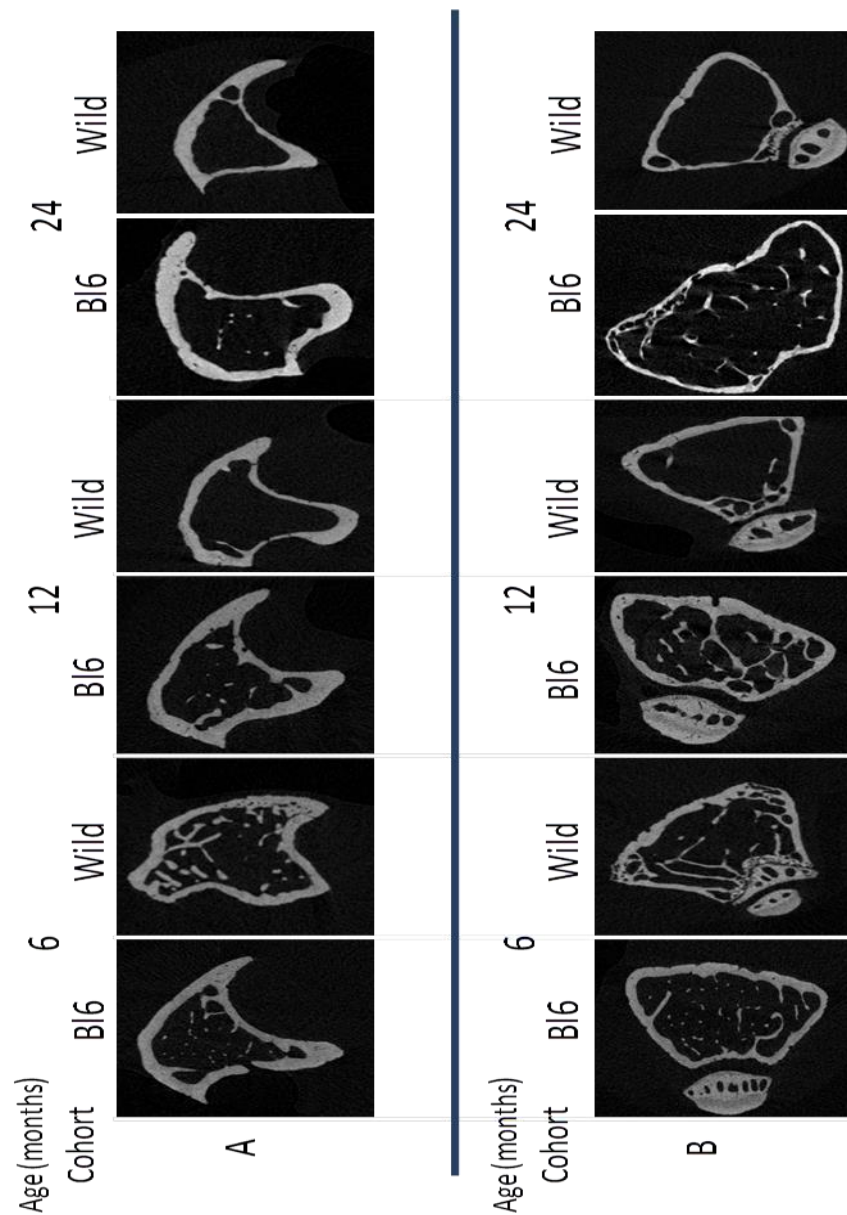
Fig 6.20 Representative microCT images of the tibia (A) and femur (B) in the male C57Bl/6 and wild mice at different ages.

A representative image from each bone site at different ages has been selected based on mean bone values. In both bones, the cortical and trabecular bone was clearly evident. A change in 2D shape of both bone sites is seen with advancing age. Loss of trabecular bone was also seen with advancing age in both wild mice and C57Bl/6 mice.
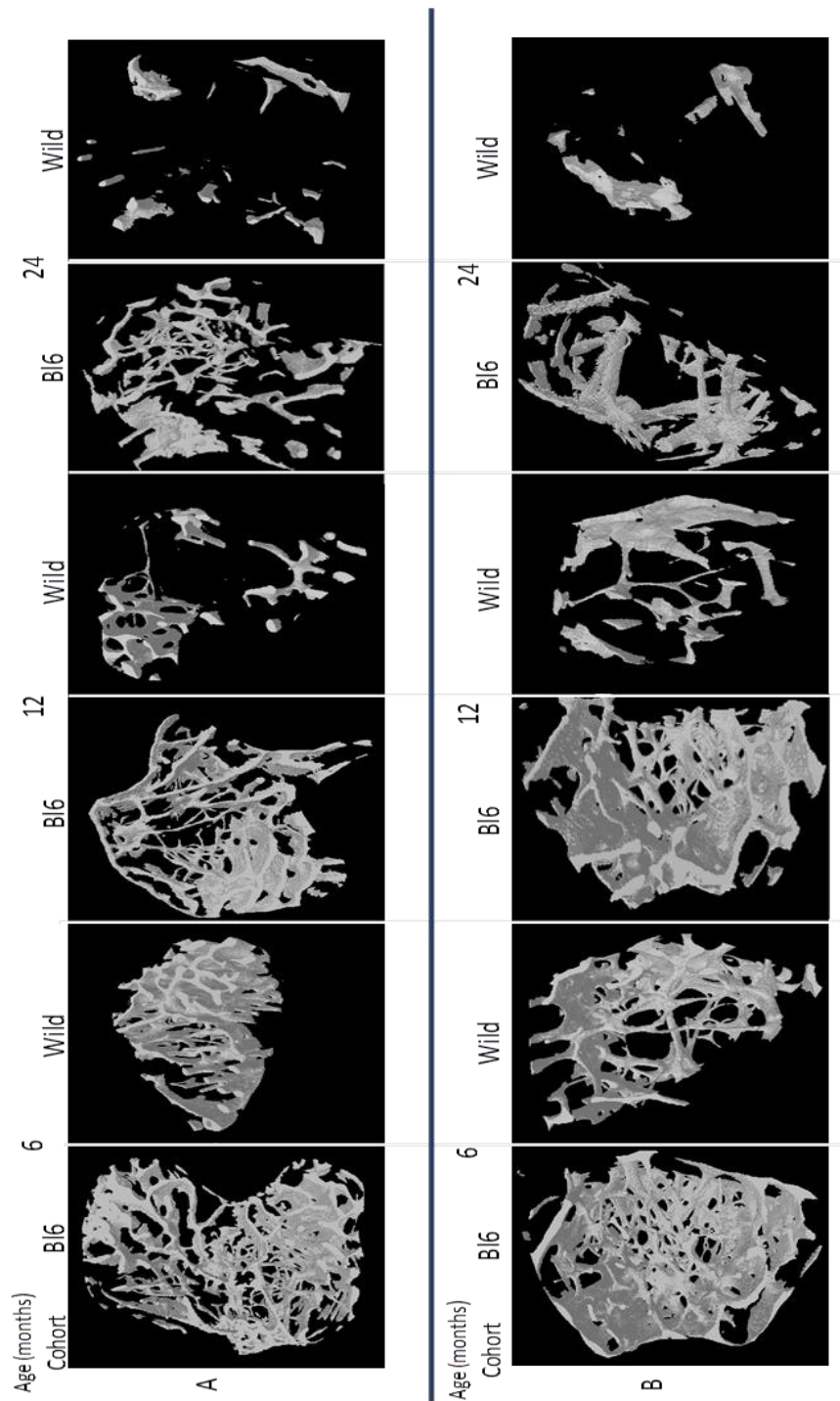
Fig 6.21 Representative 3D models of trabecular bone in tibia (A) and femur (B) through age in the wild and C57Bl/6 male mouse.

The loss of trabecular bone was seen at both bone sites. The trabecular bone mass also became less connected. Smaller trabecular bones appeared to be lost first with larger bones becoming thinner. There were clear visual differences between the wild mice and C57Bl/6 mice.

6.4.12 Comparison of age-related changes cortical and trabecular bone measures in both male C57Bl/6 and male wild mice
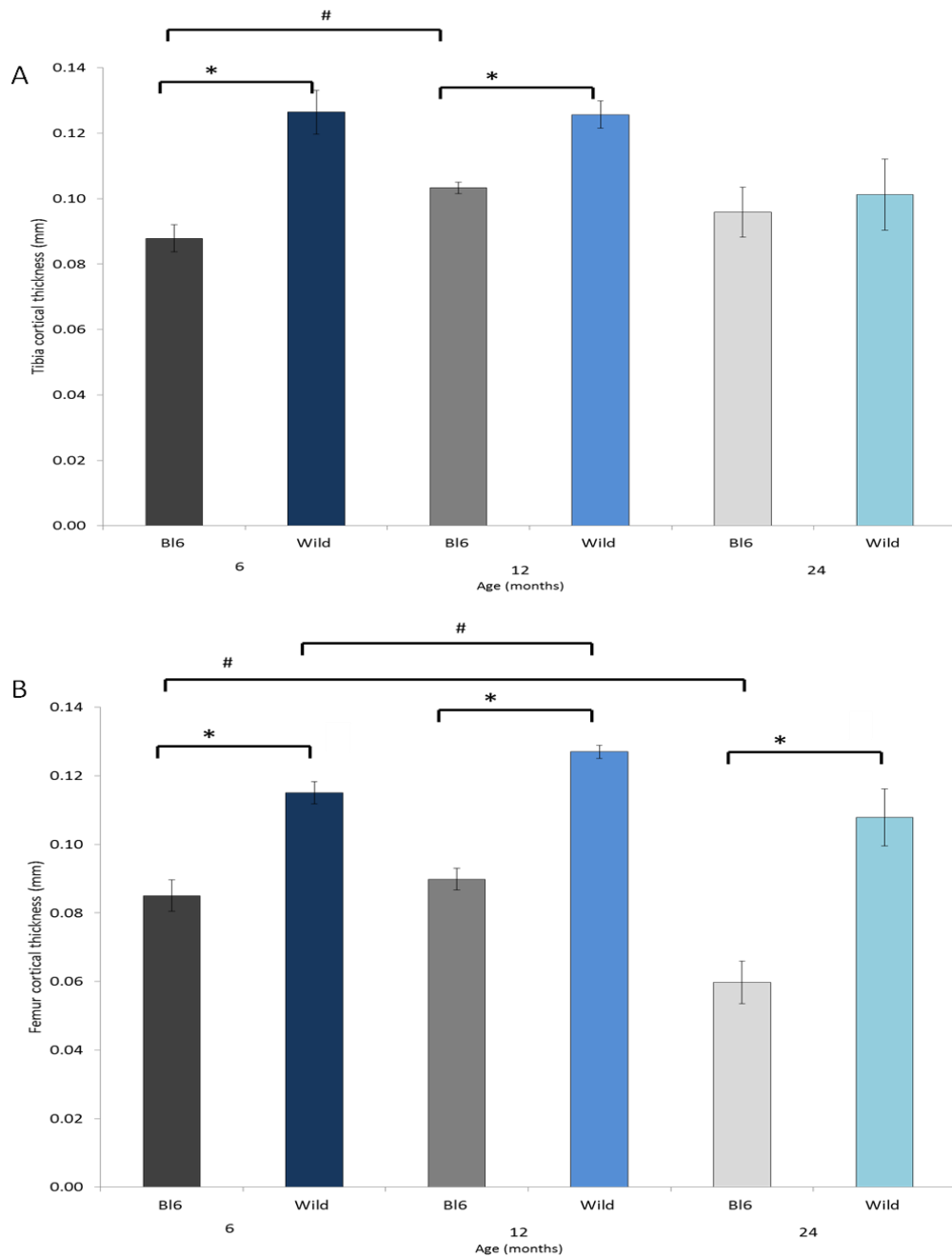
Fig 6.22 Mean cortical bone thickness values for tibia (A) and femur (B) at different ages in wild and C57Bl/6 male mice (Data presented as mean +/- SEM, n=6) * $p < 0.05$ compared with data from age matched C57Bl/6 mice, # $p < 0.05$ compared with cohort matched 6 month old values.

The wild male mice values for cortical bone thickness were significantly decreased compared with the C57Bl/6 mouse at 6 months of age in both bones. There was no age related change in the C57Bl/6 mice. There was an age-related decrease in the wild mice in the tibia only.
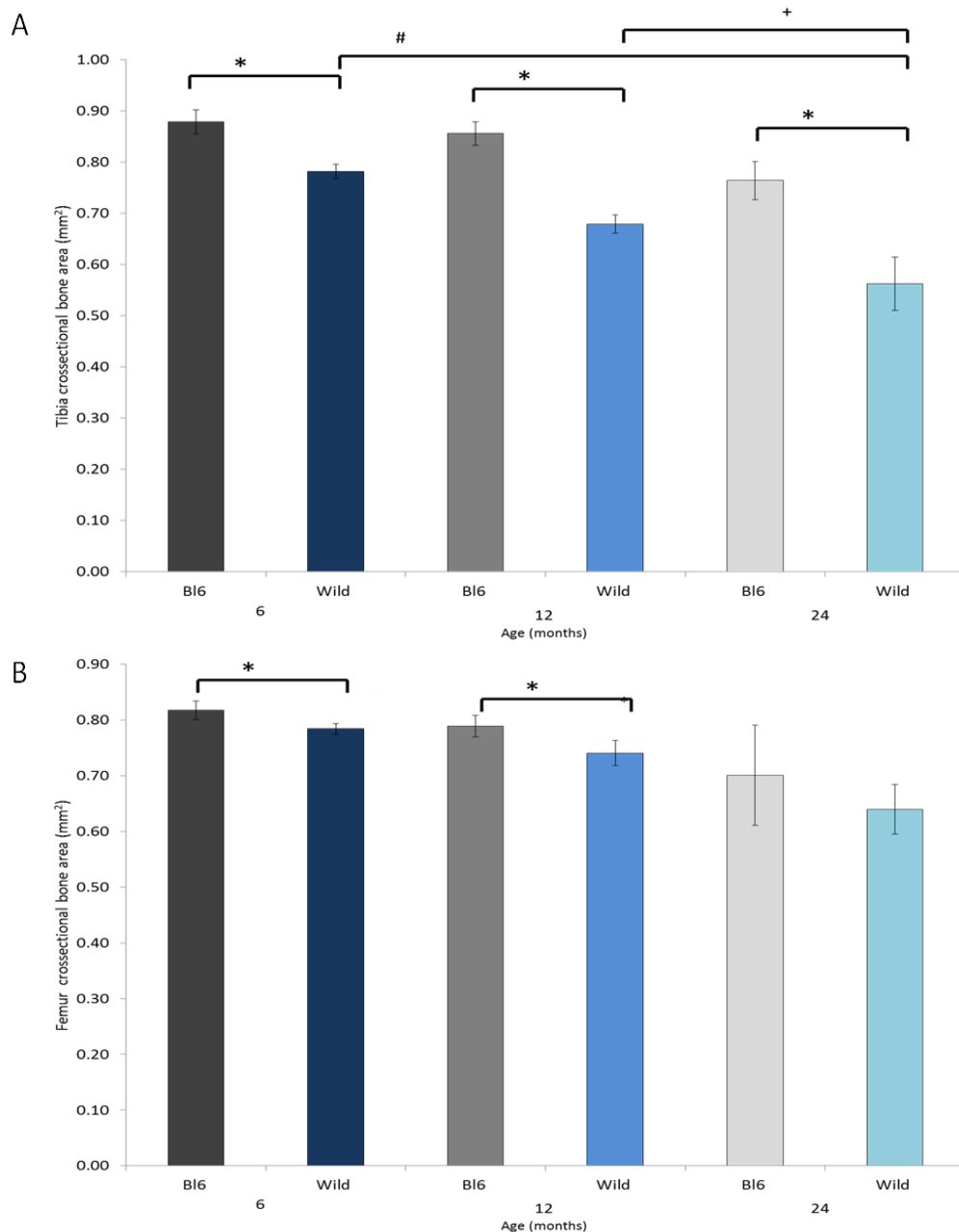
Fig 6.23 Mean cross-sectional area values for tibia (A) and femur (B) at different ages in wild and C57Bl/6 male mice (Data presented as mean +/- SEM, n=6) * p <0.05 compared with age matched C57Bl/6 values, # p <0.05 compared with cohort matched 6 month old values, + p <0.05 compared with cohort matched 12 month values.

The tibia trabecular bone surface density was significantly reduced in wild mice compared with that of C57Bl/6 mice at 6 months. The tibia and femur bone surface density decreased with increasing age although the decline in wild mice was considerably more dramatic.
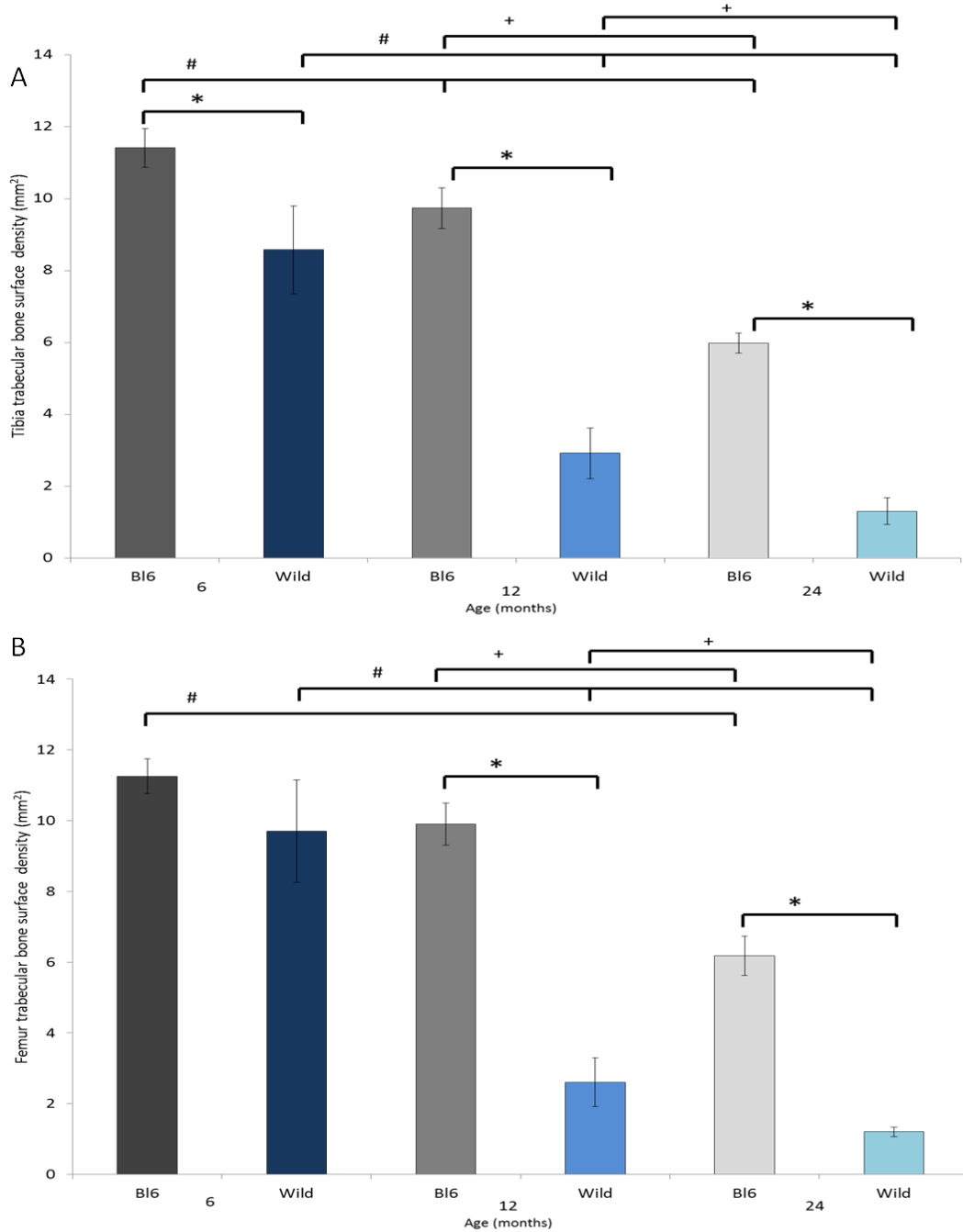
Fig 6.24 Mean bone surface density values for tibia (A) and femur (B) at different ages in wild and C57Bl/6 male mice (Data presented as mean +/- SEM, n=6) * p <0.05 compared with age matched C57Bl/6 values, # p <0.05 compared with cohort matched 6 month old values, + p <0.05 compared with cohort matched 12 month values.

The tibia trabecular bone surface density was significantly reduced in wild mice compared with that of C57Bl/6 mice at 6 months. The tibia and femur bone surface density decreased with increasing age although the decline in wild mice was considerably more dramatic.
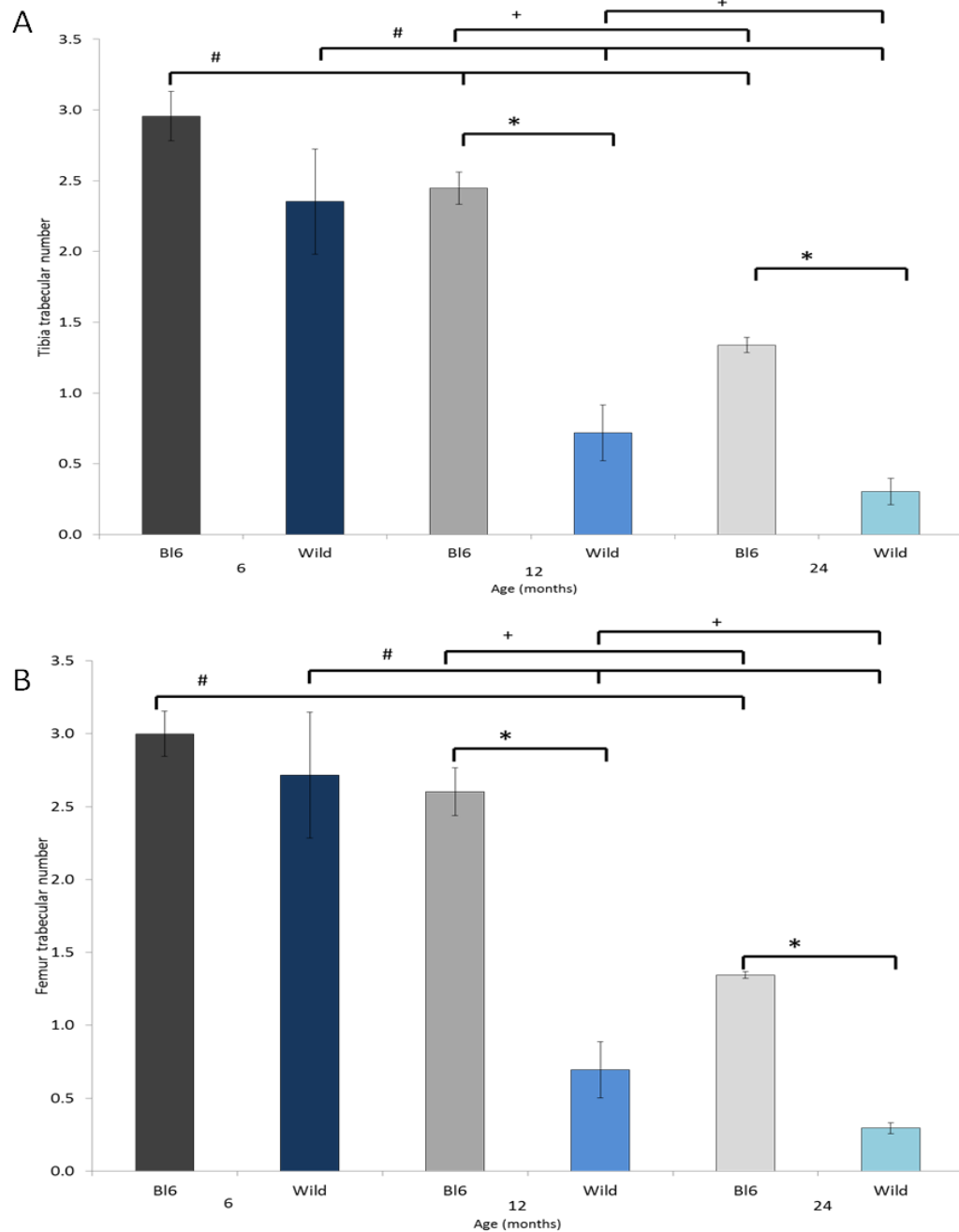
Fig 6.25 Mean trabecular number values for tibia (A) and femur (B at different ages in wild and C57Bl/6 male mice (Data presented as mean +/- SEM, n=6) * p <0.05 compared with age matched C57Bl/6 values, # p <0.05 compared with cohort matched 6 month old values, + p <0.05 compared with cohort matched 12 month values.

The trabecular bone number was not significantly reduced in wild mice compared with that of C57Bl/6 mice at 6 months. The tibia and femur bone number decreased with increasing age although the decline in wild mice was considerably more dramatic.

A

C57 Bl6	Tibia trabecular number	% Change
6 Months	2.67	22.22
12 Months	2.08	SEM ± 2.7
12 Months	2.08	38.55
24 Months	1.28	SEM ± 5.6

Wild male	Tibia trabecular number	% Change
6 Months	2.35	69.40
12 Months	0.72	SEM ± 12.8
12 Months	0.72	57.87
24 Months	0.30	SEM ± 27.7

B

C57 Bl6	Femur trabecular number	% Change
6 Months	2.78	11.31
12 Months	2.46	SEM ± 3.0
12 Months	2.46	53.14
24 Months	1.15	SEM ± 5.3

Wild male	Femur trabecular number	% Change
6 Months	2.72	74.40
12 Months	0.70	SEM ± 9.9
12 Months	0.70	57.71
24 Months	0.29	SEM ± 14.5

C

C57 Bl6	Tibia trabecular number	% Change
6 Months	2.67	50.40
24 Months	2.08	SEM ± 4.7
	Femur trabecular number	
6 Months	2.78	58.44
24 Months	1.15	SEM ± 4.2

Wild male	Tibia trabecular number	% Change
6 Months	2.35	87.11
24 Months	0.30	SEM ± 3.4
	Femur trabecular number	
6 Months	2.72	89.17
24 Months	0.29	SEM ± 2.8

Table 6.9 Percentage change of trabecular number at chosen age points in both tibia (A) and femur (B) bone and at different ages (C) in C57Bl/6 and wild male mice.

A	C57 Bl6	Gastrocnemius weight	Femur trabecular number	Pearson
	6 Months	0.17	2.78	
	12 Months	0.16	2.46	**0.97**
	24 Months	0.14	1.15	

	C57 Bl6	Anterior tibialis weight	Tibia trabecular number	Pearson
	6 Months	0.06	2.67	
	12 Months	0.06	2.08	**0.86**
	24 Months	0.05	1.28	

B	C57 Bl6	Gastrocnemius weight	Femur cortical bone thickness	Pearson
	6 Months	0.17	0.13	
	12 Months	0.16	0.13	**0.92**
	24 Months	0.14	0.09	

	C57 Bl6	Anterior tibialis weight	Tibia cortical bone thickness	Pearson
	6 Months	0.06	0.13	
	12 Months	0.06	0.13	**1.00**
	24 Months	0.05	0.12	

C	Wild male	Gastrocnemius weight	Femur trabecular number	Pearson
	6 Months	0.13	2.72	
	12 Months	0.13	0.70	**0.63**
	24 Months	0.11	0.29	

	Wild male	Anterior tibialis weight	Tibia trabecular number	Pearson
	6 Months	0.04	2.35	
	12 Months	0.04	0.72	**0.66**
	24 Months	0.03	0.30	

D	Wild male	Gastrocnemius weight	Femur cortical bone thickness	Pearson
	6 Months	0.13	0.13	
	12 Months	0.13	0.13	**1.00**
	24 Months	0.11	0.10	

	Wild male	Anterior tibialis weight	Tibia cortical bone thickness	Pearson
	6 Months	0.04	0.12	
	12 Months	0.04	0.13	**0.87**
	24 Months	0.03	0.11	

Table 6.10 Pearson correlation calculations comparing the cortical bone thickness and trabecular number and the attached muscle mass in C57Bl/6 (A,B) and wild male mice (C,D).

There were close correlations between loss of muscle mass and loss of trabecular bone in the C57Bl/6 mice. This was not seen in the wild male mice.
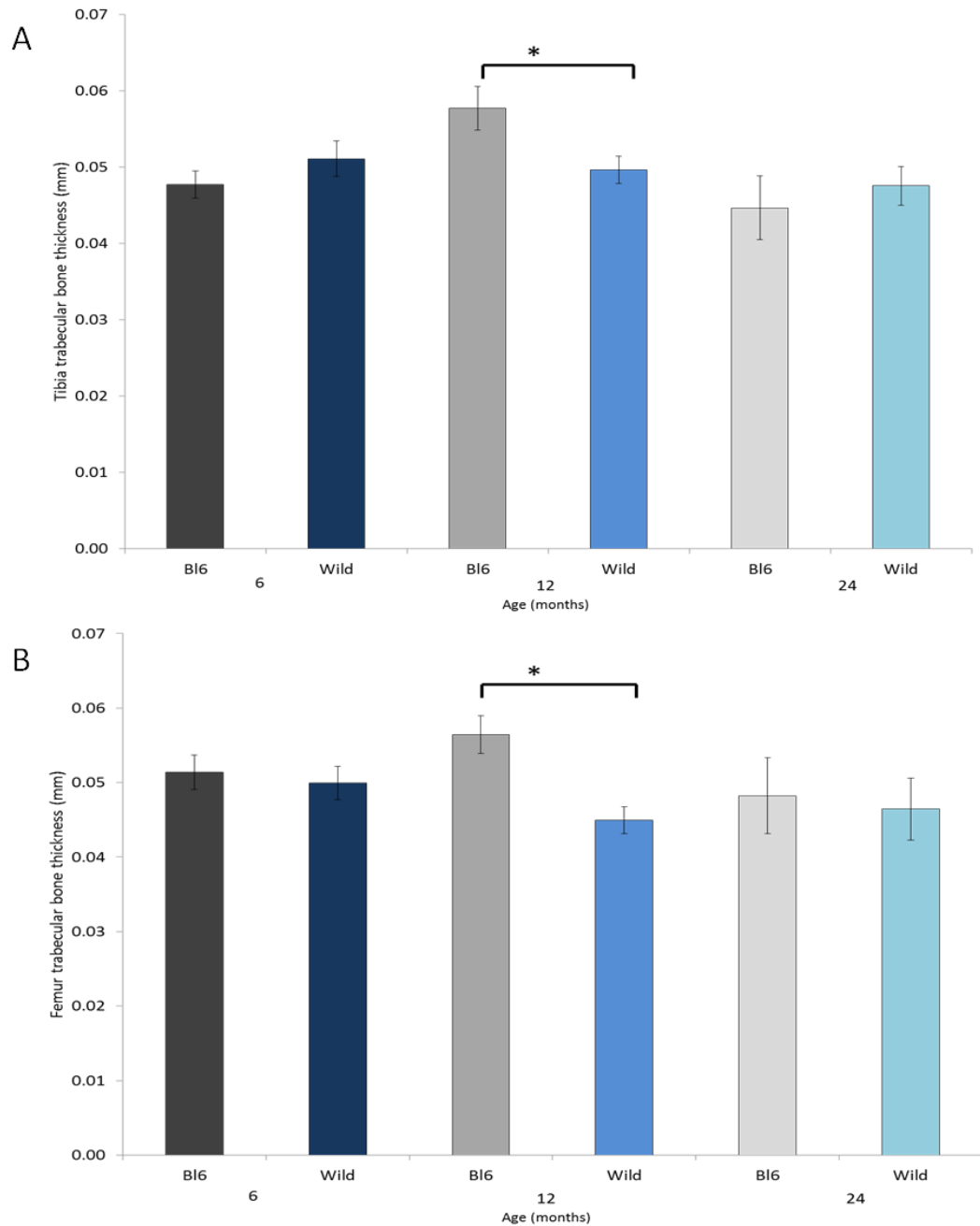
Fig 6.26 Changes in trabecular bone thickness in the tibia (A) and femur (B) at different ages in wild and C57Bl/6 male mice (Data presented as mean +/- SEM, n=6) * p <0.05 compared with age matched C57Bl/6 values.

Minor differences in trabecular bone thickness were seen when comparing to C57Bl/6 mice. Trabecular bone thickness was significantly reduced in wild male mice compared with C57Bl/6 mice at 12 months old at both sites measured. No other differences were noted between species or with advancing age.
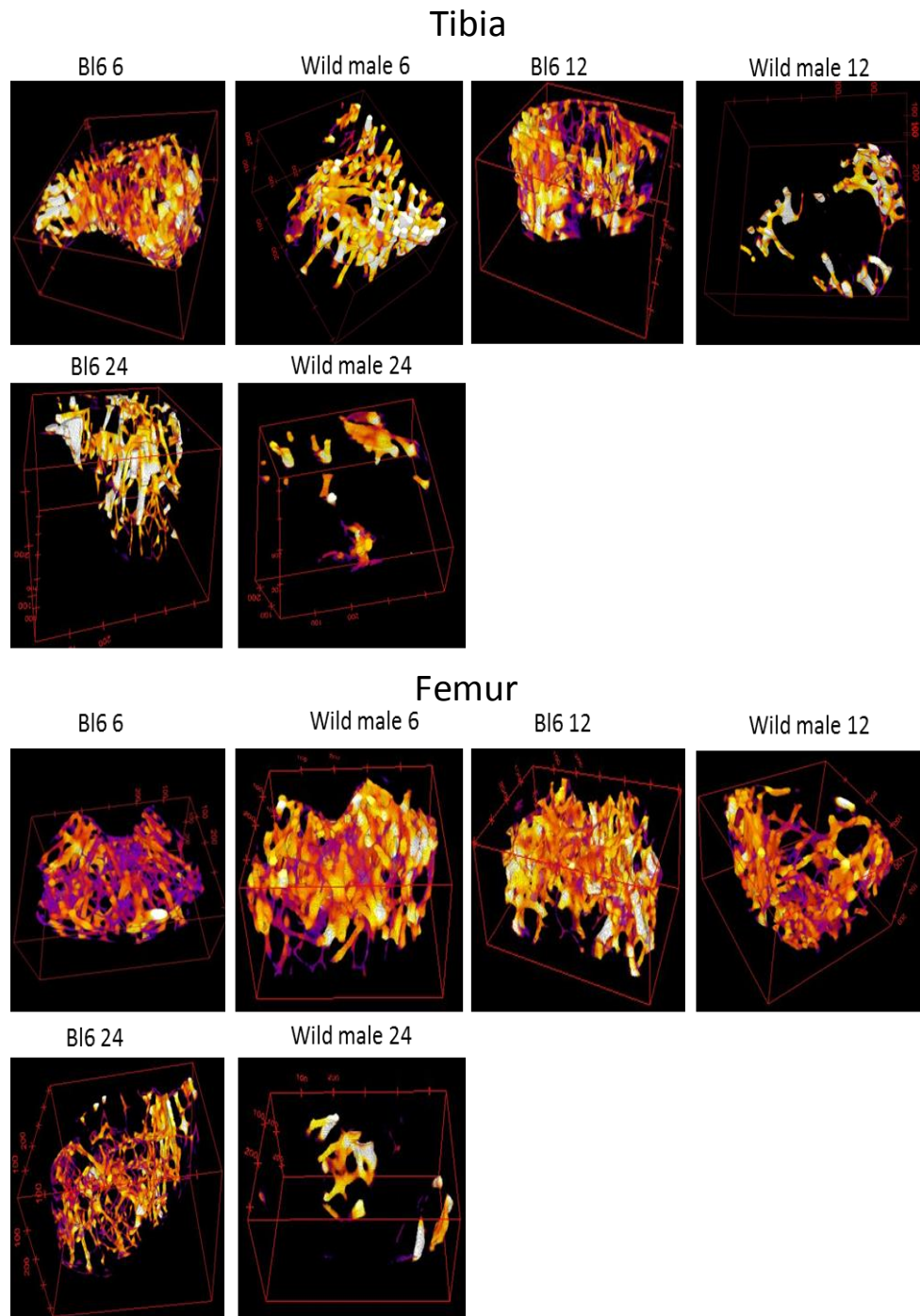
Fig 6.27 3D representation of trabecular bone thickness through age in the tibia and femur of C57Bl/6 and wild male mice. White colours represent the thickest points, purple colours representing the thinnest areas. Scale is specific to each individual bone based of mean and SEM as such, no key is present.

There were visual decreases in trabecular thicknesses between 6 and 24 months of age in both strains. The loss of trabecular bone was also evident.

6.5 Discussion

6.5.1 Comparison of age-related changes between male and female wild mice

As seen in Figure 4.1, female wild mice were generally significantly smaller in body mass at both 6 and 12 months of age however this significance was removed at later ages. This loss of significance was due to females maintaining body mass with increasing age whereas the males lost body mass with increasing age.

The effect on body mass is possibly partly due to differences in organ mass shown in Table 4.1 which showed at 6 and 12 months of age all organs were smaller in the female mice compared with the males. At 24 months, females had significantly larger brain and lung tissues. This effect remained evident when removing body mass as a factor (Table 4.2). The effect was due to the male mice losing tissue mass by this age. This would suggest that the female mice preserve tissue mass better than males with increasing age.

The hind limb muscles of the female mice at both 6 and 12 months were smaller than the male counterpart (Table 4.4). When the body mass differences were accounted for, the *anterior tibialis*, EDL and *soleus* muscle were only smaller at 6 months old (Table 4.4) with no significant differences from that point on. This would suggest that females were able to adapt to ageing and were able to preserve muscle mass to a greater extent than the males.

Mechanisms responsible for this are not understood. However, in most species studied females generally out-live the male counterpart and may therefore be primed to maintain tissue integrity for longer than the male (1999, Borras et al., 2003). One explanation may be due to the absence of breeding within the groups. A study published in 1967 examined lifespan in rats which had undergone differential breeding patterns, castration or no breeding at all (Asdell et al., 1967). This study clearly showed that females that were never bred lived much longer and conversely males that were never bred lived the shortest (Asdell et al., 1967). This study also noted the benefit of oestrogen on life span where both males and females lived longer if supplemented with oestrogen, an effect which has been show by other groups (Vina et al., 2005). It was therefore hypothesised that females that never breed have higher than normal levels of oestrogen (Asdell et

al., 1967), despite females already having a higher level of oestrogen compared with males (Goodman-Gruen and Barrett-Connor, 2000). Despite the mouse undergoing reproductive senescence between 9 and 12 months of age, the lack of breeding may also be preventing the onset of menopause within these animals, thus maintaining the production of female hormones (Mobbs et al., 1984, Bellino, 2000).

Oestrogen is classed as an 'antioxidant' and is capable of up-regulating longevity genes as well as dampening of harmful inflammatory chemicals (Kendall and Eston, 2002, Vina et al., 2005, Vina et al., 2006). However the low levels seen in circulation show that they are not chemical antioxidants but works through the oestrogen receptor which can in turn up regulate antioxidant enzymes within a given tissue (Vina et al., 2013).

Muscle deterioration and preservation in particular have been linked with oestrogen and other variants such as estradiol (Maltais et al., 2009, Moran et al., 2007). A lack of estradiol has been shown to significantly reduced muscle force generation which can be recovered with hormone replacement (Moran et al., 2007). This link between female sex hormones and muscle preservation as well as their role in improving antioxidant enzyme levels may explain why there is a lack of change in muscle tissue and even organ tissues with increasing age in this population of female wild mice. When examining the male mice, these mice lose male sex hormone levels with increasing age. The loss of these hormones is clearly linked with loss of muscle mass and force, again suggesting a role for sex hormones in muscle deterioration and preservation. It is therefore apparent that the wild male mice are undergoing a normal loss of muscle mass with increasing age and that the female wild mice are being preserved.

Both sexes displayed large disruption of the knee joint with increasing age (Figure 4.2-4.4). However there were no gross differences between the sexes. This may have been due to the low n numbers for analysis; however the severity of the disruption indicates this was a natural phenomenon within the wild mouse population. The disruption of the joints would suggest the underlying bone was also affected. As discussed previously, there were severe age-related effects on

the bone in the males and this was seen, although at a lesser extent in the female wild mice.

Although the females do show a reduced bone structure in a number of measures, they are often comparable if not significantly higher than age-matched male mice. The female mice also show a lack of age-related decline in the cortical bone in particular and significant but minor decreases in trabecular bone measures with increased age. It is more striking when compared with the male counterparts where hugely significant bone loss is seen.

This potential preservation may also be due to maintained levels of female sex hormones into later life. Oestrogen and others are known to be key in maintenance of bone mass and is often prescribed for cases of osteoporosis.

If we assume that these females are maintaining the levels of female sex hormones into later life (possibly due to a lack of breeding) then muscle mass and possibly bone mass may be being maintained. This scenario is similar to humans undergoing hormone replacement therapy. These patients have significantly stronger muscles than those not receiving the therapy and also display a higher bone mass.

6.5.2 Age-related changes in the wild male mouse compared with a laboratory strain mouse

In general, the wild male mice were significantly smaller in body mass then the C57Bl/6 lab strain (Figure 4.17), had smaller organs even when body mass was removed as a compounding factor and had smaller muscle mass. However both groups display age-related decreases in body and tissue mass, the wild male mice show this to a greater extent.

This would suggest that the laboratory grown mice are bigger in body mass and this appeared to be due to larger tissue mass. As the wild mice were more active, the fact they have smaller muscle masses compared with a sedentary laboratory strain would show that the activity was not a major factor in the muscle mass of the wild mice. However it could be that the muscles were leaner with less fatty deposits and were therefore healthier, but this was not examined in this study.

Cortical bone thickness was significantly higher in the wild male mice at both 6 and 12 months of age compared with the C57Bl/6 mouse (Figure 4.21). However the CSA is smaller than the C57Bl/6 mouse at every age (Figure 4.22). The trabecular bone of wild male mice showed dramatic reductions in number of bones at 12 and 24 months of age compared with the C57Bl/6 mouse suggesting that in terms of bone health, 12 months old is deemed very old age in the wild mice (Figure 4.23-4.25).

When examining the association with the muscle mass and number of trabecular bone, a close association between these two tissues in the C57Bl/6 mice was seen, but there was only a poor correlation in the wild male mice (Figure 4.26). This would indicate that another factor other than muscle mass and strength may be causing the dramatic reductions in trabecular bone number in wild mice. This factor is currently unknown. As mentioned previously, the hormone levels may be different between the groups but this could also be due to how the mice were housed, the genetic background and the presence of pathogens in the wild mice group and would all have an effect on the musculoskeletal health of these wild mice. Further indepth investigations must be undertaken to truly elucidate the reasons behind this abnormal decline in bone health compared to the C57Bl/6 mice.

Following on from the dramatic changes seen in the bones of wild male mice compared with the C57Bl/6 mice, there were significant gross age-related differences seen in the joint. This included loss of ligament and meniscus tissues, loss of cartilage tissue and the presence of cartilage lesions and the formation of osteophytes, none of these were present in the laboratory strain C57Bl/6 mouse, even at older ages.

This would suggest that the quadrupedal locomotion of mice was not the reason C57Bl/6 mice do not naturally develop osteoarthritis or show age-related disruption to the joints. The appearance of naturally occurring joint disruption in the wild mice suggests that joint protection in the C57Bl/6 mice was a consequence of either the inbreeding of strains, or the environment the laboratory mice compared with the wild mice.

6.6 Conclusions

The aim of this study was to examine a population of mice that have increased activity levels compared with laboratory strains. The predicted outcome was that increased activity would lead to increased muscle mass and improve bone values, ultimately leading to preservation of musculoskeletal tissues into old age.

- Female wild mice which appeared to have prolonged muscle mass until a much later age then was measured in the C57Bl/6 cohort but still showed loss with increasing age.
- Wild male mice appeared to be poorer in terms of musculoskeletal health compared to the female mice.
- Prolonged production of female sex hormones in the wild female mice due to lack of breeding may be playing a protective role on musculoskeletal tissues and may explain the lack of age-related change seen.
- One theory explaining why these wild mice did not age as well as the laboratory based strain C57Bl/6 may be due to the artificial lengthening of a wild mouse lifespan. If in the wild, these mice would only live a maximum of 12 months old, then it is reasonable to suggest that the wild mice are not genetically capable of ageing in the same way the inbred laboratory strains are, and will therefore degenerate rapidly after this point.
- The strength this cohort of mice has over the lab based strain C57Bl/6 is that they do appear to naturally develop osteoarthritis, a condition not present in the C57Bl/6 mice.

These mice provide a model to determine how 'healthy' murine ageing occurs normally, with our genetically identical laboratory strains being the abnormal ageing group. Normal ageing of a wild population of mice has been shown here, the increased activity that was initially the aim of the study should instead be considered normal activity levels.

Laboratory strains of mice are commonly used, but these may now be masking the normal ageing process for the mouse itself, therefore, any interventions and drug treatments that have affects in this cohorts (positively or negatively) may not represent a valuable result and may need to be displayed in a number of stains or

species before truly representable. Conversely, attention should be focused on laboratory bred strains of mice as successfully ageing animals, determining why they doing so.

Chapter 7

General discussion and future directions

7.1 General Discussion

The main aim of this thesis was to determine how musculoskeletal tissues age and to integrate interventions targeted at a specific musculoskeletal tissue, multiple musculoskeletal tissues or the whole body to alter age-related changes in musculoskeletal tissues. Initially, the development of a reliable and expansive model to measure age-related changes in all musculoskeletal tissues was required which then informed our approach to any possible interventions.

This multidisciplined approach to identifying age-related changes in musculoskeletal tissues has significant advantages through the use of *in vivo*, *ex vivo* and *in silico* methods, as well as interactions with engineers, computer scientists, behavioural biologists alongside cell biologists and musculoskeletal biologists to give, for the first time, a detailed representation of how musculoskeletal tissues age, and of the effects of possible treatments to improve the age-related declines that for so long have seemed inevitable.

The main hypothesis of this thesis is that with increasing age in mice, there is a decline in the mass and function of all musculoskeletal tissues. The secondary hypothesis is that this decline could be prevented through a series of interventions including increased lifelong activity, a whole body over-expression of Heat Shock Protein 10 (HSP10) or an increase in dietary nitrates.

As stated in 1.7 this aims of this study were as follows:

- To integrate methods of visualising and measuring age-related changes in musculoskeletal tissues.
- To identify changes in musculoskeletal tissues throughout the life span of C57BL/6 mice.
- To determine the effects of increased sodium nitrate intervention, both short-term and longer-term, on changes seen in the musculoskeletal system of C57Bl/6 mice with age.
- To examine the effect of lifelong overexpression of HSP10, known to preserve muscle CSA on other tissues from the musculoskeletal system.

- To compare data from C57Bl/6 mice with changes in musculoskeletal tissues of male and female wild mice which demonstrate a significant increase in activity

The first aim was addressed throughout this thesis as a number of practical and analytical methods were implemented to allow for a full analysis of musculoskeletal tissue ageing. These included identifying specific muscles and organs to reliably dissect and measure. Muscle force testing was updated and used here to gauge changes in muscle force. Through collaboration with experts in engineering we developed testing protocols for mouse tendon and a number of novel methods of measuring bone and cartilage have been used throughout.

The second aim was achieved and detailed in Chapter 3 were it were we pin pointed the specific age in mice where the decline in muscle and bone occurred. We were unable to successfully measure tendon changes (discussed further) and there were no changes in the joint cartilage to report. The methods created for the first aim were brought through to answer the third aim and measure the effect of both long and short term treatment of mice with sodium nitrate in the drinking water. As seen in Chapter 4, this had a surprising affect as there was no difference when treated from 9 months of age until 24 months old, but when treated from 18 months it had a deleterious effect on the muscle force production indicating this treatment is unsuitable to reverse the ageing process in musculoskeletal tissues.

Aim 4 was addressed in Chapter 5 following the unsuccessful dietary intervention. Effect of overexpressing HSP10 on muscle tissue has been reported previously and the results here support this. The novel finding from this was that preserving the muscle mass had no effect on the underlying bone tissue and led to the theory that both tissues may age independently from each other. Following surprising results in the previous chapters, we decided to use a different mouse strain to see if lab based strains of mice are protected from ageing to a greater extent then what is deemed normal for the species. A colony of wild bred mice was used in Chapter 6 and the methods gained through aim 1 were applied to show that indeed, lab based mice age slower than the wild mice.
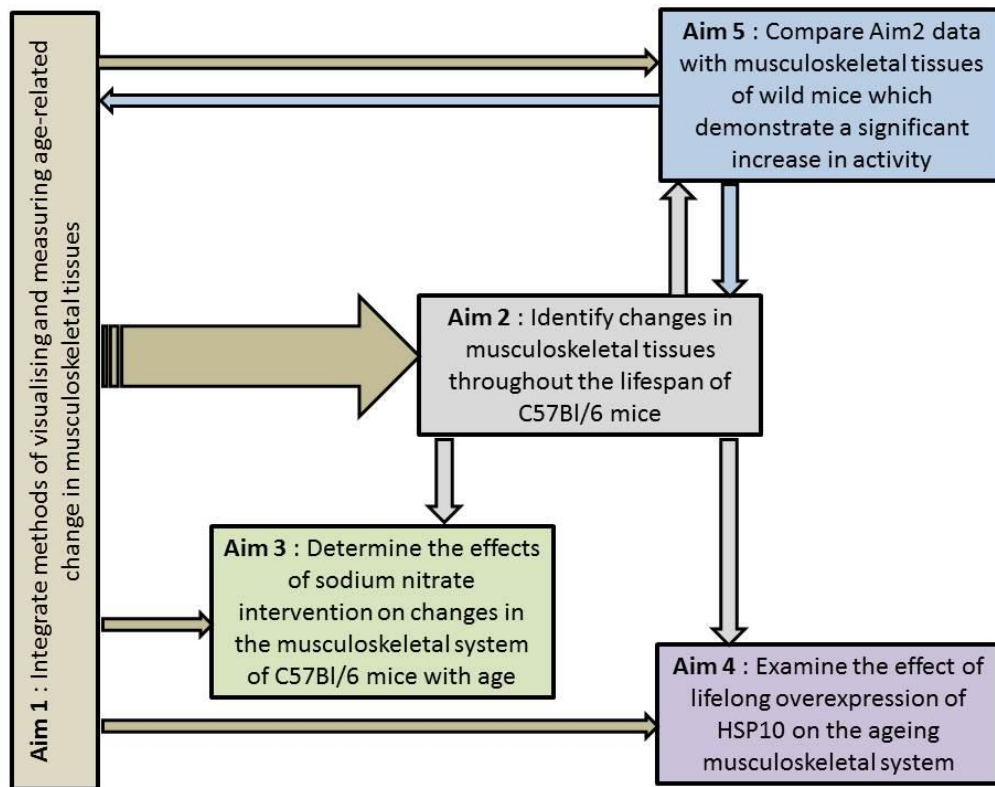
Fig 7.1 Schematic showing the flow of aims through the thesis and how they interact with each other. Aim 1 umbrellas all other aims and primarily feeds into Aim 2. Aim 2 directed the following aims. Aim 5 due to the nature of the findings led directly back to Aim 2 and potentially changes the outcomes of Aim 1.

7.2 Summary of major findings

7.2.1 Integration of methods to determine age-related changes in musculoskeletal tissues

It is possible to use a range of *in vivo*, *ex vivo* and *in silico* techniques to measure all musculoskeletal tissues through age. This study has integrated muscle force generation measured in vivo with muscle mass to show how muscle mass and muscle force changes through life. Cross-sectional analysis of muscle allowed for further analysis of the effect of age on individual muscle fibres. Tendon tissue can be dissected intact and analysed for both histological and mechanical characteristics to identify how changes in tendon size with age may impact on the mechanical forces. The joint and associated structures can be visualised and quantified and the joint can be stained for signs of osteoarthritis,

the presence of bony outgrowths and ligament morphology and cellular number can be quantified. MicroCT scanning of hindlimbs allowed for a large number of complex bone analyses to be undertaken. This gave an appreciation for the mechanical strength of both cortical and trabecular bone as well as detailing the age-related changes in bone. This allowed for a vast array of computational models to be produced for visualisation of these various measures in high resolution. The inclusion of recording body mass was necessary to eliminate age-related increases in body mass as a compounding factor when investigating changes in tissues, especially in the case of organ pathologies which may have had influences on the musculoskeletal tissues. Furthermore, the storing of blood for further analysis provides another element when considering the causes age-related changes measured in musculoskeletal tissues.

7.2.2 Changes in musculoskeletal tissues throughout the lifespan of the C57Bl/6 mouse

The muscles of the hindlimb undergo age-related loss of mass, although muscles were affected by age differently. As a general principle, the larger the muscle, the earlier age-related loss of mass was seen. The *gastrocnemius* muscle first showed significant loss of mass by 12 months of age which represent approximately 30-50 years of age in humans. The *anterior tibialis* was the next muscle to show age-related declines in mass at 24 months representing a human age of approximately 70 years of age. The remaining two muscles, the EDL and *soleus* displayed age-related loss of muscle mass at 28 months. This showed the need to study multiple muscles. When accounting for body mass as an influencing factor on muscle mass, the larger muscles again displayed age-related loss of muscle mass earlier with the *gastrocnemius* and *anterior tibialis* showing significant loss at 6 months of age although this may relate to muscle and organ remodelling between 3 and 6 months old. The remaining two muscles only showed significant declines at 28 months.

Tendons appear to be resistant to age-related changes. There were no significant changes in diameter or the cross-sectional area of the *posterior tibialis* tendon. However when measuring the ability of this tendon to handle mechanical force, at an older age, the tendon was unable to correctly manage higher stresses

and strains when applied indicating an age-related change in this index and a weaker tendon tissue. However, the inability to study other tendons was a limitation of this study

The knee joint of the C57Bl/6 mouse does not appear to undergo significant age-related changes. There was no indication of a loss of articular cartilage with increasing age. Although there did appear to be some minor reduction in cellular presence within the cartilage and ligament, as well as some disorganisation of the ligament structure, no gross alterations were seen.

There was clear evidence for age-related changes in the bone were seen in both cortical and trabecular bone compartments. There were some differential results between the tibia and femur bones indicating that external factors may be playing a role in bone ageing and again demonstrating the need to study more than one bone. Both bones measured showed significant increases in cortical bone thickness from 3 to 6 months of age which remained significantly high until 24 months of age. Both had significant declines in cortical bone thickness by 28 months of age. There was little significant change in bone cross-sectional area however until it decreased from 24 months of age indicating the cortical bone dictates the cross-sectional area. Trabecular bone values show significant age-related declines from 3 months of age including loss of trabecular bone number and bone surface density which have ramifications for the mechanical properties of the bone. Trabecular thickness appeared unaffected by increasing age but did increase significantly at 18 months only.

There was an association between muscle and bone tissues. There was a close correlation between the mass of the attached muscle and the cortical bone thickness of the underlying bone e.g. *gastrocnemius* mass change is closely correlated with femoral cortical bone thickness. There are also close correlations between muscle mass and trabecular bone number although the correlation is lower than with the cortical bone thickness.

7.2.3 Comparison of musculoskeletal ageing between C57Bl/6 laboratory mice and wild mice.

Wild male mice musculoskeletal tissues age differently to laboratory grown C57Bl/6 mice. The muscle mass data shows wild male mice muscles are significantly smaller then C57Bl/6 mice at 6 months of age and 24 months of age but muscle loss occurred in a similar pattern in both strains of mice although the percentage loss of mass appears greater in the wild mice between 12 and 24 months old

Wild male mice display age-related changes in both joint and ligament tissues with disintegration of the knee ligament and meniscus, removal of articular cartilage and bony growths. This would suggest wild male mice naturally develop knee osteoarthritis whereas the laboratory strain showed no evidence of such changes.

There were differential age-related changes between male and female wild mice. Female wild mice had significantly smaller muscle mass at 6 months old in all muscle measured, all muscles remained significantly smaller except *soleus* at 12 months and this remained in the *anterior tibialis* and *soleus* muscles at 24 months of age. When removing body mass as a factor, only the *anterior tibialis*, EDL and *soleus* muscles only remained smaller at 6 months of age suggesting that the major reason for smaller muscles was the reduced body mass.

Both sexes showed age-related disruption of the joint and signs of osteoarthritis to a similar extent.

7.2.4 Effect of muscle overexpression of HSP10 on musculoskeletal tissue structure

Overexpression of HSP10 in mice led to some preservation of muscle mass compared to C57Bl/6 mice. Absolute mass of anterior tibialis and gastrocnemius muscle was preserved in HSP10 overexpressor mice compared with wild type mice although this preservation was lost when accounting for body mass.

Bones of the HSP10 overexpressor mice displayed significantly larger cross-sectional area compared to C57Bl/6 mice at 6 months, with no significant differences at 24 months. In general terms, HSP10 overexpression in muscle had little effect on bone structure.

7.2.5 Effect of nitrate interventions on musculoskeletal tissue deterioration during ageing

Mice appear to tolerate both long and short-term interventions with 1mM sodium nitrate solution with no effects of death rates.

Long-term nitrate treatment can improve age-related muscle mass declines. Long-term nitrate treatment led to a significant increase in EDL mass, when accounting for body mass the *anterior tibialis*, EDL and *gastrocnemius* muscle masses were significantly increased.

Short-term nitrate treatment led to significantly smaller EDL compared to control. This remained even after accounting for body mass.

Neither intervention led to any major effects on cortical thickness or cross-sectional bone area. There were no significant changes in trabecular bone measures between control and long-term nitrate; short-term nitrate treatment increased trabecular number which seemed to be reflected in a larger number of smaller trabecular.

Both nitrate interventions increased heart and lung tissue weight.

7.3 Limitations of this study

7.3.1 The use of mice to study the effects of musculoskeletal ageing.

Since the sequencing of the C57Bl/6 mouse genome, it has been shown that 99% of mouse genes have human homologues (Mouse Genome Sequencing et al., 2002). There are many examples of mouse studies involving the alterations of genes/proteins to potentially resemble an ageing animal or human through accelerating the ageing process. In muscle studies this include a reduction of antioxidant enzymes level (Sakellariou et al., 2014b), deletion of maintenance proteins such as heat shock proteins (Kim et al., 2006), reduce the calorie intake (Sohal and Forster, 2014), which all aim to mimic, accelerate or delay ageing.

The mouse models used tend to be genetically identical mice which provide the most reliable information in a given genotype. This has the benefit of

reproducibility and allows for integration of data across experiments and time points (Threadgill et al., 2011) meaning the genetic pool of inbred laboratory strain mice has become smaller and smaller over time.

This study attempted to examine the effects of this lack of diversity with the inclusion of a cohort of wild mice, directly comparing the two strains. There were a number of differences between the two strains and it appears that the potential shorter life span of wild mice may be influential. This is important to note when trying to translate the findings from this study into future mouse ageing studies.

A further limitation of this study is studying a quadrupedal animal model, which is a change in locomotion compared to the bipedal human. This essentially results in body mass and muscle mass being evenly distributed amongst 4 limbs rather than two and may result in differing stresses and strains placed upon joints and bones. This may be an influential difference and a limitation to using such animal models.

Through the use of the C57Bl/6 mouse as our control group, we wanted to study ageing in a commonly used laboratory strain of mice. However these mice do not appear to naturally develop osteoarthritis, a common condition within the human population. This may be attributed to the quadrupedal movement. However, osteoarthritis does develop naturally in the wild mice. This shows that mice can develop the condition despite the differential locomotion and shows that as a control, C57Bl/6 mice may not be the ideal choice for this condition.

Ultimately despite these limitations, the positives to using animal models are outweigh the negative but caution is needed when focusing on one strain of animal, before applying findings directly to humans.

7.3.2 The use of male mice throughout this study

Although the effects of age on musculoskeletal tissues are basically the same between the sexes, the rate of development and presentation of each dysfunction can be widely different. One of the main causes of these differences is proposed to be the dimorphisms in size and body mass between males and females and the levels of sex hormones. The ideal future study would be to repeat the study in female mice.

One benefit of using solely male mice is to remove the influential effects of circulating hormones and how they change with age, especially in females. The onset of the menopause has direct consequences on skeletal health and is a key differing point in the ageing of musculoskeletal tissues between the sexes.

A study by Glatt et al. (2007) examined the age-related changes in bone of both male and female mice without surgical or pharmaceutical intervention (Glatt et al., 2007). The authors showed that changes in trabecular bone occur much earlier and to a greater extent in the female mice when compared with male. Females in general in this study displayed lower values for each bone measurements and showed a higher rate of decline with age (Glatt et al., 2007).

7.3.3 The ages at which measurements were taken, the choice against ageing a cohort continuously.

For this study, the start of testing was chosen as 3 months and end at 28 months. Although it would have been beneficial to study from birth to natural death, this was not possible for several reasons including time and financial restrictions. This meant that rapid changes seen in mice between 0-3 months were not measured and the study commenced at an age that equated to post puberty in humans. 28 months was chosen as an end point as this resembled 50% survived based on Jackson laboratory data and also avoided the development of unrelated pathologies developing such as tumours.

7.3.4 Examination of age-related changes in tendon structure and function.

The *posterior tibialis* tendon was chosen due to the ease at which it could be reproducibly dissected and analysed. The *Achilles* tendon was considered but in the mice this is very short and could not be used for functional studies.

There was a possibility that due to the tendon having such a small size, any structural or functional changes were too small to identify, in a similar manner to mass of the smaller hindlimb muscles.

Due to the small size of the tendons, stress-strain measurements may have been influenced directly by the grips causing unnatural strains. As the samples had to be glued in place, there was a risk of glue detrimentally affecting the tendon.

In conclusion, although this study has provided some useful information on tendon ageing, mouse tendons were not deemed suitable for the mechanical testing that was performed. In retrospect, structural analysis of other tendons would have been optimal.

7.3.5 Unfeasibility of gathering important data out of the base location

There are a few important pieces of data that have been unable to be recorded. These include the activity level of the wild mice, force measurements of wild mice muscles and direct measurement of dietary intake of mice under nitrate supplementation.

The activity levels were not measured due to how the cohort of wild mice were gifted to the study. There was not sufficient time to integrate an activity study prior to the need to cull the animals to gain the other data. As this cohort was not originally part of the thesis, the other studies had already been concluded and so there was no opportunity to measure activity in the other animals either.

Again, as these wild animals were housed at our facility in Leahurst and not the same location as the other animals in the thesis, the equipment used to measure muscle forces could not be transported.

Daily nitrate intake was also not measured although water bottles were weighed upon change over for the first month to gauge any animal welfare issues arising from sodium nitrate intervention. There were practical issues with availability of staff or researchers to do this process as well as a consideration of disrupting the mice excessively.

7.3.6 Wet-mass over dry-mass measurements

 Wet mass measurements were used as a standard throughout this thesis due to past experiences in this method and ease of process during periods of dissection. Dry mass measurements however are deemed much more accurate due to the removal of points of variability. For future work, dry-mass should be preferred when applicable to use.

7.3.7 Possible issues with choice of statistical analysis

One way ANOVA was used throughout this thesis. After discussions with people with more expertise in statistics, it was decided that for some of the measures, more appropriate statistical tests should have been applied in some of the analysis provided in this thesis. Although not integral to the overall results gained and the overriding conclusions, it may influence specific results and is therefore a limitation of this thesis.

7.4 Future directions

7.4.1 The inclusion of more musculoskeletal sites of interest

This study examined all of the major musculoskeletal tissues in the hindlimb of the mouse. A major site that was not included was the spine. Inclusion of spinal tissue would have been helpful to compare bone ageing within the spine, in a site of minimal stress and strain compared to the tibia and femur measured in this study as the spine is not primarily load bearing within these animals.

Despite this, spine samples were stored from each mouse and would be useful to use in a future study to ultimately compare bone loss between the spine and hindlimb with increasing age.

7.4.2 Investigation into the role of the nerve in musculoskeletal ageing.

There is a growing body of evidence that suggest a change in nervous tissue could be the trigger for age-related changes in the muscle and potentially for changes in the underlying joint and bone.

It would be advantageous to monitor age-related changes in nervous tissue. This analysis may give some insight into the mechanisms by which muscle mass is lost with increasing age and possibly why the bigger muscles lose mass at an earlier time point.

As the ability of muscle to generate force is lost, the underlying bone is likely to be affected and may lead to resorption of bone minerals, thus explaining the close association between muscle and bone.

7.4.3 The use of in silico modelling of ageing of the musculoskeletal system as a whole.

Finite element (FE) methods are computational methods which have been used since the 1940s. They are the chosen method for predicting how an object or material will perform when subjected to loads (Panagiotopoulou, 2009). A chosen object must be represented by a finite group of elements, each defined by a small number of nodes. It is the alteration or deformation of each element and associated node during an application of load that determines the placement of stresses and strains throughout the material being tested (Richmond et al., 2005, Rayfield, 2007).

Due to FE processing being able to represent highly complex structures in simpler parts, FE has been used in the study of bone tissue to show the stresses and strains through the whole or part of the bone via the muscle.

This has been shown in an earlier study aiming to display the effects of a reduction in muscle force on the underlying bone (Fig 7.2). Through this, it is possible to predict the areas of trabecular bone loss when muscle mass is reduced in this study the areas of significant loss of force appeared to be confined to the lower trabecular bones situated distally and away from the areas of high strain placed upon the cortical bone (Fig 7.2).
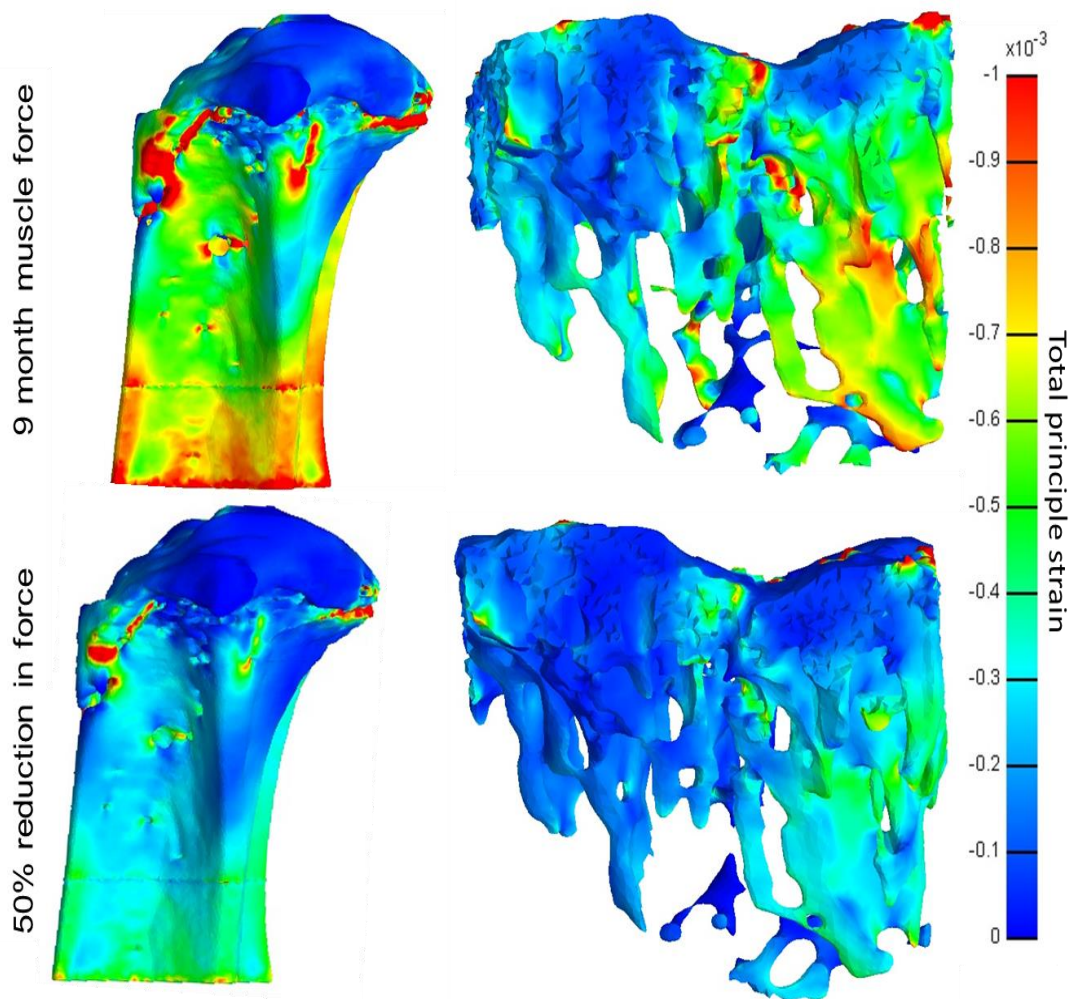
Fig 7.2 Finite element analysis of total principle strain applied to the tibia following application of force gained from 9 month muscles and following a 50% reduction in these forces. Areas of red indicate regions of high strain, regions of blue represent areas of low strain. Areas displaying large change under force reduction are predicted to be resorbed.

7.5 Conclusion

This thesis focused on how ageing in musculoskeletal tissues can be measured in the C57Bl/6 male mouse and by using these methods, can interventions be shown to improve the ageing process. Using the C57Bl/6 mouse, we can clearly see ageing occurring in the muscle and bone tissue however, it was difficult to measure changes in the tendon of these mice and they developed no age-related changes in the joint tissue. Intervention with sodium nitrate was ineffective at reversing the ageing process despite a large body of research reporting the

opposite. Overexpression of HSP10 in these mice did provide some protection against ageing in the muscle, but this protection did not apply to other musculoskeletal tissues. Due to these surprising results, we argued whether the C57Bl/6 mouse was the most suitable model to study musculoskeletal ageing. The same measures were applied to a cohort of wild mice which displayed ageing in all musculoskeletal tissues, which also occurred much earlier in their lifespan indicating that lab based strains of mice are actually ageing artificially compared to what is normal for the species. It is our conclusion that lab based strains of mice are not suitable for studying ageing processes due to the fact they are already "better" at ageing then normal wild mice.

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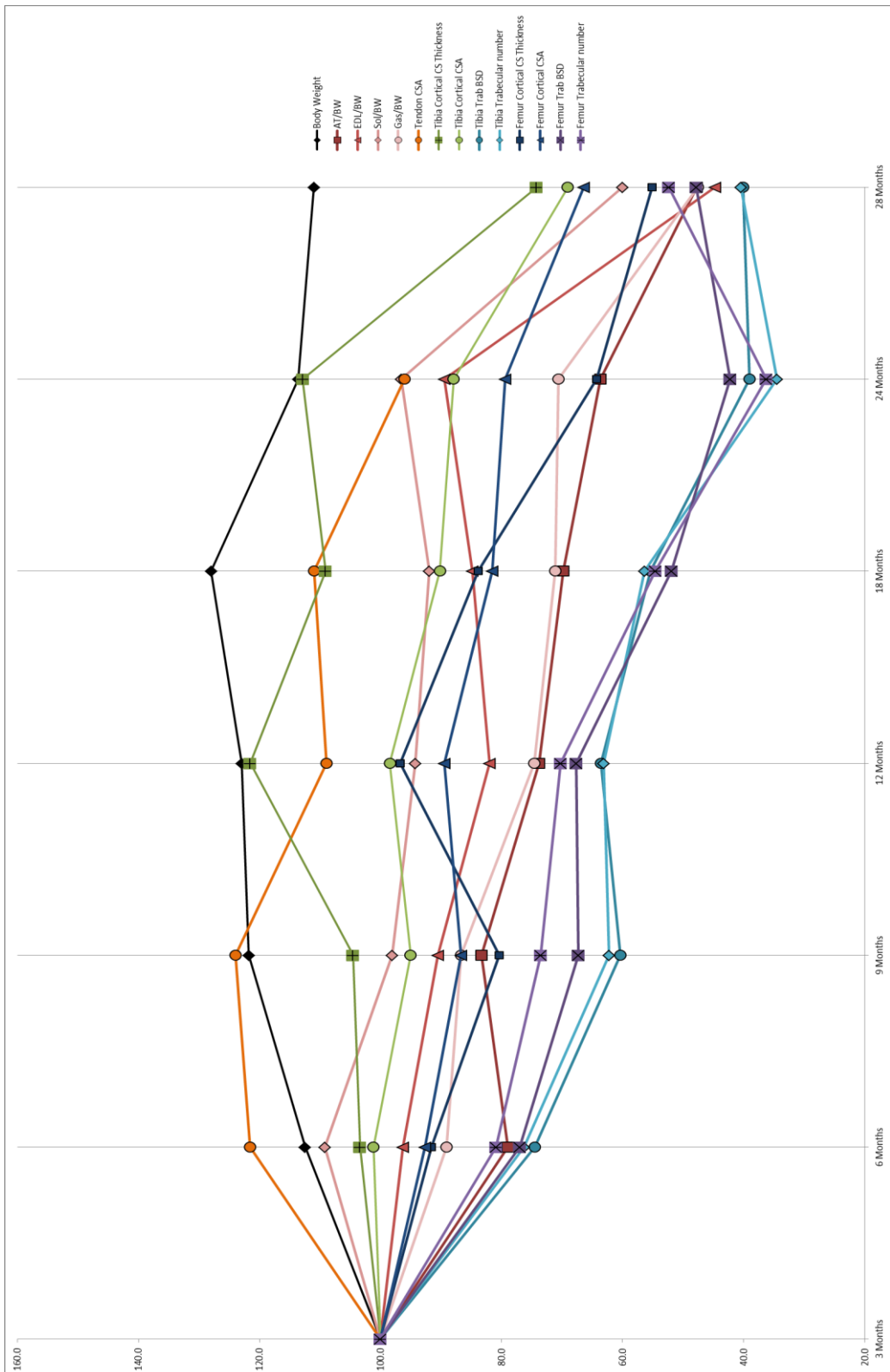
Fig SD.1. Percentage levels of measured tissues compared to 3 month values in C57Bl/6 mice.
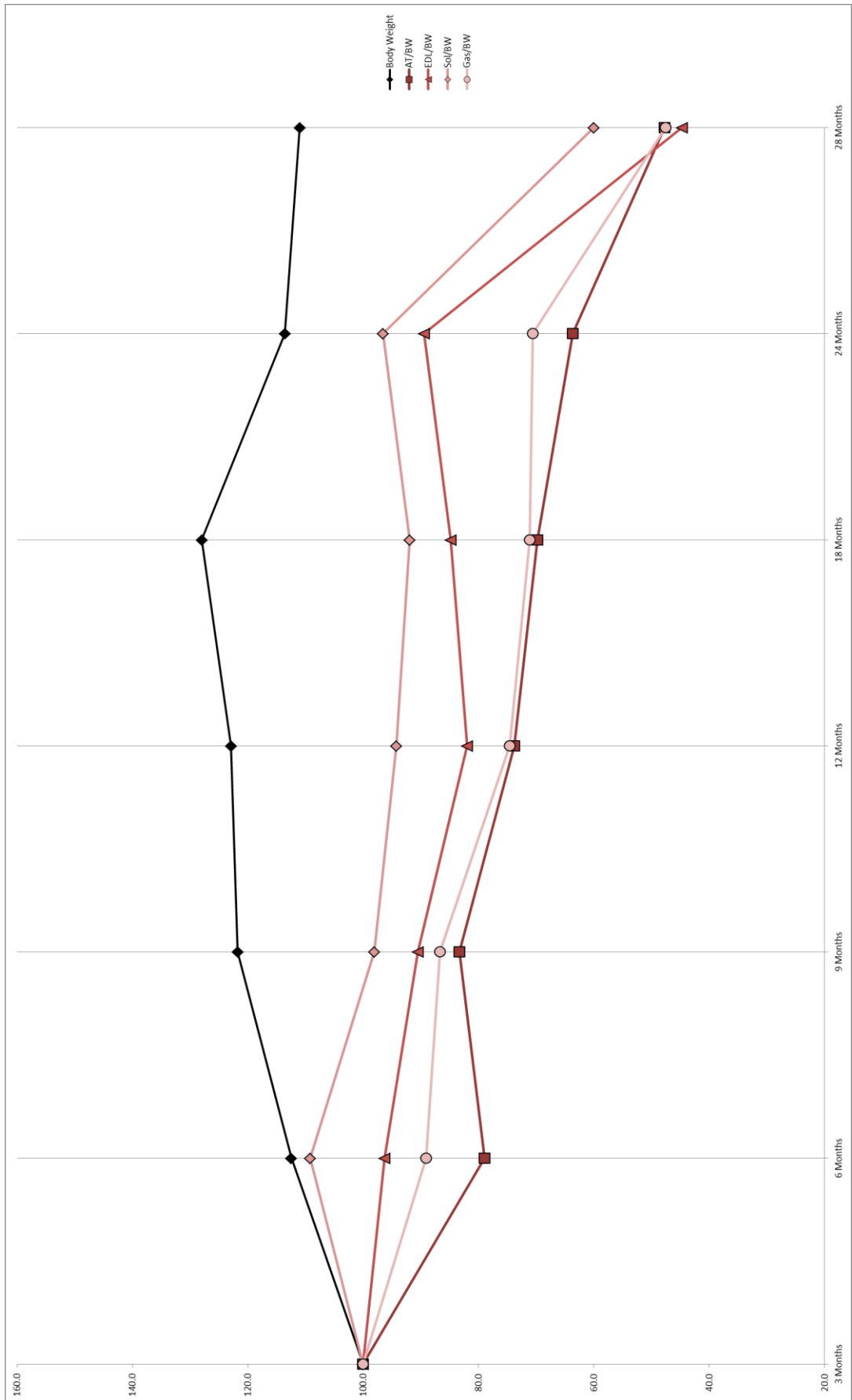
Fig SD.2. Percentage levels of body mass and muscle tissues compared to 3 month values in C57Bl/6 mice.
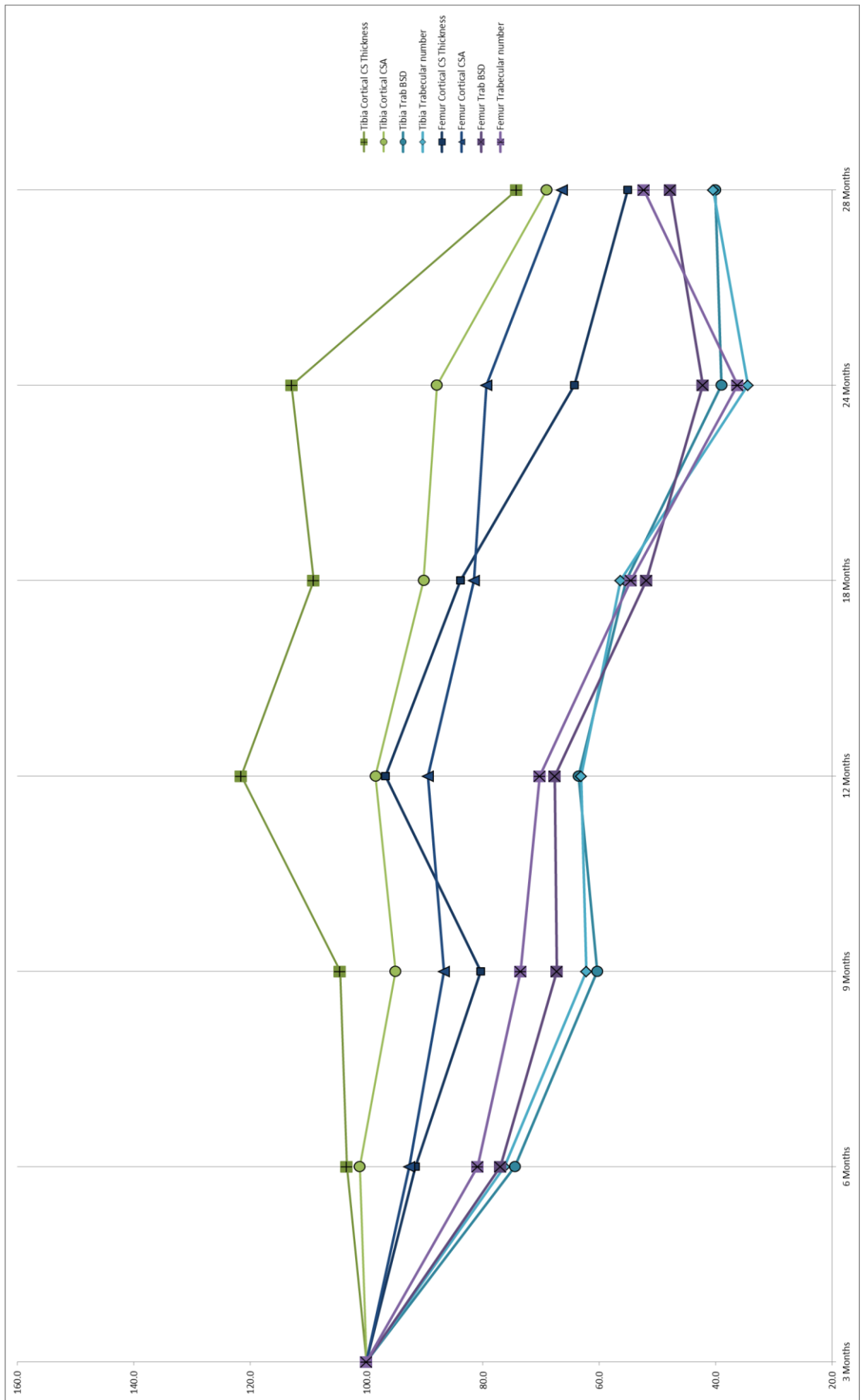
Fig SD.3. Percentage levels of bone measures compared to 3 month values in C57Bl/6 mice
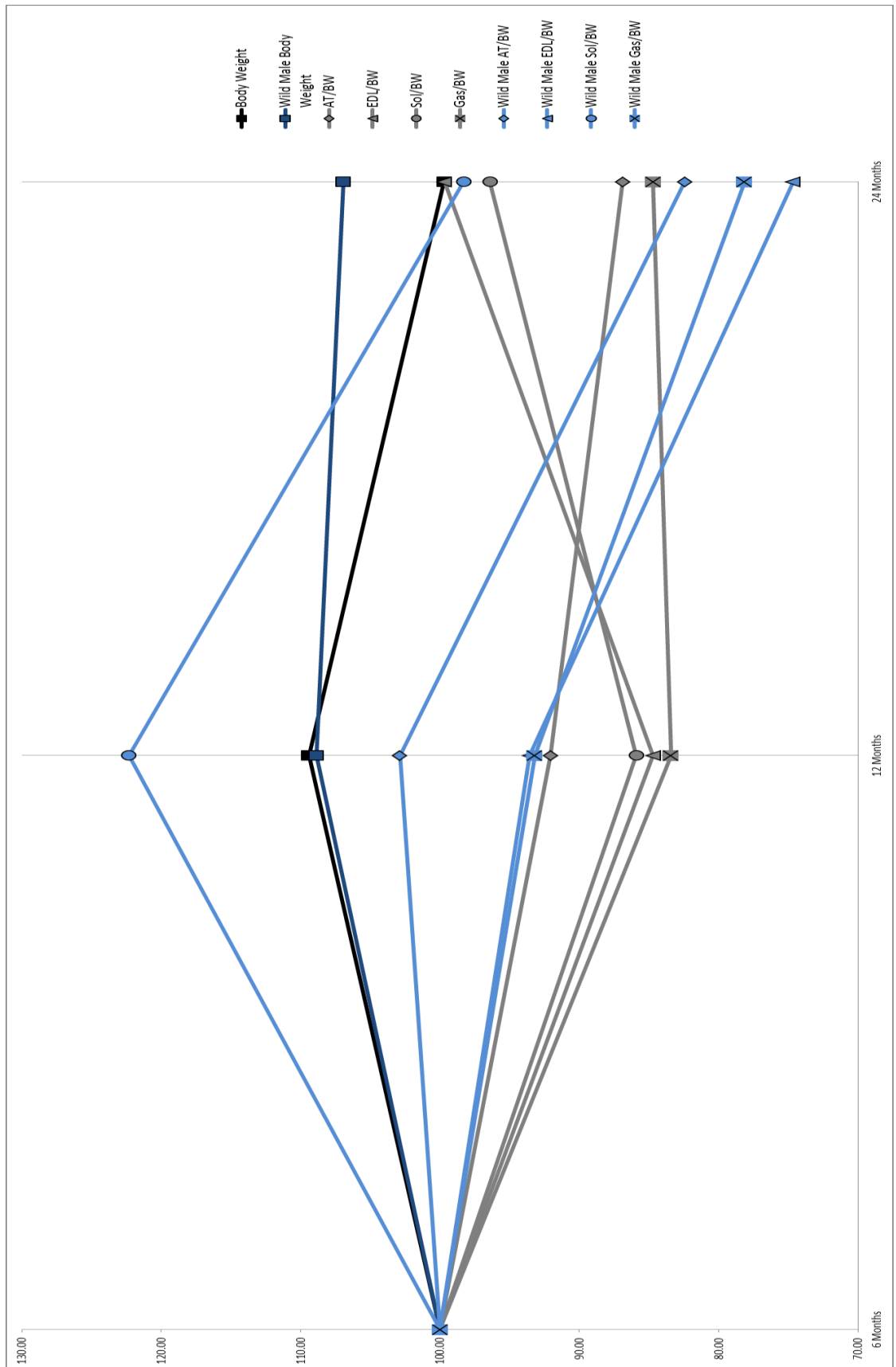
Fig SD.4. Percentage levels of body mass and muscle tissues compared to 6 month values in C57Bl/6 and wild male mice.

Fig SD.5. Percentage levels of cortical bone measures compared to 6 month values in C57Bl/6 and wild male mice.

Fig SD.6. Percentage levels of trabecular bone measures compared to 6 month values in C57Bl/6 and wild male mice.

Tibia cortical — Part 1

Age	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD
3 months	0.19	0.18	98.80	5.54	5.95	4.87	32.52	32.12	1.08	1.18	4.42	0.70	0.03	0.03	0.05	2.10
	0.01	0.01	0.14	0.20	0.24	0.16	1.12	1.07	0.04	0.06	0.16	0.13	0.00	0.00	0.00	0.01
6 months	0.19	0.19	99.51	5.31	5.45	4.81	28.28	28.14	1.20	1.11	3.23	1.68	0.02	0.02	0.04	2.07
	0.00	0.00	0.03	0.06	0.07	0.06	0.37	0.37	0.06	0.03	0.67	0.11	0.00	0.00	0.01	0.00
9 months	0.19	0.19	99.59	5.27	5.39	4.81	28.12	28.01	1.16	1.29	3.82	1.78	0.02	0.02	0.05	2.07
	0.01	0.01	0.02	0.06	0.06	0.04	1.07	1.06	0.06	0.07	0.86	0.05	0.00	0.00	0.01	0.00
12 months	0.20	0.20	99.54	5.39	5.52	4.89	28.06	27.93	1.16	1.14	2.15	1.62	0.02	0.03	0.05	2.07
	0.00	0.00	0.04	0.07	0.07	0.06	0.58	0.57	0.04	0.05	0.44	0.04	0.00	0.01	0.01	0.00
18 months	0.19	0.18	99.53	5.28	5.40	4.82	29.35	29.21	1.25	1.26	1.77	1.58	0.02	0.02	0.04	2.08
	0.00	0.00	0.04	0.11	0.12	0.08	0.57	0.56	0.07	0.07	0.37	0.05	0.00	0.00	0.00	0.00
24 months	0.16	0.16	99.48	4.96	5.08	4.53	32.34	32.16	1.21	1.24	2.00	1.43	0.03	0.03	0.06	2.09
	0.01	0.01	0.06	0.14	0.14	0.12	2.10	2.08	0.04	0.05	0.45	0.09	0.00	0.00	0.01	0.01
28 months	0.16	0.16	99.51	6.11	6.43	5.17	40.23	39.62	1.29	1.09	0.90	1.20	0.03	0.04	0.05	2.14
	0.01	0.01	0.53	0.56	0.69	0.31	1.44	1.24	0.09	0.10	0.29	0.09	0.01	0.01	0.01	0.02

Tibia cortical — Part 2

Age	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Mean total crosssectional tissue area T.Ar (mm^2)	Mean total crosssectional tissue perimeter T.Pm (mm)	Mean total crosssectional bone area B.Ar (mm^2)	Mean total crosssectional bone perimeter B.Pm (mm)	Crosssectional thickness Cs.Th (mm)	Cortical bone thickness Ct.Th (mm)
3 months	31.17	79.00	0.00	0.04	0.04	0.00	1.16	0.00	1.20	40.83	0.90	16.52	0.89	18.29	0.10	0.11
	7.54	10.34	0.00	0.00	0.01	0.00	0.14	0.00	0.14	11.29	0.05	0.57	0.05	0.76	0.00	0.00
6 months	25.60	30.00	0.00	0.01	0.01	0.00	0.48	0.00	0.49	27.80	0.94	15.09	0.94	15.65	0.12	0.13
	6.24	4.51	0.00	0.00	0.00	0.00	0.02	0.00	0.03	4.02	0.02	0.17	0.02	0.20	0.00	0.00
9 months	20.80	16.20	0.00	0.01	0.00	0.00	0.40	0.00	0.41	14.60	0.94	15.01	0.94	15.45	0.12	0.13
	3.68	3.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	5.93	0.05	0.21	0.05	0.21	0.01	0.01
12 months	22.20	19.80	0.00	0.01	0.01	0.00	0.45	0.00	0.46	24.00	0.96	15.15	0.95	15.67	0.12	0.13
	6.19	3.43	0.00	0.00	0.00	0.00	0.04	0.00	0.04	5.77	0.02	0.29	0.02	0.31	0.00	0.00
18 months	18.80	16.20	0.00	0.01	0.01	0.00	0.46	0.00	0.47	19.00	0.90	15.36	0.89	15.86	0.11	0.13
	5.14	4.05	0.00	0.00	0.00	0.00	0.04	0.00	0.04	6.23	0.02	0.40	0.02	0.46	0.00	0.00
24 months	16.33	11.83	0.00	0.02	0.01	0.00	0.50	0.00	0.52	-0.50	0.78	14.97	0.78	15.44	0.10	0.12
	3.21	3.22	0.00	0.01	0.00	0.00	0.06	0.00	0.06	9.07	0.05	0.57	0.05	0.57	0.01	0.01
28 months	31.67	9.00	0.00	0.08	0.08	0.00	1.41	0.00	1.49	-102.33	0.79	19.56	0.77	20.87	0.07	0.10
	5.34	3.52	0.00	0.08	0.08	0.00	0.57	0.00	0.53	101.53	0.07	1.62	0.06	2.21	0.00	0.00

Table SD.2.

Full table of femur cortical bone measures for the ageing cohort detailed in chapter 3 with average and standard error noted.

Femur cortical

Age		Tissue volume TV mm^3	Bone volume BV mm^3	Percent bone volume BV/TV %	Tissue surface TS mm^2	Bone surface BS mm^2	Intersection surface i.S mm^2	Bone surface / volume ratio BS/BV 1/mm	Bone surface density BS/TV 1/mm	Centroid (x) Crd.X mm	Centroid (y) Crd.Y mm	Centroid (z) Crd.Z mm	Structure model index SMI	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD
3 months	avg	0.18	0.18	99.08	5.59	5.82	4.86	32.18	31.88	1.24	1.11	9.42	1.48	0.01	0.02	0.02	2.10
	SE	0.01	0.01	0.04	0.16	0.17	0.13	0.70	0.68	0.11	0.09	0.18	0.06	0.00	0.00	0.00	0.00
6 months	avg	0.18	0.18	99.48	5.12	5.24	4.59	29.66	29.49	1.23	1.14	8.18	1.76	0.01	0.02	0.03	2.07
	SE	0.00	0.00	0.03	0.11	0.12	0.09	0.28	0.27	0.09	0.11	0.66	0.09	0.00	0.01	0.01	0.00
9 months	avg	0.19	0.19	99.41	5.44	5.59	4.86	30.35	30.17	1.30	1.18	8.79	1.78	0.01	0.01	0.02	2.07
	SE	0.01	0.01	0.04	0.16	0.18	0.13	0.28	0.27	0.12	0.11	0.85	0.16	0.00	0.00	0.00	0.00
12 months	avg	0.19	0.18	99.44	5.39	5.53	4.81	30.11	29.94	1.30	1.18	7.02	1.62	0.01	0.01	0.02	2.08
	SE	0.01	0.01	0.04	0.15	0.06	0.05	0.69	0.69	0.07	0.11	0.44	0.07	0.00	0.00	0.01	0.00
18 months	avg	0.17	0.17	99.40	5.34	5.47	4.77	31.73	31.53	1.16	1.33	6.57	1.63	0.01	0.01	0.01	2.08
	SE	0.01	0.01	0.05	0.13	0.14	0.10	0.39	0.39	0.12	0.05	0.39	0.04	0.00	0.00	0.02	0.00
24 months	avg	0.14	0.14	99.19	5.26	5.42	4.72	39.50	39.19	1.38	1.27	6.85	1.24	0.01	0.01	0.02	2.12
	SE	0.01	0.01	0.10	0.23	0.24	0.19	2.74	2.72	0.09	0.08	0.47	0.11	0.00	0.00	0.01	0.01
28 months	avg	0.15	0.15	98.78	6.17	6.42	5.37	43.38	42.86	1.38	1.09	5.76	1.06	0.01	0.01	0.02	2.15
	SE	0.02	0.02	0.12	0.27	0.31	0.19	3.47	3.48	0.21	0.08	0.32	0.23	0.00	0.00	0.00	0.01

Femur cortical

Age		Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) mm^3	Surface of closed pores Po.S(cl) mm^2	Closed porosity (percent) Po(cl) %	Volume of open pore space Po.V(op) mm^3	Open porosity (percent) Po(op) %	Total volume of pore space Po.V(tot) mm^3	Total porosity (percent) Po(tot) %	Euler number Eu.N	Mean total crosssectional bone area B.Ar mm^2	Mean total crosssectional bone perimeter B.Pm mm	Mean total crosssectional tissue area T.Ar mm^2	Mean total crosssectional tissue area T.Pm mm	Crosssectional thickness Cs.Th mm	Cortical bone thickness Ct.Th mm
3 months	avg	61.17	40.83	0.00	0.01	0.01	0.00	0.91	0.00	0.92	41.00	0.88	17.96	0.89	16.98	0.10	0.12
	SE	10.25	8.79	0.00	0.00	0.00	0.00	0.04	0.00	0.04	16.24	0.04	0.44	0.04	0.40	0.00	0.00
6 months	avg	22.00	17.40	0.00	0.01	0.01	0.00	0.51	0.00	0.52	19.00	0.86	15.79	0.86	15.32	0.11	0.13
	SE	5.00	1.85	0.00	0.00	0.00	0.00	0.03	0.00	0.03	5.24	0.02	0.38	0.02	0.35	0.00	0.00
9 months	avg	48.60	16.20	0.00	0.01	0.01	0.00	0.58	0.00	0.59	33.00	0.90	16.76	0.90	16.21	0.11	0.12
	SE	20.81	3.79	0.00	0.00	0.01	0.00	0.04	0.00	0.04	13.78	0.05	0.31	0.05	0.23	0.01	0.01
12 months	avg	31.00	14.40	0.00	0.01	0.01	0.00	0.55	0.00	0.56	18.80	0.89	16.57	0.90	16.02	0.11	0.13
	SE	4.35	4.27	0.00	0.00	0.00	0.00	0.04	0.00	0.04	7.66	0.03	0.17	0.03	0.20	0.00	0.00
18 months	avg	43.40	10.20	0.00	0.01	0.01	0.00	0.59	0.00	0.60	26.20	0.84	17.05	0.84	16.55	0.10	0.11
	SE	14.30	1.97	0.00	0.00	0.00	0.00	0.05	0.00	0.05	11.09	0.03	0.41	0.03	0.36	0.00	0.00
24 months	avg	18.83	9.33	0.00	0.01	0.02	0.00	0.79	0.00	0.81	-12.00	0.68	17.88	0.68	17.25	0.08	0.09
	SE	9.09	5.46	0.00	0.02	0.01	0.00	0.09	0.00	0.10	12.89	0.04	0.85	0.04	0.86	0.01	0.01
28 months	avg	17.67	14.33	0.00	0.02	0.05	0.00	1.17	0.00	1.22	-45.00	0.73	21.81	0.74	20.83	0.07	0.09
	SE	4.34	10.36	0.00	0.05	0.05	0.00	0.09	0.00	0.12	32.96	0.09	0.61	0.09	0.45	0.01	0.00

Table SD.3. Full table of tibia trabecular bone measures for the ageing cohort detailed in chapter 3 with average and standard error noted.

Tibia trabecular

Age	Tissue volume TV mm^3	Bone volume BV mm^3	Percent bone volume BV/TV %	Tissue surface TS mm^2	Bone surface BS mm^2	Intersection surface iS mm^2	Bone surface / volume ratio BS/BV 1/mm	Bone surface density BS/TV 1/mm	Trabecular pattern factor Tb.Pf 1/mm	Centroid (x) Crd.X mm	Centroid (y) Crd.Y mm	Centroid (z) Crd.Z mm	Structure model index SMI	Trabecular thickness Tb.Th mm	Trabecular number Tb.N 1/mm	Trabecular separation Tb.Sp mm
3 months	2.25	0.46	20.38	12.75	32.54	3.45	72.10	14.43	17.66	1.06	1.18	4.89	1.70	0.05	3.92	0.16
	0.09	0.05	1.51	0.35	1.81	0.33	3.82	0.47	1.82	0.03	0.06	0.16	0.07	0.00	0.14	0.00
6 months	2.10	0.28	13.24	12.57	20.95	2.39	75.72	9.96	22.73	1.21	1.08	3.75	2.03	0.05	2.67	0.20
	0.04	0.02	0.82	0.09	1.25	0.16	2.40	0.38	1.71	0.08	0.04	0.66	0.10	0.00	0.13	0.00
9 months	2.13	0.26	12.21	12.73	18.41	2.31	71.10	8.63	21.69	1.04	1.21	4.36	2.16	0.05	2.35	0.25
	0.05	0.04	1.57	0.28	2.41	0.14	4.76	1.06	2.14	0.09	0.10	0.87	0.25	0.01	0.36	0.03
12 months	2.05	0.26	12.61	12.99	16.01	2.40	62.88	7.86	19.61	1.08	1.20	2.68	2.19	0.06	2.08	0.25
	0.09	0.01	0.74	0.34	0.37	0.11	2.66	0.19	0.94	0.09	0.09	0.44	0.10	0.00	0.05	0.01
18 months	2.07	0.25	13.31	12.88	15.36	2.17	60.57	7.44	18.70	1.28	1.39	2.31	2.16	0.06	2.01	0.26
	0.06	0.01	0.52	0.26	0.36	0.10	0.97	0.28	1.34	0.12	0.06	0.36	0.13	0.00	0.11	0.01
24 months	1.93	0.13	6.62	12.47	9.81	1.48	78.64	5.07	26.38	1.11	1.10	2.35	2.35	0.05	1.28	0.32
	0.08	0.02	0.81	0.46	0.94	0.14	5.12	0.48	2.38	0.06	0.07	0.44	0.13	0.00	0.12	0.03
28 months	1.91	0.11	5.76	12.72	8.20	1.25	78.54	4.43	26.88	1.21	0.94	1.41	2.28	0.05	1.16	0.34
	0.17	0.02	1.37	0.57	1.35	0.35	4.35	0.91	2.04	0.13	0.10	0.34	0.06	0.00	0.23	0.03

Tibia trabecular

Age	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) mm^3	Surface of closed pores Po.S(cl) mm^2	Closed porosity (percent) Po(cl) %	Volume of open pore space Po.V(op) mm^3	Open porosity (percent) Po(op) %	Total volume of pore space Po.V(tot) mm^3	Total porosity (percent) Po(tot) %	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn 1/mm^3
3 months	7.74	15.85	20.34	2.13	723.83	10.00	0.00	0.00	0.00	1.79	79.62	1.79	79.62	-278.88	1012.67	451.48
	1.87	3.91	4.97	0.00	67.97	3.26	0.00	0.00	0.00	0.06	1.51	0.06	1.51	54.63	64.18	29.15
6 months	9.57	16.20	22.48	2.10	668.40	4.20	0.00	0.00	0.00	1.82	86.76	1.82	86.76	-71.00	743.60	353.78
	1.45	2.71	3.75	0.01	33.19	0.58	0.00	0.00	0.00	0.02	0.82	0.02	0.82	44.86	63.30	25.81
9 months	4.84	8.09	11.47	2.09	840.20	4.20	0.00	0.00	0.00	1.86	87.79	1.86	87.79	22.40	822.00	386.60
	0.66	1.65	1.49	0.02	82.94	1.15	0.00	0.00	0.00	0.04	1.57	0.04	1.57	89.84	87.93	39.68
12 months	5.11	7.61	10.48	2.08	800.80	8.80	0.00	0.00	0.00	1.79	87.39	1.79	87.39	113.80	695.80	343.38
	1.04	1.71	2.01	0.01	101.86	2.02	0.00	0.01	0.01	0.09	0.74	0.09	0.74	36.11	93.55	49.12
18 months	4.23	6.46	9.39	2.08	742.60	6.80	0.00	0.00	0.01	1.82	87.69	1.82	87.69	141.20	608.20	293.20
	1.18	2.28	2.98	0.01	134.87	2.38	0.00	0.00	0.00	0.06	0.52	0.06	0.52	107.53	81.99	38.31
24 months	7.35	10.49	13.52	2.07	623.50	3.83	0.00	0.00	0.00	1.80	93.38	1.80	93.38	0.33	627.00	321.92
	2.09	2.26	3.76	0.01	61.29	0.98	0.00	0.00	0.00	0.08	0.81	0.08	0.81	36.47	68.26	28.45
28 months	6.92	9.52	15.29	2.07	413.67	2.00	0.00	0.00	0.00	1.80	94.24	1.80	94.24	47.33	368.33	208.28
	2.97	4.02	8.22	0.00	121.42	1.53	0.00	0.00	0.00	0.18	1.37	0.18	1.37	27.94	124.45	88.02

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Table SD.4. Full table of femur trabecular bone measures for the ageing cohort detailed in chapter 3 with average and standard error noted.

Femur trabecular

Age	Tissue volume TV mm^3	Bone volume BV mm^3	Percent bone volume BV/TV %	Tissue surface TS mm^2	Bone surface BS mm^2	Intersection surface i.S mm^2	Bone surface / volume ratio BS/BV 1/mm	Bone surface density BS/TV 1/mm	Trabecular pattern factor Tb.Pf 1/mm	Centroid (x) Crd.X mm	Centroid (y) Crd.Y mm	Centroid (z) Crd.Z mm	Structure model index SMI	Trabecular thickness Tb.Th mm	Trabecular number Tb.N 1/mm	Trabecular separation Tb.Sp mm
3 months	2.34	0.57	24.22	11.95	37.02	3.94	66.38	15.80	11.48	1.04	0.97	9.01	1.40	0.06	4.35	0.15
	0.08	0.06	1.70	0.26	2.14	0.46	3.85	0.52	1.42	0.07	0.08	0.17	0.07	0.00	0.18	0.00
6 months	2.43	0.35	14.51	12.23	24.80	2.40	71.33	10.18	19.07	1.05	1.00	7.80	1.82	0.05	2.78	0.21
	0.06	0.03	1.15	0.20	1.23	0.11	3.70	0.31	1.79	0.12	0.05	0.66	0.08	0.00	0.11	0.00
9 months	2.66	0.38	14.10	13.21	23.96	2.37	65.06	8.97	16.25	1.12	1.03	8.38	1.82	0.06	2.54	0.24
	0.05	0.04	1.36	0.19	1.98	0.20	5.51	0.64	1.31	0.10	0.09	0.83	0.17	0.01	0.22	0.01
12 months	2.52	0.38	14.93	12.80	22.09	2.56	58.96	8.75	13.84	1.09	1.13	6.62	1.71	0.06	2.46	0.25
	0.09	0.02	0.51	0.23	1.25	0.11	2.77	0.21	0.97	0.04	0.13	0.43	0.13	0.00	0.09	0.01
18 months	2.65	0.31	11.54	12.97	17.55	1.98	57.71	6.66	17.25	1.09	1.22	6.17	2.03	0.06	1.85	0.28
	0.09	0.02	0.79	0.25	1.02	0.15	1.58	0.48	1.16	0.12	0.11	0.41	0.12	0.00	0.16	0.01
24 months	2.58	0.15	5.89	12.71	11.13	0.94	74.69	4.35	23.40	1.28	1.12	6.42	2.06	0.05	1.15	0.36
	0.12	0.01	0.50	0.43	0.84	0.09	4.41	0.34	2.04	0.07	0.11	0.47	0.08	0.00	0.09	0.04
28 months	2.52	0.18	7.21	12.88	13.01	1.21	75.25	5.27	19.04	1.25	1.05	5.30	1.75	0.05	1.48	0.34
	0.18	0.03	1.60	0.39	1.02	0.21	4.86	0.80	2.13	0.15	0.04	0.35	0.11	0.00	0.24	0.03

Femur trabecular

Age	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) mm^3	Surface of closed pores Po.S(cl) mm^2	Closed porosity (percent) Po(cl) %	Volume of open pore space Po.V(op) mm^3	Open porosity (percent) Po(op) %	Total volume of pore space Po.V(tot) mm^3	Total porosity (percent) Po(tot) %	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn 1/mm^3
3 months	12.47	20.87	23.21	2.15	1352.50	80.50	0.00	0.04	0.03	1.76	75.77	1.76	75.78	-13.17	1446.17	621.69
	2.44	4.42	4.56	0.01	194.11	20.83	0.00	0.01	0.01	0.02	1.71	0.02	1.70	176.33	123.48	58.20
6 months	16.70	24.17	28.72	2.10	617.00	10.40	0.00	0.01	0.01	2.08	85.49	2.08	85.49	-177.60	805.00	331.82
	3.00	4.47	5.22	0.01	83.06	2.96	0.00	0.00	0.00	0.03	1.15	0.03	1.15	83.46	40.72	17.89
9 months	14.91	20.20	24.05	2.10	791.80	17.40	0.00	0.00	0.00	2.29	85.90	2.29	85.90	83.00	726.20	273.60
	4.31	6.17	7.50	0.01	167.60	5.88	0.00	0.00	0.00	0.03	1.36	0.03	1.36	150.17	43.40	19.07
12 months	9.18	12.00	13.47	2.10	556.80	14.00	0.00	0.01	0.01	2.14	85.07	2.14	85.07	9.20	561.60	222.59
	1.47	1.48	1.66	0.01	38.83	3.66	0.00	0.01	0.01	0.08	0.51	0.08	0.51	56.71	50.77	16.34
18 months	7.15	9.54	12.34	2.07	572.20	22.20	0.00	0.01	0.01	2.35	88.45	2.35	88.46	150.80	443.60	168.75
	0.67	0.82	0.85	0.01	113.69	9.22	0.00	0.00	0.00	0.09	0.79	0.09	0.79	96.12	23.34	12.72
24 months	16.74	21.38	27.44	2.08	272.67	6.83	0.00	0.01	0.02	2.43	94.11	2.43	94.11	-17.17	296.67	115.42
	3.28	3.95	5.12	0.01	41.59	2.36	0.00	0.00	0.01	0.12	0.50	0.12	0.50	32.89	39.25	14.00
28 months	13.10	19.24	23.46	2.11	366.33	6.33	0.00	0.00	0.00	2.34	92.79	2.34	92.79	95.76	366.00	146.87
	2.36	3.59	2.33	0.01	130.00	2.85	0.00	0.00	0.00	0.21	1.60	0.21	1.60	18.12	38.35	18.12

Table SD.5. Full table of tibia cortical bone measures for the wild mouse cohort detailed in chapter 4 with average and standard error noted.

Tibia cortical

Age	Tissue volume	Bone volume	Percent bone volume	Tissue surface	Bone surface	Intersection surface	Bone surface / volume ratio	Bone surface density	Centroid (x)	Centroid (y)	Centroid (z)	Structure model index	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension
	TV	BV	BV/TV	TS	BS	iS	BS/BV	BS/TV	Crd.X	Crd.Y	Crd.Z	SMI				FD
	mm^3	mm^3	%	mm^2	mm^2	mm^2	1/mm	1/mm	mm	mm	mm					
Wild male 6	0.35	0.34	96.03	7.08	7.49	5.17	22.15	22.21	1.01	1.08	3.79	0.80	0.09	0.11	0.15	2.03
	0.01	0.01	1.30	0.32	0.38	0.04	1.19	0.82	0.05	0.04	0.21	0.14	0.02	0.03	0.04	0.00
Wild female 6	0.29	0.29	99.42	5.79	6.15	5.08	21.55	21.43	0.89	0.95	3.46	-0.68	0.06	0.08	0.10	2.04
	0.01	0.01	0.11	0.14	0.17	0.12	0.79	0.78	0.07	0.08	0.19	0.87	0.01	0.01	0.02	0.00
Wild male 12	0.30	0.29	98.39	6.32	6.48	5.02	22.08	22.72	1.10	1.09	3.33	1.14	0.08	0.11	0.16	2.04
	0.01	0.01	0.30	0.19	0.20	0.14	0.66	0.62	0.05	0.06	0.27	0.04	0.01	0.01	0.02	0.00
Wild female 12	0.28	0.28	98.98	6.05	6.74	5.04	24.36	24.11	1.06	0.91	3.78	-2.43	0.16	0.21	0.26	2.07
	0.01	0.01	0.11	0.19	0.31	0.11	0.98	0.95	0.03	0.02	0.11	0.70	0.03	0.03	0.04	0.01
Wild male 24	0.25	0.24	98.09	6.24	6.38	4.98	26.88	26.34	1.23	1.00	3.62	1.15	0.08	0.11	0.15	2.06
	0.02	0.02	0.45	0.12	0.15	0.15	2.50	2.34	0.02	0.02	0.40	0.10	0.02	0.03	0.04	0.01
Wild female 24	0.23	0.23	99.34	4.92	5.23	4.19	23.90	23.74	1.03	1.04	2.52	0.40	0.10	0.13		2.06
	0.04	0.04	0.08	0.73	0.84	0.57	1.26	1.25	0.01	0.06	0.44	1.02	0.05	0.06		0.00
Wild female 31	0.12	0.12	99.41	3.39	3.47	3.01	29.75	29.57	0.92	0.96	2.23	2.43	0.04	0.05	0.09	2.07
	0.01	0.01	0.09	0.24	0.25	0.19	1.28	1.25	0.10	0.05	1.09	0.24	0.01	0.01	0.02	0.01

Tibia cortical

Age	Number of objects	Number of closed pores	Volume of closed pores	Surface of closed pores	Closed porosity (percent)	Volume of open pore space	Open porosity (percent)	Total volume of pore space	Total porosity (percent)	Euler number	Mean total cross sectional tissue area	Mean total cross sectional tissue superimeter	Mean total cross sectional bone area	Mean total cross sectional bone perimeter	Cross sectional thickness	Cortical bone thickness
	Obj.N	Po.N(cl)	Po.V(cl)	Po.S(cl)	Po(cl)	Po.V(op)	Po(op)	Po.V(tot)	Po(tot)	Eu.N	T.Ar	T.Pm	B.Ar	B.Pm	Cs.Th	Ct.Th
			mm^3	mm^2	%	mm^3	%	mm^3	%	Eu.N	mm^2	mm	mm^2	mm	mm	mm
Wild male 6	9.40	49.40	0.00	0.03	0.02	0.01	3.95	0.01	3.97	27.00	1.62	11.00	0.78	12.51	0.13	0.15
	3.69	15.15	0.00	0.01	0.01	0.00	1.30	0.00	1.30	16.43	0.03	0.32	0.01	0.74	0.01	0.01
Wild female 6	1.75	239.25	0.00	0.10	0.06	0.00	0.51	0.00	0.58	218.25	0.67	9.64	0.66	10.32	0.13	0.14
	0.25	88.73	0.00	0.05	0.04	0.00	0.08	0.00	0.11	84.40	0.03	0.23	0.03	0.24	0.01	0.01
Wild male 12	1.80	15.60	0.00	0.01	0.01	0.00	1.61	0.00	1.61	6.00	1.46	10.37	0.68	10.84	0.13	0.16
	0.37	3.85	0.00	0.00	0.00	0.00	0.31	0.00	0.30	4.92	0.04	0.29	0.02	0.37	0.00	0.00
Wild female 12	5.00	751.33	0.00	0.16	0.09	0.00	0.94	0.00	1.02	679.33	0.65	10.05	0.64	11.34	0.11	0.12
	2.09	239.14	0.00	0.04	0.02	0.00	0.10	0.00	0.11	217.03	0.03	0.29	0.03	0.51	0.01	0.01
Wild male 24	2.25	7.00	0.00	0.01	0.01	0.00	1.90	0.00	1.91	-5.25	1.49	10.84	0.56	11.19	0.10	0.14
	0.95	1.47	0.00	0.07	0.05	0.00	0.45	0.00	0.45	3.47	0.02	0.25	0.05	0.38	0.01	0.01
Wild female 24	36.17	265.17	0.00	0.07	0.05	0.00	0.62	0.00	0.66	2573.17	0.71	10.06	0.71	10.69	0.13	0.14
	16.49	130.30	0.00	0.03	0.02	0.00	0.06	0.00	0.08	1104.48	0.03	0.34	0.03	0.49	0.01	0.01
Wild female 31	74.50	3.17	0.00	0.00	0.00	0.00	0.59	0.00	0.59	31.00	0.64	10.42	0.64	10.75	0.12	0.14
	26.76	0.83	0.00	0.00	0.00	0.00	0.09	0.00	0.09	20.27	0.03	0.68	0.03	0.74	0.01	0.01

Table SD.6. Full table of femur cortical bone measures for the wild mouse cohort detailed in chapter 4 with average and standard error noted.

Femur cortical

Age	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD
Wild male 6	0.38	0.35	92.48	7.44	8.25	5.29	23.36	21.60	1.17	0.93	8.95	0.94	0.06	0.07	0.13	2.05
	0.02	0.02	0.27	0.18	0.25	0.08	0.52	0.46	0.05	0.05	0.19	0.06	0.01	0.01	0.01	0.00
Wild female 6	0.30	0.30	99.45	6.50	6.82	5.72	22.48	22.36	0.95	1.12	7.42	0.13	0.10	0.13	0.19	2.04
	0.03	0.03	0.06	0.57	0.63	0.46	0.66	0.66	0.10	0.02	1.27	0.27	0.02	0.03	0.04	0.00
Wild male 12	0.33	0.32	97.98	6.78	6.98	5.45	21.75	21.31	1.08	0.96	8.81	1.21	0.05	0.06	0.10	2.02
	0.01	0.01	0.26	0.26	0.27	0.18	0.35	0.29	0.12	0.13	0.27	0.05	0.01	0.01	0.02	0.00
Wild female 12	0.27	0.27	99.37	6.45	6.78	5.63	25.15	24.99	1.13	0.81	9.07	0.12	0.06	0.08		2.04
	0.01	0.01	0.09	0.22	0.28	0.11	0.63	0.61	0.06	0.06	0.10	0.24	0.01	0.01	0.00	0.01
Wild male 24	0.28	0.28	98.73	6.65	6.83	5.47	24.96	24.65	1.28	0.87	8.55	1.02	0.04	0.06	0.10	2.03
	0.02	0.02	0.10	0.09	0.10	0.05	2.01	1.98	0.02	0.12	0.03	0.01	0.01	0.01	0.01	0.00
Wild female 24	0.25	0.25	99.17	6.47	6.75	5.61	29.78	29.46	1.18	0.94	8.48	0.97	0.06	0.07		2.07
	0.04	0.04	0.28	0.77	0.82	0.60	6.40	6.20	0.06	0.08	0.33	0.60	0.02	0.02		0.03
Wild female 31	0.13	0.13	99.39	4.10	4.18	3.64	34.22	34.01	1.06	1.01	6.11	1.95	0.02	0.03	0.06	2.08
	0.01	0.01	0.07	0.19	0.19	0.19	2.71	2.67	0.05	0.08	0.96	0.17	0.00	0.01	0.01	0.01

Femur cortical

Age	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Mean total crosssectional tissue area T.Ar (mm^2)	Mean total crosssectional tissue perimeter T.Pm (mm)	Mean total crosssectional bone area B.Ar (mm^2)	Mean total crosssectional bone perimeter B.Pm (mm)	Crosssectional thickness Cs.Th (mm)	Cortical bone thickness Cs.Th (mm)
Wild male 6	18.40	33.80	0.00	0.03	0.03	0.03	7.49	0.03	7.52	0.40	1.66	10.91	0.78	13.65	0.13	0.14
	6.60	5.30	0.00	0.01	0.01	0.00	0.27	0.00	0.27	5.15	0.07	0.18	0.01	0.33	0.00	0.00
Wild female 6	6.25	142.75	0.00	0.04	0.02	0.00	0.52	0.00	0.55	109.50	0.70	10.75	0.70	11.33	0.12	0.14
	1.84	30.42	0.00	0.01	0.01	0.00	0.06	0.00	0.06	201.12	0.06	0.77	0.06	0.88	0.00	0.00
Wild male 12	5.20	12.00	0.00	0.01	0.00	0.01	2.01	0.01	2.02	3.80	1.72	10.66	0.74	11.68	0.13	0.15
	1.95	2.08	0.00	0.00	0.00	0.00	0.27	0.00	0.26	2.46	0.11	0.28	0.02	0.42	0.00	0.00
Wild female 12	7.33	210.17	0.00	0.04	0.02	0.00	0.61	0.00	0.63	177.50	0.63	11.05	0.62	11.64	0.11	0.13
	4.31	106.50	0.00	0.02	0.01	0.00	0.08	0.00	0.09	97.56	0.02	0.37	0.02	0.48	0.00	0.00
Wild male 24	1.00	9.00	0.00	0.01	0.01	0.00	1.26	0.00	1.27	-0.33	1.76	11.16	0.64	11.89	0.11	0.13
	0.00	1.53	0.00	0.01	0.01	0.00	0.10	0.00	0.10	3.65	0.05	0.17	0.04	0.17	0.01	0.01
Wild female 24	78.33	89.67	0.00	0.03	0.06	0.00	0.77	0.00	0.83	98.83	0.81	15.40	0.81	16.12	0.11	0.13
	56.57	43.34	0.00	0.01	0.05	0.00	0.24	0.00	0.28	59.75	0.06	3.78	0.06	3.96	0.02	0.02
Wild female 31	72.33	4.83	0.00	0.00	0.00	0.00	0.61	0.00	0.61	52.67	0.63	12.64	0.62	12.96	0.10	0.11
	25.02	2.14	0.00	0.00	0.00	0.00	0.07	0.00	0.07	216.69	0.06	0.52	0.06	0.52	0.01	0.01

Table SD.7. Full table of tibia trabecular bone measures for the wild mouse cohort detailed in chapter 4 with average and standard error noted.

Tibia trabecular

Age	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Trabecular pattern factor Tb.Pf (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Trabecular thickness Tb.Th (mm)	Trabecular number Tb.N (1/mm)	Trabecular separation Tb.Sp (mm)
Wild male 6	1.54	0.19	11.98	10.17	13.66	1.77	72.39	8.58	19.63	1.16	0.98	4.40	1.86	0.05	2.35	0.28
	0.10	0.04	1.83	0.45	2.97	0.15	2.76	1.22	1.59	0.13	0.05	0.19	0.12	0.00	0.37	0.04
Wild female 6	1.18	0.06	5.17	8.25	5.27	1.15	84.24	4.33	28.76	0.83	0.88	4.31	2.45	0.05	1.06	0.52
	0.09	0.01	0.65	0.36	0.97	0.16	2.22	0.48	1.18	0.05	0.04	0.17	0.09	0.00	0.14	0.03
Wild male 12	1.28	0.05	3.62	9.74	3.83	0.88	87.05	2.92	37.00	1.15	1.05	4.32	2.81	0.05	0.72	0.49
	0.07	0.01	0.99	0.37	0.92	0.16	7.63	0.71	6.84	0.13	0.15	0.29	0.25	0.00	0.20	0.04
Wild female 12	1.15	0.03	2.82	8.56	2.85	0.53	81.11	2.30	24.44	0.97	0.86	4.60	2.16	0.05	0.58	0.58
	0.09	0.01	0.73	0.53	1.03	0.10	2.51	0.63	2.25	0.06	0.08	0.12	0.15	0.00	0.17	0.04
Wild male 24	1.38	0.02	1.50	10.24	1.75	0.43	95.01	1.30	39.13	1.18	0.95	4.48	2.61	0.05	0.30	0.66
	0.11	0.01	0.53	0.52	0.43	0.11	8.16	0.37	6.63	0.14	0.11	0.35	0.14	0.00	0.09	0.03
Wild female 24	1.53	0.03	3.33	9.11	2.47	0.43	73.47	2.49	23.66	0.92	0.93	5.03	2.41	0.06	0.65	0.64
	0.48	0.01	1.42	1.27	0.78	0.14	8.78	0.98	2.09	0.11	0.09	1.07	0.25	0.01	0.27	0.07
Wild female 31	1.25	0.06	4.30	8.72	4.83	0.71	84.12	3.68	29.68	0.80	0.94	3.37	2.35	0.05	0.96	0.53
	0.10	0.02	1.74	0.39	2.01	0.20	1.79	1.54	4.79	0.07	0.10	0.76	0.29	0.00	0.43	0.06

Tibia trabecular

Age	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn (1/mm^3)
Wild male 6	6.94	12.33	15.81	2.11	359.00	4.20	0.00	0.00	0.00	1.35	88.02	1.35	88.02	-105.00	468.20	299.29
	1.63	3.26	3.32	0.01	21.49	2.69	0.00	0.00	0.00	0.07	1.83	0.07	1.83	76.72	64.58	20.63
Wild female 6	17.42	24.19	40.37	2.07	506.25	7.50	0.00	0.00	0.01	1.12	94.83	1.12	94.83	48.75	465.00	403.48
	2.74	3.06	6.74	0.01	75.60	1.52	0.00	0.00	0.01	0.08	0.65	0.08	0.65	10.48	67.23	70.62
Wild male 12	6.13	8.86	12.88	2.02	451.60	0.60	0.00	0.00	0.00	1.24	96.38	1.24	96.38	47.00	405.20	311.25
	1.00	1.56	2.14	0.03	67.19	0.40	0.00	0.00	0.00	0.07	0.99	0.07	0.99	18.90	55.94	32.22
Wild female 12	7.04	9.08	15.84	2.09	157.00	17.00	0.00	0.00	0.00	1.11	97.18	1.11	97.18	-2.83	176.83	145.89
	1.07	1.31	2.63	0.02	26.88	11.00	0.00	0.00	0.00	0.08	0.73	0.08	0.73	15.93	50.34	28.80
Wild male 24	7.60	14.67	23.10	2.03	188.00	0.75	0.00	0.00	0.00	1.36	98.50	1.36	98.50	29.75	139.00	116.94
	1.23	3.16	1.75	0.02	43.09	0.48	0.00	0.00	0.00	0.11	0.53	0.11	0.53	8.17	34.97	26.69
Wild female 24	15.56	19.69	26.64	2.07	126.00	3.83	0.00	0.00	0.01	1.50	96.67	1.50	96.67	-20.83	150.67	151.36
	6.75	8.05	8.65	0.03	22.70	1.58	0.00	0.00	0.01	0.44	1.42	0.44	1.42	24.64	45.02	67.85
Wild female 31	12.50	20.20	27.74	2.08	187.83	3.33	0.00	0.00	0.00	1.19	95.70	1.19	95.70	-81.67	272.83	213.58
	1.48	4.80	9.14	0.03	37.59	1.98	0.00	0.00	0.00	0.09	1.74	0.09	1.74	77.45	112.14	89.14

Table SD.8. Full table of femur trabecular bone measures for the wild mouse cohort detailed in chapter 4 with average and standard error noted.

Femur trabecular

Age		Tissue volume TV mm^3	Bone volume BV mm^3	Percent bone volume BV/TV %	Tissue surface TS mm^2	Bone surface BS mm^2	Intersection surface iS mm^2	Bone surface / volume ratio BS/BV 1/mm	Bone surface density BS/TV 1/mm	Trabecular pattern factor Tb.Pf 1/mm	Centroid (x) Crd.X mm	Centroid (y) Crd.Y mm	Centroid (z) Crd.Z mm	Structure model index SMI	Trabecular thickness Tb.Th mm	Trabecular number Tb.N 1/mm	Trabecular separation Tb.Sp mm
Wild male 6	avg	1.69	0.23	13.69	9.81	16.48	1.83	72.49	9.70	17.22	1.02	0.85	8.30	1.62	0.05	2.72	0.26
	se	0.02	0.04	2.41	0.14	2.61	0.25	3.05	1.45	2.11	0.06	0.04	0.21	0.11	0.00	0.43	0.03
Wild female 6	avg	1.60	0.11	6.44	9.04	8.24	1.25	80.30	5.07	20.41	0.73	0.88	8.05	1.91	0.05	1.34	0.50
	se	0.06	0.03	1.40	0.32	1.83	0.21	2.26	0.96	1.39	0.04	0.13	0.17	0.09	0.00	0.28	0.08
Wild male 12	avg	1.74	0.05	3.18	9.97	4.49	0.75	85.86	2.60	26.30	1.11	1.18	8.19	2.05	0.04	0.70	0.58
	se	0.06	0.02	0.89	0.33	1.16	0.15	4.00	0.68	3.25	0.09	0.13	0.31	0.17	0.00	0.19	0.05
Wild female 12	avg	1.57	0.04	2.48	9.14	3.33	0.53	84.06	2.12	22.50	1.14	1.06	8.41	2.00	0.05	0.54	0.70
	se	0.05	0.01	0.72	0.15	1.08	0.11	4.61	0.67	2.48	0.06	0.10	0.10	0.15	0.00	0.17	0.07
Wild male 24	avg	1.82	0.03	1.40	10.52	2.17	0.52	90.15	1.20	28.47	1.40	1.07	7.84	2.20	0.05	0.29	0.81
	se	0.05	0.00	0.30	0.36	0.21	0.07	10.56	0.14	4.79	0.05	0.11	0.07	0.22	0.00	0.04	0.01
Wild female 24	avg	1.46	0.03	1.90	8.90	2.29	0.41	84.56	1.38	34.98	0.89	0.85	7.59	2.73	0.06	0.35	0.70
	se	0.16	0.02	1.18	0.66	1.42	0.18	11.74	0.83	6.10	0.12	0.23	0.24	0.37	0.01	0.23	0.06
Wild female 31	avg	1.66	0.06	3.27	9.97	4.58	0.56	91.01	2.71	33.26	0.88	0.98	5.84	2.23	0.04	0.74	0.54
	se	0.06	0.02	1.34	0.61	1.98	0.20	8.08	1.14	7.06	0.12	0.13	0.68	0.25	0.00	0.32	0.07

Femur trabecular

Age		Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) mm^3	Surface of closed pores Po.S(cl) mm^2	Closed porosity (percent) Po(cl) %	Volume of open pore space Po.V(op) mm^3	Open porosity (percent) Po(op) %	Total volume of pore space Po.V(tot) mm^3	Total porosity (percent) Po(tot) %	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn 1/mm^3
Wild male 6	avg	11.88	17.53	20.67	2.12	238.00	3.40	0.00	0.00	0.00	1.46	86.31	1.46	86.31	-236.60	478.00	281.19
	se	2.93	4.52	5.05	0.01	28.56	1.62	0.00	0.00	0.00	0.03	2.41	0.03	2.41	65.95	94.05	52.94
Wild female 6	avg	27.54	39.38	46.04	2.10	385.25	14.75	0.00	0.00	0.00	1.49	93.56	1.49	93.56	-91.50	491.50	304.11
	se	6.34	8.55	8.58	0.01	68.18	6.42	0.00	0.00	0.00	0.04	1.40	0.04	1.40	21.72	94.37	50.20
Wild male 12	avg	18.48	26.65	39.75	2.06	169.40	0.60	0.00	0.00	0.00	1.68	96.82	1.68	96.82	11.60	158.40	91.82
	se	4.80	6.03	9.71	0.02	38.47	0.40	0.00	0.00	0.00	0.06	0.89	0.06	0.89	13.10	39.01	23.72
Wild female 12	avg	19.70	34.03	51.65	2.08	184.50	30.17	0.00	0.01	0.05	1.53	97.52	1.53	97.52	10.67	204.00	127.09
	se	2.14	5.08	6.80	0.01	40.54	22.85	0.00	0.00	0.05	0.05	0.72	0.05	0.72	8.22	63.23	37.34
Wild male 24	avg	23.54	42.05	57.67	2.05	162.67	2.00	0.00	0.00	0.01	1.79	98.60	1.79	98.60	11.33	153.33	83.71
	se	10.57	18.94	17.25	0.02	24.77	1.53	0.00	0.00	0.01	0.05	0.30	0.05	0.30	23.70	26.07	12.16
Wild female 24	avg	29.89	41.95	74.48	2.01	115.00	2.17	0.00	0.00	0.01	1.43	98.10	1.43	98.10	-8.83	126.00	77.35
	se	13.96	19.73	35.72	0.03	27.61	1.17	0.00	0.00	0.01	0.15	1.18	0.15	1.18	39.44	55.70	31.70
Wild female 31	avg	25.42	42.61	59.83	2.04	133.17	2.17	0.00	0.00	1.18	1.61	96.73	1.61	96.73	-52.00	187.33	110.82
	se	7.40	7.99	14.26	0.04	36.69	0.65	0.00	0.00	1.18	0.06	1.34	0.06	1.34	45.18	74.54	42.78

Table SD.9. Full table of tibia and femur cortical bone measures for the HSP10 overexpressor mouse cohort detailed in chapter 5 with average and standard error noted.

Tibia cortical

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD
HSP10 6	0.20	0.20	99.49	5.39	5.54	4.85	27.94	27.80	1.35	1.20	3.51	1.72	0.01	0.02	0.02	2.03
	0.01	0.01	0.03	0.19	0.21	0.16	0.77	0.77	0.03	0.10	0.80	0.06	0.00	0.00	0.00	0.00
HSP10 24	0.18	0.17	99.38	5.36	5.50	4.81	32.00	31.80	1.23	1.30	4.64	1.65	0.02	0.02	0.02	2.05
	0.01	0.01	0.06	0.14	0.15	0.13	2.14	2.11	0.09	0.09	0.83	0.11	0.00	0.00	0.00	0.01

Group	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Mean total crosssectional tissue area T.Ar (mm^2)	Mean total crosssectional bone area B.Ar (mm^2)	Mean total crosssectional tissue perimeter T.Pm (mm)	Mean total crosssectional bone perimeter B.Pm (mm)	Crosssectional thickness Cs.Th (mm)	Cortical bone thickness Ct.Th (mm)
HSP10 6	17.40	26.60	0.00	0.01	0.01	0.00	0.49	0.00	0.51	24.80	1.07	1.06	15.83	16.47	0.13	0.14
	3.18	2.68	0.00	0.00	0.00	0.00	0.03	0.00	0.03	3.69	0.06	0.06	0.49	0.58	0.00	0.00
HSP10 24	27.60	13.60	0.00	0.01	0.00	0.00	0.62	0.00	0.62	10.80	0.96	0.95	16.95	17.62	0.11	0.12
	7.48	5.85	0.00	0.00	0.00	0.00	0.06	0.00	0.06	15.01	0.06	0.06	0.75	0.78	0.01	0.01

Femur cortical

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD
HSP10 6	0.18	0.18	99.30	5.28	5.45	4.67	30.07	29.86	1.32	1.19	8.32	1.66	0.01	0.02	0.02	2.05
	0.01	0.01	0.05	0.09	0.11	0.08	0.61	0.61	0.07	0.06	0.76	0.07	0.00	0.00	0.00	0.00
HSP10 24	0.16	0.16	98.29	6.34	6.70	5.39	42.53	41.75	1.45	1.38	8.12	0.95	0.01	0.01	0.01	2.11
	0.01	0.01	0.41	0.24	0.30	0.15	3.61	3.39	0.13	0.10	0.29	0.20	0.00	0.00	0.00	0.02

Group	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Mean total crosssectional tissue area T.Ar (mm^2)	Mean total crosssectional bone area B.Ar (mm^2)	Mean total crosssectional tissue perimeter T.Pm (mm)	Mean total crosssectional bone perimeter B.Pm (mm)	Crosssectional thickness Cs.Th (mm)	Cortical bone thickness Ct.Th (mm)
HSP10 6	24.80	17.60	0.00	0.01	0.01	0.00	0.69	0.00	0.70	16.20	0.97	0.97	16.38	17.10	0.11	0.13
	4.55	2.99	0.00	0.00	0.00	0.00	0.05	0.00	0.05	2.54	0.02	0.02	0.32	0.39	0.00	0.00
HSP10 24	26.00	16.33	0.00	0.00	0.00	0.00	1.71	0.00	1.71	-62.00	0.88	0.87	22.60	24.23	0.07	0.09
	11.16	5.21	0.00	0.00	0.00	0.00	0.41	0.00	0.41	19.23	0.04	0.04	1.29	1.60	0.01	0.01

Table SD.10. Full table of tibia and femur trabecular bone measures for the HSP10 overexpress or mouse cohort detailed in chapter 5 with average and standard error noted.

Tibia trabecular

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface iS (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface area density BS/TV (1/mm)	Trabecular pattern factor Tb.Pf (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Trabecular thickness Tb.Th (mm)	Trabecular number Tb.N (1/mm)	Trabecular separation Tb.Sp (mm)
HSP10.6	2.04	0.35	17.30	12.30	22.94	2.90	65.53	11.27	16.11	1.28	1.27	3.96	1.80	0.06	3.11	0.20
	0.09	0.03	0.62	0.38	0.99	0.29	2.69	0.13	1.51	0.08	0.12	0.79	0.04	0.00	0.04	0.01
HSP10.24	1.99	0.25	12.60	12.59	17.05	2.10	71.54	8.48	22.41	1.21	1.14	5.09	2.08	0.05	2.30	0.24
	0.08	0.05	2.56	0.14	2.78	0.40	5.95	1.30	4.13	0.10	0.09	0.83	0.13	0.00	0.39	0.02

Group	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn (1/mm^3)
HSP10.6	6.07	9.92	13.46	2.11	710.80	13.40	0.00	0.00	0.00	1.68	82.70	1.68	82.70	-65.40	789.60	387.38
	0.79	1.05	1.58	0.00	106.86	4.46	0.00	0.00	0.00	0.06	0.62	0.06	0.62	65.18	71.64	28.42
HSP10.24	5.59	7.91	10.97	2.08	645.40	7.00	0.00	0.00	0.00	1.74	87.40	1.74	87.40	58.00	594.40	299.07
	0.83	0.90	1.26	0.02	127.32	1.71	0.00	0.00	0.00	0.07	2.56	0.07	2.56	74.92	65.62	33.33

Femur trabecular

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface iS (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface area density BS/TV (1/mm)	Trabecular pattern factor Tb.Pf (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Trabecular thickness Tb.Th (mm)	Trabecular number Tb.N (1/mm)	Trabecular separation Tb.Sp (mm)
HSP10.6	2.36	0.45	18.82	12.07	26.68	2.58	61.11	11.29	11.36	1.13	1.16	7.89	1.45	0.06	3.19	0.21
	0.05	0.04	1.34	0.13	1.03	0.23	4.05	0.28	2.76	0.10	0.08	0.78	0.12	0.00	0.09	0.01
HSP10.24	2.56	0.29	11.56	12.95	18.53	1.77	66.25	7.29	17.86	1.25	1.07	8.22	1.84	0.06	1.98	0.28
	0.10	0.06	2.67	0.34	2.73	0.31	4.25	1.20	2.61	0.16	0.12	0.43	0.15	0.00	0.37	0.02

Group	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn (1/mm^3)
HSP10.6	12.10	15.93	18.14	2.12	556.60	27.60	0.00	0.02	0.01	1.91	81.18	1.91	81.18	-119.20	703.40	289.24
	2.46	2.94	3.44	0.00	71.94	5.54	0.00	0.01	0.01	0.08	1.34	0.03	1.34	57.07	20.32	14.71
HSP10.24	8.35	10.89	13.58	2.09	481.50	20.25	0.00	0.02	0.03	2.27	88.43	2.27	88.44	8.50	493.25	195.49
	1.23	2.30	2.65	0.01	190.69	7.86	0.00	0.01	0.01	0.13	2.67	0.13	2.67	120.95	87.69	38.98

Tibia cortical

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD
Control	0.77	0.74	95.06	18.27	17.96	14.44	24.97	23.50	1.32	1.22	1.39	0.45	0.11	0.19	0.19	0.00
	0.03	0.06	4.60	1.75	2.01	1.59	3.00	2.99	0.07	0.07	0.31	0.22	0.02	0.04	0.04	0.00
Long term nitrate	0.69	0.68	99.17	15.46	16.22	13.10	24.33	24.13	1.14	1.29	2.28	0.61	0.09	0.18	0.18	2.05
	0.10	0.09	0.12	1.89	2.05	1.54	1.24	1.23	0.08	0.07	0.70	0.23	0.02	0.05	0.05	0.01
Short term nitrate	0.45	0.45	98.20	12.73	13.48	10.59	37.94	37.09	1.52	1.33	1.16	2.11	0.06	0.12	0.12	2.07
	0.15	0.15	0.56	4.09	4.38	3.56	8.67	8.26	0.06	0.10	0.73	0.99	0.02	0.05	0.05	0.03

Tibia cortical

Group	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity [percent] Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity [percent] Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity [percent] Po(tot) (%)	Euler number Eu.N	Mean total crossectional tissue area T.Ar (mm^2)	Mean total crossectional tissue perimeter T.Pm (mm)	Mean total crossectional bone area B.Ar (mm^2)	Mean total crossectional bone perimeter B.Pm (mm)	Crossectional thickness Cs.Th (mm)	Cortical bone thickness Ct.Th (mm)
Control	2.06	252.35	55.43	0.08	0.04	0.03	4.90	0.03	4.94	155.40	0.83	14.60	0.77	15.55	0.10	0.14
	0.01	69.68	10.28	0.02	0.02	0.03	4.61	0.03	4.60	793.39	0.08	1.26	0.03	1.40	0.01	0.01
Long term nitrate	254.50	43.50	0.00	0.14	0.12	0.01	0.72	0.01	0.83	174.00	0.77	14.34	0.76	14.98	0.10	0.14
	35.66	9.96	0.00	0.04	0.03	0.00	0.10	0.00	0.12	30.31	0.04	0.77	0.03	0.84	0.00	0.00
Short term nitrate	582.00	54.00	0.00	0.06	0.04	0.01	1.77	0.01	1.80	345.00	0.74	17.31	0.73	18.61	0.08	0.12
	283.50	20.81	0.00	0.04	0.02	0.00	0.57	0.00	0.56	260.31	0.09	1.19	0.09	1.49	0.02	0.03

Femur cortical

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD
Control	0.79	0.78	98.53	19.85	21.41	16.61	27.66	27.25	1.27	1.34	7.20	0.14	0.08	0.13	0.13	2.07
	0.04	0.04	0.31	0.91	1.26	0.61	2.09	2.01	0.11	0.11	0.46	0.48	0.01	0.02	0.02	0.02
Long term nitrate	0.73	0.72	98.77	18.76	19.89	15.71	27.94	27.58	1.36	1.26	8.19	0.65	0.08	0.13	0.13	2.05
	0.03	0.03	0.23	0.67	0.88	0.70	1.73	1.66	0.13	0.07	0.77	0.30	0.02	0.04	0.04	0.01
Short term nitrate	0.74	0.73	98.54	21.02	22.43	17.28	31.51	31.02	1.39	1.21	5.99	0.32	0.07	0.10	0.10	2.09
	0.05	0.05	0.29	1.09	1.34	0.71	3.30	3.19	0.09	0.13	0.74	0.39	0.01	0.02	0.02	0.02

Femur cortical

Group	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity [percent] Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity [percent] Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity [percent] Po(tot) (%)	Euler number Eu.N	Mean total crossectional tissue area T.Ar (mm^2)	Mean total crossectional tissue perimeter T.Pm (mm)	Mean total crossectional bone area B.Ar (mm^2)	Mean total crossectional bone perimeter B.Pm (mm)	Crossectional thickness Cs.Th (mm)	Cortical bone thickness Ct.Th (mm)
Control	360.12	90.47	0.00	0.19	0.13	0.01	1.34	0.01	1.47	231.47	0.79	16.36	0.78	17.63	0.09	0.11
	113.46	26.31	0.00	0.07	0.04	0.00	0.28	0.00	0.31	99.04	0.04	0.87	0.04	1.18	0.01	0.01
Long term nitrate	442.50	66.17	0.00	0.10	0.08	0.01	1.16	0.01	1.23	316.67	0.72	15.80	0.71	16.68	0.09	0.11
	81.51	6.50	0.00	0.02	0.02	0.00	0.22	0.00	0.23	72.97	0.03	0.60	0.03	0.79	0.01	0.01
Short term nitrate	396.80	97.80	0.00	0.16	0.12	0.01	1.34	0.01	1.46	190.60	0.74	17.45	0.73	18.57	0.08	0.11
	175.68	39.16	0.00	0.02	0.01	0.00	0.29	0.00	0.29	194.58	0.05	0.95	0.05	1.16	0.01	0.01

Table SD.12. Full table of tibia and femur trabecular bone measures for the nitrate treated mouse cohort detailed in chapter 6 with average and standard error noted.

Tibia trabecular

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Trabecular pattern factor Tb.Pf (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Trabecular thickness Tb.Th (mm)	Trabecular number Tb.N (1/mm)	Trabecular separation Tb.Sp (mm)
Control	1.68	0.08	4.93	11.61	6.47	0.78	80.90	3.86	32.08	1.06	1.02	2.42	2.47	0.05	0.98	0.32
	0.12	0.01	0.71	0.71	0.77	0.10	4.39	0.38	3.02	0.09	0.08	0.28	0.13	0.00	0.11	0.02
Long term nitrate	1.79	0.11	6.08	12.06	8.63	0.79	80.94	4.90	34.39	0.98	1.08	3.22	2.60	0.05	1.24	0.26
	0.10	0.02	0.89	0.51	1.17	0.08	1.59	0.69	2.08	0.04	0.10	0.70	0.09	0.00	0.19	0.02
Short term nitrate	1.32	0.09	6.76	10.22	6.76	0.70	80.58	5.24	32.93	1.17	1.14	1.20	2.41	0.05	1.40	0.27
	0.19	0.02	1.22	0.67	1.22	0.07	4.45	0.74	11.15	0.08	0.10	0.69	0.47	0.00	0.25	0.02

Group	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn (1/mm^3)
Control	8.61	11.69	15.15	2.04	347.30	2.25	0.00	0.01	0.06	1.60	95.06	1.60	95.07	166.53	183.02	112.68
	1.55	2.14	2.11	0.01	49.33	0.99	0.00	0.00	0.06	0.12	0.71	0.12	0.71	46.91	15.36	13.94
Long term nitrate	7.60	10.42	13.45	2.03	561.00	1.67	0.00	0.00	0.00	1.68	93.92	1.68	93.92	284.50	278.17	158.05
	1.29	1.55	2.36	0.01	48.76	1.12	0.00	0.00	0.00	0.10	0.89	0.10	0.89	48.69	38.61	24.66
Short term nitrate	8.50	11.31	15.64	2.05	531.40	13.60	0.00	0.01	0.04	1.24	93.24	1.24	93.24	329.80	215.20	171.75
	1.21	1.86	2.57	0.06	246.35	7.45	0.00	0.01	0.03	0.18	1.22	0.18	1.22	219.84	40.43	35.41

Femur trabecular

Group	Tissue volume TV (mm^3)	Bone volume BV (mm^3)	Percent bone volume BV/TV (%)	Tissue surface TS (mm^2)	Bone surface BS (mm^2)	Intersection surface i.S (mm^2)	Bone surface / volume ratio BS/BV (1/mm)	Bone surface density BS/TV (1/mm)	Trabecular pattern factor Tb.Pf (1/mm)	Centroid (x) Crd.X (mm)	Centroid (y) Crd.Y (mm)	Centroid (z) Crd.Z (mm)	Structure model index SMI	Trabecular thickness Tb.Th (mm)	Trabecular number Tb.N (1/mm)	Trabecular separation Tb.Sp (mm)
Control	2.26	0.15	6.36	11.80	10.29	0.82	72.26	4.49	25.94	1.04	1.14	6.33	2.20	0.05	1.21	0.31
	0.13	0.03	1.11	0.45	1.90	0.15	3.34	0.71	3.55	0.13	0.10	0.37	0.17	0.00	0.21	0.02
Long term nitrate	2.27	0.11	4.96	11.70	8.76	0.58	77.13	3.85	31.36	1.19	1.05	7.24	2.45	0.05	0.98	0.30
	0.20	0.01	0.38	0.71	1.27	0.07	4.05	0.42	3.90	0.13	0.06	0.69	0.17	0.00	0.10	0.02
Short term nitrate	2.29	0.15	6.68	11.93	13.21	0.77	82.66	5.77	27.23	1.09	1.05	5.14	2.16	0.05	1.56	0.30
	0.16	0.03	1.08	0.54	3.15	0.11	7.02	1.46	4.09	0.17	0.09	0.72	0.24	0.00	0.39	0.02

Group	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3	Fractal dimension FD	Number of objects Obj.N	Number of closed pores Po.N(cl)	Volume of closed pores Po.V(cl) (mm^3)	Surface of closed pores Po.S(cl) (mm^2)	Closed porosity (percent) Po(cl) (%)	Volume of open pore space Po.V(op) (mm^3)	Open porosity (percent) Po(op) (%)	Total volume of pore space Po.V(tot) (mm^3)	Total porosity (percent) Po(tot) (%)	Euler number Eu.N	Connectivity Conn	Connectivity density Conn.Dn (1/mm^3)
Control	18.57	21.79	25.69	2.06	513.38	11.68	0.00	0.01	0.01	2.11	93.64	2.11	93.64	263.97	261.10	115.34
	2.76	3.09	3.97	0.02	176.15	3.94	0.00	0.00	0.01	0.11	1.11	0.11	1.11	123.14	73.10	30.36
Long term nitrate	22.60	26.07	34.82	2.04	550.33	9.17	0.00	0.01	0.04	2.16	95.04	2.16	95.04	308.83	250.67	112.94
	6.29	7.48	9.99	0.02	109.49	3.88	0.00	0.00	0.03	0.19	0.38	0.19	0.38	58.71	59.30	24.11
Short term nitrate	14.67	16.79	19.49	2.08	554.40	15.20	0.00	0.01	0.03	2.14	93.32	2.14	93.32	7.20	562.40	254.20
	2.54	2.74	3.28	0.03	159.47	8.76	0.00	0.01	0.03	0.15	1.08	0.15	1.08	227.41	350.84	166.13

Appendix

A.1 Poster – Experimental Biology, San Diego (2014)

A.2 Poster - Characterising musculoskeletal changes throughout age in C57Bl/6 mice, British Society of Research into Ageing, Liverpool (2014)

A.3 Poster - Characterising musculoskeletal changes throughout age in C57Bl/6 mice and male wild mice, CIMA annual meeting, Sheffield (2015)

A.4 Poster - Characterising musculoskeletal changes throughout age in C57Bl/6 mice and wild mice, CIMA/CMAR joint meeting, Nottingham (2015)

A.5 Poster – Characterising musculoskeletal changes throughout age in C57Bl/6 mice Faculty of Health and Life sciences Poster day, Liverpool (2016) *Prize winning*

A.6 Poster - Creating an in vivo model system to measure age-related changes in musculoskeletal tissues and test interventions to improve musculoskeletal function, British Society of Research into Ageing, Durham (2016)

UNIVERSITY OF
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Musculoskeletal changes in Superoxide Dismutase 1 (SOD1) null mice

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INTRODUCTION

As we age we experience a loss of muscle & bone function leading to frailty & a reduction in quality of life [1, 2]. Despite the inevitability of these age related changes and the large input into researching this area, there has been no clear explanation behind the causes. There is a strong indication that changes in muscle and bone, along with all other musculoskeletal tissues are related and conditions could be caused by a functional decline in only one tissue [8]. Studies from our group have shown that rapid, premature muscle loss can be achieved through a global loss of the antioxidant enzyme copper zinc superoxide dismutase (CuZnSOD) in mice (Sod1-/- mice) when compared to wild type mice [22]. This is a reliable model of premature muscle loss and ageing reported in numerous papers. SOD1 can be rescued in the nerve of SOD1-/- mice [28]. Rescue mice show 25% expression of SOD1 compared to wild type in nerves only [28]. This it is enough to reverse the muscle effects of SOD1-/-. The effect on bones have not yet been elevated.

RESULTS

Fig 2. Graph showing average weight of Gastrocnemius muscle +/- SEM in male mice aged 3 month and 9 month. Lines represent wild type, SOD1 knockout and SOD1-/-.

Fig 1. Schematic detailing SOD1 position and function within the cell

MicroCT data
SOD1 Model

Bone Mineral Density
Trabecular Thickness
Trabecular Number
Trabecular Separation

Bone Surface Density
Trabecular Thickness
Trabecular Number
Trabecular Separation

Wild Type   SOD1 -/-

A   B

C   D

E   F

Fig 3. Images showing compressive forces acting on the tibia in age matched 10 month wild type and SOD1-/- mice. A and B show whole tibial forces, C and D show forces acting upon trabecular bone only and E and F show forces on single trabeculae.

AIMS

The aims of this experiment were;

- To use the SOD1-/- mice investigate how a global rise in ROS caused by a loss of CuZnSOD effect both muscle and bone tissue.
- To use finite element analysis, a computational approach, to model how these changes may lead to an ageing phenotype within musculoskeletal tissues.
- To use a second mouse model to rescue the SOD1 enzyme in the nerves of SOD1-/- mice to explore whether the musculoskeletal system reverts to match wild type conditions

METHODS

Anterior tibialis, Extensor Digitorum longus, Soleus and Gastrocnemuis muscles were dissected and weighed from 10 month old and 25 month old C56 BL6 mice and in 10 month old SOD1 null mice and 10 month old nerve rescue. Tibia and femur were also dissected keeping knee joint intact.

This process was CuZnSOD (Sod1-/-) KO mice were generated via the insertion of LoxP sites into the introns flanking both exons 3 and 4 of SOD1 with selection via a neomycin cassette creating whole body mouse knock-out on a C57BL6 background, by the Transgenic Animal Model Core at the University of Michigan.

MicroCT analysis was performed on dissected bone tissue via Skyscan 1172 (Skyscan, Belgium) using a 0.5 Al filter setting. Height was altered to centralise region of interest and pixel size adjusted to 4.3μm. Scans were taken using medium camera setting, 0.7 rotations and averaging 2x 180° scan.

NRecon was used to reconstruct scans. Once reconstructed CTan was used to analyse each scan. Once reconstructed, 3D measurements including bone volume and density and trabecular analysis were performed.

Finite element analysis of bone tissue

Label maps were created from Tibial microCT scan data and viewed in Amira software. Using PreView, material properties were added. Final model included compartmentalised sections for cortical bone (Young's modulus – 1.79E+010, Poisson's Ratio – 0.3), trabecular bone (Young's modulus – 1.50E+010, Poisson's ratio – 0.3) and the growth plate (Bulk modulus – 856000). Rigid bodies were added at this stage to simulate the Tibialis anterior and posterior, EDL and body weight plus traversing muscles such as the Gastrocnemius. Forces were applied to each rigid body based on forces from the literature and data from our lab. Model was solved using FE Bio and viewed in PostView to analyse strain locations.

CONCLUSIONS

This is the first study to examine the sub-cellular sites (mitochondrial or extramitochondrial) that modulate cytosolic superoxide in skeletal muscle at rest and during contractile activity. Our data indicate that when superoxide production within the intermembrane space was grossly excessive, intact mitochondria from single muscle fibres released superoxide to the sarcoplasm through the VDAC & Bax channels of the outer mitochondrial membrane. Protein expression of the Bax enzyme in the cytosolic compartment of skeletal muscle was confirmed but inhibition of the enzyme did not modify DHE oxidation. In contrast, NADPH oxidase inhibition reduced DHE oxidation both at rest and during contractions. Collectively, these data provide direct evidence that NADPH oxidase located on the membrane and transverse tubule system is the major modulator of cytosolic superoxide production at rest and following contractile activity in skeletal muscle.

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Characterising musculoskeletal changes throughout age in C57Bl6 mice

Gareth A. Nye[1], Adam P. Lightfoot[1], Eithne Comerford[1], Rob van't Hoff[1], Jane Hurst[1], Ilaria Bellantuono[2], Nathan Jeffery[1], Anne McArdle[1].
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[1.] Department of Musculoskeletal Biology, University of Liverpool and MRC-Arthritis Research UK Centre for Integrated Research into Musculoskeletal Ageing (CIMA); [2.] Academic Unit in Bone Biology, University of Sheffield.

INTRODUCTION

As we age, we lose muscle and bone mass and function, leading to frailty and a reduction in quality of life (1, 2) but despite the apparent inevitability of these age-related changes, no clear mechanism has been identified. Data suggest that changes in muscle and bone (as well as other musculoskeletal tissues) are related and musculoskeletal dysfunction could be caused by a functional decline in only one tissue (3). Without detailed analysis and characterisation of musculoskeletal ageing, we can not begin to sufficiently manipulate the onset and/or development of any detrimental changes

AIMS

The aims of this experiment were;
- To characterise how ageing effects musculoskeletal tissues in the C57Bl6 mouse.
- To characterise how ageing effects musculoskeletal tissues in mice with genetic/environmental modifications known to improve lifespan.
- To devise timely pharmacological interventions to manipulate the onset and/or development of any detrimental changes in C57Bl6 mice.

METHODS

Male C57Bl6 mice were culled and weighed at a range of ages (3, 6, 9, 12, 18, 24 and 28 months old). Blood, organs, hind limb muscles (anterior tibialis, extensor digitorum longus, soleus and gastrocnemius muscles) and bones were dissected weighed and stored for further analysis.

Wild mice were culled and weighed at 6, 12, 24 and 31 months of age and the same methods applied.

MicroCT Scanning
MicroCt analysis was performed on dissected tibia, femur and spine via Skyscan 1272 (Skyscan, Belgium) using a 0.5 Al filter setting. Height was altered to centralise the region of interest and pixel size adjusted to 4.3µm. Scans were taken using medium camera setting, 0.7 rotations and averaging 2x 180° scan. NRecon was used to reconstruct scans. Once reconstructed CTan was used to analyse each scan. Once reconstructed, 3D measurements including bone volume and density and trabecular analysis were performed.

Cytokine Analysis
Blood was centrifuged at 12,000 rpm for 10 minutes and serum extracted. Cytokine analysis was performed through Luminex assays as per the manufacturers instructions.

RESULTS

Fig 1. Average body weight through age of C57BL/6 mice.
* Represents significant values compared to 3 months, # represents significant values compared to 6 months (n=6).

Fig 2. Average weight of 4 hind limb muscles through age of C57BL/6 mice.
* Represents significant values compared to 3 months, # represents significant values compared to 6 months. (n=6)

Fig 3. Average 3D bone measurements in Male C57Bl6 mice through age. * Represents significant values compared to 6 months, # represents significant values compared to 9 months. (n=6)
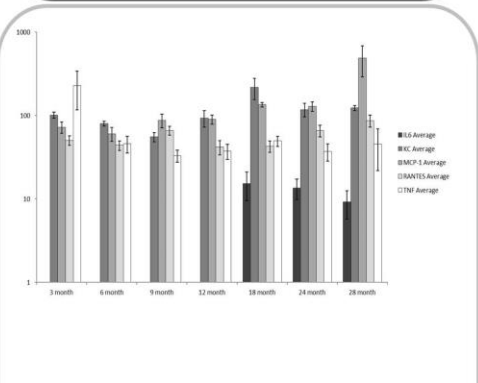
Fig 4. Compressive forces acting on the tibia in age matched 10 month wild type and SOD1-/- mice. A and B show whole tibial forces, C and D show forces acting upon trabecular bone only and E and F show forces on single trabeculae. Red colours indicate high strain, blue colours represent low strain
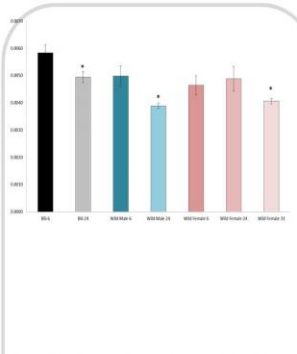
Fig 5. Computer generated model of recorded SOD1-/- force acting on the wild type tibia with microCT data from SOD1-/- mouse overlaid (red). Predicted areas of low strain and force on the tibia are missing from the SOD1-/- tibia microCT analyses.

CONCLUSIONS

- Muscle mass and function was reduced in adult SOD1-/- and old WT mice but not evident in muscles of mKO or in nerve rescued mice (Figure 1).
- Loss of muscle mass by 24 months old was associated with a 37 +/-1% loss of trabecular bone compared with adult mice (Figure 2). The loss of muscle in adult SOD1-/- mice was associated with a 29+/-1% loss of trabecular bone. FE analyses indicated that this loss of muscle led to 50% reduction in tibia strain (Figures 2 and 4).
- This reduction in strain on the tibia was associated with reabsorption of bone in the SOD1-/- mice which explains the reduction in bone quality (Figure 2 and 5).
- Rescuing SOD1 in the nerve reversed the loss of muscle seen in the SOD1-/- (Figure 1), but did not reverse the changes to bone (Figure 2).
- The marginal muscle hypertrophy reported in the muscle specific knockout of SOD1. was not sufficient to improve bone indices (Figure 3).

This is the first study to examine and compare the combined skeletal and muscular changes in old, whole body SOD1 null, muscle specific SOD1 knockout and SOD1 nerve rescue mice.
Modelling based on muscle force data is a novel way of determining changes in bone prior to experimentation.
Through the use of whole body SOD1 null mice, muscle specific knockout mice and mice with SOD1 rescued in the nerve we have demonstrated that rescue of muscle mass when SOD1 remains deficient in bone (nerve rescue mice) is not sufficient to restore bone structure and function

In summary, although the changes in muscle force directly influence bone quality, SOD1 is required for correct growth and maintenance of both the muscle and bone.

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Characterising musculoskeletal changes throughout age in C57Bl6 mice

UNIVERSITY OF LIVERPOOL
INSTITUTE OF AGEING AND CHRONIC DISEASE
Athena SWAN Silver Award

Gareth A. Nye[1], George Sakellariou[1], Adam P. Lightfoot[1], Eithne Comerford[1], Rob van't Hoff[1], Jane Hurst[1], Ilaria Bellantuono[2], Nathan Jeffery[1], Anne McArdle[1].
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CIMA
The Centre for Integrated research into Musculoskeletal Ageing

INTRODUCTION

As we age, we lose muscle and bone mass and function, leading to frailty and a reduction in quality of life (1, 2) but despite the apparent inevitability of these age-related changes, no clear mechanism has been identified. Data suggest that changes in muscle and bone (as well as other musculoskeletal tissues) are related and musculoskeletal dysfunction could be caused by a functional decline in only one tissue (3). Without detailed analysis and characterisation of musculoskeletal ageing, we can not begin to sufficiently manipulate the onset and/or development of any detrimental changes

AIMS

- To characterise how ageing effects musculoskeletal tissues in the C57Bl6 mouse.
- To characterise how ageing effects musculoskeletal tissues in mice with genetic/environmental modifications known to improve lifespan.
- To devise timely pharmacological interventions to manipulate the onset and/or development of any detrimental changes in C57Bl6 mice.

METHODS

Male C57Bl6 mice were culled and weighed at a range of ages (3, 6, 9, 12, 18, 24 and 28 months old). Blood, organs, hind limb muscles (anterior tibialis, extensor digitorum longus, soleus and gastrocnemius muscles) and bones were dissected weighed and stored for further analysis.

Wild mice were culled and weighed at 6, 12, 24 and 31 months of age and the same methods applied.

MicroCT Scanning
MicroCt analysis was performed on dissected tibia, femur and spine via Skyscan 1272 (Skyscan, Belgium) using a 0.5 Al filter setting. Height was altered to centralise the region of interest and pixel size adjusted to 4.3μm. Scans were taken using medium camera setting, 0.7 rotations and averaging 2x 180° scan. NRecon was used to reconstruct scans. Once reconstructed CTan was used to analyse each scan. Once reconstructed, 3D measurements including bone volume and density and trabecular analysis were performed.

Cytokine Analysis
Blood was centrifuged at 12,000 rpm for 10 minutes and serum extracted. Cytokine analysis was performed through Luminex assays as per the manufacturers instructions.

CONCLUSIONS

- Average body weight increased with age in C57Bl6 mice (fig 1).
- Hind limb muscles reached peak mass by 9 months and were all significantly reduced by 28 months. Larger muscles significantly reduced by 24 months (fig 2a).
- Although muscle mass did not decline until 24 months, loss of fibres occurred from 12 months (data not shown).
- Bones measures showed differing patterns through age – tibia peaked at 6 months and declined slowly, femur peaked at 9 months and declined quickly (fig 4).
- Changes in bone appears to be connected to changes in muscles that insert into them (fig 2a, 4).
- Changes in cytokine levels may play a part in the musculoskeletal changes we see in ageing (fig 5).
- Increased activity did not prevent the age related loss of muscle mass (fig 3).
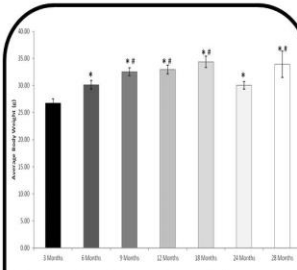
RESULTS

Fig 1. Average body weight through age of C57BL/6 mice.
* Represents significant values compared to 3 months, # represents significant values compared to 6 months (n=6).
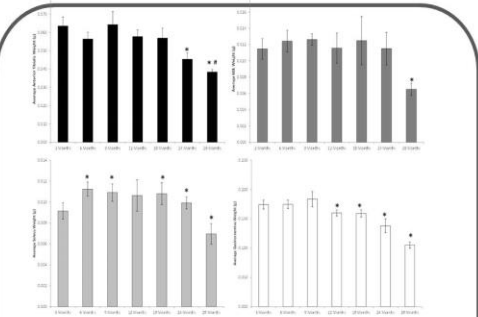

Fig 2a. Average mass of 4 hind limb muscles through age of C57BL/6 mice.
* Represents significant values compared to 3 months, # represents significant values compared to 6 months. (n=6)

Fig 3. Average mass of *gastrocnemius* muscles as proportion of body weight at 6 and 24 months of age in C57BL/6 mice and wild mice with increased activity.
* Represents significant values compared to 6 months

Tibial trabecular number
Femoral trabecular number
Tibial trabecular thickness
Femoral trabecular thickness
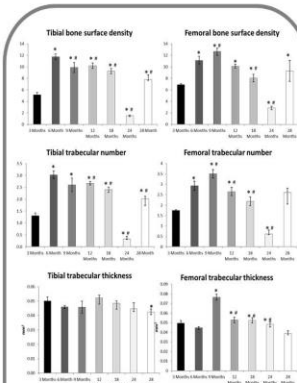Tibial bone surface density
Femoral bone surface density

Fig 4a. Average 3D bone measurements in Male C57/Bl6 mice through age. * Represents significant values compared to 6 months, # represents significant values compared to 9 months. (n=6)
Fig 4b. Comparable microCT images of femur at 9, 18 and 24 months in C57Bl6 mice

Fig 5. Mean values for 5 major cytokines found within blood serum through age. Data shown on a logarithmic scale.

Characterising musculoskeletal changes throughout age in C57Bl6 mice

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AIMS

- To characterise how ageing effects musculoskeletal tissues in the C57Bl6 mouse.
- To characterise how ageing effects musculoskeletal tissues in mice with genetic/environmental modifications known to improve activity.
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MicroCT Scanning
MicroCt analysis was performed on dissected tibia, femur and spine via Skyscan 1272 (Skyscan, Belgium) using a 0.5 Al filter setting. Height was altered to centralise the region of interest and pixel size adjusted to 4.3µm. Scans were taken using medium camera setting, 0.7 rotations and averaging 2x 180° scan. NRecon was used to reconstruct scans. Once reconstructed CTan was used to analyse each scan. Once reconstructed, 3D measurements including bone volume and density and trabecular analysis were performed.
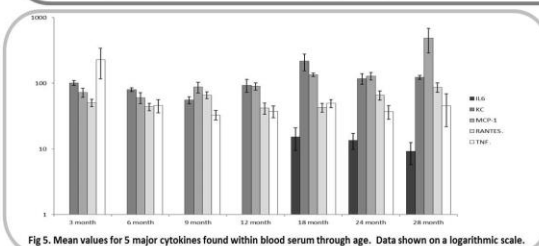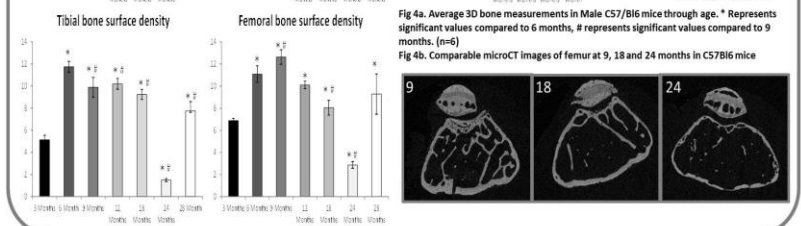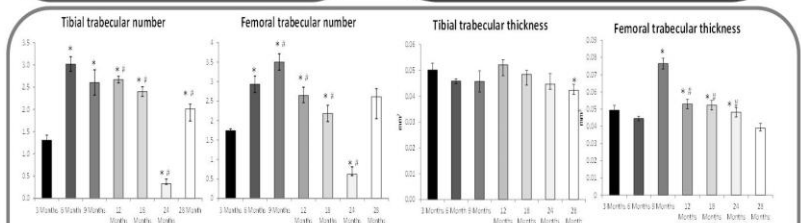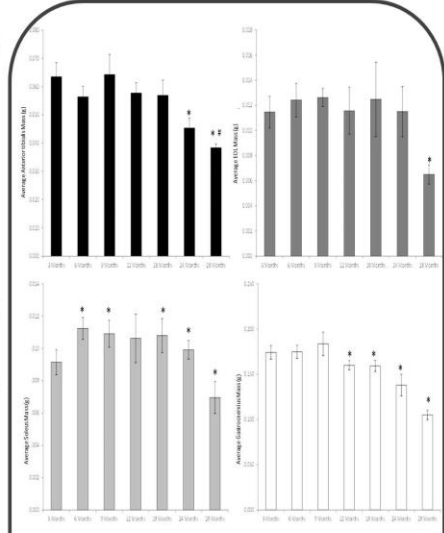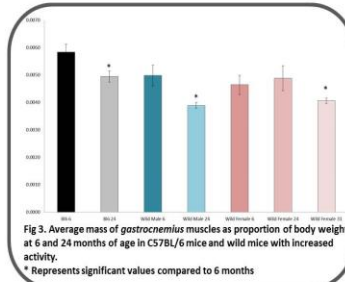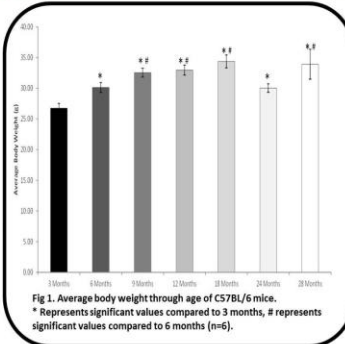
Cytokine Analysis
Blood was centrifuged at 12,000 g for 10 minutes and serum extracted. Cytokine analysis was performed through Luminex assays as per the manufacturers instructions.

CONCLUSIONS

- Average body weight reached a peak at 9 months of age in C57Bl6 mice (fig 1).
- Hind limb muscles reached peak mass by 9 months and were all significantly reduced by 28 months. Larger muscles significantly reduced by 24 months.
- When taken as a proportion of body weight, all muscle decline but this is significant earlier in larger muscles (fig 2).
- Although muscle mass did not decline until 24 months, loss of muscle fibres occurred from 12 months (data not shown).
- Bones measures show a rapid decline between 12 and 24 months in C57Bl6 mice but this was earlier in wild male mice (fig 4).
- Changes in cytokine levels may play a part in the musculoskeletal changes we see in ageing (fig 5).
- Increased activity in wild mice did not prevent the age related loss of muscle mass (fig 3) or loss of trabecular bone (fig 4).

RESULTS

Fig 1. Average body weight through age of C57BL/6 mice. * Represents significant values compared to 3 months, # represents significant values compared to 6 months (n=6).

Fig 2. Average weight as a proportion of average body weight of male C57 Bl6 mice through life in four hind limb muscles (+/- SEM)(n=6). A shows anterior tibialis, B shows EDL, C shows soleus, D shows gastrocnemius. * represents significant values compared with 3 month value, # represents significant values compared with 24 month value.

Fig 3. Average gastrocnemius weight as a proportion of average body weight of male C57 Bl6 & male wild mice at 6, 12 & 24 months of age (+/- SEM)(n=6). * Represents significant values compared with age matched C57 Bl6 value.

Fig 4a. Average 3D bone measurements in C57 Bl6 & male wild mice (+/- SEM)(n=6). * Represents significant values compared to age matched C57 Bl6 value.
Fig 4b. Comparable microCT images of femur at 9, 18 and 24 months in C57Bl6 mice
Fig 4c. Percentage trabecular bone loss in tibia and femur of male C57 Bl6 and wild mice between 6 and 24 months of age.
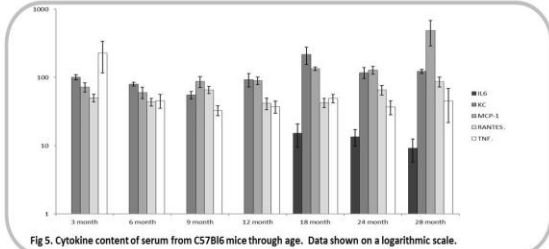
C57 Bl6	Tibia Trabecular Number	% Change
6 Months	2.95	
24 Months	1.33	54.70
	Femur Trabecular Number	
6 Months	2.99	
24 Months	1.34	55.14

Wild Male	Tibia Trabecular Number	% Change
Wild male 6	2.35	
Wild male 24	0.30	87.10
	Femur Trabecular Number	
Wild male 6	2.72	
Wild male 24	0.29	89.17

Fig 5. Cytokine content of serum from C57Bl6 mice through age. Data shown on a logarithmic scale.

ACKNOWLEDGEMENTS

[1.] Effects of ageing on the motor unit. Larsson L, 1995. [2.] Sarcopenia: causes, consequences, and preventions. Marcell TJ, 2003. [3.] A path model of sarcopenia on bone mass loss in elderly subjects. Rondanelli M, 2014. [4.] Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. Muller FL, 2006. [5.] Neuron-specific expression of CuZnSOD prevents the loss of muscle mass and function that occurs in homozygous CuZnSOD-knockout mice. Sakellariou GK, 2014.

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Characterising musculoskeletal changes throughout age in C57Bl6 mice

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CIMA
The Centre for Integrated research into Musculoskeletal Ageing

INTRODUCTION

As we age, we lose muscle and bone mass and function, leading to frailty and a reduction in quality of life (1, 2) but despite the inevitability of these changes, no clear mechanism has been identified. Data suggests that musculoskeletal dysfunction could be caused by a functional decline in only one tissue (3). Without detailed analysis and characterisation of musculoskeletal ageing, we can not begin to sufficiently alter the onset and/or development of detrimental changes with appropriate interventions.

AIMS

- To characterise how ageing effects musculoskeletal tissues in the C57Bl6 mouse.
- To characterise how ageing effects musculoskeletal tissues in mice with genetic/environmental modifications known to improve activity.
- To devise timely pharmacological interventions to manipulate the onset and/or development of any detrimental changes in C57Bl6 mice.

METHODS

Male C57Bl6 mice were culled at a range of ages (3, 6, 9, 12, 18, 24 and 28 months old). Hind limb muscles, tendons and bones were dissected and muscles weighed. Tendons were imaged at 100 μm intervals using a Nikon Eclipse Ci microscope equipped with a 10x/0.30 lens and a Nikon DS-Fi2 camera.

Male wild mice were culled and weighed at 6, 12 and 24 months of age and the same methods applied.

MicroCT Scanning

MicroCT analysis was performed on dissected tibia, femur and spine via Skyscan 1272 (Skyscan, Belgium) using a 0.5 Al filter setting. Height was altered to centralise the region of interest and pixel size adjusted to 4.3μm. Scans were taken using medium camera setting, 0.2 rotations and averaging 2x 180° scan. Nrecon was used to reconstruct scans. Once reconstructed CTan was used to analyse each scan. Once reconstructed, 3D measurements including bone volume and density and trabecular analysis were performed.

Joint Analysis

Bones transferred to decalcification solution (EDTA, pH 7), left at room temperature non agitated for 4 weeks and embedded in paraffin coronally. Joints were cut on HM3555 microtome. Sections were stained for H&E and Safranin-O.

CONCLUSIONS

- Hind limb muscles reached peak mass by 9 months and all significantly reduced by 28 months. Larger muscles significantly reduced by 24 months.
- This is still true when taken as a proportion of body weight, but larger muscles were significant earlier (fig 1).
- Although muscle mass did not decline until 24 months, loss of muscle fibres occurred from 12 months (fig 2).
- Bones measures show a lifelong decline in C57Bl6 mice in two bone sites but the femur appeared to decline earlier (fig 3).
- Increased activity in wild male mice appeared to accelerate trabecular bone loss (fig 4).
- Tendons appeared to be less affected by age and displayed no significant changes in cross sectional area (fig 5) despite changes in muscle mass.
- Age related changes in cartilage are apparent and may be related to changes in bone (fig 5).

The aim was to characterise changes that occur in muscle, bones, tendons and joints across the life course of C57Bl6 mice, to allow the testing of interventions to manipulate onset and/or development of detrimental changes. Muscle mass and bone measures peak in Bl6 mice at 6-9 months and declines are evident by 24 months old. Computer models showing strain across the tibia demonstrated a close association between the two tissues leading to a compounded effect on function. Data provided time points to develop interventions to preserve musculoskeletal function. This model has been applied to determine effects of differing mouse strains, transgenic mice and dietary interventions.

RESULTS

Fig 1. Average weight as a proportion of average body weight of male C57 Bl6 mice through life in four hind limb muscles (+/- SEM)(n=6). A shows anterior tibialis, B shows EDL, C shows soleus, D shows gastrocnemius. * represents significant values compared with 3 month value, # represents significant values compared with 24 month value.

Fig 2. A) Average number of muscle fibres from anterior tibialis at 6, 12 & 24 months (+/- SEM)(n=6). * represents significant values compared to 12 month.
B) Average anterior tibialis muscle fibre CSA at 6, 12 & 24 months (+/- SEM)(n=6). * Represents significant values compared with 12 month.
C) Representative images of anterior tibialis fibres stained for measurement

Fig 5A. Average cortical bone measures for tibia (black) and femur (grey) bones through age in C57BL/6 mice (+/- SEM)(n=6). * Represents significant values compared to 3 month value.
Fig 3B. Average trabecular bone measures for tibia (black) and femur (grey) bones through age in C57BL/6 mice (+/- SEM) (n=6). * Represents significant values compared to 3 month value.

Fig 4A. Representative sagittal microCT images of the femur from age-matched C57BL/6 and wild male mice through age.
Fig 4B. Percentage change in trabecular bone number in two bone sites in C57BL/6 and wild male mice through age.

Fig 5 A,B,C. Representative tendon images from 6 month (A) and 24 month (B) posterior tibialis tendon from C57BL/6 mice through age with average cross-sectional area through age (C).
Fig 5 D,E. Representative images taken from the knee joint of 6 month (D) and 24 month (E) C57BL/6 mice. Sections stained with Safranin-O.

Creating an in vivo model system to measure age related changes in musculoskeletal tissues and test interventions to improve musculoskeletal function

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As we age, we lose muscle and bone mass and function, leading to frailty and a reduction in quality of life (1, 2) but despite the inevitability of these changes, no clear mechanism has been identified. Data suggests that musculoskeletal dysfunction could be caused by a functional decline in only one tissue (3). Without detailed analysis and characterisation of musculoskeletal ageing, we can not begin to sufficiently alter the onset and/or development of detrimental changes with appropriate interventions.

AIMS

- To characterise how ageing effects musculoskeletal tissues in the C57Bl6 mouse.
- To characterise how ageing effects musculoskeletal tissues in mice with genetic/environmental modifications known to improve activity.
- To devise timely pharmacological interventions to manipulate the onset and/or development of any detrimental changes in C57Bl6 mice.

METHODS

Male C57Bl6 mice were culled at a range of ages (3, 6, 9, 12, 18, 24 and 28 months old). Hind limb muscles, tendons and bones were dissected and muscles weighed.

Male SOD1-/- and mKO mice were produced as published (4) to remove the antioxidant enzyme SOD1 and culled and weighed at 9 months of age and the same methods applied.

Male mice undertook daily 1mM sodium nitrate interventions from either 9 months of age or 18 months of age. Both ended at 24 months of age and the same methods applied.

MicroCT Scanning
MicroCt analysis was performed on dissected tibia, femur and spine via Skyscan 1272 (Skyscan, Belgium) using a 0.5 Al filter setting. Height was altered to centralise the region of interest and pixel size adjusted to 4.3μm. Scans were taken using medium camera setting, 0.2 rotations. NRecon was used to reconstruct scans.

FEA Modelling
Label maps were created from 9 month tibial microCT scan data and viewed in Amira software. Using PreView, material properties were added. Rigid bodies were added at this stage to simulate the attached and traversing muscles with forces from the literature and data from our lab. Model was solved using FE Bio and viewed in Postview to analyse strain locations.

CONCLUSIONS

- Hind limb muscles reached peak mass by 9 months and all significantly reduced by 28 months. Larger muscles were significantly reduced by 24 months.
- This remains significant when taken as a proportion of body weight, larger muscles showed reductions earlier (fig 1).
- Muscle forces cause strain on both cortical and trabecular bone (fig 2).
- Bones show a lifelong decline in C57Bl6 mice at two bone sites but both show differential ageing patterns (fig 3).
- Loss of the antioxidant enzyme SOD1 leads to loss of muscle weight, force and lowers bone quality. Muscle specific knockout improves muscle weight but does not improve bone (fig 4).
- Long term treatment with sodium nitrate shows improved muscle weight but no significant changes with bone. Short term treatment shows no change in the muscle but increases trabecular number.
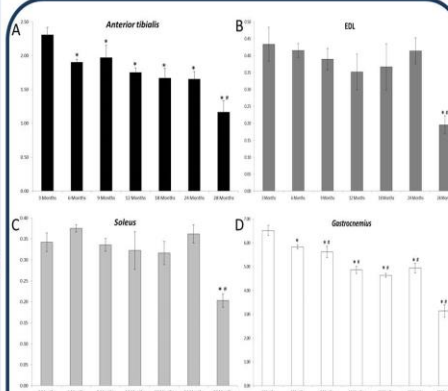
RESULTS

Fig 1. Average weight (mg) as a proportion of average body weight of male C57 Bl6 mice through life in four hind limb muscles. A shows *anterior tibialis*, B shows EDL, C shows *soleus*, D shows *gastrocnemius*. (Mean +/- SEM, n=6) * p <0.05 compared with mice aged 3 months, # p <0.05 compared with mice aged 6 months.
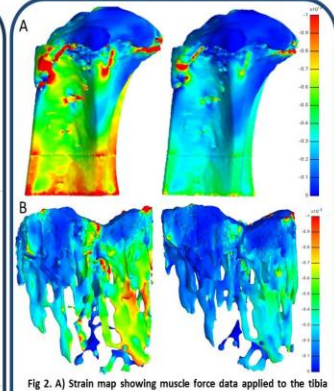

Fig 2. A) Strain map showing muscle force data applied to the tibia cortical bone
B) Strain map showing muscle force data applied to the tibia trabecular bone
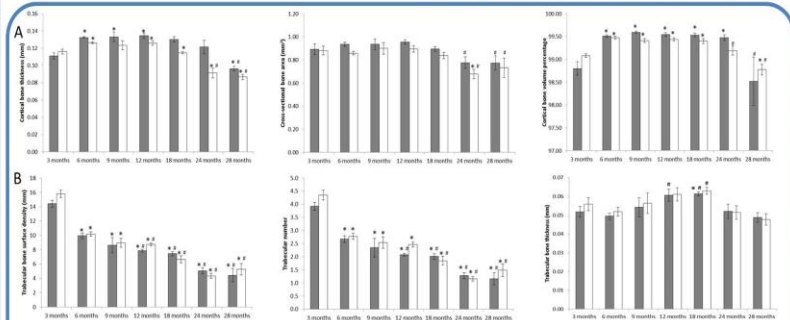Red indicates areas of high strain, blue areas of low strain. Force data taken from 9 month time point

Fig 3A. Average cortical bone measures for tibia (Grey) and femur (White) bones through age in C57BL/6 mice (Mean +/- SEM, n=6) * p <0.05 compared with mice aged 3 months, # p <0.05 compared with mice aged 6 months.
Fig 3B. Average trabecular bone measures for tibia (black) and femur (grey) bones through age in C57BL/6 mice (Mean +/- SEM, n=6) * p <0.05 compared with mice aged 3 months, # p <0.05 compared with mice aged 6 months.
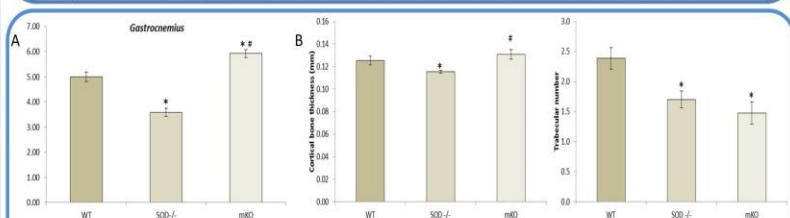

Fig 4A. Average weight (mg) of the gastrocnemius muscle as a proportion of average body weight from male C57 Bl6 (WT), male whole body SOD1 knockout (SOD-/-) and male muscle specific SOD1 knockout (mKO) mice at 10 months of age. (Mean +/- SEM, n=6) * p <0.05 compared with WT mice, # p <0.05 compared with SOD1-/- mice.
Fig 4B. Average cortical bone thickness and trabecular number from the tibia of male C57 Bl6 (WT), male whole body SOD1 knockout (SOD-/-) and male muscle specific SOD1 knockout (mKO) mice at 10 months of age. (Mean +/- SEM, n=6) * p <0.05 compared with WT mice, # p <0.05 compared with SOD1-/- mice.
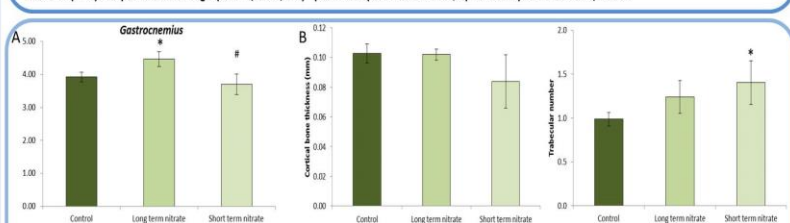

Fig 5A. Average weight (mg) of the gastrocnemius muscle as a proportion of average body weight from male C57 Bl6 (Control), male C57 Bl6 mice treated with sodium nitrate for 18 months (Long term nitrate) and male C57 Bl6 mice treated with sodium nitrate for 6 months (Short term nitrate) aged 24 months of age. (Mean +/- SEM, n=6) * p <0.05 compared with control mice, # p <0.05 compared with mice treated for 18 months.
Fig 5B. Average cortical bone thickness and trabecular number from the tibia of male C57 Bl6 (Control), male C57 Bl6 mice treated with sodium nitrate for 18 months (Long term nitrate) and male C57 Bl6 mice treated with sodium nitrate for 6 months (Short term nitrate) aged 24 months of age. (Mean +/- SEM, n=6) * p <0.05 compared with control mice