MECHANICAL PROPERTIES OF BONE DUE TO SOST EXPRESSION:
A 3-POINT BENDING ASSESSMENT OF MURINE FEMURS

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TITLE: Mechanical Properties of Bone Due to SOST
Expression: A 3-Point Bending Assessment of Murine
Femurs

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ABSTRACT

Mechanical Properties of Bone Due to SOST Expression:

A 3-Point Bending Assessment of Murine Femurs

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Sclerostin, a protein coded for by the SOST gene, is an osteocyte-expressed negative regulator of bone formation. The absence of SOST in the genome may have an effect on bone formation both during skeletal maturation and full maturity. This study attempts to determine significant differences in the mechanical properties of bone that expresses SOST compared to bone that does not. One hundred femur samples from 6, 8, and 12 month old mice were obtained from Lawrence Livermore National Labs and loaded until failure using three-point bending. Results showed significant differences in treatment group effects for cross sectional area, yield force, and ultimate force. SOST knockout (KO) mice were found to have significantly higher values for these properties in comparison to transgenic (TG) and wildtype (WT) littermates. In addition, there was a noted effect dependent on the primary axis of loading, anterior-posterior versus medial-lateral. Lastly, data from this study support the existing hypothesis that there is no systematic side-to-side (left-right) difference in bone formation. This data may aid understanding of the role SOST has in bone formation. If the structural integrity and quality of bone resulting from the removal of the SOST gene is shown to be comparable to that of normal, healthy bone, the use of gene therapy to combat diseases/disorders such as osteoporosis may lead to important contributions to medical therapy.

Keywords: SOST, three-point bending, mechanical properties

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I. INTRODUCTION

1.1 Bone Physiology

The main functions of bone include locomotion, support of organs, protection, attachment sites for muscles, mineral homeostasis, and blood cell formation [1]. According to the primary locomotive function of the skeleton, mechanical competence of bones represents the ultimate measure of bone phenotype [2]. Structural rigidity of the skeleton is principally maintained by a mechanosensory feedback system that senses the loading induced deformations within the bones and copes with the locomotive challenges through modifications in bone size and shape [3]. New bone is laid on regions that are subject to loading that exceeds the customary loading range, whereas bone is removed from regions that experience reduced loading well below the customary loading range [4]. The cellular mechanisms involved in directing new bone formation to the high strain regions of a loaded bone are unclear. Further understanding of these mechanisms could provide an attractive target for pharmaceutical intervention aimed at mimicking the adaptive response to loading [5].

A simple yet effective means to enhance bone mass and architecture is through mechanical stimulation of the resident bone cell population [6-7]. Mechanical loading (e.g. exercise) improves bone mass and strength by stimulating the addition of new bone onto surfaces experiencing high strains, whereas surfaces that experience small strains remain largely quiescent [8]. In line with the established functional bone adaptation to loading, first described as Wolff's Law, bones – as locomotive organs – adapt their structural rigidity and strength to incident loading through changes in structural

particulars (mineral mass, geometry, architecture, material properties) [4, 9]. Simplified, form follows function [10].

1.2 Bone Structure

Bones are highly calcified structures which provide a support and protective function for the rest of the body. In addition to the structural framework and protective functions of bone, it also serves as a mineral reservoir for the rest of the body. Long bones make up the majority of bones and primarily consist of two different types; cortical and trabecular. These types of bone have differing structures which affect associated mechanical properties.

1.2.1 Cortical Bone

Cortical bone (Figure 1.1), also referred to as compact bone, is dense bone found in the shafts or diaphyses of long bone. This bone is characterized by low, 5-10%, porosity and is often tested to determine the mechanical properties of bone. Cortical bone is primarily made up of primary or secondary bone. The ratio of primary to secondary bone greatly affects the properties of the bone. The main structural component of this type of bone is the osteon which gives bone the ability to withstand torsion and bending stresses [11]. The remodeling of compact bone, or replacement of primary bone with secondary bone (discussed in Section 1.2.3.2), reduces its flexural fatigue strength and resistance to tension, compression, shear, and bending [12].

Figure 1.1. Representation of compact (cortical) and spongy (trabecular) bone in the femur. <http://www.gla.ac.uk/ibls/US/fab/tutorial/generic/bone2.html>

1.2.2 Trabecular Bone

Trabecular bone (Figure 1.1), also referred to as either cancellous or spongy bone, is characterized by largely spongy and porous network. This type of bone is found at the ends or epiphyses of long bones and has a disorganized, random bone structure comprised of interconnected pores containing bone marrow which incorporate approximately 75-95% of trabecular bone volume [11]. Due to its porous structure, this type of bone is much weaker and more compliant than cortical bone. Mineral content and tissue density of cancellous bone have been found to be less than that of cortical bone [13-15]. The mechanical properties (e.g. modulus, strength, etc.) of trabecular bone are also different from those of cortical bone [16-18].

1.2.3 Modeling and Remodeling of Bone

Throughout life, bone is constantly changing. As the bone experiences greater or lesser forces, the overall structure is altered to maintain its level of function while using the least amount of bone material. This process of adaptation occurs in two different phases; modeling and remodeling.

1.2.3.1 Bone Cells

Bone is made up of cellular components which function to either form or resorb bone. Osteoclasts (Figure 1.2) resorb bone and are multinuclear cells formed by the fusion of monocytes which originate in the bone marrow. These cells respond to stresses or lack thereof and erode bone by demineralizing and dissolving the resulting collagen [11].

The voids left behind following resorption by osteoclasts either remain quiescent due to lack of stresses or are filled with new bone formed by osteoblasts. Osteoblasts (Figure 1.2) are mononuclear, cuboidal cells which respond to the presence of mechanical stresses. These cells are differentiated from mesenchymal cells and deposit the organic portion of bone matrix, osteoid [11].

Osteocytes (Figure 1.2) are terminally differentiated cells derived from osteoblasts, which first become embedded and surrounded by osteoid matrix that subsequently mineralizes [19]. These cells compose over 90-95% of all bone cells in the adult mammal [20].

Osteocytes are historically implicated with sensing and initiating mechanical signaling [8]. Thus, osteocytes have been regarded as the “mechano sensor” in bone [21-22].

Figure 1.2. Bone cells and their role in the remodeling cycle. Hill 1998.

1.2.3.2 Modeling versus Remodeling

As long bones develop, they must increase in both diameter and length. However, as different stresses are applied to the bone, the formed bone must be shaped to continue properly performing its intended function. The process of removing bone in certain areas and replacing it with bone in others is known as bone modeling. Modeling as a whole involves independent actions of osteoclasts and osteoblasts, results in a change of bone size and/or shape, and occurs continuously at particular sites before skeletal maturity is reached. As bone experiences stresses, fatigue damage occurs and these regions of the bone must be repaired. The process of repairing resulting damage is known as remodeling (Figure 1.2). This is characterized by interaction between osteoblasts and osteoclasts, little to no change in the size and/or shape of bone, and continuous, episodic occurrence throughout life [11]. Thus, modeling is typically the formation of primary bone as the

body adjusts to the strains of growth, while remodeling creates secondary bone which serves to strengthen and repair damaged bone as fatigue and increased stresses are experienced by the bone following skeletal maturity.

1.3 Bone Disorders

There are a number of disorders which are attributed to altered modeling and remodeling of bone. A few of these disorders of interest to this project are osteoporosis, Van Buchem disease, and Sclerosteosis.

1.3.1 Osteoporosis

A consensus development conference statement defined osteoporosis as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue" [23].

The incident of low bone mass and poor bone structure are two major risk factors for osteoporotic fracture [24-25]. This condition is prevalent in the adult skeleton as bone remodeling becomes less efficient. In addition to skeletal abnormality causes for osteoporotic fracture, a variety of non-skeletal factors contribute to increased fracture risk, including trauma resulting from falls [26].

Increased porosity associated with aging and osteoporosis is largely confined to the endosteal, inner, regions near the neutral axis of bone [11]. In recent years, a number of therapies have been developed to attempt to combat osteoporosis. Thus far there is no treatment that has been established to effectively mitigate the risks of osteoporosis, although recent research is attempting to develop better understanding of the gene networks regulating osteocytes which may provide a more efficient avenue for development of new therapies for osteoporosis [27].

1.3.2 Van Buchem Disease

Van Buchem is a homozygous recessive disorder [28-30] that results in progressive increase in bone density [31]. Van Buchem patients carry a homozygous 52kb non coding deletion ~35kb downstream of SOST (described in Section 1.4) transcript and ~10kb upstream of the downstream gene, MEOX1, on human chromosome 17p21 [29-30, 32]. Patient complications include vast accumulation of bone mass which gives rise to facial distortions, enlargement of the mandible and head, entrapment of the cranial nerves, increase in bone strength, and excessive weight [28-30]. There have been approximately 40 reported cases of Van Buchem disease [33].

1.3.3 Sclerosteosis

Sclerosteosis is a cranio-tubular hyperosteosis that is phenotypically indistinguishable from Van Buchem disease except that it is more severe and occasionally displays syndactyly of the digits, a trait absent in Van Buchem disease [34-37]. Observed intracranial pressure sometimes leads to sudden death [38]. Shared clinical similarities between sclerosteosis and Van Buchem disease, along with their strong genetic linkage to the SOST locus on chromosome 17q12, suggest that they are allelic [39]. There have been less than 100 reported cases of patients experiencing this disease [33].

1.4 SOST/Sclerostin

Sclerostin, a protein coded for by the SOST gene [40], is an osteocyte-expressed negative regulator of bone formation that is structurally most closely related to the DAN/Cerberus family of bone morphogenic protein (BMP) antagonists [41-42]. Mutations in the SOST gene, or in its distant regulatory elements, cause sclerosing bone disorders such as sclerosteosis and Van Buchem disease [29-30, 43].

Currently there is no clear understanding of how the lack of sclerostin promotes osteogenesis; therefore determining its transcriptional regulation is essential to understanding the interconnection between its expression in osteogenic cells and its mechanism of action either as a BMP antagonist [41] or Wnt antagonist [44]. Sclerostin was originally classified as a BMP antagonist because of its sequence homology to other members of the DAN family of cysteine knot proteins, but more recently has been shown to bind low-density lipoprotein receptor related protein 5/6 (LRP5/6) with high affinity [44-46].

Data suggest Wnt/LRP5 signaling is an integral part of the mechanotransduction cascade in normal bone tissue [47]. Recent research provides evidence that sclerostin is an indirect inhibitor of BMP, but specifically antagonizes the Wnt pathway [42] as an antagonist of LRP5, a gene shown to be important as a positive regulator of bone mass [44]. Additional support for the connection between SOST and Wnt signaling pathway is the regulation of both Wnt and SOST by mechanical strain in osteocytes [48].

1.5 Use of Animal Models to Mimic Human Bone Disorders

Due to ethical and logistic concerns, the use of human bones for research testing is limited. The need for effective methods to enhance bone repair persists and appropriate models are needed for evaluating new therapies. Despite problems resulting from their small size, mice have advantages over larger species that justify their introduction as models for skeletal research [49-50]. Genetic engineering has introduced an increasing number of transgenic mouse models with genetically modified skeletal functions [51]. The genetic factors that contribute to susceptibility to bone loss are extremely heterogeneous; therefore murine models that affect bone development and growth can

provide invaluable insights into the molecular mechanisms of progressive bone loss in humans [39].

Multiple bones have been used in mouse models with the tibia and femur being the most prevalent [52]. Although the tibia is easier to access than the femur, the tibia is not an ideal model for fracture studies because of its curved major axis which complicates mechanical testing [53-54]. The femur, however, is able to be positioned on the flat posterior surface, and thus is more conducive to repeatable biomechanical testing. A common concern with testing of murine bones is the small size, and while the murine femur is small in comparison to larger animal models, it is the largest long bone in the body, providing a testable region of cortical bone. Researchers view the femur as an exciting fracture model for modern orthopedic research [52].

1.6 Hybridization of Mice

Researchers are continually creating new knockout (KO) or transgenic (TG) mouse models to explore the genetic basis of various skeletal traits [55]. Sclerostin deficiency in humans has the same phenotypic outcome regardless of sex and the phenotype of SOST KO mice was essentially the same across sexes with differences only in magnitude for some effects [56]. The effects of sclerostin deficiency on bone seen in the SOST KO mice are the opposite of and biologically consistent with the effects described for sclerostin overexpression in transgenic mice (Figure 1.3) [56].

Figure 1.3. Increased BMD in *SOST* KO mice. (A) Whole body radiographs of 4-mo-old female WT and KO mice showing increased radiodensity throughout the skeleton of KO mice. (B) Enlargement of radiographs showing pelvic region. (C) Enlargement of radiographs showing skull. Li et al., 2008.

Lawrence Livermore National Labs, located in Livermore, California, has discovered a novel function for sclerostin during limb development, demonstrating that Van Buchem (VB) disease is caused by the removal of bone-specific distant enhancer elements and is allelic to sclerosteosis [39]. These researchers have characterized human bacterial artificial chromosome (BAC) *SOST* transgenic mice carrying either a normal (*SOST*^{wt}) or an allele with the VB-associated deletion (*SOST*^{vbΔ}). Only the *SOST*^{wt} allele faithfully expresses human *SOST* in the adult bone and has an impact on bone metabolism, consistent with the model that the VB noncoding deletion removes a *SOST*-specific regulatory element [39]. All lines of *SOST*^{wt} transgenic animals reliably expressed human *SOST* in the mineralized bone of neonatal and adult mice (skull, rib, femur) while all *SOST*^{vbΔ} lines had dramatically reduced levels of human *SOST* mRNA

expression. All lines also expressed human SOST in adult kidney and heart (Figure 1.4) [39].

Figure 1.4. Generation and characterization of Van Buchem transgenic mouse models. (A) A 158-kb human BAC (*SOST*^{wt}) spanning *SOST* and *MEOX1* was engineered using in vitro BAC recombination in *Escherichia coli* (Lee et al. 2001) by deleting the 52-kb noncoding region missing in VB patients (*SOST*^{vbΔ}). Human *SOST* expression was analyzed by rtPCR in adult tissues (B), embryos (C), and measured by quantitative rtPCR in E10.5 embryos (D) from two independent lines of each *SOST*^{wt} and *SOST*^{vbΔ} transgene. Loots et al., 2005.

Data from Loots et al. [39] demonstrate that in vivo, the VB allele confers dramatically reduced SOST expression in the adult bone and suggests the vbΔ contains essential bone-specific enhancer elements. This data strengthens evidence that lack of SOST expression in vbΔ is due to a 52kb noncoding deletion opposed to an artifact due to transgene copy number or site-of-integration position effect.

SOST^{wt} mice displayed decreased bone volume and mineral density at skeletal maturity. The *SOST*^{vbΔ} mice were indistinguishable from their non-transgenic litter mates and the

observed osteopenia was gene-dose dependent. The successful hybridization of these mice effectively shows that the SOST gene has an effect on bone formation in the mammalian skeleton, with overexpression of human SOST negatively modulating bone mass once skeletal maturity is reached [39].

1.6.1 Lawrence Livermore National Labs Mouse Treatment Groups

Murine femur samples obtained from Lawrence Livermore National Labs were classified as one of two genotypes; SOST KO (knockout) or SOST TG (transgenic). SOST TG mice carried a bacterial artificial chromosome (BAC 209M4) which resulted in an overexpression of human SOST. Within the genotype grouping of SOST TG, there was a sub-breakdown of TG, DEF (animals with limb defects), and littermate WT (wild type) controls. The classification of TG or DEF was based on the presence of limb defects, which was assumed to be caused by higher levels of SOST transgenic expression in the DEF mice. SOST TG mice were hemizygous, signifying the integration of the BAC at a single site, while SOST DEF mice were offspring from the mating of two SOST TG mice. The SOST DEF mice were presumed to have twice the amount of human SOST expression in comparison to the SOST TG mice. The SOST KO genotype also had a sub-breakdown consisting of KO and littermate WT controls. The SOST KO mice were homozygous for SOST deficiency and displayed high bone mass as previously characterized by Li et al [56]. Wild type mice were the control, non-transgenic littermates of the alleles described above; these mice had normal levels of mouse SOST expression.

1.7 Mechanical Testing of Bone

The mechanical properties of bone can be determined by application of bending, torsional, tensile, compression and shear tests [57]. Although biomechanical testing of bones, similar to any other in vitro measurement, represents an oversimplification of the complexity of the actual in vivo situation, the intention is to test the skeletal structure of interest as closely as possible in terms of the predominant loading environment [9].

Material properties of mouse cortical bone are difficult to measure directly and are often estimated based on engineering theory and measurements of whole bone mechanical properties and cross sectional geometry [58-60]. There may be considerable error in estimating material properties because mouse bones do not meet the ideal conditions of the engineering theory, such as constant geometric and material properties along their length [61-62]. Despite the limitations, bench top testing of mouse bone serves as a useful tool for determining significant differences so long as testing procedures are conducted under consistent conditions.

1.7.1 Three-Point Bending

Mechanical testing can provide researchers with important information regarding properties of bone. A load-deformation test is conducted and results are recorded in deformation as a function of the applied load, typically tension, compression, torsion, or bending [11]. In recent bone research, the most frequently used method for characterization of long bone biomechanical properties of the appendicular skeleton is 3-point bending of the femoral midshaft [2, 9]. This test, coupled with measured geometric dimensions can be extrapolated to give the resulting stress and strain experienced by the bone. From this information, the elastic modulus, stiffness, can be determined. These

measured material properties allow for bone stiffness and strength to be evaluated both with and without normalization for geometric effects.

1.7.1.1 Rational for Testing

The majority of 3-point bending tests are conducted in the anterior-posterior (AP) direction. The femur is usually placed onto the test apparatus on its flat posterior surface and tested in the AP direction due to anatomical and practical reasons [9]. Although this method of testing is considered the current “gold standard”, testing the bone in the medial-lateral (ML) direction would provide useful information regarding the maximum mechanical properties of the femur. Studies have been conducted to determine the feasibility of testing in the ML direction and results from Leppanen et al. [9] show no reason why the ML testing model cannot be applied to the mouse model. According to this study, testing should be carried out in the ML direction, otherwise, there remains a risk that some essential information regarding the effects of any intervention, particularly that of altered mechanical loading, on the femur structure and mechanical competence might be missed. Regarding biological validation, loading induced increase in bone cross sectional moment of inertia (CSMI) and structural strength solely seen in the ML direction provides proof of appropriateness of ML testing direction [9].

Along the principle of Wolff’s Law (previously described in Section 1.1), one can assume that the ML direction, given the widest diameter of the elliptic cross section of the midfemur (Figure 1.5), represents the apparent primary direction of skeletal adaptation to locomotive loading [63]. If true, the common AP testing direction of the femora can be argued suboptimal, and thus the testing should be carried out in the ML direction [9]. This assumption may be incomplete as useful information can be obtained

from both bending directions. The ML direction will provide values in the principle axis of loading and can be taken as the maximum strength of bone, while the AP direction can be used to determine reduced mechanical property limits. These reduced values are useful in the case of impact or trauma occurring in the non-principle axis of loading. Potential fractures may occur in this direction and if only ML data is obtained, inaccurate assumptions may be made regarding the forces which bone can withstand. This is further supported by the finding that whole bone bending provides a measure at the continuum scale and is strongly influenced by porosity [64-65]. Since the porosity of bone may vary between the primary and non-primary axis of loading, conducting testing in both directions is necessary to provide a complete picture of the mechanical properties of bone.

Figure 1.5. Anterior-Posterior (AP) and Medial-Lateral (ML) testing directions.

There has been concern regarding differences between right and left leg analysis. Current approaches make an inherent assumption that right-to-left biomechanical properties and geometry of femora are equal, which may not be entirely accurate [66-67]. However it

can be anticipated that, under normal circumstances, there is no systematic difference between structural and mechanical characteristics of right and left femora [9]. One group attempted to determine the repeatability of biomechanical testing of rat femora by comparing data from left and right femora of nonoperated rats. Following this study, there was no indication of systematic side to side differences between right and left femora [9].

1.7.1.2 Testing Conditions

As described in Section 1.6, 3-point bending has been readily used for the biomechanical testing of bones. There are conditions which are maintained in the majority of these tests including removal of all soft tissue from the bone, wrapping bones in a saline-soaked gauze bandage to prevent dehydration, and storage at -20°C until testing. This procedure has been shown not to affect the mechanical properties of bone [68-69]. It has been shown that the elastic modulus of bone is quite sensitive to its mineral content [70] and that the ionic content of storage baths can alter the mechanical properties of bone [71]. Some fatigue tests which last up to a few hours may not require a calcium buffering bath, however, experiments done at physiologic strains and under the best simulation of in vivo conditions will result in greater clinical significance and thus bone samples should be kept moist while undergoing short testing procedures [72]. Support placement for AP testing is located beneath the distal head of the femur and at 90° rotation from the greater trochanter, while ML testing support placement is located under greater trochanter and at 90° rotation to the distal femur head (Figure 1.6).

Figure 1.6. (A) Three-point bending in the mediolateral direction. Side and top view. (B) Three-point bending in the anteroposterior direction. Side and top view. Leppanen et al., 2006.

Although storage conditions and testing orientations are commonly conducted uniformly between studies, the loading rate and distance between the supports varies between research groups. Loading rates of 1.0 mm/sec [9] and 0.03 mm/sec [73] have been reported in previous studies. Support gap distances also vary, but are within a confined range of values due to the fairly consistent length of murine femurs. Gaps of 6 mm [56], 7 mm [52], and 10 mm [74] have been reported.

1.8 Study Goals

The goals of this study are to determine any mechanical property differences between the various murine treatment groups described in Section 1.6.1, as determined by 3-point bending. Mechanical properties for comparison have been discussed in Section 1.7.1 and include modulus, stress, and strain. Additional goals include determination of the effect of age and bend direction (AP versus ML) on the mechanical properties along with any resulting geometric differences of the tested murine femurs. Data from this study should aid current, ongoing research by providing laboratory data regarding the mechanical

properties of mice hybridized by Lawrence Livermore National Labs. Determination of significant differences in the strength of bone which does or does not express the SOST gene can support useful follow on research which may result in potential new gene therapies targeted for the treatment of osteoporosis.

II. MATERIALS AND METHODS

100 murine hindlimbs were obtained from Lawrence Livermore National Labs (LLNL) located in Livermore, California. Mice were hybridized to display various genotypes and phenotypes (SOST TG/DEF/WT 6 month, SOST TG/DEF/WT 8 month, SOST KO/WT 6 month, SOST KO/WT 12 month) at LLNL (Table 1, full breakdown of mouse numbers and treatments can be found in Appendix A). SOST transgenic (TG), wild type (WT), and deficient (DEF) mice were Friend leukemia virus B (FVB) mixed with C57/Bl/6J, backcrossed 4 generations to C57/Bl/6J while SOST knockout (KO) and wild types (WT) were a mixture of C57/Bl/6J and 129 background, primarily showing body characteristics of the 129 strain. Femurs of both the left and right legs were dissected from the remaining portion of the hindlimb and were cleansed of any remaining soft tissue that may have had an effect on the mechanical testing. Bones were wrapped in phosphate buffered saline (PBS) soaked gauze and stored in individually labeled vials in a sub-zero, -20°C, freezer until the time of testing. Samples were removed from the freezer one at a time and allowed to thaw until ready for testing.

Table 1. Treatment Group Distribution

<u>Treatment Group</u>	<u>Number of Mice</u>	<u>Description</u>
SOST TG 6 month	2	Express human SOST.
SOST DEF 6 month	9	Express twice the amount of human SOST as TG.
WT 1	11	6 month old wildtype littermates of SOST TG/DEF mice. Faithfully express murine SOST.
SOST TG 8 month	2	Express human SOST.
SOST DEF 8 month	4	Express twice the amount of human SOST as TG.
WT 2	4	8 month old wildtype littermates of SOST TG/DEF mice. Faithfully express murine SOST.
SOST KO 6 month	5	Do not express any SOST.
WT 3	6	6 month old wildtype littermates of SOST KO mice. Faithfully express murine SOST.
SOST KO 12 month	5	Do not express any SOST.
WT 4	2	12 month old wildtype littermates of SOST KO mice. Faithfully express murine SOST.
<i>TOTAL</i>	<i>50</i>	-

2.1 Selection of Testing Direction

3-point bending tests are commonly carried out with testing occurring in the anterior-posterior (AP) direction. Since the medial-lateral (ML) diameter of bones is larger and more robust, as it is the principal axis of load bearing, it has been speculated that testing should be done in this direction [9]. To allow for a complete analysis of mechanical property differences in the various bone treatment types femurs were tested in both AP and ML directions (Figure 2.1). Each femur was randomly assigned a bend direction using Minitab 16 Statistical Software resulting in 50 bones being broken AP and 50 bones broken ML. In order to make relevant and accurate comparisons of these testing orientations, one femur from each mouse (right or left) was broken in the AP direction while the other was done in the ML direction.

Figure 2.1: A cross section view of the femur with labeling of anatomical locations. A: Anterior, P: Posterior, M: Medial, L: Lateral.

2.2 Three-Point Bending

Once thawed, bones were measured and marked at the midpoint of the length for identification of probe (load) location under a microscope and placed in a fixture to allow

for three-point bending of samples (Figure 2.4a). The 3-point bending apparatus (Figure 2.2) was linked to a computer and needed calibration to ensure proper testing location (Figure 2.3). A computer program written by Dr. Lanny Griffin was used to calibrate the testing procedure and allowed for customization of testing parameters (testing speed and probe distance moved). Measurement Velocity was set to 20,000 nm/second (1.2 mm/min) and the Max Probe Displacement was 1,000 μm (74). During the testing procedure, bones were kept moist using a phosphate buffered solution to help account for a more physiologic condition of bone. The test was conducted (Figure 2.4b depicts a test in progress), resulting in stored values of force and displacement for the time which bone was contacted by the measurement probe. These values were exported as .FIB files, analyzed using MATLAB, and imported into Microsoft Excel for creation of graphical representations of force and displacement.

Figure 2.2: Three-point bend apparatus and computer used for calibration and data capture.

Figure 2.3: Microscope image of bone place in holding tray during programming of three point bend test.

Figure 2.4: A) Applied force points during a three-point bend test [75]. B) Three-point bend test in progress at time of impact between probe and bone.

Immediately following testing, broken femur halves were removed from the apparatus and bone marrow was removed using fine tipped forceps (Figure 2.5). Bones were then placed in a custom made holding fixture (Figure 2.6) to allow for diameter measurements to be taken under a microscope. Anterior-posterior and medial-lateral inner and outer diameters were measured for later use in data analysis. Measurements were taken with the use of a crosshair reticle and a moveable stage controlled by micromanipulators. The center of the crosshair was positioned at either the inner or outer edge of the bone and the

stage was moved to the opposite edge. The amount of movement was recorded by the micromanipulator and the resulting value was recorded. This process was used opposed to imaging and analysis with Image J due to limitations of the camera/microscope system. Following measurement, bones were wrapped in PBS soaked gauze and re-frozen until embedding in epoxy, which would allow for additional analyses to be performed.

Figure 2.5: Marrow extraction following three-point bend test using fine tip forceps.

Figure 2.6: Custom fabricated bone holding fixture for analysis of fracture surface.

2.3 Data Analysis

Design parameters of a MATLAB code were developed and the working code was written with the help of an undergraduate student, Brian Daugherty. Individual mouse data was compiled based on treatment type and compared to identify important differences in mechanical properties. Load maximum and yield points were determined from the force data obtained during testing and allowed for calculation of post-yield displacement as defined by the distance moved by the measurement probe between yield and fracture (see Appendix B for code). Figure 2.7 depicts an example graph of force versus displacement for each bone in a given treatment group while Figure 2.8 shows the measured values from this graph.

Using obtained geometric and testing data, stress and strain values were calculated using the following mathematical equations [76]:

Stress

$$\sigma = \frac{My}{I} = \left(\left(F * \left(\frac{d}{4} \right) \right) * \frac{APo}{2} \right) / Ixx \text{ for bones broken in AP direction.}$$

or

$$\sigma = \frac{My}{I} = \left(\left(F * \left(\frac{d}{4} \right) \right) * \frac{MLo}{2} \right) / Ixx \text{ for bones broken in ML direction.}$$

Strain

$$\varepsilon = (12x * (\frac{APo}{2})) / d^2 \text{ for bones broken in AP direction.}$$

or

$$\varepsilon = (12x * (\frac{MLo}{2})) / d^2 \text{ for bones broken in ML direction.}$$

Elastic modulus was calculated using a best fit to the slope of the stress-strain curve until yield. Yield stress was calculated with a 0.2% offset from 0 strain using the slope of the

elastic modulus [76]. Ultimate stress was determined using the MATLAB code in the same fashion as the maximum loads. Figure 2.9 depicts an example graph of stress versus strain (see Appendix B for code).

The following mathematical equations were used for CSA and Ixx calculation [76-77]:

Cross Sectional Area

$$CSA = \left(\frac{\pi}{4}\right) * ((APo * MLo) - (APi * MLi))$$

Area Moment of Inertia

$$Ixx = \left(\frac{\pi}{64}\right) * ((MLo * (APo^3)) - (MLi * (APi^3))) \text{ for bones broken in AP direction.}$$

or

$$Ixx = \left(\frac{\pi}{64}\right) * ((APo * (MLo^3)) - (APi * (MLi^3))) \text{ for bones broken in ML direction}$$

The following list details the definition of symbols used above:

APi = inner diameter in anterior-posterior direction

APo = outer diameter in anterior-posterior direction

CSA = cross sectional area

d = distance between supports

E = elastic modulus

ϵ = strain

F = force

I and Ixx = area moment of inertia

MLi = inner diameter in medial-lateral direction

MLo = outer diameter in medial-lateral direction

σ = stress

x = measurement probe displacement

y = distance from farthest point in cross section from neutral axis

Figure 2.7: Force versus displacement graph for right legs of 6 month SOST KO mice.

Figure 2.8: MatLab output graph of force versus displacement. Point A marks the yield force, point B marks the ultimate force, and the distance between points A and fracture is the post-yield displacement.

Figure 2.9: Stress versus strain graph for left legs of 6 month SOST TG mice.

2.3.1 Statistical Analysis

Minitab 16 Statistical Software was used for the statistical analysis. Compiled data was organized by mouse treatment type, bend direction, and leg. Each effect (CSA, Ixx, E, yield force, ultimate force, yield stress, ultimate stress, post-yield displacement) was compared for statistical significance between the treatment groups listed in Table 1 (i.e. SOST KO 6 month versus SOST TG 6 month versus etc.).

Initially, a general linear model (GLM) was run with three-way interactions including treatment group, bend direction, and leg (left or right). However, due to the absence of replication in a few of the treatment groups, one of these factors was left out of subsequent analyses. For CSA and Ixx the GLM was run with the two-way interaction of treatment group and leg. Bend direction was excluded due to the assumption that this factor would not affect the outcome of bone geometry. For the remaining analyses, leg was excluded and bend direction was included in the GLM, as this testing parameter was hypothesized to significantly affect the resulting data.

Prior to running of the GLM, a test for equal variances was performed. This test verified or discounted the assumption that variances were equal. Following this test, a test for normality was conducted on the standardized residuals to check the assumption that the data was distributed normally. Any outliers, as defined by a standard residual value >2.75 , were excluded from the data set in an attempt to regain a normal distribution.

Once equal variances and normal distribution were checked and outliers excluded, the GLM was run with the inclusion of Tukey comparison groups at a 95% confidence interval to determine the significant effect that treatment type and the secondary variable of either leg or bend direction had on the desired parameter. Residual and interaction plots were generated to help visually show any differences. All Minitab outputs can be found in Appendix C.

There was one data file (for bone 8007L) which was corrupted and could not be salvaged. As such all data from this bone was not included in the statistical analysis. In addition, one bone set (9200L/R) was lost due to improper testing and data collection.

2.4 Post Analysis

Three-point bending generally results in an oblique fracture which is not suitable for analysis techniques such as nanoindentation. In order to allow for future work to be conducted on the samples, bones were embedded in epoxy and metallographically polished to an appropriate level of smoothness that would be suitable for nanoindentation. A description of the embedding and polishing is located in Appendix D.

III. RESULTS

Following three-point bending, proximal bone halves were measured for geometric data. Using data obtained during testing and measurement, values were calculated for CSA, Ixx, E, yield force, ultimate force, post-yield displacement, yield stress, and ultimate stress. Data was organized by treatment group and each group was compared versus the other treatments. The information below shows the results of treatment groups based upon the criteria specified. It was hypothesized that either bend direction or leg (left or right) would have a significant effect on some of the comparisons and as such, these parameters were compared for interaction with the treatment type (labeled Genotype+Phenotype below). Complete Minitab outputs and graphs for all comparisons can be found in the appendix.

3.1 Cross Sectional Area

Cross sectional area was predicted to be only affected by the treatment type. No effect was predicted for the difference between left and right hindlimbs as it is not known for mice to have a dominant limb. Leg was included in the analysis and bend direction was excluded as mechanical testing direction has no effect on cross sectional area. Three values had standardized residuals of greater than ± 2.75 and were excluded from the analysis for normality. Figure 3.1 shows the associated p-values for the general linear model (GLM) analysis for this data set. Figure 3.2 displays the different treatment groups and their associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for CSA_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	78.8471	77.0300	8.5589	140.78	0.000
Leg	1	0.1876	0.1548	0.1548	2.55	0.115
Genotype+Phenotype*Leg	9	0.8704	0.8704	0.0967	1.59	0.134
Error	74	4.4989	4.4989	0.0608		
Total	93	84.4039				

Figure 3.1: GLM of cross sectional area across treatment groups and associated p-values. Data shown reflects statistical analysis and excludes 3 outlier values.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month	7	4.0197	A
SOST KO 6 month	8	2.8581	B
WT 3	12	1.7129	C
WT 4	4	1.3174	C D
WT 1	22	1.0365	D
WT 2	8	1.0280	D
SOST DEF 6 month	18	0.9375	D
SOST TG 8 month	4	0.9038	D
SOST TG 6 month	4	0.8793	D
SOST DEF 8 month	7	0.8354	D

Means that do not share a letter are significantly different.

Figure 3.2: Treatment group averages and significantly different value groupings for cross sectional area. Data shown reflects statistical analysis and excludes 3 outlier values. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

The treatment group was determined to be the only factor which significantly affected cross sectional area (CSA) ($p = 0.00$). There was not a significant interaction between treatment group and the leg broken. Observations from these results show that there was no significant difference between any treatment groups of 6 month old SOST TG, SOST DEF, and and their control littermate (WT 1) mice (Figure 3.3). This observation was also true for the 8 month old SOST TG, DEF, and their control littermate (WT 2) mice (Figure 3.4). There was also no significant difference between these two age groups (Figure 3.8). Opposite of these observations, there were significant differences between

both 6 and 12 month SOST KO mice and their respective wildtype (WT) control littermates (Figures 3.5 and 3.6). Interestingly, there was also a difference found between 6 month old WT for the SOST TG and SOST KO littermates (WT 1 and WT 3) (Figure 3.7).

Figure 3.3: Histogram displaying the comparison of cross sectional area for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.4: Histogram displaying the comparison of cross sectional area for 8 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.5: Histogram displaying the comparison of cross sectional area for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.6: Histogram displaying the comparison of cross sectional area for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.7: Histogram displaying the comparison of cross sectional area for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.8: Histogram displaying the comparison of cross sectional area for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.9: Histogram displaying the comparison of cross sectional area for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

3.2 Area Moment of Inertia

Area moment of inertia was predicted to be significantly affected by only the treatment type. The bone formation differences were predicted to be the cause of this difference.

The leg (left or right) was not predicted to have an influence on the outcome of the test.

One value had a standardized residual of greater than ± 2.75 and was excluded from the analysis for normality. Figure 3.10 shows the associated p-values for the general linear

model (GLM) analysis for this data set. Figures 3.11 and 3.12 display the different treatment groups and leg distributions along with their associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for Ixx_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	11.70817	11.31846	1.25761	21.75	0.000
Leg	1	0.15947	0.19304	0.19304	3.34	0.072
Genotype+Phenotype*Leg	9	0.49026	0.49026	0.05447	0.94	0.494
Error	76	4.39524	4.39524	0.05783		
Total	95	16.75314				

Figure 3.10: GLM of area moment of inertia across treatment groups and associated p-values. Data shown reflects statistical analysis and excludes 1 outlier value.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month	9	1.3	A
SOST KO 6 month	8	0.8	B
WT 3	12	0.6	B C
WT 4	4	0.4	B C D
WT 2	8	0.3	C D
WT 1	22	0.2	D
SOST TG 6 month	4	0.2	C D
SOST TG 8 month	4	0.2	C D
SOST DEF 6 month	18	0.2	D
SOST DEF 8 month	7	0.2	D

Means that do not share a letter are significantly different.

Figure 3.11: Treatment group averages and significantly different value groupings for area moment of inertia. Data shown reflects statistical analysis and excludes 1 outlier value. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

Grouping Information Using Tukey Method and 95.0% Confidence

Leg	N	Mean	Grouping
R	49	0.5	A
L	47	0.4	A

Means that do not share a letter are significantly different.

Figure 3.12: Average values by leg and significantly different value groupings for area moment of inertia. Data shown reflects statistical analysis and excludes 1 outlier value.

The treatment group was determined to be the only factor which significantly affected the area moment of inertia (Ixx) ($p = 0.00$). There was, however, a marginally significant effect due to the leg (left or right) broken ($p = 0.072$). There was not a significant interaction between treatment group and the leg broken. Observations from these results

show that there was no significant difference between any treatment groups of 6 month old SOST TG, SOST DEF, and their control littermate (WT 1) mice (Figure 3.13). This observation was also true for the 8 month old SOST TG, DEF, and their control littermate (WT 2) mice (Figure 3.14). There was also no significant difference between these two age groups (Figure 3.18). Opposite of these observations, there was a significant difference between 12 month SOST KO mice and their WT control littermates (Figure 3.16). Interestingly, there was no difference found between 6 month old SOST KO and WT littermates (Figure 3.15).

Figure 3.13: Histogram displaying the comparison of area moment of inertia for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.14: Histogram displaying the comparison of area moment of inertia for 6 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.15: Histogram displaying the comparison of area moment of inertia for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.16: Histogram displaying the comparison of area moment of inertia for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.17: Histogram displaying the comparison of area moment of inertia for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.18: Histogram displaying the comparison of area moment of inertia for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.19: Histogram displaying the comparison of area moment of inertia for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

Figure 3.20: Histogram of the average values of area moment of inertia for all bones in by leg.

3.3 Elastic Modulus

Elastic modulus was predicted to be significantly affected by both treatment type and bend direction. Bone formation differences between the treatment groups along with the difference in principle axis of loading between anterior-posterior (AP) and medial-lateral (ML) were the predicted reasons for this difference. The leg (left or right) was not predicted to have an influence on the outcome of the test and as such was replaced with bend direction in the GLM. There were two values at a standard residual of 2.81 and - 2.81 respectively. These values were determined to be marginal outliers, and were included in the analysis. Figure 3.21 shows the associated p-values for the general linear model (GLM) analysis for this data set. Figures 3.22 and 3.23 display the different treatment groups and bend directions with associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for E, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	320.357	315.522	35.058	7.93	0.000
Bend Direction	1	55.869	58.883	58.883	13.33	0.000
Genotype+Phenotype*Bend Direction	9	22.948	22.948	2.550	0.58	0.812
Error	77	340.203	340.203	4.418		
Total	96	739.377				

Figure 3.21: GLM of elastic modulus across treatment groups and associated p-values. There were no outliers in the data set.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST DEF 6 month	18	6.1665	A
SOST DEF 8 month	7	6.0325	A
WT 2	8	5.4316	A
WT 1	22	5.0449	A
SOST TG 8 month	4	4.8171	A B
WT 4	4	4.6147	A B
SOST TG 6 month	4	2.8387	A B
WT 3	12	2.0889	B
SOST KO 12 month	10	1.6407	B
SOST KO 6 month	8	1.3179	B

Means that do not share a letter are significantly different.

Figure 3.22: Treatment group averages and significantly different value groupings for elastic modulus. There were no outliers in the data set. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend Direction	N	Mean	Grouping
AP	49	4.9170	A
ML	48	3.0817	B

Means that do not share a letter are significantly different.

Figure 3.23: Bend direction averages and significantly different value groupings for elastic modulus. There were no outliers in the data set.

Both treatment group ($p = 0.00$) and bend direction ($p = 0.00$) were determined to significantly affect the modulus (E) individually. There was not a significant interaction between treatment group and the bend direction. Observations from these results show

that there was no significant difference in elastic modulus between treatment groups of 6 month old SOST TG, SOST DEF, and their control littermate (WT 1) mice (Figure 3.24). This observation was also true for the 8 month old SOST TG, DEF, and their control littermate (WT 2) mice (Figure 3.25). There was also no significant difference between these two age groups (Figure 3.29). Interestingly, there was no difference in elastic modulus found between 6 and 12 month old SOST KO mice, nor their respective WT littermates (WT3 and WT4) (Figures 3.26, 3.27, and 3.30).

Figure 3.24: Histogram displaying the comparison of elastic modulus for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.25: Histogram displaying the comparison of elastic modulus for 8 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.26: Histogram displaying the comparison of elastic modulus for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.27: Histogram displaying the comparison of elastic modulus for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.28: Histogram displaying the comparison of elastic modulus for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.29: Histogram displaying the comparison of elastic modulus for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.30: Histogram displaying the comparison of elastic modulus for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

Figure 3.31: Histogram of the average values of elastic modulus for all bones by bend direction. AP: anterior-posterior; ML: medial-lateral.

3.4 Yield Force

Yield force was predicted to be significantly affected by both treatment type and bend direction. Bone formation differences between the treatment groups along with the difference in principle axis of loading between anterior-posterior (AP) and medial-lateral (ML) directions were the hypothesized reasons for this variation. The leg (left or right) was not predicted to have an influence on the outcome of the test. Three values had standardized residuals of greater than ± 2.75 and were excluded from the analysis for normality. Figure 3.32 shows the associated p-values for the general linear model (GLM) analysis for this data set. Figure 3.33 displays the interaction between different treatment groups and bend direction along with associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for Yield Force_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	26456.2	27663.0	3073.7	36.62	0.000
Bend Direction	1	1686.8	1325.8	1325.8	15.79	0.000
Genotype+Phenotype*Bend Direction	9	3460.1	3460.1	384.5	4.58	0.000
Error	74	6211.4	6211.4	83.9		
Total	93	37814.5				

Figure 3.32: GLM of yield force across treatment groups and associated p-values. Data shown reflects statistical analysis and excludes 3 outlier values.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Bend Direction	N	Mean	Grouping
SOST KO 6 month	ML	3	73.145	A
SOST KO 12 month	ML	4	69.912	A
SOST KO 12 month	AP	4	60.861	A
WT 3	ML	6	25.746	B
SOST KO 6 month	AP	4	23.582	B C
WT 4	ML	2	18.396	B C
WT 4	AP	2	15.416	B C
WT 1	ML	11	14.708	B C
SOST DEF 6 month	ML	9	13.824	B C
WT 3	AP	6	13.226	B C
WT 1	AP	11	11.042	B C
SOST DEF 8 month	ML	3	10.517	B C
WT 2	ML	4	9.670	B C
SOST DEF 8 month	AP	4	9.626	B C
WT 2	AP	4	9.375	B C
SOST TG 8 month	AP	2	8.730	B C
SOST TG 6 month	ML	2	7.927	B C
SOST TG 8 month	ML	2	7.651	B C
SOST DEF 6 month	AP	9	7.139	C
SOST TG 6 month	AP	2	4.027	B C

Means that do not share a letter are significantly different.

Figure 3.33: Treatment group averages and significantly different value groupings for yield force. Data shown reflects statistical analysis and excludes 3 outlier values. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

Individually, both treatment group ($p = 0.00$) and bend direction ($p = 0.00$) were determined to significantly affect the yield force. There was also a significant interaction between the combination of treatment group and bend direction ($p = 0.00$). Observations from these results show that there was no significant difference caused by the interaction

of treatment group and bend direction between 6 month old SOST TG, SOST DEF, and their control littermate (WT 1) mice (Figure 3.42). This observation was also true for the 8 month old SOST TG, DEF, and their control littermate (WT 2) mice (Figure 3.43). There was also no significant difference between these two age groups (Figure 3.47). The yield force of the bones broken in the AP and ML bend direction for the 12 month SOST KO mice were statistically equivalent to each other as well as the yield force for the 6 month SOST KO ML bones (Figure 3.48). There was an observed statistical difference between 12 month SOST KO mice and their WT littermates, regardless of bend direction (Figure 3.45). Interestingly, there was a significant difference in yield force between 6 month SOST KO mice when the loading direction was ML or AP, which was found to be statistically equivalent to the AP and ML values for the WT littermates (Figure 3.44).

Figure 3.34: Histogram displaying the comparison of yield force for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.35: Histogram displaying the comparison of yield force for 6 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.36: Histogram displaying the comparison of yield force for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.37: Histogram displaying the comparison of yield force for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.38: Histogram displaying the comparison of yield force for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.39: Histogram displaying the comparison of yield force for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.40: Histogram displaying the comparison of yield force for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

Figure 3.41: Histogram of the average values of yield force for all bones by bend direction.

Figure 3.42: Histogram displaying the comparison of yield force in the AP or ML bend direction for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.43: Histogram displaying the comparison of yield force in the AP or ML bend direction for 8 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.44: Histogram displaying the comparison of yield force in the AP or ML bend direction for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.45: Histogram displaying the comparison of yield force in the AP or ML bend direction for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.46: Histogram displaying the comparison of yield force in the AP or ML bend direction for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.47: Histogram displaying the comparison of yield force in the AP or ML bend direction for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.48: Histogram displaying the comparison of yield force in the AP or ML bend direction for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

3.5 Ultimate Force

Ultimate force was predicted to be significantly affected by both treatment type and bend direction. Bone formation differences between the treatment groups along with the difference in principle axis of loading between anterior-posterior (AP) and medial-lateral (ML) directions were the hypothesized reasons for this variation. The leg (left or right) was not predicted to have an influence on the outcome of the test. Three values had standardized residuals of greater than ± 2.75 and were excluded from the analysis for normality. Figure 3.49 shows the associated p-values for the general linear model (GLM) analysis for this data set. Figure 3.50 displays the interaction between different treatment groups and bend direction along with associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for Ultimate Force_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	24875.1	25483.9	2831.5	134.31	0.000
Bend Direction	1	551.1	502.2	502.2	23.82	0.000
Genotype+Phenotype*Bend Direction	9	615.1	615.1	68.3	3.24	0.002
Error	74	1560.1	1560.1	21.1		
Total	93	27601.5				

Figure 3.49: GLM of ultimate force across treatment groups and associated p-values. Data shown reflects statistical analysis and excludes 3 outlier values.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Bend Direction	N	Mean	Grouping
SOST KO 6 month	ML	2	69.807	A
SOST KO 12 month	ML	4	65.603	A
SOST KO 12 month	AP	5	59.675	A B
SOST KO 6 month	AP	4	49.346	B
WT 3	ML	6	35.429	C
WT 4	ML	2	29.335	C D
WT 3	AP	6	24.718	D
WT 4	AP	2	21.747	C D E
WT 1	ML	11	20.568	D E
WT 1	AP	11	16.691	D E
SOST DEF 6 month	ML	9	15.408	E
SOST TG 8 month	ML	2	14.859	D E
SOST DEF 8 month	AP	4	13.798	E
SOST TG 6 month	ML	2	13.612	D E
WT 2	AP	4	13.600	E
SOST DEF 6 month	AP	9	13.436	E
WT 2	ML	4	13.074	E
SOST TG 8 month	AP	2	12.839	D E
SOST DEF 8 month	ML	3	12.322	E
SOST TG 6 month	AP	2	9.180	E

Means that do not share a letter are significantly different.

Figure 3.50: Treatment group averages and significantly different value groupings for ultimate force. Data shown reflects statistical analysis and excludes 3 outlier values. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

Individually, both treatment group ($p = 0.00$) and bend direction ($p = 0.00$) were determined to significantly affect the ultimate force. There was also a significant

interaction between the combination of treatment group and bend direction ($p = 0.002$).

Observations from these results show that there was no significant difference due to the interaction of treatment group and bend direction between 6 month old SOST TG, SOST DEF, and their control littermate (WT 1) mice (Figure 3.59). This observation was also true for the 8 month old SOST TG, DEF, and their control littermate (WT 2) mice (Figure 3.60). There was also no significant difference between these two age groups (Figure 3.64). The ultimate force for 6 and 12 month SOST KO bones tested in the ML direction were found to be statistically equivalent and the ultimate force for bones tested in the AP

direction for the same groups were also found to be statistically equivalent (Figure 3.65).

In additional, all SOST KO mice were significantly different from their WT littermates

(Figures 3.61 and 3.62).

Figure 3.51: Histogram displaying the comparison of ultimate force for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.52: Histogram displaying the comparison of ultimate force for 6 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.53: Histogram displaying the comparison of ultimate force for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.54: Histogram displaying the comparison of ultimate force for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.55: Histogram displaying the comparison of ultimate force for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.56: Histogram displaying the comparison of ultimate force for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.57: Histogram displaying the comparison of ultimate force for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

Figure 3.58: Histogram of the average values of ultimate force for all bones by bend direction.

Figure 3.59: Histogram displaying the comparison of ultimate force in the AP or ML bend direction for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.60: Histogram displaying the comparison of ultimate force in the AP or ML bend direction for 8 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.61: Histogram displaying the comparison of ultimate force in the AP or ML bend direction for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.62: Histogram displaying the comparison of ultimate force in the AP or ML bend direction for 6 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.63: Histogram displaying the comparison of ultimate force in the AP or ML bend direction for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.64: Histogram displaying the comparison of ultimate force in the AP or ML bend direction for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.65: Histogram displaying the comparison of ultimate force in the AP or ML bend direction for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

3.6 Post-Yield Displacement

Post-yield displacement was predicted to be significantly affected by both treatment type and bend direction. Bone formation differences between the treatment groups along with the difference in principle axis of loading between anterior-posterior and medial-lateral were the hypothesized reasons for this variation. The leg (left or right) was not predicted to have an influence on the outcome of the test. One value had a standardized residual of greater than ± 2.75 and was excluded from the analysis for normality. Figure 3.66 shows the associated p-values for the general linear model (GLM) analysis for this data set.

Figure 3.67 displays the interaction between different treatment groups and bend direction along with associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for Post Yield Displacement_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	0.31893	0.33884	0.03765	1.04	0.416
Bend Direction	1	0.00130	0.01296	0.01296	0.36	0.551
Genotype+Phenotype*Bend Direction	9	0.36949	0.36949	0.04105	1.14	0.349
Error	76	2.74814	2.74814	0.03616		
Total	95	3.43786				

Figure 3.66: GLM of post-yield displacement across treatment groups and associated p-values. Data shown reflects statistical analysis and excludes 1 outlier value.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST TG 6 month	4	0.29479	A
WT 2	8	0.26133	A
SOST TG 8 month	4	0.25399	A
SOST DEF 8 month	7	0.24441	A
WT 4	4	0.21078	A
SOST KO 6 month	8	0.18372	A
WT 1	22	0.16650	A
WT 3	12	0.16014	A
SOST DEF 6 month	17	0.13713	A
SOST KO 12 month	10	0.06117	A

Means that do not share a letter are significantly different.

Figure 3.67: Treatment group averages and significantly different value groupings for post-yield displacement. Data shown reflects statistical analysis and excludes 1 outlier value. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

There were no factors which significantly affected the post-yield displacement. Post-yield behavior for all treatment groups and bend directions were considered statistically equivalent. There was no significant interaction between treatment group and bend direction.

Figure 3.68: Histogram displaying the comparison of post yield displacement for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.69: Histogram displaying the comparison of post yield displacement for 6 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.70: Histogram displaying the comparison of post yield displacement for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.71: Histogram displaying the comparison of post yield displacement for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.72: Histogram displaying the comparison of post yield displacement for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.73: Histogram displaying the comparison of post yield displacement for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.74: Histogram displaying the comparison of post yield displacement for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

3.7 Yield Stress

Yield stress was predicted to be significantly affected by both treatment type and bend direction. Bone formation differences between the treatment groups along with the difference in principle axis of loading between anterior-posterior (AP) and medial-lateral (ML) directions were the hypothesized reasons for this variation. The leg (left or right) was not predicted to have an influence on the outcome of the test. Figure 3.75 shows the associated p-values for the general linear model (GLM) analysis for this data set. Figure 3.76 displays the interaction between different treatment groups and bend direction along with associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for Yield Stress, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	26321	26285	2921	0.64	0.757
Bend Direction	1	3815	196	196	0.04	0.836
Genotype+Phenotype*Bend Direction	9	31676	31676	3520	0.77	0.640
Error	77	350029	350029	4546		
Total	96	411840				

Figure 3.75: GLM of yield stress across treatment groups and associated p-values. There were no outliers in the data set.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
WT 4	4	124.47	A
WT 1	22	119.00	A
SOST KO 6 month	8	115.72	A
SOST KO 12 month	10	109.64	A
SOST DEF 6 month	18	109.49	A
SOST DEF 8 month	7	107.86	A
WT 3	12	93.57	A
WT 2	8	86.82	A
SOST TG 8 month	4	80.30	A
SOST TG 6 month	4	49.66	A

Means that do not share a letter are significantly different.

Figure 3.76: Treatment group averages and significantly different value groupings for yield stress. There were no outliers in the data set. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

There were no factors which significantly affected the yield stress. The interaction of treatment group and bend direction was also found to not significantly affect the yield stress. The yield stresses for all treatment groups and bend directions were considered statistically equivalent.

Figure 3.77: Histogram displaying the comparison of yield stress for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.78: Histogram displaying the comparison of yield stress for 6 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.79: Histogram displaying the comparison of yield stress for 6 month old SOST Transgenic mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.80: Histogram displaying the comparison of yield stress for 12 month old SOST Transgenic mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.81: Histogram displaying the comparison of yield stress for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.82: Histogram displaying the comparison of yield stress for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.83: Histogram displaying the comparison of yield stress for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

3.8 Ultimate Stress

Ultimate stress was predicted to be significantly affected by both treatment type and bend direction. Bone formation differences between the treatment groups along with the difference in principle axis of loading between anterior-posterior (AP) and medial-lateral (ML) directions were the hypothesized reasons for this variation. The leg (left or right) was not predicted to have an influence on the outcome of the test. Figure 3.84 shows the associated p-values for the general linear model (GLM) analysis for this data set. Figure 3.85 displays the interaction between different treatment groups and bend direction along with associated means and significantly different groupings, as determined by a Tukey's comparison with a 95% confidence interval.

Analysis of Variance for Ultimate Stress, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	37589	37721	4191	1.97	0.054
Bend Direction	1	2877	3329	3329	1.57	0.214
Genotype+Phenotype*Bend Direction	9	12980	12980	1442	0.68	0.726
Error	77	163621	163621	2125		
Total	96	217066				

Figure 3.84: GLM of ultimate stress across treatment groups and associated p-values. There were no outliers in the data set.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 6 month	8	175.11	A
WT 1	22	168.58	A
WT 4	4	166.16	A
SOST DEF 6 month	18	154.54	A
SOST DEF 8 month	7	144.34	A
WT 3	12	139.86	A
SOST TG 8 month	4	139.56	A
SOST KO 12 month	10	134.99	A
WT 2	8	116.33	A
SOST TG 6 month	4	98.74	A

Means that do not share a letter are significantly different.

Figure 3.85: Treatment group averages and significantly different value groupings for ultimate stress. There were no outliers in the data set. WT 1-4 are control littermates of SOST TG 6 month, SOST TG 8 month, SOST KO 6 month, and SOST KO 12 month respectively. These mice faithfully express murine SOST.

There were no factors which significantly affected the ultimate stress, although treatment group was marginally significant. The interaction of treatment group and bend direction was also found to not significantly affect the ultimate stress. The ultimate stresses for all treatment groups and bend directions were considered statistically equivalent.

Figure 3.86: Histogram displaying the comparison of ultimate stress for 6 month old SOST Transgenic mice. WT 1 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.87: Histogram displaying the comparison of ultimate stress for 6 month old SOST Transgenic mice. WT 2 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.88: Histogram displaying the comparison of ultimate stress for 6 month old SOST Knockout mice. WT 3 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.89: Histogram displaying the comparison of ultimate stress for 12 month old SOST Knockout mice. WT 4 mice were control littermates and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.90: Histogram displaying the comparison of ultimate stress for 6 month old mice. WT 1 and 3 mice were control littermates (SOST TG/DEF and SOST KO respectively) and faithfully express murine SOST. No animals were excluded from this analysis.

Figure 3.91: Histogram displaying the comparison of ultimate stress for 6 month old Transgenic mice and 8 month old Transgenic mice. Wild type controls were not included in this analysis.

Figure 3.92: Histogram displaying the comparison of ultimate stress for 6 month old Knockout mice and 12 month old Knockout mice. Wild type controls were not included in this analysis.

IV. DISCUSSION

The primary goal of this study was to identify biomechanical properties of bone resulting from expression of the SOST gene. Secondary goals were to test differences in bending strength between anterior-posterior (AP) and medial-lateral (ML) axes of loading along with potentially elucidating any difference in left and right limbs. Femurs from hybridized mice were obtained from Lawrence Livermore National Labs and tested using 3-point bending until fracture. Force and displacement data were captured along with measured geometric data of the bones in order to calculate and analyze mechanical properties of bone.

The hypothesis was that there would be a significant difference between bones which expressed SOST (TG) and those which had genomes devoid of the region coding for SOST expression (KO). Since SOST expression negatively regulates bone formation, it was anticipated that the KO mice would have larger geometric measurements along with an increased resistance to fracture due to additional bone formation caused by the absence of SOST. In addition, femurs tested in the ML direction were predicted to have a greater breaking force as this axis is the principle direction of loading under normal skeletal conditions. It was also anticipated that there would be no difference between left and right limbs, consistent with previous reports [9].

Outcomes were predicted to be useful for further evidence and validation of current bone property knowledge. Potential differences could provide supporting evidence for continued research into the mechanisms of action of SOST which may lead to strategic utilization of gene therapy to combat prevalent bone diseases including osteoporosis.

4.1 Analysis of Results

4.1.1 Cross Sectional Area

Results showed treatment group to be the only factor which significantly affected CSA ($p < 0.001$). The absence of side-to-side differences in limb cross sectional area for all mice was also observed ($p = 0.448$). In addition, no interaction was found between treatment group and the leg broken ($p = 0.648$). Bending direction was not analyzed for this geometric property, as cross-sectional area is independent of the direction the limbs are loaded during mechanical testing.

Significant differences between the KO mice, their respective WT controls, and TG mice appeared to be closely related to SOST expression. It was found that 12 month old KO mice had the largest cross sectional area, which may have resulted from the increased amount of time for unregulated bone growth. Cross sectional area of 6 month old KO mice was found to be significantly less than 12 month KO along with being significantly greater than all WT and TG mice. This difference was also consistent with the absence of SOST which would lead to greater bone formation in comparison to the mice with regulated bone formation. Interestingly, DEF mice (a subset of TG mice expressing twice the amount of SOST as TG mice) did not have a significantly different cross sectional area than TG mice or their WT control littermates. In addition, there was no cross sectional area difference in the age groups (6 and 8 months old) of TG mice.

These results validate the successful hybridization of mice, in such that mice with no SOST expression formed significantly more bone than those with SOST expression. The absence of a significant difference between TG, DEF, and WT mice suggests that SOST uptake is limited to the number of receptors available and not resultant of the total

amount of SOST transcription. In this situation, a gross overexpression of SOST may not directly result in reduced bone formation or bone growth regulation during skeletal maturation. Although this whole-bone assessment suggests that there is not a direct correlation between SOST overexpression and reduced cortical bone formation in comparison to normal levels of SOST, data from Loots et al. [39] have shown that trabecular bone may be much more significantly impacted by SOST overexpression.

The lack of significance between left and right femora adds to the hypothesis that there is no systematic side-to-side difference in bone formation. The most interesting and potentially significant finding was the absence of difference in cross sectional area due to age of TG mice while there was an observed difference due to age in SOST KO mice. As mice of most strains typically reach skeletal maturity at 4-5 months old [78], this finding suggests an increase in bone formation following maturity resulting from the elimination of SOST.

4.1.2 Area Moment of Inertia

Results showed treatment group to be the only factor which significantly affected area moment of inertia ($p < 0.001$). Interestingly, there was a marginal effect on area moment of inertia due to the leg broken ($p = 0.072$). No interaction observed between treatment group and the leg broken ($p = 0.494$). For this calculated parameter, bending direction was not analyzed, as moment of inertia is independent of the direction the limbs are loaded during mechanical testing.

It was anticipated that there would be a significant difference in area moment of inertia between treatment groups, however the marginal difference of left versus right limbs was

not predicted. Interesting observations included the absence of a difference between all 6 and 8 month old TG mice (TG, DEF, and WT littermates), the absence of a difference between 6 month old KO mice and WT littermates, and the presence of a difference between 12 month old KO and WT mice.

Larger calculated area moment of inertia values suggest an increase in the resistance to bending stress. KO mice were predicted to have the highest values due to added bone formation leading to fracture resistance. This prediction was observed as the KO mouse groups had the two highest area moment of inertia values. The marginal difference in side-to-side may have resulted from unequal sample sizes, as there were 49 right legs included in this analysis while there were only 47 left legs. The difference in values of 0.5 and 0.4, respectively, is close enough to have been impacted by the difference in sample size, as the one excluded outlier was in the 12 month KO group which displayed the highest values of area moment of inertia.

4.1.3 Elastic Modulus

Both treatment group ($p < 0.001$) and bend direction ($p < 0.001$) were determined to significantly affect the calculated elastic modulus. There was no observed interaction between treatment group and the bend direction ($p = 0.812$). Leg was excluded from this analysis and bend direction was analyzed, as side-to-side differences were assumed to be negligible in respect to resistance to elastic deformation.

It was anticipated that the SOST KO mice would have increased resistance to elastic deformation due to the increased bone accumulation. In addition, since more bone is formed in the ML regions of the femoral diaphysis, where there are increased stresses, it

was also anticipated that bones broken in the ML direction would yield a higher modulus. Interesting observations included the absence of a difference in elastic modulus between all 6 and 8 month old TG mice (TG, DEF, and WT littermates) along with no difference between 6 and 12 month KO mice and their respective WT littermates. Also, contrary to the expected results, it was found that bones broken in the AP direction were significantly more resistant to elastic deformation than bones in the ML direction.

The elastic modulus was calculated from the slope of the stress-strain curve using a 0.02% offset. It was found that the elastic modulus was higher in all TG mice in comparison to the KO mice. This observation was unusual when combined with the hypothesis that increased bone formation would allow for greater elastic resistance to applied force. Potentially, the additional bone formation resulted in the ability for KO bones to resist fracture while experiencing increased microfracture damage due to applied loads. This observation may correlate with the difference seen in the AP and ML bend directions, as additional bone in the ML regions may allow for increased damage prior to fracture rather than a greater resistance to deformation.

4.1.4 Yield Force

Individually, both treatment group ($p < 0.001$) and bend direction ($p < 0.001$) were determined to significantly affect yield force. There was also a significant interaction between the combination of treatment group and bend direction ($p < 0.001$). Bend direction was included in this analysis, as side-to-side differences were assumed to be negligible in respect to resistance to yield force.

The unregulated bone growth in KO mice was predicted to have a significant effect on the amount of force the bone could withstand prior to yield. Consistent with this hypothesis, bones broken in the ML direction were expected to behave the same way in comparison to those broken in the AP direction. Important observations included the similarity of 12 month KO bones broken in the AP and ML directions along with the observed difference of 6 month KO bones in these directions. All bones broken for both 6 month and 8 month TG mice were found to be statistically equivalent. Lastly, bones broken in the ML direction were found to have a significantly higher yield force than those in the AP direction.

As expected, bones broken in the ML direction for the 6 month and 12 month KO bones were found to give the highest value for yield force. The additional bone formed due to the absence of SOST is predicted to be the driving factor behind the high yield force observations. The most interesting and unpredicted observation was the low yield force value for the 6 month KO bones broken in the AP direction, which was not found to be different from values of the TG mice and WT littermates. Overall, the high yield force of bones broken in the ML direction confirmed the hypothesis and is also assumed to be attributed to the increased bone formation in these regions.

4.1.5 Ultimate Force

As with yield force, both treatment group ($p < 0.001$) and bend direction ($p < 0.001$) were determined to individually affect ultimate force. There was also a significant interaction between the combination of treatment group and bend direction ($p = 0.002$). Bend direction was included in this analysis, as side-to-side differences were assumed to be negligible in respect to resistance to ultimate force.

Observed results were consistent with the overall hypotheses that KO mice would display the highest values for ultimate force as well as bones broken in the ML direction having larger values than bones broken in the AP direction. Consistent with the yield force observation, 6 month KO bones broken in the ML direction gave the highest values for ultimate force and were statistically equivalent to values of the 12 month KO ML bones. The hypothesis was supported as 6 and 12 month KO bones broken in the ML direction were found to be statistically equivalent to each other and significantly different from corresponding treatment group bones broken in the AP direction. The KO bones were also significantly different from all TG and WT mice, independent of bend direction. There was no observed difference between 6 and 8 month TG mice.

These results are mostly consistent with the yield force results for the corresponding treatment groups. The added bone in both KO mice as well as in the ML direction is assumed to be the cause of the increased resistance to overall fracture. These results may provide a better assessment of whole bone fracture properties in comparison to yield force, as they tend to be in line with the overall assumption that increased bone mass will result in increased resistance to fracture.

4.1.6 Post Yield Displacement

Results showed that there were no factors which significantly affected the post-yield displacement. No significant difference was detected for treatment groups ($p = 0.416$) and bend directions ($p = 0.551$). There was no significant interaction between treatment group and bend direction ($p = 0.349$).

These observations did not support the hypothesis that each of the factors (treatment group and bend direction) would have some significant effect on the post yield displacement. Considering that the elastic modulus was found to be dependent on the treatment group, the finding that there was no difference between treatment groups is surprising. It was assumed that an increase in resistance to elastic deformation would correlate with bones being more brittle and thus a reduction in the displacement between yield and fracture. In addition, bend direction was assumed to impact the post yield behavior of bone due to the increased amount of bone in the ML direction and thus the potential ability to withstand fracture following damage.

4.1.7 Yield Stress

There were no factors which significantly affected the yield stress, with all treatment groups ($p = 0.757$) and bend directions ($p = 0.836$) showing no difference. There was no significant interaction between treatment group and bend direction ($p = 0.640$).

These observations did not support the hypothesis that each of the factors (treatment group and bend direction) would have some significant effect on the yield stress.

Considering that the area moment of inertia was found to be dependent on the treatment group, the finding that there was no difference between treatment groups is surprising. It was assumed that the larger values for area moment of inertia would correspond to the lowest stress values. This prediction was inconsistent with the observed results. There may have been significant error in the yield stress data due to unequal sample sizes or the low number of samples in some treatment groups.

4.1.8 Ultimate Stress

There were no factors which significantly affected the ultimate stress, however the treatment groups were shown to have a marginally significant effect ($p = 0.054$). There was no difference in bend direction ($p = 0.214$) and there was no significant interaction between treatment group and bend direction ($p = 0.726$).

These observed results did not support the hypothesis that each of the factors (treatment group and bend direction) would have significant effect on the ultimate stress. Concurrent with the assumption for yield stress, the finding that area moment of inertia was dependent on the treatment group results in the surprising observation of no difference between treatment groups for ultimate stress. It was again assumed that the larger values for area moment of inertia would correspond to the lowest stress values. Although the results are marginally significant, this assumption proved to be incorrect in comparison to the results from the two parameters. There may have been significant error in the yield stress data due to the large difference in sample sizes or the low number of samples in some treatment groups.

4.2 Challenges/Limitations

There were a few challenges/limitations experienced during the conduct of this study. Most importantly, there were small and unequal sample sizes for the different treatment groups, including varying numbers of male and female mice in each group. These inequalities may have resulted in non-significant differences between some of the treatment groups. When conducting statistical comparisons the lack of repetition, caused by small sample sizes within some treatment groups, made it necessary to run the general linear model (GLM) with only 2 factors (Treatment Group and either Leg or Bend

Direction). Had there been repetition within all of the groups, the analysis could have been conducted with all three factors allowing for a more in depth analysis of the effect that each factor had on the others. If all three factors were included in the analysis the randomization used would have been inadequate. Minitab was used to randomize all 50 left legs to either the AP or ML bend direction (25 each direction) and the right leg was broken in the opposite direction. In order to have obtained truly equal sample sizes, this randomization would have needed to be conducted on a per treatment group basis rather than on the entire sample set.

When conducting the 3-point bend test, it was often difficult to keep bones broken in the ML direction in place. Clay was used to help secure these bones in the proper orientation, although there were instances when this was not sufficient. On a few occasions the bone was contacted by the probe and at some point during the test it would slip from the intended orientation. This would typically occur at a time where plastic deformation had occurred, thus rendering the bone unable to be retested, and as such the test was completed with the bone out of its initial orientation. This is expected to have had some effect on the non-observance of differences in AP versus ML bend directions for some of the parameters.

Measuring of geometric data at the fracture site was conducted under a microscope with the use of a crosshair reticle and a moveable stage controlled by micromanipulators. This process was used, rather than Image J analysis, due to limitations of the microscope and camera combination. Using the available equipment, the magnification was too large and as such it was not possible to take a single image of the entire bone at the fracture site.

Had this been possible, potentially more accurate measurements would have been made

using the Image J software for the cross sectional area of the bones. One possible advantage of measuring the geometric data using the microscope was that bones were often broken in an oblique fracture, which may not have allowed for clear capturing of an image at the fracture site. The microscope often required refocusing to allow for clear identification of the edge of the major axis as the stage was moved from anterior to posterior or medial to lateral regions. Both possible measurement techniques have advantages and disadvantages which may result in slightly different results.

Lastly, in calculating many of the parameters, generalizations were made when writing the Matlab code. This was done to allow for calculation of multiple bone samples rather than individually identifying and calculating each parameter by hand. These generalizations combined with the previously mentioned difficulty of maintaining bones in their intended orientation during testing may have led to inaccurate force-displacement curves and thus inaccurate results for the calculated parameters.

4.3 Significance of Results

Comparison of data obtained from this study with that of a previous 3-point bend study reported by Wergedal et al [79] showed similar results for many biomechanical properties. Wergedal et al [79] performed 3-point bending tests on 29 different strains of inbred mice, including 3 different genetic variations of C57 mice. The following table shows the comparison of the average of the 29 mouse strains analyzed by Wergedal et al [79] and the different treatment groups in this study.

Table 2: Comparison of findings from this study to those of Wergedal et al 2009 [79].

Treatment Group	CSA (mm²)	Ixx (mm⁴)	E (GPa)	Ultimate Force (N)	Ultimate Stress (MPa)
SOST TG 6 month	0.88	0.216	2.84	11.4	98.7
WT 1	1.04	0.219	5.04	18.6	169
SOST DEF 6 month	0.94	0.183	6.17	14.4	155
SOST TG 8 month	0.9	0.194	4.82	13.8	140
WT 2	1.03	0.285	5.43	13.3	116
SOST DEF 8 month	0.84	0.172	6.2	13.2	147
SOST KO 6 month	2.86	0.822	1.32	57.5	175
WT 3	1.71	0.56	2.09	30.1	140
SOST KO 12 month	3.95	1.89	1.64	64.6	135
WT 4	1.32	0.394	4.61	25.5	166
Wergedal et al 2009	1.47	0.134	6.56	18.7	176

Although there may have been calculation errors due to geometric measuring and computer analysis, many of the large differences in values shown above are consistent with the hypothesis that the enhanced bone formation due to removal of the SOST gene increases cross sectional area, yield force, and ultimate force. There may have been significant error in the calculation of the area moment of inertia, modulus, and stress values, although the differences seen may accurately reflect the impact SOST has on bone properties. Further analysis and computation of calculated values (area moment of inertia, elastic modulus, post yield displacement, yield stress, ultimate stress) by hand may be useful in determining the overall validity of the findings of this study.

4.4 Future Work

The work completed in this study was done to assess the whole-bone biomechanical properties of murine femurs which either did or did not express the SOST gene. Further analysis of the resulting fracture site can be performed to enhance understanding of the effect SOST has on bone formation.

4.4.1 Imaging

Imaging of the fracture site, resulting from 3-point bending, would allow for additional analysis and comparison of treatment groups. Potential imaging with a scanning electron microscope (SEM) may provide insight into the different bone structural units and characterization of bone integrity in the various treatment groups. This information may be useful in understanding the bone modeling and remodeling effect that the SOST gene has on bone. In addition, imaging using polarized light could allow for identification of differences in collagen fiber orientation which may be altered due to the expression of SOST. Collagen fiber orientation has previously been thought to be an important predictor of bone strength [80-81].

4.4.2 Nanoindentation

Nanoindentation has been applied in recent years to measure elastic properties of bone tissue at a microscopic length scale, overcoming the limitations of traditional mechanical testing techniques for small samples [82-84]. This method is precise and accurate [85] and appears well suited for measuring material properties in mouse bones [73].

Nanoindentation provides a measure of the modulus of the solid phase of bone at the microstructural scale, with no influence of porosity (where porosity includes vascular and resorption but not lacunar or canicular voids) [11].

Samples which have previously undergone 3-point bending can be embedded and used for nanoindentation testing to obtain a more complete representation of mechanical properties of cortical bone. Samples are typically dehydrated in ethanol, embedded in poly(methyl methacrylate) (PMMA) [73, 85-86], and polished to a mirrored surface. Once these preparation steps are completed, nanoindentation can be conducted.

4.4 Conclusions

Identification of significant differences in biomechanical properties of bones from mice that express the SOST gene and those that do not can help advance the understanding of how SOST works and determine if it is a suitable target for applied gene therapy. The findings of this study show that mice hybridized by Lawrence Livermore National Labs that do not express SOST have significantly more bone formation and some enhanced biomechanical properties in comparison to littermate controls and transgenic mice.

Further analysis of these bones needs to be conducted in order to generate an accurate picture of the overall impact of SOST on bone formation. If the structural integrity and quality of bone resulting from the removal of the SOST gene is shown to be comparable to that of normal, healthy bone, the use of gene therapy to combat diseases/disorders such as osteoporosis may lead to important medical breakthroughs in and has the potential to impact millions of individuals.

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APPENDIX A: Mouse Treatment Groups and Data Values

<u>SOST TG, 6 months</u>		
<u>#</u>	<u>Sex</u>	<u>Treatment</u>
8587	M	DEF
8601	M	DEF
8641	M	DEF
8642	M	DEF
8658	M	DEF
9294	F	DEF
9295	F	DEF
9528	F	DEF
9530	F	DEF
9293	F	TG
9296	F	TG
8599	M	WT
8600	M	WT
8640	M	WT
8656	M	WT
8657	M	WT
8602	F	WT
8603	F	WT
8643	F	WT
9526	F	WT
9527	F	WT
9529	F	WT

<u>SOST TG, 8 months</u>		
<u>#</u>	<u>Sex</u>	<u>Treatment</u>
5588	F	DEF
5589	F	DEF
8007	F	DEF
8008	F	DEF
5592	F	TG
5599	F	TG
5590	F	WT
5593	F	WT
5597	F	WT
5598	F	WT

<u>SOST KO, 6 months</u>		
<u>#</u>	<u>Sex</u>	<u>Treatment</u>
9198	M	KO
9200	M	KO
9201	M	KO
9202	M	KO
9203	M	KO
9207	M	WT
9208	M	WT
9209	M	WT
9238	M	WT
9239	M	WT
9240	M	WT

<u>SOST KO, 12 months</u>		
<u>#</u>	<u>Sex</u>	<u>Treatment</u>
7544	M	KO
7565	M	KO
7592	M	KO
7593	M	KO
7594	M	KO
7545	M	WT
7564	M	WT

Number	Genotype+Phenotype	Sex	Bend Direction	Leg	CSA	Ixx	E	Yield Force	Ultimate Force	Post Yield Displacement	Yield Stress	Ultimate Stress
8587L	SOST TG 6 month DEF	M	ML	L	1.07E+00	3.38E-01	3.84E+00	1.45E+01	1.61E+01	1.35E-01	9.99E+01	1.11E+02
8587R	SOST TG 6 month DEF	M	AP	R	1.12E+00	1.44E-01	5.74E+00	8.80E+00	9.38E+00	9.84E-02	9.36E+01	9.98E+01
8599L	SOST TG 6 month WT	M	AP	L	1.04E+00	1.41E-01	4.37E+00	5.80E+00	1.59E+01	2.83E-01	6.37E+01	1.74E+02
8599R	SOST TG 6 month WT	M	ML	R	9.31E-01	2.21E-01	7.41E+00	1.26E+01	2.20E+01	3.73E-01	1.16E+02	2.02E+02
8600L	SOST TG 6 month WT	M	AP	L	1.39E+00	2.09E-01	6.32E+00	1.75E+01	1.88E+01	4.63E-02	1.37E+02	1.48E+02
8600R	SOST TG 6 month WT	M	ML	R	1.09E+00	3.46E-01	2.21E+00	4.40E+00	2.32E+01	4.54E-01	3.02E+01	1.59E+02
8601L	SOST TG 6 month DEF	M	ML	L	9.73E-01	2.61E-01	4.90E+00	2.12E+01	2.15E+01	1.00E-02	1.79E+02	1.81E+02
8601R	SOST TG 6 month DEF	M	AP	R	8.96E-01	1.13E-01	6.24E+00	1.59E+01	1.61E+01	1.62E-02	2.07E+02	2.11E+02
8602L	SOST TG 6 month WT	F	AP	L	9.60E-01	1.16E-01	8.08E+00	1.39E+01	1.40E+01	3.30E-03	1.79E+02	1.80E+02
8602R	SOST TG 6 month WT	F	ML	R	7.94E-01	1.63E-01	7.13E+00	1.94E+01	1.98E+01	1.29E-02	2.26E+02	2.31E+02
8603L	SOST TG 6 month WT	F	AP	L	9.86E-01	1.53E-01	4.66E+00	5.15E+00	1.62E+01	2.72E-01	5.56E+01	1.75E+02
8603R	SOST TG 6 month WT	F	ML	R	9.79E-01	2.16E-01	7.41E+00	1.75E+01	1.78E+01	2.17E-01	1.60E+02	1.63E+02
8640L	SOST TG 6 month WT	M	ML	L	9.42E-01	2.14E-01	6.95E+00	1.70E+01	2.05E+01	1.39E-01	1.56E+02	1.88E+02
8640R	SOST TG 6 month WT	M	AP	R	1.14E+00	1.65E-01	5.21E+00	1.77E+01	1.86E+01	0.00E+00	1.68E+02	1.76E+02
8641L	SOST TG 6 month DEF	M	ML	L	9.48E-01	2.79E-01	1.88E+00	4.03E+00	1.09E+01	7.05E-01	3.22E+01	8.70E+01
8641R	SOST TG 6 month DEF	M	AP	R	7.44E-01	9.50E-02	9.06E+00	6.57E+00	7.24E+00	3.57E-02	9.59E+01	1.06E+02
8642L	SOST TG 6 month DEF	M	AP	L	1.21E+00	1.20E-01	1.05E+01	1.22E+01	1.46E+01	7.41E-02	1.35E+02	1.62E+02
8642R	SOST TG 6 month DEF	M	ML	R	9.26E-01	2.51E-01	3.48E+00	8.05E+00	1.11E+01	1.69E-01	6.87E+01	9.44E+01
8643L	SOST TG 6 month WT	F	ML	L	1.04E+00	2.74E-01	4.14E+00	1.56E+01	1.56E+01	6.72E-03	1.24E+02	1.24E+02
8643R	SOST TG 6 month WT	F	AP	R	9.15E-01	1.30E-01	8.52E+00	1.43E+01	1.43E+01	0.00E+00	1.73E+02	1.73E+02
8656L	SOST TG 6 month WT	M	ML	L	1.05E+00	2.56E-01	4.09E+00	2.00E+01	2.04E+01	2.33E-02	1.63E+02	1.66E+02
8656R	SOST TG 6 month WT	M	AP	R	1.10E+00	1.83E-01	3.68E+00	1.10E+01	1.10E+01	0.00E+00	9.96E+01	9.96E+01
8657L	SOST TG 6 month WT	M	AP	L	9.61E-01	1.18E-01	6.36E+00	1.14E+01	1.14E+01	0.00E+00	1.39E+02	1.39E+02
8657R	SOST TG 6 month WT	M	ML	R	1.01E+00	3.98E-01	3.12E+00	2.27E+01	2.27E+01	0.00E+00	1.47E+02	1.47E+02
8658L	SOST TG 6 month DEF	M	ML	L	6.97E-01	1.16E-01	8.56E+00	1.19E+01	1.26E+01	3.92E-02	1.78E+02	1.87E+02
8658R	SOST TG 6 month DEF	M	AP	R	8.49E-01	1.01E-01	9.14E+00	1.37E+00	1.77E+01	4.38E-01	1.93E+01	2.49E+02
9293L	SOST TG 6 month TG	F	AP	L	8.54E-01	1.44E-01	4.08E+00	6.52E+00	6.88E+00	3.60E-02	7.35E+01	7.76E+01
9293R	SOST TG 6 month TG	F	ML	R	8.74E-01	3.13E-01	3.35E-01	5.61E+00	1.27E+01	5.48E-01	2.20E+01	4.99E+01
9294L	SOST TG 6 month DEF	F	AP	L	9.48E-01	1.13E-01	6.29E+00	1.92E+00	1.48E+01	2.71E-01	2.55E+01	1.96E+02
9294R	SOST TG 6 month DEF	F	ML	R	8.42E-01	2.38E-01	5.08E+00	1.42E+01	1.47E+01	2.38E-02	1.28E+02	1.32E+02
9295L	SOST TG 6 month DEF	F	ML	L	7.76E-01	1.34E-01	9.60E+00	1.65E+01	1.68E+01	2.85E-02	2.11E+02	2.15E+02
9295R	SOST TG 6 month DEF	F	AP	R	9.01E-01	1.58E-01	5.50E+00	8.20E+00	9.78E+00	7.46E-02	8.58E+01	1.02E+02
9296L	SOST TG 6 month TG	F	AP	L	8.75E-01	1.13E-01	3.47E+00	1.54E+00	1.15E+01	3.88E-01	2.01E+01	1.50E+02
9296R	SOST TG 6 month TG	F	ML	R	9.13E-01	2.92E-01	3.47E+00	1.02E+01	1.45E+01	2.07E-01	8.30E+01	1.17E+02
9526L	SOST TG 6 month WT	F	AP	L	8.36E-01	1.51E-01	4.08E+00	4.54E+00	2.16E+01	5.21E-01	5.20E+01	2.47E+02
9526R	SOST TG 6 month WT	F	ML	R	1.11E+00	2.84E-01	2.40E+00	9.37E+00	2.46E+01	2.77E-01	7.09E+01	1.86E+02
9527L	SOST TG 6 month WT	F	AP	L	1.20E+00	2.37E-01	1.88E+00	2.91E+00	2.31E+01	3.68E-01	2.23E+01	1.77E+02
9527R	SOST TG 6 month WT	F	ML	R	1.15E+00	3.78E-01	1.21E+00	2.98E+00	1.89E+01	5.91E-01	1.87E+01	1.19E+02
9528L	SOST TG 6 month DEF	F	AP	L	9.36E-01	1.25E-01	9.02E+00	7.28E+00	1.83E+01	4.01E-01	8.92E+01	2.24E+02
9528R	SOST TG 6 month DEF	F	ML	R	7.15E-01	1.54E-01	6.64E+00	1.51E+01	1.51E+01	0.00E+00	1.87E+02	1.87E+02
9529L	SOST TG 6 month WT	F	ML	L	1.05E+00	2.82E-01	4.98E+00	2.02E+01	2.09E+01	1.01E-02	1.54E+02	1.59E+02
9529R	SOST TG 6 month WT	F	AP	R	1.11E+00	1.78E-01	6.79E+00	1.73E+01	1.88E+01	6.52E-02	1.63E+02	1.77E+02

9530L	SOST TG 6 month DEF	F	ML	L	1.18E+00	3.70E-01	3.18E+00	1.89E+01	2.01E+01	2.23E-01	1.18E+02	1.26E+02
9530R	SOST TG 6 month DEF	F	AP	R	1.09E+00	1.92E-01	2.28E+00	2.06E+00	1.31E+01	3.51E-01	1.78E+01	1.13E+02
5588L	SOST TG 8 month DEF	F	AP	L	8.76E-01	1.41E-01	8.18E+00	1.28E+01	1.70E+01	8.60E-02	1.46E+02	1.93E+02
5588R	SOST TG 8 month DEF	F	ML	R	9.96E-01	3.07E-01	3.82E+00	1.42E+01	1.60E+01	1.78E-01	1.05E+02	1.18E+02
5589L	SOST TG 8 month DEF	F	ML	L	7.70E-01	1.86E-01	6.61E+00	1.48E+01	1.50E+01	2.21E-01	1.60E+02	1.62E+02
5589R	SOST TG 8 month DEF	F	AP	R	9.04E-01	1.42E-01	6.64E+00	7.57E+00	1.81E+01	1.74E-01	8.65E+01	2.07E+02
5590L	SOST TG 8 month WT	F	ML	L	1.37E+00	7.85E-01	1.01E+00	7.05E+00	1.34E+01	3.09E-01	2.77E+01	5.27E+01
5590R	SOST TG 8 month WT	F	AP	R	9.55E-01	1.83E-01	6.32E+00	1.18E+01	1.29E+01	2.28E-01	1.14E+02	1.25E+02
5592L	SOST TG 8 month TG	F	AP	L	1.03E+00	1.51E-01	7.99E+00	1.43E+01	1.51E+01	3.57E-02	1.53E+02	1.61E+02
5592R	SOST TG 8 month TG	F	ML	R	7.98E-01	1.98E-01	2.98E+00	3.06E+00	1.71E+01	4.31E-01	3.17E+01	1.77E+02
5593L	SOST TG 8 month WT	F	AP	L	1.23E+00	1.99E-01	6.21E+00	3.13E+00	1.80E+01	4.96E-01	2.71E+01	1.55E+02
5593R	SOST TG 8 month WT	F	ML	R	8.33E-01	1.91E-01	6.13E+00	1.16E+01	1.33E+01	2.85E-01	1.19E+02	1.36E+02
5597L	SOST TG 8 month WT	F	AP	L	7.20E-01	1.00E-01	1.21E+01	1.01E+01	1.09E+01	2.57E-02	1.50E+02	1.64E+02
5597R	SOST TG 8 month WT	F	ML	R	8.74E-01	2.54E-01	4.76E+00	1.20E+01	1.32E+01	2.62E-01	1.03E+02	1.13E+02
5598L	SOST TG 8 month WT	F	ML	L	9.53E-01	3.54E-01	2.79E+00	8.00E+00	1.24E+01	4.85E-01	5.62E+01	8.69E+01
5598R	SOST TG 8 month WT	F	AP	R	1.30E+00	2.17E-01	4.16E+00	1.26E+01	1.26E+01	0.00E+00	9.71E+01	9.71E+01
5599L	SOST TG 8 month TG	F	AP	L	9.25E-01	1.51E-01	3.80E+00	3.18E+00	1.06E+01	3.37E-01	3.42E+01	1.14E+02
5599R	SOST TG 8 month TG	F	ML	R	8.60E-01	2.77E-01	4.50E+00	1.22E+01	1.26E+01	2.12E-01	1.02E+02	1.05E+02
8007R	SOST TG 8 month DEF	F	AP	R	1.05E+00	2.11E-01	5.24E+00	7.24E+00	7.24E+00	0.00E+00	6.21E+01	6.21E+01
8008L	SOST TG 8 month DEF	F	AP	L	7.02E-01	1.08E-01	8.68E+00	1.09E+01	1.28E+01	1.66E-01	1.60E+02	1.88E+02
8008R	SOST TG 8 month DEF	F	ML	R	5.99E-01	1.10E-01	4.21E+00	2.55E+00	6.03E+00	7.49E-01	4.13E+01	9.79E+01
9198L	SOST KO 6 month KO	M	ML	L	2.80E+00	1.06E+00	3.01E-01	6.18E+01	6.18E+01	0.00E+00	1.68E+02	1.68E+02
9198R	SOST KO 6 month KO	M	AP	R	3.36E+00	7.12E-01	2.29E+00	5.19E+01	5.27E+01	0.00E+00	1.58E+02	1.61E+02
9201L	SOST KO 6 month KO	M	ML	L	2.55E+00	8.41E-01	3.38E-01	7.98E+01	8.01E+01	0.00E+00	2.53E+02	2.54E+02
9201R	SOST KO 6 month KO	M	AP	R	3.27E+00	8.04E-01	3.20E+00	6.74E-01	5.68E+01	3.98E-01	1.93E+00	1.63E+02
9202L	SOST KO 6 month KO	M	AP	L	2.95E+00	7.08E-01	1.38E+00	1.12E+00	4.73E+01	6.29E-01	3.58E+00	1.51E+02
9202R	SOST KO 6 month KO	M	ML	R	2.70E+00	1.03E+00	1.51E+00	7.78E+01	7.78E+01	0.00E+00	2.17E+02	2.17E+02
9203L	SOST KO 6 month KO	M	ML	L	2.33E+00	6.45E-01	3.01E-01	1.77E-01	4.30E+01	4.43E-01	6.74E-01	1.64E+02
9203R	SOST KO 6 month KO	M	AP	R	2.92E+00	7.74E-01	1.22E+00	4.06E+01	4.06E+01	0.00E+00	1.23E+02	1.23E+02
9207L	SOST KO 6 month WT	M	AP	L	1.42E+00	2.25E-01	5.40E-01	3.10E+01	3.10E+01	0.00E+00	2.36E+02	2.36E+02
9207R	SOST KO 6 month WT	M	ML	R	2.04E+00	1.29E+00	3.21E-01	1.54E+01	4.05E+01	4.16E-01	1.98E+01	5.21E+01
9208L	SOST KO 6 month WT	M	AP	L	1.74E+00	4.34E-01	3.26E+00	3.01E+01	3.01E+01	0.00E+00	1.46E+02	1.46E+02
9208R	SOST KO 6 month WT	M	ML	R	2.07E+00	1.45E+00	3.78E-01	3.58E+01	3.73E+01	0.00E+00	8.64E+01	9.00E+01
9209L	SOST KO 6 month WT	M	ML	L	1.62E+00	4.65E-01	1.05E+00	3.48E+01	3.48E+01	0.00E+00	1.73E+02	1.73E+02
9209R	SOST KO 6 month WT	M	AP	R	1.53E+00	3.48E-01	3.69E+00	7.42E+00	2.25E+01	1.67E-01	4.18E+01	1.27E+02
9238L	SOST KO 6 month WT	M	ML	L	1.31E+00	4.16E-01	3.56E+00	3.06E+01	3.06E+01	0.00E+00	1.76E+02	1.76E+02
9238R	SOST KO 6 month WT	M	AP	R	1.83E+00	4.18E-01	3.98E+00	2.48E+00	2.66E+01	2.84E-01	1.21E+01	1.30E+02
9239L	SOST KO 6 month WT	M	ML	L	1.46E+00	5.43E-01	2.69E+00	3.12E+01	3.12E+01	0.00E+00	1.47E+02	1.47E+02
9239R	SOST KO 6 month WT	M	AP	R	1.52E+00	2.45E-01	2.00E+00	5.29E+00	1.51E+01	3.42E-01	3.76E+01	1.07E+02
9240L	SOST KO 6 month WT	M	ML	L	1.58E+00	4.82E-01	2.52E+00	6.60E+00	3.82E+01	3.78E-01	3.26E+01	1.89E+02
9240R	SOST KO 6 month WT	M	AP	R	2.45E+00	4.09E-01	1.09E+00	3.09E+00	2.30E+01	3.34E-01	1.41E+01	1.05E+02
7544L	SOST KO 12 month KO	M	AP	L	3.55E+00	9.71E-01	1.03E+00	1.48E+01	5.10E+01	3.54E-01	3.82E+01	1.32E+02
7544R	SOST KO 12 month KO	M	ML	R	3.82E+00	2.43E+00	1.17E+00	5.90E+01	5.90E+01	0.00E+00	9.30E+01	9.30E+01

7545L	SOST KO 12 month WT	M	AP	L	1.59E+00	3.78E-01	2.18E+00	7.35E+00	1.92E+01	5.42E-01	3.97E+01	1.04E+02
7545R	SOST KO 12 month WT	M	ML	R	1.53E+00	6.51E-01	3.03E+00	1.27E+01	3.24E+01	2.58E-01	5.18E+01	1.33E+02
7564L	SOST KO 12 month WT	M	AP	L	1.06E+00	1.36E-01	1.05E+01	2.35E+01	2.43E+01	4.22E-02	2.59E+02	2.68E+02
7564R	SOST KO 12 month WT	M	ML	R	1.09E+00	4.10E-01	2.69E+00	2.41E+01	2.63E+01	0.00E+00	1.47E+02	1.60E+02
7565L	SOST KO 12 month KO	M	AP	L	4.71E+00	1.05E+00	1.69E+00	7.04E+01	7.04E+01	0.00E+00	1.57E+02	1.57E+02
7565R	SOST KO 12 month KO	M	ML	R	3.80E+00	1.80E+00	1.55E+00	6.08E+01	6.08E+01	0.00E+00	1.13E+02	1.13E+02
7592L	SOST KO 12 month KO	M	ML	L	3.28E+00	1.28E+00	2.82E+00	7.47E+01	7.49E+01	0.00E+00	1.75E+02	1.76E+02
7592R	SOST KO 12 month KO	M	AP	R	2.99E+00	5.58E-01	3.58E+00	5.69E+01	5.98E+01	0.00E+00	2.08E+02	2.19E+02
7593L	SOST KO 12 month KO	M	ML	L	2.97E+00	1.24E+00	1.91E+00	7.35E+00	6.77E+01	2.57E-01	1.78E+01	1.65E+02
7593R	SOST KO 12 month KO	M	AP	R	4.57E+00	9.94E-01	1.46E+00	5.61E+01	5.61E+01	0.00E+00	1.27E+02	1.27E+02
7594L	SOST KO 12 month KO	M	ML	L	5.28E+00	6.98E+00	1.17E-01	8.52E+01	8.52E+01	0.00E+00	6.29E+01	6.29E+01
7594R	SOST KO 12 month KO	M	AP	R	4.59E+00	1.59E+00	1.09E+00	6.01E+01	6.12E+01	0.00E+00	1.05E+02	1.06E+02

APPENDIX B: Matlab Code

```
clear

%choose manual input of bone geometries or entry from .csv file

prompt = {'Manual entry (enter "0") or Entry from .csv file (enter "1"):'};

dlg_title = 'Bone Geometry Dimensions';

num_lines = 1;

def = {'1'};

answer = inputdlg(prompt,dlg_title,num_lines,def);

man_auto = str2num(answer{1}); %state: manual = 0, automatic = 1

%request path (to save outputs) from user

path = uigetdir('','Choose or create a location to save outputs:');

%create data file for outputs

datafile = uiputfile('*.csv', 'Save output data file as:', 'datafile.csv');

fid = fopen(datafile, 'a');

labels = {'Bone Name:' 'Bending Direction:' 'Cross-sectional Area (mm^2):' 'Eobs (GPa):'
'Yield Load (N):' 'Ultimate Load (N):' 'Post Yield Displacement (mm):' 'Yield Stress
(MPa):' 'Ultimate Stress (MPa):' 'R^2:'};

for n = 1:size(labels)

    fprintf(fid,'%s',labels{n,1:end-1});

    fprintf(fid,'%s\n',labels{n,end});

end

fclose(fid);
```

```

if man_auto == 0

    %request directory location from user

    [PathName] = uigetdir('','Select directory of data files for analysis:');

    %load data files

    allfiles = dir([PathName, '*.FIB']);

end

if man_auto == 1

    %request directory location from user

    [PathName] = uigetdir('','Select directory of data files for analysis:');

    %load data files

    allfiles = dir([PathName, '*.FIB']);

    %request bone geometry file location from user

    [FileName_geo,PathName_geo] = uigetfile('*.FIB','Select file containing bone geometry
data');

    %load bone geometry data file

    fid=fopen([PathName_geo,FileName_geo]);

    bonedata = textscan(fid,'%s %s %s %f %f %f %f %f %f
%f','HeaderLines',1,'Delimiter',' ','CollectOutput',1);

    fclose(fid);

```

```

%create name matching array

for i = 1:length(bonedata{1,1})

    matchname(i,1) = strcat(bonedata{1,1}(i,1), bonedata{1,1}(i,2));

end

end

%open waitbar

wait = waitbar(0,'Please Wait...','Name','Generating
Plots...','CreateCancelBtn','setappdata(gcf,"canceling",1)');

setappdata(wait,'canceling',0)

for e = 1:length(allfiles)

    %stop calculations if 'Canel' is clicked on waitbar

    if getappdata(wait,'canceling')

        break

    end

    waitbar(e/length(allfiles),wait)

    %clear working variables

    clear F M cx cy fdx fdy maxind maxval regx regy strain stress x yfit yresid indval;

```



```

FileName = allfiles(e).name;

file = csvread([PathName, '\', FileName]);


%remove extension from filename

FileName = strep(FileName, '.FIB', '');


%establish arrays

x = zeros(size(file(),1),1); %displacement

F = zeros(size(file(),1),1); %force

M = zeros(size(file(),1),1); %bending moment

stress = zeros(size(file(),1),1); %stress

strain = zeros(size(file(),1),1); %strain

avg = zeros(size(file(),1),1); %average difference in stress


%populate arrays

for n = 1:size(file(),1)

    x(n,1) = file(n,1);

    F(n,1) = file(n,2);

end


%unit conversion

x = x*(1E-3); %um to mm

F = F*(1E-3); %mN to N


comp = 0;

```

```

if man_auto == 1

    for i = 1:length(bonedata{1,1})

        if strcmp(matchname(i,1), FileName) == 1

            L = bonedata{1,2}(i,2); %bone length

            APi = bonedata{1,2}(i,6); %anterior-posterior inner diameter

            APo = bonedata{1,2}(i,5); %anterior-posterior outer diameter

            MLi = bonedata{1,2}(i,4); %medial-lateral inner diameter

            MLo = bonedata{1,2}(i,3); %medial-lateral outer diameter

            d = bonedata{1,2}(i,1); %distance between supports


            %warn user if inner/outer dimensions are reversed

            if APi > APo

                errordlg('Inner AP dimension must be less than outer AP dimension. Check
values in "bone measurements" data file.', 'AP Size Error');

                error('Inner AP dimension must be less than outer AP dimension. Check
values in "bone measurements" data file.');
```

end

```

            if MLi > MLo

                errordlg('Inner ML dimension must be less than outer ML dimension. Check
values in "bone measurements" data file.', 'ML Size Error');
```

error('Inner ML dimension must be less than outer ML dimension. Check values in "bone measurements" data file.');

```

            end

            AP_ML_state = strcmp(bonedata{1,1}(i,3), 'ML');
```

comp = 1;

```

elseif strcmp(matchname(i,1), FileName) == 0 && i == length(bonedata{1,1})
&& comp == 0

    %request measured geometry values from user

    prompt = {'Length (mm):','AP inner diameter (mm):','AP outer
diameter(mm):','ML inner diameter (mm):','ML outer diameter (mm):','Distance between
supports (mm):'};

    dlg_title = [FileName, ' -- Geometry'];

    num_lines = 1;

    def = {' ',' ',' ',' ',' '};

    answer = inputdlg(prompt,dlg_title,num_lines,def);

    L = str2num(answer{1}); %bone length

    APi = str2num(answer{2}); %anterior-posterior inner diameter

    APo = str2num(answer{3}); %anterior-posterior outer diameter

    MLi = str2num(answer{4}); %medial-lateral inner diameter

    MLo = str2num(answer{5}); %medial-lateral outer diameter

    d = str2num(answer{1}); %distance between supports

    %warn user if inner/outer dimensions are reversed

    if APi > APo

        errordlg('Inner AP dimension must be less than outer AP dimension', 'AP
Size Error');

        error('Inner AP dimension must be less than outer AP dimension');

    end

    if MLi > MLo

```

```
        errordlg('Inner ML dimension must be less than outer ML dimension', 'ML  
Size Error');
```

```
        error('Inner ML dimension must be less than outer ML dimension');
```

```
    end
```

```
    %request bending direction from user
```

```
    prompt = {'Anterior-Posterior (enter "0") or Medial-Lateral (enter "1"):'};
```

```
    dlg_title = [FileName, ' -- Bending Direction'];
```

```
    num_lines = 1;
```

```
    def = {'0'};
```

```
    answer = inputdlg(prompt,dlg_title,num_lines,def);
```

```
    AP_ML_state = str2num(answer{1}); %state: 0 for AP, 1 for ML
```

```
    end
```

```
end
```

```
else
```

```
    %request measured geometry values from user
```

```
    prompt = {'Length (mm):','AP inner diameter (mm):','AP outer diameter(mm):','ML  
inner diameter (mm):','ML outer diameter (mm):','Distance between supports (mm):'};
```

```
    dlg_title = [FileName, ' -- Geometry'];
```

```
    num_lines = 1;
```

```
    def = {' ',' ',' ',' ',' '};
```

```
    answer = inputdlg(prompt,dlg_title,num_lines,def);
```

```

L = str2num(answer{1}); %bone length

APi = str2num(answer{2}); %anterior-posterior inner diameter
APo = str2num(answer{3}); %anterior-posterior outer diameter
MLi = str2num(answer{4}); %medial-lateral inner diameter
MLo = str2num(answer{5}); %medial-lateral outer diameter
d = str2num(answer{1}); %distance between supports


%warn user if inner/outer dimensions are reversed
if APi > APo
    errordlg('Inner AP dimension must be less than outer AP dimension', 'AP Size
Error');
    error('Inner AP dimension must be less than outer AP dimension');
end
if MLi > MLo
    errordlg('Inner ML dimension must be less than outer ML dimension', 'ML Size
Error');
    error('Inner ML dimension must be less than outer ML dimension');
end


%request bending direction from user
prompt = {'Anterior-Posterior (enter "0") or Medial-Lateral (enter "1"):'};
dlg_title = [FileName, ' -- Bending Direction'];
num_lines = 1;
def = {'0'};
answer = inputdlg(prompt,dlg_title,num_lines,def);

```

```

AP_ML_state = str2num(answer{1}); %state: 0 for AP, 1 for ML

end

%calculate cross-sectional area of bone
A = (pi/4)*((APo*MLo)-(APi*MLi));

%calculate area moment of inertia
if AP_ML_state == 0
    Ixx = (pi/64)*((MLo*(APo^3))-(MLi*(APi^3))); %anterior-posterior
else
    Ixx = (pi/64)*((APo*(MLo^3))-(APi*(MLi^3))); %medial-lateral
end

%calculate bending moment
for n = 1:size(file(),1)
    M(n,1) = F(n,1)*(d/4);
End

%calculate bending stress (in units of MPa) and strain for each data point
if AP_ML_state == 0
    for n = 1:size(file(),1)
        stress(n,1) = ((M(n,1)*(APo/2))/Ixx); %also convert GPa to MPa
        strain(n,1) = (12*x(n,1)*(APo/2))/(d^2);
    end
end

```

```

    end
else
    for n = 1:size(file(),1)
        stress(n,1) = ((M(n,1)*(MLo/2))/Ixx);
        strain(n,1) = (12*x(n,1)*(MLo/2))/(d^2);
    end
end

%remove irrelevant data
cy(1,1) = 0;
cx(1,1) = 0;
fdy(1,1) = 0;
fdx(1,1) = 0;

for n = 1:size(file(),1)-1
    if strain(n+1,1)-strain(n,1) > 0
        cy(n+1,1) = stress(n,1);
        cx(n+1,1) = strain(n,1);
    end
    if x(n+1,1)-x(n,1) > 0
        fdy(n+1,1) = F(n,1);
        fdx(n+1,1) = x(n,1);
    end
end
end

```

```

n = 1;

k = 0;

while k ~= 1

    if cy(n,1)-cy(n+1,1) > (0.5)*mean(cy()) %if the difference between adjacent y
values is greater than half of the current average

        cy = cy(1:n+3,1);

        cx = cx(1:n+3,1);

        fdy = fdy(1:n+3,1);

        fdx = fdx(1:n+3,1);

        k = 1;

    end

    n = n+1;

end

% determine ultimate tensile strength

UTS = cy(1,1);

for n = 1:size(cy(),1)

    if cy(n,1) > UTS

        UTS = cy(n,1);

        indval(1,1) = n;

    end

end

end

```



```

%linear regression to determine elastic modulus and yield strength
n = 50;          %start at point 50 to start with 'established' R^2 value
k = 0;
while n < size(cy(),1) && k == 0
    regx = zeros(n,1);
    regy = zeros(n,1);
    for m = 1:n
        regx(m,1) = cx(m,1);
        regy(m,1) = cy(m,1);
    end
    reg = regx\regy;
    yfit = polyval([reg,0],regx);
    yresid = regy - yfit;
    SSresid = sum(yresid.^2);
    SStotal = (length(regy)-1)*var(regy);
    rsq(n,1) = 1 - (SSresid/SStotal);

    if rsq(n,1) < rsq(n-1,1) && rsq(n,1) > 0.4 %if the current R^2 value is less than the
previous one, and greater than 0.4 exit the loop -- it's not necessary to re-calculate
        k = 1;
        rsq_ind = n;
    end

    n = n+1;
end

```

```

%calculate yield strength using 0.2% offset method

n = 50;

k = 0;

while n <= size(cx(),1) && k == 0

    if cy(n,1) > 0.93*reg*(cx(n,1) - 0.002) && cy(n,1) < 1.07*reg*(cx(n,1) - 0.002);

        YS = cy(n,1);

        indval(2,1) = n;

        k = 1;

    elseif n == size(cx(),1)

        YS = cy(n-3,1);

        indval(2,1) = n - 3; %so that yield isn't 0

    end

    n = n+1;

end

```

```

Eobs = reg/1000; %observed elastic modulus (GPa)

```

```

Eobsx(1,1) = 0;

```

```

Eobsy(1,1) = 0;

```

```

Eobsx(2,1) = cx(rsq_ind);

```

```

Eobsy(2,1) = reg*Eobsx(2,1);

```

```

%other values to display

```

```

yl = fdy(indval(2,1),1); %yield load (N)

```

```

ul = fdy(indval(1,1),1); %ultimate load (N)

```

```

pyd = fdx(end-3,1) - fdx(indval(2,1),1); %post yield displacement (mm)

%plot force vs. displacement
figure('visible','off'); %suppress graphics output
p1 = plot (fdx,fdy);
xlabel('Displacement (mm)')
ylabel('Force (N)')
title(['Force vs. Displacement, Bone ' FileName])
set(p1,'Color','red','LineWidth',2) %change the line color and set its width to 2]
print('-dbmp','-r150',[path,'\',FileName,'_FD']); %save figure to output file

%plot stress vs. strain
figure('visible','off');
p2 = plot
(cx,cy,Eobsx,Eobsy,cx(indval(2,1)),cy(indval(2,1)),'^',cx(indval(1,1)),cy(indval(1,1)),'v');
xlabel('Strain (mm/mm)')
ylabel('Stress (MPa)')
title(['Stress vs. Strain, Bone ' FileName])
set(p2,'LineWidth',2) %set width to 2
print('-dbmp','-r150',[path,'\',FileName,'_SS']);
%print relevant calculated values to data file
fid = fopen(datafile, 'a');
%   fprintf(fid, '\r\n');
if AP_ML_state == 0
    AP_ML = 'AP';

```

```

else
    AP_ML = 'ML';
end
values = {FileName, AP_ML, A, Eobs, yl, ul, pyd, YS, UTS, Ixx, rsq(rsq_ind,1)};
for n = 1:size(values)
    fprintf(fid,'%s',values{n,1:2});
    fprintf(fid,'%d',values{n,3:end-1});
    fprintf(fid,'%d\n',values{n,end});
end
fclose(fid);
end

delete(wait)

```

APPENDIX C: Minitab Statistical Outputs

Results for: BONE DATA__BLANK.MTW

General Linear Model: CSA versus Genotype+Phenotype, Bend Direction, Leg

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML
Leg	fixed	2	L, R

Analysis of Variance for CSA, using Adjusted SS for Tests

Source	Model DF	Reduced DF	Seq SS
Genotype+Phenotype	9	9	93.7382
Bend Direction	1	1	0.3173
Leg	1	1	0.1031
Genotype+Phenotype*Bend Direction	9	9	0.4223
Genotype+Phenotype*Leg	9	6+	0.5387
Bend Direction*Leg	1	1	0.0034
Genotype+Phenotype*Bend Direction*Leg	9	6+	0.1800
Error	57	63	7.7023
Total	96	96	103.0053

+ Rank deficiency due to empty cells, unbalanced nesting, collinearity, or an undeclared covariate. No storage of results or further analysis will be done.

S = 0.349656 R-Sq = 92.52% R-Sq(adj) = 88.61%

Test for Equal Variances: CSA versus Genotype+Phenotype, Bend Direction, Leg

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend Direction	Leg	N	Lower	StDev	Upper
SOST KO 12 month KO	AP	L	2	0.244778	0.82015	811.434
SOST KO 12 month KO	AP	R	3	0.344564	0.91960	32.376
SOST KO 12 month KO	ML	L	3	0.468945	1.25155	44.063
SOST KO 12 month KO	ML	R	2	0.004917	0.01647	16.300
SOST KO 12 month WT	AP	L	2	0.111056	0.37210	368.149
SOST KO 12 month WT	ML	R	2	0.092843	0.31108	307.772
SOST KO 6 month KO	AP	L	1	*	*	*
SOST KO 6 month KO	AP	R	3	0.087430	0.23334	8.215
SOST KO 6 month KO	ML	L	3	0.089483	0.23882	8.408
SOST KO 6 month KO	ML	R	1	*	*	*
SOST KO 6 month WT	AP	L	2	0.067568	0.22639	223.987
SOST KO 6 month WT	AP	R	4	0.183775	0.43387	5.181
SOST KO 6 month WT	ML	L	4	0.057620	0.13603	1.624
SOST KO 6 month WT	ML	R	2	0.006890	0.02309	22.841
SOST TG 6 month DEF	AP	L	3	0.056994	0.15211	5.355
SOST TG 6 month DEF	AP	R	6	0.070939	0.14542	0.741

SOST TG 6 month DEF	ML	L	6	0.088371	0.18115	0.923
SOST TG 6 month DEF	ML	R	3	0.048725	0.13004	4.578
SOST TG 6 month TG	AP	L	2	0.004430	0.01484	14.684
SOST TG 6 month TG	ML	R	2	0.008237	0.02760	27.305
SOST TG 6 month WT	AP	L	7	0.095057	0.18599	0.766
SOST TG 6 month WT	AP	R	4	0.042941	0.10138	1.211
SOST TG 6 month WT	ML	L	4	0.022156	0.05231	0.625
SOST TG 6 month WT	ML	R	7	0.063178	0.12362	0.509
SOST TG 8 month DEF	AP	L	2	0.036697	0.12296	121.651
SOST TG 8 month DEF	AP	R	2	0.031682	0.10615	105.026
SOST TG 8 month DEF	ML	L	1	*	*	*
SOST TG 8 month DEF	ML	R	2	0.083828	0.28087	277.886
SOST TG 8 month TG	AP	L	2	0.022497	0.07538	74.579
SOST TG 8 month TG	ML	R	2	0.013044	0.04370	43.239
SOST TG 8 month WT	AP	L	2	0.106878	0.35810	354.299
SOST TG 8 month WT	AP	R	2	0.072463	0.24279	240.213
SOST TG 8 month WT	ML	L	2	0.087053	0.29168	288.580
SOST TG 8 month WT	ML	R	2	0.008537	0.02860	28.301

Bartlett's Test (Normal Distribution)
Test statistic = 80.70, p-value = 0.000

Levene's Test (Any Continuous Distribution)
Test statistic = 1.13, p-value = 0.337

Test for Equal Variances: CSA versus Genotype+Phenotype, Bend Direction, Leg

DUE TO LACK OF REPETITION CANNOT RUN GLM WITH ALL FACTORS. BEND DIRECTION WILL BE EXCLUDED AS CSA SHOULD NOT DEPEND ON THIS FACTOR.

Test for Equal Variances: CSA versus Genotype+Phenotype, Leg

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Leg	N	Lower	StDev	Upper
SOST KO 12 month KO	L	5	0.465962	0.987673	6.194
SOST KO 12 month KO	R	5	0.313128	0.663719	4.162
SOST KO 12 month WT	L	2	0.115301	0.372102	237.516
SOST KO 12 month WT	R	2	0.096392	0.311077	198.563
SOST KO 6 month KO	L	4	0.119556	0.274316	2.829
SOST KO 6 month KO	R	4	0.134463	0.308519	3.181
SOST KO 6 month WT	L	6	0.076218	0.152434	0.710
SOST KO 6 month WT	R	6	0.177635	0.355266	1.655
SOST TG 6 month DEF	L	9	0.094006	0.168023	0.498
SOST TG 6 month DEF	R	9	0.077993	0.139402	0.413
SOST TG 6 month TG	L	2	0.004599	0.014841	9.473
SOST TG 6 month TG	R	2	0.008552	0.027599	17.616
SOST TG 6 month WT	L	11	0.086897	0.147959	0.375
SOST TG 6 month WT	R	11	0.066959	0.114010	0.289
SOST TG 8 month DEF	L	3	0.033896	0.087638	2.478
SOST TG 8 month DEF	R	4	0.088213	0.202402	2.087
SOST TG 8 month TG	L	2	0.023357	0.075379	48.115
SOST TG 8 month TG	R	2	0.013542	0.043704	27.896
SOST TG 8 month WT	L	4	0.125280	0.287449	2.964
SOST TG 8 month WT	R	4	0.092164	0.211467	2.180

Bartlett's Test (Normal Distribution)

Test statistic = 73.79, p-value = 0.000

Levene's Test (Any Continuous Distribution)

Test statistic = 3.09, p-value = 0.000

Test for Equal Variances: CSA versus Genotype+Phenotype, Leg

P-VAL < 0.05 TELLS THAT CSA IS DIFFERENT BASED ON GENOTYPE+PHENOTYPE AND LEG.

General Linear Model: CSA versus Genotype+Phenotype, Leg

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Leg	fixed	2	L, R

Analysis of Variance for CSA, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	93.7382	93.7417	10.4157	95.29	0.000
Leg	1	0.0916	0.0637	0.0637	0.58	0.448
Genotype+Phenotype*Leg	9	0.7585	0.7585	0.0843	0.77	0.643
Error	77	8.4170	8.4170	0.1093		
Total	96	103.0053				

S = 0.330622 R-Sq = 91.83% R-Sq(adj) = 89.81%

Unusual Observations for CSA

Obs	CSA	Fit	SE Fit	Residual	St Resid
90	4.70690	3.95674	0.14786	0.75015	2.54 R
92	3.28288	3.95674	0.14786	-0.67387	-2.28 R
93	2.98779	3.95264	0.14786	-0.96485	-3.26 R
94	2.96983	3.95674	0.14786	-0.98692	-3.34 R
95	4.57234	3.95264	0.14786	0.61970	2.10 R
96	5.27709	3.95674	0.14786	1.32034	4.46 R
97	4.58869	3.95264	0.14786	0.63604	2.15 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month KO	10	3.9547	A
SOST KO 6 month KO	8	2.8581	B
SOST KO 6 month WT	12	1.7129	C
SOST KO 12 month WT	4	1.3174	C D
SOST TG 6 month WT	22	1.0365	D
SOST TG 8 month WT	8	1.0280	D
SOST TG 6 month DEF	18	0.9375	D
SOST TG 8 month TG	4	0.9038	D
SOST TG 6 month TG	4	0.8793	D

SOST TG 8 month DEF 7 0.8354 D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Leg	N	Mean	Grouping
R	49	1.5765	A
L	48	1.5162	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Leg	N	Mean	Grouping
SOST KO 12 month KO	L	5	3.9567	A
SOST KO 12 month KO	R	5	3.9526	A
SOST KO 6 month KO	R	4	3.0604	B
SOST KO 6 month KO	L	4	2.6557	B C
SOST KO 6 month WT	R	6	1.9059	C D
SOST KO 6 month WT	L	6	1.5199	D E
SOST KO 12 month WT	L	2	1.3241	D E
SOST KO 12 month WT	R	2	1.3108	D E
SOST TG 8 month WT	L	4	1.0660	E
SOST TG 6 month WT	L	11	1.0420	E
SOST TG 6 month WT	R	11	1.0310	E
SOST TG 8 month WT	R	4	0.9899	E
SOST TG 8 month TG	L	2	0.9787	D E
SOST TG 6 month DEF	L	9	0.9711	E
SOST TG 6 month DEF	R	9	0.9039	E
SOST TG 6 month TG	R	2	0.8936	E
SOST TG 8 month DEF	R	4	0.8884	E
SOST TG 6 month TG	L	2	0.8650	E
SOST TG 8 month TG	R	2	0.8288	E
SOST TG 8 month DEF	L	3	0.7825	E

Means that do not share a letter are significantly different.

Interaction Plot for CSA

Probability Plot of SRES1

TEST FOR NORMALITY SHOWS OUTLIERS WELL ABOVE 2 STDEV FROM THE MEAN. THESE 3 OBSERVATIONS WILL BE EXCLUDED FROM THIS DATA SET (7592R, 7593L, 7594L).

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Leg

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Leg	N	Lower	StDev	Upper
SOST KO 12 month KO	L	5	1.57570	3.33992	20.95
SOST KO 12 month KO	R	5	1.05887	2.24444	14.08
SOST KO 12 month WT	L	2	0.49319	1.59164	1015.96
SOST KO 12 month WT	R	2	0.41231	1.33061	849.34
SOST KO 6 month KO	L	4	0.41755	0.95805	9.88
SOST KO 6 month KO	R	4	0.46961	1.07751	11.11

SOST KO 6 month WT	L	6	0.25253	0.50506	2.35
SOST KO 6 month WT	R	6	0.58856	1.17710	5.48
SOST TG 6 month DEF	L	9	0.30158	0.53903	1.60
SOST TG 6 month DEF	R	9	0.25021	0.44721	1.33
SOST TG 6 month TG	L	2	0.01967	0.06348	40.52
SOST TG 6 month TG	R	2	0.03658	0.11805	75.35
SOST TG 6 month WT	L	11	0.27566	0.46936	1.19
SOST TG 6 month WT	R	11	0.21241	0.36166	0.92
SOST TG 8 month DEF	L	3	0.12556	0.32464	9.18
SOST TG 8 month DEF	R	4	0.30809	0.70689	7.29
SOST TG 8 month TG	L	2	0.09991	0.32243	205.81
SOST TG 8 month TG	R	2	0.05793	0.18694	119.32
SOST TG 8 month WT	L	4	0.43754	1.00392	10.35
SOST TG 8 month WT	R	4	0.32188	0.73855	7.62

Bartlett's Test (Normal Distribution)
Test statistic = 75.05, p-value = 0.000

Levene's Test (Any Continuous Distribution)
Test statistic = 3.20, p-value = 0.000

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Leg

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Leg

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Leg	N	Lower	StDev	Upper
SOST KO 12 month KO	L	3	0.990766	2.56159	72.43
SOST KO 12 month KO	R	4	0.658238	1.51030	15.57
SOST KO 12 month WT	L	2	0.493193	1.59164	1015.96
SOST KO 12 month WT	R	2	0.412308	1.33061	849.34
SOST KO 6 month KO	L	4	0.417550	0.95805	9.88
SOST KO 6 month KO	R	4	0.469613	1.07751	11.11
SOST KO 6 month WT	L	6	0.252531	0.50506	2.35
SOST KO 6 month WT	R	6	0.588556	1.17710	5.48
SOST TG 6 month DEF	L	9	0.301578	0.53903	1.60
SOST TG 6 month DEF	R	9	0.250208	0.44721	1.33
SOST TG 6 month TG	L	2	0.019671	0.06348	40.52
SOST TG 6 month TG	R	2	0.036580	0.11805	75.35
SOST TG 6 month WT	L	11	0.275658	0.46936	1.19
SOST TG 6 month WT	R	11	0.212408	0.36166	0.92
SOST TG 8 month DEF	L	3	0.125565	0.32464	9.18
SOST TG 8 month DEF	R	4	0.308086	0.70689	7.29
SOST TG 8 month TG	L	2	0.099910	0.32243	205.81
SOST TG 8 month TG	R	2	0.057926	0.18694	119.32
SOST TG 8 month WT	L	4	0.437541	1.00392	10.35
SOST TG 8 month WT	R	4	0.321885	0.73855	7.62

Bartlett's Test (Normal Distribution)
Test statistic = 46.16, p-value = 0.000

Levene's Test (Any Continuous Distribution)
Test statistic = 2.77, p-value = 0.001

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Leg

THERE IS NO EQUAL VARIANCE.

General Linear Model: CSA_1 versus Genotype+Phenotype, Leg

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Leg	fixed	2	L, R

Analysis of Variance for CSA_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	78.8471	77.0300	8.5589	140.78	0.000
Leg	1	0.1876	0.1548	0.1548	2.55	0.115
Genotype+Phenotype*Leg	9	0.8704	0.8704	0.0967	1.59	0.134
Error	74	4.4989	4.4989	0.0608		
Total	93	84.4039				

S = 0.246569 R-Sq = 94.67% R-Sq(adj) = 93.30%

Unusual Observations for CSA_1

Obs	CSA_1	Fit	SE Fit	Residual	St Resid
83	2.44513	1.90594	0.10066	0.53919	2.40 R
90	4.70690	3.84560	0.14236	0.86129	4.28 R
92	3.28288	3.84560	0.14236	-0.56273	-2.80 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month KO	7	4.0197	A
SOST KO 6 month KO	8	2.8581	B
SOST KO 6 month WT	12	1.7129	C
SOST KO 12 month WT	4	1.3174	C D
SOST TG 6 month WT	22	1.0365	D
SOST TG 8 month WT	8	1.0280	D
SOST TG 6 month DEF	18	0.9375	D
SOST TG 8 month TG	4	0.9038	D
SOST TG 6 month TG	4	0.8793	D
SOST TG 8 month DEF	7	0.8354	D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Leg	N	Mean	Grouping
R	48	1.6007	A
L	46	1.5051	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Leg	N	Mean	Grouping
SOST KO 12 month KO	R	4	4.1939	A
SOST KO 12 month KO	L	3	3.8456	A
SOST KO 6 month KO	R	4	3.0604	B
SOST KO 6 month KO	L	4	2.6557	B
SOST KO 6 month WT	R	6	1.9059	C
SOST KO 6 month WT	L	6	1.5199	C D
SOST KO 12 month WT	L	2	1.3241	C D E
SOST KO 12 month WT	R	2	1.3108	C D E
SOST TG 8 month WT	L	4	1.0660	D E
SOST TG 6 month WT	L	11	1.0420	E
SOST TG 6 month WT	R	11	1.0310	E
SOST TG 8 month WT	R	4	0.9899	D E
SOST TG 8 month TG	L	2	0.9787	D E
SOST TG 6 month DEF	L	9	0.9711	E
SOST TG 6 month DEF	R	9	0.9039	E
SOST TG 6 month TG	R	2	0.8936	D E
SOST TG 8 month DEF	R	4	0.8884	E
SOST TG 6 month TG	L	2	0.8650	D E
SOST TG 8 month TG	R	2	0.8288	D E
SOST TG 8 month DEF	L	3	0.7825	E

Means that do not share a letter are significantly different.

Results for: BONE DATA__BLANK.MTW

General Linear Model: Ixx_1 versus Genotype+Phenotype, Leg

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Leg	fixed	2	L, R

Analysis of Variance for Ixx_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	11.70817	11.31846	1.25761	21.75	0.000
Leg	1	0.15947	0.19304	0.19304	3.34	0.072
Genotype+Phenotype*Leg	9	0.49026	0.49026	0.05447	0.94	0.494
Error	76	4.39524	4.39524	0.05783		
Total	95	16.75314				

S = 0.240483 R-Sq = 73.76% R-Sq(adj) = 67.21%

Unusual Observations for Ixx_1

Obs	Ixx_1	Fit	SE Fit	Residual	St Resid
49	0.78461	0.35937	0.12024	0.42524	2.04 R
73	1.28804	0.69323	0.09818	0.59482	2.71 R
75	1.45154	0.69323	0.09818	0.75831	3.45 R

81	0.24481	0.69323	0.09818	-0.44842	-2.04	R
85	2.42895	1.47377	0.10755	0.95518	4.44	R
93	0.55750	1.47377	0.10755	-0.91626	-4.26	R
95	0.99449	1.47377	0.10755	-0.47928	-2.23	R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month KO	9	1.3	A
SOST KO 6 month KO	8	0.8	B
SOST KO 6 month WT	12	0.6	B C
SOST KO 12 month WT	4	0.4	B C D
SOST TG 8 month WT	8	0.3	C D
SOST TG 6 month WT	22	0.2	D
SOST TG 6 month TG	4	0.2	C D
SOST TG 8 month TG	4	0.2	C D
SOST TG 6 month DEF	18	0.2	D
SOST TG 8 month DEF	7	0.2	D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Leg	N	Mean	Grouping
R	49	0.5	A
L	47	0.4	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Leg	N	Mean	Grouping
SOST KO 12 month KO	R	5	1.5	A
SOST KO 12 month KO	L	4	1.1	A B
SOST KO 6 month KO	R	4	0.8	B C
SOST KO 6 month KO	L	4	0.8	B C D
SOST KO 6 month WT	R	6	0.7	B C D
SOST KO 12 month WT	R	2	0.5	B C D E
SOST KO 6 month WT	L	6	0.4	C D E
SOST TG 8 month WT	L	4	0.4	C D E
SOST TG 6 month TG	R	2	0.3	C D E
SOST KO 12 month WT	L	2	0.3	C D E
SOST TG 6 month WT	R	11	0.2	E
SOST TG 8 month TG	R	2	0.2	C D E
SOST TG 8 month WT	R	4	0.2	C D E
SOST TG 6 month DEF	L	9	0.2	E
SOST TG 6 month WT	L	11	0.2	E
SOST TG 8 month DEF	R	4	0.2	D E
SOST TG 6 month DEF	R	9	0.2	E
SOST TG 8 month TG	L	2	0.2	C D E
SOST TG 8 month DEF	L	3	0.1	D E
SOST TG 6 month TG	L	2	0.1	C D E

Means that do not share a letter are significantly different.

Results for: BONE DATA__BLANK.MTW

General Linear Model: E versus Genotype+Phenotype, Bend Direction

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for E, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	320.357	315.522	35.058	7.93	0.000
Bend Direction	1	55.869	58.883	58.883	13.33	0.000
Genotype+Phenotype*Bend Direction	9	22.948	22.948	2.550	0.58	0.812
Error	77	340.203	340.203	4.418		
Total	96	739.377				

S = 2.10196 R-Sq = 53.99% R-Sq(adj) = 42.63%

Unusual Observations for E

Obs	E	Fit	SE Fit	Residual	St Resid
31	9.6009	5.2415	0.7007	4.3594	2.20 R
44	2.2839	7.0915	0.7007	-4.8076	-2.43 R
55	12.0800	7.1925	1.0510	4.8875	2.68 R
86	2.1841	6.3668	1.4863	-4.1827	-2.81 R
88	10.5495	6.3668	1.4863	4.1827	2.81 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST TG 6 month DEF	18	6.1665	A
SOST TG 8 month DEF	7	6.0325	A
SOST TG 8 month WT	8	5.4316	A
SOST TG 6 month WT	22	5.0449	A
SOST TG 8 month TG	4	4.8171	A B
SOST KO 12 month WT	4	4.6147	A B
SOST TG 6 month TG	4	2.8387	A B
SOST KO 6 month WT	12	2.0889	B
SOST KO 12 month KO	10	1.6407	B
SOST KO 6 month KO	8	1.3179	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend Direction	N	Mean	Grouping
AP	49	4.9170	A
ML	48	3.0817	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Bend		N	Mean	Grouping
	Direction				
SOST TG 8 month WT	AP		4	7.1925	A B C
SOST TG 8 month DEF	AP		4	7.1837	A B C
SOST TG 6 month DEF	AP		9	7.0915	A
SOST KO 12 month WT	AP		2	6.3668	A B C D E
SOST TG 8 month TG	AP		2	5.8945	A B C D E
SOST TG 6 month WT	AP		11	5.4497	A B C D
SOST TG 6 month DEF	ML		9	5.2415	A B C D E
SOST TG 8 month DEF	ML		3	4.8813	A B C D E
SOST TG 6 month WT	ML		11	4.6402	A B C D E
SOST TG 6 month TG	AP		2	3.7737	A B C D E
SOST TG 8 month TG	ML		2	3.7397	A B C D E
SOST TG 8 month WT	ML		4	3.6708	A B C D E
SOST KO 12 month WT	ML		2	2.8627	A B C D E
SOST KO 6 month WT	AP		6	2.4255	C D E
SOST KO 6 month KO	AP		4	2.0228	B C D E
SOST TG 6 month TG	ML		2	1.9037	A B C D E
SOST KO 12 month KO	AP		5	1.7698	D E
SOST KO 6 month WT	ML		6	1.7523	D E
SOST KO 12 month KO	ML		5	1.5117	D E
SOST KO 6 month KO	ML		4	0.6131	E

Means that do not share a letter are significantly different.

Probability Plot of SRES1

DUE TO TEST FOR NORMALITY SHOWING NORMAL DISTRIBUTION WITH NO OUTLIERS, NO DATA POINTS WILL BE EXCLUDED.

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend		N	Lower	StDev	Upper
	Direction					
SOST KO 12 month KO	AP		5	0.26325	0.55800	3.50
SOST KO 12 month KO	ML		5	0.24872	0.52720	3.31
SOST KO 12 month WT	AP		2	1.23321	3.97985	2540.37
SOST KO 12 month WT	ML		2	0.05061	0.16332	104.25
SOST KO 6 month KO	AP		4	0.21908	0.50266	5.18
SOST KO 6 month KO	ML		4	0.14360	0.32949	3.40
SOST KO 6 month WT	AP		6	0.37231	0.74461	3.47
SOST KO 6 month WT	ML		6	0.35239	0.70478	3.28
SOST TG 6 month DEF	AP		9	0.72357	1.29328	3.83
SOST TG 6 month DEF	ML		9	0.72441	1.29479	3.84
SOST TG 6 month TG	AP		2	0.09024	0.29123	185.89
SOST TG 6 month TG	ML		2	0.46251	1.49263	952.76
SOST TG 6 month WT	AP		11	0.58110	0.98944	2.51
SOST TG 6 month WT	ML		11	0.67158	1.14349	2.90
SOST TG 8 month DEF	AP		4	0.37368	0.85738	8.84
SOST TG 8 month DEF	ML		3	0.34045	0.88021	24.89
SOST TG 8 month TG	AP		2	0.61790	1.99410	1272.85
SOST TG 8 month TG	ML		2	0.22465	0.72501	462.78
SOST TG 8 month WT	AP		4	0.81556	1.87128	19.29

SOST TG 8 month WT ML 4 0.53679 1.23164 12.70

Bartlett's Test (Normal Distribution)
Test statistic = 29.59, p-value = 0.057

Levene's Test (Any Continuous Distribution)
Test statistic = 2.37, p-value = 0.004

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

THERE IS NO EQUAL VARIANCE.

General Linear Model: E versus Genotype+Phenotype, Bend Direction

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for E, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	320.357	315.522	35.058	7.93	0.000
Bend Direction	1	55.869	58.883	58.883	13.33	0.000
Genotype+Phenotype*Bend Direction	9	22.948	22.948	2.550	0.58	0.812
Error	77	340.203	340.203	4.418		
Total	96	739.377				

S = 2.10196 R-Sq = 53.99% R-Sq(adj) = 42.63%

Unusual Observations for E

Obs	E	Fit	SE Fit	Residual	St Resid
31	9.6009	5.2415	0.7007	4.3594	2.20 R
44	2.2839	7.0915	0.7007	-4.8076	-2.43 R
55	12.0800	7.1925	1.0510	4.8875	2.68 R
86	2.1841	6.3668	1.4863	-4.1827	-2.81 R
88	10.5495	6.3668	1.4863	4.1827	2.81 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST TG 6 month DEF	18	6.1665	A
SOST TG 8 month DEF	7	6.0325	A
SOST TG 8 month WT	8	5.4316	A
SOST TG 6 month WT	22	5.0449	A
SOST TG 8 month TG	4	4.8171	A B
SOST KO 12 month WT	4	4.6147	A B
SOST TG 6 month TG	4	2.8387	A B

SOST KO 6 month WT	12	2.0889	B
SOST KO 12 month KO	10	1.6407	B
SOST KO 6 month KO	8	1.3179	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend			
Direction	N	Mean	Grouping
AP	49	4.9170	A
ML	48	3.0817	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

		Bend		
Genotype+Phenotype	Direction	N	Mean	Grouping
SOST TG 8 month WT	AP	4	7.1925	A B C
SOST TG 8 month DEF	AP	4	7.1837	A B C
SOST TG 6 month DEF	AP	9	7.0915	A
SOST KO 12 month WT	AP	2	6.3668	A B C D E
SOST TG 8 month TG	AP	2	5.8945	A B C D E
SOST TG 6 month WT	AP	11	5.4497	A B C D
SOST TG 6 month DEF	ML	9	5.2415	A B C D E
SOST TG 8 month DEF	ML	3	4.8813	A B C D E
SOST TG 6 month WT	ML	11	4.6402	A B C D E
SOST TG 6 month TG	AP	2	3.7737	A B C D E
SOST TG 8 month TG	ML	2	3.7397	A B C D E
SOST TG 8 month WT	ML	4	3.6708	A B C D E
SOST KO 12 month WT	ML	2	2.8627	A B C D E
SOST KO 6 month WT	AP	6	2.4255	C D E
SOST KO 6 month KO	AP	4	2.0228	B C D E
SOST TG 6 month TG	ML	2	1.9037	A B C D E
SOST KO 12 month KO	AP	5	1.7698	D E
SOST KO 6 month WT	ML	6	1.7523	D E
SOST KO 12 month KO	ML	5	1.5117	D E
SOST KO 6 month KO	ML	4	0.6131	E

Means that do not share a letter are significantly different.

Results for: BONE DATA__BLANK.MTW

General Linear Model: Yield Force versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Yield Force, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	20476.1	20457.0	2273.0	11.64	0.000

Bend Direction	1	1232.7	783.2	783.2	4.01	0.049
Genotype+Phenotype*Bend Direction	9	1584.2	1584.2	176.0	0.90	0.528
Error	77	15036.9	15036.9	195.3		
Total	96	38329.9				

S = 13.9744 R-Sq = 60.77% R-Sq(adj) = 51.09%

Unusual Observations for Yield Force

Obs	Yield Force	Fit	SE Fit	Residual	St Resid
65	51.9074	23.5825	6.9872	28.3249	2.34 R
66	79.8187	54.9027	6.9872	24.9161	2.06 R
70	0.1770	54.9027	6.9872	-54.7256	-4.52 R
84	14.7553	51.6400	6.2495	-36.8848	-2.95 R
94	7.3456	57.3987	6.2495	-50.0531	-4.00 R
96	85.2417	57.3987	6.2495	27.8430	2.23 R

R denotes an observation with a large standardized residual.

Probability Plot of SRES1

TEST FOR NORMALITY SHOWS OUTLIERS FOR 9203L, 7544L, and 7593L WHICH WILL BE EXCLUDED FROM THE ANALYSIS.

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend Direction	N	Lower	StDev	Upper
SOST KO 12 month KO	AP	4	0.229423	0.52640	5.428
SOST KO 12 month KO	ML	4	0.432059	0.99134	10.222
SOST KO 12 month WT	AP	2	0.357629	1.15415	736.701
SOST KO 12 month WT	ML	2	0.254434	0.82112	524.124
SOST KO 6 month KO	AP	4	0.957883	2.19782	22.662
SOST KO 6 month KO	ML	3	0.315984	0.81696	23.100
SOST KO 6 month WT	AP	6	0.530191	1.06037	4.939
SOST KO 6 month WT	ML	6	0.467183	0.93435	4.352
SOST TG 6 month DEF	AP	9	0.208100	0.37195	1.102
SOST TG 6 month DEF	ML	9	0.223995	0.40036	1.186
SOST TG 6 month TG	AP	2	0.110368	0.35618	227.353
SOST TG 6 month TG	ML	2	0.102924	0.33216	212.019
SOST TG 6 month WT	AP	11	0.247209	0.42092	1.067
SOST TG 6 month WT	ML	11	0.290562	0.49474	1.254
SOST TG 8 month DEF	AP	4	0.096449	0.22130	2.282
SOST TG 8 month DEF	ML	3	0.234258	0.60567	17.125
SOST TG 8 month TG	AP	2	0.245976	0.79382	506.700
SOST TG 8 month TG	ML	2	0.203683	0.65733	419.578
SOST TG 8 month WT	AP	4	0.154630	0.35479	3.658
SOST TG 8 month WT	ML	4	0.090328	0.20725	2.137

Bartlett's Test (Normal Distribution)
Test statistic = 43.01, p-value = 0.001

Levene's Test (Any Continuous Distribution)
Test statistic = 3.39, p-value = 0.000

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Bend Direction

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend Direction	N	Lower	StDev	Upper
SOST KO 12 month KO	AP	5	0.80744	1.71149	10.733
SOST KO 12 month KO	ML	5	1.13113	2.39759	15.036
SOST KO 12 month WT	AP	2	0.35763	1.15415	736.701
SOST KO 12 month WT	ML	2	0.25443	0.82112	524.124
SOST KO 6 month KO	AP	4	0.95788	2.19782	22.662
SOST KO 6 month KO	ML	4	1.34566	3.08756	31.836
SOST KO 6 month WT	AP	6	0.53019	1.06037	4.939
SOST KO 6 month WT	ML	6	0.46718	0.93435	4.352
SOST TG 6 month DEF	AP	9	0.20810	0.37195	1.102
SOST TG 6 month DEF	ML	9	0.22399	0.40036	1.186
SOST TG 6 month TG	AP	2	0.11037	0.35618	227.353
SOST TG 6 month TG	ML	2	0.10292	0.33216	212.019
SOST TG 6 month WT	AP	11	0.24721	0.42092	1.067
SOST TG 6 month WT	ML	11	0.29056	0.49474	1.254
SOST TG 8 month DEF	AP	4	0.09645	0.22130	2.282
SOST TG 8 month DEF	ML	3	0.23426	0.60567	17.125
SOST TG 8 month TG	AP	2	0.24598	0.79382	506.700
SOST TG 8 month TG	ML	2	0.20368	0.65733	419.578
SOST TG 8 month WT	AP	4	0.15463	0.35479	3.658
SOST TG 8 month WT	ML	4	0.09033	0.20725	2.137

Bartlett's Test (Normal Distribution)

Test statistic = 77.97, p-value = 0.000

Levene's Test (Any Continuous Distribution)

Test statistic = 1.99, p-value = 0.018

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

ALTHOUGH P-VALUE INCLUDING OUTLIERS IS GREATER THAN WITHOUT THE OUTLIERS, THE RESULT DOES NOT CHANGE SIGNIFICANCE AND AS SUCH THE OUTLIERS WILL BE EXCLUDED.

General Linear Model: Yield Force_ versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Yield Force_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
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Genotype+Phenotype	9	26456.2	27663.0	3073.7	36.62	0.000
Bend Direction	1	1686.8	1325.8	1325.8	15.79	0.000
Genotype+Phenotype*Bend Direction	9	3460.1	3460.1	384.5	4.58	0.000
Error	74	6211.4	6211.4	83.9		
Total	93	37814.5				

S = 9.16179 R-Sq = 83.57% R-Sq(adj) = 79.36%

Unusual Observations for Yield Force_1

	Yield					
Obs	Force_1	Fit	SE Fit	Residual	St Resid	
65	51.9074	23.5825	4.5809	28.3249	3.57	R
67	0.6744	23.5825	4.5809	-22.9080	-2.89	R
68	1.1169	23.5825	4.5809	-22.4655	-2.83	R
71	40.6311	23.5825	4.5809	17.0486	2.15	R
72	31.0272	13.2258	3.7403	17.8014	2.13	R
74	30.0507	13.2258	3.7403	16.8249	2.01	R
82	6.5979	25.7456	3.7403	-19.1477	-2.29	R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month KO	8	65.387	A
SOST KO 6 month KO	7	48.363	B
SOST KO 6 month WT	12	19.486	C
SOST KO 12 month WT	4	16.906	C
SOST TG 6 month WT	22	12.875	C
SOST TG 6 month DEF	18	10.481	C
SOST TG 8 month DEF	7	10.072	C
SOST TG 8 month WT	8	9.523	C
SOST TG 8 month TG	4	8.190	C
SOST TG 6 month TG	4	5.977	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend			
Direction	N	Mean	Grouping
ML	46	25.150	A
AP	48	16.302	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

	Bend			
Genotype+Phenotype	Direction	N	Mean	Grouping
SOST KO 6 month KO	ML	3	73.145	A
SOST KO 12 month KO	ML	4	69.912	A
SOST KO 12 month KO	AP	4	60.861	A
SOST KO 6 month WT	ML	6	25.746	B
SOST KO 6 month KO	AP	4	23.582	B C
SOST KO 12 month WT	ML	2	18.396	B C
SOST KO 12 month WT	AP	2	15.416	B C

SOST TG 6 month WT	ML	11	14.708	B C
SOST TG 6 month DEF	ML	9	13.824	B C
SOST KO 6 month WT	AP	6	13.226	B C
SOST TG 6 month WT	AP	11	11.042	B C
SOST TG 8 month DEF	ML	3	10.517	B C
SOST TG 8 month WT	ML	4	9.670	B C
SOST TG 8 month DEF	AP	4	9.626	B C
SOST TG 8 month WT	AP	4	9.375	B C
SOST TG 8 month TG	AP	2	8.730	B C
SOST TG 6 month TG	ML	2	7.927	B C
SOST TG 8 month TG	ML	2	7.651	B C
SOST TG 6 month DEF	AP	9	7.139	C
SOST TG 6 month TG	AP	2	4.027	B C

Means that do not share a letter are significantly different.

Results for: BONE DATA__BLANK.MTW

General Linear Model: Ultimate For versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Ultimate Force, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	30530.1	30524.8	3391.6	99.38	0.000
Bend Direction	1	698.9	524.7	524.7	15.37	0.000
Genotype+Phenotype*Bend Direction	9	607.2	607.2	67.5	1.98	0.053
Error	77	2627.7	2627.7	34.1		
Total	96	34463.9				

S = 5.84180 R-Sq = 92.38% R-Sq(adj) = 90.49%

Unusual Observations for Ultimate Force

Obs	Ultimate Force	Fit	SE Fit	Residual	St Resid
66	80.1300	65.6761	2.9209	14.4539	2.86 R
69	77.8290	65.6761	2.9209	12.1529	2.40 R
70	42.9596	65.6761	2.9209	-22.7165	-4.49 R
85	58.9935	69.5306	2.6125	-10.5371	-2.02 R
90	70.3674	59.6747	2.6125	10.6927	2.05 R
96	85.2417	69.5306	2.6125	15.7111	3.01 R

R denotes an observation with a large standardized residual.

Probability Plot of SRES1

TEST FOR NORMALITY SHOWED OUTLIERS FOR 9201L, 9203L, and 7594L WHICH WILL BE EXCLUDED FROM ANALYSIS.

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend		N	Lower	StDev	Upper
	Direction					
SOST KO 12 month KO	AP		5	0.64565	1.36855	8.583
SOST KO 12 month KO	ML		5	0.97616	2.06912	12.976
SOST KO 12 month WT	AP		2	0.26742	0.86301	550.864
SOST KO 12 month WT	ML		2	0.32326	1.04323	665.904
SOST KO 6 month KO	AP		4	0.60239	1.38216	14.252
SOST KO 6 month KO	ML		4	1.48196	3.40028	35.061
SOST KO 6 month WT	AP		6	0.54941	1.09881	5.118
SOST KO 6 month WT	ML		6	0.36818	0.73635	3.430
SOST TG 6 month DEF	AP		9	0.39397	0.70416	2.087
SOST TG 6 month DEF	ML		9	0.37605	0.67213	1.992
SOST TG 6 month TG	AP		2	0.24378	0.78673	502.179
SOST TG 6 month TG	ML		2	0.09243	0.29829	190.401
SOST TG 6 month WT	AP		11	0.41149	0.70064	1.776
SOST TG 6 month WT	ML		11	0.27008	0.45986	1.166
SOST TG 8 month DEF	AP		4	0.42440	0.97378	10.041
SOST TG 8 month DEF	ML		3	0.44355	1.14678	32.426
SOST TG 8 month TG	AP		2	0.23698	0.76479	488.173
SOST TG 8 month TG	ML		2	0.23925	0.77211	492.842
SOST TG 8 month WT	AP		4	0.26196	0.60107	6.198
SOST TG 8 month WT	ML		4	0.04048	0.09288	0.958

Bartlett's Test (Normal Distribution)

Test statistic = 50.85, p-value = 0.000

Levene's Test (Any Continuous Distribution)

Test statistic = 3.18, p-value = 0.000

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend		N	Lower	StDev	Upper
	Direction					
SOST KO 12 month KO	AP		5	0.645649	1.36855	8.58
SOST KO 12 month KO	ML		4	0.607226	1.39325	14.37
SOST KO 12 month WT	AP		2	0.267415	0.86301	550.86
SOST KO 12 month WT	ML		2	0.323261	1.04323	665.90
SOST KO 6 month KO	AP		4	0.602393	1.38216	14.25
SOST KO 6 month KO	ML		2	0.694812	2.24231	1431.28
SOST KO 6 month WT	AP		6	0.549410	1.09881	5.12
SOST KO 6 month WT	ML		6	0.368180	0.73635	3.43
SOST TG 6 month DEF	AP		9	0.393967	0.70416	2.09
SOST TG 6 month DEF	ML		9	0.376045	0.67213	1.99
SOST TG 6 month TG	AP		2	0.243781	0.78673	502.18
SOST TG 6 month TG	ML		2	0.092429	0.29829	190.40
SOST TG 6 month WT	AP		11	0.411491	0.70064	1.78
SOST TG 6 month WT	ML		11	0.270077	0.45986	1.17

SOST TG 8 month DEF	AP	4	0.424404	0.97378	10.04
SOST TG 8 month DEF	ML	3	0.443550	1.14678	32.43
SOST TG 8 month TG	AP	2	0.236982	0.76479	488.17
SOST TG 8 month TG	ML	2	0.239249	0.77211	492.84
SOST TG 8 month WT	AP	4	0.261964	0.60107	6.20
SOST TG 8 month WT	ML	4	0.040479	0.09288	0.96

Bartlett's Test (Normal Distribution)
 Test statistic = 26.61, p-value = 0.114

Levene's Test (Any Continuous Distribution)
 Test statistic = 1.71, p-value = 0.053

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Bend Direction

General Linear Model: Ultimate For versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Ultimate Force_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	24875.1	25483.9	2831.5	134.31	0.000
Bend Direction	1	551.1	502.2	502.2	23.82	0.000
Genotype+Phenotype*Bend Direction	9	615.1	615.1	68.3	3.24	0.002
Error	74	1560.1	1560.1	21.1		
Total	93	27601.5				

S = 4.59158 R-Sq = 94.35% R-Sq(adj) = 92.90%

Unusual Observations for Ultimate Force_1

Obs	Ultimate Force_1	Fit	SE Fit	Residual	St Resid
64	61.7859	69.8074	3.2467	-8.0215	-2.47 R
69	77.8290	69.8074	3.2467	8.0215	2.47 R
71	40.6311	49.3462	2.2958	-8.7151	-2.19 R
81	15.1245	24.7182	1.8745	-9.5937	-2.29 R
84	51.0223	59.6747	2.0534	-8.6523	-2.11 R
90	70.3674	59.6747	2.0534	10.6927	2.60 R
92	74.9481	65.6029	2.2958	9.3452	2.35 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month KO	9	62.639	A

SOST KO 6 month KO	6	59.577	A
SOST KO 6 month WT	12	30.074	B
SOST KO 12 month WT	4	25.541	B C
SOST TG 6 month WT	22	18.630	C D
SOST TG 6 month DEF	18	14.422	D
SOST TG 8 month TG	4	13.849	D
SOST TG 8 month WT	8	13.337	D
SOST TG 8 month DEF	7	13.060	D
SOST TG 6 month TG	4	11.396	D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend

Direction	N	Mean	Grouping
ML	45	29.002	A
AP	49	23.503	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Bend			Grouping
	Direction	N	Mean	
SOST KO 6 month KO	ML	2	69.807	A
SOST KO 12 month KO	ML	4	65.603	A
SOST KO 12 month KO	AP	5	59.675	A B
SOST KO 6 month KO	AP	4	49.346	B
SOST KO 6 month WT	ML	6	35.429	C
SOST KO 12 month WT	ML	2	29.335	C D
SOST KO 6 month WT	AP	6	24.718	D
SOST KO 12 month WT	AP	2	21.747	C D E
SOST TG 6 month WT	ML	11	20.568	D E
SOST TG 6 month WT	AP	11	16.691	D E
SOST TG 6 month DEF	ML	9	15.408	E
SOST TG 8 month TG	ML	2	14.859	D E
SOST TG 8 month DEF	AP	4	13.798	E
SOST TG 6 month TG	ML	2	13.612	D E
SOST TG 8 month WT	AP	4	13.600	E
SOST TG 6 month DEF	AP	9	13.436	E
SOST TG 8 month WT	ML	4	13.074	E
SOST TG 8 month TG	AP	2	12.839	D E
SOST TG 8 month DEF	ML	3	12.322	E
SOST TG 6 month TG	AP	2	9.180	E

Means that do not share a letter are significantly different.

Results for: BONE DATA__BLANK.MTW

General Linear Model: Post Yield D versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Post Yield Displacement, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	0.29652	0.31080	0.03453	0.86	0.565
Bend Direction	1	0.00869	0.02048	0.02048	0.51	0.478
Genotype+Phenotype*Bend Direction	9	0.31422	0.31422	0.03491	0.87	0.557
Error	77	3.09713	3.09713	0.04022		
Total	96	3.71655				

S = 0.200555 R-Sq = 16.67% R-Sq(adj) = 0.00%

Unusual Observations for Post Yield Displacement

Obs	Post Yield Displacement	Fit	SE Fit	Residual	St Resid
15	0.705207	0.148239	0.066852	0.556968	2.95 R
38	0.591390	0.191330	0.060470	0.400060	2.09 R
63	0.748680	0.382418	0.115791	0.366263	2.24 R
68	0.629456	0.256752	0.100278	0.372704	2.15 R

R denotes an observation with a large standardized residual.

Probability Plot of SRES1

TEST FOR NORMALITY IS VIOLATED AND SHOWS AN OUTLIER FOR 8641L WHICH WILL BE EXCLUDED FROM THE ANALYSIS.

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend		N	Lower	StDev	Upper
	Direction					
SOST KO 12 month KO	AP		5	0.416800	0.88347	5.54
SOST KO 12 month KO	ML		5	0.302717	0.64165	4.02
SOST KO 12 month WT	AP		2	0.772921	2.49438	1592.18
SOST KO 12 month WT	ML		2	0.399296	1.28861	822.53
SOST KO 6 month KO	AP		4	0.780953	1.79186	18.48
SOST KO 6 month KO	ML		4	0.555489	1.27454	13.14
SOST KO 6 month WT	AP		6	0.432483	0.86496	4.03
SOST KO 6 month WT	ML		6	0.561167	1.12232	5.23
SOST TG 6 month DEF	AP		9	0.499176	0.89221	2.64
SOST TG 6 month DEF	ML		9	0.660985	1.18142	3.50
SOST TG 6 month TG	AP		2	0.544319	1.75664	1121.27
SOST TG 6 month TG	ML		2	0.527356	1.70189	1086.33
SOST TG 6 month WT	AP		11	0.571560	0.97319	2.47
SOST TG 6 month WT	ML		11	0.641777	1.09275	2.77
SOST TG 8 month DEF	AP		4	0.203935	0.46792	4.82
SOST TG 8 month DEF	ML		3	0.750937	1.94152	54.90
SOST TG 8 month TG	AP		2	0.465948	1.50371	959.83
SOST TG 8 month TG	ML		2	0.339199	1.09467	698.74
SOST TG 8 month WT	AP		4	0.576610	1.32301	13.64
SOST TG 8 month WT	ML		4	0.254897	0.58485	6.03

Bartlett's Test (Normal Distribution)

Test statistic = 13.63, p-value = 0.805

Levene's Test (Any Continuous Distribution)

Test statistic = 0.73, p-value = 0.780

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend		N	Lower	StDev	Upper
	Direction					
SOST KO 12 month KO	AP		5	0.416800	0.88347	5.54
SOST KO 12 month KO	ML		5	0.302717	0.64165	4.02
SOST KO 12 month WT	AP		2	0.772921	2.49438	1592.18
SOST KO 12 month WT	ML		2	0.399296	1.28861	822.53
SOST KO 6 month KO	AP		4	0.780953	1.79186	18.48
SOST KO 6 month KO	ML		4	0.555489	1.27454	13.14
SOST KO 6 month WT	AP		6	0.432483	0.86496	4.03
SOST KO 6 month WT	ML		6	0.561167	1.12232	5.23
SOST TG 6 month DEF	AP		9	0.499176	0.89221	2.64
SOST TG 6 month DEF	ML		8	0.243112	0.44800	1.48
SOST TG 6 month TG	AP		2	0.544319	1.75664	1121.27
SOST TG 6 month TG	ML		2	0.527356	1.70189	1086.33
SOST TG 6 month WT	AP		11	0.571560	0.97319	2.47
SOST TG 6 month WT	ML		11	0.641777	1.09275	2.77
SOST TG 8 month DEF	AP		4	0.203935	0.46792	4.82
SOST TG 8 month DEF	ML		3	0.750937	1.94152	54.90
SOST TG 8 month TG	AP		2	0.465948	1.50371	959.83
SOST TG 8 month TG	ML		2	0.339199	1.09467	698.74
SOST TG 8 month WT	AP		4	0.576610	1.32301	13.64
SOST TG 8 month WT	ML		4	0.254897	0.58485	6.03

Bartlett's Test (Normal Distribution)

Test statistic = 19.48, p-value = 0.426

Levene's Test (Any Continuous Distribution)

Test statistic = 1.00, p-value = 0.470

Test for Equal Variances: SRES1_1 versus Genotype+Phenotype, Bend Direction

ALTHOUGH INCLUDING OUTLIER RESULTS IN MORE EQUAL VARIANCE, EXCLUDING THIS POINT DOES NOT EFFECT THE SIGNIFICANCE AND AS SUCH WILL CONTINUE TO BE EXCLUDED.

General Linear Model: Post Yield D versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT

Bend Direction fixed 2 AP, ML

Analysis of Variance for Post Yield Displacement_1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	0.31893	0.33884	0.03765	1.04	0.416
Bend Direction	1	0.00130	0.01296	0.01296	0.36	0.551
Genotype+Phenotype*Bend Direction	9	0.36949	0.36949	0.04105	1.14	0.349
Error	76	2.74814	2.74814	0.03616		
Total	95	3.43786				

S = 0.190157 R-Sq = 20.06% R-Sq(adj) = 0.08%

Unusual Observations for Post Yield Displacement_1

Obs	Post Yield Displacement_1	Fit	SE Fit	Residual	St Resid
35	0.521067	0.141663	0.057335	0.379403	2.09 R
38	0.591390	0.191330	0.057335	0.400060	2.21 R
63	0.748680	0.382418	0.109787	0.366263	2.36 R
68	0.629456	0.256752	0.095079	0.372704	2.26 R
70	0.442741	0.110685	0.095079	0.332056	2.02 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST TG 6 month TG	4	0.29479	A
SOST TG 8 month WT	8	0.26133	A
SOST TG 8 month TG	4	0.25399	A
SOST TG 8 month DEF	7	0.24441	A
SOST KO 12 month WT	4	0.21078	A
SOST KO 6 month KO	8	0.18372	A
SOST TG 6 month WT	22	0.16650	A
SOST KO 6 month WT	12	0.16014	A
SOST TG 6 month DEF	17	0.13713	A
SOST KO 12 month KO	10	0.06117	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend Direction	N	Mean	Grouping
ML	47	0.21103	A
AP	49	0.18376	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Bend Direction	N	Mean	Grouping
SOST TG 8 month DEF	ML	3	0.38242	A
SOST TG 6 month TG	ML	2	0.37741	A
SOST TG 8 month WT	ML	4	0.33519	A

SOST TG 8 month TG	ML	2	0.32152	A
SOST KO 12 month WT	AP	2	0.29234	A
SOST KO 6 month KO	AP	4	0.25675	A
SOST TG 6 month TG	AP	2	0.21218	A
SOST TG 6 month DEF	AP	9	0.19564	A
SOST TG 6 month WT	ML	11	0.19133	A
SOST KO 6 month WT	AP	6	0.18786	A
SOST TG 8 month WT	AP	4	0.18747	A
SOST TG 8 month TG	AP	2	0.18646	A
SOST TG 6 month WT	AP	11	0.14166	A
SOST KO 6 month WT	ML	6	0.13241	A
SOST KO 12 month WT	ML	2	0.12922	A
SOST KO 6 month KO	ML	4	0.11069	A
SOST TG 8 month DEF	AP	4	0.10640	A
SOST TG 6 month DEF	ML	8	0.07862	A
SOST KO 12 month KO	AP	5	0.07087	A
SOST KO 12 month KO	ML	5	0.05147	A

Means that do not share a letter are significantly different.

Results for: BONE DATA__BLANK.MTW

General Linear Model: Yield Stress versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Yield Stress, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	26321	26285	2921	0.64	0.757
Bend Direction	1	3815	196	196	0.04	0.836
Genotype+Phenotype*Bend Direction	9	31676	31676	3520	0.77	0.640
Error	77	350029	350029	4546		
Total	96	411840				

S = 67.4228 R-Sq = 15.01% R-Sq(adj) = 0.00%

Unusual Observations for Yield Stress

Obs	Yield Stress	Fit	SE Fit	Residual	St Resid
70	0.674	159.668	33.711	-158.993	-2.72 R
72	236.161	81.305	27.525	154.856	2.52 R
86	39.709	149.394	47.675	-109.684	-2.30 R
88	259.078	149.394	47.675	109.684	2.30 R

R denotes an observation with a large standardized residual.

Probability Plot of SRES1

TEST FOR NORMALITY IS BORDERLINE, BUT SHOWS NORMAL DISTRIBUTION WITH NO OUTLIERS.

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend		N	Lower	StDev	Upper
	Direction					
SOST KO 12 month KO	AP		5	0.49299	1.04496	6.55
SOST KO 12 month KO	ML		5	0.45715	0.96900	6.08
SOST KO 12 month WT	AP		2	1.00818	3.25363	2076.82
SOST KO 12 month WT	ML		2	0.43859	1.41544	903.49
SOST KO 6 month KO	AP		4	0.60431	1.38656	14.30
SOST KO 6 month KO	ML		4	0.83292	1.91111	19.71
SOST KO 6 month WT	AP		6	0.73530	1.47058	6.85
SOST KO 6 month WT	ML		6	0.56604	1.13206	5.27
SOST TG 6 month DEF	AP		9	0.53894	0.96328	2.85
SOST TG 6 month DEF	ML		9	0.52753	0.94288	2.79
SOST TG 6 month TG	AP		2	0.24544	0.79209	505.60
SOST TG 6 month TG	ML		2	0.28073	0.90596	578.28
SOST TG 6 month WT	AP		11	0.52152	0.88798	2.25
SOST TG 6 month WT	ML		11	0.56674	0.96498	2.45
SOST TG 8 month DEF	AP		4	0.34851	0.79965	8.25
SOST TG 8 month DEF	ML		3	0.41880	1.08280	30.62
SOST TG 8 month TG	AP		2	0.54497	1.75875	1122.62
SOST TG 8 month TG	ML		2	0.32528	1.04974	670.06
SOST TG 8 month WT	AP		4	0.38602	0.88569	9.13
SOST TG 8 month WT	ML		4	0.31422	0.72096	7.43

Bartlett's Test (Normal Distribution)

Test statistic = 11.97, p-value = 0.887

Levene's Test (Any Continuous Distribution)

Test statistic = 0.94, p-value = 0.539

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

THERE ARE EQUAL VARIANCES.

General Linear Model: Yield Stress versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Yield Stress, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	26321	26285	2921	0.64	0.757
Bend Direction	1	3815	196	196	0.04	0.836

Genotype+Phenotype*Bend Direction	9	31676	31676	3520	0.77	0.640
Error	77	350029	350029	4546		
Total	96	411840				

S = 67.4228 R-Sq = 15.01% R-Sq(adj) = 0.00%

Unusual Observations for Yield Stress

Obs	Yield Stress	Fit	SE Fit	Residual	St Resid
70	0.674	159.668	33.711	-158.993	-2.72 R
72	236.161	81.305	27.525	154.856	2.52 R
86	39.709	149.394	47.675	-109.684	-2.30 R
88	259.078	149.394	47.675	109.684	2.30 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 12 month WT	4	124.47	A
SOST TG 6 month WT	22	119.00	A
SOST KO 6 month KO	8	115.72	A
SOST KO 12 month KO	10	109.64	A
SOST TG 6 month DEF	18	109.49	A
SOST TG 8 month DEF	7	107.86	A
SOST KO 6 month WT	12	93.57	A
SOST TG 8 month WT	8	86.82	A
SOST TG 8 month TG	4	80.30	A
SOST TG 6 month TG	4	49.66	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend Direction	N	Mean	Grouping
ML	48	101.33	A
AP	49	97.98	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Bend Direction	N	Mean	Grouping
SOST KO 6 month KO	ML	4	159.67	A
SOST KO 12 month WT	AP	2	149.39	A
SOST TG 6 month DEF	ML	9	133.49	A
SOST KO 12 month KO	AP	5	126.97	A
SOST TG 6 month WT	ML	11	124.14	A
SOST TG 6 month WT	AP	11	113.86	A
SOST TG 8 month DEF	AP	4	113.50	A
SOST KO 6 month WT	ML	6	105.84	A
SOST TG 8 month DEF	ML	3	102.22	A
SOST KO 12 month WT	ML	2	99.55	A
SOST TG 8 month WT	AP	4	97.15	A
SOST TG 8 month TG	AP	2	93.54	A

SOST KO 12 month KO	ML	5	92.32	A
SOST TG 6 month DEF	AP	9	85.50	A
SOST KO 6 month WT	AP	6	81.30	A
SOST TG 8 month WT	ML	4	76.48	A
SOST KO 6 month KO	AP	4	71.78	A
SOST TG 8 month TG	ML	2	67.07	A
SOST TG 6 month TG	ML	2	52.50	A
SOST TG 6 month TG	AP	2	46.81	A

Means that do not share a letter are significantly different.

Results for: BONE DATA__BLANK.MTW

General Linear Model: Ultimate Str versus Genotype+Phe, Bend Directi

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Ultimate Stress, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	37589	37721	4191	1.97	0.054
Bend Direction	1	2877	3329	3329	1.57	0.214
Genotype+Phenotype*Bend Direction	9	12980	12980	1442	0.68	0.726
Error	77	163621	163621	2125		
Total	96	217066				

S = 46.0971 R-Sq = 24.62% R-Sq(adj) = 6.02%

Unusual Observations for Ultimate Stress

Obs	Ultimate Stress	Fit	SE Fit	Residual	St Resid
61	62.133	162.670	23.049	-100.537	-2.52 R
72	236.161	141.903	18.819	94.258	2.24 R
73	52.105	137.824	18.819	-85.720	-2.04 R
86	103.847	185.806	32.596	-81.959	-2.51 R
88	267.766	185.806	32.596	81.959	2.51 R

R denotes an observation with a large standardized residual.

Probability Plot of SRES1

NORMALITY IS VERIFIED.

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

95% Bonferroni confidence intervals for standard deviations

Genotype+Phenotype	Bend Direction	N	Lower	StDev	Upper
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SOST KO 12 month KO	AP	5	0.49657	1.05255	6.60
SOST KO 12 month KO	ML	5	0.54568	1.15664	7.25
SOST KO 12 month WT	AP	2	1.10186	3.55595	2269.79
SOST KO 12 month WT	ML	2	0.18697	0.60339	385.15
SOST KO 6 month KO	AP	4	0.19776	0.45376	4.68
SOST KO 6 month KO	ML	4	0.46975	1.07781	11.11
SOST KO 6 month WT	AP	6	0.57795	1.15589	5.38
SOST KO 6 month WT	ML	6	0.65096	1.30190	6.06
SOST TG 6 month DEF	AP	9	0.76096	1.36011	4.03
SOST TG 6 month DEF	ML	9	0.60005	1.07251	3.18
SOST TG 6 month TG	AP	2	0.48675	1.57086	1002.69
SOST TG 6 month TG	ML	2	0.45346	1.46340	934.10
SOST TG 6 month WT	AP	11	0.47433	0.80764	2.05
SOST TG 6 month WT	ML	11	0.43955	0.74842	1.90
SOST TG 8 month DEF	AP	4	0.73690	1.69079	17.43
SOST TG 8 month DEF	ML	3	0.33729	0.87206	24.66
SOST TG 8 month TG	AP	2	0.31761	1.02501	654.27
SOST TG 8 month TG	ML	2	0.48283	1.55820	994.61
SOST TG 8 month WT	AP	4	0.33142	0.76042	7.84
SOST TG 8 month WT	ML	4	0.39148	0.89824	9.26

Bartlett's Test (Normal Distribution)
Test statistic = 16.06, p-value = 0.653

Levene's Test (Any Continuous Distribution)
Test statistic = 1.37, p-value = 0.167

Test for Equal Variances: SRES1 versus Genotype+Phenotype, Bend Direction

EQUAL VARIANCES IS CONFIRMED.

General Linear Model: Ultimate Str versus Genotype+Phe, Bend Direction

Factor	Type	Levels	Values
Genotype+Phenotype	fixed	10	SOST KO 12 month KO, SOST KO 12 month WT, SOST KO 6 month KO, SOST KO 6 month WT, SOST TG 6 month DEF, SOST TG 6 month TG, SOST TG 6 month WT, SOST TG 8 month DEF, SOST TG 8 month TG, SOST TG 8 month WT
Bend Direction	fixed	2	AP, ML

Analysis of Variance for Ultimate Stress, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype+Phenotype	9	37589	37721	4191	1.97	0.054
Bend Direction	1	2877	3329	3329	1.57	0.214
Genotype+Phenotype*Bend Direction	9	12980	12980	1442	0.68	0.726
Error	77	163621	163621	2125		
Total	96	217066				

S = 46.0971 R-Sq = 24.62% R-Sq(adj) = 6.02%

Unusual Observations for Ultimate Stress

Ultimate

Obs	Stress	Fit	SE Fit	Residual	St Resid
61	62.133	162.670	23.049	-100.537	-2.52 R
72	236.161	141.903	18.819	94.258	2.24 R
73	52.105	137.824	18.819	-85.720	-2.04 R
86	103.847	185.806	32.596	-81.959	-2.51 R
88	267.766	185.806	32.596	81.959	2.51 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	N	Mean	Grouping
SOST KO 6 month KO	8	175.11	A
SOST TG 6 month WT	22	168.58	A
SOST KO 12 month WT	4	166.16	A
SOST TG 6 month DEF	18	154.54	A
SOST TG 8 month DEF	7	144.34	A
SOST KO 6 month WT	12	139.86	A
SOST TG 8 month TG	4	139.56	A
SOST KO 12 month KO	10	134.99	A
SOST TG 8 month WT	8	116.33	A
SOST TG 6 month TG	4	98.74	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Bend			
Direction	N	Mean	Grouping
AP	49	150.72	A
ML	48	136.92	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Genotype+Phenotype	Bend		N	Mean	Grouping
	Direction				
SOST KO 6 month KO	ML		4	200.67	A
SOST KO 12 month WT	AP		2	185.81	A
SOST TG 6 month WT	AP		11	169.64	A
SOST TG 6 month WT	ML		11	167.51	A
SOST TG 8 month DEF	AP		4	162.67	A
SOST TG 6 month DEF	AP		9	162.45	A
SOST KO 6 month KO	AP		4	149.56	A
SOST KO 12 month KO	AP		5	148.20	A
SOST TG 6 month DEF	ML		9	146.64	A
SOST KO 12 month WT	ML		2	146.51	A
SOST KO 6 month WT	AP		6	141.90	A
SOST TG 8 month TG	ML		2	141.38	A
SOST KO 6 month WT	ML		6	137.82	A
SOST TG 8 month TG	AP		2	137.73	A
SOST TG 8 month WT	AP		4	135.41	A
SOST TG 8 month DEF	ML		3	126.00	A
SOST KO 12 month KO	ML		5	121.79	A
SOST TG 6 month TG	AP		2	113.85	A
SOST TG 8 month WT	ML		4	97.24	A
SOST TG 6 month TG	ML		2	83.64	A

Means that do not share a letter are significantly different.

APPENDIX D: Epoxy Embedding and Metallographic Polishing

Epoxy Embedding

Three-point bending generally results in an oblique fracture which is not suitable for analysis techniques such as nanoindentation. In order to allow for future work to be conducted on the samples, bones were embedded in epoxy and metallographically polished to an appropriate level of smoothness that would be suitable for nanoindentation.

Sample Preparation

Following bending and refreezing, proximal femur halves were dehydrated in increasing concentrations of alcohol, to remove remaining moisture from the samples, after being allowed to thaw. 70% isopropyl alcohol was used and was diluted to varying percentages with distilled water. Bones were initially placed in individual vials of 50% isopropyl and 50% distilled water for one hour, immediately followed by increasing concentrations of 60%, 70%, 80%, 90%, and 100% isopropyl. After this process had been completed, bones were grouped based on treatment type for embedding. The amount of bones per group varied between the treatments and the epoxy pucks that were made contained either all left or right femurs to allow for easier measurement and record keeping. Figure D.1 depicts a breakdown of the different epoxy pucks with the corresponding mouse numbers.

Figure D.1: Individual pucks with bone groupings.

Aluminum cylindrical tubes were used to hold and effectively separate each bone during embedding. Each sample was checked to ensure that anatomical variations would not compromise or impede proper fit in the holding fixture (to be discussed in detail later).

Improper fit was typically caused by a diaphysial cortical diameter greater than that of the sample holder or a large protrusion of the third (lateral) trochanter (Figure D.2). Many of the SOST KO samples had a diameter that was too large to fit into the most frequently used tubing size, a larger size aluminum cylindrical tube was used. This technique was useful for the larger, thicker samples however, there were incidences where the smaller

SOST TG bones did not fit into the initial size tubes. This issue was the result of an excessive protrusion from the third trochanter and was alleviated by carefully removing this anatomical feature using a razor blade. Small slices from the lateral side of the trochanter were removed until the bone fit into the holding tube. Larger tubes were not used in this instance because the instability of the bone within the holding tube was found to cause issues during embedding. Additionally, future work will be primarily interested in the cortical surfaces of the diaphysis, the removal of the third trochanter was not believed to negatively affect the integrity and/or future testability of the samples.

Figure D.2: Third trochanter of the mouse femur [74].

Custom Sample Holder

Custom sample holders were fabricated to allow for proper and consistent placement of bones when embedded in epoxy. Holders also allowed for accurate identification of individual bones. Aluminum cylindrical tube was cut using a tubing cutter to the appropriate length of each bone, such that the head of the femur rested on the upper edge of the tube (Figure D.3). The cut pieces were bored using a 30 gauge screw driver to

widen the crimped end(s) that occurred due to the cutting process. In order to remove any remaining debris artifacts from cutting, tubes were sonicated in an ethanol bath for 15 minutes. Bones were checked for fit, as described previously, and tubes were glued together using super glue in order to create one object that would support all bones from a given treatment group (Figure D.4). One tube in each group was marked with a sharpie. This mark was visible through the hardened epoxy puck and allowed for identification of individual bones following embedding.

Figure D.3: Sample holding tube containing bone during test fit.

Figure D.4: Sample holding object with bones in place prior to embedding.

Sample Casting

After dehydration, the mouse femurs were removed and placed in aluminum tubing (Diameter_{Inner} = 5/32" or 3/16"), the diaphysis contained within the tube and the femoral head resting just outside and above the tube. Immediately prior to casting, samples were placed and centered, fracture surface down, in a clean casting mold cup, Part No.

(Buehler, Lake Bluff, IL). Samples were cast using EPO-Thin Low Viscosity Epoxy Resin, Part No. 20-8140-032 (Buehler, Lake Bluff, IL) and EPO-Thin Low Viscosity Epoxy Hardener, Part No. 20-8142-064 (Buehler, Lake Bluff, IL), which were combined at a ratio of 100 parts resin to 36 parts hardener by weight.

A clean cup was placed on a digital scale and tared. Epoxy was then poured into the cup to the appropriate weight to make the desired number of pucks (Figure D.5). Hardener was then added to the weighed epoxy until the final weight was reached such that the 100:36 ratio was achieved (Figure D.6). The epoxy mixture was then gently stirred with a popsicle stick, avoiding creation of air bubbles, until the mixture became consistent in color (Figure D.7). At this point, working time was approximately 20 minutes which allowed ample time sample placement and pouring. Samples holders, already containing bones, were then placed in their respective mounting cups within a vacuum container/pump assembly (Model #: UN7260.3, KNF Neuberger, Inc., Trenton, NJ) (Figure D.8). Epoxy was slowly poured into each cup until the samples were completely covered by approximately 1 centimeter of epoxy. Following the addition of epoxy, many samples began floating out of their respective mounting tubes. These bones were gently pushed back into place using forceps to regain their initial positioning. This was done to insure that all bones surfaces to be polished and subsequently tested were close in

proximity, alleviating the need for repolishing between testing. Once all bones were returned to their correct place, a gentle vacuum was applied for 5 minutes followed by a period of no vacuum, which allowed for verification that all bones were still in the proper place. This process was repeated 4 times resulting in a total time of 20 minutes that samples were exposed to the vacuum. Vacuuming was conducted to remove air bubbles that were present within the epoxy puck. Once this was complete, samples were removed from the pump assembly and allowed to cure for a period of 24-48 hours and removed from molding cups. This resulted in hardened epoxy pucks which could then be polished to the appropriate level of smoothness required for future testing (Figure D.9).

Figure D.5: Digital scale showing final weight of epoxy added.

Figure D.6: Digital scale showing final weight of epoxy and hardener mixture.

Figure D.7: Mixing of cup filled with epoxy and hardener using popsicle stick.

Figure D.8: Vacuum container/pump assembly used to remove air bubbles from samples.

Figure D.9: A sample cast in epoxy mold (unpolished).

Metallographic Polishing

In order to create a flat, smooth testing surface for nanoindentation, sample pucks were polished metallographically. To obtain this mirrored surface, multiple grinding and polishing steps were conducted. Initially, pucks were ground using a 120 grit belt sander. This course grit allowed for exposure of cortical bone surface, which was the region of interest. Following exposure of the cortical surface, successive grits of 240, 320, 400, and 600 were used. Each step was conducted until the previous sanding lines were removed.

This was accomplished with unidirectional strokes along the sand paper. Each subsequent sanding was done at 90 degrees to the previous grit. This allowed for identification of when each step was complete. In order to prevent deep scoring of samples, the sand paper belt was adequately wetted and minimal pressure was applied to the sample during sanding. Visual assessment of the samples was done using a microscope following washing with ethanol.

Once the samples were adequately sanded using the 5 grits, they were then metallographically polished with 0.3 micron (Buehler # 40-6249) and 0.05 micron suspension (Buehler # 40-6377-064). Polishing pads (Buehler # 40-1008) were placed on the polishing wheel and 0.03 micron suspension was mixed with distilled water for application to pad. The pad was then lightly moistened with Forgeng Solution and the motor was set to 200 rpm. Samples were polished in a circular or figure eight pattern. Following liberal polishing at the 0.3 micron level, samples were visually assessed for removal of all 600 grit sanding lines. Once these lines were completely removed from the cortical bone surface samples were moved to the 0.05 Alumina Oxide micron polishing step. The suspension formulation and pad preparation was conducted the same as the 0.3 micron level, except the polishing pad (Allied High Tech # 90-500-350) was a magnetic pad, rather than adhesive backed. The sample was polished until there was uniform polishing as evident by visual assessment. The 0.05 step provided allowed for identification of bone structures and sites of remodeling and is recognized as an adequate level of surface roughness for nanoindentation.

Imaging

Images of polished pucks were taken using a microscope and camera assembly to show the progression through the polishing steps (Figures D.10 – D.16). Upon microscope assessment, there were apparent differences between treatment types and images were taken to show these observations (Figures D.17 - D.36). Utilization of this system did not allow for capture of the entire bone, but image analysis was not a primary goal of this study. As such, only representative images were taken.

Figure D.10: Epoxy Puck not finished polishing as indicated by inconsistent line patterns.

Figure D.11: Epoxy Puck 6 polished at 240 grit.

Figure D.12: Epoxy Puck 11 polished at 320 grit.

Figure D.13: Epoxy Puck 9 polished at 400 grit.

Figure D.14: Epoxy Puck 10 polished at 600 grit.

Figure D.15: Epoxy Puck 1 polished at 0.3 micron.

Figure D.16: Epoxy Puck 1 polished at 0.05 micron.

Figure D.17: SOST TG 6 month bone (# 9296) polished to 0.3 micron.

Figure D.18: SOST TG 6 month bone (# 9296) polished to 0.05 micron.

Figure D.19: SOST DEF 6 month bone (# 8601) polished to 0.3 micron.

Figure D.20: SOST DEF 6 month bone (# 8601) polished to 0.05 micron.

Figure D.21: WT 1 bone (# 8599) polished to 0.3 micron.

Figure D.22: WT 1 bone (# 8599) polished to 0.05 micron.

Figure D.23: SOST TG 8 month bone (# 5592) polished to 0.3 micron.

Figure D.24: SOST TG 8 month bone (# 5592) polished to 0.05 micron.

Figure D.25: SOST DEF 8 month bone (# 5589) polished to 0.3 micron.

Figure D.26: SOST DEF 8 month bone (# 5589) polished to 0.05 micron.

Figure D.27: WT 2 bone (# 5598) polished to 0.3 micron.

Figure D.28: WT 2 bone (# 5598) polished to 0.05 micron.

Figure D.29: SOST KO 6 month bone (# 9198) polished to 0.3 micron.

Figure D.30: SOST KO 6 month bone (# 9198) polished to 0.05 micron.

Figure D.31: WT 3 bone (# 9207) polished to 0.3 micron.

Figure D.32: WT 3 bone (# 9207) polished to 0.05 micron.

Figure D.33: SOST KO 12 month bone (# 7592) polished to 0.3 micron.

Figure D.34: SOST KO 12 month bone (# 7592) polished to 0.05 micron.

Figure D.35: WT 4 bone (# 7564) polished to 0.3 micron.

Figure D.36: WT 4 bone (# 7564) polished to 0.05 micron.