THE EFFECT OF DIETARY SALT ON BONE IN A GENETICALLY-DEFINED RAT UNDERLOADING / OVERLOADING MODEL

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ABSTRACT

One the most serious health hazards of aging and of long term space flight is the loss of bone. The most important determinant of the debilitation due to bone loss is the peak bone mass achieved during late adolescence, which itself may be influenced by gender and environmental factors such as mineral balance. Sodium intake is considered a risk factor for both hypertension and osteoporosis. It is estimated that 30-60% of the population is hypertensive and 30-40% of the population is salt sensitive. The primary purpose of the present studies was to both delineate and combine the effects between salt intake and salt sensitive hypertensive genotype on bone. Our hypothesis was that hypertensive rats would have more severely affected bone than normotensive rats due to salt supplementation and/or genotype. In addition, how these effects might be altered by immobilization/overloading stress was examined as this further burdens NASA space pioneers.

This study investigated the possible effects of an ad libidum 1% or 2% saline instead of water on the normotensive (W) and salt sensitive hypertensive (SS) young female rats. A total of 46 weight-matched female rats (7 weeks old) were used. Treated rats in the 1% study drank 1% saline ad libitum for a 42 day salt supplementation period, beginning at day 7 (after arrival) to day 49. Treated rats in the 2% study drank 2% saline ad libitum for a 42 day salt supplementation period, beginning at day 7 (after arrival) to day 49. The right hindlimb of each animal was immobilized by binding to the abdomen with 4 layers of elastic bandage tape, the hip joint in flexion and the knee and ankle joint

in extension for the 42 day salt experimental period. Body weight and urine volume was measured biweekly. Food and fluid intake was monitored daily. After sacrifice, three sites (both the underloaded and the overloaded tibiae, as well as the L-2 vertebrae) were processed for histomorphometric analysis. The wet weight (g) and length (mm) of the excised right immobilized and left overloaded femur and the ulna were measured. A 3-point bending test was applied to femurs only. Immediately after the femur breaking strength measurements, bone was cut transversely, one mm from breaking point (fracture location), and a 1.0 mm cross-section was cut for morphological measurement. In addition, a 5 mm high cylinder section from each femur was cut and used for bone composition measurements along with the right ulna bone. A number of elements were analyzed at one time with Induced Coupled Plasma (ICP) spectrometry. The systolic blood pressure and heart rate were measured in the 6^{th} week of study by the tail-cuff sphygmomanometer method.

A more robust result was seen with increased concentration of saline treatment from a 1% saline threshold level, to the 2% saline level. Using two-way ANOVA, both hypertensive genotype and 2% saline treatment significantly increased blood pressure and heart rate, and decreased femur magnesium. The SS rat had significant reductions in bone mass, femur cross-sectional area and zinc concentrations with simultaneous elevations in femur stiffness, strength and calcium concentrations. Two percent saline treatment markedly increased both blood pressure and heart rate and decreased both femurs magnesium and cancellous bone in the weight-bearing tibia bone. After 6 weeks of immobilization (to simulate space weightlessness), reductions in cancellous tibia bone volume, with elevations in femur bone stiffness, mineral concentration (calcium and

phosphorus) and in trace elements (zinc and manganese) were found in the underloaded femur. Our findings suggest genotype, and saline treatment, and immobilization adversely affect bone in adolescent female rats. In addition, the deleterious bone effects are site specific, affecting each site differently.

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ABBREVIATIONS

ARS	active resorption surface
B	outside major diameter, m
b	inside major diameter, m
BFR	bone formation rate
bp	blood pressure
bpm	beats per minute
BS	bone surface
BV	bone volume
C	distance from neutral axis to outer fiber, mm
cm	centimeter
C-S	cross-sectional
D	outside minor diameter, m
d	inside minor diameter, m
E	apparent modulus of elasticity, GPa
F	applied force, N
g	gram
GPa	gigapascals
H&E	hemotoxylin and eosin stain (also H+E)
hr	heart rate

I	moment of inertia, m^4
L	distance between supports, mm
Mar	mineral apposition rate
ml	mililiter
mm	millimeter
mmHg	millimeters of mercury
MPa	megapascals
MS	mineralizing surface
N	newtons
n	number
NaCl	sodium chloride
O	overloading
Oc. S	osteoclast surface
p	p-value used in statistics
ppm	parts per million
SEM	standard error of the mean
SR	salt resistant rat
SS	salt sensitive hypertensive rat
SSC	salt sensitive hypertensive water control rat
SSS	salt sensitive hypertensive treated with saline rat
Tb.N	trabecular number

Tb.Sp	trabecular separation
Tb.Th	trabecular thickness
TV	tissue volume
U	underloading
UV	ultraviolet
W	wistar normotensive rat
WC	wistar normotensive water control rat
WS	wistar normotensive treated with saline

WITH SYMBOLS

Ar/Le	area divided by length
+	plus
H+E	hemotoxylin and eosin stain
V+H+E	Van Kossa with hemotoxylin and eosin stain
*	times
σ	ultimate bending stress, MPa
μm	micron

CHAPTER I

INTRODUCTION

1.1 OVERVIEW

As the American population grows older, osteoporosis is becoming an even more serious problem. Coupled with this, we are as a civilization planning major expeditions into space, first to the moon, then to Mars, then to Jupiter's moon, Europa. Extended space travel is known to have serious effects on bone. To exacerbate an already difficult situation, the American diet is changing. Increasing amounts of fast and highly processed foods are being consumed. The salt contained in an urban diet has increased several fold over what was consumed a generation ago, even though a high salt diet has long been postulated to be detrimental to bone (Shortt and Flynn, 1990; MacGregor and Cappuccio, 1993; Chan et al., 1992; McCarron et al., 1980; McParland et al., 1989; Goulding and Campbell, 1983; Goulding and Campbell, 1984; Chan and Swaminathan, 1998). Even with this high risk diet and increasing bone loss particularly in women of western societies, people are living longer, in part, due to pharmaceutical interventions.

The group most at risk for the additive effects of old age, high salt and potential immobilization associated with weightlessness in long space voyages is women. It is unclear when the damage to female bones occurs and which factors are the most significant. This is the goal of the present study. Four factors will be specifically addressed in growing rats: dietary salt intake, genetic predisposition, overloading (in part simulating orbital recovery) and immobilization (in part simulating weightlessness). This

study will look at the onset of adverse bone development, beginning in adolescence. By beginning the study at an early age, it is possible to see the initial stages of what may become osteoporotic bone.

Most older women now get some form of bone scan to check for osteoporosis. Unfortunately, current techniques rarely give a full picture of actual damage. It is also very difficult in late adulthood to quantify the factors leading to the problem. Human adolescent studies are marred by the fact that a precise quantification of salt intake and genetic make-up is unlikely. For this reason, the present studies selected a rat model.

In general, one week of a rat's life reflects one year of human life in terms of physiological change. The weightbearing hindlimb bones of the Wistar rats closely resemble the human analog. Unlike a human study, the study of rats can be terminated at the appropriate ages and bone extracted for detailed morphometry and microscopy. This study selected healthy adolescent female rats to model the onset of adverse bone development in women. We treated rats in the 1% study to 1% saline ad lib instead of drinking water and increased the salt in the drinking water from 1% to 2% in the second study. Because much of the human population has a hypertensive ancestry (as many as 50% of today's population becomes hypertensive), genetic salt sensitive hypertensive rats to model this portion of the population were also used.

To model the effect of space flight, a simple elastic bandaging technique was used on the right leg of the growing female rats. The immobilized hindlimb was compared to the left unbandaged (or overloaded hindlimb). The rat was forced to excessively use the left limb to get around. This simulates recovery from spaceflight upon reintroduction to gravity. The right immobilized limb, which could not be moved, simulates a lack of

gravity considered to be part of the bone loss problem in space. What factors contribute to the severity of this space risk?

1.2 PREVIOUS WORK

Does salt intake or a genetic predisposition to the physiological effect of salt adversely affect bone in space or upon return to earth? The present studies of the effects of salt on the overloaded and underloaded bones of genetically defined growing rats may begin to answer this question. Recently, Navidi et al. (1995) investigated the effect of excess dietary salt in a rat spaceflight model; no further reduction in calcium content was found apart from that induced by unloading the hind limbs. However, the examination of young growing male genetically-defined spontaneously hypertensive rats (SHR) pointed to early adverse bone morphological changes induced by salt that would be missed by standard measurements of bone calcium content (Furuse et al., 1992). It appears that salt damage to bone may be structural phenomena, not strictly calcium content modulated. These early adverse structural changes can precede calcium content change leading to fracture. Bone mineral-salt association and hypertension in adult and elderly human populations are difficult to assess accurately because genetics, blood pressure therapies and other environmental factors influence reductions in bone mineral and elevations in blood pressure.

Generally speaking, females in America have chronic subnormal intake of dietary calcium and excessive intake of sodium. Likewise, dietary trends for very young American females continue to be low in calcium and high in sodium (Albertson et al., 1992). According to Law et al. (1991) adults in industrially developed countries like

Japan and America have average intakes of dietary salt far above the maximal daily recommendations set by the U.S. Food and Nutrition Board of approximately 6 grams of dietary salt. Daily intake from selected regions in America ranged between 7-11 grams per day, while regions in Japan may reach 20 grams per day (Law et al., 1991).

Sodium intake is considered a risk factor for both hypertension and osteoporosis (Goulding and McParland, 1990; McParland et al., 1989; Law et al., 1992) though some researchers indicate this is not strong enough for intervention (Short and Flynn, 1990). Researchers estimate that 30-60% of the population is hypertensive and 30-40% of the population is salt sensitive (Muntzel and Drueke, 1992; Dyer et al., 1995). Some astronauts may be salt sensitive. There is great controversy on the mechanism involved in increasing blood pressure in salt-sensitive subjects. DeWardener (2001) has suggested abnormal kidney function in Dahl salt sensitive rats elevates plasma sodium in response to very high salt intake (8%) and this increase may alter hypothalamic function, thereby elevating sympathetic nervous activity and blood pressure. In addition, the Dahl salt sensitive rat's more porous blood brain barrier to excess sodium, may be a genetic abnormality (DeWardener, 2001). Grisk and Retigg performed renal cross transplantation experiments that demonstrated arterial hypertension can be transferred with a renal graft from the salt sensitive hypertensive rat to a normotensive recipient, and lowered blood pressure followed renal grafts from a normotensive to a salt sensitive hypertensive rat.

Very high sodium intake (8%) has been suggested to cause concurrent adverse changes in calcium and bone metabolism (Shortt and Flynn, 1990; MacGregor and Cappuccio, 1993). Sodium intake (1.8%) may lead to extensive bone loss in rats if given for a prolonged period of time because of calcium lost in urine (Chan and Swarminathan,

1997). In experimental animals, calcium excretion is thought to depend on urinary sodium excretion due to common resorption pathways for ions in the convoluted portion of the proximal tubule and the loop of Henle (Shortt and Flynn, 1990). In humans, elevations in sodium intake have been associated with elevations in blood pressure, urinary calcium excretion, and urinary excretion of hydroxyproline [a bone resorption marker in humans (Chan et al., 1992; McCarron et al., 1980; McParland et al., 1989). McCarron et al. has reported human hypertensives excrete more calcium than normotensive controls for any given level of urinary sodium excretion. Normotensive young adult sons (average age 21) of hypertensive parents when compared to young adult controls from normotensive parents, exhibit an increase in urinary calcium excretion when high salt diet of 20g/day was administered for 7 days suggesting genetic difference in renal calcium handling (Yamakawa et al., 1992). In addition, salt restriction has been shown to lower blood pressure in patients with mild and moderate hypertension (MacGregor et al. 1982). Salt restriction may also lower urinary calcium excretion and hydroxyproline in normal postmenopausal females (Need et al., 1991).

Calcium metabolism in humans is linked to both hypertension and osteoporosis. However, human studies are difficult to access accurately because genetics and other environmental factors influence bone mineral content and blood pressure. Historically, mineral metabolism has been studied in many defined genetic models. Of interest to our study is definitive research begun by Dahl et al. (1962b). After long experience working with rats and hypertension, Dahl et al. (1962a) reported that approximately 2-3% of unselected rats (later named salt sensitive) will die of hypertension after only a few months' ingestion of a high salt diet. However, approximately 25% (later named salt

resistant) from the same grouping did not develop any increase in blood pressure even after a lifetime long, high salt diet. All other rats fall in various degrees between these ends of the spectrum.

What differences exist between the normotensive rat and hypertensive rat? In just three generations of rats, Dahl et al. (1962b) developed salt sensitive (SHR) and salt resistant (SR) strains of rats with a differential response to high salt loading (7.3% NaCl added to dietary chow). After a six month high-salt experiment, not a single salt resistant rat was hypertensive, but in three months, 49 of 60 salt sensitive rats were hypertensive, some severely so. However, when maintained on a low salt diet, salt sensitive rats did not develop hypertension in a three month study. Dahl et al. (1965) have argued that, no matter how strong the genetic tendency for salt sensitive hypertension, the addition of excess dietary salt is required to uncover it in early life.

Historically, a Wistar-Kyoto normotensive (WKY) and a spontaneously hypertensive (SHR) were developed from inbred Wistar stock. These were most commonly used in blood pressure testing and were supplied from NIH. When the present studies began, the NIH supplier was not able to supply us and advised us to use the salt sensitive spontaneously hypertensive from Sprague Dawley (SD). Their salt sensitive spontaneously hypertensive (SSJr) were inbred from SD stock. It is generally recognized that an increase in high salt in the experimental diet is responsible for the increased blood pressure of the SS and of SHR (Krari and Allain, 1991; and Rapp, 1987). The suppliers also developed a salt resistant normotensive variety (SRJr). These inbred salt resistant rats were not appropriate as controls for the present bone studies as they typically weigh much less than SSJr and have medical problems. Our objective required a standard

healthy normotensive control rat. For this reason, a normotensive wistar (W) from Sprague Dawley was chosen.

In Young et al.'s (1988) review of calcium metabolism and hypertension, all studies cited used SHR (spontaneously hypertensive rat) and WKY (Wistar-Kyoto normotensive control rat) for testing purposes. Occasionally, Sprague Dawley (SD) normotensive rats were used instead of WKY rats in previous studies. Specific breeder information for the present studies' use of salt sensitive spontaneously hypertensive (SS) and wistar normotensive (W) rats is in the Methods section. In the Results section, the following group designations (with their abbreviations for easier reading) are used: wistar normotensive water control, WC; wistar normotensive, treated with saline, WS; salt sensitive hypertensive water control, SSC; salt sensitive hypertensive treated with saline, SSS. In order to now be able to understand each section of the discussion's many comparisons, the commonly used term SHR (spontaneously hypertensive rat) and WKY (wistar-kyoto normotensive rat) will be used to compare our SS (salt sensitive spontaneously hypertensive rat) and W (wistar normotensive rat) with other historical studies.

Progressive changes have been seen in mineral metabolism with aging in hypertensive genetically-defined animal models. Bindels et al. (1987) found young SHR male rat's femoral bone calcium content, ash weight, dry weight and volume to be similar to WKY control rats at age 6 weeks. Using radiology, Umemura et al. (1992) found significant differences in bone length between sedentary growing male SHR (15 weeks of age) and sedentary growing Wistar normotensive rats. The tibia, femur, vertebral column and coccyges were significantly shorter in the SHR strain. However, voluntary running

exercise training in SHR resulted in significant increases in bone length and transient decreases in blood pressure when compared to SHR sedentary controls; however, SHR trained animals did not reach the bone lengths of Wistar trained or untrained rats.

In examining SHR older adult males of age 23, 26, and 54 weeks, researchers reported reduced bone mineralization suggesting a genetic influence as SHR rodents age (Izawa et al., 1985; Lucas et al., 1986; Metz et al., 1990). Lucas et al. (1986) reported significantly decreased cortical bone density in SHR (age 23 weeks) and significantly decreased bone calcium content in SHR (age 31weeks) as compared to WKY controls. Izawa et al. (1985) found reductions in cortical thickness, and trabecular bone mass in SHR (age 26 weeks). In addition, Izawa et al. demonstrated femoral bone length decreases in older SHR rats in comparison to WKY controls; corroborating and extending Umemura et al.'s (1992) measurements of young SHR.

There are few studies on growing female SHR rat bones but in male rats, Metz et al. (1990) found significantly reduced bone density after one year in SHR rats over normotensive WKY rats. Bone calcium content was not significantly different between the two groups. However, when both were exposed to .65%-2.6% NaCl supplementation in their diet, SHR rats demonstrated significant reductions in bone density. On the other hand, male WKY rats administered supplementation, exhibited no bone or calcium response to these salt loading regimen, when compared to controls. Seven weeks of salt supplementation [1% saline and 2%(1% saline and 1%NaCl in food)] in young growing male SHR (age 16 weeks) had no effect on the calcium content of bone (Furuse et al., 1992). However, 3 of 15 rats on salt supplementation in Furuse et al.'s SHR developed fractured bones. Furuse et al.'s examination of these young growing male animals pointed

to early adverse bone osteoclastic and magnesium changes induced by salt that would be missed by standard measurements of bone calcium content.

Furuse et al. 's data indicate early severe abnormalities in bone in the SHR male model. In humans, only difficult bone biopsy studies could have uncovered this important finding which may partially explain why human subjects with seemingly normal to high readings for calcium content and bone mineral (as measured by Dual Photon Absorptiometry / Dual X-ray Absorptiometry), experience fractures (Gluer, 1994).

At the beginning of our studies only one genetic study (Lau et al., 1984) had used the female model to look at bone mineral association with hypertension. Lau et al.'s findings indicate adult female SHR treated for hypertension did not exhibit any effect on calcium content in one year's time when compared to WKY, however, in their study a blood pressure medication to lower blood pressure was used complicating the interpretation (Lau et al., 1984).

To sum up previous work, SHR rats exhibit bone abnormalities when compared to WKY rats. Predominately, these studies use mature female rats (Lau et al., 1984; Liang et al., 1997) or male rats (Bindels et al., 1987; Furuse et al., 1992; Hsu et al., 1986; Izawa et al., 1985; Lucas et al., 1986; McCarron et al., 1981; Metz et al., 1990). Male studies have found reduced trabecular bone (Yamori et al., 1991) and lower cortical bone (Izawa et al., 1985; Yamori et al., 1991) in SHR when compared to WKY rats. Previous male studies on a bone–hypertension association have most often focused on the density and calcium metabolism of bone (Bindels et al., 1987; Hsu et al., 1986; Lucas et al., 1986; McCarron et al., 1981).

1.3 OBJECTIVES OF THE PRESENT STUDY

To expand on previous data in assessing peak bone mass (a primary determinant in osteoporosis) in an experimental design, the present study looked at bone mass, strength, mineral/element concentrations, morphometry, and cancellous microstructure, in response to a dietary salt supplementation (1% saline and 2% saline) and limb underloading / overloading (42 days) in 2 different genetically-defined (normotensive wistar and salt sensitive hypertensive) female rat models. In addition, to further investigate bone metabolism, parameters were examined at several bone sites instead of just one, to provide a more complete assessment of an individual rat's overall bone status. Multi-site examination findings could have ramifications on screenings for osteoporosis as well as therapeutic implications. By using normotensive and salt sensitive hypertensive rat models, the present study was able to address questions relating to bone in two female strains of differing salt sensitivity and questions relating to bone in space or recovery upon return to earth.

The present studies focused on the combination of three characteristics in a rat model: 1) young female 2) chronic (42 day) hindlimb underloading / overloading 3) salt sensitivity. The study worked with rats of two genetic groups mirroring salt sensitivity in the American population. The rat bones were examined to quantify bone metabolism in relation to these characteristics. This gave a statistically valid result for the bone damage due to genotype and excess salt consumption, which may lead to osteoporosis later in life. One of the major contributing factors to osteoporosis is theorized to be high dietary salt intake from processed foods. In America, with a large population consuming a typically high salt diet, this may be of particular significance.

In summary, the purpose of study was to investigate: 1) the effect of salt on bone quality in adolescent W and SS rats 2) the effect of genetics on bone quality in adolescent W and SS rats 3) the effect of underloading/overloading on bone quality in adolescent W and SS rats and 4) if the combined factors have additive or synergistic effects..

Although the present study obtained data pertaining to young female rats during the development and accumulation of peak bone mass, it is hoped that the implications may have a wider application. It may be that early bone damage from excessive consumption of salt occurs initially in adolescence and to those who are salt sensitive. This has never been tested but is clearly critical information to formulating any effective osteoporosis treatment and prevention plan here in the U.S and to those pioneers in space travel.

CHAPTER II

METHODS

2.1 INTRODUCTION AND EXPERIMENTAL PERIOD

A preliminary study of six rats [3 Wistar (W) from Sprague Dawley and 3 Salt Sensitive Hypertensive (SS) from Sprague Dawley] was conducted to establish protocols and procedures. In addition to six rats that were used in the preliminary test to establish protocols, 2 untreated W rats (age 14 weeks) were used to pre-test a calcein labeling technique. Subsequently, twenty-two rats (11 W and 11 SS) for the 1% saline ad libitum (instead of water) study followed by twenty-four rats (12 W and 12 SS) for the 2% saline ad libitum (instead of water) study were purchased at 7 weeks of age, and housed in individual stainless steel wire-bottomed cages by Laboratory Animal Services at standard room temperature (68°-72°) with a standard light-dark (12-12) cycle. All rats were initially fed a control diet ad lib with distilled water for 7 days (to acclimatize to new surroundings and personnel). Treated rats in the 1% study drank 1% saline ad libitum instead of water for a 42 day salt supplementation period, beginning at day 7 (after arrival) to day 49. Treated rats in the 2% study drank 2% saline ad libitum instead of water for a 42 day salt supplementation period, beginning at day 7 (after arrival) to day 49. Control rats in the 1% and 2% studies drank distilled water ad libitum.

All animals were fed a diet of AIN 93M (ICN Biomedicals, Aurora, OH). All animals were subjected to the immobilization bandage procedure on day 7, post-control period (see Section 2.3). Systolic blood pressure (BP) was measured in prewarmed,

restrained unanesthetized rats by the tail-cuff method using an available electrosphygmomanometer with a physiological recorder (see section 2.4). For the preliminary study, BP was measured 5 times and one time by direct carotid artery cannulation (prior to death). The method of euthanasia used in the preliminary study (rats were anaesthetized with 35-45 mg/kg sodium pentobarbital (I.P.) used for a left carotid artery cannula; followed by exsanguinations) was changed to carbon dioxide asphyxiation for both the 1% and 2% study. In addition, only one systolic blood pressure reading was taken in the 6th week of study, instead of multiple readings throughout the study. In both the 1% and 2% study, systolic blood pressure was measured once in the 6^{th} week of study by the tail-cuff method. Immediately following asphyxiation, femur, tibia, and ulna bones were dissected, freed of soft tissue, and prepared for analysis and storage (usually within one hour of death) in the1% and the 2% studies (see Section 2.5).

Preliminary findings indicated that to coordinate breeding of these animals, meet new Laboratory Animal Services requirements, use available special cages, it was necessary to split the study of 1% and 2% saline into two separate groups of 22 rats and 24 rats, respectively.

2.2 FOOD INTAKE, FLUID INTAKE, BODY WEIGHT, AND URINE

2.2.1 Food Intake

Composition of the experimental diet (ICN Biomedicals, Aurora, Ohio) contained standard AIN-93M MX Mineral mix, standard AIN-93 -VX Vitamin Mix in standard AIN-93M mix (Reeves et al., 1993). While attempting to model this study after Furuse et al. (1992), the newer AIN-93M mix was chosen instead of the AIN 76 mix. In personal

communication with Greg Sidley/Technical Service ICN Biomedicals, it was pointed out that differences in the new formulation posed no problem for my study and was a better formulation providing a better balance of essential nutrients. A comparison of essential nutrients provided in AIN-93M, making it a better diet than AIN-76A, is found in P.G. Reeves (1997) study.

In the preliminary study, a weight record of food eaten and the remains that fell through the cage bottom cage, was kept. It was hoped that if animals were weight and age matched initially, that the genetic differences in weight would not be significant at the end of this short study. In both the 1% and 2% study, food intake was measured twice a week. Body weight was measured biweekly. A revision to study was submitted to the Institutional Animal Care and Use Committee to restrict food up to 10% should this become necessary to maintain similar body weights of the groups.

2.2.2 Body Weight

Body weight was measured biweekly during bandage change (Ohaus Triple Beam Balance, Florham Park, NJ $\pm$ 0.1 gm).

Because the controls between the 1% Study and 2% Study were significantly different in body weight, thus affecting outcomes, 1% and 2% results could not be combined. Results between the two studies are compared in most general discussions.

2.2.3 Fluid Intake

Saline treatment (used in place of drinking water for treated rats) was prepared from distilled water with the addition of sodium chloride equal to 1% of solution (Furuse

et al., 1992). Saline was administered to the experimental groups ad lib for the 6 week experimental period. Distilled water was administered to control groups ad lib during that period.

For the 1% study, ten grams of NaCl (Sodium Chloride from Sigma (Acs Reagent assay> or= 99.0%) was weighed and placed in an acid washed 1 liter flask. Distilled water was added until the solution reached one liter mark. For the 2% study, twenty grams of NaCl was weighed and placed in a one liter acid washed flask. Distilled water was added until the solution reached one liter mark. Water and saline were monitored daily. Both were changed and measured at least once a week.

Unexpectedly, two W died on the 2% saline treatment. One W died in the third week of study. While cause of death of this rat was unknown, saline intake had risen from 16 ml per day to 185 ml per day in one rat, which may have accounted for the death. Another W died in the fifth week of study. Saline intake of this rat rose from 21 ml to 37 ml per day. It was unanticipated that a W rat would die on this amount of saline in this short experimental period as Chan and Swaminathan female Spague Dawley rats survived drinking 1.8% saline ad lib for four months (Chan and Swaminathan, 1993) and one year (Chan and Swaminathan, 1998) with sham surgery or oophorectomy without premature deaths. However, total salt per day of these two rats were above average. In the W rat to die first, salt intake was 4 times than the average W on saline. The second W rat's salt intake, while similar to another surviving W, was almost 50% higher than the average intake of surviving W rats.

2.2.4 Urinary Volume

Three individual urine volume measurements (24 hour period per measurement) were taken from each rat in both the 1% study and 2% study. Each urine sample was measured in an acid washed 100 ml graduated cylinder to determine volume for each 24 hour period. After this measurement, 1.5 ml increments were pipetted into 1.5 ml snap seal microtubes. Each individual sample was placed in numbered, color-coded tube rack/collection plate with 80 wells. Additionally, each microtube labeled with waterproof colored tape of genetic/saline grouping with assigned number written in ink. The number of rows assigned per animal is dependent on each animal's urine volume collected from individual metabolic cage (stainless steel, racks of 6 x 4 individual cages, Hoeltge Inc., Cincinnati, OH) from each assigned 24 hour period. All collected urine was saved for further analysis in a later study. Though labor intensive, urine was collected three times from each animal and stored for deoxypyridinoline analysis (Metro Biosystems, Mountain View, CA) when funding and time become available. This marker is used clinically as well as in the rat model and has potential in monitoring bone turnover rate differences in both animals and humans (Fisher et al., 1995; Delmas et al., 1991). The organic matrix of bone is approximately 90% Type I Collagen. Deoxypyridinoline (Metro Biosystems, 1996) is one of two crosslinks of Type I Collagen (Pyridiline is the other crosslink), providing rigidity and strength. During resorption, Deoxypyridinoline (Dpd) is released by the action of lysyl oxidase (an enzyme involved in collagen and elastin cross-linking) acting on the amino acid lysine into the circulation. Dpd is then excreted by the kidney. If differences occurred between the saline treated versus water controls, these may further be differentiated by future bone turnover rate measurements.

2.3 IMMOBILIZATION BANDAGE TECHNIQUE

Following the Li et al. (1990) protocol, the right hindlimb was immobilized by binding to the abdomen the hip joint in flexion and the knee and ankle joint in extension. A brief non-surgical isoflurane anesthesia was administered during the bandaging procedure. The bandage of at least 4 layers of elastic bandage tape was changed approximately every 2 weeks or as needed. After the procedure, the bandage and the right hindlimb did not touch the floor of the cage during movement. The left hindlimb supported the weight previously supported by the two hindlimbs. Rats were able to adapt in one day to this modification by walking and hopping (Li et al., 1990). This immobilization technique, used in many studies, has been noted for its ease of application and reversibility (Bagi et al., 1992). Personal communication with Dr. Kimmel (Maede et al., 1993) advised to massage and monitor daily the immobilized (underloaded) limb for blueness or signs of lost circulation. Adhesive elastic bandage tape Elastikon by Johnson and Johnson 1" x 5 yd. was used for bandage technique. Conform Kendall, a stickier bandage, was not useful for the immobilization technique. We used gauze and bacitracin on redness in two rats with good results overnight. There was much more than expected hair breakage. Some depilation did occur in rewrapping. IACUC members Drs. Manuel Himenes and Sylvia Kondo discussed stipulations for protocol #97-002-2. LAS staff worked closely with investigators providing care and maintenance of the rats. The bandage technique required constant daily monitoring and periodic replacement. Though it was felt that this was the best method to use for the study, it became apparent that individual rats reacted differently to the underloaded stress, which could not be

measured as objectively as food/salt intake or body weight. Some rats were able to escape overnight to various degrees. Other rats were able to do isometric movements beneath the stretchy, elastic material making the underloaded limbs incompletely immobilized, but fully non-weightbearing.

2.4 TAIL CUFF BLOOD PRESSURE MEASUREMENT

The indirect tail cuff technique is both an accurate and reliable method for the determination of systolic arterial pressure (Pfeffer et al., 1971; Williams et al., 1939) in prewarmed, restrained, unanaesthetized SHR models. In the preliminary study, the rat was placed between adjustable plastic housings and rat holder base with built-in warming element for raising the environmental temperature of each constrained rat for 5-10 minutes. The occlusion cuff was placed as close to the base of tail as possible with firm fixation of pneumatic pulse transducer to the ventral surface of tail immediately distal to tail cuff. A careful acclimation protocol was followed to insure rats were not overheated or overexcited, which can cause restlessness and false pressure readings (Williams et al., 1939). Determinations were made at the same hour of day with each procedure taking approximately 20 minutes.

Researchers have reported a high degree of correlation (r =.975) between the indirect tail cuff method and directly recorded left carotid systolic pressure by cannula (attached to Century Technology pressure transducer and recorded on a Beckman Type R Dynograph (Pfeffer et al., 1971). Our laboratory validated the tail cuff pressure measurement at the time of left carotid artery cannulation with a direct measurement before autopsy in 5 rats. Three to five readings from each rat were taken simultaneously

by both methods and averaged. We found similarly good agreement as reported by others. A programmed electro-sphygmomanometer was initially used. Maximum cuff pressure was set at 200 mmHg. Calibration (Internal) was 100 mmHg. Inflation /deflation rate set at 2.5 mmHg/sec. Cycling interval on manual, approximately one per minute. In all studies, three readings from each rat were taken and averaged, using the electro-sphygmomanometer. From the zero line, 100 mmHg was set at 26 mm. For heart rate, bpm (beats per minute), arterial pressure pulses were counted. Recorder chart speed was set at 2.5 cm per sec.

Deltaphase isothermal pads instead of electric heaters were used in both 1% and 2% study, as electrical noise, overheating and oscillation in temperature can occur in electric heaters, and the pads cannot overheat the rat. The acrylic restrainer rested on the isothermal pad. Additionally, another pad was placed on top of restrainer partially covering the rat.

As noise of electro-sphygmomanometer disturbed relaxation of rat and vibration also randomly produced occasional artifacts, a manual sphygmomanometer was attached to physiograph recorder, replacing electro-sphygmomanometer. The Internal Calibration button was calibrated against pressure from manual sphygmomanometer.

2.5 CORTICAL AND TRABECULAR SAMPLE PREPARATION AND STORAGE

2.5.1 Cortical Bone Sample Collection and Handling

2.5.1.1 Ulna Preparation

The upper limbs from each animal were removed and freed from skin. Both the right and left ulna bones were cleaned of soft tissue. The wet weight (g) of the left ulna (Figure 2.1) was measured by balance (Mettler Instrument Corp, scale Type H6, Hightstown) to the nearest 0.1 mg. The length of left ulna was measured with Mitutoyo digital calipers from the tip of the olecranon process to the distal ulnar notch. Untreated ulna bone samples were stored separately in sealed petri dishes (50mm x 9mm) in sealed plastic bags at -20 ^{O}C until further analysis (eg. element compositional testing).

2.5.1.2 Femur Preparation

The hindlimbs from each animal were removed and freed from skin. Both the right and left femur bones (Figure 2.1) were cleaned of soft tissue taking approximately 15 minutes per animal. Labeled femurs were immediately placed in freezer (-20°C). Each sample was individually contained in snap top labeled petri dishes (50mm x 9mm, pre-weighed) with one drop isotonic saline solution added to humidify chamber upon thawing. Small plastic sealed containers of six petri dishes each were frozen at -20°C until further analysis. After 24-hour thawing on the day of breaking strength measurements, the labeled dissected bone tissue was immediately placed in humidified chamber, 37°, (according to methods, Shaw et al., 1988) and transferred to weighing room. Bone tissue was exposed as briefly as possible to air and subsequent drying. The

wet weight (g) of the right and left femur was measured in weighing dish and weighed (Mettler Instrument Corp. Type H6, 0.001g Hightstown, NJ). The length of each femur was measured with Mitutoyo digital calipers from the tip of greater trochanter to the distal end of the femur and from the proximal head of femur to the distal end of femur. A felt tipped pen was used to mark midpoint of the length of bone in preparation for breaking strength measurements. Immediately following marking, each bone sample was photographed and returned to the humidified chamber for subsequent strength measurements (Figure 2.2). Further bone handling is discussed under Femur Strength Testing.

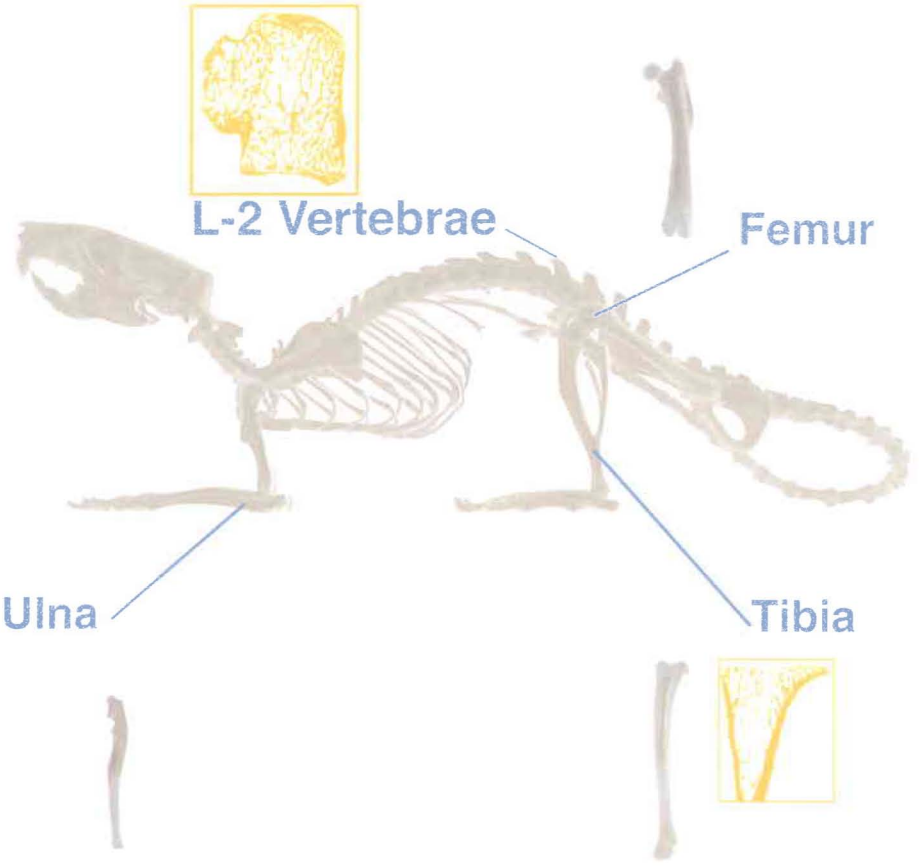

Figure 2.1 Cortical bone samples (femurs and ulna) and cancellous bone samples (L-2 vertebra and tibias) excised for measurement. Yellow identifies bone area in undecalcified 5μm thick section of proximal tibia and L-2 vertebrae.

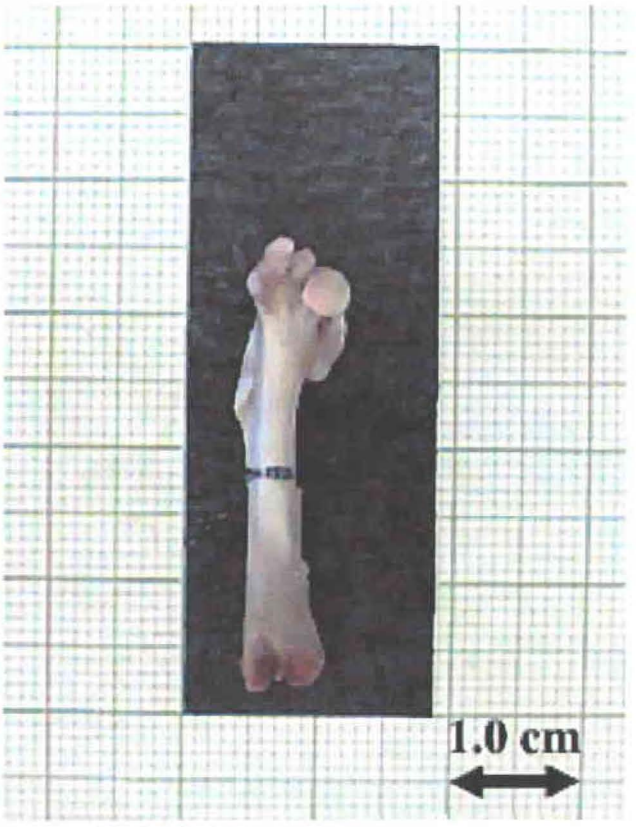

Figure 2.2 Femur bone tissue photographed and marked in preparation for strength measurements.

2.5.1 Trabecular Bone Sample Collection and Handling

Following removal from each rat, both the right and left tibia whole bone samples were stored separately in cold ethyl alcohol at 4°C until sectioning (Figure 2.1). In addition, a large section of approximately 5 vertebrae was removed with soft tissue attached from each animal in the 2% study and stored separately in cold ethyl alcohol at 4°C until sectioning (Figure 2.1). Commonly used fixatives such as EDTA

(ethylenediaminetetraacetic acid, disodium salt), formic acid-citrate and embedding solution MMA (methyl methacrylate) inactivate bone alkaline phosphatase (AIP) and acid phosphatase (AcP) activity (Liu et al., 1987). Liu et al. (1987) found that using 70% ethanol, acetone or GMA (glycol methacrylate) allowed AIP and AcP activities to be shown simultaneously. Ruegg (Personal communication, 1996) advised 100% alcohol storage for tetracycline or calcein-labeled bone biopsies (see Figure 2.9), as our lab must similarly transport samples between labs for various procedures. Ruegg has found that ethyl alcohol at the temperature of regular melting ice provides excellent visualization of calcein in unstained sections as well as excellent visualization in stained sections of acid phosphatase for identifying osteoclastic activity, alkaline phosphatase associated with bone formation activity, and the Van Kossa technique for identifying insoluble calcium.

2.5.2 Slicing of Fixed Tibia and Vertebrae

Protocols developed by Ruegg and Smith (1996) under Dr. W. Huffer for embedding in glycol methacrylate and staining were strictly followed. As soon as possible, the tibias of each animal were removed from alcohol storage. With soft tissue attached to aid in slicing, a low speed diamond saw (Buehler, Lake Bluff, IL) longitudinally sliced each proximal tibia into 500 μm thick sections. Slicing to these dimensions provided adequate infiltration with GMA (Ruegg and Smith, 1996).

If possible, each vertebrae section and each tibia were removed as one piece with scissors. Muscle, tendon and ligaments were left attached to act as an aid in slicing. In the preliminary study, the epiphysis of the tibia tended to slide off these young growing rats with dissection. All muscle and ligaments thereafter were left attached to keep bone

in place. These limbs were then placed in cold ethyl alcohol because of needed transport time to slicing equipment (Buehler low speed diamond wheel saw with precision diamond wheel - 3"diameter, 0.006 in. thickness, medium diamond size and high diamond concentration and 4"diameter, 0.015 in. thickness, fine diamond size and high diamond concentration) located in another building (HIGA lab); with occasional use of diamond wire saw model #850- using .010"diameter, diamond impregnated wireblade (South Bay Technology, San Clemente, CA).

At HIGA lab the whole tibia or vertebrae section was removed from the cold vial of ethanol storage and was briefly blotted on a paper towel. Each sample was glued to a flat block of wood (2.5 cm x5 cm x 0.8 cm) with instant superglue (ethyl cyanoacrylate, World Precision Instruments, Sarasota, FL), and placed on the stage of the diamond saw. The saw cooling tray was filled with 70% alcohol as recommended in Ruegg and Smith protocol to help keep the sample wet and to set the glue in the preliminary study. The saw cooling solution in the 1% and 2% study, was changed to less toxic cold water and was found to work well. Cold alcohol can be added at gluing site if sample begins to fall off.

While the diamond saw differed from that presently used in Ruegg and Smith lab, the sample can be lined up to the diamond saw using the first cut as the reference cut for creating a parallel plane. The micrometer on the saw can advance the sample 500μm for slicing. By using a combination of slow speed and alcohol/water coolant, slices can be prepared with a minimal amount of heat generation and sample damage.

Sections were sliced longitudinally through each sample in 500μm thick increments depending on the sample thickness. In humans, vertical slices of bone have

been found to have statistically higher values of bone formation and resorption than horizontal slices (Malluche et al., 1982). Ruegg and Smith have found that slices thicker than 400 microns need an extended infiltration time. For this reason, samples were infiltrated as long as possible in cold room (4°C) on a shaker for approximately 2 days. As it was considered the most important step in the process, this longer period was chosen. A minimal 6-12 hours shaking in cold room had been recommended for these 500μm thick sections of mineralized bone. The infiltrating solution was removed and replaced with pre-mixed infiltrating solution at least twice. Each slice was then processed without decalcification into glycol methacrylate (GMA). This processing method required a relatively short time because of the thinness of the sample. It was required that a plastic embedding medium be used instead of the commonly used quick clinical paraffin embedding medium for bone tissue. Plastic (Sorvall, Norwalk CT, 1972) offered far better resolution with no compression, thinner sections, and a reduction in damage from xylene, benzene, fixatives, heat, etc. Undecalcified sections obtained by plastic embedding offered the detection of unmineralized osteiod (used to quantify areas of active bone formation, from the lining of active osteoblasts which can form osteiod matrix and areas of inactive unmineralized osteiod, without osteoblasts or collagen synthesis) (Albright and Skinner, 1987). Since GMA was water soluble, it did not require removal from the section after the sample was cut. However, the most important reason for using plastic was the rigid nature of plastic itself, which allowed bone to remain in undecalcified state (necessary for detection of mineral content, mineral distribution, calcein labeling, and for cutting a 5μm thickness with the Sorvall microtome for a microscopic viewing).

2.5.4 Sectioning and Staining of Fixed Tibia and Vertebra

Each tibia or vertebra sample was processed, and embedded in GMA accordingly to Ruegg and Smith Protocols, 1996 (See Appendix A, Section B). Using a JB-4 microtome (Sorvall, Norwalk, CT) with tungsten carbide knives (Tabeling, 1997), at least 3 sections, 5 microns thick, were cut from the center of proximal tibia. In addition, 3 sections from the L-2 vertebrae sample, placed in a longitudinal direction in the 2% study, were cut by JB-4 microtome. Each section was collected from the microtome with very fine tweezers and stretched by floating into room-temperature deionized water. Sections were then mounted to glass microscope charged slides (Fisher Scientific). Depending on stain to be applied, slides were either air dried or heated on hot plate to dry.

One section was stained with a Van Kossa Stain (see Appendix A, Section B) combined with Hemotoxylin and Eosin Stain (VK+H+E) for light microscopy. One section was stained with a Trap (Tartrate Resistant Acid Phosphatase) stain with a green counterstain for light microscopy (see Appendix A, Section B). The last section was left unstained for epifluorescent microscopy measurements (see Figure 2.7, 2.8 and 2.9).

2.6 FEMUR STRENGTH TESTING

Rat left and right femurs were rapidly dissected, freed from soft tissue and stored separately in plastic containers in sealed labeled petri dishes (50 mm x 9 mm) at -20 ^{o}C until further analysis (e.g. strength testing). Freeze-thaw protocols do not change the mechanical properties of bone (Seldin, 1965; Pelker et al., 1984).

After 24 hour thawing, the labeled dissected bone tissue was immediately placed in a humidified chamber, 37°, (Shaw et al., 1988) and transferred to a weighing room. Bone tissue was exposed as briefly as possible to air and subsequent drying. The wet weight (g) of the right and left femur was measured (Mettler Instrument Corp. Type H6, 0.001 g). The length of both femurs was measured with Mitutoyo digital calipers from the tip of the greater trochanter to the distal femur. Immediately following these measurements each bone sample was returned to the humidified chamber.

Immediately following photography of femur bones, each sample was placed between two support plates for the 3 point bending test. The test of femur bending was performed using an Instron Universal Testing Machine (Model No.4206-004, Instron Corp., Canton, MA) with a 50 pound load scale, 0.4in/min crosshead speed and 10in/min chart speed. Force was applied at the midpoint by a one mm plunger. The bottom intra-support length distance was 18 mm. The sample was placed carefully on a support fixture with flattest side down in a consistent manner similar to photographic position (see Figure 2.3).

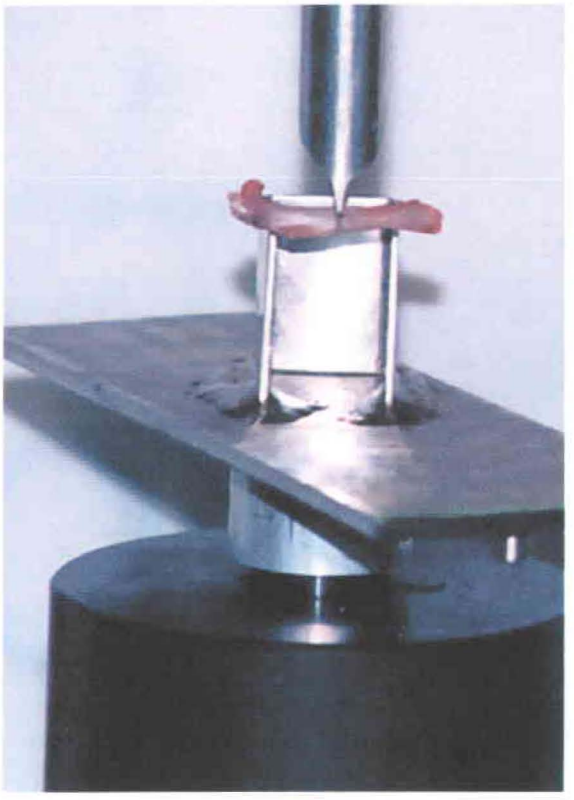

Figure 2.3 Femur 3 point bending test

Immediately after the breaking strength measurement, bone was cut transversely (Buehler saw and 4" precision diamond wheel, 0.006 in. thickness, medium diamond size, with high diamond concentration) one mm from breaking point (fracture location) toward proximal end. From proximal end, a 1.0 mm section was cut for the calculation of each bone cross-sectional diameters. In addition, these photographed 1.0 mm sample sections were used for femur cortical bone /marrow measurements [Bone area/perimeters were measured by computerized Image Analysis (IPTK 3 Tool Kit, 1999, John C. Russ, Materials Science and Engineering Department, North Carolina State University,

Raleigh, North Carolina and Photoshop 6.0)]. In addition, a 5 mm cylinder section was cut from the distal end (see Figure 2.4). The 5 mm section was used for bone composition measurements.

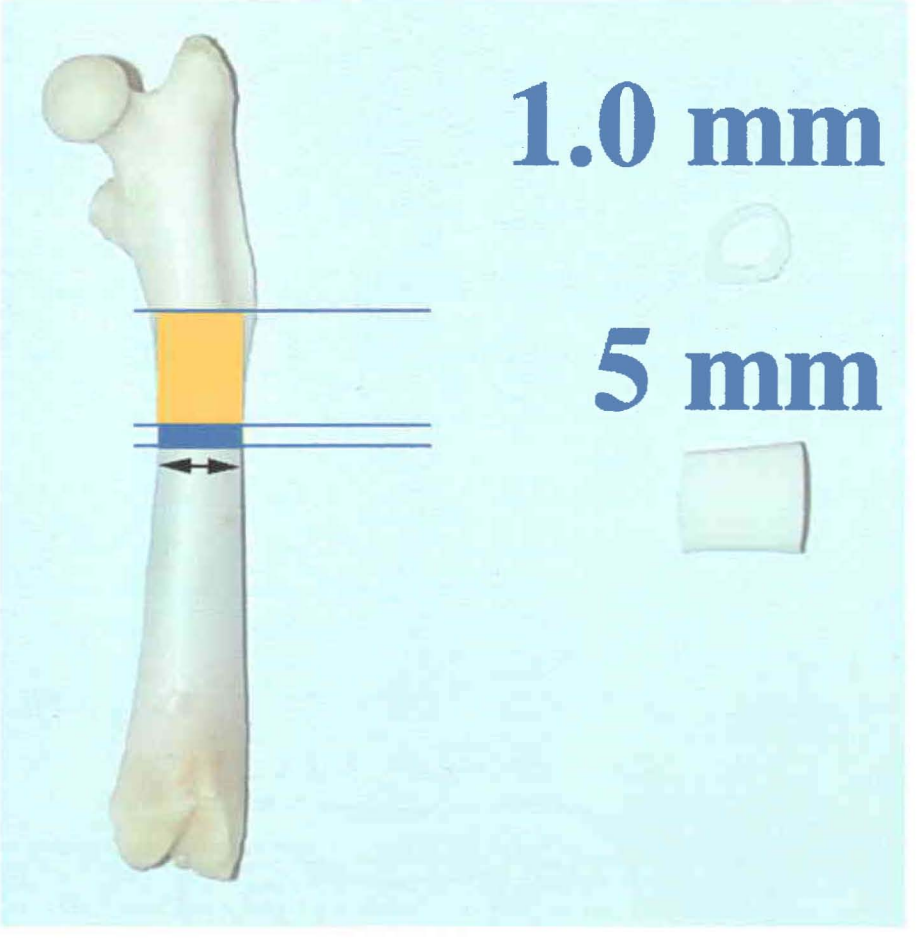

Figure 2.4 Femur slices for compositional testing (5 mm length) and morphology (1.0 mm thick). Black arrows indicate breaking point fracture location. Toward proximal end (Top), blue section is 1 mm slice location. Yellow area is 5 mm slice location.

To determine the ultimate bending strength and the apparent modulus of elasticity, American National Standards Institute / American Society of Agricultural Engineers ANSI/ASAE (1995) standard for three-point bending test of animal bone was used.

Measured parameters for ASAE Standards 1995, three point bending test

o = ultimate bending stress, MPa

C= distance from neutral axis to outer fiber, mm

I = moment of inertia, m^4

L = distance between supports, mm

F = applied force, N

Derived parameters for ASAE Standards 1995, three point bending test

1) The ultimate bending strength, o, (stress) was determined by: $o = FLC/4I$

ASAE state most bone sections can be modeled from a hollow ellipse or a quadrant of an ellipse. This young growing rat femur bone matched best the quadrant of an ellipse. Therefore, for calculation of each bone cross-sectional diameters, the quadrant of an ellipse model (Figure 2.5) was used (ASAE, 1995).

Measured parameters (Figure 2.5) for ANSI/ASAE Standards 1995, quadrant of an ellipse calculation:

B = outside major diameter, mm

b = inside major diameter, mm

D = outside minor diameter, mm

d = inside minor diameter, mm

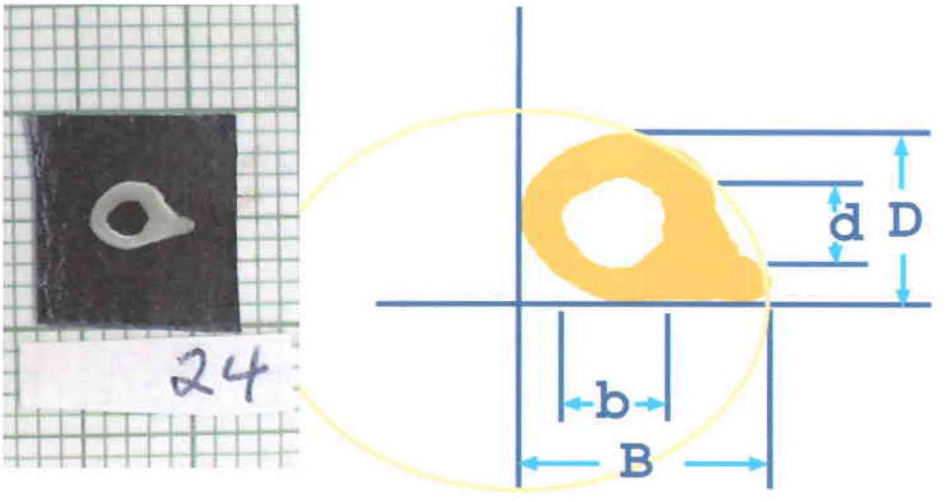

Figure 2.5 Femur 1.0 mm thick cross-section used to measure parameters for ANSI/ASAE Standards 1995, quadrant of an ellipse calculation.

Derived Parameters for ANSI/ASAE Standards 1995, quadrant of an ellipse calculation:

C= distance from neutral axis to outer fiber, mm

I = moment of inertia, m^4

$I = .0549 [(B*D^3) - (b*d^3)]$

$C = 0.57559 D$

2) Apparent modulus of elasticity for a 3 point bending test calculations:

$E = FL^3 / 48 Id$

E = apparent modulus of elasticity, GPa

df =deformation, mm

2.7 FEMUR AND ULNA BONE COMPOSITION

A number of elements can be measured at one time with an Induced Coupled Plasma (ICP) spectrometer, thus reducing the cost of testing, when compared to atomic absorptiometry. ICP measurements were used for all element analyses. To defat femur bone, each 5 mm femur section was placed in 3 ml chloroform/methanol (2:1) in 7 ml vial for solvents for 24 hours. To defat ulna bone, each left ulna was placed in 6 ml chloroform/methanol (2:1) in 7 ml vial for solvents for 24 hours. All bones were placed in fresh chloroform/methanol (2:1) 24 hours later. After defatting technique, femur sections and ulna bones were dried in an available oven at 80°C for 72 hours and weighed for dry weight. Sections were not ashed as they were too small for equipment available. All glassware was double washed and acid-rinsed. Following drying, the fat

free dry weight was recorded, followed by digestion with concentrated nitric acid. Tissue element/mineral content was analyzed by Inductively Coupled Plasma (Thermo Jarrell Ash, Atom Scan 16, Franklin, MA, in collaboration with Xue Xin Huang, Agricultural Diagnostic Service Center, paid service) for calcium (10 mg/g), magnesium (10 mg/g), phorphorus (10 mg/g), sodium (10 mg/g), potassium (10 (mg/g), boron (μg/g), manganese (μg/g), iron (μg/g), copper (μg/g), and zinc (μg/g). Tissues were digested at 150 degrees C for approximately 2 hours or until material totally digested. Digestion was based on the Environmental Protection Agency's Method 3050B, 1996. Dilution was made only for calcium, since levels were high. Standard samples were completed before each run of the instrument.

2.8 MORPHOMETRIC CHANGES IN RAT FEMUR CORTICAL BONE

Immediately after the breaking strength measurement, bone was cut transversely (Buehler saw and 4" precision diamond wheel, .006 thickness, medium diamond size, with high diamond concentration) one mm from breaking point (fracture location) toward proximal end. From proximal end, a 1.0 mm section was cut for bone cross-sectional calculations. These photographed sample sections (see Figure 2.5) would serve as femur cortical bone /marrow size and shape measurements (Table 2.1).

Cortical bone area (cross sectional area minus medullary area) and medullary area (area delineated by endocortical surface of sample) (Evans et al., 1994) were measured separately by computerized Image Analysis (IPTK 3 Tool Kit, 1999, John C. Russ, Materials Science and Engineering Department, North Carolina State University, Raleigh, North Carolina and Photoshop 6.0).

Table 2.1 Definitions of Measurements of 1 mm Femur Cross-Sections

Area(cm^2)	no. of pixels
Convex Area(cm^2)	area measurements including holes and exteior indentations
Perimeter(cm)	length of edges
Convex Perim(cm)	perimeter measurements including holes and exteior indentations
Equiv.Diam.(cm)	square root of$4/\pi$ * area
FormFactor	4 π* area / perimeter^2
Roundness	4 * area / π * max diameter^2
AspectRatio	max diameter / min diameter
Convexity	convex perimeter/perimeter
Solidity	area / convex area
Breadth(cm)	calipers dimensions
Length(cm)	calipers dimensions
X-Feret	Longest distance between any two points on the peripheral
Y-Feret	Longest distance between any two points on the peripheral
Adj. Count	image width*image height/(image width- feature width) * (image height- feature height)
Holes	no. of holes
X-Cent.Grav.(cm)	centroid or center of gravity, Âi xi / area
Y-Cent.Grav.(cm)	centroid or center of gravity, Âi yi / area
Moment Angle	moment axis of a feature

2.9 MICROSCOPY AND IMAGE ANALYSIS OF 5 MICRON GLYCOL METHACRYLATE TIBIA AND VERTEBRA SECTIONS

Each proximal tibia and entire L-2 vertebra were photographed in 2.2MB sections by manually panning a microscopic stage across a stained section for brightfield microscopy or fluorescently labeled section for UV microscopy. A digital camera tethered to computer (Figure 2.6) grabbed sets of overlapping images from microscope to stitch or tile together to make a large montage approximately 20-60 MB (Figure 2.6).

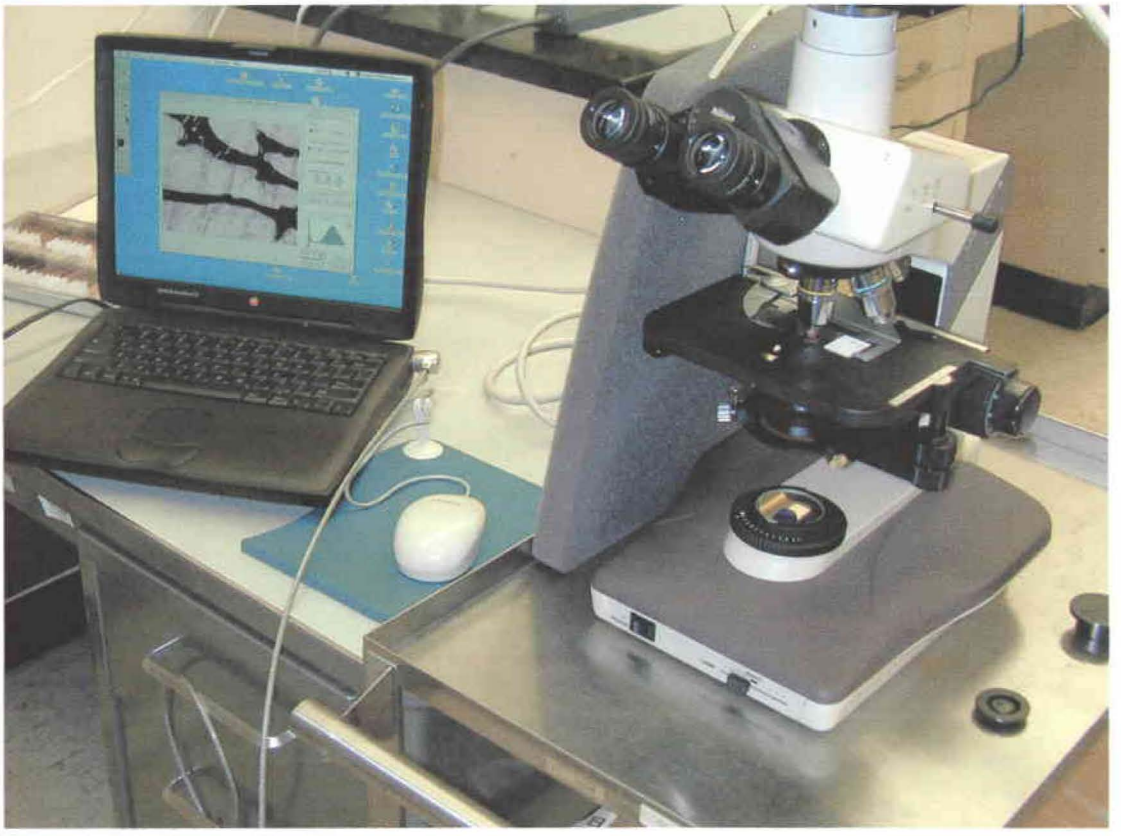

Figure 2.6 Computer image (detail from Van Kossa stained slide) from microscope

The SV Micro camera (not shown) was attached to microscopes' camera ports by standard Nikon C-mount adapter. An SV Micro digital camera, tethered to SCSI (DB50) interface of G3 power book, was used to capture images. The SV Micro is a 3-shot camera (multi-exposure, composite imaging camera with a 1000x800 pixel array, 24 or 48 bit color depth). Microscopic slides were viewed with live video focusing and preview in camera. Processing and analysis was done using IPTK software plugs-in inside Photoshop operating with a Macintosh G4. A 0.1mm graticule with 0.01 mm divisions (B&L Graticule) was used for calibration.

To create the final montage of a whole vertebrae or proximal end of tibia, the images were labeled in numerical order and placed in an individual file with rat's identification number. From each file, small images (2.2MB) were stitched together and aligned at a high magnification in Photoshop to make one large seamless view of bone forming a large microscopic composite image (see Figure 2.6 and 2.7)

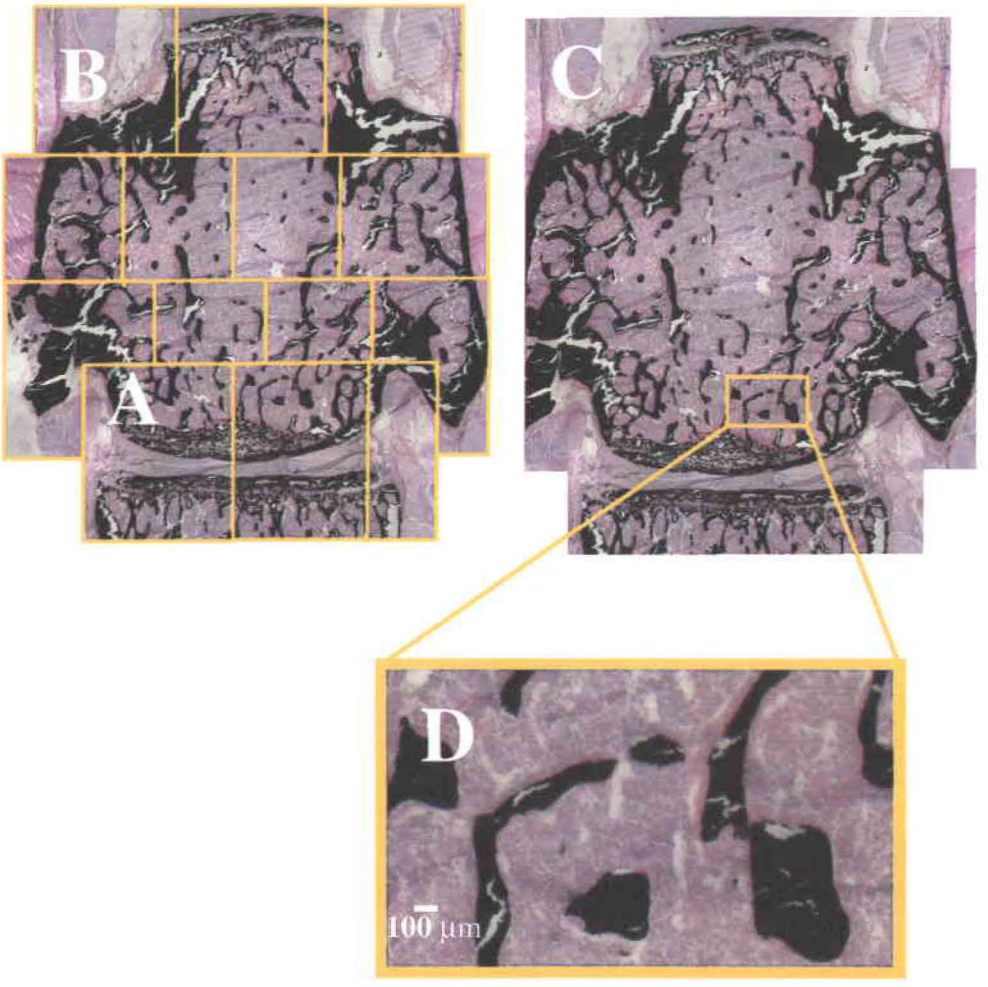

Figure 2.7 Stained vertebra with Van Kossa Stain (black) defining bone, combined with Hemotoxylin and Eosin Stains defining marrow (VK+H+E). A is the last single frame of overlapping frames. B is the initial overlapped tile in sequence of frames tiled together for final composite. C is completed montage. D is a close-up of cancellous bone and marrow. White area (small cracks) in black is a common artifact. Undecalcified, 5µm thick section of L-2 vertebrae, brightfield microscopy x 40.

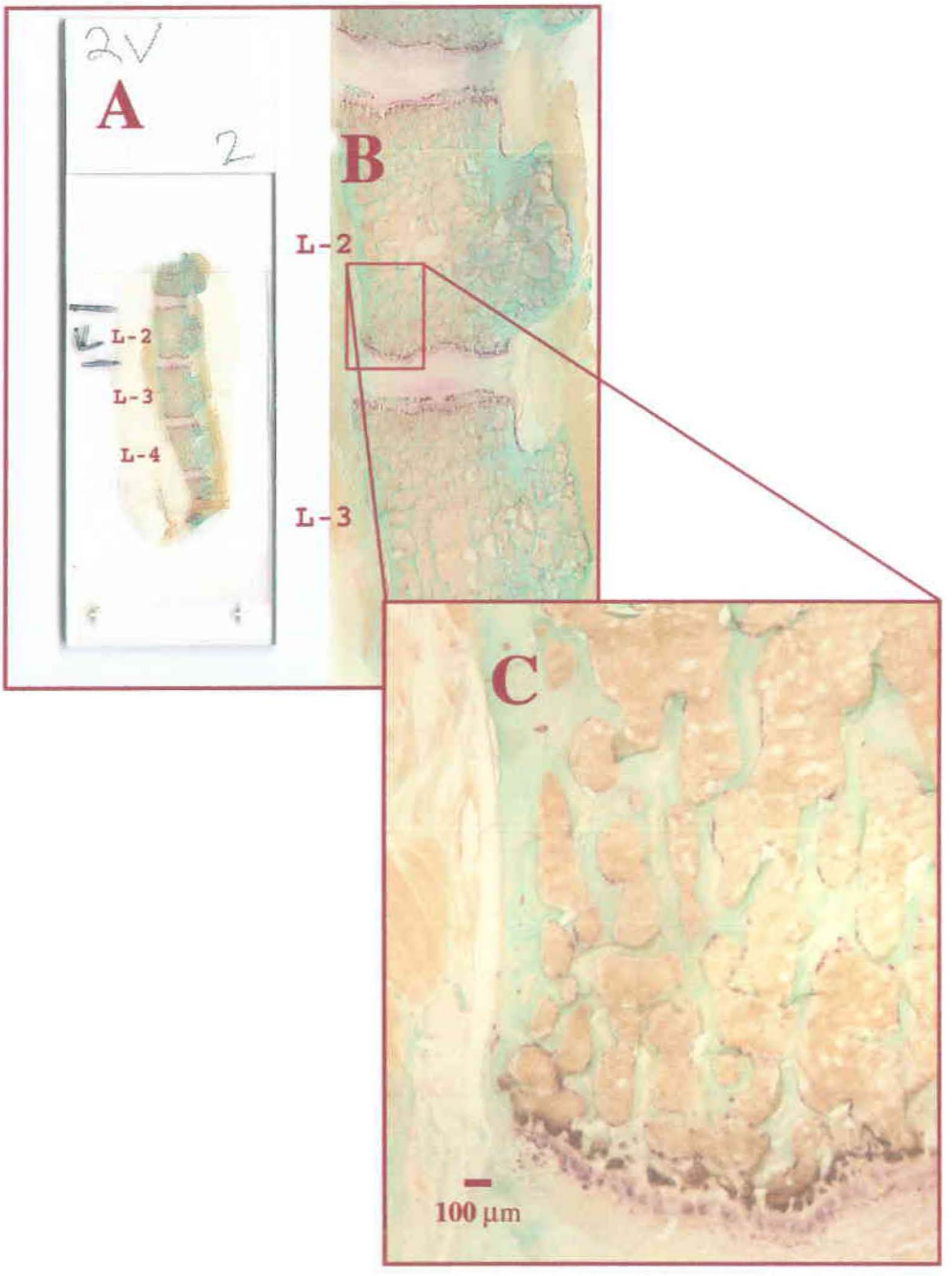

Figure 2.8 Microscopic slide demonstrating excised vertebra section showing red osteoclasts (A-B). Scanned enlargement for illustration (C). Standard 2.2MB single frame enlargement. Undecalcified, 5µm thick section of L-2 vertebrae, brightfield microscopy x 40, Tartrate Resistant Acid Phosphatase (Trap) stain identifying red osteoclasts with a green bone counterstain.

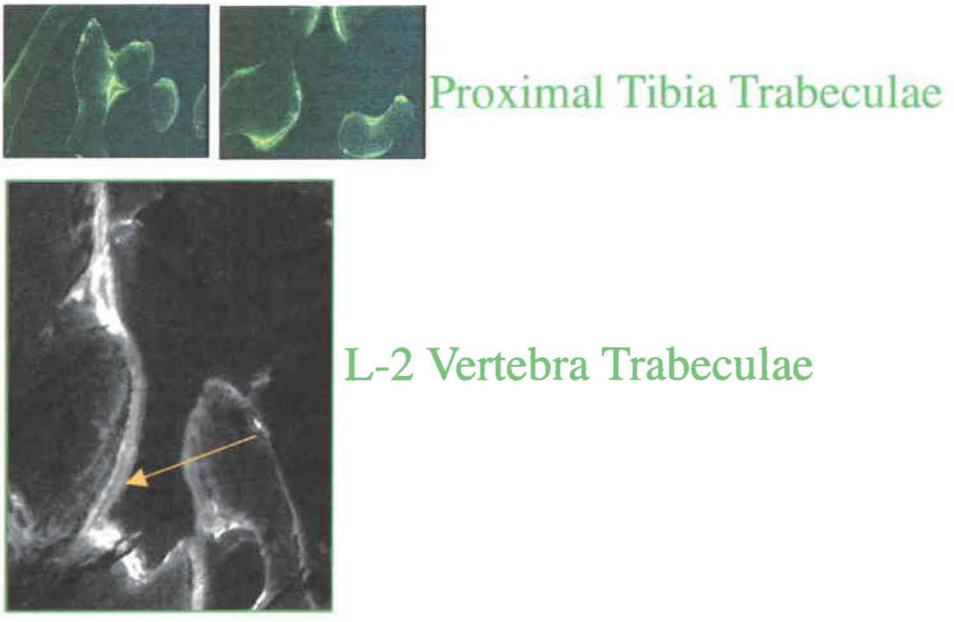

Figure 2.9 Proximal tibia and L-2 vertebrae detail of the trabecular surface double bands due to calcein labels at 2 day interval. Undecalcified, unstained, 5μm thick section. Fluorescent light microscopy x 40.

After thresholding (see Figure 2.10) with the image analysis software (Image Processing Tool Kit) to separate the marrow and background pixels from bone pixels [the method is detailed in The Image Processing Handbook (Russ, 1999)] each sample's composite image, a rectangular window was drawn for processing (Maeda et al., 1993). The top line of the rectangle was 1mm distal to the growth cartilage-metaphyseal junction. The bottom line was parallel to top line and [3mm for vertebrae (Figure 2.10), 2.5mm for tibia (Figure 2.11)] distal to top line. The sides of the rectangle were place as close to endocortical surface as possible but not touching. The rectangular window area of the L-2 vertebrae measured 4.37-9.47mm^2 (6.63±0.28 mm^2). The rectangular window area of the proximal tibias measured 2.26-8.28mm^2 (4.7±0.14 mm^2).

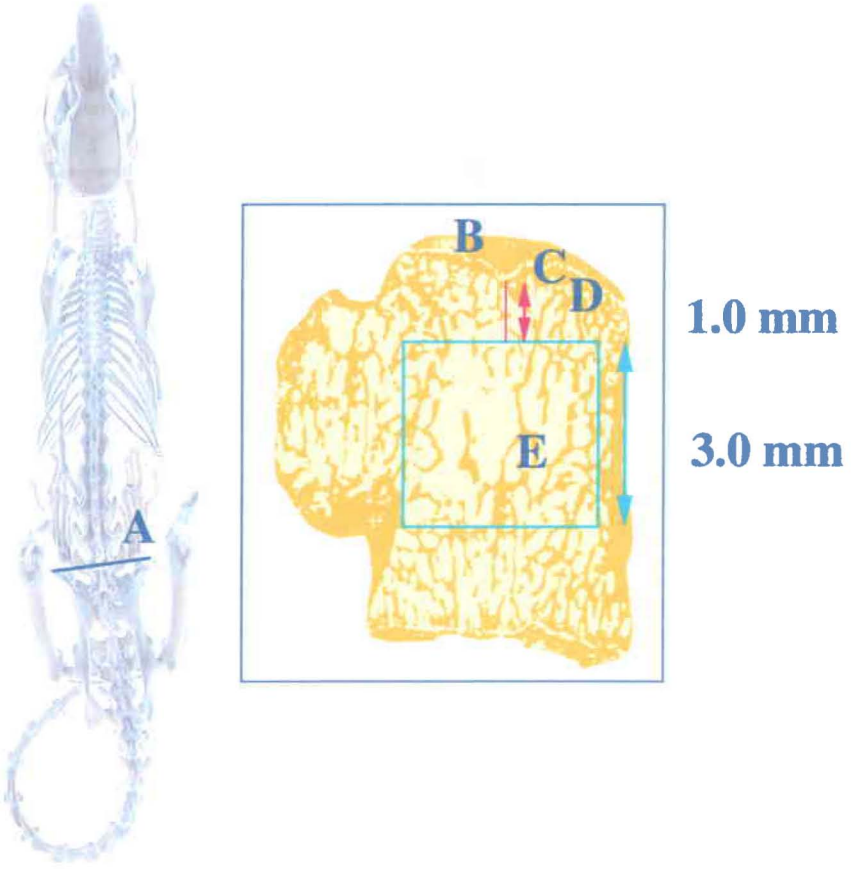

Figure 2.10 Section of L-2 vertebrae with the rectangular window used in analysis delineated in the 2% study. A shows the excision line at top or iliac crest through the L-1 vertebra. B is the epiphysis area. C is the epiphyseal growth cartilage. D is the primary spongiosa. E is the secondary spongiosa. Trabeculae and cortex bone are darker shade. Marrow is lighter shade. Analysis consisted of 4.37-9.47mm^2 rectangular area of secondary spongiosa which enclosed only cancellous bone and marrow. The top line of the rectangle was 1mm distal to the growth cartilage-metaphyseal junction. The bottom line of the vertebra rectangle was parallel to top line and 3mm distal to top line. The sides of the rectangle were placed as close to endocortical surface as possible but not touching.

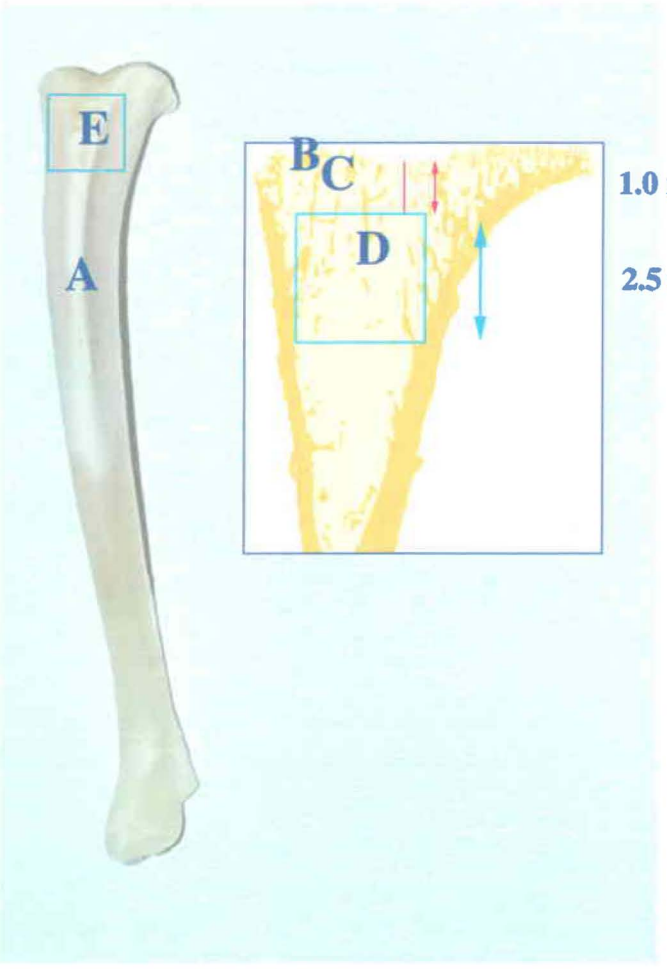

Figure 2.11 Proximal tibia (A) with approximate location of rectangular window (E) in the 1% study and 2% study. B, C and D frontal section of proximal tibia. B is the epiphyseal growth cartilage (the epiphysis area not shown). C is the primary spongiosa. D is the secondary spongiosa. Trabeculae and cortex bone are darker shade. Marrow is lighter shade. Analysis consisted of 2.26-8.28 mm^2 rectangular area of secondary spongiosa which enclosed only cancellous bone and marrow. The top line of the rectangle was 1mm distal to the growth cartilage-metaphyseal junction. The bottom line of the tibia rectangle was parallel to top line and 2.5 mm distal to top line. The sides of the rectangle were place as close to endocortical surface as possible but not touching.

Total tissue area (TtCn.Ar), cancellous bone area (Cnb.Ar) and bone surface (Cn.Pm.) were measured on computer with the image analysis software (Image Processing Tool Kit 3.0) from slides [40x magnification using a Nikon Optiphot brightfield microscope (Nikon, Inc., Instrument Group, Melville, N.Y.)] stained with Van Kossa and H+ E (Figure 2.7). In addition, the osteoclast surface (Oc.S) was measured at 40x magnification using a Nikon Optiphot brightfield microscope from slides stained with Tartrate Resistant Acid Phosphatase (Figure 2.8). Epifluorescent microscopy measurements from unstained slide sections (Figure 2.9) were obtained using Nikon Eclipse E 400 (with Y-FL Epi-Fluorescence attachment) microscope with High Pressure mercury lamp power supply model: HB-10103AF (Nikon, Inc., Instrument Group, Melville, N.Y.).

2.10 MEASUREMENTS FROM 5 MICRON GLYCOL METHACRYLATE TIBIA SECTIONS

Histomorhometric parameters (Table 2.2) were recorded at this standard sampling site according to the standardization of the American Society of Bone Mineral Research Histomorphometric Nomenclature Committee (Parfitt, 1987). Data for trabecular bone volume, trabecular thickness, trabecular number, trabecular separation, osteoclast surface and mineralizing surface was calculated according to formulas given (Parfitt, 1987). In addition, calcein calculations and measurements utilized Area/Length method (Huffer and Lepoff, 1992).

Stains and analysis techniques separate the region of interest, and lower the amount of editing necessary to differentiate bone from adjacent tissues as bone marrow

(Figure 2.7, 2.8, and 2.9). For example, editing an image with a Van Kossa Stain requires only small corrections for artifacts as holes, cracks or wrinkles.

In microscopic measurement, the convention of using 3D Terminology (Albright and Brand, 1987) is applied (see Parfitt, 1987 for objectives). This convention allows linear surface measurement to be changed to volume measurement because the vertical section of bone is extremely thin in comparison with it's area. This is referred to as the infinitely thin principle (Albright and Brand, 1987). Thereafter, formulas set by Standardization of the American Society of Bone Mineral Research Histomorphometric Nomenclature Committee (Parfitt, 1987) were used for bone analysis (Table 2.3).

Table 2.2 List Of Measurements in 2 D and 3 D Terminology Obtained From Stained And Unstained Sections

Measurements Obtained From Stained And Unstained Sections		
3 D Terminology		*Stain*
BV	Bone Volume	VK+H+E
BS	Bone Surface	VK+H+E
TV	Tissue Volume	
VK+H+E		
Tb.Th	Trabecular Thickness	VK+H+E
Tb.N	Trabecular Number	VK+H+E
TbSp	Trabecular Separation	VK+H+E
Oc. S	Osteoclast Surface	Trap
Calcein Label		*Unstained*
MS	Mineralizing Surface	with UV light
Mar	Mineral Apposition Rate	with UV light
BFR	Bone Formation Rate	with UV light
2 D Terminology		*Stain*
Perimeter		VK+H+E
Area		VK+H+E
Total Sample Area		VK+H+E

VK+H+E is Van Kossa Stain with Hemotoxylin and Eosin Stain. Trap is Tartrate Resistant Acid Phosphatase Stain.

Table 2.3 Formulas for Histomorphormetric Calculations

Calculation/Abbreviation	Units	3 D Terminology	Formula
BV/TV	%	Bone Volume	
BS/TV	mm^2/mm^3	Bone Surface, Sv	
TV	mm^3	Tissue Volume	
Tb.Th	μm	Trabecular Thickness	2/(BS/BV)
Tb.N	/mm	Trabecular Number	(BV/TV)/TBTh
Tb.Sp	μm	Trabecular Separation	(1/Tb.N)-Tb.Th
Oc.S/BS	%	Osteoclast Surface	
Tet. Label			
MS/BS	%	Mineralizing Surface	(dLS+sLS/2)/BS
Mar	μm/d	Mineral Apposition Rate	Ir.L.Th/ Ir.L.t
BFR	$\mu m^3/\mu m^3/d$	Bone Formation Rate per unit of Tissue Volume,	Mar*(MS/BS)

Formulas set by Standardization of the American Society of Bone Mineral Research Histomorphometric Nomenclature Committee (Parfitt, 1987). Volume density=B.A./T.A.*1.273

2.11 DYNAMIC HISTOMORPHOLOGIC ANALYSIS OF TIBIA TRABECULAR BONE

For trabecular labeling in the preliminary study, one dose of tetracycline 25mg/kg of body weight and one dose of calcein 8mg/kg of body weight was administered to each animal by intraperitoneal injection on separate days respectively before rat autopsy (Fisher et al., 1995). The two markers separated by 7 d allowed for an assessment of bone growth. Tetracycline and its derivative calcein bind reversibly to all bone surfaces.

When administration ceases, it will diffuse back into circulation except for chelated calcium trapped by new mineral deposition (Parfitt, 1992). Tetracycline labels are observed in yellow fluorescent layers and calcein labels as bright green layers. An estimation of mineral appositional rate followed, quantified by the distance between two labelled layers by the following formula, MAR (mineral appositional rate) equals ILW (interlabel width) divided by ITL (interlabel time)(Frost et al., 1983).

In personal communication with Patsy Ruegg, pathologist and lab manager at the Metabolic Bone Biopsy Service at the University of Colorado Health Sciences Center, and R. Birchman (from the Shen et al.'s, 1995 study and the Regional Bone Center, Helen Hayes Hospital, West Haverstraw, NY lab procedure), both recommended a shorter separation than 7 d between markers in growing young rats and felt using calcein labeling twice was the best way as tetracycline did not flouresce nearly as well as calcein. Ruegg had used 5 mg/ kg while Birchman 20 mg/kg. In our preliminary study we tried the more popular 10 mg/kg, dose of calcein on day 5 and one dose of calcein on day 2 before rat autopsy. This, again did not work in a growing female rat model for trabecular bone measurements, as labels were too diffuse. Upon examination of the 5 μm slides, Patsy Ruegg felt that even a shorter time was necessary. Since the calcein measurements would not be measured until after both the 1% study and the 2% study were complete, two 13 week old Ws were ordered to test calcein before the 1% study started. A 2 day labeling period was chosen for both studies based on the comparison between two rats. While this short labeling time worked well for trabecular bone of the unstressed test rat, for subtle trabecular bone labeling of our overloaded/ underloaded study, this could still

be optimized. This may have occurred because the growth rate of both tibias were suppressed due to loading technique, forming label that often blurred together.

Calcein labeling procedure for animals used in preliminary study:

Reagents:

1. Calcein (Sigma C0875)	10	g
2. NaC1	8.5	g
3. NaHC03	20	g
4. DH20	1	liter

Dosage prescribed by Ruegg and Smith:

10 mg/kg body weight i.p.

l ml solution = l0 mg calcein

2.12 STATISTICAL ANALYSIS

The null hypothesis was that there were no differences between groups. Descriptive analysis provided means±SE. Analysis of variance using the General Linear Model, (GLM) was performed followed by a Tukey-Kramer Honestly Significant Difference test. Hypotheses were rejected at 0.05 level of significance. All data was analyzed using Minitab 13 statistical software.

1) A two-way ANOVA with two "main effects" was applied to compare genetic differences as one of the main effects (W or SS), and to compare drinking fluid treatment

as the second main effect (water or saline), and to check for interactions between genotype and treatment.

2) A different two-way ANOVA with two "main effects" was applied to compare group differences as one main effect (WC, WS, SSC, SSS), and loading differences as the second main effect (overloading/underloading), and to check for interactions between groups and loading.

3) Tukey-Kramer Honestly Significant Difference pairwise post-hoc tests were applied. Using the Tukey tests, all possible comparisons of loaded versus unloaded could be made between 4 groups with genetic and saline treatment.

4) One-way ANOVA with four levels was used to compare overall variability between the 4 groups. The four groups were defined by genotype and drinking fluid treatment. The groups were: WC, wistar water control, WS, wistar, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with saline. If the overall comparison was significant, Tukey pairwise relevant tests were reported.

CHAPTER III

RESULTS

3.1 BODY WEIGHT WITH FOOD AND DRINKING FLUID INTAKE

3.1.1 Genetic Effects

3.1.1.1 1% Study Genetic Effects

3.1.1.1.1 Body Weight

Biweekly body weight measurements are shown in Figure 3.1 and Figure 3.4. Significant differences were found in our 1% saline and 2% saline study. Because the controls between the 1% and 2% were significantly different in body weight (affecting other outcomes), the 1% and 2% results could not be combined. However, results between the two studies are compared in most general discussions. W and SS rats were age and weight-matched at the beginning of the experimental period for the 1% study and the 2% study.

When rats arrived for the 1% study, a 3% difference in initial weight was observed with W weighing more than SS rats. At the study midpoint SS rats slightly outweighed W by 0.4%, with all rats eating ad lib. In the last biweekly weighing, a 4% difference in weight was observed with W weighing more. At autopsy, the W weighed significantly 6.7% more than SS rats (Figure 3.1).

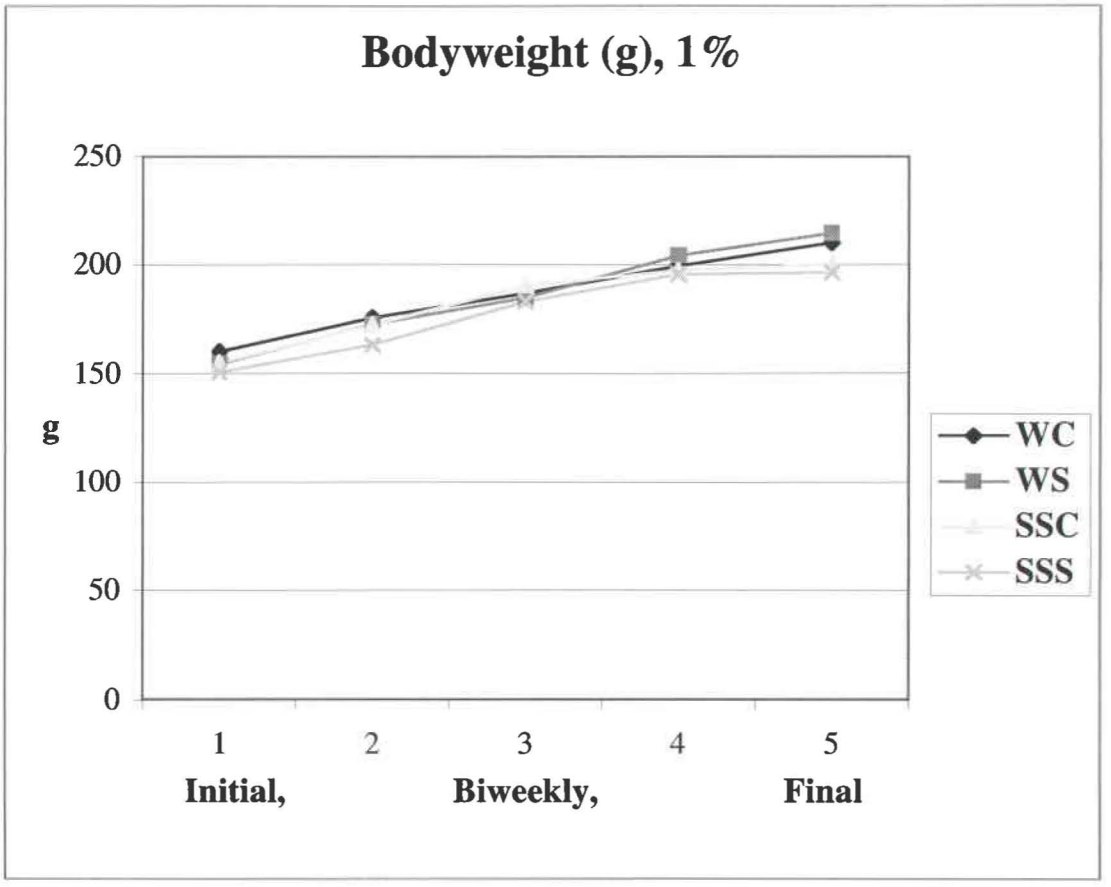

Figure 3.1. Body weight in young Wistar (W) or salt sensitive (SS) female rats given water (C) or 1% saline (S) ad libitum. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with 1% saline.

3.1.1.1.2 Food Intake

In the 42 day experimental period, the total food eaten in 1% the study averaged 666.6 g per W and 666.3 g per SS Food intake records (Figure 3.2) were kept, as well as biweekly weighings of each animal. It was found that W gained 6.7% more weight than SS rats on the same amount of food eaten.

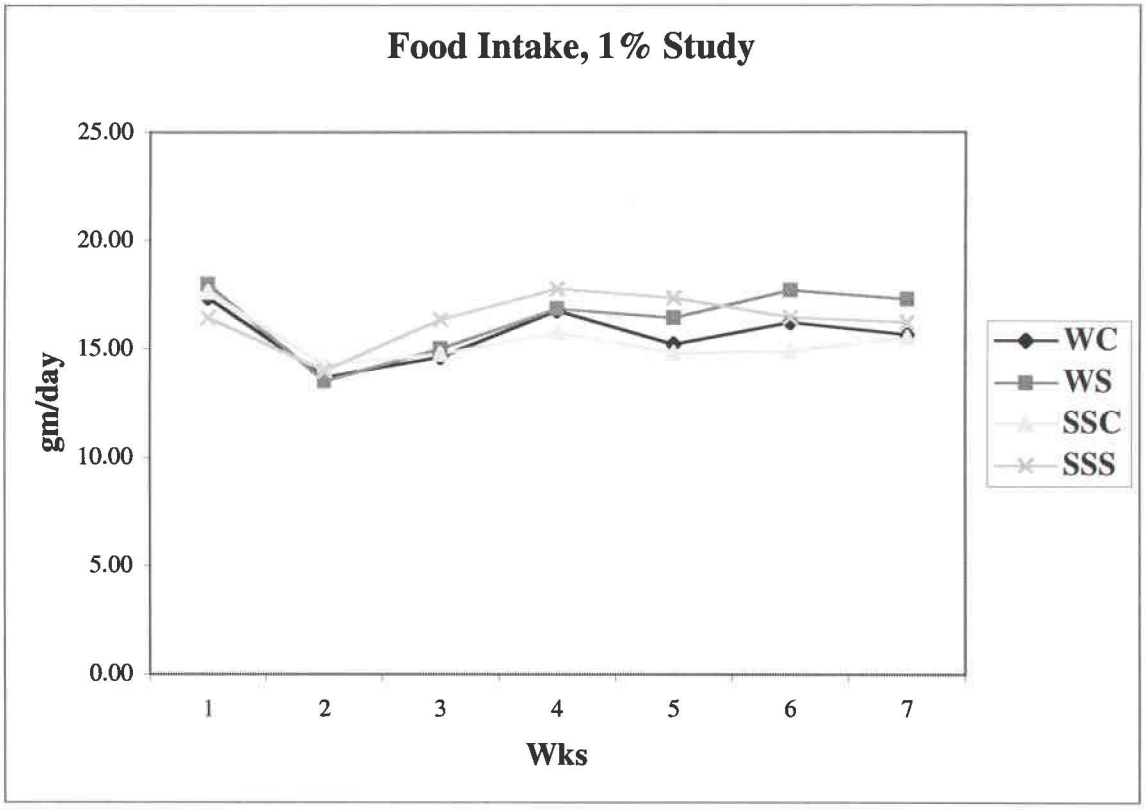

Figure 3.2. Food intake for Wistar (W) or salt sensitive (SS) young female rats given water (C) or 1% saline (S) ad libitum. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with 1% saline.

3.1.1.1.3 Fluid (Water or Saline) Intake

Rats on 1% saline drank 94% more fluid compared to those on water. No significant differences in saline fluid intake between SS and W groups were found in response to the 1% saline supplementation, however, water drinking W outdrank the water drinking SS consistently, though modestly (Figure 3.3). Unexpectedly, when drinking saline, salt appetites between rats varied considerably. No rat was restricted in the amount that it drank. Because the added NaCl was in the drinking water, and each rat consumed a different amount of saline water, the dose of salt differed between individual rats. Individual average fluid intake among rats ranged from 9 to 51 ml per day (Figure 3.3) during the 42 day experiment. WC average daily water consumption ranged between 9-18 ml; while WS average daily saline consumption ranged between 11-32 ml. SSC average daily water consumption ranged between 8-12 ml; while SSS average daily saline consumption ranged between 9-30 ml.

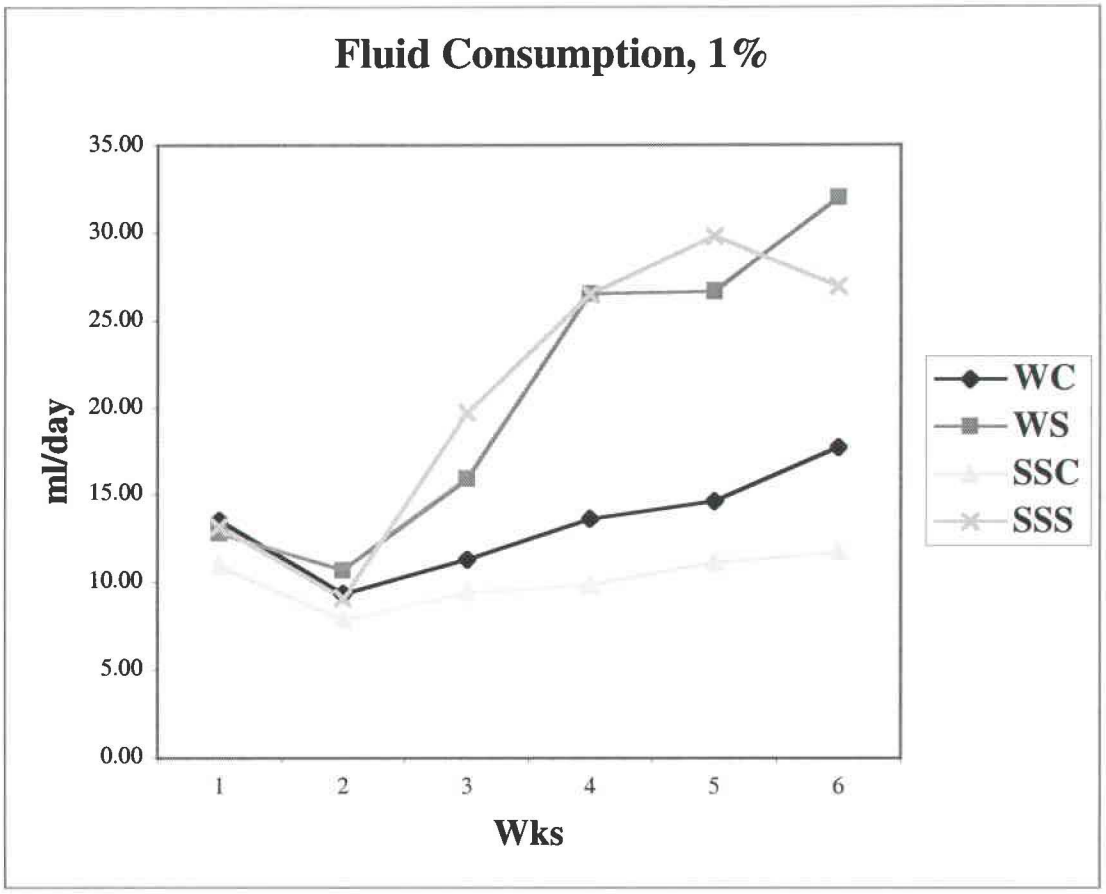

Figure 3.3. Fluid consumption in Wistar (W) or salt sensitive (SS) young female rats given water (C) or 1% saline (S) ad libitum. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with 1% saline.

3.1.1.2 2% Study Genetic Effects

3.1.1.2.1 BodyWeight

As in the 1% study, W and SS rats were age and weight-matched at the beginning of the experimental period for the 2% study. When rats arrived for the 2% study, a 0.8% difference in initial weight was observed with W weighing more than SS rats (Figure 3.4). In the first acclimation week, a significant 9% difference in weight was observed with W outweighing SS rats. In response to this unanticipated early weight gain in W, the W of the 2% study were fed the same amount of food as SS rats of the 2% study (Roudebush, 1993). In addition, for one week W were food restricted (approximately 10% of food intake to balance extra food eaten during acclimation period). On reduced food, W initially retained weight, however, at autopsy, the W weighed 1.5% less than SS rats (statistically non-significant). In the 2% study total food eaten averaged 654.8 g per W rat and 657.5 g per SS rat, compared to 666.6 g per W and 666.3 g per SS rat in 1% study.

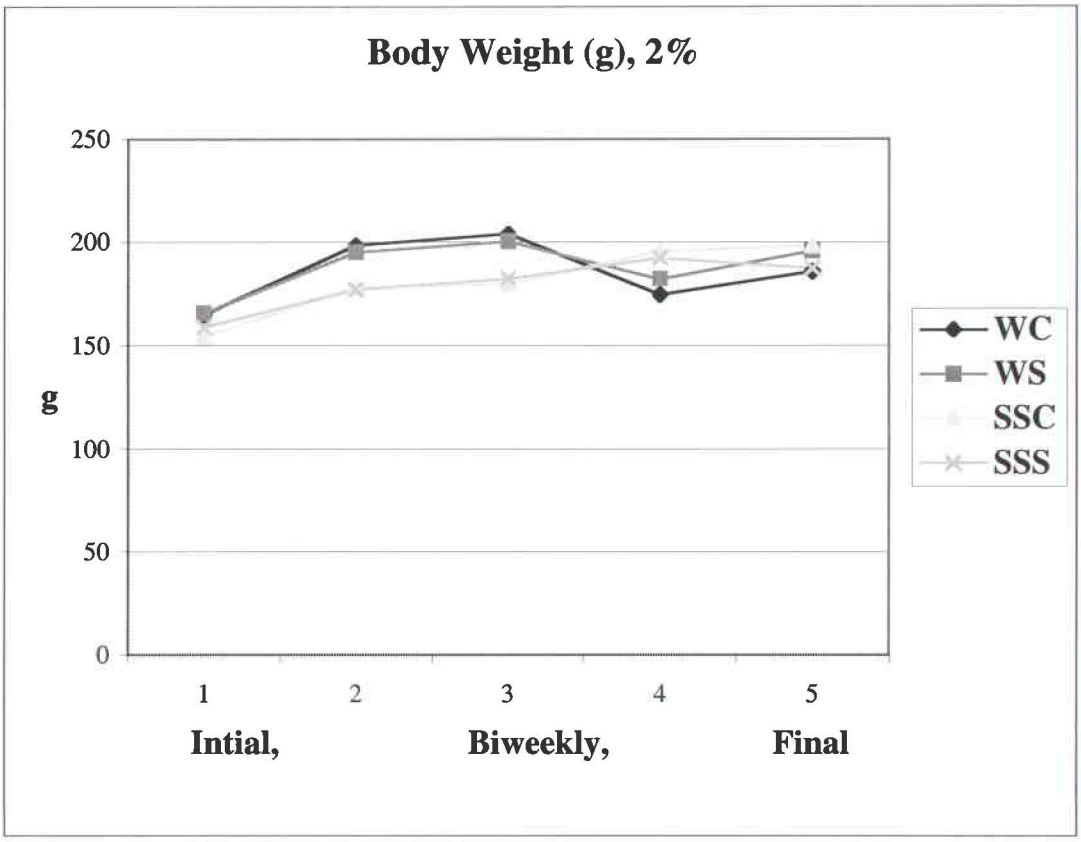

Figure 3.4. Body weight in Wistar (W) or salt sensitive (SS) young female rats given water (C) or 2% saline (S) ad libitum. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with 2% saline.

3.1.1.2.2 Food Intake

The total food eaten in 2% study averaged 654.8 g per W and 657.8 g per SS rat (Figure 3.5) in the 42 day experimental period. At autopsy SS weighed a non-significant 1.5% more than W.

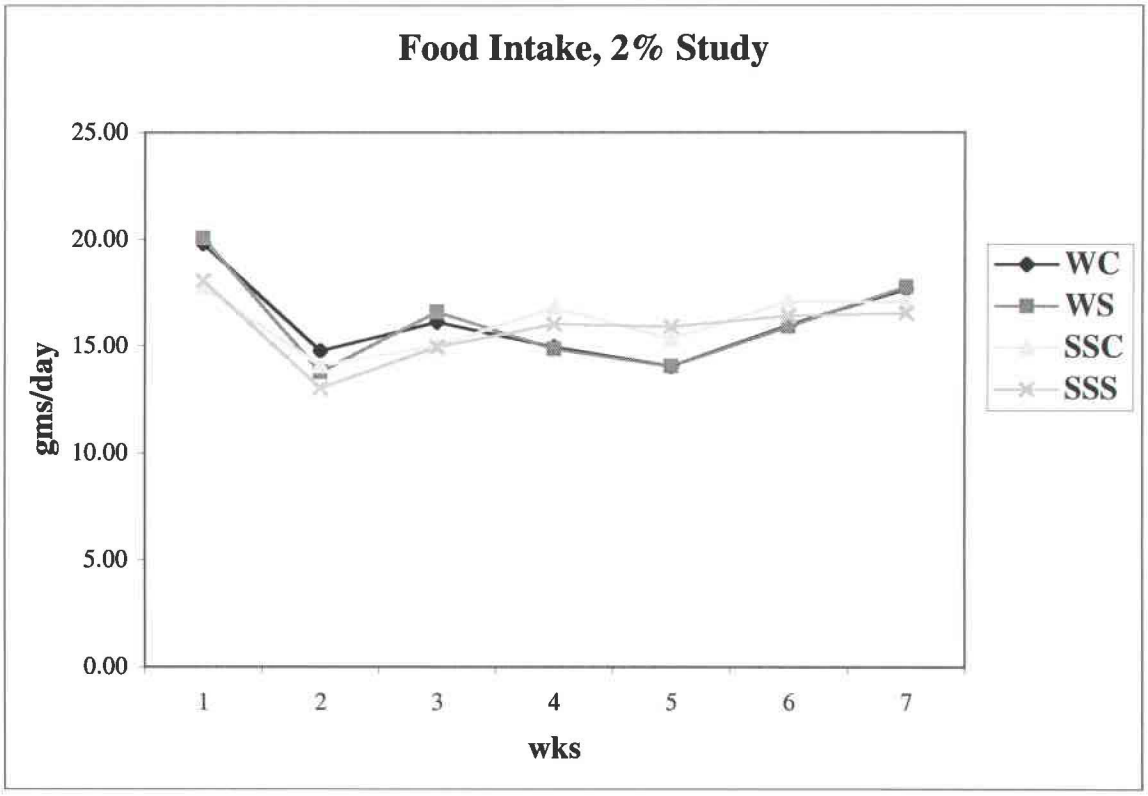

Figure 3.5. Food intake for Wistar (W) or salt sensitive (SS) young female rats given water (C) or 2% saline (S) ad libitum. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with 2% saline.

3.1.1.2.3 Fluid (Water or Saline) Intake

As in the 1% study, there were highly variable salt appetites between female rats when drinking 2% saline. Again, no rat was restricted in the amount that it drank. Thus, each dose of salt differed between individual rats. Individual rats' fluid intake ranged from 8 to 55 ml per day in 2% saline study (Figure 3.6). Rats on saline drank 78% more fluid compared to those on water in 2% study. WC average daily fluid consumption in 2% study ranged between 10-15 ml. WS average daily fluid consumption ranged between 18-25 ml. SSC average daily fluid consumption ranged between 8-12 ml. SSS average daily fluid consumption ranged between 12-26 ml. No significant differences in saline fluid intake between SS and W groups were found here in response to the 2% saline supplementation, or between water drinking controls.

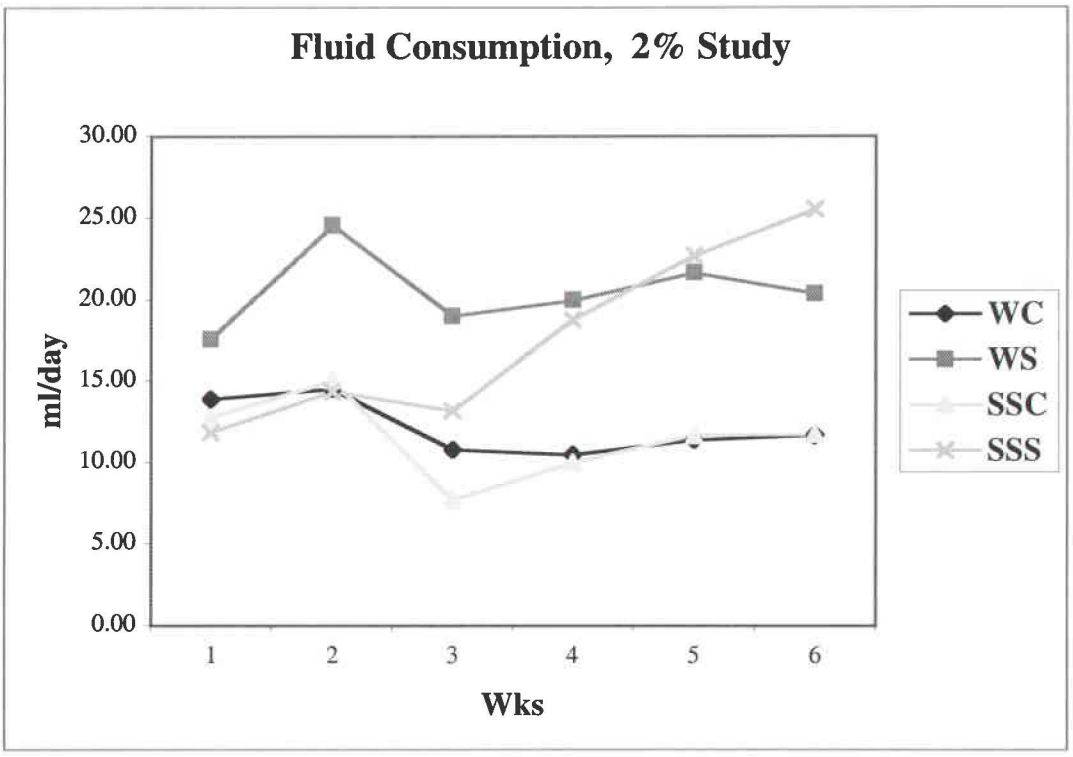

Figure 3.6. Fluid consumption in Wistar (W) or salt sensitive (SS) young female rats given water (C) or 2% saline (S) ad libitum. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with 2% saline.

3.1.2 Drinking Fluid Treatment

3.1.2.1 1% Study Saline Effect

3.1.2.1.1 Body Weight and the Intake of Food and Fluid

There was not a significant difference in body weight between rats on saline and rats on water in 1% saline study (Table 3.1). In the 1% saline study, a significant difference in food intake between rats on water and rats on saline was observed ($P< 0.005$). Saline treated rats ate 7% more than rats on water (Figure 3.2). This may have contributed to results noted in1% study, but not in 2% study as food was controlled in W rats.

Rats on saline drank significantly more than those on water ($P<0.001$). In the 1% study, rats on saline consumed 94% more fluid than those on water. Mean salt intake per day of rats on saline and maintenance diet averaged 7 times more than mean salt intake of those on water and maintenance diet ($p<0.001$).

Table 3.1. The Effect of Saline Treatment on Body Weight, Food, Fluid and Salt Intake, 1% Study

Variable	*p*	Treatment	n	Mean	SEM
Final body weight, g	ns	water	10	205.11	± 4.46
		saline	12	205.51	± 5.34
Food intake, g	< 0.005	water	10	640.47	± 9.30
		saline	12	685.35	± 9.74
Food intake per day, g	< 0.005	water	10	15.25	± 0.221
		saline	12	16.32	± 0.232
Fluid intake, ml	< 0.001	water	10	517.8	± 45.2
		saline	12	1005.3	± 99.2
Fluid intake per day, ml	< 0.001	water	10	12.33	± 1.08
		saline	12	23.93	± 2.36
Total NaCl intake, g	< 0.001	water	10	1.66	± 0.025
		saline	12	11.83	± 1.000
NaCl intake per day, g	< 0.001	water	10	0.040	± 0.000
		saline	12	0.281	± 0.024

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA; ns, not significant.

3.1.2.2 2% Study Saline Effect

3.1.2.2.1 Body Weight and the Intake of Food and Fluid

There was not a significant difference in body weight between rats on saline and rats on water in 2% saline study (Table 3.2). In the 2% saline study, there was no significant difference in food intake between rats on water and rats on saline. Rats on saline drank significantly more than rats on water ($P<0.001$). In the 2% study, rats consumed 78% more saline fluid than water. Mean salt intake per day of rats on saline and the maintenance diet averaged 11 times more than those on water and the maintenance diet ($P<0.001$).

Table 3.2. The Effect of Saline Treatment on Body Weight, Food, Fluid and Salt Intake, 2% Study

Variable	p	Treatment	n	Mean	SEM
Final body weight, g	ns	water	12	191.99	± 3.93
		saline	10	190.71	± 3.80
Food intake, g	ns	water	12	661.59	± 5.88
		saline	10	649.95	± 7.78
Food intake per day, g	ns	water	12	15.75	± 0.140
		saline	10	15.47	± 0.186
Fluid intake, ml	< 0.001	water	12	490.7	± 20.4
		saline	10	874.2	± 83.2
Fluid intake per day, ml	< 0.001	water	12	11.68	± 0.486
		saline	10	20.82	± 1.980
Total sodium intake, g	< 0.001	water	12	1.71	± 0.015
		saline	10	19.17	± 1.670
Sodium intake per day, g	< 0.001	water	12	0.040	± 0.000
		saline	10	0.457	± 0.040

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA; ns, not significant.

3.1.3 Tukey Pairwise Post Hoc Results and One-way ANOVA for Both Genotype and Saline Treatment

Using the Tukey tests, pairwise post-hoc tests were applied. In addition, one-way ANOVA with four levels was used to compare overall variability between the 4 groups. If the overall comparison was significant, Tukey pairwise relevant tests were reported. See Chapter 2, Section 2.12 for complete method description.

3.1.3.1 1% Study Using One-way ANOVA for Both Genotype and Saline Treatment

In order to determine if there were differences between the means of the WC, WS, SSC, and SSS groups (Table 3.3), a one-way ANOVA was performed. Significant overall variation between the 4 groups were seen for food, fluid and salt intake in the 1% Study group comparison (Table 3.3). In addition, each group (WC, WS, SSC, SSS) was compared to each other, pairwise (Table 3.3). Food intake was increased in both groups of rats on 1% saline fluid, with slightly more intake by SS than by W rats. Noteworthy is that fluid intake in rat strains treated with 1% saline was very similar. No significant pairwise differences in fluid per day between SS and W groups were found here in response to the 1% saline treatment and no difference occurred between groups in overall genetic comparison.

Table 3.3 Body Weight, Food, Fluid and Salt Intake for the 1% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	P	Group	n	Mean		SEM
Final body weight, g	ns	WC	5	209.92	±	8.13
		WS	6	214.57	±	8.30
		SSC	5	200.30	±	3.45
		SSS	6	196.45	±	4.89
Food intake, g	<0.030	WC	5	649.4	±	17.9
		WS	6	680.9	±	14.9
		SSC	5	631.5	±	5.44 [d]
		SSS	6	689.8	±	13.70
Food intake per day, g	<0.030	WC	5	15.46	±	0.425
		WS	6	16.21	±	0.355
		SSC	5	15.04	±	0.131 [d]
		SSS	6	16.43	±	0.326
Fluid intake, ml	<0.006	WC	5	599	±	62.5
		WS	6	1019	±	164.0
		SSC	5	437	±	44.9 [d]
		SSS	6	992	±	128.0
Fluid intake per day, ml	<0.006	WC	5	14.26	±	1.49
		WS	6	24.26	±	3.90
		SSC	5	10.40	±	1.07 [d]
		SSS	6	23.61	±	3.05
Total sodium intake, g	<0.001	WC	5	1.68	±	0.048 [a]
		WS	6	11.95	±	1.660
		SSC	5	1.64	±	0.015 [d]
		SSS	6	11.70	±	1.300
Sodium intake per day, g	<0.001	WC	5	0.040	±	0.001 [a]
		WS	6	0.285	±	0.040
		SSC	5	0.040	±	0.000 [d]
		SSS	6	0.276	±	0.031

For overall comparison of variability between the 4 groups the p-value is shown; ns, not significant. WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.1.3.2 2% Study Using One-way ANOVA for Both Genotype and Saline Treatment

Significant overall variation between the 4 groups were seen for fluid and salt intake in the 2% Study group comparison (Table 3.4). In addition, each group (WC, WS, SSC, SSS) was compared to each other, pairwise (Table 3.4). Food intake was not increased in either group of rats on 2% saline compared to water controls, with slightly less intake by SS than in W rats. This was a slightly different response than in the 1% study, possibly due to concentration of saline treatment. Noteworthy is that the saline intake in rat groups treated with saline is not similar as in 1% study. No significant differences in mean salt per day between SSS and WS groups were found here in response to the 2% saline supplementation, however the SSS rats drank more saline than WS rats.

Table 3.4 Body Weight, Food, Fluid and Salt Intake for the 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	P	Group	n	Mean	SEM
Final body weight, g	ns	WC	6	185.77	± 5.78
		WS	4	195.85	± 5.88
		SSC	6	198.22	± 4.38
		SSS	6	187.28	± 4.85
Food intake, g	ns	WC	6	656.69	± 7.49
		WS	4	652.07	± 8.01
		SSC	6	666.5	± 9.30
		SSS	6	648.5	± 12.40
Food intake per day, g	ns	WC	6	15.64	± 0.178
		WS	4	15.52	± 0.192
		SSC	6	15.87	± 0.222
		SSS	6	15.44	± 0.297
Fluid intake, ml	<0.001	WC	6	491.2	± 24.9
		WS	4	799.0	± 115.0
		SSC	6	490.1	± 34.8 [d]
		SSS	6	924.0	± 119.0
Fluid intake per day, ml	<0.001	WC	6	11.70	± 0.594
		WS	4	19.03	± 2.740
		SSC	6	11.67	± 0.828 [d]
		SSS	6	22.00	± 2.840
Total sodium intake, g	<0.001	WC	6	1.70	± 0.019 [a]
		WS	4	17.68	± 2.320
		SSC	6	1.73	± 0.024 [d]
		SSS	6	20.16	± 2.400
Sodium intake per day, g	<0.001	WC	6	0.040	± 0.000 [a]
		WS	4	0.421	± 0.055
		SSC	6	0.041	± 0.000 [d]
		SSS	6	0.480	± 0.057

For overall comparison of variability between the 4 groups the p-value is shown; ns, not significant. WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.1.3.3 Examination of 1% and 2% Study Using ANOVA for Both Genotype and Saline Treatment Effects

3.1.3.3.1 *Body Weight and Food Intake*

On first glance, it would appear that WC rats' final body weight was lower than saline treated rats in both the 1% and 2% study (Table 3.5). However, 2 WS died in 2% study which would have brought down the body weight average as they lost weight on saline. The SS final body weight was reduced with saline treatment in both the 1% and 2% study, when compared to water drinking SS rats in spite of similar food intakes, suggesting an increased metabolic rate or poor absorption of nutrients in SS rats on saline.

Table 3.5 Body Weights in the 4 Groups of the 1% Study and the 2% Study

	group	n	Mean		SEM
1% study final body weight, (g)	WC	5	209.92	±	8.13
	WS	6	214.57	±	8.30
	SSC	5	200.30	±	3.45
	SSS	6	196.45	±	4.89
2% study final body weight, (g)	WC	6	185.77	±	5.78
	WS	4	195.85	±	5.88
	SSC	6	198.22	±	4.38
	SSS	6	187.28	±	4.85

Values are the means ±*SEM*. n, no. of rats. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with saline.

Initally, at the start of the acclimation period 2% study rats weighed 4% more than 1% study rats weighed. However, the final body weight in 1% study rats was 7% more than the final body weights of 2% rats. This suggests either the 1% saline had a positive effect on weight gain and/or 2% saline had an adverse effect on weight gain. This could suggest that increased cardiac load and renal excretion of 2% salt load increased the metabolic rate in the 2% study. Total food intake difference between the studies was only 1.55%, which would not account for a suppression of weight in 2% study.

Previous studies have reported body weight loss when using the overloading/underloading bandaging technique. Body weight was significantly decreased by approximately 10% in Maeda et al. (1993) study of adult female rats. Thereafter, reduced levels of food were eaten by overloaded/underloaded rats compared to controls. Body weight was also reduced by 6% during the initial two weeks in Li et al. study (1990) then remained constant. The overloaded/underloaded technique has not been previously applied to growing female rats. Harlan Sprague Dawley provided Female Growth Data Charts (1996) for W and SS rats; at 14 weeks, Harlan Sprague Dawley SS rats weighed approximately 257 g. At 14 weeks, Harlan Sprague Dawley W rats were approximately 275 g. In our two studies, at 14 weeks rats average body weight was 199 g, not reaching the projected weight of Sprague Dawley control W or SS rats. The suppressed growth may be due to decreased food intake due to physical restriction or emotional stress to restraint (Maeda et al., 1993). In addition, a brief non-surgical

isoflurane anesthesia was administered during the bandaging procedure every 2 weeks. Eating did not usually resume for 24 hours after anesthesia.

3.1.3.3.2 Fluid (Water or Saline) Intake

Many studies have shown spontaneously hypertensive rats (SHR) to have a stronger preference for saline over water when compared to Wistar-Kyoto (WKY) normotensive rats in brief 24 hour testing periods using a two bottle choice (Ferrell and Gray, 1985; Furuse et al., 1992). While a two bottle choice was not used in our studies, these results suggest salt sensitive hypertensive consumption of saline might exceed wistar rats' consumption of saline. Based on these observations, it was expected that the dose of saline would be elevated in SS rats due to the rat's salt preference, perhaps affecting bone differences between SS and W rats. Examination of this behavioral pattern was carefully monitored by recording the total salt in food and fluid intake. However, no significant difference in saline fluid intake between SSS and WS groups were found here in response to either the 1% saline supplementation or to the 2% saline supplementation. The dose of NaCl consumed, as measured by mean salt intake/day, was not significantly different between strains. These observations suggest genetic female bone differences detected may be due to type of genetic strain, body weight characteristics and/or salt sensitivity in strain, but not excessive intake of saline by SS rats over W rats.

3.2 BLOOD PRESSURE AND HEART RATE

3.2.1 Genetic Effect

Blood pressure was measured in the sixth week of the experimental period. A two-way ANOVA was applied to look at: 1) differences between strains 2) the effect of saline and 3) to check for any interactions.

3.2.1.1 1% Study Genetic Effect

Tail cuff measurements of blood pressure and heart rate were significantly higher in salt sensitive hypertensive rat (SS) strain than in the wistar rat (W) strain, with saline and water drinking groups pooled (Table 3.6). That is, the "main effect" of genetic strain, in the two-way ANOVA, was statistically significant.

Table 3.6 Systolic Blood Pressure and Heart Rate in Female Wistar and Salt Sensitive Hypertensive Rats, 1% Study

Variable	*p*	strain	n	Mean		SEM
Blood pressure, mmHg	<0.022	W	10	117.0	±	3.96
		SS	10	137.2	±	6.23
Heart rate, bpm	<0.004	W	11	390.5	±	38.5
		SS	10	511.5	±	12.9

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.2.1.2 2% Study Genetic Effect

Blood pressure was also measured in the sixth week of the experimental period to verify inbred strains in the 2% study (Table 3.7). Tail cuff measurements of blood pressure and heart rate were significantly higher in the salt sensitive strain. The SS strain had significantly higher blood pressure when compared to W strain. In addition, the SS strain had significantly higher heart rate when compared to W strain.

Table 3.7 Systolic Blood Pressure and Heart Rate in Female Wistar and Salt Sensitive Hypertensive Rats, 2% Study

Variable	p	strain	n	Mean		SEM
Blood pressure, mmHg	<0.001	W	10	109.6	±	1.89
		SS	12	133.3	±	4.34
Heart rate, bpm	<0.001	W	10	391.0	±	13.5
		SS	12	464.2	±	9.0

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.2.2 Drinking Fluid Treatment

In addition to differences between strains, blood pressure and heart rate data were recorded in the sixth week of each experimental period to look at the effect of a 1% saline treatment and a 2% saline treatment.

3.2.2.1 1% Study Saline Effect

There was not a significant difference in systolic blood pressure or heart rate between rats on 1% saline and rats on water, when SS and W strain data were pooled (Table 3.8).

Table 3.8 The Effect of 1% Saline Treatment on Systolic Blood Pressure and Heart Rate

Variable	p	Treatment	n	Mean	SEM
Blood pressure, mmHg	ns	water	9	128.2 ±	4.97
		saline	11	126.2 ±	6.84
Heart rate, bpm	ns	water	10	421.6 ±	47.2
		saline	11	472.2 ±	19.3

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.2.2.2 2% Study Saline Effect

In the 2% saline study, a significant difference (Table 3.9) in systolic blood pressure between all rats on water versus all rats on saline was observed. While the heart rate tended to be higher in rats on saline in the 2% study, there was not a significant difference between 2% saline treated and water controls.

Table 3.9 The Effect of 2% Saline Treatment on Systolic Blood Pressure and Heart Rate

Variable	p	Treatment	n	Mean	SEM
Blood pressure, mmHg	<0.005	water	12	115.2 ±	3.58
		saline	10	131.2 ±	5.55
Heart rate, bpm	<0.15	water	12	416.7 ±	14.3
		saline	10	448.0 ±	16.4

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.2.2.3 Summary of 1% and 2% Study Genetic and Saline Effects from 2-Way ANOVA

The genetic results in 2% study for heart rate and blood pressure were similar to results from the 1% study. Both are somewhat unique, as little measurement of rat blood pressure has been done on growing, genetically-defined female rats under stress due to bandage restraint. The sedentary nature of the study, the bandage restraint stress, as well as anticipatory response to the (warmed) tail-cuff test may have raised all rat' heart rate

measurements (Nieman, 1990). Both studies genetic results indicate elevated blood pressure and heart rate for the salt sensitive strain in comparison to the wistar strain.

There was not a significant difference in systolic blood pressure or heart rate between rats on 1% saline and rats on water. However, there was a significant difference in systolic blood pressure between rats on saline and rats on water in the 2% Study. Evaluation of the saline treatment and genetics found no interactions by a two-way ANOVA in either the 1% or 2% study.

3.2.3 Tukey Pairwise Post Hoc Results and One-way ANOVA for Both Genotype and Saline Treatment

See Chapter 2, Section 2.12 for complete method description.

3.2.3.1 1% Study Using One-way ANOVA for Both Genotype and Saline Treatment

In order to determine if there were differences between the means of the WC, WS, SSC, and SSS groups (Table 3.10), a one-way ANOVA was performed. Unlike the two-way ANOVA (Table 3.6), a significant overall variation between the 4 groups was not seen for blood pressure in the 1% study ($p < 0.12$). The reason for this apparent discrepancy is that the variance in the two-way ANOVA is factored into two overall main effects, not four, as is done in the one-way. However, a significant overall variation between the 4 groups was seen for heart rate ($p < 0.029$). A Tukey–Kramer Multiple Comparisons Test was applied to each group (WC, WS, SSC, and SSS,) however, no significant differences were found in relevant pairwise combinations (Table 3.10).

Table 3.10 Systolic Blood Pressure and Heart Rate for the 1% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	P	Group	n	Mean	SEM
Blood pressure, mmHg	ns	WC	4	118.5 ±	5.74
		WS	6	116.0 ±	5.77
		SSC	5	136.0 ±	5.93
		SSS	5	138.4 ±	11.8
Heart rate, bpm	<0.029	WC	5	339.0 ±	31.5
		WS	6	433.3 ±	20.3
		SSC	5	504.2 ±	16.6
		SSS	5	518.8 ±	21.0

For overall comparison of variability between the 4 groups the p-value is shown; ns, not significant. WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.2.3.2 2% Study Using One-way ANOVA for Both Genotype and Saline Treatment

Significant overall variation between the 4 groups were seen for both blood pressure and heart rate in the 2% Study group comparison (Table 3.11). In addition, each group (WC, WS, SSC, SSS) was compared to each other, pairwise (Table 3.11). Blood pressure was significantly higher in the SSS than in SSC group. In addition, blood pressure was significantly higher in the SSC than in WC group. Blood pressure was significantly higher in the SSS than in WS group. Pairwise ANOVA show that SSS group blood pressure measurements were raised by saline treatment. Also, the pairwise

ANOVA indicate that the SS genetic strain had higher blood pressure than the W strain, both with and without saline treatment (Figure 3.8).

Heart rate in the 2% study was significantly higher in the SSS than in WS group (Table 3.11). Heart rate was significantly higher in the SSC than in WC group. These pairwise ANOVA demonstrate that the SS strain had a higher heart rate than the W strain, both with and without saline treatment (Figure 3.11).

Table 3.11 Systolic Blood Pressure and Heart Rate for the 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	P	Group	n	Mean	SEM
Blood pressure, mmHg		WC	6	106.7 ±	2.36[b]
	<0.001	WS	4	114.0 ±	1.47[c]
		SSC	6	123.8 ±	4.64[d]
		SSS	6	142.7 ±	5.08
Heart rate, bpm		WC	6	383.3 ±	18.0[b]
	<0.001	WS	4	402.5 ±	22.1[c]
		SSC	6	450.0 ±	11.3
		SSS	6	478.3 ±	12.2

For overall comparison of variability between the 4 groups the p-value is shown; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.2.3.3 Summary of 1% and 2% Study Using ANOVA for Both Genetic and Saline Treatment Effects

The 1% Study and the 2% Study results are compared below (Figure 3.7 to 3.10). One percent saline treatment had no effect on blood pressure (Figure 3.7). When the saline was increased to a 2% concentration (Figure 3.8), ANOVA indicated that there was significant overall variation between the 4 groups for blood pressure. Figure 3.8 graphically shows the increased blood pressure in the SS strain both with and without saline treatment. In addition, saline treatment significantly increased blood pressure in the SS rats but only non-significantly enhanced the blood pressure in W rats.

ANOVA indicated that there was significant overall variation between the 4 groups for the heart rate in both 1% and 2% studies (Figure 3.9 and 3.10). Further pairwise analysis did not demonstrate differences between the pairs in the 1% Study (Figure 3.9). Pairwise ANOVA for heart rate indicate a significant genetic effect in the 2% Study with and without saline treatment and an enhanced effect by saline treatment (Figure 3.10).

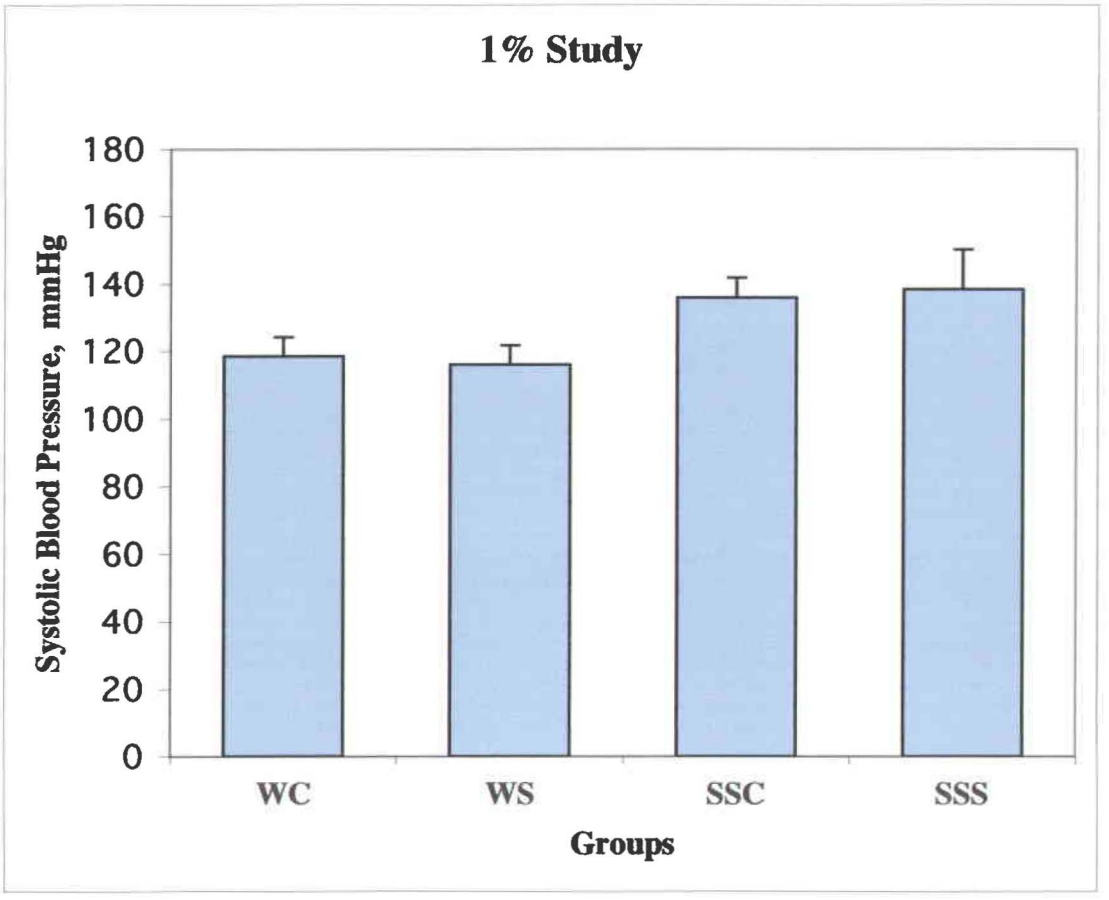

Figure 3.7 The effects of genetic strain and added dietary salt on blood pressure in the 1% Study. Shown are the means ± SEM. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with saline. An overall one-way analysis of variance comparison between the 4 groups was not significant, no pairwise comparisons were tested. There were significant differences overall between genetic strains (see Table 3.6).

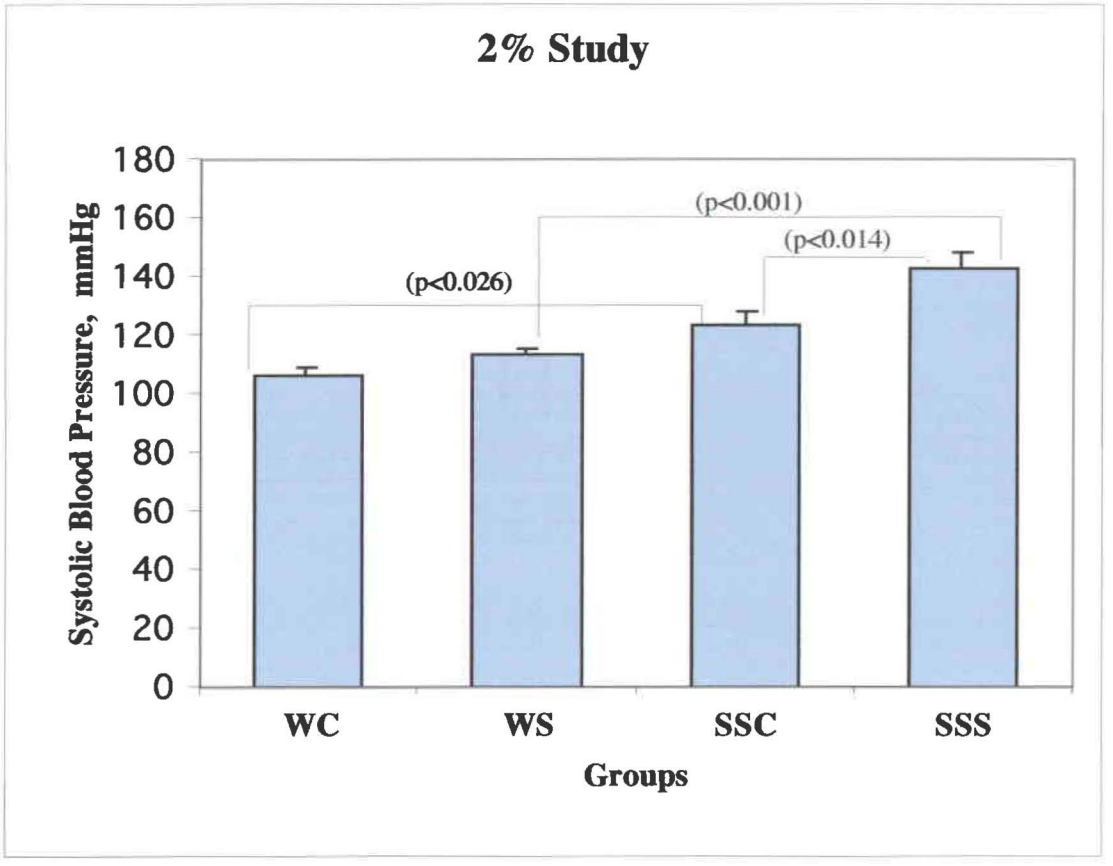

Figure 3.8. The effects of genetic strain and added dietary salt on blood pressure in the 2% Study. Shown are the means ± SEM. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with saline. An overall one-way analysis of variance comparison between the 4 groups was significant (Table 3.11). For pairwise comparison significant levels $p < 0.05$; WC vs SSC; WS vs SSS; SSC vs SSS were significant. There were significant differences overall between genetic strains and saline treated (see Table 3.7 and 3.9).

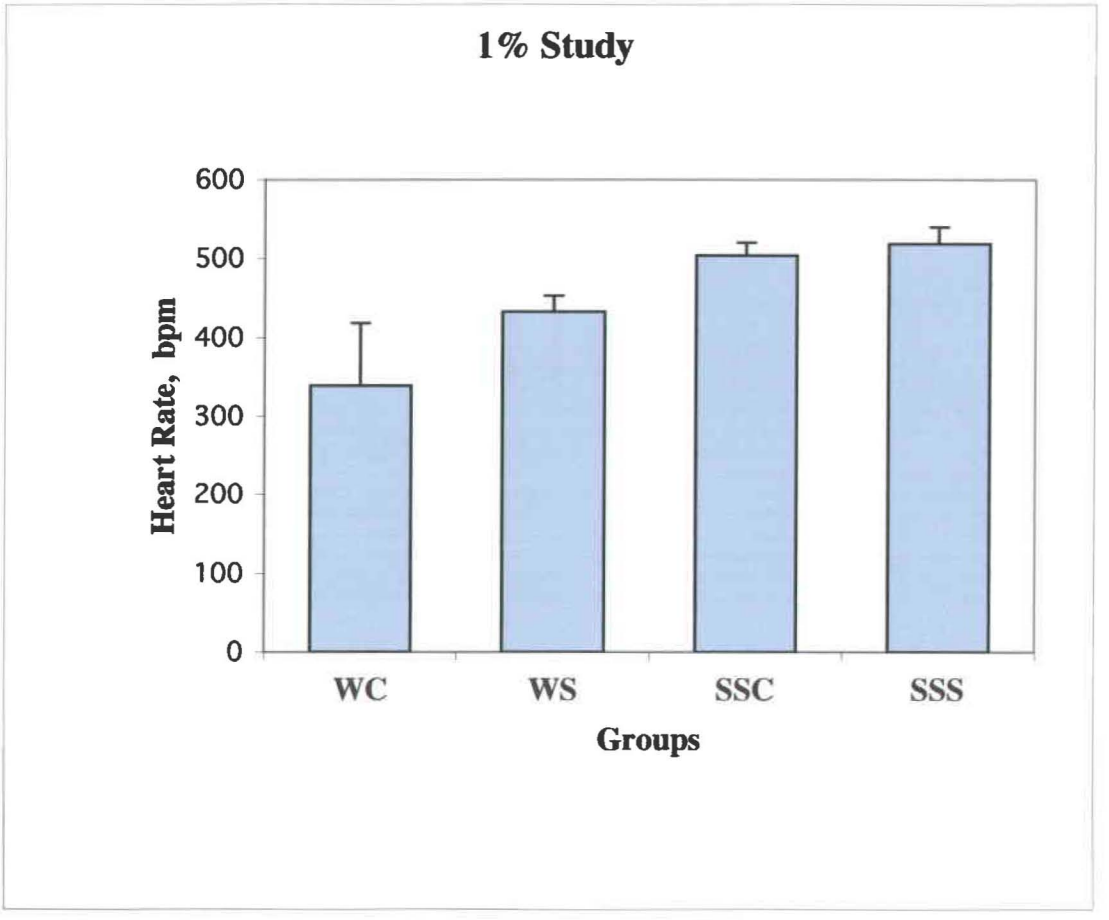

Figure 3.9. The effects of genetic strain and added dietary salt on heart rate in the 1% Study. Also shown are the means ± SEM. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with saline. An overall one-way analysis of variance comparison between the 4 groups was significant, however, no pairwise comparisons were significant. There were significant differences overall between genetic strains (see Table 3.6).

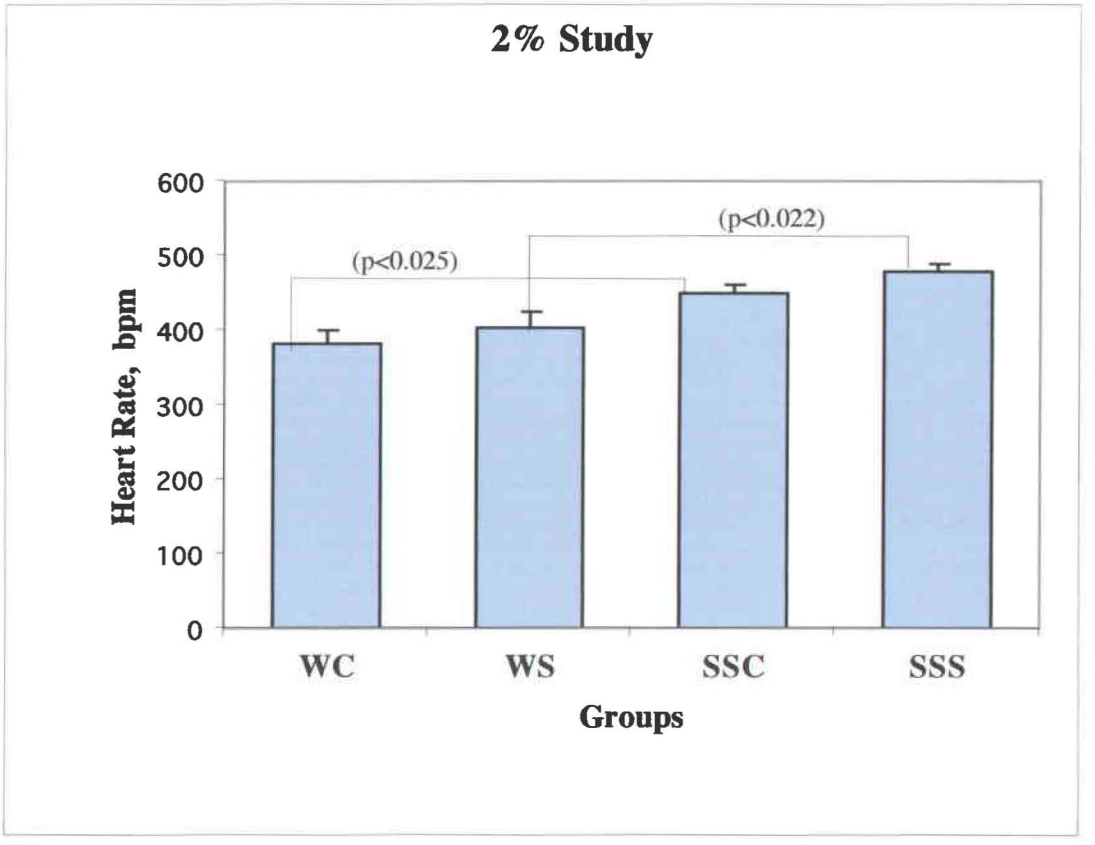

Figure 3.10. The effects of genetic strain and added dietary salt on heart rate in the 2% Study. Also shown are the means ± SEM. WC, wistar normotensive water control, WS, wistar normotensive, treated with saline, SSC, salt sensitive hypertensive water control, SSS, salt sensitive hypertensive treated with saline. For pairwise comparison significant levels $p < 0.05$; WC vs SSC and WS vs SSS were significant. There were significant differences overall between genetic strains (see Table 3.7).

3.3 BONE MASS, LENGTHS, AND MORPHOMETIC ANALYSES

3.3.1 Genetic Effects

3.3.1.1 1% Study Genetic Effects

For each rat, the left ulna, the left femur and right femur fresh weight (Table 3.12) and length (Table 3.13) were measured. Many genetic bone differences were recorded. In the 1% study with saline and water drinking groups pooled, the wet weight in both femur limbs of the W rats were significantly heavier than the SS femur weights. It was found that W gained 6.7% more weight than SS rats (see Section 3.1.1.1.1). For this reason the wet weight/body weight X 100 = % calculation was used. When expressed as a percentage of body weight, both the overloaded and the underloaded femur limbs of the W were heavier than the SS strain femur weights (p <0.007 and p <0.062, respectively). Body weight is often seen as a significant influence on bone, however, genetic strain in this study affected bone even though expressed as a percentage of body weight. The left ulna bone was heavier in the W over the SS, but not significantly (Table 3.12).

Table 3.12 Body Weight, Femur and Ulna Weight Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 1% Study

Parameter	p	strain	n	Mean	SEM
Body wt, g	< 0.052	W	11	212.45 ±	5.60
		SS	11	198.20 ±	3.01
Overloaded femur wet weight, g	< 0.001	W	11	0.583 ±	0.017
		SS	11	0.493 ±	0.014
Overloaded femur wet weight/body weight, %	< 0.002	W	11	0.275 ±	0.003
		SS	11	0.249 ±	0.007
Underloaded femur wet weight, g	< 0.007	W	11	0.601 ±	0.021
		SS	11	0.514 ±	0.018
Underloaded femur wet weight/body weight, %	< 0.062	W	11	0.283 ±	0.005
		SS	11	0.259 ±	0.008
Left ulna bone weight, g	ns	W	11	0.124 ±	0.003
		SS	11	0.122 ±	0.001

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA; ns, not significant.

Two femur length measurements were taken, from the ball of the proximal femur to the distal end of femur and from the trochanter end of proximal femur to the distal end. A single length measurement was made of each left ulna. While the W average femur measurements were longer than the SS femur averages, only the overloaded femurs were significant (with saline and water drinking groups pooled). This extra femur growth may have been due to extra 6.7% food eaten in 1% study by W over SS.

Table 3.13 Femur and Ulna Length Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 1% Study

Parameter	p	strain	n	Mean		SEM
Overloaded femur length,	< 0.001	W	11	31.24	±	0.229
trochanter to distal, mm		SS	11	30.08	±	0.127
Overloaded femur length,	< 0.001	W	11	30.47	±	0.212
ball to distal, mm		SS	11	29.38	±	0.178
Underloaded femur length,	ns	W	11	31.25	±	0.244
trochanter to distal, mm		SS	11	30.49	±	0.374
Underloaded femur length,	ns	W	11	30.18	±	0.214
ball to distal, mm		SS	11	29.56	±	0.386
Left ulna bone length, mm	ns	W	11	28.86	±	0.338
		SS	11	29.39	±	0.249

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA; ns, not significant.

3.3.1.2 2% Study Genetic Effects

As in the 1% study, the wet weight of the underloaded and the overloaded femur limbs of the W rats in the 2% study were significantly heavier than the SS femur weights with saline and water drinking groups pooled (Table 3.14). In the 2% study, W and SS body weights were not significantly different. When expressed as a percentage of body weight, both femurs of the W rats were significantly heavier than the SS femur weights (Table 3.14) indicating genetic difference.

Table 3.14 Body Weight, Femur and Ulna Weight Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 2% Study

Parameter	p	strain	n	Mean	SEM
Body wt, g	ns	W	10	189.80 ±	4.300
		SS	12	192.75 ±	3.520
Overloaded femur wet weight, g	< 0.002*	W	10	0.595 ±	0.015
		SS	12	0.537 ±	0.008
Overloaded femur wet weight/body weight, %	< 0.001*	W	10	0.314 ±	0.006
		SS	12	0.280 ±	0.005
Underloaded femur wet weight, g	< 0.001*	W	10	0.582 ±	0.012
		SS	12	0.520 ±	0.010
Underloaded femur wet weight/body weight, %	< 0.003*	W	10	0.307 ±	0.007
		SS	12	0.271 ±	0.007
left ulna bone weight, g	ns	W	10	0.141 ±	0.004
		SS	12	0.138 ±	0.002

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

As in 1% study, two femur length measurements were taken (from the ball of the proximal femur to distal end of femur and from the trochanter end of proximal femur to the distal femur) and one single length measurement of the left ulna. In addition, the L-2 vertebrae lengths were measured. No significant differences (Table 3.15) were seen in the lengths of femurs or vertebrae, however, the ulna bones were longer in SS when compared to W (with saline and water drinking groups pooled).

Table 3.15 Femur, Ulna, and L-2 Vertebrae Length Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 2% Study

Parameter	p	strain	n	Mean		SEM
Overloaded femur length,	ns	W	10	30.12	±	0.212
trochanter to distal, mm		SS	12	30.22	±	0.135
Overloaded femur length,	ns	W	10	29.24	±	0.199
ball to distal, mm		SS	12	29.49	±	0.175
Underloaded femur length,	ns	W	10	30.47	±	0.160
trochanter to distal, mm		SS	12	30.36	±	0.122
Underloaded femur length,	ns	W	10	29.48	±	0.141
ball to distal, mm		SS	12	28.87	±	0.326
Left ulna bone length, mm	< 0.030	W	10	28.25	±	0.131
		SS	12	28.62	±	0.128
Vetebrae length, mm	ns	W	10	6.018	±	0.078
		SS	12	5.678	±	0.148

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.; ns not significant.

3.3.1.3 Summary of 1% and 2% Genetic Results

Unlike the 1% study, body weights between the W and the SS rats were not significantly different at end of study, suggesting the genetic difference in femur weight was not attributed to differing body weights. Femur weight remained significantly higher in the W rats compared to SS rats despite their similar body weight in 2% study.

No significant differences in length between W and SS rats were found in 2% study in either the femur or the L-2 vertebra. While the ulna weights were not significantly different, the SS had longer ulnas in the 2% study than W. The overloaded

femur lengths of the W in the 1% study were both longer than the SS, which might be expected as the W weighed 6.7% more SS in the 1% study.

3.3.2 Drinking Fluid Treatment

3.3.2.1 1% Study and 2% Study Summary

There was not a significant difference in femur weight or length between rats on saline and rats on water, when SS and W strain data were pooled in the 1% study or 2% study.

3.3.3 Tukey Pairwise Post Hoc Results and One-way ANOVA for Both Genotype and Saline Treatment

Tukey's pairwise testing indicated a predominately genetic influence on wet bone weight irrespective of saline consumed in both the 1% and 2% study.

3.3.3.1 1% Study Using ANOVA for Both Genotype and Saline Treatment

Significant overall variation between the 4 groups was seen for femur weight in the 1% study, Tukey post-hoc pairwise tests further indicated these were genetic (Table 3.16). The overloaded femur wet weight in the 1% study was higher in the WC than in the SSC group ($p < 0.05$). The overloaded and the underloaded femur wet weights tended to be higher in the WS than in the SSS group ($p < 0.055$ and $p < 0.076$, respectively). The results indicate that the W strain had higher femur weight than the SS strain, both with and without saline treatment. The overloaded femur wet bone weight to body weight ratio was significantly higher in the WC than in the SSC group. The results suggest a predominately genetic influence on overloaded and underloaded femur wet bone weight

in the 1% study (Table 3.16). Small group size and Tukey's conservative estimates may be the reason these results were just short of significance.

Table 3.16 Femur Weight Measurements for the 1% Study using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean	SEM
Overloaded femur wet weight, g		WC	5	0.585	± 0.025[b]
	< 0.009	WS	6	0.582	± 0.024
		SSC	5	0.489	± 0.021
		SSS	6	0.496	± 0.020
Underloaded femur wet weight, g		WC	5	0.599	± 0.024
	< 0.044	WS	6	0.602	± 0.035
		SSC	5	0.527	± 0.036
		SSS	6	0.502	± 0.014
Overloaded femur wet weight/body weight, %		WC	5	0.278	± 0.005[b]
	< 0.028	WS	6	0.272	± 0.004
		SSC	5	0.244	± 0.007
		SSS	6	0.253	± 0.012
Underloaded femur wet weight/body weight, %		WC	5	0.286	± 0.004
	0.186	WS	6	0.280	± 0.009
		SSC	5	0.263	± 0.014
		SSS	6	0.257	± 0.011

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

Significant overall variation between the 4 groups was seen for femur length in the 1% study, Tukey post-hoc pairwise tests further indicated these were genetic (Table 3.17).

Table 3.17 Femur Length Measurements for the 1% Study using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean	SEM
Overloaded femur length, trochanter to distal, mm	< 0.003	WC	5	31.11	± 0.431
		WS	6	31.34	± 0.250[c]
		SSC	5	30.26	± 0.121
		SSS	6	29.92	± 0.195
Overloaded femur length, ball to distal, mm	< 0.003	WC	5	30.39	± 0.374
		WS	6	30.54	± 0.264[c]
		SSC	5	29.76	± 0.138
		SSS	6	29.06	± 0.243
Underloaded femur length, ball to distal, mm	0.090	WC	5	30.22	± 0.338
		WS	6	30.14	± 0.301
		SSC	5	30.29	± 0.709
		SSS	6	28.96	± 0.224
Underloaded femur length, trochanter to distal, mm	0.189	WC	5	31.17	± 0.432
		WS	6	31.31	± 0.305
		SSC	5	31.01	± 0.751
		SSS	6	30.06	± 0.228

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

The overloaded femur lengths, (both trochanter to distal and ball to distal) in the 1% study were significantly longer in the WS than in the SSS group (Table 3.17). As the WS group had the highest body weight of any group, this extra femur growth may have been due to extra food eaten.

3.3.3.2 2% Study Using ANOVA for Both Genotype and Saline Treatment

Significant overall variation between the 4 groups was seen for femur weight in the 2% study, Tukey post-hoc pairwise tests further indicated these were genetic (Table 3.18).

Table 3.18 Femur Weight Measurements for the 2% Study using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean	SEM
Overloaded femur wet weight, g		WC	6	0.596	± 0.024
	< 0.011	WS	4	0.594	± 0.013[c]
		SSC	6	0.553	± 0.010
		SSS	6	0.522	± 0.010
Underloaded femur wet weight, g		WC	6	0.581	± 0.020
	< 0.013	WS	4	0.583	± 0.012
		SSC	6	0.523	± 0.016
		SSS	6	0.517	± 0.013
Overloaded femur wet weight/body weight, %	< 0.004	WC	6	0.320	± 0.008[b]
		WS	4	0.304	± 0.008
		SSC	6	0.280	± 0.010
		SSS	6	0.279	± 0.006
Underloaded femur wet weight/body weight, %	< 0.014	WC	6	0.314	± 0.010[b]
		WS	4	0.298	± 0.008
		SSC	6	0.265	± 0.012
		SSS	6	0.277	± 0.009

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

The underloaded femur wet weight in the 2% study was higher in the WC than in the SSC group (p <0.072). The overloaded and underloaded femur wet weights were higher in the WS than in the SSS group (p <0.036 and p <0.070, respectively). The

results indicate that W had higher femur weight than the SS strain, both with and without saline treatment. When body weight was factored in, the overloaded and underloaded femur wet bone weight to body weight ratios were significantly higher in the WC than in the SSC group (p <0.013 and p <0.008, respectively). The overloaded and underloaded femur wet bone weight to body weight ratios tended to be higher in the WS than in the SSS group, but not significantly.

The L-2 vertebra length in the 2% study (Table 3.19) was significantly longer in the WS than in the SSS group (p <0.007). In addition, the L-2 vertebra length was significantly longer in the SSC than in the SSS group (p <0.016). This may suggest that the salt sensitive rats' L-2 vertebra length measurements were lowered by saline treatment and by their genotype.

Table 3.19 L-2 Vertebra Length Measurements for the 2% Study using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean		SEM
L-2 vertebra length, mm	< 0.005	WC	6	5.93	±	0.096
		WS	4	6.15	±	0.112[c]
		SSC	6	6.01	±	0.197[d]
		SSS	6	5.35	±	0.119

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.3.3.3 Summary of 1% and 2% Study Using ANOVA for Both Genetic and Saline Treatment Effects

There was not a significant saline treatment difference between saline treated and water controls in either the 1% or 2% study for femur weight and length. The heaviest bone/body weights were found in overloaded femur WC group on water. The lightest bone/ body weights were found in overloaded femur SSS group on saline. While there is some variation, the progressive reduction in weights in three bone sites suggest the significant genetic effect was further enhanced by the saline treatment.

Lengths demonstrated few significant effects, and only in the 1% study overloaded femur and 2% vertebra. The 1% study femur growth may have been due to extra body weight. The 2% study difference suggests 2% saline treatment and genetic difference may influence vertebra bone length adversely.

3.3.4 Femur Overloading/Underloading

No weight or length differences were observed between the overloaded and underloaded femurs in 1% or 2% study.

3.4 BONE STRENGTH AND MORPHOLOGIC ANALYSES

3.4.1 2% Study Genetic Effects

A three point bending test was used in 2% study to determine mechanical properties of the overloaded and underloaded femur bones (See Chapter 2 Methods, Section 2.6). Three biomechanical parameters were assessed to investigate femur breaking strength:

1) Applied force

This is the force necessary to break bone. A recording chart was used, from which force was read directly from the chart. The deflection was calculated by multiplying the chart reading by the crosshead speed and dividing by chart speed.

2) Ultimate bending stress

Since the applied force measurement relies on both a material and geometric result, a calculation using the moment of inertia (taken from a 1.0 mm transverse cross-section of femur) was used to separate out the geometric property (includes size, area and length distribution) leaving only the material property, ultimate bending stress (Mpa). This is commonly called the breaking strength measurement.

3) Modulus of elasticity

A second calculation was used to separate out another material property, the apparent modulus of elasticity, using the applied force measurement (the slope of the straight line portion of the stress-strain diagram) and transverse cross-sectional measurements (GPa). This property is commonly called stiffness.

The applied force measurements were not significantly different when W and SS strains were compared (Table 3.20). The SS femurs had increased breaking strength (as measured by ultimate bending stress) in both the overloaded and underloaded femurs compared to W rats. In addition, a marked increase in stiffness (as measured by the modulus of elasticity) was found for SS overloaded and underloaded femurs.

Table 3.20 Femur Biomechanical Properties in Female Wistar and Salt Sensitive Hypertensive Rats, 2% Study

Parameter	p	strain	n	Mean ±SEM
Applied force, underloaded femur, N	<0.282	W	10	94.97 ± 2.15
		SS	12	91.18 ± 2.28
Ultimate bending stress, underloaded femur, MPa	<0.050	W	10	99.17 ± 3.72
		SS	12	116.7 ± 6.44
Modulus of elasticity, underloaded femur, GPa	<0.003	W	10	0.485 ±0.027
		SS	12	0.696 ±0.049
Applied force, overloaded femur, N	<0.140	W	10	99.75 ± 2.13
		SS	12	96.37 ± 1.14
Ultimate bending stress, overloaded femur, MPa	<0.001	W	10	90.26 ± 4.48
		SS	12	119.00± 2.84
Modulus of elasticity, overloaded femur, GPa	<0.001	W	10	0.355 ±0.021
		SS	12	0.525 ±0.027

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

The significant increase in breaking strength in both the overloaded femur and the underloaded femur of the SS rats is shown in Figure 3.11. The lengths of the femur diameters in transverse section calculations were greatly reduced in the SS femurs in the 2% study, creating significant increases in the final ultimate bending stress result (Figure 3.11), in both the underloaded and overloaded SS femurs.

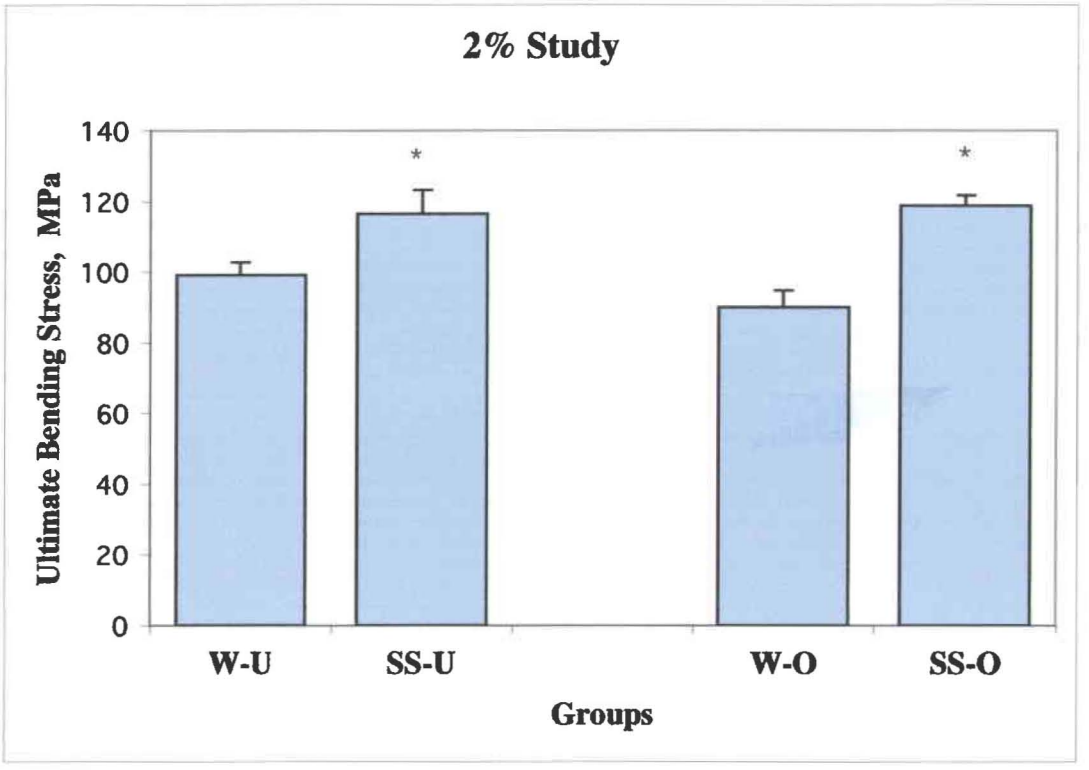

Figure 3.11 Ultimate bending stress (MPa, megapascals) of underloaded (U) and overloaded (O) femurs in wistar and salt sensitive rats, with saline and water drinking groups pooled, in the 2% Study. The data shown are means ± SEM. Groups are W-U, wistar underloaded femur; SS-U, salt sensitive hypertensive underloaded femur; W-O, wistar overloaded femur; SS-O, salt sensitive hypertensive overloaded femur. Significant effects of groups are displayed by *$p < 0.05$ (SS-U vs W-U) and *$p < 0.05$ (SS-O vs W-O).

Figure 3.12 demonstrates the marked increase found in stiffness (i.e. apparent modulus of elasticity) in SS in both the overloaded femur and the underloaded femurs when compared to W.

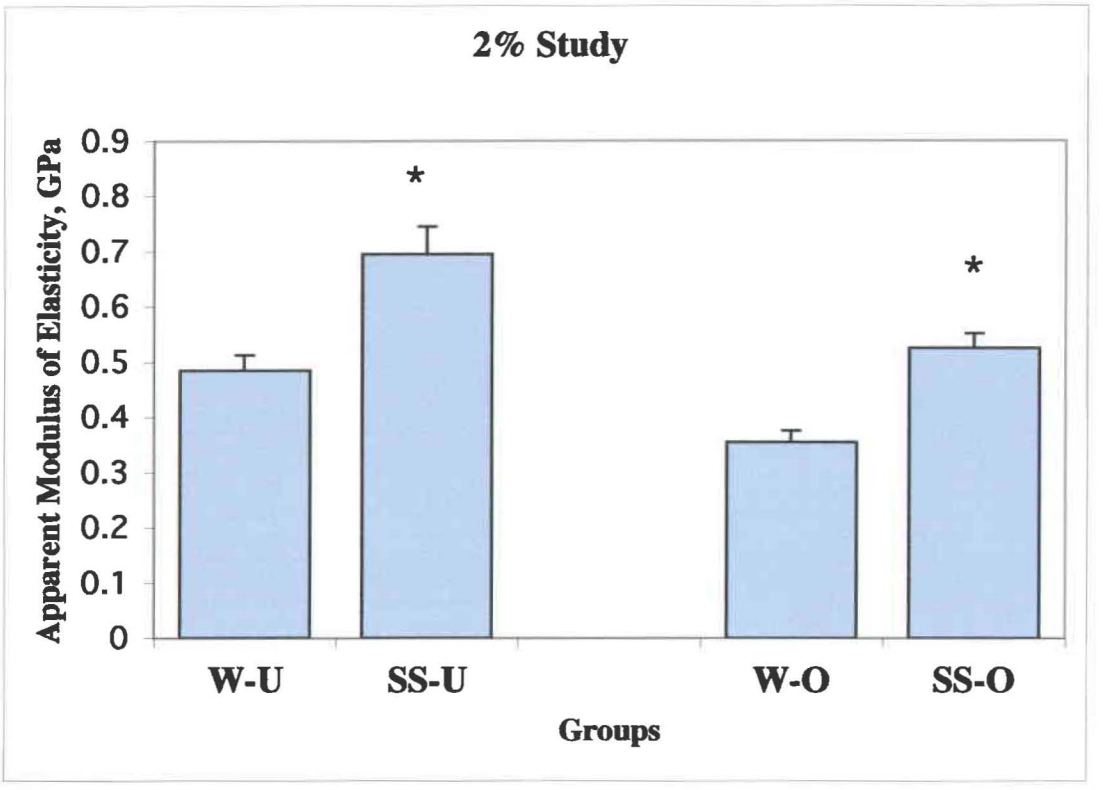

Figure 3.12 Apparent modulus of elasticity (GPa, gigapascals) of underloaded (U) and overloaded (O) femurs in wistar and salt sensitive rats, with saline and water drinking groups pooled, in the 2% Study. The data shown are means ± SEM. Groups are W-U, wistar underloaded femur; SS-U, salt sensitive hypertensive underloaded femur; W-O, wistar overloaded femur; SS-O, salt sensitive hypertensive overloaded femur. Significant effects of groups are displayed by *$p < 0.05$ (SS-U vs W-U) and *$p < 0.05$ (SS-O vs W-O).

3.4.2 Summary of 2% Study Genetic and Saline Results

For breaking strength, the ultimate bending stress in the underloaded femur tended to be increased in rats on saline over those on water (117.62MPa±6.12 vs 101.32MPa±5.2), but the difference was shy of significance ($p < 0.068$) when tested by 2 way ANOVA.

As previously displayed in Table 3.20, when size of individual bone was factored in, using the ASAE calculation for ultimate bending stress, the SS strain displayed a significant increase in femur breaking strength and stiffness in both the overloaded and underloaded femurs compared to the W strain.

3.4.3 Tukey Pairwise Post Hoc Results and One Way ANOVA for Both Genotype and Saline Treatment

Comparing the WC, WS, SSC, and SSS groups (Table 3.21), significant overall variation between the 4 groups was not seen for ultimate bending stress in the underloaded femur limb, however, in the overloaded femur limb, significant overall variation between the 4 groups was seen for ultimate bending stress. In addition, significant overall variation between the 4 groups was seen for the apparent modulus of elasticity in both the underloaded and overloaded femur limb.

Tukey post-hoc tests were applied to each pair from the 4 groups (WC, WS, SSC, and SSS). Significant differences were found in relevant pairwise combinations (Table 3.21). The ultimate bending stress in the overloaded femurs was higher in the SSC than in WC group suggesting that the SS strain had a higher ultimate bending stress in the overloaded femur than the W strain. The ultimate bending stress in the overloaded femur

was significantly higher in the SSS than in WS group suggesting that the SS strain had a higher ultimate bending stress in the overloaded femur than the W strain with saline treatment.

The apparent modulus of elasticity results (Table 3.21) in the underloaded and the overloaded femurs tended to be higher in the SSC than in WC group (p <0.098 and p <0.009, respectively). The apparent modulus of elasticity in the overloaded femurs was higher in the SSS than in WS group (p <0.061).

Table 3.21 Femur Biomechanical Properties in the 2% Study using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean	SEM
Ultimate bending stress, Mpa, underloaded femur limb	<0.054	WC	6	93.04	± 2.78
		WS	4	108.36	± 6.12
		SSC	6	109.59	± 9.16
		SSS	6	123.78	± 8.85
Apparent modulus of elasticity, GPa, underloaded femur limb	<0.014	WC	6	0.457	± 0.033
		WS	4	0.528	± 0.042
		SSC	6	0.657	± 0.060
		SSS	6	0.735	± 0.080
Ultimate bending stress, Mpa, overloaded femur limb	<0.001	WC	6	87.17	± 5.75[b]
		WS	4	94.90	± 7.48[c]
		SSC	6	119.73	± 3.88
		SSS	6	118.21	± 4.49
Apparent modulus of elasticity, GPa, overloaded femur limb	<0.002	WC	6	0.342	± 0.029[b]
		WS	4	0.375	± 0.033
		SSC	6	0.523	± 0.044
		SSS	6	0.527	± 0.036

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.4.4 2% Study Femur Overloading/Underloading

The overloaded femurs supporting the rear body weight of animals had a significantly higher applied force reading than underloaded femur (Table 3.22). However, there was no significant difference in the breaking strength measurement [ultimate bending stress (Mpa) calculation]. All groups tended to have smaller transverse cross-sectional areas in the underloaded femurs compared to the overloaded femurs, though these were not significantly different. This geometric difference may account for the lack of breaking strength difference between the underloaded and overloaded femurs. The apparent modulus of elasticity was significantly higher in the underloaded femurs than the overloaded femurs (Table 3.22).

Table 3.22 Significant Effect of Underloading and Overloading on Femur Biomechanical Properties in All Rats of the 2% Study

Parameter		U/O	n	Mean	± SEM
Applied force, N, femur	< 0.018	O	22	97.91	± 1.18
		U	22	92.90	± 1.60
Modulus of elasticity, GPa, femur limb	< 0.001	O	22	0.448	± 0.025
		U	22	0.600	± 0.037

The main effects assessed in this table are for the loading stress and include the 4 groups (WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline). n, no. of rats; O, overloaded femur ; U, underloaded femur. Significance levels were set at 0.05, and determined by two-way ANOVA.

An overall analysis between the 8 groups demonstrated significant differences between groups. An enhanced effect of saline treatment in the salt sensitive strain is demonstrated in Table 3.23, however, the conservative Tukey post-hoc pairwise testing indicated only one significant difference between relevant pairs.

Table 3.23 The Femur Modulus of Elasticity Properties for the 2% Study Using ANOVA for Groups and Loading

Group	Variable	p	U/O	n	Mean	SEM
2%WC	Femur modulus of elasticity, GPa	ns	O	6	0.342	0.029
			U	6	0.457	0.033
2%WS	Femur modulus of elasticity, GPa	< 0.013	O	4	0.375	0.033
			U	4	0.528	0.042
2%SSC	Femur modulus of elasticity, GPa	ns	O	6	0.523	0.044
			U	6	0.657	0.060
2%SSS	Femur modulus of elasticity, GPa	< 0.064	O	6	0.527	0.036
			U	6	0.735	0.080

The overall comparison of variability between the 8 groups was $p < 0.001$ (not shown). WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline; O, overloaded femur ; U, underloaded femur. Values are the means ±SEM; n, no. of rats. For pairwise comparison significant levels $p < 0.05$. WS-U vs WS-O was significant.

3.4.5 Bone Morphology

As body weight was not significantly different in the 2% study, the cross-sectional results may present a more accurate picture of the transverse bone size and shape, compared to the 1% study. The primary use of transverse cross-sectional values initially was for calculations of the material strength measurement. First, transverse diameters were measured and calculated according to ASAE standards for the modulus of elasticity and ultimate bending stress measurement. However, because of significant differences found, one mm sections were subjected to a more expanded investigation. Area, equivalent circular diameter, length, perimeter, breadth, form factor and roundness were measured or calculated from the 1.0 mm transverse section.

Genetic differences played a dominant role in bone cross-sectional area as well as the other morphology parameters measured (Table 3.24). Because of the larger number of these parameters and a similar result in all, a brief discussion of only the bone area and marrow area parameter results follow.

3.4.5.1 Cross-Sectional Transverse Area Measurements Genetic Effects

Cross-sectional transverse area results by two-way ANOVA of both femurs were significantly higher in the wistar rat (W) strain than in the salt sensitive hypertensive rat (SS) strain, with saline and water drinking groups pooled, in both the 1% Study and 2% Study (Table 3.24).

The main effect of saline treatment by two-way ANOVA was not significant in the 1% Study or the 2% Study femur area measurements.

Table 3.24 Cross-Sectional Transverse Area Measurements of Femur in Female Wistar and Salt Sensitive Hypertensive Rats, 1% and 2% Study

Variable	p	Group	n	Mean	SEM
Area (cm^2) 1% Study	< 0.007	W	11	0.088	± 0.003
Underloaded femur transverse section		SS	11	0.076	± 0.002
Area (cm^2) 1% Study	< 0.001	W	10	0.082	± 0.003
Overloaded femur transverse section		SS	11	0.067	± 0.001
Area (cm^2), 2% Study	< 0.009	W	10	0.079	± 0.002
Underloaded femur transverse section		SS	12	0.068	± 0.003
Area (cm^2) 2% Study	< 0.001	W	10	0.085	± 0.003
Overloaded femur transverse section		SS	12	0.069	± 0.001

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.4.5.2 Tukey Pairwise Post Hoc Results and One-way ANOVA for Both Genotype and Saline Treatment

When a one-way ANOVA was used, there were significant differences between the means of the WC, WS, SSC, and SSS groups (Table 3.25) When each group was compared to each other pairwise, the area of the overloaded femur transverse sections in the 1% Study was larger in the WC than in SSC group (p <0.008). In addition, the area of the underloaded and the overloaded femur transverse sections were larger in the WS than in SSS group (p <0.090) and p <0.017, respectively). These pairwise tests indicate that the bone area of the underloaded and the overloaded femur transverse sections of the

W strain was larger than the SS strain, both with and without salt treatment. These pairwise Tukey tests indicate a genetic effect in adolescent female rats of the 1% Study.

A significant overall variation between the 4 groups (Table 3.24) was not seen in the underloaded femur in the 2% Study (p <0.053). As previously seen for blood pressure, the reason for this apparent discrepancy is that the variance in the two-way ANOVA is factored into two overall main effects, not four, as is done in the one-way. However, a significant overall variation between the 4 groups was seen for the overloaded femur (p <0.003). The area of the overloaded femur transverse sections in the 2% Study was significantly larger in the WC than in SSC group. The area the overloaded femur transverse sections was significantly larger in the WS than in SSS group. These tests indicate that the bone area of the overloaded femur transverse section of the W strain was larger than the SS strain, both with and without saline treatment in the 2% Study.

Table 3.25 Cross-Sectional Transverse Area Measurements of Femur for the 1% Study and 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean		SEM
Area (cm^2) 1% Study		WC	5	0.087	±	0.005
Underloaded femur transverse section	< 0.035	WS	6	0.088	±	0.002
		SSC	5	0.077	±	0.004
		SSS	6	0.076	±	0.003
Area (cm^2) 1% Study		WC	4	0.082	±	0.005[b]
Overloaded femur transverse section	< 0.001	WS	6	0.082	±	0.004[c]
		SSC	5	0.065	±	0.002
		SSS	6	0.068	±	0.001
Area (cm^2), 2% Study		WC	6	0.079	±	0.003
Underloaded femur transverse section	< 0.053	WS	4	0.08	±	0.003
		SSC	6	0.07	±	0.005
		SSS	6	0.067	±	0.003
Area (cm^2) 2% Study		WC	6	0.086	±	0.006[b]
Overloaded femur transverse section	< 0.003	WS	4	0.084	±	0.001[c]
		SSC	6	0.071	±	0.002
		SSS	6	0.068	±	0.002

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.4.5.3 Cross-Sectional Transverse Area Marrow Measurements Genetic and Saline Effects

Femur marrow area results were not significantly different in the 1% Study, however, femur marrow area in the 2% Study were significantly higher in W than in SS, with saline and water drinking groups pooled. There were no significant effects from saline treatment in the 1% Study or the 2% Study femur marrow area measurements.

3.4.5.4 Tukey Pairwise Post Hoc Marrow Results and One-way ANOVA for Both Genotype and Saline Treatment

There was not a significant overall difference for the 4 groups in the 1% Study marrow absolute area measurements of the overloaded and underloaded femurs (Table 3.26). However, marrow as a percentage of the total cross-sectional (CS) bone area of the overloaded femur was significantly different (Table 3.26). The 2% Study indicated a significant overall difference for the 4 groups in absolute area measurements of the overloaded and underloaded femurs. However, marrow as a percentage of the total cross-sectional (CS) bone area of the underloaded and overloaded femur was not significantly different.

In response to post hoc testing, the area of the underloaded and the overloaded femur transverse marrow sections were significantly larger in the WS than in SSS groupings of the 2% Study. However, marrow as a percentage of the total cross-sectional (CS) bone area of these underloaded and overloaded femurs were not significantly different (Table 3.26).

Table 3.26 Cross-Sectional Transverse Area Marrow Measurements of Femur for the 1% Study and 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	P	Group	n	Mean	SEM
Area, underloaded femur transverse marrow section, cm^2, 1% Study	< 0.113	WC	5	0.027	± 0.001
		WS	6	0.026	± 0.001
		SSC	5	0.025	± 0.001
		SSS	6	0.024	± 0.001
Area, overloaded femur transverse marrow section, cm^2, 1% Study	ns	WC	4	0.025	± 0.002
		WS	6	0.026	± 0.002
		SSC	5	0.027	± 0.001
		SSS	6	0.026	± 0.001
Underloaded marrow/total CS area %, 1% Study	ns	WC	5	0.239	± 0.008
		WS	6	0.228	± 0.006
		SSC	5	0.248	± 0.016
		SSS	6	0.241	± 0.011
Overloaded marrow/total CS area %, 1% Study	< 0.014	WC	4	0.234	± 0.007
		WS	6	0.239	± 0.015
		SSC	5	0.289	± 0.014
		SSS	6	0.279	± 0.010
Area, underloaded femur transverse marrow section, cm^2, 2% Study	< 0.016	WC	6	0.034	± 0.003
		WS	4	0.034	± 0.003
		SSC	6	0.027	± 0.002
		SSS	6	0.026	± 0.001
Area, overloaded femur transverse marrow section, cm^2, 2% Study	< 0.004	WC	6	0.034	± 0.003[b]
		WS	4	0.035	± 0.002[c]
		SSC	6	0.027	± 0.001
		SSS	6	0.026	± 0.001
Underloaded marrow/total CS area %, 2% Study	ns	WC	5	0.301	± 0.024
		WS	6	0.300	± 0.022
		SSC	5	0.278	± 0.027
		SSS	6	0.279	± 0.009
Overloaded marrow/total CS area %, 2% Study	ns	WC	4	0.287	± 0.024
		WS	6	0.291	± 0.008
		SSC	5	0.276	± 0.007
		SSS	6	0.279	± 0.013

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.5 BONE COMPOSITION

3.5.1 Genetic Effects

3.5.1.1 1% Study Genetic Effect

Major bone mineral elements were measured in the dissolved left ulna bone, a 5 mm section of the left overloaded femur and a 5 mm section of the right underloaded femur. Concentrations (mg/gm of dry fat free bone) of calcium, magnesium, phosphorus, potassium and sodium were obtained, as well as, concentrations (μg/g of bone) of boron, manganese, iron, copper, and zinc (Appendix B). Only genetic differences in bone composition of major and/or significantly different elements are reported.

Genetic strains in the 1% Study accounted for significant differences in femur zinc concentrations, with saline and water drinking groups pooled (Table 3.27). The reduced zinc concentration in SS rats was not statistically significant in the ulnas (Table 3.27)

Table 3.27 Femur and Ulna Zinc Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 1% Study

Variable (ppm)	p	strain	n	Mean	SEM
Zinc, underloaded femur	<0.002	W	11	344.5	± 16.8
		SS	11	280.3	± 3.6
Zinc, overloaded femur	<0.001	W	11	352.9	± 19.9
		SS	11	273.1	± 3.2
Zinc, ulna	0.115	W	11	250.1	± 15.5
		SS	11	215.7	± 18.4

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

In the only comparable (but of one year duration) published study, higher calcium content has been observed in adult female spontaneously hypertensive femur bone tissue over wistar bone tissue (Lau et al., 1984). In the 1% Study, SS had significantly higher calcium, phosphorus and sodium in the underloaded femur. Magnesium, on the other hand, was higher in W than in SS overloaded femur. The only genetic difference found in the ulna bone, with saline and water drinking groups pooled was for potassium (Table 3.28).

Table 3.28 Femur and Ulna Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 1% Study

Variable (%)	p	strain	n	Mean	SEM
Calcium, underloaded femur	<0.001	W	11	28.53	± 0.321
		SS	11	30.03	± 0.165
Phosphate, underloaded femur	<0.017	W	11	13.33	± 0.136
		SS	11	13.77	± 0.089
Sodium, underloaded femur	<0.020	W	11	0.676	± 0.020
		SS	11	0.606	± 0.018
Magnesium, overloaded femur	<0.004	W	11	0.541	± 0.004
		SS	11	0.523	± 0.005
Potassium, ulna	< 0.011	W	11	0.15	± 0.009
		SS	11	0.19	± 0.012

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.5.1.2 2% Study Genetic Effect

As in the 1% Study, a complete examination of major bone elements was performed on the dissolved left ulna bone, a 5 mm section of the left overloaded femur and a 5 mm section of the right underloaded femur (Appendix B). Calcium, magnesium, phosphorus, sodium, potassium, boron, manganese, iron, copper, and zinc concentrations were obtained from each animal. Wistar rats again had higher zinc levels in the three samples measured, with saline and water drinking groups pooled (Table 3.29).

Table 3.29 Femur and Ulna Zinc Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 2% Study

Variable (ppm)	p	strain	n	Mean	SEM
Zinc, underloaded femur	<0.029	W	10	348.1	± 19.7
		SS	12	297.3	± 8.8
Zinc, overloaded femur	<0.537	W	10	283.7	± 17.5
		SS	12	271.6	± 4.8
Zinc, ulna	<0.044	W	10	264.7	± 20.1
		SS	12	222.5	± 3.9

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

In the 2% Study overloaded femur, calcium was significantly higher in SS rats than in W rats, with saline and water drinking groups pooled (Table 3.30). In the underloaded femur, calcium was only negligibly higher in SS than in W rats. It is unclear why the underloaded bones in the 1% study were significantly differently but may be due to the effect of body weight between the 1% Study and the 2% Study. In both studies consistent with Lau et al.'s (1984) study, the hypertensive strain had significantly higher femur calcium over the normotensive strain. Magnesium was higher in the underloaded femur of the W over the SS rats. Potassium was higher in W than SS (Table 3.30).

Mineral concentrations were somewhat higher overall than Furuse's male rats. This may reflect differences in methods. Lower values were found in Furuse' study because the entire bone was used. Our studies used 5mm fat free cortical shaft femur sections from which higher concentrations of mineral were obtained. In addition, Liang

has found that female rats generally have higher mineral content than males and lower body weights.

Table 3.30 Femur and Ulna Compositional Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 2% Study

Variable (%)	p	strain	n	Mean	SEM
Calcium, overloaded femur	<0.015	W	10	28.55	± 0.115
		SS	12	29.24	± 0.212
Calcium, underloaded femur	ns	W	10	29.40	± 0.176
		SS	12	29.43	± 0.186
Magnesium, underloaded femur	<0.034	W	10	0.486	± 0.005
		SS	12	0.472	± 0.004
Potassium, ulna	<0.011	W	10	0.093	± 0.006
		SS	12	0.063	± 0.008

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.5.2 Drinking Fluid Treatment

3.5.2.1 1% Study Saline Effect

Significant differences in femur magnesium concentrations were found in overloaded but not underloaded femur or ulna in the 1% Study (Table 3.31). As the

concentration of saline increased from 1% Study to 2% Study, this magnesium difference became more robust.

Table 3.31 The Effect of Saline Treatment on Femur and Ulna Magnesium, 1% Study

Variable (%)	p	Treatment	n	Mean	SEM
Magnesium, ulna	ns	water	11	0.422	±0.010
		saline	11	0.415	±0.014
Magnesium, underloaded femur	ns	water	11	0.535	±0.006
		saline	11	0.567	±0.027
Magnesium, overloaded femur	< 0.003	water	11	0.542	±0.004
		saline	11	0.523	±0.005

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA.

As previously noted, the 1% salt treatment was insufficient to overcome the range of variability, which was clearer in the 2% Study. Nonetheless, the 1% Study is important, as it shows the level below which the salt signal strength becomes masked by many other physiological variables. However, significant reductions in femur magnesium concentrations between the rats on saline and the rats on water were found in the overloaded femur of the 1% Study.

3.5.2.2 2% Study Saline Effect

Major bone mineral elements were measured as above in the 1% Study (see section 3.5.1.1). Two percent saline markedly reduced magnesium levels in the saline treated over the water controls (Table 3.32) in the overloaded and the underloaded femur

bone (p <0.001 and p <0.001, respectively). As in the 1% Study, ulna magnesium was non-significantly reduced in rats on 2% saline (Table 3.32).

Table 3.32 The Effect of Saline Treatment on Femur and Ulna Magnesium, 2% Study

Variable (%)	p	Treatment	n	Mean	SEM
Magnesium, ulna	ns	water	12	0.416	±0.003
		saline	10	0.407	±0.006
Magnesium, underloaded femur	< 0.001	water	12	0.493	±0.005
		saline	10	0.463	±0.005
Magnesium, overloaded femur	< 0.001	water	12	0.478	±0.004
		saline	10	0.455	±0.003

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.5.3 Tukey Pairwise Post Hoc Results and One-way ANOVA for Both Genotype and Saline Treatment

3.5.3.1 1% Study Using One-way ANOVA for Both Genotype and Saline Treatment

In order to determine if there were differences between the means of the WC, WS, SSC, and SSS groups (Table 3.33), a one-way ANOVA was performed. A significant overall variation between the 4 groups was seen for magnesium in the overloaded femur ($p < 0.002$). A Tukey–Kramer Multiple Comparisons Test was applied to each group (WC, WS, SSC, and SSS). In relevant pairwise combinations (Table 3.33) magnesium in the overloaded femur tended to be higher in the WS than in the SSS group ($p < 0.066$) suggesting that magnesium concentration of the W strain was higher than the SS strain with the saline treatment. Magnesium in the overloaded femur tended to be higher in the SSC than in SSS group ($p < 0.070$) suggesting that SS groups' overloaded femur magnesium was lowered by saline treatment.

Table 3.33 Overloaded Femur Bone Magnesium Measurement for the 1% Study Using ANOVA for Both Genetic and Saline Treatment

Variable (%)	p	group	n	Mean	SEM
Magnesium, overloaded femur	<0.002	WC	5	0.550	± 0.004
		WS	6	0.533	± 0.006
		SSC	5	0.534	± 0.005
		SSS	6	0.513	± 0.006

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

In order to determine if there were differences between the means of the WC, WS, SSC, and SSS groups (Table 3.34), a one-way ANOVA was performed for zinc at the three sites. A significant overall variation between the 4 groups was seen for zinc in the overloaded and underloaded femur. A Tukey Test was applied to each group (WC, WS, SSC, and SSS). Zinc in the underloaded and the overloaded femurs tended to be higher in the WS than in the SSS group (p <0.093 and p <0.015, respectively). Zinc in the underloaded femur tended to be higher in the WC than in the SSC group (p <0.079). These results suggest that zinc concentration in the overloaded and the underloaded femurs of the W strain was higher than the SS strain, both with and without saline treatment.

Table 3.34 Femur and Ulna Bone Zinc Measurements for the 1% Study Using ANOVA for Both Genetic and Saline Treatment

Variable (ppm)	p	group	n	Mean	SEM
Zinc, underloaded femur		WC	5	340.4 ±	32.0
	< 0.016	WS	6	347.8 ±	18.5
		SSC	5	271.8 ±	5.1
		SSS	6	287.3 ±	2.7
Zinc, overloaded femur		WC	5	332.4 ±	30.6
	< 0.006	WS	6	370.0 ±	26.5[c]
		SSC	5	269.8 ±	5.1
		SSS	6	275.8 ±	4.2
Zinc, ulna	ns	WC	5	227.6 ±	17.5
		WS	6	268.8 ±	22.9
		SSC	5	199.4 ±	5.0
		SSS	6	229.3 ±	33.8

The p-value is shown for overall comparison of variability between the 4 groups the p-value is shown; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

Calcium in the underloaded femur tended to be higher in the SSC than in the WC group (p <0.097) and in the SSS than in the WS group (p <0.015), suggesting that the SS strain had a higher calcium concentration in the underloaded femur limb than the W strain, both with and without saline treatment (Table 3.35).

Table 3.35 Femur Bone Calcium for the 1% Study Using ANOVA for Both Genetic and Saline Treatment

Variable (%)	p	group	n	Mean	SEM
Calcium, underloaded femur	<0.003	WC	5	28.34	± 0.397
		WS	6	28.68	± 0.511 [c]
		SSC	5	29.66	± 0.175
		SSS	6	30.33	± 0.196

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.5.3.2 2% Study Using One-way ANOVA for Both Genotype and Saline Treatment

As in the 1% Study, to determine if there were differences between the means of the WC, WS, SSC, and SSS groups (Table 3.36), a one-way ANOVA was performed in the 2% study. A significant overall variation between the 4 groups was seen for magnesium in all three sites tested: the underloaded femur (p <0.001), the overloaded femur (p <0.009), and the ulna (p <0.043).

A Tukey Test was applied to each group (WC, WS, SSC, and SSS). Magnesium in the overloaded and the underloaded femurs tended to be higher in the WC than in the WS group (p <0.13 and p <0.12, respectively), suggesting magnesium was lowered by saline treatment. Magnesium (%) in the overloaded and the underloaded femurs was significantly higher in the SSC than in the SSS group (p <0.027 and p <0.004, respectively), indicating, magnesium was lowered by saline treatment. Magnesium (%)

in the ulna was significantly higher in the SSC than in the SSS group (p <0.049), again indicating, magnesium was lowered by saline treatment. Magnesium in both femurs was significantly higher in the SSC than in the SSS group. While post hoc tests for the W vs SS femurs were not quite significant, two rats had died from the W group on saline treatment. One more animal may have made results significant.

Table 3.36 Femur and Ulna Bone Magnesium Measurements for the 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable (%)	p	group	n	Mean	SEM
Magnesium, ulna		WC	6	0.412 ±	0.005
	< 0.043	WS	4	0.420 ±	0.011
		SSC	6	0.420 ±	0.004[d]
		SSS	6	0.398 ±	0.004
Magnesium, underloaded femur		WC	6	0.497 ±	0.008
	< 0.001	WS	4	0.475 ±	0.009
		SSC	6	0.488 ±	0.005[d]
		SSS	6	0.455 ±	0.002
Magnesium, overloaded femur		WC	6	0.473 ±	0.006
	< 0.009	WS	4	0.453 ±	0.003
		SSC	6	0.482 ±	0.007[d]
		SSS	6	0.457 ±	0.006

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

Zinc, which was previously noted in both the 1% Study and the 2% Study for its significant genetic effect, did not demonstrate significant overall variation between groups in the 2% Study though a higher trend is seen for W than SS (Table 3.37).

Table 3.37 Femur and Ulna Bone Zinc Measurements for the 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable (ppm)	p	group	n	Mean		SEM
Zinc, ulna		WC	6	261.3	±	32.4
	ns	WS	4	269.8	±	19.9
		SSC	6	220.5	±	6.8
		SSS	6	224.5	±	4.1
Zinc, underloaded femur		WC	6	348.8	±	29.1
	ns	WS	4	347.0	±	28.2
		SSC	6	287.0	±	9.1
		SSS	6	307.7	±	14.6
Zinc, overloaded femur		WC	6	287.5	±	26.6
	ns	WS	4	278.0	±	23.0
		SSC	6	265.8	±	8.4
		SSS	6	277.3	±	4.4

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS. No significant differences were seen.

Significant overall variation between the 4 groups was seen for bone calcium and sodium in the 2% Study's overloaded femur, but no pairwise comparisons were significant (Table 3.38).

Table 3.38 Overloaded Femur Bone Calcium and Sodium for the 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable (%)	p	group	n	Mean	SEM
Calcium, overloaded femur	<0.050	WC	6	28.54	± 0.186
		WS	4	28.56	± 0.111
		SSC	6	28.99	± 0.332
		SSS	6	29.49	± 0.251
Sodium, overloaded femur	<0.049	WC	6	0.673	± 0.019
		WS	4	0.623	± 0.023
		SSC	6	0.607	± 0.020
		SSS	6	0.682	± 0.023

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS. No significant differences were seen.

3.5.3.3 Summary of 1% and 2% Study Using ANOVA for Both Genetic and Saline Treatment Effects

Saline in the 1% Study significantly reduced femur magnesium concentrations in the overloaded femur as indicated by two-way ANOVA. In the 2% Study, saline treated had reduced magnesium levels compared to water controls in both the overloaded and underloaded femur bones (p <0.001 and p <0.001, respectively).

All 3 bone sites tested in the 2% Study were found to have significant overall variation in bone magnesium between the 4 groups: Tukey pairwise testing indicated significant reductions due to 2% saline at all 3 sites in the SS group but only trends due to salt for smaller W group.

3.5.4. Femur Overloading/Underloading

3.5.4.1 1% Study

The 1% Study was influenced by the weight gain of W over SS, perhaps affecting underloading/overloading technique results in Table 3.39.

Table 3.39 Effect of Underloading and Overloading on Femur Bone Composition in All Rats of the 1% Study

Variable	p	U/O	n	Mean		SEM
Calcium (%)	<0.001	O	22	30.03	±	0.116
		U	22	29.28	±	0.240
Phosphorus (%)	<0.009	O	22	13.87	±	0.082
		U	22	13.55	±	0.093
Sodium (%)	<0.001	O	22	0.574	±	0.010
		U	22	0.641	±	0.015
Potassium (%)	<0.039	O	22	0.222	±	0.016
		U	22	0.277	±	0.018
Boron (ppm)	<0.028	O	22	47.95	±	3.97
		U	22	59.27	±	3.13

The main effects assessed in this table are for the loading stress and include the 4 groups (WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline). n, no. of rats; O, overloaded femur ; U, underloaded femur. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.5.4.2 2% Study

Mineral concentration of calcium, magnesium, and phosphorus were significantly higher in the underloaded femur than in the overloaded femur (Table 3.40). While more mineral concentration is usually thought of as stronger bone, an excess of mineral (as calcium, magnesium, and phosphorus) with less collagen in the lattice would create stiffer and much less flexible bone. This is consistent with our finding of stiffer bone in underloaded femurs when compared to overloaded femurs in the modulus of elasticity (Table 3.22).

Table 3.40 Effect of Underloading and Overloading of Femur Bone Composition in All Rats of the 2% Study

Variable	p	U/O	n	Mean		SEM
Calcium (%)	<0.009	O	22	28.93	±	0.145
		U	22	29.42	±	0.126
Magnesium (%)	<0.005	O	22	0.467	±	0.004
		U	22	0.479	±	0.005
Phosphorus (%)	<0.001	O	22	13.00	±	0.051
		U	22	13.33	±	0.062
Zinc (ppm)	<0.002	O	22	277.09	±	8.27
		U	22	320.40	±	11.30

The main effects assessed in this table are for the loading stress and include the 4 groups (WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline). n, no. of rats; O, overloaded femur ; U, underloaded femur. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.6 TIBIA AND VERTEBRA HISTOMORPHOMETIC ANALYSES

3.6.1. Genetic Structural Effect

3.6.1.1 1% Study Genetic Effect

When W rats allowed to eat ad lib significant increases in cancellous bone structure over SS rats were recorded (Table 3.41).

Table 3.41 Underloaded Tibia Histomorphometric Measurements in Female Wistar and Salt Sensitive Hypertensive Rats, 1% Study

Variable	p	strain	n	Mean		SEM
Bone volume/tissue volume, %	<0.005	W	11	18.56	±	3.47
		SS	11	8.16	±	1.37
Bone surface, mm^2/mm^3	<0.006	W	11	6.579	±	0.468
		SS	11	4.189	±	0.500
Trabecular thickness, μm	<0.046	W	11	72	±	13
		SS	11	47	±	2
Trabecular number	<0.005	W	11	2.584	±	0.184
		SS	11	1.645	±	0.196
Trabecular separation, μm	<0.019	W	11	335	±	33
		SS	11	686	±	121

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA

3.6.1.2 2% Study Genetic Effect

When weight was controlled no significant differences were found between W and SS rats in cancellous bone structure (Table 3.42).

3.6.1.3 Summary of 1% and 2% Study Genetic Effects from 2-Way ANOVA

For some perspective on female rat cancellous bone volume, controls in Iwamoto et al.'s 1999 study are reported. Their 12 and 16 week old female Sprague Dawley rat control vertebrae bone volume ranged from 25-26% of tissue volume. Their average female Sprague Dawley rats' control tibial bone volume ranged from 15% of tissue volume at week 12 to 16% at week 16.

The present study's Sprague Dawley female rats were 14 weeks of age at the end of study. Noteworthy in the 2% Study is that the vertebrae of both W and SS, contained 25-27% cancellous bone by volume, which is normal cancellous bone growth for female Sprague Dawley rats. However, the range of cancellous volume was 8-19% in tibias of the 1% Study (Table 3.41). The 19% group was the W underloaded tibia from the 1% Study. The 1% Study W group's body weight was 6.7% heavier than the 1% Study SS group. When W in the 2% Study were food restricted to the amounts eaten by SS rats, their tibia bone volume fell to the 6-12% range of SS rats (Table 3.42). This decreased range (6-12%) may have resulted from the loading stress technique applied to SS rats and from loading stress plus food restrictions applied to the W rats of the 2% Study. The technique may have reduced adolescent cancellous bone growth in tibias, by as much as 61% in underloaded and as much as 33% in overloaded when compared to Iwamoto et al. (1999) study's female Sprague Dawley rat controls.

Table 3.42 Vertebrae and Tibia Bone Volumes / Tissue Volume in Female Wistar and Salt Sensitive Rats, 1% and 2% Study

Bone volume/tissue volume, %	p	strain	n	Mean		SEM
Underloaded tibia, (1% Study)	<0.005	W	11	18.56	±	3.47
		SS	11	8.16	±	1.37
Overloaded tibia, (1% Study)	ns	W	11	11.40	±	1.34
		SS	11	11.56	±	2.45
Underloaded tibia, (2% Study)	ns	W	10	6.18	±	0.96
		SS	12	6.26	±	1.52
Overloaded tibia, (2% Study)	ns	W	10	11.90	±	2.54
		SS	12	9.76	±	1.17
L-2 vertebra, (2% Study)	ns	W	10	24.91	±	1.65
		SS	12	26.75	±	2.24

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±*SEM*; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA

3.6.2 Drinking Fluid Treatment Structural Effect

3.6.2.1 1% Study Saline Effect

There was not a significant difference in tibia cancellous bone between rats on 1% saline and rats on water, when SS and W strain data were pooled. Tabled data for individual group differences is listed (Table 3.44), but no discussion follows.

3.6.2.2 2% Study Saline Effect

As the concentration of saline increased from the 1% Study to the 2% Study differences in bone volume and structure became more apparent. Marked reductions from the 2% saline treatment were seen in tibia cancellous bone in the W and SS (Table 3.43). Significant differences were found only in the overloaded but not the underloaded tibia in the 2% Study.

Table 3.43 The Effect of Saline Treatment on Overloaded Tibia Histomorphometric Measurements, 2% Study

Variable	p	Treatment	n	Mean		SEM
Bone vol./tissue vol., %	<0.018	water	12	13.51	±	1.87
		saline	10	7.39	±	1.15
Bone surface, mm^2/mm^3	<0.014	water	12	5.614	±	0.596
		saline	10	3.916	±	0.504
Trabecular thickness, μm	<0.012	water	12	60	±	4
		saline	10	47	±	2
Trabecular number	<0.042	water	12	2.205	±	0.234
		saline	10	1.538	±	0.198
Trabecular separation, μm	<0.139	water	12	448	±	50
		saline	10	830	±	237

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.6.3 Tukey Pairwise Post Hoc Results and One-way ANOVA for Both Genotype and Saline Treatment

3.6.3.1 1% Study Using One-way ANOVA for Both Genotype and Saline Treatment

While one-way ANOVA with four levels was used to compare overall variability between the 4 groups in the 1% Study were significant, only one pairwise Tukey result was significant (Table 3.44). As there was also an overall genetic difference in the underloaded femur, this result may reflect partially on differing body weights between strains in the 1% study.

Table 3.44 Underloaded Tibia Histomorphometric Measurements for the 1% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean		SEM
Bone vol./tissue vol., %	<0.013	WC	5	24.71	±	6.20[b]
		WS	6	13.44	±	2.62
		SSC	5	8.16	±	1.03
		SSS	6	8.15	±	2.49
Bone surface, mm^2/mm^3	<0.029	WC	5	6.821	±	0.411
		WS	6	6.377	±	0.820
		SSC	5	4.228	±	0.409
		SSS	6	4.156	±	0.896
Trabecular thickness, μm	<0.049	WC	5	94.80	±	26.90
		WS	6	52.14	±	3.49
		SSC	5	48.54	±	1.63
		SSS	6	46.48	±	4.19
Trabecular number	<0.029	WC	5	2.679	±	0.161
		WS	6	2.505	±	0.322
		SSC	5	1.661	±	0.161
		SSS	6	1.632	±	0.352

The p-value is shown for overall comparison of variability between the 4 groups; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline. n, no. of rats; For pairwise comparison significant levels $p < 0.05$; [a]WC vs WS; [b]WC vs SSC; [c]WS vs SSS; [d]SSC vs SSS.

3.6.3.2 2% Study Using One-way ANOVA for Both Genotype and Saline Treatment

In order to determine if there were differences between the means of the WC, WS, SSC, and SSS groups, a one-way ANOVA was performed. Unlike the two-way ANOVA (Table 3.43), significant overall variation between the 4 groups was not seen for in tibia cancellous bone between rats in the 2% study (Table 3.45). The reason for this apparent discrepancy is that the variance in the two-way ANOVA is factored into two overall main effects, not four, as is done in the one-way. Change in the structural histomorphometry of the tibias or in the vertebrae was not expected, as cancellous bone is usually thought of as deteriorating with aging after menopause.

Table 3.45 Overloaded Tibia Histomorphometric Measurements for the 2% Study Using ANOVA for Both Genetic and Saline Treatment

Variable	p	Group	n	Mean		SEM
Bone vol./tissue vol. , %		WC	6	15.28	±	3.51
	< 0.077	WS	4	6.84	±	1.86
		SSC	6	11.75	±	1.37
		SSS	6	7.76	±	1.59
Bone surface, mm^2/mm^3		WC	6	6.420	±	1.050
	< 0.080	WS	4	3.625	±	0.627
		SSC	6	4.812	±	0.448
		SSS	6	4.110	±	0.765
Trabecular thickness, μm		WC	6	57.06	±	5.15
	< 0.066	WS	4	45.86	±	5.03
		SSC	6	62.46	±	5.06
		SSS	6	47.88	±	2.47
Trabecular number		WC	6	2.52	±	0.413
	< 0.107	WS	4	1.42	±	0.246
		SSC	6	1.89	±	0.176
		SSS	6	1.61	±	0.301

Values are the means ±*SEM;* n, no. of rats. WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Significant levels: * $p < 0.05$.

3.6.4 Tibia Overloading/Underloading Structural Effect

3.6.4.1 Tibia 1% Study

In the 1% Study tibia histomorphometric analysis, no significant differences were found due to underloading or overloading.

3.6.4.2 Tibia 2% Study

Bone volume/tissue volume and trabecular number in the overloaded bone was significantly greater than in the underloaded bone (Table 3.46). Trabecular separation in the overloaded bone was significantly reduced when compared to the underloaded bone. Our 2% Study is consistent with previous findings (Maeda et al., 1993) that the overloaded bone displayed significantly more volume and higher trabecular number in the overloaded tibia and less separation between trabecular bone in the underloaded bone. Maeda et al. in a similar 6 week immobilization study on 3 month old adult female Sprague-Dawley rats found a significant 76% difference between the immobilized limb and the control rat limbs. Non-bandaged control rats were not used for the underloading / overloading techniques in our study.

Table 3.46 Significant Effect of Underloading and Overloading on Tibia Histomorphometric Measurements in All Rats of the 2% Study

Variable	p	U/O	n	Mean	SEM
Bone Volume/Tissue Volume, %	<0.011	O	22	10.730	± 1.300
		U	22	6.222	± 0.916
Trabecular Number	<0.001	O	22	1.902	± 0.169
		U	22	1.052	± 0.113
Trabecular Separation, μm	<0.011	O	22	0.622	± 0.116
		U	22	1.253	± 0.196

The main effects assessed in this table are for the loading stress and include animals from the 4 groups (WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive, treated with saline). n, no. of rats; O, overloaded femur ; U, underloaded femur. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.6.5 Tibia Genetic Turnover Effect

3.6.5.1 1% Study Genetics, Active Resorption and Formation

As this study also looked at genetic developments, only one difference was found in the active resorption (osteoclast surface / bone surface) of tibia bone measurements (Table 3.47). While more osteoclasts are found in W, this may be due to the growth differences from increased body weight in 1% Study. When body weight was controlled (2% Study), no genetic differences were found in active resorption (ARS) in tibias of rats.

No bone formation differences were observed in any groupings.

Table 3.47 Active Resorption (Osteoclast Surface / Bone Surface,%) of Underloaded Tibia Bone in Female Wistar and Salt Sensitive Rats, 1% Study

Variable (%)	p	Strain	n	Mean	SEM
Active resorption, underloaded tibia	< 0.020	W	11	0.516	± 0.036
		SS	11	0.341	± 0.059

The main effects of genetic strain are shown, which includes animals with and without saline added to their diet. Values are the means ±SEM; n, no. of rats. W, wistar; SS, salt sensitive. Significance levels were set at 0.05, and determined by two-way ANOVA.

3.6.5.2 2% Study Genetics Active Resorption and Formation

In looking at genetic developments, no differences in resorption and formation measurements were found in the 2% Study.

3.6.6 Tibia Saline Turnover Effect

3.6.6.1 1% Study Active Resorption and Formation

Because Furuse et al. (1992) found differences in osteoclast measurements in adolescent male SHR on 1% saline in a longer 7 week study, histomorphometric measurements were made for both the 1% and 2% Study. While a salt trend was observed in the 1% Study, there were no overall differences between groups in female tibia cancellous bone turnover. Saline treated rats did show an increase in active resorption over water control in the underloaded tibia. Vertebrae were not looked at in this study. No bone formation differences were observed in any groupings.

3.6.6.2 2% Study Active Resorption and Formation

Histomorphometric active resorption measurements were made in the underloaded tibia, the overloaded tibia and the vertebrae in the 2% Study. While a salt trend was observed, there was not a significant difference in active resorption (ARS) in vertebrae or in tibias of rats on saline and rats on water in 2% Study. Saline treated rats did show a non-significant increase over water control at all sites tested (Table 3.48). The active resorption of L-2 vertebrae in saline treated was 41% more than water control. The active resorption of underloaded tibia was 24% more than water control and the active resorption overloaded tibia was 28% more than water control. No bone formation differences were observed in any groupings.

Table 3.48 The Effect of Saline Treatment on Active Resorption (Osteoclast Surface / Bone Surface), 2% Study

Variable, %	p	Treatment	n	Mean	SEM
L-2 vertebrae	< 0.177	water	12	0.086	± 0.016
		saline	10	0.121	± 0.016
Underloaded tibia	< 0.142	water	12	0.370	± 0.033
		saline	10	0.458	± 0.072
Overloaded tibia	< 0.133	water	12	0.391	± 0.045
		saline	10	0.501	± 0.050

The main effects assessed in this table are for the addition of saline to the diet, and comprise animals of both W and SS genetic strains. Values are the means ±*SEM*; n, no. of rats. Significance levels were set at 0.05, and determined by two-way ANOVA.

CHAPTER IV

DISCUSSION

4.1 OVERVIEW

The purpose of the present study was to examine genetic predisposition to physiological change induced by salt and what, if any, application this would have to a spaceflight mission. Two modest levels of dietary saline treatment (1% and 2% saline ad libitum) were combined with two differing genetic strains of rats [a normotensive (W) and a salt sensitive hypertensive (SS)]. All animals were subjected to an immobilization bandage procedure to simulate spaceflight. Significant changes, due either to genetics, saline treatment, the bandage technique were found in the following parameters:

Reduction in peak bone mass

Reduction in femur bone cross -sectional area

Loss of tibia cancellous bone

Elevation in femur stiffness

Elevation in femur strength

Reduction in bone magnesium concentration

Reduction in bone zinc concentration

Elevation in bone calcium concentration

Elevation in blood pressure and heart rate

To make the results easier to interpret, ANOVA tests were applied to all data to estimate with more precision the overall effects of 1) genetics, 2) saline treatment and/or 3) underloading/overloading technique (Box et al., 1978). The results of these ANOVA

suggest both genetics and saline treatment had significant effects on bone, and that often saline treatment enhanced the genetic effect. Two percent saline generally had an expanded and more pronounced effect without interactions, than the one percent saline treatment, as the concentration of saline was increased. All of the discussion centers on the 2% saline study's results except where noted. In addition, the immobilized and weightbearing femurs simulating adjustments to microgravity and recovery when returning from space, produced marked results. Interestingly, loading effects also combined with saline treatment and genetic groups, producing a stronger cumulative adverse effect. Individual results are discussed under the following headings.

Genetics and bone

Salt and bone

Salt enhanced genetic effects

Space and bone

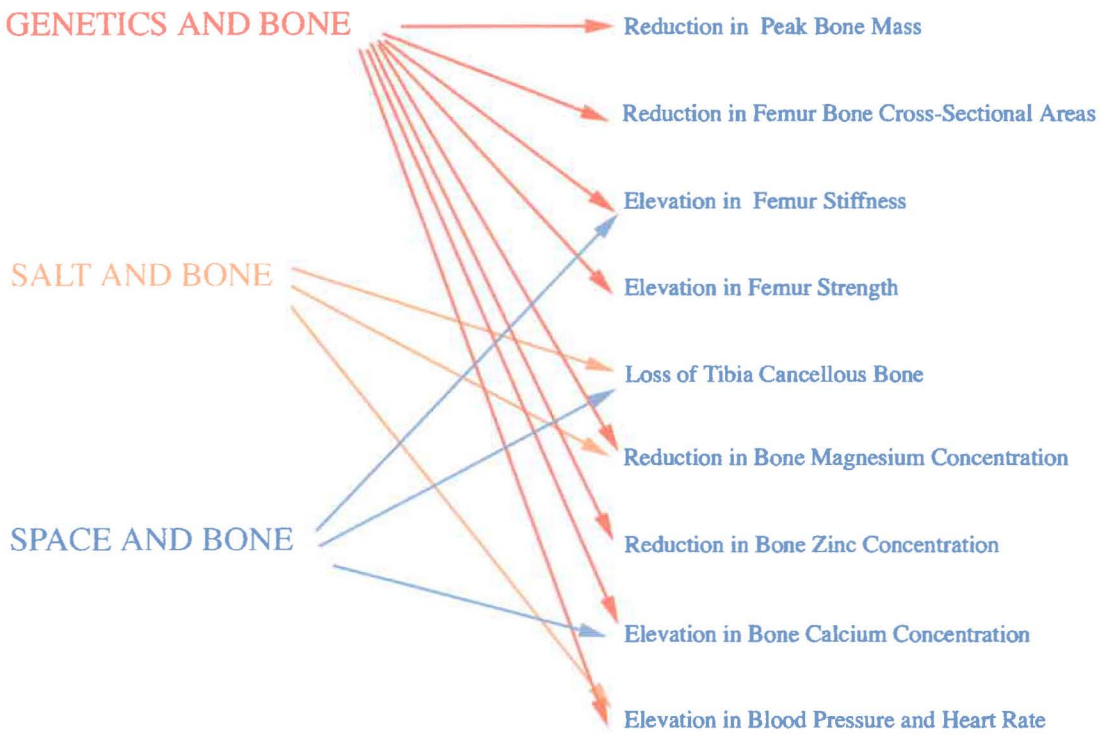

Figure 4.1. Summary chart of significant effects, due to genetics, saline treatment, the underloading/overloading technique

Genotype and high salt intake have both been associated with hypertension and osteopenia in the adult rat, but there may be differences in the effects at a very early age in females before a developed hypertension can be detected and before peak bone mass is reached. Many studies only look at urine excretions; however, damage may occur before urine analysis can detect an abnormal calcium leak or a bone calcium concentration decrease. At some sites, genotype, salt, and inactivity combine contributing to bone

anormalities that increase risk to fracture (Figure 4.1). Bones at various sites with differing ranges of mechanical usage were also examined. Adverse effects were seen with immobilization which itself interacted with salt and genotype enhancing deleterious effects.

4.2 GENETICS AND BONE

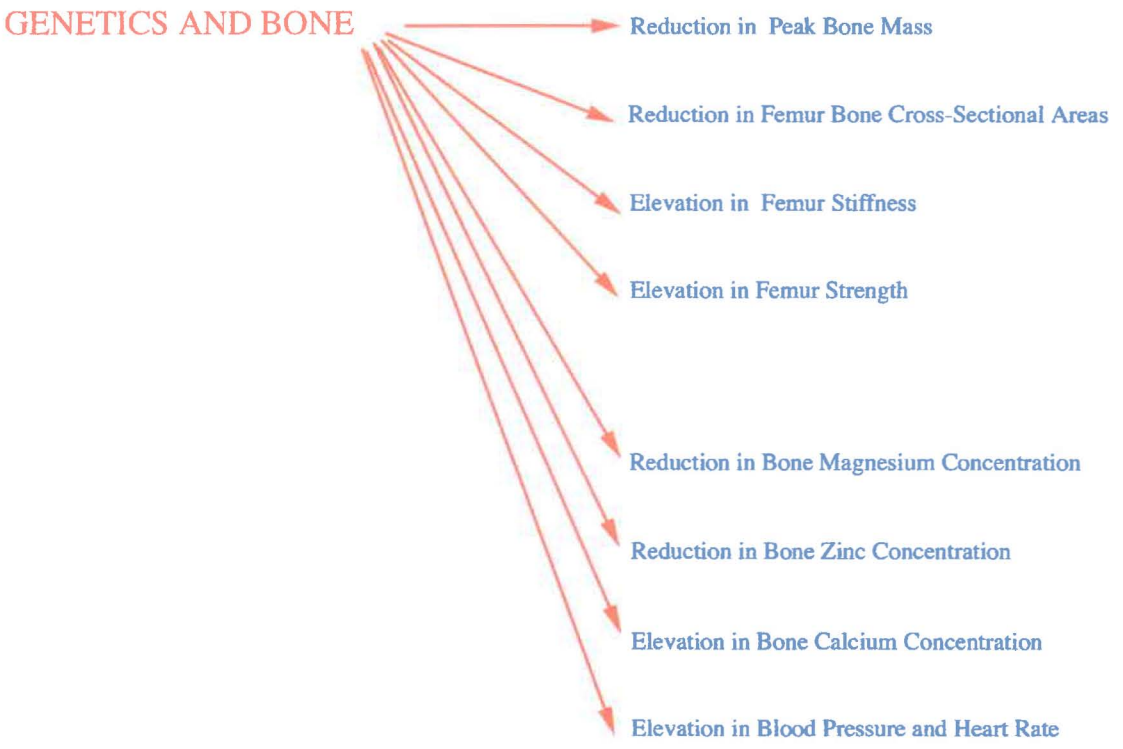

Figure 4.2. Summary chart of significant effects due to genotype

Most of the genetic differences in the present study are unique data for an adolescent female model and may contribute toward our understanding of the

mechanisms involved in attaining peak bone mass and the of pathophysiology of bone fracture in aging females (Figure 4.2).

4.2.1 Blood Pressure, Heart Rate and Body Weight

Only a few female rat studies (Lau et al., 1984; Liang et al., 1997) have compared skeletal parameters of female SHR and WKY rats at differing bone sites. Liang et al.'s study also included the measurements of blood pressure and heart rate. Previously Lau et al. (1984) had examined female SHR rat's bone calcium metabolism, however, it used a blood pressure medication to lower blood pressure complicating the interpretation (Lau et al., 1984). Lau et al.'s study of SHR treated for hypertension did not demonstrate an adverse effect in female SHR calcium per gram of tibial and femoral bone in one year's time.

Liang et al. examined female WKY and SHR age 23-31 weeks of age. Liang's rats arrived at 12 weeks and began a lengthy acclimation period during which biweekly weight, heart rate and blood pressure were made. During Liang's study, SHR maintained the lowered body weight upon their arrival below their age matched WKY, when fed ad lib. As expected, SHR exhibited and maintained elevated blood pressure and heart rate over WKY rats. Liang,'s control rats at 14 weeks had a systolic blood pressure of approximately 210 mmHg. This is congruent with Rapp and Dene's (1985) control Spague Dawley SHR rats (also from the present studies' supplier), at age14 weeks, had systolic blood pressure readings averaging 199 mmHg.

In the present studies, SS control rats in the 1% study averaged 136 mmHg and in the 2% study 123 mmHg, substantially lower than readings from Liang's and Rapp and Dene's studies. Because the present studies were measuring each individual rat's food

and fluid intake, each rat had to be kept in an individual cage and socially isolated. Rapp and Dene's (1985) control rats were housed 4-6 rats per cage. The lowered blood pressures of young female control rats (in the present studies) at a similar age, in comparison to other SHR controls in other studies (Liang et al., 1997; Rapp and Dene, 1985) without saline treatment, may indicate that chronic social isolation can temporarily reduce blood pressure levels to normotensive levels. This is consistent with previous studies (Hallback, 1975) in which social isolation lowered the development of SHR hypertension, whereas, there was no effect on WKY. This may indicate that lowering stress levels can lower blood pressure in females, but it is unknown if the beneficial effect on female SHR blood pressure is linked to, and/or had a beneficial effect on the simultaneous adverse effect on bone that resulted in this strain.

The results in 2% saline study strain examination for heart rate and blood pressure were similar to observations recorded from 1% study. Both are novel, as little measurement of rat blood pressure/bone parameters has been done on growing, genetically-defined SS and W female rats under immobilization and chronic social isolation stress. It appears that the rats had adapted to the chronic immobilization stress because those without saline treatment had near normal blood pressure readings in both studies. Yamori et al. (1969) found an elevated blood pressure response in SHR over WKY in response to a differing immobilization stress (2 limbs supported) than the present studies.

The present studies indicated markedly elevated blood pressure and heart rates for the SS controls as compared with the W controls. However the systolic blood pressure readings were only marginally different (17 and 18 mmHg points in the 1% and 2%

studies, respectively). Whereas, in Liang's study, SHR controls were approximately 75 mmHg apart from WKY at the same age indicating in Liang's study advanced hypertension in SHR at 14 weeks.

When rats arrived for the 1% study, the age matched strains exhibited a 3% difference in initial weight with wistar normotensive weighing more than hypertensive rats. At autopsy, the normotensive weighed significantly (6.7%) more than hypertensive rats. Researchers have reported body weight may affect the results of bone studies (Liang et al., 1997). Metz et al. (1990) suggest that increased body weight of their male normotensive rats over hypertensive rats accounted for increased bone density, rather than genetic difference. For example, if normotensive rats had heavier bones this could be directly or proportionately linked to a heavier body weight. As in the 1% study, W and SS rats were age and successfully (breeder) weight-matched at beginning of experimental period for the 2% study. In order to insure no difference in body weight between the two strains, W were fed the same amount of food as SS rats in 2% study. At autopsy, the W weighed 1.5% less than SS rats (statistically non-significant).

4.2.2 Bone

The left ulna, the left femur and right femur fresh weight and length were obtained from each rat (Table 3.12 to Table 3.18) in an effort to accrue simultaneous multisite bone measurements. W body weight was higher than SS in the 1% study ($P<0.052$), however, W body weight was non significantly different from SS in the 2% study. In both the 1% and the 2% studies, the wet weight of the underloaded and the overloaded femur limbs of the W rats were significantly heavier than the SS rat femur

bones (Table 3.12 and Table 3.14). This result suggests that bone wet weight is most influenced by genetic strain irrespective of body weight.

Two femur length measurements were taken, from the ball of the proximal femur to distal end of femur and from the trochanter end of proximal femur to the distal femur. A single length measurement was made of each left ulna. All W femur length averages were non-significantly different than SS femur averages. The exception was that the overloaded ball to distal femur was significantly longer in the W ($p< 0.001$) in the 1% study. This extra growth may have been due to extra 6.7% food eaten in 1% study by W over SS rats and the overloaded exercise effect. As the extra bone weight was not closely associated with length, the morphometry of a 1mm cross-sectional transverse section of the midpoint of femur was examined. Bone area was significantly larger in both W femurs in both the 1% and 2% study. This indicates that the heavier W femur bone expanded more significantly in the transverse direction rather than in length. These results with the femur weights of previous paragraph suggest SS rats have thinner, lighter bones.

Liang's more mature SHR rats also exhibited differences in bone parameters. Liang et al. found less cortical cross-sectional tissue bone area in SHR tibial shaft than WKY controls. Less cortical bone in the cross sectional samples in Liang's SHR was consistent with the present 1% and 2% study SS controls compared to W femur cross-sectional area.

Liang et al. found more cancellous bone in SHR than WKY controls. In the present 2% study, no difference was found between the SS control and the W control tibia cancellous structural parameters or in the 1% overloaded tibia. In the 1% study's

immobilized tibia the underloading effect interacted with the salt effect producing more cancellous bone in W when compared to SS rats. Both Liang's and the present study differs from male SHR in Yamori et al. (1991) where less cancellous bone was found when compared to WKY bone. Interestingly, Liang's SHR lost cancellous bone from 23 to 31 weeks, suggesting earlier age related changes, as WKY did not lose bone. In addition, Liang's SHR exhibited reduced cancellous bone when treated by ovariectomy (simulating postmenopausal conditions) compared to WKY. Reductions (as found in the present study) in cortical peak bone mass in a young, growing SS female rats (followed by the potential risk of continued abnormalities in cancellous bone as shown in Liang's study) suggest the salt sensitive strain is at much greater risk to osteopenia than the normotensive beginning in adolescence.

The SS strain was found to display a significant increase in breaking strength in both the overloaded femur and the underloaded femur (Table 3.20). In addition, a marked increase in stiffness was found in SS strain in both the overloaded femur and the underloaded femurs (Table 3.20). Intially, the 3 point bending strength test found applied force measurements of each group not to be significantly different. However, to obtain the breaking strength and stiffness measurement (which separates the material from the size properties), diameters from the transverse section must be included in the calculations. These two calculations, which take into account the differences in transverse bone area, indicate that transverse bone area played a key role in genetic strength difference in both an overloaded and underloaded limb. As the results indicate, the SS strain exhibited a thinner, but stronger and stiffer bone which may indicate more rapid aging and acceleration of the maturation process (Matsuda et al., 1986; Currey,

1984). Thus, bone mechanical properties may not only be dependent on chronological age, but on genotype as well.

Previously, Inoue et al. (1995) has found peak bone mass in the tibia was reached at 18 weeks in SHR male and female rats and in 30-36 week in male and female SD rats. In Inoue's study, normotensive Sprague Dawley rats weighed more than SHR and had more Ca mineral (mg/cm^2) than SHR. In the present 2% study, more calcium mineral was not seen in our normotensives as they were fed the same nutrients and calcium as salt sensitive rats and perhaps because of their femur's large size and their own small body weight did not need additional calcium material support. A genetically derived template may be in place, in which, if proper nutrients are provided, a normotensive rat may be able to eat enough to fill with calcium in 30 or more weeks. On the other hand, the SHR is eating ad lib and approaching peak bone mass at 18 weeks.

When confronted with small bone weight and size, the physiology must adapt. The physiology adapts in the optimal way available. With a larger bone, lesser amounts of calcium may be necessary to support a femur, so until the normotensive rat gains more weight or acquires more calcium (as when it's growing time is extended), a stronger bone is not necessary. However, the potential is there in the future, but not for the SHR. The present studies indicate the SS may have a predetermined genetic limit for femur small bone size and small bone weight. When confronted with this genetic deadline and to minimize current damage, modifications are made at the expense of possible future damage. In other words, the SS rat's bone maintained strength, a necessary factor for the growing rat, but at the time may have sacrificed other bone qualities.

Breaking strength and stiffness differences may be a consequence of material differences resulting from anomalies found in measuring genetic mineral properties. Higher calcium content (mg/g) has been observed in adult female SHR femur bone tissue over WKY femur bone tissue previously by Lau et al.'s long term (one year) study. In the present studies, the overloaded femur, calcium was significantly higher in SS rat femurs than in W rat femurs (Table 3.30). In the underloaded femur, calcium was only slightly higher in SS rats than in W rats. This genotype calcium abnormality in overloaded bone may explain increased stiffness in overloaded SS bone over W but does not explain the result in underloaded bone. Calcium modulation in underloaded bone seems to be under the control of salt sensitivity. Similar to the present studies, in the LeBondel and Allain (1988) study, male SHR versus WKY tibia element analysis, no significant differences were found for bone calcium. SHR also showed reduced concentrations in zinc and magnesium in their study, as found with our present studies. Mineral and element concentrations were measured in three bone sites per rat in the 1% study and in the same 3 bone sites per rat in the 2% study. Femur zinc concentrations were higher in W bone than in SS in the six samples measured, 4 samples were significant.

Dietary zinc positively affects the outer periosteal envelope increasing bone area and tissue area in this region in growing rats (Ovsen et al., 2001). This area is particularly important as bone growth accelerates here during adolescence (Recker, 1992). While our studies did not delineate the periosteal envelope, we did examine the cross-sectional transverse cortical femur bone area which includes this section. W was significantly increased over SS suggesting more zinc available in W bone may be

necessary for periosteal bone area growth and development. Comparatively, the SS had significantly reduced cross-sectional transverse cortical area of femur bone here as well as reduced zinc concentrations in the cortical area of femur bone, which may indicate zinc had a role in cross sectional dimensions.

A few measurements from the 2% study suggest a genetic magnesium difference. Magnesium in the underloaded femur was significantly higher in the W than in the SS group (which included rats with and without saline treatment). As noted previously, in the LeBondel and Allain (1988) study, male SHR versus WKY element analysis, SHR exhibited reductions in magnesium but unlike our present studies were not on saline treatment. Decreased magnesium bone content has been reported in postmenopausal women with osteoporosis (Rude et al., 1999). How magnesium differences impact bone is discussed at length in the upcoming Salt and Bone section and the Saline Enhanced Genetic Effect section.

In summary, the hypertensive genotype significantly increased blood pressure and heart rate. The spontaneously hypertensive rat had significant reductions in overall bone mass, femur cross-sectional area, magnesium and zinc concentrations, with simultaneous elevations in femur stiffness, strength, and calcium. Despite no apparent weight difference between strains, SS bone was lighter and smaller and at the same time stiffer and stronger (perhaps to accommodate equal body weight stress). Interpretation and understanding of bone measurements and quality should be at the forefront. If only bone mineral content/density is investigated as done in many experimental studies and the majority of human studies, the meaning of calcium content results could be misinterpreted, if an overall assessment of genetics is not taken into consideration.

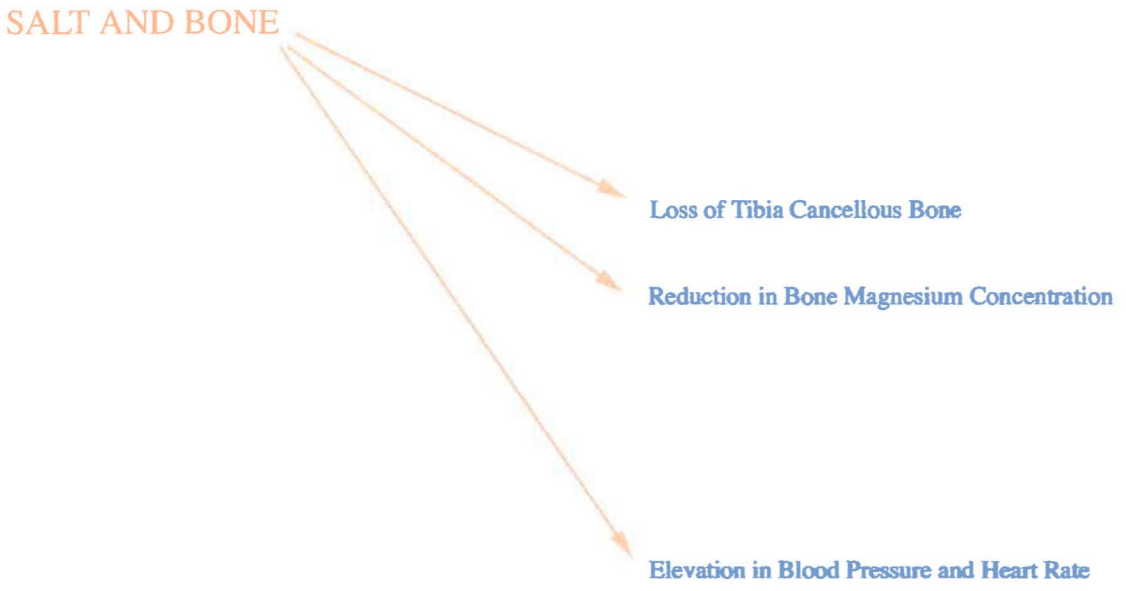

Figure 4.3. Summary chart of significant effects of saline treatment.

4.3 SALT AND BONE

The dose of NaCl consumed, as measured by mean salt intake/day, was not significantly different between W and SS strains in either the 1% or 2% study. Based on previous observations in salt preference testing (Ferrell and Gray, 1985; Furuse et al., 1992), it was expected the dose of saline would be elevated in SS rats due to SHR rat's salt preference, thereby effecting genetic strain bone and blood pressure differences between SS and W rats. The present study suggests that the SS genotype did not play a significant role in determining the amount of salt each rat consumed. While the present study did not give each rat a choice between water or saline treatment (such as in a

standard salt preference test) random assignment may be more relevant to today's urban society where little choice is available in the amount of salt processed food contains. Thus, importantly for the present study, differences detected (Figure 4.3) may be due to type of genetic strain, individual dose of saline, body weight characteristics, not excessive intake of saline by SS rats over W rats.

4.3.1 Blood Pressure and Heart Rate

There is great controversy on the mechanism involved in increasing blood pressure in salt-sensitive subjects. High sodium intake has been suggested to cause concurrent adverse changes in calcium and bone metabolism (Shortt and Flynn, 1990; MacGregor and Cappuccio, 1993). Elevations in sodium intake have been associated with elevations in blood pressure, urinary calcium excretion and urinary excretion of hydroxyproline [a bone resorption marker in humans (Chan et al., 1992; McCarron et al., 1980; McParland et al., 1989). Salt restriction has been shown to lower blood pressure in patients with mild and moderate hypertension (MacGregor et al., 1982). In addition, salt restriction may also lower urinary calcium excretion and hydroxyproline in normal postmenopausal females (Need et al., 1991).

A salt effect was not seen in the 1% study for blood pressure or for heart rate. In the 2% study, elevations from saline treatment were seen for blood pressure in both the W and SS. Heart rate was elevated with salt but did not reach significance. In both the 1% study and the 2% study, SS heart rates were higher than W heart rates consistent with Liang's (1997) observations.

Ferrell and Gray (1985) found blood pressure in rats begins to plateau around the 12 or 13th week. As discussed in genetic and bone chapter, 6 weeks of social isolation

may have lowered the development of SS controls' hypertension, whereas, there was no effect on W. This favorable effect on those rats on water appears to have been carried over even to those SS that were saline treated. While there was a significant increase in rat blood pressure between those on water and those on 2% saline, only 3 out of 12 rat blood pressures were in the 150 mmHg range, all other rats on saline treatment were below 140 mmHg. On the other hand, Liang,'s female SHR control rats at 14 weeks had a systolic blood pressure of approximately 210 mmHg without saline treatment. In response to salt treatment for a short period of time, Furuse's SHR male rats elevated blood pressure approximately 45 mmHg. Furuse et al. also isolated their SHR rats in individual cages, however, a more stressful 16 hour on / 8 hour off light-cycle was used. Furuse's special light-cycle (standard LAS light-cycles are 12 hour on / 12 hour off) may have had a more adverse effect on blood pressure in their study, as SHR are reported to have attenuated blood pressures when raised in dark (Lawler et al., 1993). In addition, it may be a gender difference that female rats respond to isolation better than males or have lower blood pressure than males. While the mechanism for gender difference is unclear, Reckelhoff et al. (2000) suggest that androgens promote the exacerbation of hypertension in male SHR via a mechanism involving the renin-angiotensin system, and Crews et al. (1999) points to gender-specific changes in vascular reactivity and Ca2+ entry which are increased in hypertension. In summary, SS in the present study on 2% saline treatment had significantly higher blood pressures than those on water but not nearly to the degree of other studies.

The present study may indicate that lowering stress levels for SS can lower blood pressure, but it is unknown if the beneficial effect on female SS blood pressure is linked

to, and/or had a beneficial effect on the simultaneous adverse effect on bone that resulted in this strain.

4.3.2 Bone

In a 1995 study, Navidi et al. investigated the effect of excess dietary salt in a rat SD normotensive, spaceflight model. No further reduction in calcium content was found apart from that induced by unloading the hind limbs. However, work by others on young growing male genetically defined SHR rats pointed to early adverse bone morphological changes induced by salt that would be missed by standard measurements of bone calcium content. It appears that salt structural damage to bone may not be strictly calcium content modulated, since these early adverse structural changes can preceed calcium content change.

In normotensive animal studies, high salt (as high as 8%) has been shown to augment calcium urinary excretion and reduce calcium content in bone (Goulding and Campbell, 1983; Goulding and Campbell, 1984) in studies of less than 3 months. In addition, 1.8% salt concentrations, as used in Chan and Swaminathan's (1993) four month study, produced increments in calcium excretion and hydroxyproline in normotensive rats. Previously, Chan and Swaminathan (1998) found that 1.8% salt concentrations increased calcium excretion, and moderately decreased calcium content of bone in a year long study. If the saline treatment is high enough (8%) as in short studies or at lower levels (2%) for approximately 20-50% of a normotensive rat's lifespan, reduced calcium content has been found.

Furuse et al. (1992) measured mineral content (calcium and magnesium) and mineral balance (intake, fecal excretion and urinary excretion) in a short (7 week), 1%

and 2% saline treatment (in food and saline) study on young growing male SHR. Salt treatment raised calcium excretion in 1% treatments but not significantly in 2% treatment. There was no effect on magnesium in any excretion. However, magnesium bone content was significantly decreased by salt intake (and calcium was not) in Furuse's rat femurs. The present study found significant reductions between the rats on saline and the rats on water in femur magnesium concentration in the overloaded femur of the 1% study and 2% in both strains. In addition, the 2% study demonstrated a more system–wide influence than 1% study. In the 1% study, magnesium was only significantly reduced in the overloaded femur. In the 2 percent study, reduced magnesium levels were found in the saline treated over water controls in both the femurs and ulna. While the overall variation between the 4 groups for magnesium in the 2% study was significant, post hoc tests on magnesium in the overloaded and the underloaded femurs only tended to be higher in the WC than in the WS group (p <0.13 and p <0.12, respectively). Magnesium in the ulna, in the overloaded and the underloaded femurs was significantly higher in the SSC than in the SSS group, indicating, magnesium was lowered by saline treatment. While little study had been done on adolescent female SHR bone, bone magnesium depletion was found in young growing SHR male rats (Furuse et al., 1992). However, the more systemic–wide magnesium depletion response to saline treatment (overloaded femur, underloaded femur and ulna) of the SS in comparison to W was interesting. The present study did not examine mineral balance and urinary excretion but if Furuse et al. study is correct, it is quite possible that urine analysis would not have been a sensitive enough test to pick up these early adverse changes in magnesium metabolism. This is quite important because presently Antonios and Macgregor (1995)

report increasing research between high salt intake and elevated urinary calcium excretion which may be associated with bone loss, but in fact abnormalities leading to earlier fracture risk may be occurring sooner and go undetected by urine analysis and bone density testing, (an indication of bone calcium content).

Furuse et al. did not find a difference in wet weight of the femur bone in either water controls or those with saline treatment. This is consistent with the present studies. No difference was found in wet weight of the femur bones between water controls or those with saline treatment. While Furuse et al. did not measure cancellous bone, their photographs (Furuse et al., 1992) exhibited low development in femur trabecular bone cut transversely in salt treated groups. To address this issue, the present studies measured and looked at standard sampling sites in longitudinal tibia sections. In the 2% study differences were found. This is probably due to the amount of 1% saline that Furuse et al.'s male rats drank in proportion to their body weight. Furuse et al.'s rats drank larger amounts of saline in proportion to body weight than our female rats. Other possibilities are that their lack of response reflects a bone site difference, a gender difference or a loading difference.

The present study examined cancellous bone at three sites. With the increased concentration of saline in the 2% study and control of body weight between groups, a more robust result was seen making differences in bone volume and structure more apparent. Significant reductions were found in the overloaded femur structural parameters of the 2% study between the rats on saline and the rats on water. Marked reductions from the 2% saline treatment were seen in tibia cancellous bone in the wistar and salt sensitive rats (Table 3.43). Significant differences were found only in the

overloaded but not the underloaded tibia in the 2% study. This probably was due to immobilization suppressing cancellous growth to the 5.66-6.85% range. In overloaded limbs bone volume of rats on water was a 11.75 to 15.28% range and 6.84 to 7.76% range for the rats on saline. In addition to both tibia, the cancellous bone of the L-2 vertebrae of the 2% study was examined. No difference from salt treatment was detected.

Histological evidence of salt's effects was only clearly seen in the overloaded cancellous tibia bone. This was site specific to weight bearing bone with the effect apparently influenced by mechanical loading in growing rats. In the lumbar L-2 vertebral bone, the short term saline treatment had no effect indicating a site specific or a loading specific location is necessary for the cancellous bone to respond. Vertebral cancellous bone is slightly differing structure than cancellous tibia bone with heavier platelike trabecular structures. As magnesium is mostly contained near the surface of bone (Rude et al., 1999), the tibia cancellous bone with greater surface to bone ratio may be more susceptible to a more rapid adverse effect. Change in the structural histomorphometry of the tibias was not expected, as cancellous bone is usually thought of as deteriorating only with aging, particularly after menopause (Rizzoli et al., 2001). However, Rude et al. (1999) found by inducing magnesium deficiency in female adult rats, magnesium content fell in bone. At the same time, a significant reduction in femur cancellous bone volume and elevation in osteoclastic activity were noted. In the present 2% study, saline treatment decreased magnesium in bone, lowered cancellous bone volume and increased resorption activity of osteoclasts (Table 3.48).

The present studies show that 2% saline treatment had a an adverse effect on the SS stain, and more importantly point to a modest effect on both W bone and on bone of

SS rats, which had not yet demonstrated hypertension. The present 2% study magnesium mineral reduction, tibia cancellous volume reduction and an elevation in osteoclastic activity may point to sodium working with magnesium, possibly a mechanism for sodium's initial effects on bone.

As will be seen in the next sections, salt expands its' adverse influence by combining with other factors to produce many enhanced effects important to future fracture risk.

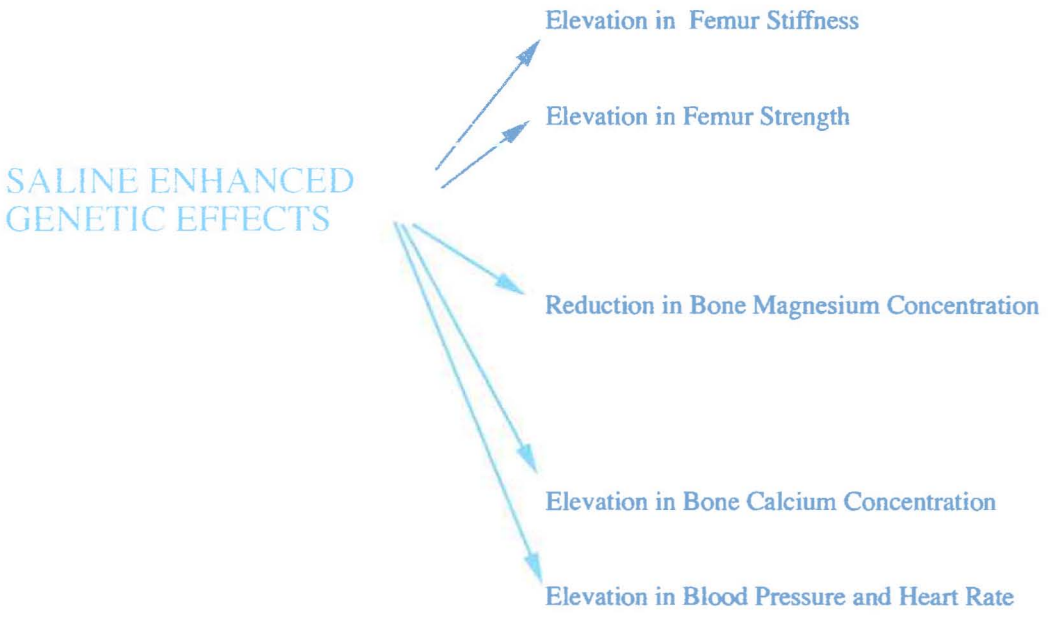

Figure 4.4. Summary chart of saline enhanced genetic effects

4.4 SALINE ENHANCED GENETIC EFFECTS

Because of the on-going salt-bone association controversy (Cohen and Roe, 2000; Antonio and McGregor, 1995), the primary purpose of the present studies was to both delineate (section 4.2 and 4.3) and combine (section 4.4) the effects between salt intake and salt sensitive genotype on bone. A brief discussion on the enhancement by saline treatment to genetic differences (Figure 4.4), using main effects plots, follows. Genetic differences were further enhanced by saline treatment in increasing femur stiffness, femur calcium and femur strength, blood pressure and heart rate, and simultaneously decreasing femur magnesium (Figure 4.4). Note that saline treatment by itself had no significant

effect on peak bone mass, femur stiffness, femur calcium and femur strength. Perhaps, if the number of rats per group had been higher, the concentration of salt higher, initiated at an earlier age, or the duration of study extended, then the salt by itself may have had a more robust influence. Reductions in femur cross sectional transverse area and bone zinc were primarily from strain difference. The reductions in cancellous bone volume were primarily from the 2% saline treatment. The result of two-way ANOVA in the 2% study demonstrated that both saline treatment and strain of rat had significant effects on blood pressure, heart rate, and magnesium.

4.4.1 Blood Pressure and Heart Rate

As previously discussed, there is great controversy on the mechanism involved in hypertension). Researchers estimate that 30-60% of the population is hypertensive and 30-40% of the population is salt sensitive (Muntzel and Drueke, 1992; Dyer et al., 1995). The present adolescent studies do confirm a salt effect on the standard normotensive rats blood pressure and heart rate and a greater increase in the hypertensive rats' blood pressure and heart rate. Following are illustrations graphically indicating the main effects of the statistical analysis from the 2% saline treatment studies, previously discussed at length in the Results, Blood Pressure and Heart Rate Section in Chapter 3. Means of the water versus saline treatment result is in the first rectangle, followed by means from the two genotypes (normotensive and salt sensitive hypertensive) in the second rectangle, with the last rectangle displaying the groups delineated into 4 groups (WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt

sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline).

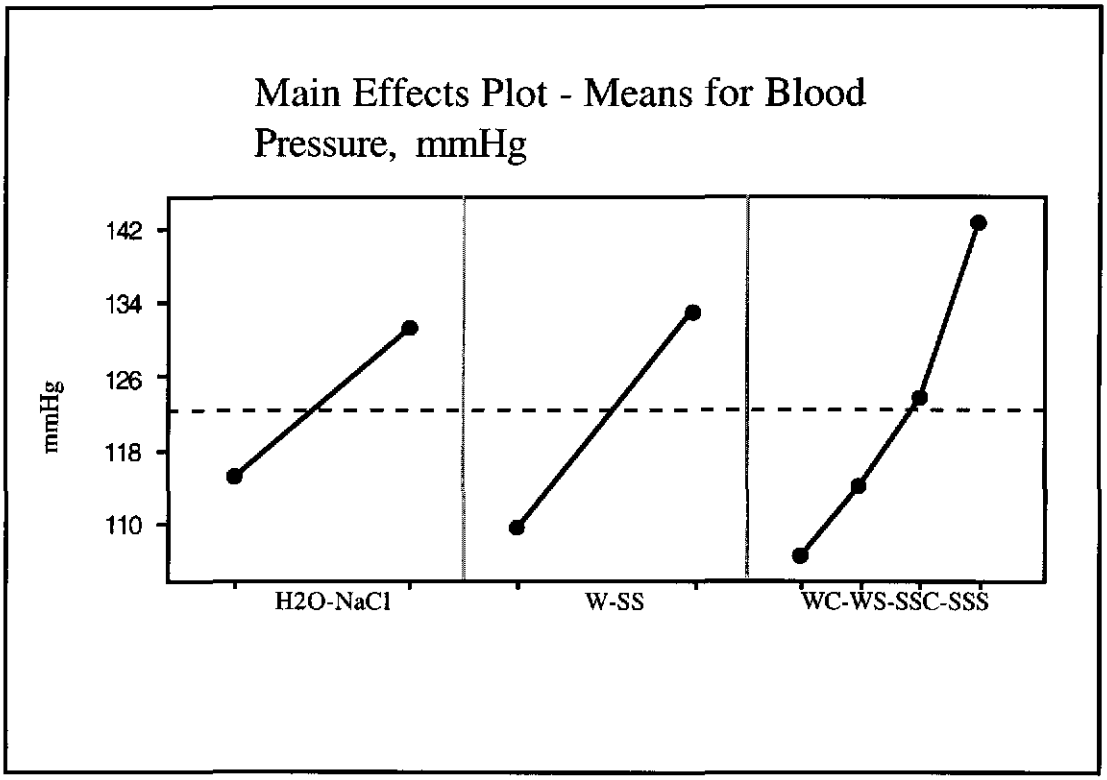

Figure 4.5 Main Effects Plot for blood pressure (mmHg) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

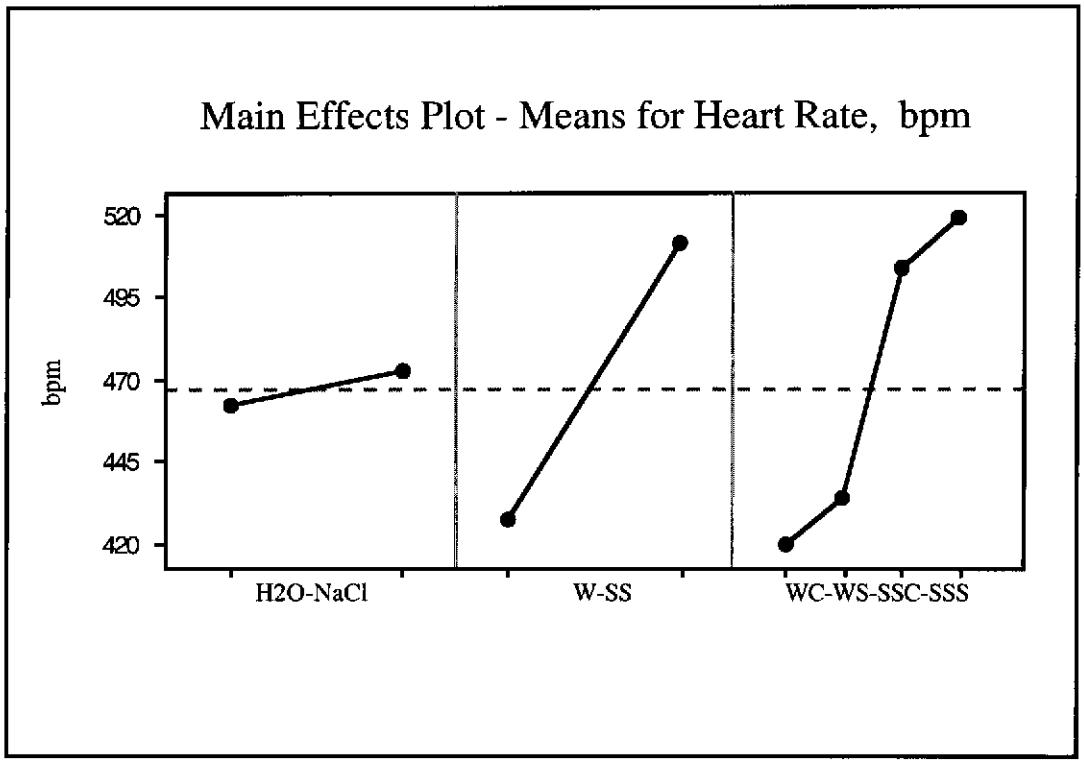

Figure 4.6 Main Effects Plot for heart rate (bpm) in 1% Study. H2O, water control; Na, 1% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

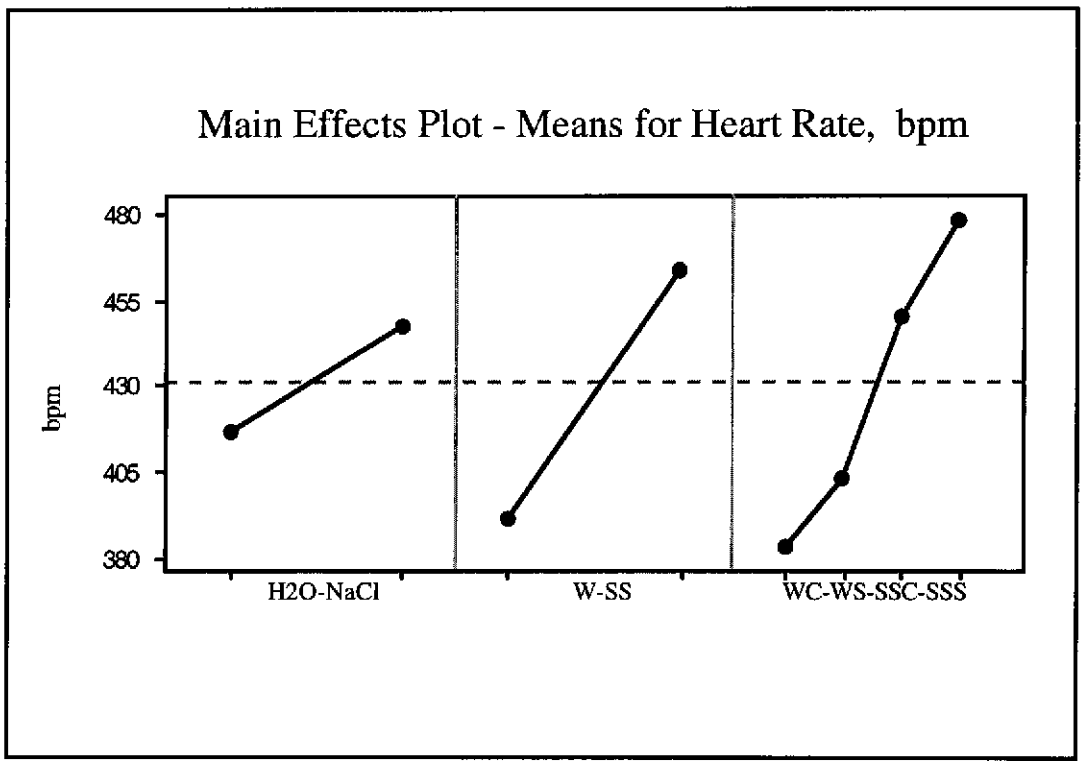

Figure 4.7 Main Effects Plot for heart rate (bpm) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

Significant differences in blood pressure were produced by differing genetic strains and salt concentrations in the 2% study (Figure 4.5). Tail cuff measurements of blood pressure and heart rate in the 2% study were higher in salt sensitive hypertensive strain with saline and water drinking groups pooled, than in the wistar normotensive strain. Low salt concentrations (such as the 1% used in the initial study), were at near threshold concentrations and had no effect on blood pressure, however, there was a salt effect on the heart rate in both 1% and 2% studies (Figure 4.6 and 4.7). When the salt

was increased to a 2% concentration, there was a marked difference in blood pressure between saline treated and water controls in both strains.

4.4.2 Bone

Saline treatment acted mainly by reducing magnesium and cancellous bone volume, while genotype accounted for significant differences in zinc, calcium, peak bone weight, femur stiffness, and femur strength (Figure 4.8-4.16). The combination of the hypertensive genotype with saline treatment increased effects on female adolescent bone beyond either factor alone, in some measurements.

The W strain had a higher percentage of magnesium in the underloaded femur limb when compared to the SS strain. Magnesium showed site variation with some changes more genetically induced and some more saline induced in both W and SS. All 3 bone sites tested in the 2% study were found to have significant overall differences. Saline treatment reduced magnesium in the underloaded femur, magnesium in the overloaded femur, and magnesium in the ulna. Differences ranged from 5% reduction in the ulna, 7% reduction in overloaded femur and an 8% reduction in underloaded femur. In humans, osteoporotic's bone has about a 10% reduction in magnesium when compared to normal bone (Wallach, 1990).

4.4.2.1 Strength Parameters

The apparent modulus of elasticity (stiffness) and the ultimate bending stress (strength) measurements of the underloaded femurs (Table 3.21) were found to have saline enhanced effects (Figure 4.8 and Figure 4.10). In the overloaded femur of the SSS rats, loading appears to have a beneficial effect by reducing bone strength and stiffness toward the level of SSC rat (Figure 4.9 and Figure 4.11).

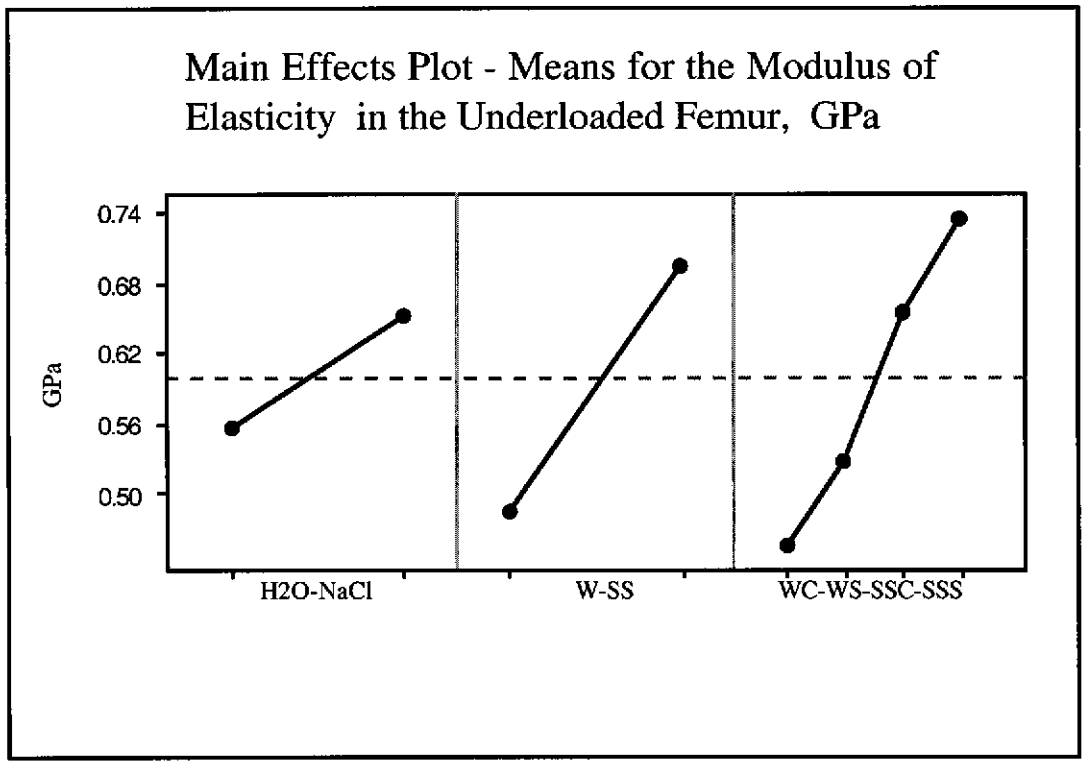

Figure 4.8 Main Effects Plot for the modulus of elasticity in the underloaded femur (GPa) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

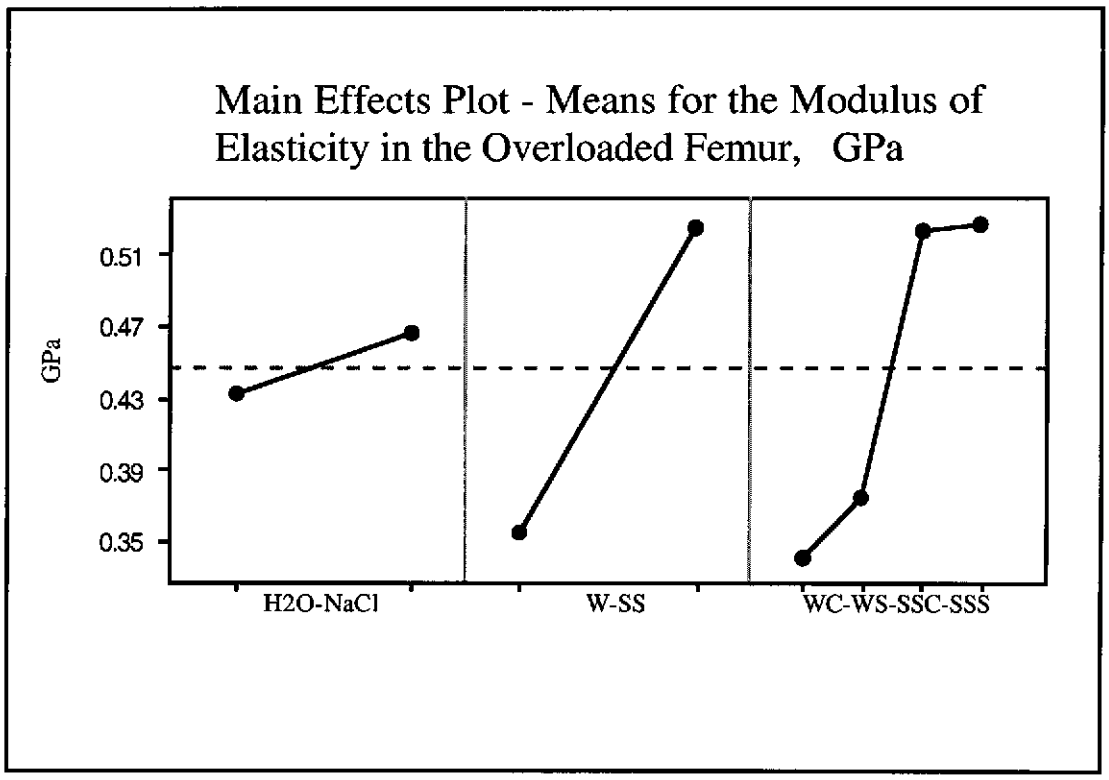

Figure 4.9 Main Effects Plot for the modulus of elasticity in the overloaded femur (GPa) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

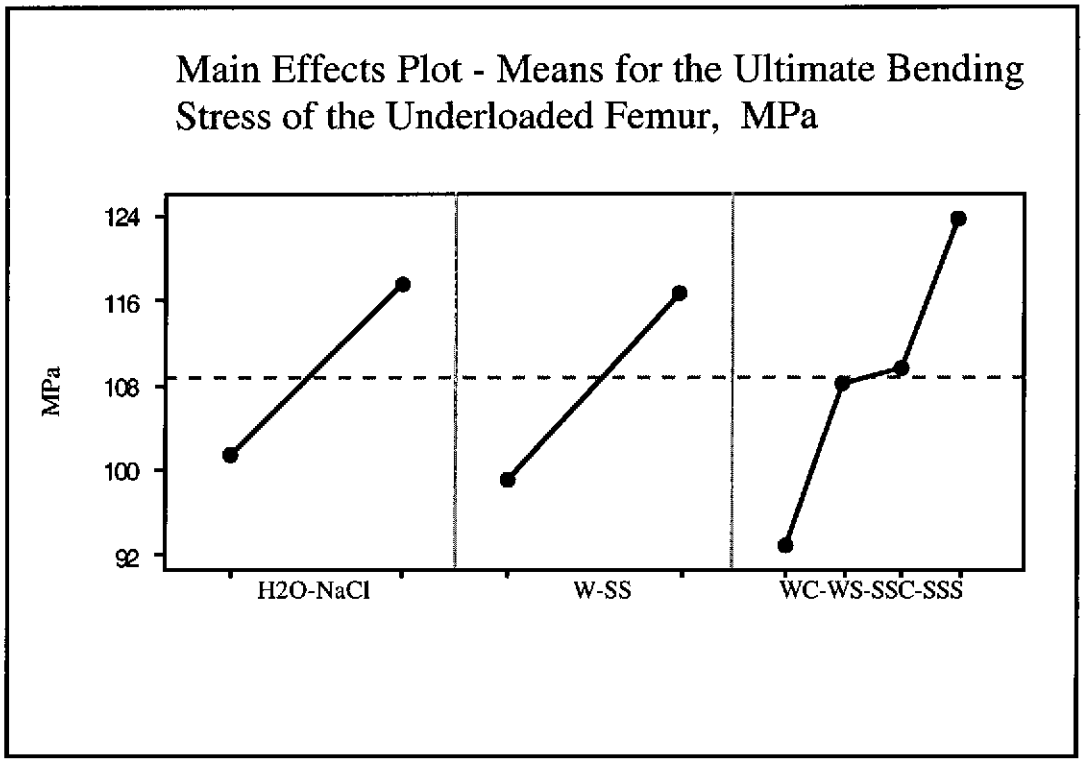

Figure 4.10 Main Effects Plot for the ultimate bending stress in the underloaded femur (MPa) in 2% study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

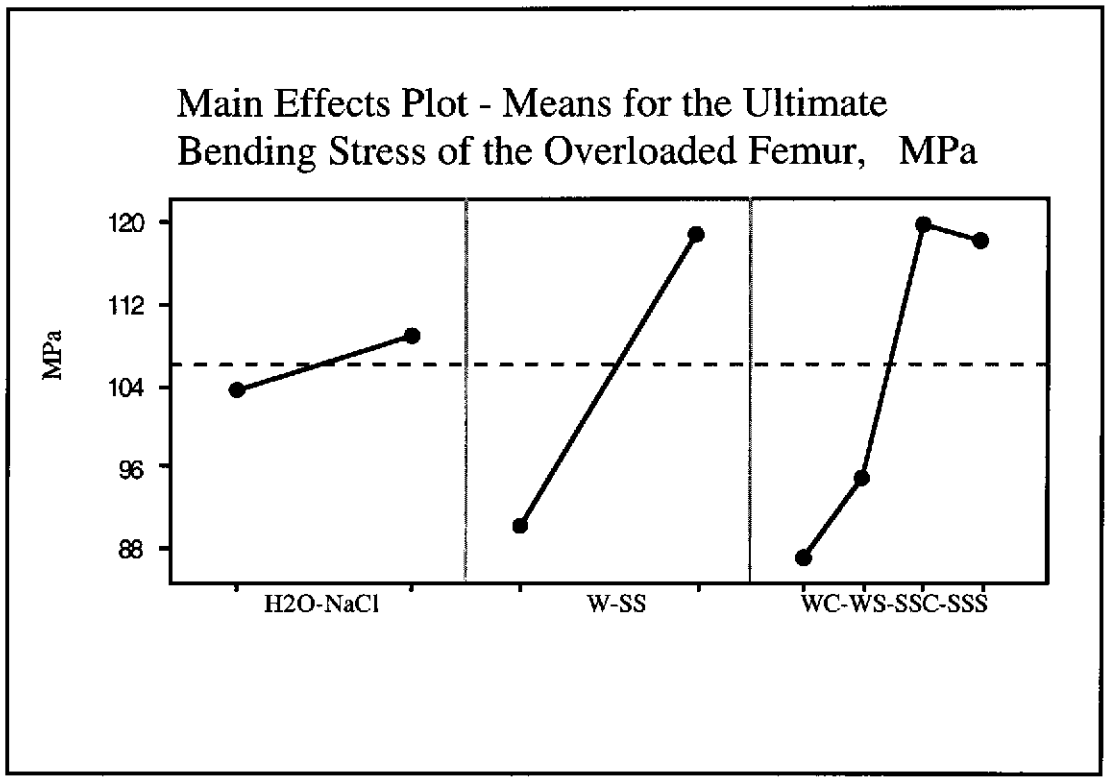

Figure 4.11 Main Effects Plot for the ultimate bending stress in the overloaded femur (MPa) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

As shown in these 4 graphs (Figure 4.8 to 4.11), salt's range of effect was less in the overloaded compared to the underloaded femur. This may suggest loading has a beneficial effect on the adverse effect of salt.

4.4.2.2 Composition

In water, certain minerals exhibit stronger bonding power or strength (Cuff, 1996). Of particular interest to skeletal integrity in the present studies is calcium showing greater bonding strength than magnesium (Cuff, 1996). Magnesium has been

reported to destabilize hydroxyapatite crystallization both in vivo and in vitro, possibly by calcium and magnesium competing for attachments to phospholipid-PO_4 fraction (Wallach, 1990). This cation exchange may be a possible mechanism for material modifications in strength and stiffness altering bone quality before peak bone mass is reached.

The Ca+2 ionic radius is 0.99 Angstrom. The Mg+2 ionic radius is 0.65 Angstrom. Genotype and saline treatment combined, lowered bone magnesium in the underloaded femur (Figure 4.12). Magnesium's close proximity near the surface of bone (Neuman and Neuman, 1958), smaller ionic size, and its weaker bonding strength may make it more susceptible to loss which can initially compromise the skeletal structural integrity. In water, the magnesium loss may offer sites where non-lattice forming elements may deposit on damaged edges of crystal. These adsorbed (lying on the surface not absorbed) ions would not be bonded as securely as ions of the hydroxyapatite structure. This newly destabilized structure is now at risk to further assault if the salt concentration or the duration of saline treatment increases. Newly altered hydroxyapatite may now be more easily vulnerable to attack even though calcium has a slightly larger ionic radius and slightly stronger bonding power than magnesium.

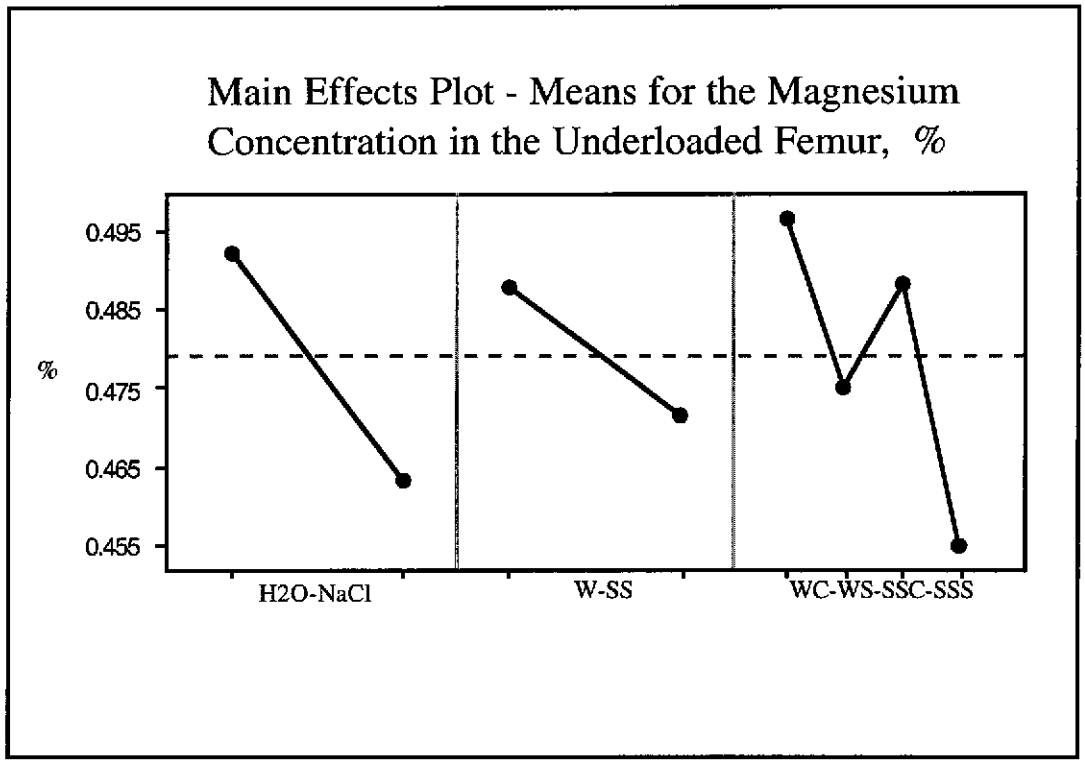

Figure 4.12 Main Effects Plot for the magnesium concentration in the underloaded femur (%) in 2% study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

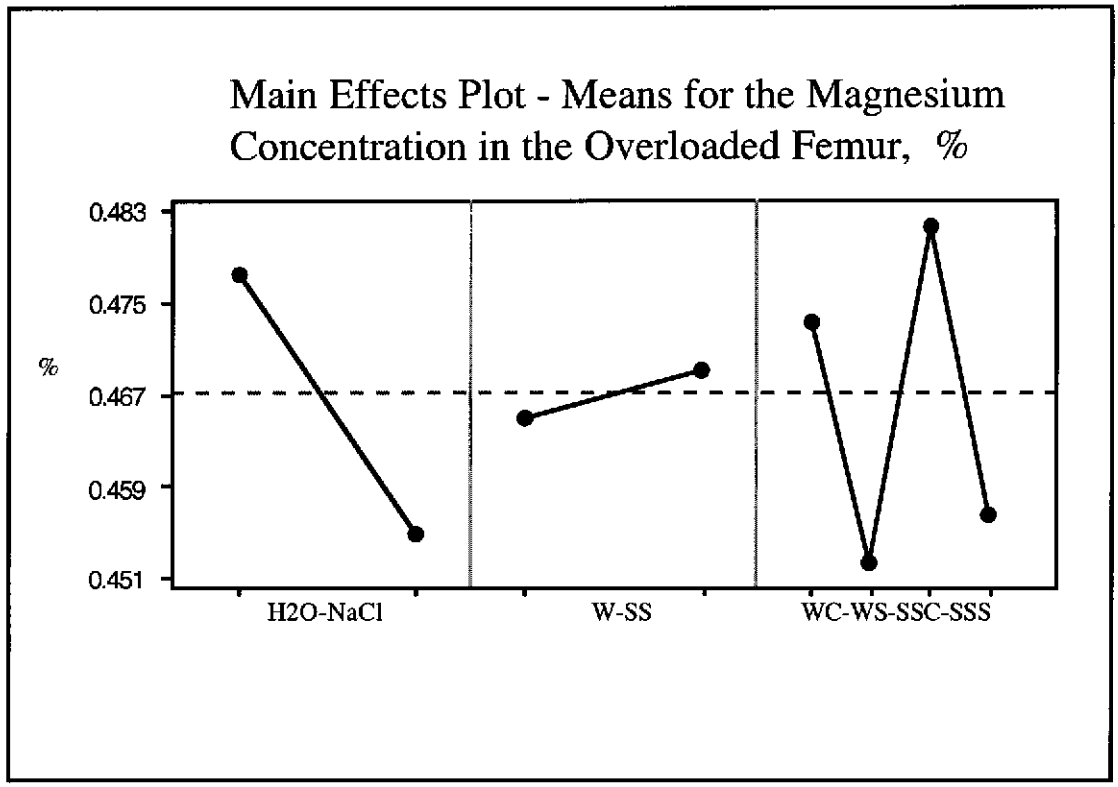

Figure 4.13 Main Effects Plot for the magnesium concentration in the overloaded femur (%) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

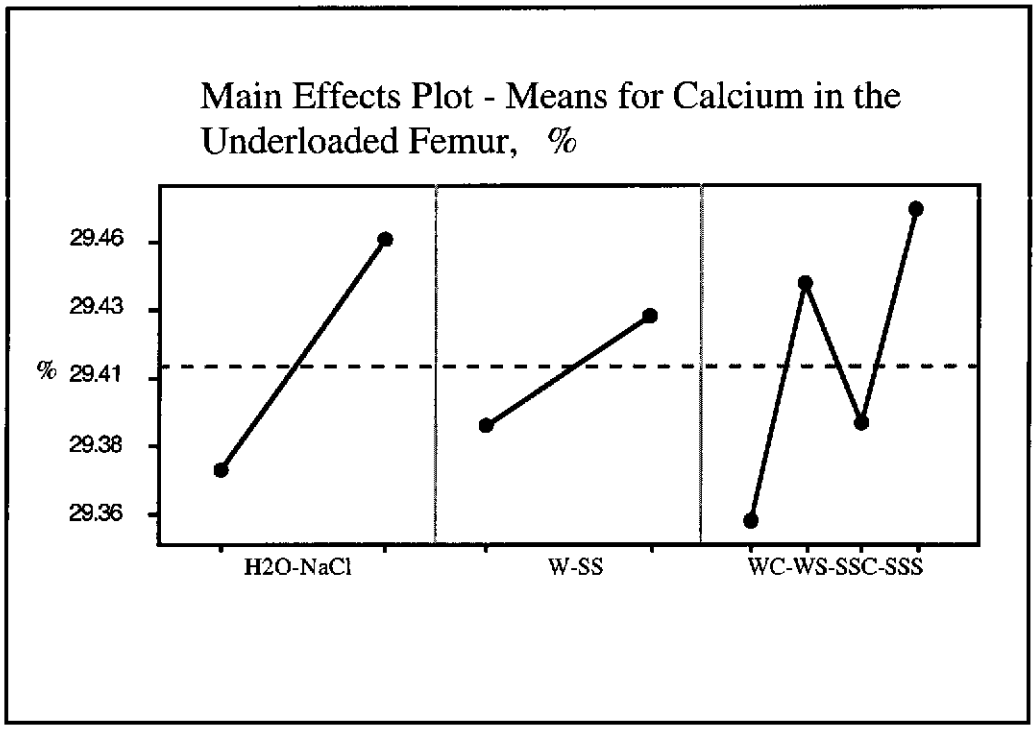

Figure 4.14 Main Effects Plot for the calcium concentration in the underloaded femur (%) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

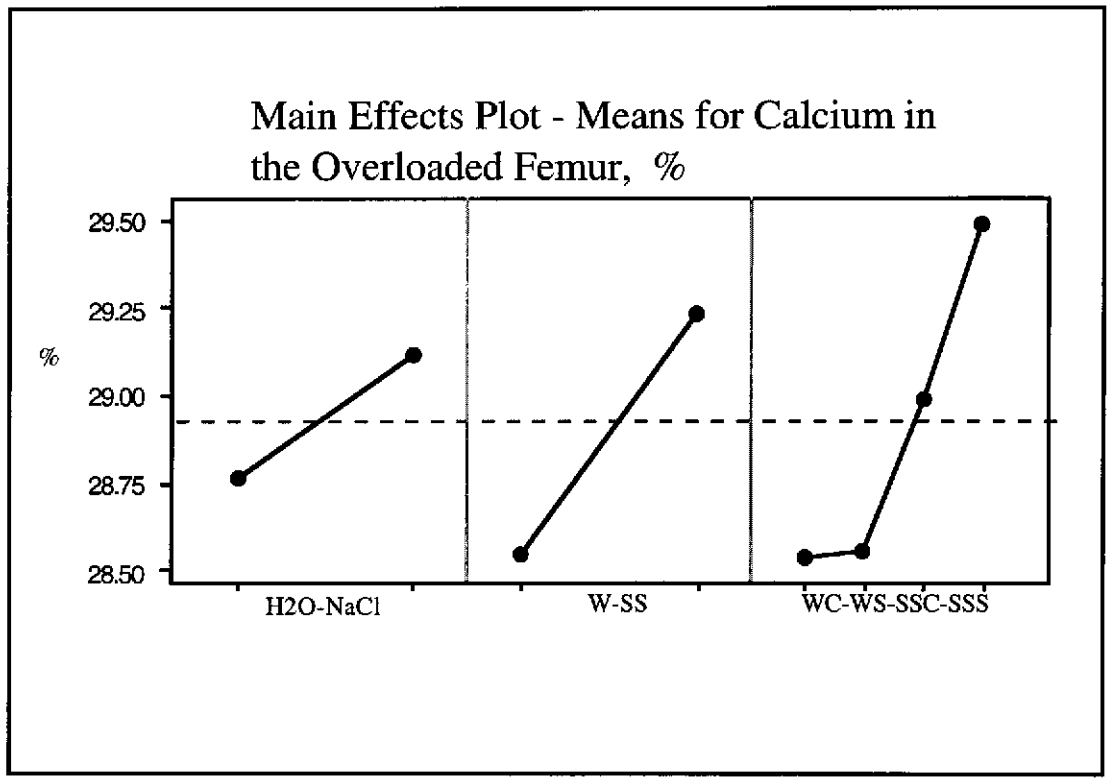

Figure 4.15 Main Effects Plot for the calcium concentration in the underloaded femur (%) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

4.4.2.3 Morphology

There was not a significant salt difference between treated and water controls in the 2% study for femur weight and length. The result of the 2-way ANOVA indicates genetics had the most effect. However, the main effect plot for the overloaded limb (Figure 4.16) suggests that saline treatment may have exaggerated the decrease in femur weight seen in SS. As noted previously, the heaviest bone/body weight was found in the overloaded femurs of wistar control group on water. The lightest bone/ body weight was

found in the overloaded femur of the salt sensitive rat group on saline. While there is some variation, the progressive reduction in weights of three bone sites suggests a saline treatment effect on bone weights overall.

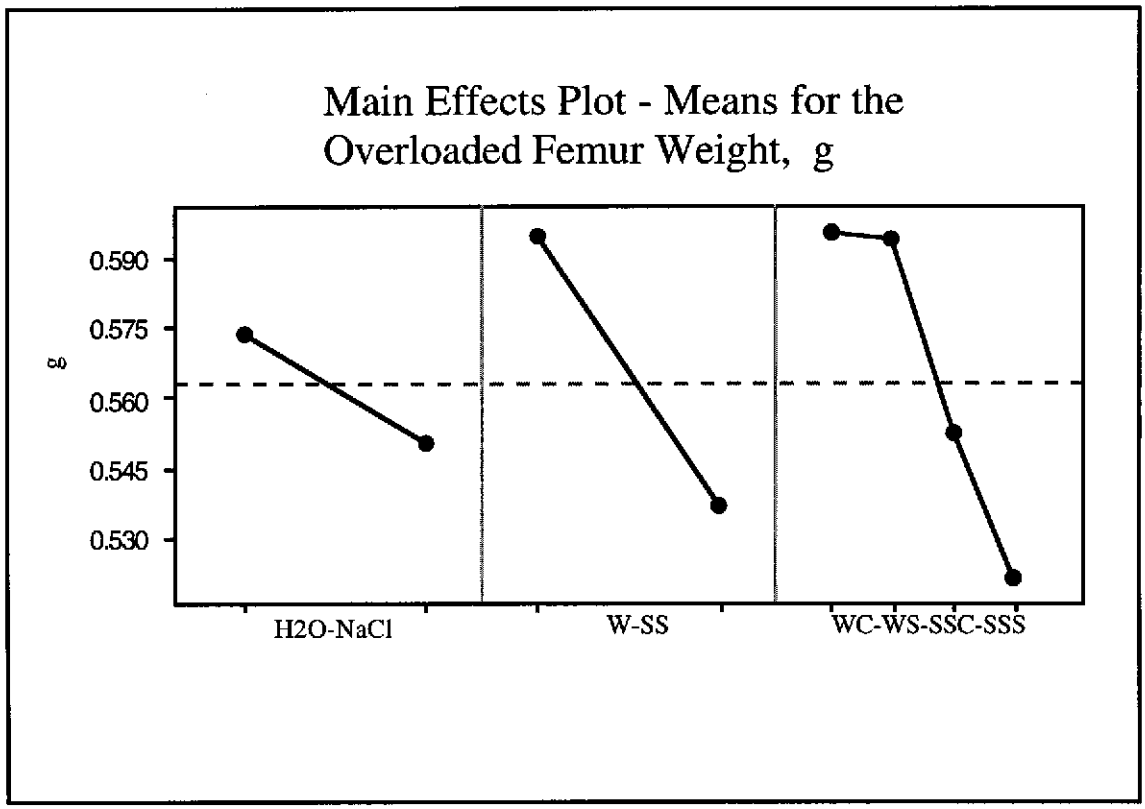

Figure 4.16 Main Effects Plot for overloaded femur weight (g) in 2% Study. H2O, water control; Na, 2% saline treated; W, wistar normotensive; SS, salt sensitive hypertensive; WC, wistar normotensive water control; WS, wistar normotensive, treated with saline; SSC, salt sensitive hypertensive water control; SSS, salt sensitive hypertensive treated with saline. Dashed line is the grand mean.

4.4.2.4 Summary

To summarize, SS genotype with enhancement by saline treatment resulted in an additional increase in femur stiffness (U), and femur strength (U), femur calcium (U and

O), blood pressure and heart rate, and simultaneously a decrease in femur magnesium (U). The SS genotype saline enhancements were generally more pronounced than that of W rats.

As noted previously, approximately 30-60% of the population is hypertensive and 30-40% of the population is salt sensitive (Muntzel and Drueke, 1992; Dyer et al., 1995). Our results suggest that genetic effects act concurrently on blood pressure and bone. How tightly are these measurements coupled together in response to both genotype and salt? Interestingly, regression analysis in the present study (all groups included) with bone parameters indicate a strong correlation with heart rate/overloaded bone weight (r^2 = .679 in 2% study and r^2 = .441 in 1% study) but a weaker correlation (r^2 = .342 in 2% study and r^2 = .224 in 1% study with blood pressure/overloaded bone weight. Because the immobilized limb demonstrated weaker correlations, one could speculate the bandage technique dampened the beneficial effects of the heart beat pulse-pressure on bone remodeling in the immobilized limb. In addition, it should be noted, that adverse bone effects were less related to blood pressure and more to heart rate in the present studies.

In Macgregor and Cappuccio (1993) put forward a hypothesis linking osteoporosis and hypertension. However two years later, Antonios and Macgregor (1995) suggest that perhaps salt intake has independent adverse effects on blood pressure and bone. In our 2% study, total salt per day was significantly correlated with the adverse effects of both magnesium and blood pressure. While the overall variation between the 4 groups for magnesium and blood pressure was significant, post hoc tests on magnesium in the overloaded and the underloaded femurs tended to be higher in the WC than in the WS group (p <0.13 and p <0.12, respectively). Likewise, blood pressure

was higher, but not significantly, in the WS group compared to the WC group. On the other hand, magnesium in the ulna, in the overloaded and the underloaded femurs was significantly higher in the SSC than in the SSS group, indicating, magnesium was lowered by saline treatment. At the same time, blood pressure was significantly increased by saline treatment in the SSC when compared the SSS group. Because no significant differences were found between water and saline W groups, these combined results suggest that an independent adverse effect of salt intake on W bone and W blood pressure cannot be firmly established in this short period of time in the present studies, although the trends were in the same direction as seen for the SS groups.

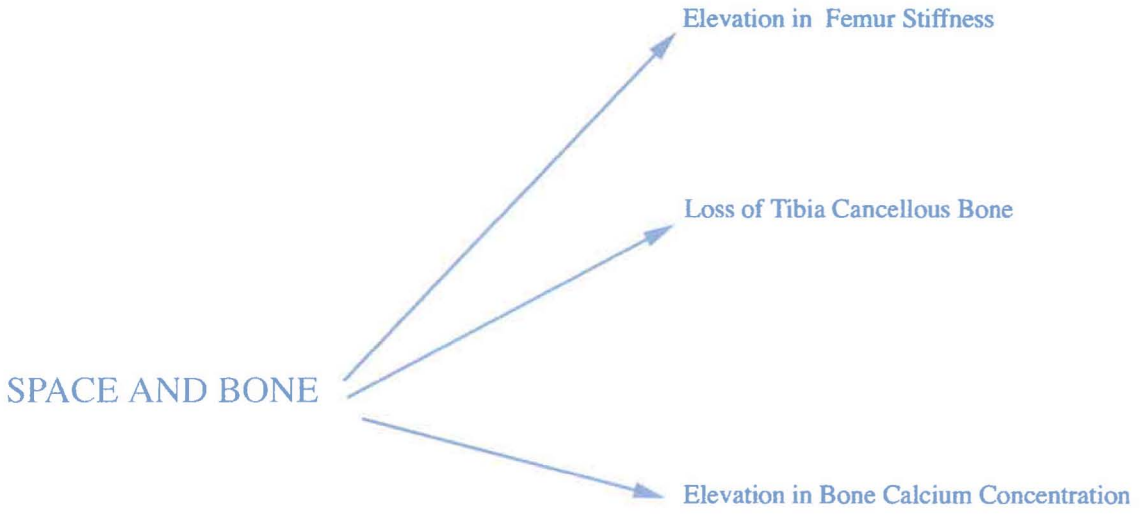

Figure 4.17 Significant Underloading/ Overloading Effects

4.5 SPACE AND BONE

After 6 weeks of right hindlimb immobilization, we found elevations in the underloaded femur bone stiffness with elevations in minerals (calcium and phosphorus) and trace elements (zinc and manganese). Importantly, the tibia cancellous bone volume was reduced in the underloaded bone.

In a 1995 study, Navidi et al. investigated the effect of excess dietary salt in a rat spaceflight model. No further reduction in calcium content was found apart from that induced by unloading the hindlimbs. However, work by others on young growing male genetically defined SHR rats pointed to early adverse bone morphological changes induced by salt that would be missed by standard measurements of bone calcium content. It appears that salt damage to bone may be a structural phenomenon, not strictly calcium

content modulated. Early loss of cancellous bone volume by either underloading or the previously discussed saline treatment, can precede calcium content change and may lead to unexpected fracture. The mechanism of this novel finding is unknown and deserves additional study.

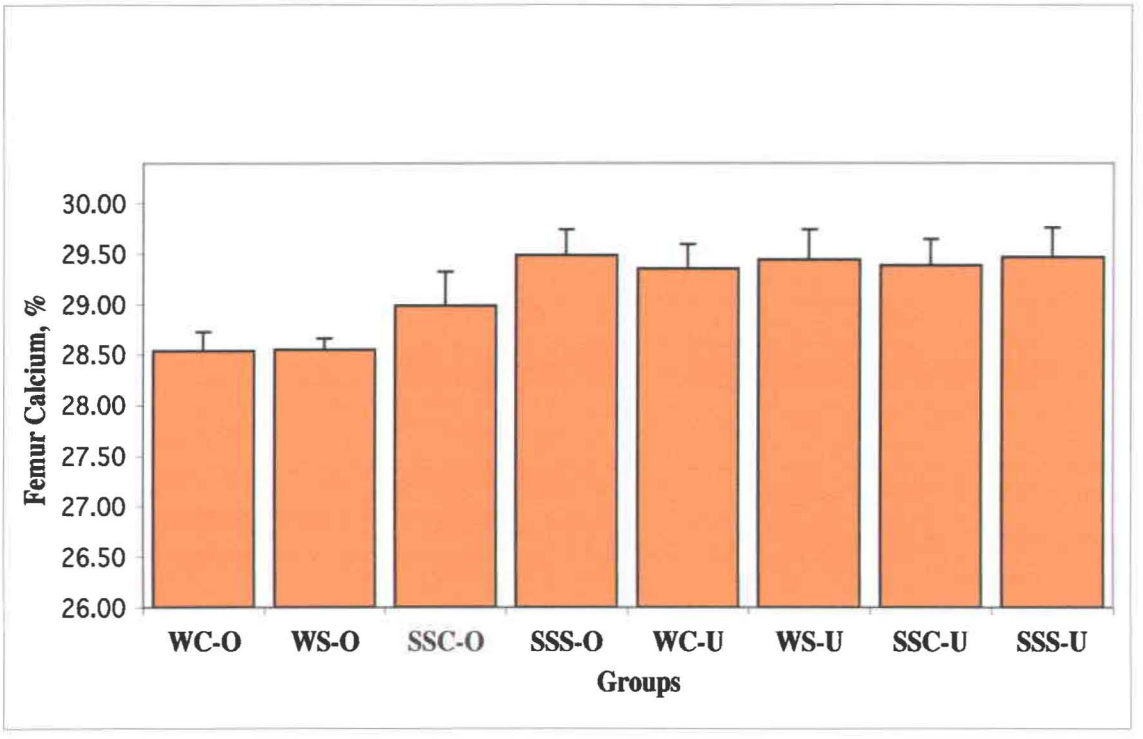

Figure 4.18 The effects of loading (underloading and overloading) and groups on femur calcium in the 2% Study. Shown are the means ± SEM. WC-O, wistar normotensive water control, overloaded femur; WS-O, wistar normotensive, treated with saline, overloaded femur; SSC-O, salt sensitive hypertensive water control, overloaded femur; SSS-O, salt sensitive hypertensive treated with saline overloaded femur; WC-U, wistar normotensive water control, underloaded femur; WS-U, wistar normotensive, treated with saline, underloaded femur; SSC-U, salt sensitive hypertensive water control, underloaded femur; SSS-U, salt sensitive hypertensive, treated with saline, underloaded femur. An overall one way analysis of variance comparison between the 8 groups was not quite significant ($p < 0.051$), no pairwise comparisons were tested. There were significant differences overall between the underloaded and overloaded limbs (see Table 3.40).

In order to determine if there were differences between the means of the WC-0, WS-0, SSC-0, SSS-0, WC-U, WS-U, SSC-U, and SSS-U groups (Figure 4.18), a one-way ANOVA was performed. Unlike the two-way ANOVA (Table 3.40), a significant overall variation between the 8 groups was not seen for calcium in the 2% study ($p < 0.051$). The reason for this apparent discrepancy is that the variance in the two way ANOVA is factored into two overall main effects, not eight, as is done in the one way.

Mineral concentrations (calcium, magnesium, phosphorus, zinc and manganese) were significantly higher in the underloaded femur than in the overloaded femur in the 2% study (Table 3.40). While more mineral concentration is usually thought of as stronger bone, an excess of mineral (as calcium, and phosphorus) with less collagen in the lattice could create stiffer and much less flexible bone. This is consistent with our finding of stiffer bone in underloaded bone when compared to overloaded bone in the strength modulus of elasticity measurements.

Stiffness was significantly greater in the underloaded femurs. As expected, overloaded bone demonstrated a higher overall strength than underloaded bone, however, when cross-sectional area was calculated in, no significant difference in femurs was seen in breaking strength.

As previously discussed, the SS strain exhibited stronger and stiffer bone, which may indicate more rapid aging and acceleration of the maturation process (Matsuda et al., 1986; Currey, 1984). Thus, the SS genotype may include an accelerated bone maturation leading to an early peak in bone mass. In addition, the effect of salt and SS genotype enhanced stiffness. To further delineate the effects of each factor and separate out the treated groups, we graphed the 8 groups (Figure 4.18; 4.19; 4.20). The underloaded

femur calcium concentration appeared high regardless of a salt effect, whereas, the overloaded femur calcium appeared to increase due to an increasing salt effect only in SS group (Figure 4.18). If no weight is placed on bone, as in immobilization or weightlessness, increased stiffness results. Thus the most at risk bone is the SS genotype, with salt and less activity. The combination of these three factors produced a 214% increase in bone stiffness (Figure 4.19).

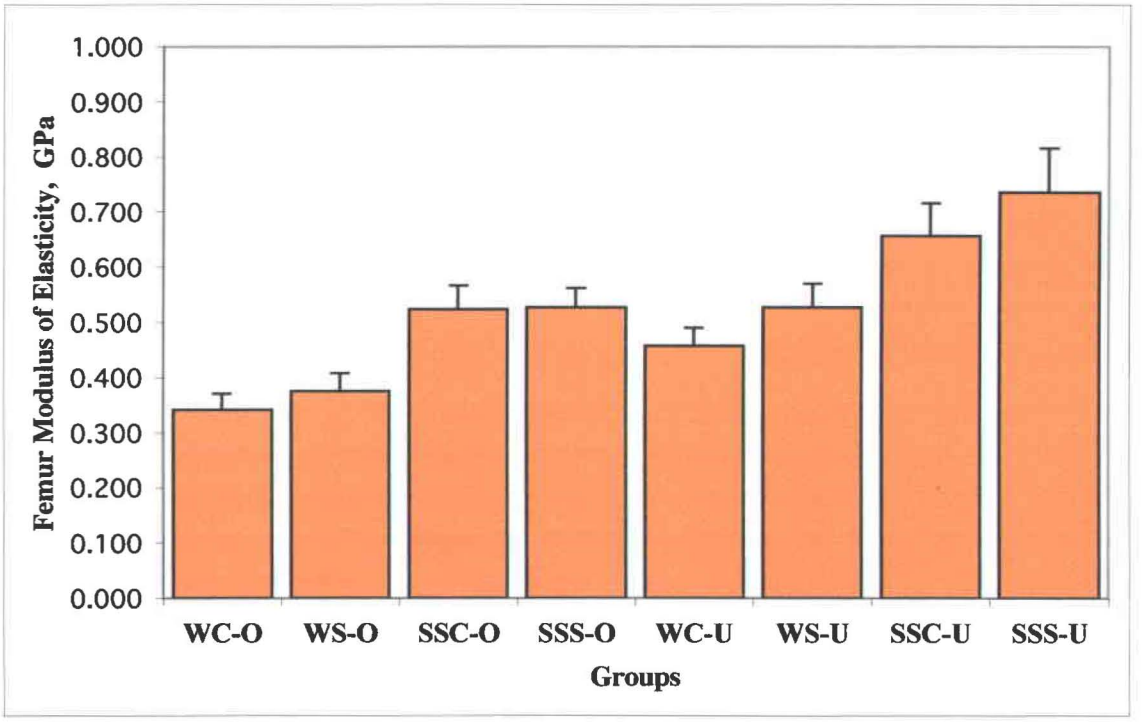

Figure 4.19 The effects of loading (underloading and overloading) and groups on femur modulus of elasticity, GPa in the 2% Study. Shown are the means ± SEM. WC-O, wistar normotensive water control, overloaded femur; WS-O, wistar normotensive, treated with saline, overloaded femur; SSC-O, salt sensitive hypertensive water control, overloaded femur; SSS-O, salt sensitive hypertensive treated with saline overloaded femur; WC-U, wistar normotensive water control, underloaded femur; WS-U, wistar normotensive, treated with saline, underloaded femur; SSC-U, salt sensitive hypertensive water control, underloaded femur; SSS-U, salt sensitive hypertensive, treated with saline, underloaded femur. An overall one way analysis of variance comparison between the 8 groups was significant. For significant pairwise comparison (see Table 3.21). There were significant differences overall between underloading and overloading, groups (see Table 3.22 and 3.23) and genetic strain (see Table 3.20).

In the 1% study tibia histomorphometric analysis no significant differences were found due to overloading or underloading. The 2% study is consistent with previous findings in that the overloaded tibia bone displayed significantly more cancellous volume and higher trabecular number and less separation between trabeculae than in the underloaded tibia bone (Maeda et al., 1993). Cancellous bone volume/tissue volume and trabecular number in the overloaded bone was significantly greater than in the underloaded bone (Table 3.46). Trabecular separation in the overloaded bone was significantly reduced when compared to the underloaded bone (Table 3.46). Loss of trabecular bone enhances the risk of future fractures. For example, trabecular architecture did not return to normal when a 6 week remobilization time period was applied after 6 weeks of immobilization (Maeda et al., 1993). Maeda et al. (1993) suggest that the loss of potential sites for bone formation during recovery from immobilization lead to thickening trabeculae, which may increase rat bone fragility with time (Maeda et al. 1993). The added adverse effect of salt on an overloaded spaceflight model, in both the W and SS (Figure 4.20; Table 3.43; Table 3.46) was perhaps one of our most important findings; and suggest the possibility that the unexplained epidemic of osteoporosis in active female humans may be partly related to salt intake in adolescence.

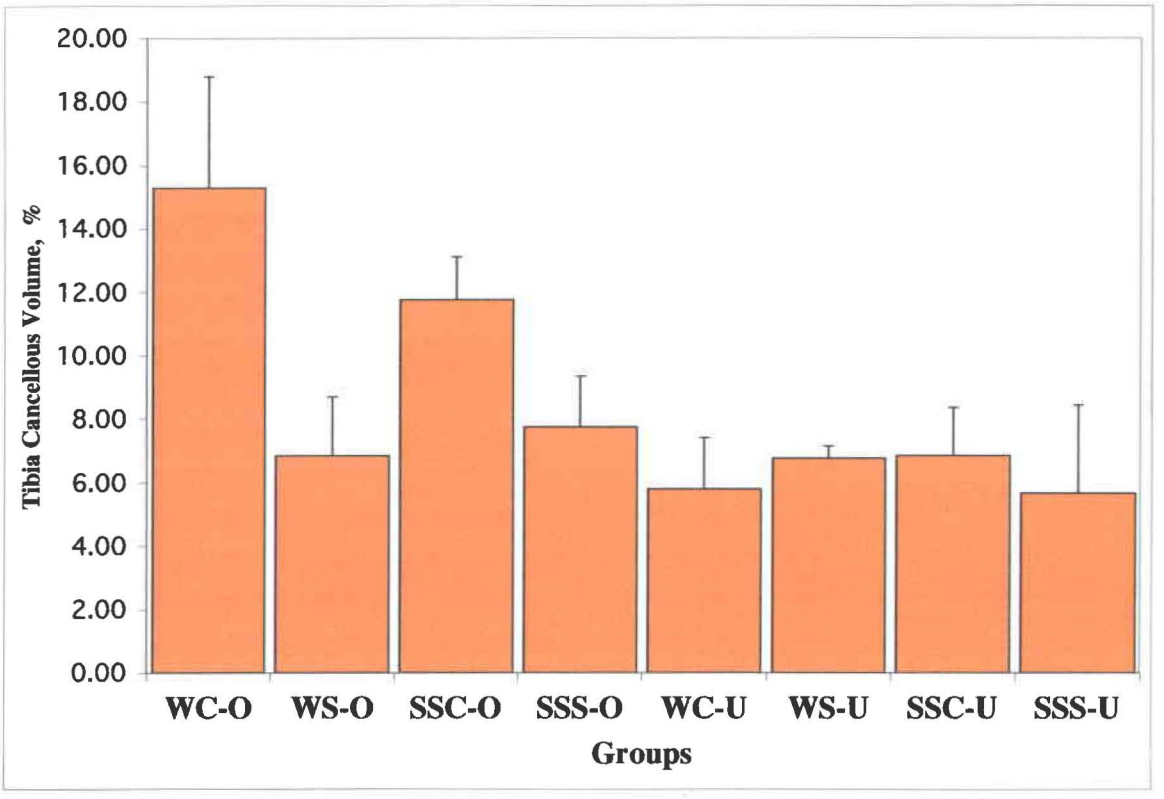

Figure 4.20 The effects of loading (underloading and overloading) and groups on tibia cancellous bone volume in the 2% Study. Shown are the means ± SEM. WC-O, wistar normotensive water control, overloaded femur; WS-O, wistar normotensive, treated with saline, overloaded femur; SSC-O, salt sensitive hypertensive water control, overloaded femur; SSS-O, salt sensitive hypertensive treated with saline overloaded femur; WC-U, wistar normotensive water control, underloaded femur; WS-U, wistar normotensive, treated with saline, underloaded femur; SSC-U, salt sensitive hypertensive water control, underloaded femur; SSS-U, salt sensitive hypertensive, treated with saline, underloaded femur. An overall one-way analysis of variance comparison between the 8 groups was significant. There were significant differences overall between underloaded and overloaded, (see Table 3.46). In addition, here were significant differences overall between saline treated (see Table 3.43).

Despite some weight loss with the bandage technique, overloading was able to maintain cancellous bone at a normal level (Figure 4.20). However, it was unable to do this when treated with 2% saline supplementation (Figure 4.20). In addition, with

immobilization in young growing female rats maintenance of cancellous was inhibited significantly when compared to overloaded tibia cancellous bone, though salt had no additive effect on the underloaded femur cancellous bone volume.

In summary, underloading, salt, and genotype appear to increase bone calcium retention (Figure 4.18). Calcium manipulation and prediction is very important to the measurement, screening, and testing of therapies and drugs for osteoporosis. The doubling in femur stiffness (which cannot be measured directly by densitometry) could have an adverse outcome with respect to future fracture risk particularly to those who work regularly in orbital weightless environments. In addition, it appears that underloading (space time) reduced cancellous bone growth while salt decreased bone in overloading (recovery time). While astronaut's diet is carefully controlled in orbit, this latter salt treatment result may have implications for looking at astronaut diets in recovery time.

CHAPTER V

CONCLUSION

5.1 SUMMARY

Our findings suggest genetics, salt, exercise loading/immobilization, affect peak bone mass, bone quality, blood pressure and heart rate. These deleterious bone effects are site specific, affecting different bone sites individually. When applied simultaneously, each factor may act alone and/or in combination with the other adverse factors, or as in the case of magnesium loss, independently and additively. As illustrated in Figures 4.5-4.15 each factor had a specific contribution to the result, which were quite diverse.

Each experimental period was six weeks. A longer period of time may have had profound effects leading to increased fracture risk in the hypertensive rats of our study. Furuse et al. (1992) had 3 of 15 SHR on saline treatment, suffer bone fractures before the 7^{th} week of the study. By immobilizing our rats with the bandage technique and with their somewhat isolated environment, we may have prevented the resulting stiff bones from fracturing by suppressing normal adolescent play. Overloading the femur may have helped prevent fractures from occurring, as well as the immobilized position may in another way have prevented fractures in the other femur. However, as indicated in Figure 4.19, the immobilized hypertensive bone with 2% supplementation demonstrated the most accumulated stiffness. Our experimental results, loss of bone elasticity (stiffness), loss of cancellous bone, abnormal mineral distribution, small bones with an early peak bone mass maturation period) may also explain why Furuse's rats fractured in

adolescence despite no change in calcium content. Furuse's rats were also on the special AIN diet used here to be sure minerals were optimal for a bone study.

In summary, a more robust result was seen with increased concentration of saline treatment from a 1% saline threshold level, to the 2% saline level. In addition, control of body weight made genetic differences more evident. Using two-way ANOVA, the spontaneously hypertensive rat had significant reductions in overall bone mass, femur cross-sectional area and zinc concentrations, with simultaneous elevations in femur stiffness, strength, and calcium. Both hypertensive genotype and saline treatment significantly increased blood pressure and heart rate, and decreased femur magnesium in the 2% study. Further, 2% saline treatment decreased magnesium in 3 differing bone sites. There was extensive loss of cancellous bone due to 2% saline treatment in the weight-bearing tibia bone. Saline treatment by itself had no significant effect on femur stiffness, femur calcium and femur strength. However, SS genotype effect was enhanced by saline treatment and resulted in an additional increase in femur stiffness (underloaded femur), femur calcium, femur strength, blood pressure and heart rate, and simultaneously a decrease in peak bone mass and femur magnesium. In addition, the W strain with saline treatment, had an additional increase in femur stiffness, femur calcium (underloaded femur) and femur strength, blood pressure and heart rate, and simultaneously a decrease femur magnesium. Femur cross sectional transverse area and zinc exhibited only a genetic influence when comparing the two strains. After 6 weeks of immobilization, marked reductions in unloaded tibia cancellous bone volume with increased femur bone stiffness and mineral concentration (calcium and phosphorus) were found in the 2% study.

5.2 BONE QUALITY MEASURES

The present studies provided morphometric and histological data about the response of rat bone to salt treatment, genotype and limb underloading/overloading. These findings may have implications in evaluating human bone as bone quality may be altered early in life before peak bone mass is achieved. Bone may be damaged by dietary salt. Genetic predisposition to hypertension is a major factor in bone development. Transient periods of immobilizations (space, sedentary job, lack of exercise, castings, hospitalizations) may interact with the salt and genetic factors to increase future fracture risks. Initial bone susceptibility may be indicated by loss of bone elasticity, loss of cancellous bone, abnormal mineral distribution, and small bones with an early maturation of peak bone mass. Screening and an adequate salt diet may be both advisable for females and especially female space travelers. Salt, genotype, and limb underloading/overloading (simulating space travel with return to earth) may combine to accelerate adverse effect on bone with a deleterious effect on earth and worse if combined with space travel bone stress. Initial bone damage (as detected by autopsy) may not be detected by bone calcium loss, and therefore bone scans. If standard bone scans were used, the stiffer, underloaded hypertensive rat bone might appear to be the strongest.

Drozdzowska et al. (2002) suggest that newer methods of measuring with DXA (dual energy X-ray absorptiometry) and ultrasound velocity may improve the prediction of the biomechanical properties of cancellous bone. Ultrasound has shown great promise in that it is comparatively inexpensive, less invasive (as no radiation), and may improve the accuracy of bone measurement when combined with other bone density

measurements. However, even if one gets a very accurate result, how will this be interpreted? The present studies indicate how bones with elevated calcium may be the most at risk for fracture. Increased stiffness and strength is often seen as a better quality bone. However, if the two qualities are generated in adolescents from smaller boned populations with above average salt intakes, salt sensitive hypertensive ancestry, or a sedentary life style, these measurements should be suspect. While having the appearance of a high quality bone with higher strength, higher stiffness and higher calcium or density, in fact, these samples could be regarded with the highest risk for future fracture risk. If transient patterns of behavior, as hospitalizations, exposure to weightlessness, a sedentary job or just inactivity are added, stiffness may increase as well as the risk for fracture. If only bone mineral content were investigated as is done in many experimental studies and the majority of human studies, these deleterious unmeasured qualitative factors that impact bone strength and stiffness would not be factored in. While the results of the current study are preliminary, they do document a critical finding. Current techniques of bone assessment are not adequate. The future of healthy space travel will depend on some of the strategies suggested here.

5.3 FUTURE EXPERIMENTAL STUDIES

Given the importance of the findings of this study, future work is clearly merited. Future work needs to take two directions. The first of these directions involves more lab animal studies. The second direction would involve applying the present findings to humans in human based studies.

Additional animal studies are needed to expand and confirm these findings. Such studies would involve more rats and more levels of saline treatment, 1.5%, 2%, 2.5%, and 3%. A future expanded study would also need to be initiated earlier and to extract bones at regular intervals of 3, 6, 9, 12, 24, 36 and 48 weeks.

Beyond rat studies other animal species such as rabbit or pigs could be considered, as an intermediate step prior to human studies. The advantage of a slightly larger species would be larger bone sizes facilitating bone strength and elasticity measurements by in vivo ultrasound/DXA followed by extraction comparisons. Finally, the genetic basis (genes) for salt sensitivity ought to be addressed.

5.4 FUTURE HUMAN STUDIES

Following confirmation of the results of this study on larger animals and in larger test numbers, human studies would be needed. Such studies would take two very different directions.

The first direction would be direct bone testing from autopsies of young female accident victims. Such autopsies would require special permission and necessarily be limited in number. Bone sections removed from these cadavers could be used for direct strength and elasticity measurements on an Instron machine. Morphology and microscopy could be done on bone sections using the techniques perfected in these studies. Of prime importance would be to take ultrasound velocity and DXA measurements. This data along with existing data would then need to be put together to determine the exact signature related to specific bone states.

Once a non-invasive truly accurate technique of bone measurement is established, a longer human statistical study could be done on females approaching peak bone mass (age 20-35). This larger study would need to include dietary parameters to assess the effects of various salt levels, exercise parameters to assess various activity levels and genetic screening for hypertension and salt sensitivity. The study would have to be quite large to statistically separate all of these variables. Ideally, the study would involve questionnaires and ultrasound measurements at several points perhaps over a decade. The study would need several hundred people and optimally, would maintain contact into the age of post menopause.

The outcome of such a study would be an accurate profile of someone likely to suffer bone loss/fracture. Using this model, early intervention studies could be applied. Initially such intervention would likely involve increasing certain kinds of exercise, restricting salt, possibly supplementing magnesium and calcium and restricting space travel.

For NASA, a screening profile could be developed to identify astronauts most likely to develop bone loss. Once identified, these people could be scheduled for shorter flights or put on a bone-building program. Experience with a bone-building program could be profitably applied to all astronauts returning from extended space travel. In addition, knowledge of the hypertensive and/or salt sensitive trait may allow early detection for those astronauts at risk for specific inherited vulnerabilities and provide ample time for specific strategies/interventions to address each specific defect. Several suspected genes are currently being studied, but at this time the molecular genetic basis for salt sensitivity remains unknown (Liang et al., 2002). More recent studies on SHR

and genetic hypertension have implicated the kidney (Grisk and Rettig, 2001; Johnson et al., 2002) and now genes (Grisk and Rettig, 2001), but the underlying cause remains unknown.

APPENDIX A

GLYCOL METHACRYLATE PROCESSING FOR TIBIA TRABECULAE

A. Materials, Solution, and Storage (Ruegg and Smith Protocols, 1996)

2-hydroxyethyl methacrylate (HEMA or GMA) low acid, CR2D2
Polysciences, Inc., Warrington, PA Cat. # 03699-0 keep in cold room

Polymerization initiating component "B" 1 ml NN Dimethyl Aniline in 15 ml
EGME, Polysciences, Inc. Warrington, PA Cat. # 0226B

Ethylene glycol monobutyl ether (EGME, CELLOSOLVE)
Fisher Scientific, Santa Clara, CA Cat. # E179-4 keep in cabinet below staining counter

Benzoyl peroxide
Aldrich Chemical Co., St. Louis, Missouri Cat. # 17,998-1 keep in refrigerator

Metal Block Holders
BH Tool Co., Bruce Houbaugh, Strasburg, CO

Peel-A- Way Disposable Molds, 22 x 40 mm, 20mm deep; 22 x 30 mm, 20mm deep
Polysciences, Inc., Warrington, PA Cat. # 18646C-1

I. Infiltration Solution:
The infiltrating solution for processing was prepared from **400 ml HEMA, 120 ml EGME** (less is used for harder blocks, more is used for softer blocks), and **9 gm Benzoyl Peroxide**. Solution was stirred until the benzoyl peroxide was completely dissolved, then stored at 4 degrees C. This solution can be used for three weeks (Ruegg and Smith, 1996).

Infiltration solution was prepared from this protocol from Ruegg and Smith, 1996.

1. **400 ml of 2-hydroxyethyl methacrylate (HEMA)** (which should be stored in cold room) was poured into a one liter beaker.

2. **120 ml ethylene glycol monobutyl (EGME)** was added.

3. **9 gm benzoyl peroxide** was added to the solution.

4. A beaker was placed on a stir plate (approximately 20 minutes was needed to dissolve) and solution stirred.

5. The solution was labeled, dated, and stored in refrigerator for up to 3 weeks.

II. Polymerizing Solution:
The polymerizing solution for processing was prepared from **20 ml infiltrating solution** and **10 drops polymerization initiating component "B"**. Ruegg and Smith recommended that this proportion could be used for what ever volume of reagent needed. As directed, solution was mixed well and work proceeded rapidly as polymerization begins within 5 minutes at room temperature. Additionally as advised, samples were polymerized at 4°C to slow the polymerization process, thus allowing the reagent to penetrate bone tissue.

APPENDIX A (cont.)

B. Tissue Processing, (Ruegg and Smith Protocols, 1996)

I. Fixation and Dehydration

Samples were sliced as soon as possible after removal from rat and / or promptly immersed in cold absolute ethanol. Temperature was reported since critical to bone temporary storage, transportation, and slicing. Dehydration of samples through typical graded alcohol protocol was not used in order to see calcein label (Personal communication, Patsy Ruegg). A brief exposure to acetone (15 minutes) was necessary to remove fat which can inhibit GMA infiltration.

II. Infiltration

Samples were infiltrated as long as possible in cold room (4°C) on a shaker ranging approximately 2 days. As it was considered the most important step in the process, this longer period was chosen. A minimal 6-12 hours shaking in cold room was used with these 500 micron thick sections of mineralized bone. The infiltrating solution was removed and replaced with pre-mixed infiltrating solution at least twice. Each labeled sample was contained in 20 ml glass vial with foil-lined screw top filled approximately 1/2 full of infiltrate.

III. Embedding

Peel away disposable molds, 25 x45 mm rectangular, 20 mm deep 25 x30 mm rectangular, 20 mm deep were used as the embedding molds for most of these samples. For smaller samples, embedding mold trays with 16 mm x 8 mm and 19 mm x 13 mm were used. A rectangular piece of filter paper (approximately 16 mm x 36 mm in size for 25 x45 mm rectangular mold) was lain flat on the bottom of the embedding mold prior to the addition of mixed polymerization solution. As recommended by Ruegg and Smith, this piece of filter paper provided an additional layer of polymerized plastic placed between the bottom exposed surface and sample. This preventive step avoided accidental tissue exposure to air without coverage in the polymerized layer. The sliced sample was placed flat over paper in the mold. Any air bubbles under the paper or under the sample were gently pushed out with tweezers. A block holder was placed on top of embedding mold. In our first trail this placement was unsuccessful because the solution slow to polymerize (two years old), which caused the metal block holder to sink unevenly. In our second attempt labeled metal blocks (with 0.5 cm x 4 cm long colored tape was placed around top portion of holder) were placed on partially filled polymerized blocks and more solution was added. These labeled holders with specimen in solution were placed in closed container in cold refrigerator (4°C) until it polymerized in 1-4 hours (overnight). According to Ruegg and Smith, the larger the volume of polymerizing solution the hotter it will get and the quicker it will polymerize. The processing method, that was adopted here for blocks, used no more than 10 ml of solution per mold.

Embedding Procedures for (GMA) Plastic Blocks (Ruegg and Smith)

1. The samples were previously processed with several changes of infiltration solution with shaking. This properly prepared them for embedding.

2. Embedding molds for samples and metal blocks were pre-labeled.

3. Filter paper was placed in bottom of mold.

4. Embedding solution was prepared. Enough solution was made for 5 ml per sample. Solution was made in disposable plastic beaker and mixed well on magnetic stirrer.

APPENDIX A (cont.)

Only 8 samples were prepared at one time because of short polymerizing time:
-20 ml infiltration solution, A&C plus (Pre-mixed HEMA, benzoyl peroxide and EGME from refridgerator)
-20 ml GMA plus (HEMA)
-20 drops solution "B"
-40 drops EGME (Ethylene Glycol Monomethyl Ether)

This worked out to equal parts A&C and GMA:
0.5 drop of solution B per ml of A&C
0.5 drop of solution B per ml of GMA solution
1 drop of Ethlene Gycol Monomethyl Ether (EGME) per ml of A&C
1 drop of Ethlene Gycol Monomethyl Ether (EGME) per ml of GMA solution

Solution was mixed very well on a magnetic stirrer, and poured into 8 embedding molds. Air bubbles were gently pushed out with a forceps as sample is positioned in mold over filter paper. Saran wrap was placed over solution to prevent rapid polymerzation by atmospheric oxygen. Embedding molds were placed in a pre-cooled closed container in 4°C refrigerator. In addition, a thin layer of crushed ice can keep polymeration temperature as low as possible. Polymerization time was approximately 2-5 hours, however, ice can extend to overnight. Embedding procedure was repeated without filter paper in order to place aluminum block holder on top of specimen in proper alignment.

Note: If sample was large, more polymerization time was required to prevent bubbling from overheating. Blocks should be placed in freezer rather than the refrigerator, or polymerization could be slowed by decreasing the amount of solution"B" used in the mixture (Ruegg and Smith,1996).

IV. Sectioning
Polymerized blocks were removed from the embedding mold. Blocks were cut using a heavy JB-4 microtome (Sorvall, Porter Blum - cutting range 0.25 micron to 10 microns) with the steel knife block holder accessory to the microtome. As recommended, the block holder, stage holder and knife holder were tighten with pliars to insure all holding systems were secure when cutting. A sharp tungsten carbide tipped cutting edge knife (Shandon), which minimized fragmentation cut 5 um and 10 um sections. Cutting was done slowly and as smoothly as possible. Each section was collected from microtome with very fine tweezers and stretched by floating into room-temperature deionized water. Tabeling (1996) noted that in a 60°C water bath, stretching of section can only reach 7-9%, while at a reduced temperature of 20°C, 10-13% stretching was possible. Once stretched in this manner less than 1% of the compression in vertical dimension caused during sectioning would be detectable. Sections were now mounted to glass microscope slides charged or silane coated to keep section from falling off during staining. Depending on stain to be applied, slides were either air dried or heated on hot plate to dry.

As directed by Ruegg and Smith protocol, 8-10 perfect sections of 5 microns were collected, placed on Silane (+) coated slides, and air dried. Air dried slides were used for acid phosphatase and Van Kossa staining. Additionally, four perfect sections of 5 microns were picked up on (+) slides and blotted with labobsorb paper and placed in a coplin jar of deionized water without drying. These wet slides were used for Von Kossa staining. Lastly, 3 perfect sections were picked up onto uncoated slides, and air dried. These sections were left unstained and without coverslips for Calcein examination.

V. Staining
The plastic was not removed before staining but can be penetrated by the staining reagents. The reagent concentrations and staining times may need to be increased for GMA sections, because staining through the plastic was necessary and the sections are unusually thin which means there is not much tissue there to take on the stain.

APPENDIX A (cont.)

Trap Stain (Tartrate Resistant Acid Phosphatase) (Ruegg and Smith Protocols, 1996)

Reagents:
Trap Stain (Tartrate Resistant Acid Phosphatase) Kit (Sigma Kit, Cat. No. 387-A)
Stored in refrigerator

1. Three air dried slides were rinsed from each specimen.

2. TRAP solution was mixed according to manufacturer's directions.

50 ml of distilled water was place in a beaker in a beaker

a. 0.5 ml sodium nitrite solution was placed in a test tube
b. 0.5 ml fast garnet was placed in a test tube
c. 2 ml of acetate buffer solution was added to beaker of distilled water. Two minutes later the test tube of fast garnet and nitrite were added to beaker. At this time, 0.5 ml of naphol AS-B1 solution was added to the beaker of distilled water. The last step was to add the 0.5 ml of tartrate solution.
d. The mixture was then stirred on a magnetic stirrer.

3. Solution was filtered onto pre-rinsed slides. Slides were then stained in 37°C oven for at least an hour.

4. Staining solution was removed and poured into waste can. Slides were rinsed with distilled water.

5. Ruegg and Smith have recommended using fast green straight from the bottle (.2% at 10 sec intervals up to 30 seconds for staining bone), rather than a diluter version.

6. Reusable, excess stain was poured into waste can. Slides were rinsed in distilled water.

7. Slides were air dried and coverslipped.

This resulted in osteoclastic staining a bright reddish purple (in granules and cytoplasm). TRAP measurements are used to quantify. 1) osteoclast no., 2) osteoclast surface and 3) eroded surface. Fast green identifies nucleii of cells. Bone area stains a very pale green.

In osteoclastic counts, globs of cells were counted as one as well as small cells lining (can be a bit in) the surface of bone. Red cells in marrow were not counted. Scalloped areas were reported.

To mount slides:
1) One drop Cytoseal 60 mounting medium (Cat. No. 8310-4,40Z) was dispensed from container on side to avoid getting bubbles. Each drop was placed on lower portion of coverslip, which is next to sample on charged glass slide. Each glass slide was pressed down onto coverslip (which was flat on padded counter cover), spreading mounting medium to all parts of coverslip. Slides were allowed to harden on tray plate with paper towels. Slides were dried in cardboard holder for several days, so mounting medium would not stick.

Van Kossa Stain (Ruegg and Smith Protocols, 1996)

1. Five micron section were cut, immediately dropped into water, retrieved with tweezers and placed on charged slides. These slides were blotted partially dry and stored in a coplin jar of water until ready to begin to stain. These slides were not dried as it will cause bone to crack. If dry, re-wet.

APPENDIX A (cont.)

2. Slides were labeled with identification no. and marked VK.

3. Slides were then placed in 7-15 slide coplin jars and rinsed in deioned water (deioned water, rather than distilled water was necessary to use with silver nitrate).

4. Water was poured off and 2% silver nitrate (kept in dark bottle in refrigerator) poured in to cover sections).

5. The coplin jar with silver nitrate solution covering slides was placed under UV light on counter for 15 minutes.

6. Silver nitrate solution was poured into separate silver nitrate container for disposal. Slides were rinsed completely with deioned water (5-10 changes of deioned water).

7. The H & E protocol used for bone:

   a). Slides were counterstained with Gill's #3 hemotoxylin for 5 minutes. The solution was poured back into brown bottle as reuseable. Slides were rinsed in deioned water.

   b). Slides were blued for 10 seconds in .02% ammonium hydroxide. Slides were rinsed well in deioned water.

   c). Slides were then placed for 10 minutes in a .02% aqueous Eosin Y solution which was made fresh each week and filtered before each use. Slides were placed in clean deioned water.

8. Slides were dipped in 95% isopropyl, 15 times. If ethyl alcohol was used here or in Eosin solution, samples fell off slides. This could happen with isopropyl solution , but far less likely. Patsy had used a reagent (mixture of alcohols) alcohol, which worked as well as isopropyl.

9. Slides were dipped 15 times in 100% isopropyl x 3.

10. Slides were dipped 15 times in non-toxic replacement for xylene x 2 (this did not work quite as well as xylene).

11. Slides were coverslipped wet with Cytoseal 60 mounting medium. Slides were not allowed to air dry.

APPENDIX B

Abbreviations key for Table 1

bdyinc	body weight increase in grams
bdywt1	initialbody weight in grams
bdywtf	final body weight in grams
B-l	overloaded femur boron (ppm)
bp	blood pressure
B-r	underloaded femur boron (ppm)
B-u	left ulna boron (ppm)
CA-l	overloaded femur calcium %
CA-r	underloaded femur calcium %
CA-u	left ulna calcium %
cl%/Yr	overloaded tibia bone formation rate expressed as % per year
clAdjC	overloaded femur adj. count, image width*image height/(image width- feature width) * (image height- feature height)
clarea	overloaded femur area (cm^2)
clAsp	overloaded femur aspect ratio, max diameter / min diameter
clBFR	overloaded tibia bone formation rate
clBFR/TV	overloaded tibia bone formation rate/tissue area
clBrea	overloaded femur breadth (cm), calipers dimensions
clConAr	overloaded femur convex area (cm^2), area measurements including holes and exteior indentations
clConPer	overloaded femur convex perim (cm), perimeter measurements including holes and exteior indentations
clconv	overloaded femur convexity, convex perimeter/perimeter
cldiam	overloaded femur equiv. diam. (cm)

Abbreviations key for Table 1 (cont.)

clform	overloaded femur form factor
clhole	overloaded femur holes
clleng	overloaded femur length (cm)
clLeOcbs	overloaded tibia osteoclast surface/bone surface
clIrLTh	overloaded tibia cancellous interlabel thickness
clMA	overloaded femur moment angle
clMar	overloaded tibia mineral apposition rate (MAR), distance between two calcein label lines/days in between
clMS	overloaded tibia mineralizing surface unstained section with UV light
clMS/BS	overloaded tibia mineralizing surface / bone surface
clNoOcBS	overloaded tibia osteoclast number/bone surface
clOcNoBA	overloaded tibia osteoclast number/bone area
clOcNoTA	overloaded tibia osteoclast number/tissue area
clperi	overloaded femur perimeter(cm)
clroun	overloaded femur roundness
clsoli	overloaded femur solidity
clx	overloaded femur x-feret, longest distance between any two points on the peripherally
clxc	overloaded femur x-cent.grav.(cm), centroid or center of gravity, Âi xi / area
cly	overloaded femur y-feret, longest distance between any two points on the peripherlly
clyc	overloaded femur
cmarr	underloaded femur marrow area
cr%/Yr	underloaded tibia bone formation rate expressed as % per year crBFR

Abbreviations key for Table 1 (cond.)

crAdjC	underloaded femur Adj. Count, image width*image height/(image width- feature width) * (image height- feature height)
crarea	underloaded femur area (cm^2)
craspR	underloaded femur aspect ratio
clBFR	overloaded tibia bone formation rate
clBFR/TV	overloaded tibia bone formation rate/tissue area
crBrea	underloaded, femur breadth (cm)
crConAr	underloaded femur convex area (cm^2)
crConPer	underloaded femur convex perim. (cm)
crconvx	underloaded femur convexity
crdiam	underloaded femur equiv. diam. (cm)
crform	underloaded femur form factor
crhole	underloaded femur holes
crleng	underloaded femur length (cm)
crLeOcbs	underloaded tibia osteoclast surface / bone surface
crMA	underloaded femur moment angle, moment axis of a feature
crMar	underloaded tibia mineral apposition rate (MAR), distance between two calcein label lines/days in between
crMS	underloaded tibia mineralizing surface unstained section with UV light
crMS/BS	underloaded tibia mineralizing surface MS = (dLS + sLS/2)/BS
crNoOcBS	underloaded tibia osteoclast number/bone surface
crOcNoBA	underloaded tibia osteoclast number /bone area
crOcNoTA	underloaded tibia osteoclast number /tissue area
crperi	underloaded femur perimeter (cm)
crrLTh	underloaded tibia cancellous interlabel thickness
crroun	underloaded femur roundness

Abbreviations key for Table 1 (cond.)

crsoli	underloaded femur solidity
crum/d	underloaded femur adjusted apposition rate
crx	underloaded femur x-feret, longest distance between any two points on the peripherlly
crxc	underloaded femur x-cent. grav. (cm)
cry	underloaded femur y-feret
cryc	underloaded femur y-cent. grav. (cm)
Cu-l	overloaded femur copper, ppm
cum/d	underloaded tibia adjusted apposition rate
Cu-r	underloaded femur copper, ppm
Cu-u	left ulna copper, ppm
fdday	food intake per day
fdsum	food intake total
Fe-l	overloaded femur iron, ppm
Fe-r	underloaded femur iron, ppm
Fe-u	left ulna iron, ppm
flday	fluid consumption per day
flsum	fluid consumption total
gml	overloaded femur weight in grams
gml/bw%	overloaded femur weight in grams/body weight in grams, %
gmr	underloaded femur weight in grams
gmr/bw%	underloaded femur weight in grams/body weight in grams, %
H2O/Na	water control and saline treated factors
hlAdjC	overloaded femur marrow section adj. count, image width*image height/(image width- feature width) * (image height- feature height)
hlarea	overloaded femur marrow section area(cm^2)
hlAsp	overloaded femur marrow section aspect ratio

Abbreviations key for Table 1 (cond.)

hlBrea	overloaded femur marrow section breadth (cm),
hlConAr	overloaded femur marrow section convex area (cm^2), area measurements including holes and exteior indentations
hlConPer	overloaded femur marrow section convex perim (cm), perimeter measurements including holes and exteior indentations
hlconv	overloaded femur marrow section convexity
hldiam	overloaded femur marrow section equiv.diam.(cm)
hlform	overloaded femur marrow section form factor
hlhole	overloaded femur marrow section holes
hlleng	overloaded femur marrow section length (cm)
hlMA	overloaded femur marrow section moment angle
hlperi	overloaded femur marrow section perimeter (cm)
hlroun	overloaded femur marrow section roundness
hlsoli	overloaded femur marrow section solidity
hlx	overloaded femur marrow section x-feret, longest distance between any two points on the peripherally
hlxc	overloaded femur marrow section x-cent. grav. (cm), centroid or center of gravity, Âi xi / area
hly	overloaded femur marrow section y-feret, longest distance between any two points on the peripherally
hlyc	overloaded femur marrow section y-cent.grav.(cm), centroid or center of gravity, Âi yi / area
hr	heart rate
hrAdjC	underloaded femur marrow section adj. count
hrarea	underloaded femur marrow section area (cm^2)
hraspR	underloaded femur marrow section aspect ratio
hrBrea	underloaded, femur breadth (cm)
hrConAr	underloaded femur marrow section convex area (cm^2)

Abbreviations key for Table 1 (cond.)

hrConPer	underloaded femur marrow section convex perim. (cm)
hrconvx	underloaded femur marrow section convexity
hrdiam	underloaded femur marrow section equiv. diam. (cm)
hrform	underloaded femur marrow section form factor
hrhole	underloaded femur marrow section length (cm)
hrMA	underloaded femur marrow section moment angle
hrperi	underloaded femur marrow section perimeter (cm)
hrroun	underloaded femur marrow section roundness
hrsoli	underloaded femur marrow section solidity
hrx	underloaded femur marrow section x-feret, longest distance between any two points on the peripherally
hrxc	underloaded femur marrow section x-cent. grav. (cm)
hry	underloaded femur marrow section y-feret
hryc	underloaded femur marrow section y-cent. grav. (cm)
K-l	overloaded femur potassium, %
K-r	underloaded femur potassium, %
K-u	left ulna potassium, %
lBA/TA	overloaded tibia bone volume (bone area / sample area) in mm^3
lbs	overloaded tibia bone surface in mm^2
lBS/BV	overloaded tibia bone surface/ bone volume in mm^2/mm^3
lBV	overloaded tibia bone volume in mm^3
lBV/TVpc	overloaded tibia bone volume expressed as a percent (bone area / sample area x 100)
lF,N	F is applied force in newtons of overloaded femur
lGPa	overloaded femur apparent modulus of elasticity in Gpa (gigapascals)
lmarr	overloaded femur marrow area cm^2
lMPa	overloaded femur ultimate bending stress in Mpa (megapascals)

Abbreviations key for Table 1 (cond.)

lsv	overloaded tibia bone surface, mm^2/mm^3
lTbN	overloaded tibia trabecular number
lTbSp	overloaded tibia trabecular separation in microns
lTbTh	overloaded tibia trabecular thickness in microns
lTV	overloaded tibia tissue volume
Mg-l	overloaded femur magnesium %
Mg-r	underloaded femur magnesium %
Mg-u	left ulna magnesium %
mmbl	left femur length in mm, ball to distal, overloaded
mmbr	right femur length in mm, ball to distal, underloaded
mmtl	left femur length in mm, trochanter to distal, overloaded
mmtr	right femur length in mm, trochanter to distal, underloaded
Mn-l	overloaded femur manganese, ppm
Mn-r	underloaded femur manganese, ppm
Mn-u	left ulna manganese, ppm
Nafdintk	NaCl in food intake
Naflintk	Nacl in fluid intake
NA-l	overloaded femur sodium, ppm
NA-r	underloaded femur sodium, ppm
NA-u	left ulna sodium, ppm
P-l	overloaded femur phorphorus %
P-r	underloaded femur phorphorus %
P-u	left ulna phorphorus %
rBA/TA	underloaded tibia bone volume (bone area / sample area) in mm^3
rbs	underloaded tibia bone surface in mm^2
rBS/BV	underloaded tibia bone surface/ bone volume in mm^2/mm^3
rBV	underloaded tibia bone volume in mm^3

Abbreviations key for Table 1 (cond.)

rBV/TVpc	underloaded tibia bone volume expressed as a percent (bone area / sample area x 100)
rF,N	F is applied force in newtons of underloaded femur
rGPa	underloaded femur apparent modulus of elasticity in Gpa (gigapascals)
rMPa	underloaded femur ultimate bending stress in Mpa (megapascals)
rsv	underloaded tibia bone surface, mm^2/mm^3
rTbN	underloaded tibia trabecular number
rTbSp	underloaded tibia trabecular separation in microns
rTbTh	underloaded tibia tibia trabecular thickness in microns
rTV	underloaded tibia tissue volume
TLNa	total NaCl consumed in food and fluid
TLNa Day	total NaCl consumed per day in food and fluid
ulnaff	fat free dry weight weight of full ulna bone in grams
ulnalen	left full ulna bone length in mm
ulnawt	left full ulna bone weight in grams
ur av	urine test 24 hours average
ur1	urine test 24 hours
ur2	urine test 24 hours
ur3	urine test 24 hours
w/ss	wistar and hypertensive genetic factors for statistical analysis
wc/ws/ssc/sss	WC,WS,SSC, and SSS(see Abbreviations) factors for statistical analysis (see Abbreviations)
Zn-l	overloaded femur zinc, ppm
Zn-r	underloaded femur zinc, ppm
Zn-u	left ulna zinc, ppm

Abbreviations key for Table 2

cv%/Yr	vertebra bone formation rate expressed as % per year
cvBFR	vertebra bone formation rate
cvBFR/TV	vertebra bone formation rate/tissue area
cvIrLTh	vertebra cancellous interlabel thickness
cvMar	vertebra mineral apposition rate (MAR), distance between two calcein label lines/days in between
cvMS	vertebra mineralizing surface unstained section with UV light
cvMS/BS	vertebra mineralizing surface / bone surface
cvum/d	vertebra adjusted apposition rate
ID	individual number of each rat in 2% study
lF,N	F is applied force in newtons of overloaded femur
lGPa	overloaded femur apparent modulus of elasticity in Gpa (gigapascals)
lMPa	overloaded femur ultimate bending stress in Mpa (megapascals)
rF,N	F is applied force in newtons of underloaded femur
rGPa	underloaded femur apparent modulus of elasticity in Gpa (gigapascals)
rMPa	underloaded femur ultimate bending stress in Mpa (megapascals)
vARS	vertebra osteoclast surface/bone surface
vBPm/TA	vertebra bone surface/ bone volume in mm^2/mm^3
vBSs	vertebra bone surface in mm^2
vBV	vertebra bone volume in mm^3
vBV/TV%	vertebra bone volume expressed as a percent (bone area / sample area x 100)
vderSv	vertebra bone surface, mm^2/mm^3
VLength	vertebra length in mm

Abbreviations key for Table 2 (cont.)

vLeOc	vertebra osteoclast surface
vNOc	vertebra osteoclast number
vNoOc/BS	vertebra osteoclast number/bone surface
vOcNo/TA	vertebra osteoclast number/tissue area
vTbN	vertebra trabecular number
vTbSp	vertebra trabecular separation in microns
vTbTh	vertebra trabecular thickness in microns
vTV	vertebra tissue volume

APPENDIX B

Table 1 Data for each rat in 1 % Study and 2% Study

ID	ID2	H2O/Na	w/ss	wc/ws/ssc/sss	%Code	bdywt1	bdyinc	bwtf	ur1	ur2	ur3
1	1	2	1	2	1	175	52.5	227.5	4	3	3
2	2	1	1	1	1	182	33.6	215.6	1.5	14	13
3	3	2	1	2	1	176.7	32.1	208.8	3	4.5	7
4	4	1	1	1	1	166.2	27.3	193.5	3	4.3	10
5	5	2	1	2	1	176.2	42.1	218.3	7.5	25	16
6	6	1	1	1	1	181.6	46.6	228.2	4.3	9	8
7	7	2	1	2	1	180	65.7	245.7	12.5	24.5	25
8	8	1	1	1	1	169.4	54.9	224.3	4.5	6.5	8.5
9	9	2	1	2	1	167.7	25.3	193	12.5	9.5	15
10	10	1	1	1	1	180.4	7.6	188	3.7	3.5	4
11	11	2	1	2	1	160.9	33.2	194.1	5	21	39
12	12	2	2	4	1	161	21.3	182.3	6	25	9
13	13	1	2	3	1	175	29.7	204.7	3	3	3
14	14	2	2	4	1	155.5	49.5	205	27	15	22.5
15	15	1	2	3	1	171	27.5	198.5	4.5	3.8	14
16	16	2	2	4	1	170	11.7	181.7	5	8.5	5
17	17	1	2	3	1	175.1	36.2	211.3	4.7	5	3.5
18	18	2	2	4	1	166.8	28.7	195.5	6.5	10	14.5
19	19	1	2	3	1	170	24.5	194.5	4	4.3	3
20	20	2	2	4	1	155	52	207	16	30	31
21	21	1	2	3	1	172.2	20.3	192.5	3	5.5	3.5
22	22	2	2	4	1	171.9	35.3	207.2	8.5	19.5	20
23	1	1	1	1	2	167.8	37.9	205.7	2	3.1	2.9
24	2	2	1	2	2	163.4	49.7	213.1	12.6	24.5	26
25	3	1	1	1	2	165.2	19.7	184.9	4	11.3	11.3
26	4	2	1	2	2	166.8	26.7	193.5	9	10.9	10.1
27	5	1	1	1	2	158	7.8	165.8	3	4.9	5.7
28	6	2	1	2	2	168.3	20.5	188.8	6.5	6.4	14.7
29	7	1	1	1	2	169.7	27.2	196.9	3	7.2	8.5
30	8	2	1	2	2	164	24	188	7.4	11.7	9.3
31	9	1	1	1	2	167.2	17.3	184.5	7.6	17.6	7.2
32	11	1	1	1	2	161.2	15.6	176.8	9.5	4.6	3.6
33	13	1	2	3	2	150.4	64.1	214.5	4	3.2	3.5
34	14	2	2	4	2	164.4	42.1	206.5	13.5	16.7	18.3
35	15	1	2	3	2	154.5	46	200.5	7.5	5.1	6.2
36	16	2	2	4	2	154.2	39.4	193.6	20.5	18	6.7
37	17	1	2	3	2	157.4	35.6	193	4.5	3.8	9.7
38	18	2	2	4	2	155.6	34.4	190	7	19.2	27
39	19	1	2	3	2	152.1	35.9	188	4	5.2	11.5
40	20	2	2	4	2	165.1	13.4	178.5	6	8.6	27.6
41	21	1	2	3	2	154	51.8	205.8	2	4.3	8.7
42	22	2	2	4	2	154.4	26.6	181	14.5	7.2	16.3
43	23	1	2	3	2	157	30.5	187.5	4	3.1	5.1
44	24	2	2	4	2	159.8	14.3	174.1	18.5	38.6	34.8

Table 1 (cont.)

ID	ur av	fdsum	fdday	flsum	flday	Nafdintk	Naflintk	TLNa	TLNa Day	gml	mmtl
1	3.3	722.39	17.2	668.39	15.91	1.87	6.68	8.55	0.20	0.636	31.79
2	9.5	681.68	16.23	717.37	17.08	1.77	0.00	1.77	0.04	0.636	31.5
3	4.8	631.22	15.03	575.35	13.7	1.63	5.75	7.38	0.18	0.544	31.37
4	5.8	600.13	14.29	661.92	15.76	1.55	0.00	1.55	0.04	0.521	29.99
5	16.2	686.37	16.34	1524.73	36.3	1.78	15.25	17.03	0.41	0.58	31.62
6	7.1	693.57	16.51	672.32	16.01	1.8	0.00	1.80	0.04	0.617	32.3
7	20.7	714.24	17.01	1342.13	31.96	1.85	13.42	15.27	0.36	0.668	32.06
8	6.5	653.17	15.55	576.13	13.72	1.69	0.00	1.69	0.04	0.624	31.5
9	12.3	644.77	15.35	752.72	17.92	1.67	7.53	9.20	0.22	0.556	30.63
10	3.7	618.49	14.73	365.87	8.71	1.6	0.00	1.60	0.04	0.525	30.25
11	21.7	686.36	16.34	1251.08	29.79	1.78	12.51	14.29	0.34	0.508	30.58
12	13.3	676.78	16.11	916.2	21.81	1.75	9.16	10.91	0.26	0.505	29.39
13	3.0	631.1	15.03	385.11	9.17	1.63	0.00	1.63	0.04	0.511	30.2
14	21.5	681.3	16.22	1423.16	33.88	1.76	14.23	15.99	0.38	0.571	30.46
15	7.4	620.81	14.78	591.28	14.08	1.61	0.00	1.61	0.04	0.501	30.38
16	6.2	660.27	15.72	555.27	13.22	1.71	5.55	7.26	0.17	0.477	29.33
17	4.4	647.13	15.41	326.12	7.76	1.68	0.00	1.68	0.04	0.551	30.66
18	10.3	657.98	15.67	849.29	20.22	1.7	8.49	10.19	0.24	0.518	29.87
19	3.8	639.9	15.24	469.01	11.17	1.66	0.00	1.66	0.04	0.441	30.14
20	25.7	736.13	17.53	1281.17	30.5	1.91	12.81	14.72	0.35	0.423	30.35
21	4.0	618.77	14.73	413.32	9.84	1.6	0.00	1.60	0.04	0.441	29.94
22	16.0	726.43	17.3	924.36	22.01	1.88	9.24	11.12	0.26	0.48	30.11
23	2.7	654.99	15.6	397.5	9.46	1.7	0.00	1.70	0.04	0.655	31.04
24	21.0	675.79	16.09	1141	27.17	1.75	22.82	24.57	0.59	0.621	30.44
25	8.9	653.2	15.55	495	11.79	1.69	0.00	1.69	0.04	0.573	29.02
26	10.0	647.35	15.41	711.5	16.94	1.68	14.23	15.91	0.38	0.578	30.39
27	4.5	631.37	15.03	495	11.79	1.64	0.00	1.64	0.04	0.519	29.51
28	9.2	644.04	15.33	704	16.76	1.67	14.08	15.75	0.38	0.565	30.27
29	6.2	688.49	16.39	449.8	10.71	1.78	0.00	1.78	0.04	0.615	30.1
30	9.5	641.12	15.26	641.4	15.27	1.66	12.83	14.49	0.35	0.612	29.45
31	10.8	653.23	15.55	554	13.19	1.69	0.00	1.69	0.04	0.664	31.07
32	5.9	658.84	15.69	555.8	13.23	1.71	0.00	1.71	0.04	0.547	29.86
33	3.6	677.07	16.12	486.5	11.58	1.75	0.00	1.75	0.04	0.539	29.86
34	16.2	648.69	15.45	1122.5	26.73	1.68	22.45	24.13	0.58	0.53	30.25
35	6.3	682.98	16.26	532.5	12.68	1.77	0.00	1.77	0.04	0.529	30.18
36	15.1	652.27	15.53	725.4	17.27	1.69	14.51	16.20	0.39	0.528	29.96
37	6.0	637.71	15.18	582.6	13.87	1.65	0.00	1.65	0.04	0.534	30.28
38	17.7	688.66	16.4	808	19.24	1.78	16.16	17.94	0.43	0.562	30.83
39	6.9	641.84	15.28	487.4	11.6	1.66	0.00	1.66	0.04	0.567	30.97
40	14.1	622.93	14.83	557.6	13.28	1.61	11.15	12.77	0.30	0.493	29.63
41	5.0	694.32	16.53	520	12.38	1.8	0.00	1.80	0.04	0.554	30.58
42	12.7	606.19	14.43	960.8	22.88	1.57	19.22	20.79	0.50	0.512	29.72
43	4.1	665.09	15.84	331.8	7.9	1.72	0.00	1.72	0.04	0.592	30.69
44	30.6	672.46	16.01	1370.2	32.62	1.74	27.40	29.15	0.69	0.506	29.67

Table 1 (cont.)

ID	mmbl	gmr	mmtr	mmbr	ulnalen	ulnawt	ulnaff	CA-u	Mg-u	P-u	NA-u	K-u
1	31.05	0.704	31.98	30.9	28.34	0.143	0.115	24.7	0.48	11.2	0.6	0.19
2	30.95	0.626	31.74	30.3	29.69	0.133	0.105	22.8	0.48	9.31	0.57	0.1
3	30.6	0.533	31.1	29.82	27.8	0.123	0.101	21	0.42	9.7	0.52	0.1
4	29.18	0.555	30.18	29.69	28.96	0.116	0.88	20.5	0.41	9.19	0.56	0.13
5	30.84	0.623	31.08	30.28	28.92	0.124	0.108	20.8	0.4	9.4	0.56	0.16
6	31.11	0.616	32.22	31.03	29.2	0.116	0.1	21.4	0.44	9.43	0.56	0.18
7	31.22	0.688	32.4	31.07	28.82	0.138	0.11	20	0.38	8.98	0.55	0.16
8	30.84	0.666	31.61	30.85	29.99	0.126	0.111	19.9	0.4	9.13	0.53	0.16
9	29.65	0.571	30.95	29.47	30	0.127	0.111	20.7	0.4	9.47	0.52	0.15
10	29.85	0.533	30.11	29.25	29.58	0.12	0.104	21	0.41	9.43	0.53	0.15
11	29.85	0.494	30.35	29.3	26.16	0.103	0.95	21.4	0.42	9.84	0.53	0.17
12	28.64	0.524	29.53	28.74	28.44	0.114	0.101	21.5	0.4	9.8	0.52	0.13
13	29.9	0.499	30.38	29.94	28.26	0.124	0.105	22.3	0.44	9.67	0.52	0.19
14	29.95	0.531	30.62	29.43	30.3	0.126	0.111	21.3	0.4	9.59	0.54	0.15
15	29.9	0.542	30.51	29.92	29.51	0.123	0.11	21.1	0.39	9.58	0.59	0.22
16	28.49	0.494	29.43	28.2	29.86	0.121	0.108	22.1	0.42	10.04	0.54	0.15
17	30.08	0.659	33.99	33.04	30.59	0.13	0.114	19.9	0.4	9.64	0.48	0.16
18	28.9	0.539	29.72	28.65	30.38	0.118	0.109	20	0.39	9.7	0.5	0.22
19	29.63	0.453	30.3	28.98	29.2	0.12	0.102	21.5	0.4	9.41	0.53	0.23
20	28.72	0.472	30.38	29.01	28.58	0.124	0.104	20.9	0.39	9.84	0.57	0.18
21	29.29	0.484	29.89	29.58	28.69	0.123	0.101	22.2	0.38	10.15	0.56	0.22
22	29.64	0.453	30.65	29.71	29.44	0.122	0.105	30	0.55	13.52	0.78	0.25
23	30	0.612	30.83	30	28.64	0.164	0.12	25.66	0.42	11.65	0.68	0.09
24	29.33	0.6	30.27	29.5	28.45	0.146	0.103	25.49	0.43	11.68	0.63	0.1
25	28.27	0.55	30.6	29.98	28.34	0.125	0.108	24.58	0.42	11.08	0.54	0.1
26	29.6	0.588	30.47	28.91	27.6	0.143	0.107	25.9	0.44	11.92	0.68	0.09
27	28.92	0.569	30.15	29.05	28.76	0.13	0.102	21.97	0.4	10	0.49	0.09
28	29.63	0.547	30.37	29.51	27.83	0.133	0.103	26.47	0.42	11.76	0.62	0.09
29	28.7	0.613	30.53	29.21	28.12	0.157	0.107	24.52	0.39	10.96	0.72	0.06
30	28.61	0.595	30.35	28.89	28.05	0.14	0.107	23.94	0.39	11.2	0.62	0.12
31	30.22	0.638	31.54	30.02	28.8	0.145	0.114	24.2	0.42	11.13	0.6	0.12
32	29.1	0.505	29.55	29.69	27.89	0.125	0.095	25.1	0.42	11.32	0.64	0.07
33	28.8	0.514	30.03	26.13	28.73	0.142	0.104	25.07	0.43	11.55	0.66	0.05
34	29.72	0.513	30.36	28.73	28.86	0.134	0.109	24.66	0.41	11.45	0.57	0.1
35	29.35	0.47	30.58	29.64	29.41	0.13	0.107	23.96	0.43	11.09	0.52	0.08
36	29.24	0.488	30.1	28.3	28.37	0.131	0.103	24.39	0.4	11.05	0.55	0.1
37	29.71	0.492	30.37	28.67	28.44	0.139	0.104	23.79	0.42	11.94	0.53	0.1
38	30.37	0.566	31.17	30.24	29.31	0.147	0.112	24.25	0.39	11.25	0.59	0.08
39	30.09	0.553	30.93	30.11	28.92	0.143	0.108	25.83	0.42	11.61	0.68	0.04
40	28.17	0.523	30.09	29.28	28.24	0.142	0.103	23.42	0.39	10.82	0.56	0.07
41	29.91	0.534	30.54	27.67	28.69	0.144	0.106	23.72	0.41	10.76	0.62	0.03
42	29.38	0.532	29.94	29.39	28	0.129	0.092	25.61	0.41	11.45	0.65	0.04
43	29.96	0.575	30.48	29.38	28.32	0.14	0.102	23.7	0.41	11.03	0.58	0.04
44	29.16	0.48	29.67	28.92	28.2	0.139	0.104	22.84	0.39	10.74	0.61	0.02

Table 1 (cont.)

ID	B-u	Mn-u	Fe-u	Cu-u	Zn-u	CA-r	Mg-r	P-r	NA-r	K-r	B-r	Mn-r	Fe-r
1	11	4	63	36	366	26.3	0.5	12.6	0.66	0.38	45	2	40
2	6	1	11	6	230	26.8	0.53	12.7	0.6	0.33	70	1	34
3	1	1	8	4	212	28.7	0.54	13.1	0.65	0.42	77	3	47
4	3	1	9	1	189	28.4	0.53	12.8	0.72	0.45	80	3	222
5	2	1	16	1	262	29.7	0.58	13.6	0.66	0.3	62	2	36
6	1	1	10	1	220	28.8	0.51	13.7	0.63	0.15	51	1	23
7	1	1	15	5	277	29.2	0.53	13.7	0.66	0.27	40	1	29
8	1	1	13	1	292	29	0.55	13.6	0.67	0.24	71	2	24
9	1	1	13	1	216	28.6	0.54	13.4	0.6	0.22	58	1	21
10	1	1	12	4	207	28.7	0.53	13.5	0.79	0.33	70	3	412
11	1	1	5	1	280	29.6	0.54	13.9	0.79	0.21	74	1	43
12	1	1	6	1	203	30.9	0.81	13.8	0.63	0.37	41	22	1211
13	1	1	23	1	187	29	0.51	13.2	0.52	0.15	35	1	21
14	1	1	20	1	192	30	0.66	13.6	0.67	0.2	47	8	455
15	1	1	12	1	201	29.9	0.53	13.8	0.71	0.16	73	2	37
16	1	1	114	1	192	30	0.55	14.1	0.56	0.33	51	3	69
17	1	1	23	1	207	29.9	0.54	13.9	0.62	0.35	76	2	32
18	1	1	10	2	189	30.9	0.53	14.2	0.63	0.23	49	1	30
19	1	1	10	1	189	29.6	0.54	13.4	0.6	0.26	38	1	48
20	1	1	22	1	202	29.8	0.53	13.7	0.63	0.24	68	1	46
21	1	1	10	1	213	29.9	0.51	14	0.54	0.27	75	2	30
22	1	1	8	1	368	30.4	0.53	13.8	0.55	0.23	53	1	21
23	9	1	46	5	387	30.34	0.48	13.66	0.63	0.33	9	1	26
24	7	1	22	1	303	29.86	0.5	13.57	0.64	0.5	7	1	26
25	8	1	28	3	208	29.23	0.5	12.96	0.58	0.19	9	2	16
26	7	1	43	5	290	28.6	0.47	13.34	0.61	0.15	10	1	14
27	1	1	24	1	200	28.69	0.52	12.86	0.61	0.37	13	1	40
28	7	1	46	9	273	29.45	0.47	13.37	0.67	0.33	10	1	19
29	4	1	22	14	335	29.22	0.47	13.47	0.65	0.43	9	2	14
30	2	1	32	4	213	29.87	0.46	13.67	0.66	0.69	6	2	51
31	2	12	335	1	216	28.97	0.5	13.4	0.64	0.36	8	2	29
32	6	9	337	7	222	29.7	0.51	13.46	0.62	0.27	14	1	16
33	12	3	173	6	231	29.78	0.5	13.66	0.57	0.19	7	1	7
34	2	3	194	1	234	29.95	0.45	13.76	0.75	0.36	34	2	30
35	1	6	380	2	205	29.98	0.5	13.61	0.63	0.25	5	1	30
36	1	1	57	1	221	30.6	0.46	13.53	0.6	0.15	4	1	11
37	3	1	41	4	214	28.83	0.47	12.69	0.6	0.3	15	1	12
38	4	2	226	4	230	28.97	0.45	13.19	0.72	0.32	16	1	13
39	5	1	108	4	246	30.02	0.48	13.25	0.77	0.39	12	1	17
40	2	1	38	6	217	29.41	0.46	12.96	0.7	0.26	10	1	16
41	8	1	180	4	202	29.21	0.5	13.31	0.62	0.45	6	1	20
42	5	2	120	4	235	29.32	0.45	13.29	0.64	0.4	4	1	23
43	3	2	321	9	225	28.54	0.48	13.23	0.6	0.4	12	2	30
44	4	4	255	7	210	28.58	0.46	13.03	0.66	0.58	5	2	35

Table 1 (cont.)

ID	Cu-r	Zn-r	CA-l	Mg-l	P-l	NA-l	K-l	B-l	Mn-l	Fe-l	Cu-l	Zn-l	bp
1	104	372	29.9	0.54	13.8	0.5	0.13	35	2	45	16	458	128
2	30	328	29.8	0.55	13.9	0.71	0.41	50	2	41	85	372	132
3	59	284	30.9	0.55	14.5	0.6	0.23	51	1	28	36	294	108
4	53	256	30	0.56	14.2	0.5	0.19	40	3	42	32	269	*
5	53	328	30.8	0.54	14.1	0.54	0.15	23	3	65	107	365	112
6	27	386	29.8	0.56	13.8	0.58	0.17	58	1	25	15	286	118
7	66	383	30.1	0.51	12.7	0.6	0.14	31	1	26	22	393	138
8	26	436	30.2	0.54	13.2	0.6	0.23	73	1	31	18	433	120
9	29	317	30.4	0.53	14	0.63	0.17	51	1	34	26	298	100
10	37	296	30.7	0.54	14.3	0.56	0.25	92	1	32	26	302	104
11	36	403	29.5	0.53	13.4	0.54	0.25	36	1	230	61	412	110
12	25	280	29.7	0.51	13.9	0.53	0.26	66	1	25	43	281	162
13	21	256	29.3	0.52	13.8	0.53	0.35	52	1	23	16	271	132
14	45	282	28.4	0.5	13.8	0.58	0.36	60	2	45	58	272	*
15	69	276	30.4	0.54	13.8	0.55	0.22	74	2	34	30	264	116
16	50	288	29.9	0.54	13.7	0.57	0.24	25	1	35	97	260	130
17	42	264	30	0.53	14.2	0.53	0.18	38	2	33	12	264	136
18	46	284	30.5	0.51	14.2	0.57	0.15	29	1	35	18	270	110
19	61	279	29.9	0.53	14	0.6	0.15	31	1	50	16	261	148
20	17	294	30.3	0.52	14	0.61	0.17	70	1	65	17	286	170
21	17	284	29.9	0.55	14	0.58	0.2	40	1	49	53	289	148
22	87	296	30.2	0.5	13.9	0.61	0.29	30	2	79	14	286	120
23	2	471	28.15	0.46	13.07	0.63	0.52	6	1	38	17	390	106
24	9	406	28.29	0.46	12.83	0.65	0.84	8	2	60	10	314	116
25	50	320	28.49	0.49	12.86	0.72	0.27	20	1	23	11	239	116
26	2	363	28.51	0.45	12.93	0.62	0.36	6	1	21	11	314	117
27	7	363	28.83	0.49	12.59	0.71	0.45	12	2	18	3	255	104
28	6	348	28.59	0.45	12.98	0.56	0.03	9	1	5	1	266	111
29	13	374	29.15	0.47	13.08	0.66	0.16	7	3	8	1	346	102
30	1	271	28.83	0.45	13.1	0.66	0.19	14	1	21	1	218	112
31	6	285	27.91	0.47	12.65	0.61	0.4	11	2	38	3	230	101
32	1	280	28.72	0.46	12.98	0.71	0.77	10	3	43	24	265	111
33	14	326	28.24	0.49	12.96	0.62	0.38	8	6	24	5	269	112
34	16	356	29.29	0.44	12.7	0.72	0.42	9	1	22	11	295	154
35	10	287	28.9	0.51	13.49	0.57	0.24	5	3	21	7	263	132
36	14	349	30	0.46	13.03	0.71	0.27	13	1	17	1	279	132
37	6	274	29.55	0.48	12.88	0.56	0.23	12	1	24	5	244	124
38	6	277	29.77	0.46	13.45	0.69	0.45	13	2	14	1	267	154
39	11	297	30.13	0.46	13.41	0.68	0.32	9	2	14	5	302	133
40	8	301	30.25	0.47	13.15	0.69	0.37	9	2	8	6	266	130
41	4	265	29.17	0.48	13.15	0.57	0.19	7	1	29	10	249	134
42	17	281	28.74	0.44	12.89	0.57	0.24	6	2	18	1	282	132
43	17	273	27.95	0.47	12.72	0.64	0.26	5	1	29	10	268	108
44	1	282	28.88	0.47	13.01	0.71	0.39	4	2	11	5	275	154

Table 1 (cont.)

ID	hr	crarea	crConAr	crperi	crConPer	crdiam	crform	crroun	craspR	crconvx
1	367	0.093	0.121	2.038	1.323	0.343	0.280	0.433	1.580	0.649
2	380	0.085	0.116	2.040	1.263	0.330	0.258	0.455	1.442	0.619
3	460	0.086	0.109	1.929	1.281	0.331	0.291	0.410	1.649	0.664
4	360	0.080	0.108	1.963	1.258	0.319	0.260	0.407	1.553	0.641
5	460	0.090	0.119	2.022	1.306	0.339	0.277	0.435	1.479	0.646
6	380	0.106	0.137	2.192	1.421	0.367	0.276	0.417	1.644	0.648
7	480	0.095	0.121	2.019	1.326	0.348	0.293	0.433	1.642	0.657
8	450	0.091	0.120	2.023	1.302	0.341	0.280	0.448	1.495	0.644
9	460	0.087	0.116	1.999	1.305	0.333	0.275	0.416	1.586	0.653
10	530	0.075	0.100	1.865	1.188	0.309	0.271	0.455	1.502	0.637
11	373	0.078	0.106	1.907	1.208	0.316	0.271	0.495	1.337	0.634
12	570	0.086	0.113	1.977	1.311	0.331	0.277	0.374	1.728	0.663
13	541	0.068	0.097	1.855	1.165	0.293	0.247	0.427	1.432	0.628
14	*	0.072	0.096	1.895	1.229	0.302	0.250	0.353	1.667	0.649
15	474	0.085	0.110	1.937	1.301	0.329	0.285	0.380	1.750	0.672
16	485	0.080	0.105	1.933	1.263	0.319	0.269	0.387	1.638	0.653
17	460	0.083	0.109	1.960	1.272	0.324	0.270	0.389	1.660	0.649
18	460	0.072	0.101	1.937	1.259	0.302	0.240	0.342	1.695	0.650
19	506	0.068	0.095	1.852	1.169	0.294	0.249	0.396	1.496	0.631
20	559	0.067	0.096	1.871	1.172	0.293	0.242	0.405	1.469	0.626
21	540	0.082	0.111	2.092	1.368	0.323	0.235	0.314	1.947	0.654
22	520	0.078	0.103	1.946	1.281	0.314	0.257	0.358	1.722	0.658
23	340	0.078	0.112	2.238	1.262	0.316	0.196	0.430	1.421	0.564
24	360	0.081	0.116	2.107	1.278	0.321	0.229	0.435	1.361	0.607
25	400	0.080	0.107	2.153	1.281	0.319	0.216	0.396	1.568	0.595
26	430	0.075	0.113	2.120	1.232	0.308	0.209	0.463	1.287	0.581
27	450	0.090	0.116	2.064	1.353	0.338	0.264	0.381	1.722	0.655
28	450	0.075	0.097	1.879	1.201	0.309	0.267	0.428	1.589	0.639
29	360	0.088	0.123	2.210	1.339	0.335	0.226	0.411	1.503	0.606
30	370	0.088	0.121	2.154	1.333	0.334	0.237	0.399	1.567	0.619
31	340	0.068	0.108	2.087	1.204	0.294	0.195	0.436	1.303	0.577
32	410	0.073	0.102	1.994	1.229	0.304	0.230	0.390	1.542	0.617
33	440	0.075	0.106	2.122	1.221	0.308	0.208	0.443	1.365	0.575
34	480	0.068	0.091	1.776	1.112	0.294	0.271	0.507	1.310	0.626
35	440	0.084	0.113	2.023	1.295	0.328	0.259	0.422	1.525	0.640
36	450	0.053	0.074	1.680	1.045	0.260	0.236	0.400	1.490	0.622
37	460	0.072	0.094	1.798	1.161	0.302	0.279	0.478	1.370	0.645
38	500	0.070	0.094	2.013	1.196	0.298	0.217	0.393	1.642	0.594
39	500	0.071	0.095	2.077	1.200	0.300	0.206	0.397	1.590	0.578
40	440	0.066	0.094	1.903	1.180	0.289	0.228	0.388	1.493	0.620
41	440	0.049	0.080	1.869	1.097	0.250	0.177	0.318	1.583	0.587
42	480	0.072	0.095	1.884	1.193	0.302	0.253	0.409	1.578	0.633
43	420	0.069	0.095	1.822	1.155	0.296	0.261	0.464	1.354	0.634
44	520	0.071	0.099	1.900	1.219	0.302	0.249	0.378	1.657	0.641

Table 1 (cont.)

ID	crsoli	crBrea	crleng	crx	cry	crAdjC	crhole	crxc	cryc	crMA	clarea	clConAr
1	0.764	0.330	0.522	60	39	1.200	1	0.488	0.926	5.7	0.080	0.105
2	0.738	0.339	0.489	56	40	1.191	1	1.361	0.932	173.8	*	*
3	0.789	0.313	0.517	60	37	1.197	1	2.340	0.956	174.2	0.079	0.102
4	0.742	0.322	0.500	58	38	1.193	1	3.459	0.953	177.3	0.089	0.122
5	0.755	0.347	0.514	59	41	1.201	1	0.530	1.647	3.6	0.081	0.114
6	0.773	0.346	0.568	66	41	1.220	1	1.407	1.637	178.7	0.084	0.120
7	0.784	0.322	0.529	60	38	1.198	1	2.488	1.605	5.3	0.098	0.130
8	0.762	0.340	0.509	59	41	1.201	1	3.517	1.623	174.8	0.088	0.115
9	0.751	0.326	0.517	60	39	1.200	1	0.544	2.242	1.9	0.082	0.108
10	0.751	0.305	0.458	51	36	1.171	1	1.442	2.234	9.2	0.068	0.094
11	0.741	0.336	0.449	52	41	1.182	1	2.448	2.218	172.7	0.070	0.101
12	0.765	0.313	0.542	59	37	1.194	1	0.601	3.111	21.6	0.068	0.100
13	0.694	0.313	0.449	52	37	1.175	1	1.604	3.091	178.4	0.066	0.092
14	0.747	0.305	0.508	59	36	1.192	1	2.576	3.097	4.0	0.067	0.096
15	0.772	0.305	0.534	62	36	1.200	1	3.538	3.107	179.3	0.059	0.088
16	0.760	0.313	0.514	59	37	1.194	1	0.593	3.832	176.3	0.072	0.100
17	0.757	0.313	0.520	60	37	1.197	1	1.562	3.816	175.1	0.068	0.098
18	0.709	0.305	0.517	59	36	1.192	1	2.574	3.786	176.1	0.070	0.098
19	0.714	0.312	0.467	54	37	1.181	1	3.516	3.799	175.5	0.069	0.093
20	0.699	0.313	0.460	53	37	1.178	1	0.565	4.577	172.9	0.065	0.095
21	0.735	0.296	0.576	67	36	1.214	1	1.563	4.545	179.8	0.064	0.094
22	0.754	0.305	0.525	61	36	1.198	1	2.608	4.522	0.6	0.067	0.095
23	0.696	0.339	0.481	102	75	1.348	1	0.318	0.266	5.8	0.092	0.130
24	0.696	0.358	0.487	102	77	1.352	1	0.936	0.261	174.1	0.087	0.122
25	0.742	0.323	0.506	107	70	1.348	1	1.481	0.258	9.0	0.104	0.135
26	0.662	0.352	0.453	96	79	1.342	1	2.062	0.255	168.6	0.085	0.120
27	0.770	0.318	0.547	115	68	1.364	1	2.641	0.264	167.3	0.088	0.116
28	0.772	0.297	0.472	100	65	1.319	1	0.299	0.722	7.0	0.081	0.108
29	0.716	0.347	0.522	108	75	1.363	1	0.881	0.709	12.3	0.090	0.129
30	0.724	0.338	0.529	111	74	1.368	1	1.513	0.696	175.3	0.085	0.124
31	0.629	0.341	0.445	94	73	1.323	1	2.118	0.696	173.3	0.077	0.118
32	0.712	0.316	0.488	102	68	1.331	1	2.656	0.697	8.8	0.063	0.092
33	0.703	0.339	0.463	98	73	1.333	1	0.826	1.134	10.4	0.065	0.091
34	0.751	0.316	0.413	86	70	1.297	1	1.374	1.185	163.0	0.073	0.098
35	0.745	0.331	0.505	107	72	1.353	1	1.932	1.140	178.5	0.072	0.099
36	0.715	0.276	0.411	86	59	1.273	1	2.551	1.160	172.5	0.059	0.091
37	0.764	0.319	0.437	92	71	1.314	1	0.251	1.629	172.7	0.067	0.092
38	0.746	0.290	0.476	100	62	1.312	1	0.807	1.603	175.8	0.074	0.101
39	0.746	0.300	0.477	101	65	1.322	1	1.411	1.593	4.3	0.074	0.095
40	0.698	0.311	0.464	98	67	1.319	1	1.973	1.620	7.7	0.066	0.089
41	0.611	0.280	0.444	94	60	1.293	1	2.543	1.606	2.9	0.071	0.098
42	0.752	0.299	0.472	100	64	1.317	1	0.271	2.070	177.4	0.065	0.089
43	0.727	0.321	0.435	92	69	1.309	1	0.859	2.063	0.6	0.075	0.103
44	0.721	0.296	0.491	104	65	1.329	1	1.462	2.079	5.3	0.071	0.098

Table 1 (cont.)

ID	clperi	clConPer	cldiam	clform	clroun	clAsp	clconv	clsoli	clBrea	clleng	clx	cly
1	1.920	1.284	0.319	0.272	0.369	1.722	0.669	0.758	0.305	0.525	61	36
2	*	*	*	*	*	*	*	*	*	*	*	*
3	1.869	1.241	0.317	0.284	0.400	1.646	0.664	0.779	0.305	0.502	58	36
4	2.049	1.342	0.337	0.267	0.399	1.658	0.655	0.735	0.322	0.534	62	38
5	2.043	1.301	0.321	0.243	0.395	1.506	0.637	0.706	0.339	0.510	59	40
6	2.478	1.340	0.328	0.173	0.373	1.625	0.541	0.706	0.330	0.537	62	39
7	2.246	1.437	0.353	0.243	0.394	1.579	0.640	0.753	0.356	0.562	65	42
8	1.938	1.267	0.335	0.294	0.478	1.393	0.654	0.764	0.347	0.484	56	41
9	1.972	1.275	0.324	0.266	0.392	1.743	0.647	0.763	0.297	0.517	59	35
10	2.110	1.183	0.295	0.193	0.385	1.603	0.561	0.731	0.297	0.475	55	35
11	1.942	1.189	0.298	0.233	0.429	1.398	0.612	0.693	0.326	0.455	49	39
12	1.914	1.205	0.294	0.232	0.376	1.487	0.630	0.680	0.322	0.479	55	38
13	1.802	1.129	0.291	0.257	0.474	1.319	0.626	0.717	0.320	0.422	42	41
14	1.953	1.145	0.293	0.222	0.458	1.342	0.586	0.700	0.322	0.432	50	38
15	1.816	1.121	0.273	0.224	0.382	1.491	0.617	0.663	0.297	0.442	51	35
16	1.849	1.197	0.304	0.266	0.409	1.442	0.647	0.725	0.329	0.474	55	39
17	1.835	1.148	0.295	0.256	0.463	1.348	0.626	0.701	0.322	0.434	50	38
18	1.863	1.169	0.299	0.255	0.466	1.346	0.628	0.720	0.326	0.439	50	42
19	1.779	1.146	0.297	0.276	0.438	1.432	0.644	0.746	0.313	0.449	52	37
20	2.074	1.144	0.289	0.191	0.431	1.401	0.552	0.686	0.313	0.439	49	37
21	1.825	1.124	0.286	0.242	0.456	1.316	0.616	0.684	0.322	0.424	49	38
22	2.007	1.162	0.292	0.209	0.417	1.441	0.579	0.703	0.313	0.452	51	37
23	2.264	1.385	0.343	0.226	0.407	1.494	0.612	0.711	0.360	0.537	114	77
24	2.314	1.365	0.333	0.205	0.365	1.648	0.590	0.714	0.335	0.551	117	73
25	2.197	1.456	0.364	0.270	0.385	1.756	0.663	0.770	0.334	0.586	124	73
26	2.457	1.293	0.329	0.177	0.442	1.403	0.526	0.711	0.353	0.495	105	76
27	2.133	1.325	0.334	0.242	0.414	1.563	0.621	0.756	0.332	0.519	110	73
28	2.068	1.289	0.320	0.237	0.375	1.699	0.623	0.744	0.308	0.523	111	68
29	2.281	1.395	0.339	0.218	0.397	1.479	0.611	0.702	0.364	0.538	113	78
30	2.186	1.361	0.329	0.223	0.389	1.492	0.623	0.686	0.353	0.527	109	80
31	2.181	1.308	0.313	0.204	0.385	1.485	0.600	0.654	0.340	0.505	107	75
32	2.106	1.139	0.283	0.179	0.434	1.400	0.541	0.682	0.307	0.430	91	66
33	1.815	1.122	0.288	0.249	0.469	1.304	0.618	0.716	0.322	0.421	89	69
34	1.865	1.189	0.305	0.264	0.434	1.435	0.638	0.743	0.322	0.463	98	69
35	1.879	1.151	0.303	0.257	0.515	1.311	0.613	0.731	0.322	0.423	89	69
36	1.882	1.165	0.274	0.210	0.352	1.505	0.619	0.651	0.307	0.463	98	66
37	1.838	1.115	0.293	0.250	0.495	1.329	0.606	0.734	0.313	0.416	86	67
38	1.973	1.233	0.306	0.238	0.390	1.567	0.625	0.729	0.313	0.491	104	67
39	1.830	1.145	0.306	0.276	0.518	1.300	0.626	0.771	0.327	0.425	90	70
40	1.990	1.154	0.290	0.210	0.417	1.438	0.580	0.743	0.313	0.450	95	67
41	1.917	1.183	0.301	0.243	0.441	1.408	0.617	0.721	0.322	0.453	95	69
42	1.795	1.125	0.288	0.255	0.435	1.418	0.627	0.733	0.308	0.437	92	66
43	1.933	1.195	0.309	0.253	0.461	1.392	0.618	0.732	0.327	0.456	95	72
44	1.884	1.205	0.302	0.253	0.393	1.561	0.640	0.726	0.308	0.481	102	66

Table 1 (cont.)

ID	clAdjC	clhole	clxc	clyc	clMA	hrarea	hrConAr	hrperi	hrConPer	hrdiam	hrform
1	1.198	1	0.415	0.854	178.7	0.029	0.029	0.650	0.623	0.192	0.857
2	*	*	*	*	*	0.029	0.030	0.745	0.644	0.194	0.666
3	1.190	1	2.147	0.878	179.0	0.023	0.023	0.576	0.549	0.170	0.866
4	1.204	1	3.078	0.882	172.2	0.028	0.028	0.641	0.610	0.188	0.851
5	1.199	1	0.409	1.444	175.9	0.028	0.028	0.640	0.615	0.189	0.860
6	1.206	1	1.213	1.467	169.9	0.029	0.030	0.689	0.639	0.193	0.777
7	1.219	1	2.192	1.448	168.6	0.025	0.025	0.633	0.588	0.178	0.780
8	1.193	1	3.094	1.427	174.7	0.027	0.027	0.643	0.608	0.186	0.824
9	1.190	1	0.445	1.990	172.5	0.027	0.027	0.621	0.601	0.184	0.864
10	1.180	1	1.268	1.998	175.7	0.023	0.023	0.597	0.565	0.172	0.816
11	1.171	1	2.132	2.003	158.8	0.025	0.025	0.614	0.581	0.179	0.834
12	1.185	1	0.432	3.165	174.5	0.022	0.023	0.583	0.556	0.169	0.829
13	1.157	1	1.455	3.171	137.4	0.027	0.027	0.622	0.597	0.186	0.878
14	1.172	1	2.469	3.162	0.9	0.021	0.021	0.574	0.530	0.164	0.806
15	1.169	1	3.256	3.147	7.7	0.021	0.021	0.552	0.523	0.163	0.865
16	1.187	1	0.460	3.841	2.0	0.025	0.025	0.595	0.575	0.177	0.875
17	1.172	1	1.461	3.817	172.7	0.026	0.027	0.626	0.593	0.183	0.844
18	1.179	1	2.451	3.793	158.7	0.025	0.025	0.607	0.574	0.177	0.841
19	1.175	1	3.349	3.788	177.8	0.026	0.026	0.619	0.591	0.182	0.856
20	1.168	1	0.466	4.457	166.0	0.027	0.027	0.631	0.603	0.185	0.844
21	1.169	1	1.400	4.421	174.3	0.025	0.025	0.631	0.598	0.179	0.794
22	1.173	1	2.428	4.435	171.2	0.024	0.024	0.593	0.566	0.174	0.848
23	1.383	1	0.279	0.213	2.5	0.038	0.039	0.788	0.722	0.221	0.774
24	1.381	1	0.923	0.212	1.5	0.038	0.039	0.770	0.720	0.221	0.813
25	1.400	1	1.563	0.233	172.5	0.029	0.031	0.705	0.640	0.193	0.744
26	1.357	1	2.239	0.231	179.7	0.040	0.040	0.799	0.740	0.225	0.784
27	1.363	1	0.294	0.652	178.4	0.025	0.026	0.633	0.589	0.178	0.785
28	1.354	1	0.902	0.645	3.7	0.025	0.026	0.640	0.612	0.179	0.768
29	1.383	1	1.544	0.659	4.8	0.037	0.038	0.766	0.719	0.216	0.785
30	1.377	1	2.183	0.660	160.7	0.034	0.034	0.715	0.681	0.209	0.841
31	1.360	1	0.277	1.090	4.9	0.043	0.044	0.838	0.759	0.235	0.773
32	1.300	1	0.840	1.091	3.8	0.032	0.033	0.713	0.669	0.203	0.797
33	1.302	1	1.930	1.086	177.0	0.029	0.030	0.696	0.642	0.193	0.761
34	1.324	1	2.471	1.101	179.6	0.026	0.026	0.628	0.589	0.180	0.814
35	1.302	1	0.253	1.500	3.2	0.026	0.027	0.632	0.591	0.182	0.821
36	1.317	1	0.772	1.508	0.7	0.022	0.023	0.583	0.546	0.168	0.821
37	1.290	1	1.307	1.475	170.1	0.021	0.021	0.567	0.538	0.162	0.809
38	1.334	1	1.841	1.522	178.2	0.026	0.026	0.628	0.587	0.181	0.816
39	1.307	1	2.428	1.498	178.5	0.023	0.023	0.588	0.553	0.171	0.831
40	1.312	1	0.232	1.898	0.3	0.030	0.030	0.676	0.629	0.194	0.815
41	1.316	1	0.821	1.920	9.1	0.033	0.033	0.730	0.689	0.206	0.788
42	1.302	1	1.334	1.931	174.2	0.024	0.025	0.617	0.565	0.175	0.794
43	1.323	1	1.849	1.919	167.3	0.027	0.027	0.624	0.592	0.184	0.862
44	1.326	1	2.418	1.920	171.9	0.027	0.027	0.630	0.601	0.186	0.860

Table 1 (cont.)

ID	hrroun	hraspR	hrconvx	hrsoli	hrBrea	hrleng	hrx	hry	hrAdjC	hrhole	hrxc	hryc
1	0.656	1.349	0.958	0.998	0.175	0.237	26	21	1.086	0	0.534	0.954
2	0.657	1.354	0.865	0.977	0.176	0.239	25	21	1.084	0	1.390	0.954
3	0.703	1.263	0.954	0.997	0.161	0.203	23	19	1.076	0	2.385	0.979
4	0.718	1.249	0.951	0.990	0.178	0.222	24	21	1.082	0	3.501	0.962
5	0.703	1.238	0.960	0.997	0.182	0.225	24	22	1.083	0	0.561	1.661
6	0.690	1.249	0.927	0.965	0.186	0.233	26	22	1.088	0	1.455	1.645
7	0.620	1.335	0.928	0.979	0.169	0.226	25	20	1.082	0	2.555	1.616
8	0.638	1.399	0.944	0.992	0.166	0.233	26	20	1.085	0	3.552	1.649
9	0.691	1.305	0.966	0.984	0.169	0.221	25	20	1.082	0	0.584	2.256
10	0.623	1.427	0.945	1.000	0.152	0.218	23	18	1.075	0	1.474	2.237
11	0.672	1.353	0.945	0.999	0.161	0.218	24	19	1.078	0	2.479	2.231
12	0.692	1.334	0.953	0.983	0.152	0.203	22	18	1.072	0	0.638	3.099
13	0.749	1.266	0.960	1.000	0.169	0.214	23	20	1.078	0	1.634	3.108
14	0.761	1.167	0.925	0.985	0.161	0.188	21	19	1.072	0	2.628	3.084
15	0.730	1.254	0.949	1.000	0.152	0.191	21	18	1.070	0	3.594	3.114
16	0.681	1.266	0.967	0.987	0.169	0.215	24	20	1.080	0	0.639	3.847
17	0.686	1.305	0.946	0.992	0.169	0.221	25	20	1.082	0	1.593	3.834
18	0.694	1.256	0.945	0.989	0.169	0.213	22	20	1.076	0	2.617	3.802
19	0.688	1.298	0.955	0.995	0.169	0.220	24	20	1.080	0	3.543	3.812
20	0.667	1.335	0.955	0.993	0.169	0.226	25	20	1.082	0	0.592	4.592
21	0.567	1.559	0.946	0.993	0.152	0.238	27	18	1.084	0	1.628	4.560
22	0.645	1.354	0.955	0.991	0.160	0.216	24	19	1.078	0	2.651	4.539
23	0.789	1.181	0.917	0.974	0.210	0.248	49	45	1.165	0	0.277	0.284
24	0.718	1.268	0.935	0.977	0.206	0.261	52	44	1.169	0	0.897	0.271
25	0.723	1.249	0.908	0.960	0.182	0.228	44	39	1.144	0	1.442	0.268
26	0.699	1.310	0.926	0.983	0.206	0.269	53	44	1.171	0	2.035	0.226
27	0.735	1.202	0.931	0.970	0.173	0.208	42	38	1.138	0	2.554	0.250
28	0.649	1.483	0.956	0.957	0.150	0.222	45	32	1.132	0	0.266	0.711
29	0.684	1.243	0.939	0.968	0.210	0.261	50	45	1.167	0	0.828	0.747
30	0.717	1.319	0.952	0.998	0.187	0.247	51	40	1.159	0	1.484	0.669
31	0.775	1.240	0.906	0.980	0.215	0.266	54	46	1.177	0	2.090	0.676
32	0.758	1.208	0.938	0.979	0.193	0.233	45	42	1.151	0	2.635	0.694
33	0.667	1.254	0.922	0.963	0.189	0.237	42	41	1.143	0	0.778	1.135
34	0.740	1.150	0.939	0.967	0.182	0.210	40	39	1.136	0	1.355	1.187
35	0.758	1.201	0.935	0.976	0.174	0.209	42	38	1.138	0	1.869	1.121
36	0.773	1.241	0.937	0.987	0.154	0.191	38	33	1.121	0	2.509	1.137
37	0.677	1.362	0.948	1.000	0.145	0.197	38	31	1.117	0	0.204	1.636
38	0.772	1.222	0.934	0.980	0.168	0.206	41	36	1.132	0	0.750	1.586
39	0.744	1.187	0.941	0.980	0.167	0.198	37	36	1.125	0	1.355	1.585
40	0.729	1.280	0.931	0.985	0.178	0.227	47	38	1.147	0	1.930	1.626
41	0.582	1.654	0.944	0.999	0.164	0.270	56	35	1.159	0	2.510	1.621
42	0.774	1.183	0.916	0.970	0.168	0.199	41	36	1.132	0	0.222	2.077
43	0.825	1.114	0.949	0.987	0.182	0.203	39	39	1.134	0	0.817	2.033
44	0.759	1.270	0.953	0.991	0.168	0.214	43	36	1.136	0	1.405	2.068

Table 1 (cont.)

ID	hrMA	hlarea	hlConAr	hlperi	hlConPer	hldiam	hlform	hlroun	hlAsp	hlconv
1	20.824	0.021	0.021	0.562	0.535	0.165	0.855	0.675	1.359	0.953
2	22.184	*	*	*	*	*	*	*	*	*
3	11.154	0.021	0.021	0.562	0.533	0.163	0.835	0.659	1.397	0.949
4	12.618	0.027	0.027	0.660	0.603	0.185	0.778	0.653	1.426	0.913
5	27.746	0.030	0.030	0.670	0.634	0.195	0.839	0.736	1.280	0.946
6	168.413	0.028	0.030	0.793	0.639	0.189	0.562	0.576	1.438	0.806
7	20.447	0.026	0.026	0.619	0.598	0.183	0.865	0.667	1.394	0.965
8	11.808	0.025	0.025	0.609	0.577	0.177	0.830	0.694	1.253	0.946
9	4.286	0.025	0.026	0.634	0.585	0.177	0.769	0.641	1.374	0.922
10	23.002	0.021	0.022	0.588	0.534	0.163	0.760	0.723	1.191	0.910
11	3.348	0.030	0.030	0.662	0.635	0.195	0.851	0.633	1.363	0.958
12	3.906	0.028	0.027	0.633	0.608	0.187	0.863	0.639	1.384	0.959
13	12.410	0.025	0.025	0.614	0.574	0.178	0.829	0.711	1.246	0.935
14	10.455	0.028	0.028	0.638	0.606	0.188	0.859	0.725	1.243	0.950
15	6.705	0.029	0.029	0.638	0.620	0.192	0.890	0.659	1.393	0.971
16	166.146	0.023	0.024	0.586	0.563	0.173	0.860	0.681	1.302	0.962
17	174.558	0.028	0.028	0.633	0.606	0.187	0.863	0.723	1.238	0.957
18	157.661	0.026	0.026	0.628	0.593	0.182	0.824	0.674	1.305	0.944
19	163.477	0.023	0.023	0.576	0.550	0.171	0.871	0.677	1.291	0.955
20	169.309	0.030	0.031	0.737	0.638	0.195	0.692	0.702	1.249	0.866
21	3.547	0.029	0.028	0.638	0.613	0.191	0.883	0.724	1.262	0.960
22	9.822	0.024	0.024	0.597	0.570	0.174	0.841	0.670	1.324	0.954
23	18.217	0.038	0.039	0.776	0.737	0.221	0.801	0.634	1.607	0.950
24	172.948	0.036	0.037	0.735	0.692	0.214	0.837	0.822	1.149	0.941
25	24.948	0.026	0.027	0.642	0.594	0.182	0.793	0.745	1.187	0.926
26	174.965	0.037	0.039	0.794	0.711	0.217	0.733	0.754	1.177	0.895
27	149.796	0.027	0.027	0.633	0.594	0.185	0.840	0.767	1.187	0.939
28	1.797	0.029	0.030	0.685	0.628	0.194	0.787	0.751	1.291	0.916
29	28.139	0.039	0.040	0.785	0.725	0.223	0.798	0.714	1.285	0.924
30	162.292	0.037	0.038	0.754	0.707	0.217	0.820	0.791	1.137	0.937
31	172.984	0.041	0.042	0.805	0.743	0.229	0.798	0.773	1.238	0.923
32	25.297	0.033	0.035	0.785	0.693	0.204	0.665	0.613	1.359	0.882
33	41.811	0.027	0.027	0.646	0.603	0.184	0.797	0.697	1.345	0.933
34	170.918	0.026	0.026	0.625	0.585	0.181	0.826	0.790	1.166	0.936
35	177.522	0.028	0.029	0.677	0.622	0.189	0.768	0.689	1.282	0.918
36	9.847	0.031	0.031	0.678	0.645	0.198	0.838	0.682	1.422	0.951
37	1.551	0.026	0.026	0.642	0.595	0.181	0.781	0.725	1.296	0.927
38	1.625	0.027	0.028	0.648	0.608	0.187	0.818	0.769	1.264	0.939
39	155.064	0.024	0.024	0.616	0.566	0.173	0.779	0.785	1.161	0.919
40	11.764	0.024	0.025	0.625	0.578	0.175	0.772	0.676	1.381	0.924
41	178.772	0.027	0.029	0.672	0.619	0.187	0.763	0.686	1.342	0.921
42	5.903	0.023	0.024	0.606	0.564	0.172	0.794	0.792	1.147	0.931
43	177.706	0.030	0.031	0.700	0.642	0.197	0.780	0.682	1.308	0.917
44	172.467	0.027	0.027	0.629	0.595	0.185	0.854	0.800	1.164	0.946

Table 1 (cont.)

ID	hlsoli	hlBrea	hlleng	hlx	hly	hlAdjC	hlhole	hlxc	hlyc	hlMA	rBV/TVpc	rBA/TA
1	1.000	0.148	0.201	22	18	1.072	0	0.600	0.568	169.2	12.521	0.125
2	*	*	*	*	*	*	*	*	*	*	18.549	0.185
3	0.990	0.144	0.201	22	17	1.071	0	2.329	0.570	171.3	8.463	0.085
4	0.993	0.161	0.229	26	19	1.083	0	3.224	0.618	4.0	20.048	0.200
5	0.999	0.178	0.228	25	21	1.084	0	0.629	1.459	168.8	10.263	0.103
6	0.953	0.173	0.249	28	21	1.091	0	1.419	1.471	174.7	14.097	0.141
7	1.000	0.161	0.224	25	19	1.081	0	2.365	1.488	8.0	14.906	0.149
8	0.988	0.169	0.212	22	21	1.077	0	3.316	1.438	142.5	21.907	0.219
9	0.962	0.161	0.221	25	19	1.081	0	0.725	2.451	166.9	8.876	0.089
10	0.968	0.161	0.192	21	19	1.072	0	1.550	2.447	170.1	48.973	0.490
11	0.988	0.179	0.244	26	22	1.088	0	2.478	2.374	158.3	25.597	0.256
12	1.000	0.169	0.234	26	20	1.085	0	0.397	3.168	169.4	2.409	0.024
13	0.991	0.169	0.211	23	20	1.078	0	1.444	3.135	158.7	4.834	0.048
14	0.989	0.178	0.221	25	21	1.084	0	2.443	3.168	174.1	9.941	0.099
15	1.000	0.169	0.236	26	20	1.085	0	3.229	3.156	4.6	8.046	0.080
16	0.991	0.161	0.209	23	19	1.076	0	0.440	3.854	165.0	3.977	0.040
17	0.995	0.178	0.220	25	21	1.084	0	1.429	3.827	162.7	8.057	0.081
18	0.982	0.169	0.221	25	20	1.082	0	2.424	3.780	177.1	4.792	0.048
19	1.000	0.161	0.208	23	19	1.076	0	3.320	3.793	12.0	8.572	0.086
20	0.962	0.186	0.233	26	22	1.088	0	0.447	4.445	177.2	8.649	0.086
21	1.000	0.178	0.224	25	21	1.084	0	1.372	4.425	170.7	11.311	0.113
22	0.984	0.161	0.213	24	19	1.078	0	2.393	4.433	167.6	19.141	0.191
23	0.994	0.173	0.278	57	37	1.165	0	0.246	0.190	0.9	2.565	0.026
24	0.982	0.206	0.236	48	44	1.161	0	0.866	0.212	15.0	7.527	0.075
25	0.972	0.178	0.211	38	38	1.130	0	1.502	0.209	51.9	3.788	0.038
26	0.950	0.212	0.249	52	46	1.173	0	2.191	0.235	8.1	6.759	0.068
27	0.983	0.178	0.211	43	38	1.140	0	0.230	0.670	5.8	13.031	0.130
28	0.989	0.173	0.223	45	37	1.142	0	0.860	0.655	173.5	7.071	0.071
29	0.985	0.206	0.264	52	44	1.169	0	1.496	0.686	18.4	7.753	0.078
30	0.979	0.215	0.244	47	46	1.163	0	2.144	0.646	169.0	5.699	0.057
31	0.982	0.210	0.260	54	45	1.175	0	0.238	1.087	4.4	4.099	0.041
32	0.937	0.192	0.260	54	41	1.167	0	0.811	1.080	179.4	3.512	0.035
33	0.983	0.164	0.220	45	35	1.138	0	1.919	1.079	10.7	7.931	0.079
34	0.978	0.174	0.203	40	39	1.136	0	2.427	1.111	47.4	1.059	0.011
35	0.971	0.178	0.228	45	38	1.144	0	0.214	1.517	21.7	6.872	0.069
36	0.992	0.168	0.239	49	36	1.147	0	0.747	1.504	6.3	1.488	0.015
37	0.971	0.164	0.212	42	35	1.132	0	1.291	1.466	171.8	2.918	0.029
38	0.979	0.168	0.213	44	36	1.138	0	1.799	1.535	172.6	18.767	0.188
39	0.961	0.168	0.195	40	36	1.130	0	2.391	1.511	46.4	2.340	0.023
40	0.970	0.154	0.213	44	33	1.132	0	0.224	1.897	7.6	4.220	0.042
41	0.957	0.168	0.226	45	36	1.140	0	0.772	1.933	6.1	12.069	0.121
42	0.973	0.168	0.193	37	36	1.125	0	1.316	1.921	107.6	1.441	0.014
43	0.985	0.182	0.238	46	39	1.147	0	1.797	1.893	26.6	8.970	0.090
44	0.989	0.178	0.207	42	38	1.138	0	2.369	1.928	13.9	7.011	0.070

Table 1 (cont.)

ID	rbs	rsv	rTV	rBV	rBS/BV	rTbTh	rTbN	rTbSp	crMS	crMS/BS	crrLTh
1	27.901	6.328	5.613	0.703	39.697	0.050	2.485	0.352	5.137	0.184	0.032
2	19.768	6.204	4.056	0.752	26.276	0.076	2.437	0.334	0.956	0.048	0.028
3	7.243	4.077	2.262	0.191	37.840	0.053	1.601	0.572	1.521	0.210	0.026
4	25.518	6.824	4.761	0.954	26.737	0.075	2.680	0.298	9.834	0.385	0.030
5	9.614	5.227	2.342	0.240	40.009	0.050	2.053	0.437	6.128	0.637	0.025
6	33.898	6.490	6.649	0.937	36.164	0.055	2.549	0.337	7.515	0.222	0.029
7	23.588	6.853	4.382	0.653	36.116	0.055	2.692	0.316	8.827	0.374	0.032
8	38.907	8.399	5.897	1.292	30.115	0.066	3.299	0.237	2.273	0.058	0.028
9	12.731	5.799	2.795	0.248	51.322	0.039	2.278	0.400	1.956	0.154	0.029
10	17.964	6.189	3.695	1.809	9.928	0.201	2.431	0.210	16.429	0.915	0.030
11	44.613	9.981	5.690	1.457	30.630	0.065	3.920	0.190	10.876	0.244	0.030
12	6.222	1.448	5.471	0.132	47.202	0.042	0.569	1.716	1.692	0.272	0.027
13	13.969	2.869	6.199	0.300	46.612	0.043	1.127	0.845	3.618	0.259	0.032
14	13.988	5.488	3.245	0.323	43.365	0.046	2.156	0.418	1.557	0.111	0.029
15	12.938	4.247	3.878	0.312	41.461	0.048	1.668	0.551	7.920	0.612	0.027
16	12.926	2.499	6.584	0.262	49.361	0.041	0.982	0.978	6.995	0.541	0.034
17	17.938	4.151	5.501	0.443	40.478	0.049	1.631	0.564	3.314	0.185	0.030
18	13.886	2.985	5.921	0.284	48.937	0.041	1.173	0.812	0.929	0.067	0.025
19	18.027	4.445	5.163	0.443	40.731	0.049	1.746	0.524	7.279	0.404	0.029
20	18.668	5.246	4.530	0.392	47.648	0.042	2.061	0.443	9.169	0.491	0.030
21	20.399	5.428	4.784	0.541	37.697	0.053	2.132	0.416	5.725	0.281	0.029
22	27.620	7.268	4.838	0.926	29.830	0.067	2.855	0.283	8.555	0.310	0.027
23	4.837	1.025	6.010	0.154	31.376	0.064	0.402	2.421	0.590	0.122	0.025
24	11.729	3.786	3.943	0.297	39.517	0.051	1.487	0.622	0.933	0.080	0.028
25	9.176	2.349	4.974	0.188	48.704	0.041	0.922	1.043	0.829	0.090	0.030
26	14.260	3.006	6.039	0.408	34.931	0.057	1.181	0.790	3.419	0.240	0.034
27	10.955	3.491	3.994	0.521	21.046	0.095	1.371	0.634	0.793	0.072	0.027
28	8.314	2.568	4.121	0.291	28.530	0.070	1.009	0.921	5.539	0.666	0.035
29	14.180	3.257	5.542	0.430	33.003	0.061	1.279	0.721	0.913	0.064	0.026
30	4.847	2.260	2.730	0.156	31.157	0.064	0.888	1.062	2.086	0.430	0.033
31	14.035	2.343	7.626	0.313	44.892	0.045	0.920	1.042	6.083	0.433	0.032
32	7.359	1.904	4.921	0.173	42.583	0.047	0.748	1.290	5.683	0.772	0.040
33	12.588	4.296	3.730	0.296	42.549	0.047	1.687	0.546	4.346	0.345	0.034
34	2.215	0.604	4.672	0.049	44.775	0.045	0.237	4.173	0.571	0.258	0.026
35	12.190	3.477	4.463	0.307	39.745	0.050	1.366	0.682	2.703	0.222	0.030
36	2.981	0.898	4.228	0.063	47.401	0.042	0.353	2.794	1.916	0.643	0.037
37	5.516	1.477	4.755	0.139	39.756	0.050	0.580	1.674	0.286	0.052	0.031
38	22.870	5.812	5.009	0.940	24.329	0.082	2.283	0.356	5.074	0.222	0.031
39	4.887	1.703	3.653	0.085	57.156	0.035	0.669	1.460	1.288	0.263	0.033
40	5.992	1.932	3.948	0.167	35.964	0.056	0.759	1.262	1.689	0.282	0.028
41	16.348	4.612	4.512	0.545	30.022	0.067	1.812	0.485	5.722	0.350	0.035
42	4.458	1.150	4.932	0.071	62.711	0.032	0.452	2.181	1.757	0.394	0.036
43	14.929	4.223	4.500	0.404	36.983	0.054	1.659	0.549	3.398	0.228	0.032
44	5.727	2.773	2.629	0.184	31.071	0.064	1.089	0.854	4.437	0.775	0.023

Table 1 (cont.)

ID	crum/d	crMar	crBFR	crBFR/TV	cr%/Yr	crNoOcBS	crLeOcbs	crOcNoBA
1	0.016	15.840	2.916	0.520	190	4.2	0.554	166.5
2	0.014	13.979	0.676	0.167	61	6.1	0.492	160.8
3	0.013	12.851	2.699	1.193	435	8.7	0.767	329.1
4	0.015	15.224	5.867	1.232	450	5.8	0.395	155.1
5	0.013	12.614	8.040	3.434	1253	4.5	0.402	178.9
6	0.014	14.465	3.207	0.482	176	5.4	0.427	194.2
7	0.016	16.053	6.007	1.371	500	5.2	0.394	188.3
8	0.014	13.790	0.806	0.137	50	5.8	0.526	175.7
9	0.014	14.453	2.221	0.795	290	4.1	0.608	209.6
10	0.015	14.798	13.533	3.663	1337	5.2	0.652	51.4
11	0.015	14.984	3.653	0.642	234	5.3	0.455	162.0
12	0.014	13.744	3.738	0.683	249	13.2	0.548	622.1
13	0.016	15.753	4.081	0.658	240	2.6	0.154	123.5
14	0.015	14.590	1.624	0.501	183	6.4	0.419	279.0
15	0.014	13.625	8.340	2.150	785	4.5	0.253	185.9
16	0.017	16.930	9.162	1.391	508	5.3	0.523	259.7
17	0.015	14.846	2.743	0.499	182	2.2	0.105	90.3
18	0.013	12.659	0.847	0.143	52	7.9	0.664	387.7
19	0.014	14.449	5.834	1.130	412	5.0	0.467	203.4
20	0.015	15.154	7.443	1.643	600	1.3	0.059	63.8
21	0.015	14.621	4.103	0.858	313	3.9	0.265	146.0
22	0.013	13.308	4.122	0.852	311	3.8	0.297	114.5
23	0.013	12.609	1.538	0.256	93	7.0	0.256	220.6
24	0.014	13.753	1.094	0.277	101	5.4	0.572	212.3
25	0.015	14.798	1.337	0.269	98	5.0	0.330	244.2
26	0.017	16.875	4.046	0.670	245	4.0	0.382	139.6
27	0.014	13.591	0.984	0.246	90	3.7	0.393	76.8
28	0.017	17.415	11.601	2.815	1027	14.9	0.759	425.5
29	0.013	12.894	0.830	0.150	55	4.1	0.340	135.0
30	0.017	16.565	7.128	2.612	953	18.6	0.639	578.6
31	0.016	15.843	6.867	0.900	329	4.6	0.529	207.9
32	0.020	19.917	15.382	3.126	1141	4.6	0.305	196.7
33	0.017	16.825	5.808	1.557	568	4.7	0.401	199.4
34	0.013	12.791	3.300	0.706	258	2.3	0.145	101.1
35	0.015	15.050	3.337	0.748	273	5.9	0.511	234.7
36	0.018	18.266	11.741	2.777	1014	17.4	0.668	826.9
37	0.016	15.562	0.806	0.169	62	7.8	0.265	309.9
38	0.016	15.632	3.468	0.692	253	5.2	0.574	127.7
39	0.017	16.706	4.402	1.205	440	8.4	0.566	479.5
40	0.014	14.034	3.955	1.002	366	4.5	0.426	162.1
41	0.017	17.497	6.125	1.357	495	3.1	0.214	91.8
42	0.018	17.879	7.049	1.429	522	1.6	0.066	98.5
43	0.016	15.920	3.624	0.805	294	5.9	0.329	218.0
44	0.011	11.328	8.777	3.339	1219	5.1	0.351	157.3

Table 1 (cont.)

ID	crOcNoTA	IBV/TVpc	IBA/TA	Ibs	Isv	ITV	IBV	IBS/BV	ITbTh	ITbN
1	20.8	9.83	0.098	10.299	5.259	2.493	0.245	42.042	0.048	2.066
2	29.8	7.09	0.071	11.246	3.377	4.239	0.301	37.400	0.053	1.326
3	27.9	17.63	0.176	48.823	9.774	6.359	1.121	43.541	0.046	3.839
4	31.1	8.09	0.081	15.717	4.261	4.695	0.380	41.398	0.048	1.674
5	18.4	4.91	0.049	19.230	2.955	8.284	0.407	47.262	0.042	1.161
6	27.4	16.76	0.168	28.360	7.320	4.932	0.827	34.305	0.058	2.875
7	28.1	11.77	0.118	26.162	4.470	7.451	0.877	29.828	0.067	1.756
8	38.5	13.64	0.136	32.388	8.059	5.116	0.698	46.420	0.043	3.166
9	18.6	17.67	0.177	25.752	6.491	5.051	0.893	28.849	0.069	2.549
10	25.2	9.75	0.097	26.049	4.466	7.425	0.724	35.987	0.056	1.754
11	41.5	8.28	0.083	15.980	3.986	5.104	0.422	37.837	0.053	1.566
12	15.0	11.74	0.117	14.594	5.633	3.298	0.387	37.692	0.053	2.213
13	6.0	8.81	0.088	12.780	4.468	3.641	0.321	39.822	0.050	1.755
14	27.7	8.19	0.082	19.274	4.160	5.897	0.483	39.920	0.050	1.634
15	15.0	9.16	0.092	19.539	4.218	5.897	0.540	36.162	0.055	1.657
16	10.3	11.00	0.110	21.531	5.414	5.063	0.557	38.653	0.052	2.127
17	7.3	8.65	0.087	14.058	4.836	3.701	0.320	43.894	0.046	1.899
18	18.6	3.03	0.030	4.656	1.918	3.091	0.094	49.702	0.040	0.753
19	17.4	15.84	0.158	23.911	6.747	4.512	0.715	33.450	0.060	2.650
20	5.5	33.45	0.334	22.962	12.342	2.368	0.792	28.986	0.069	4.848
21	16.5	13.07	0.131	18.091	6.164	3.736	0.488	37.055	0.054	2.421
22	21.9	4.18	0.042	11.984	2.613	5.839	0.244	49.086	0.041	1.026
23	5.7	21.59	0.216	24.450	8.879	3.505	0.757	32.312	0.062	3.488
24	16.0	5.52	0.055	6.864	3.236	2.700	0.149	46.087	0.043	1.271
25	9.2	24.15	0.241	44.444	9.083	6.229	1.504	29.548	0.068	3.568
26	9.4	12.09	0.121	21.041	5.457	4.909	0.594	35.449	0.056	2.143
27	10.0	9.49	0.095	25.733	5.171	6.336	0.601	42.810	0.047	2.031
28	30.1	6.36	0.064	14.558	3.200	5.791	0.368	39.558	0.051	1.257
29	10.5	23.33	0.233	18.698	8.056	2.955	0.689	27.119	0.074	3.164
30	33.0	3.38	0.034	13.049	2.607	6.372	0.216	60.512	0.033	1.024
31	8.5	6.27	0.063	10.607	3.172	4.257	0.267	39.722	0.050	1.246
32	6.9	6.83	0.068	9.653	4.142	2.966	0.203	47.645	0.042	1.627
33	15.8	10.04	0.100	14.332	3.809	4.790	0.481	29.808	0.067	1.496
34	1.1	12.43	0.124	27.621	5.944	5.915	0.735	37.559	0.053	2.335
35	16.1	8.26	0.083	19.407	4.164	5.933	0.490	39.590	0.051	1.636
36	12.3	1.74	0.017	3.524	0.865	5.187	0.090	39.083	0.051	0.340
37	9.0	9.20	0.092	21.018	4.320	6.193	0.570	36.896	0.054	1.697
38	24.0	10.28	0.103	33.196	5.751	7.348	0.755	43.954	0.046	2.259
39	11.2	12.99	0.130	16.398	4.157	5.021	0.652	25.150	0.080	1.633
40	6.8	4.76	0.048	11.398	3.295	4.404	0.210	54.402	0.037	1.294
41	11.1	17.48	0.175	17.760	6.119	3.695	0.646	27.506	0.073	2.403
42	1.4	9.02	0.090	23.035	4.663	6.288	0.567	40.607	0.049	1.832
43	19.6	12.55	0.125	17.616	6.302	3.559	0.447	39.449	0.051	2.475
44	11.0	8.36	0.084	11.123	4.144	3.416	0.285	38.964	0.051	1.628

Table 1 (cont.)

ID	lTbSp	clMS	clMS/BS	clIrLTh	cum/d	clMar	clBFR	clBFR/TV	cl%/Yr	clNoOcBS
1	0.437	1.863	0.181	0.032	0.016	15.840	2.866	1.150	420	5.7
2	0.700	1.488	0.132	0.028	0.014	13.979	1.850	0.436	159	9.4
3	0.215	1.271	0.026	0.026	0.013	12.851	0.334	0.053	19	1.6
4	0.549	1.408	0.090	0.030	0.015	15.224	1.363	0.290	106	7.3
5	0.819	0.598	0.031	0.025	0.013	12.614	0.392	0.047	17	4.2
6	0.290	17.865	0.630	0.029	0.014	14.465	9.112	1.848	674	3.3
7	0.503	17.222	0.658	0.032	0.016	16.053	10.568	1.418	518	3.7
8	0.273	4.103	0.127	0.028	0.014	13.790	1.747	0.342	125	4.9
9	0.323	5.082	0.197	0.029	0.014	14.453	2.852	0.565	206	4.2
10	0.515	2.216	0.085	0.030	0.015	14.798	1.259	0.170	62	4.0
11	0.586	2.411	0.151	0.030	0.015	14.984	2.260	0.443	162	4.9
12	0.399	1.227	0.084	0.027	0.014	13.744	1.156	0.350	128	6.1
13	0.520	6.278	0.491	0.032	0.016	15.753	7.739	2.125	776	6.3
14	0.562	6.529	0.339	0.029	0.015	14.590	4.942	0.838	306	3.4
15	0.548	3.167	0.162	0.027	0.014	13.625	2.209	0.375	137	2.4
16	0.419	17.770	0.825	0.034	0.017	16.930	13.973	2.760	1007	5.1
17	0.481	1.998	0.142	0.030	0.015	14.846	2.109	0.570	208	2.0
18	1.288	0.087	0.019	0.025	0.013	12.659	0.237	0.077	28	4.1
19	0.318	2.858	0.120	0.029	0.014	14.449	1.727	0.383	140	2.8
20	0.137	6.852	0.298	0.030	0.015	15.154	4.522	1.909	697	2.5
21	0.359	2.839	0.157	0.029	0.015	14.621	2.295	0.614	224	2.7
22	0.934	2.692	0.225	0.027	0.013	13.308	2.989	0.512	187	5.0
23	0.225	10.471	0.428	0.045	0.023	22.539	9.652	2.754	1005	4.6
24	0.743	0.554	0.081	0.037	0.018	18.459	1.489	0.551	201	13.7
25	0.213	8.241	0.185	0.032	0.016	15.902	2.949	0.473	173	5.3
26	0.410	2.732	0.130	0.034	0.017	16.896	2.194	0.447	163	5.3
27	0.446	9.972	0.388	0.035	0.017	17.394	6.741	1.064	388	4.3
28	0.745	2.117	0.145	0.032	0.016	16.015	2.329	0.402	147	7.3
29	0.242	1.682	0.090	0.034	0.017	16.936	1.523	0.516	188	2.7
30	0.944	0.897	0.069	0.038	0.019	18.826	1.294	0.203	74	2.7
31	0.752	1.791	0.169	0.029	0.014	14.294	2.413	0.567	207	5.4
32	0.573	1.284	0.133	0.028	0.014	14.009	1.863	0.628	229	5.8
33	0.601	10.056	0.702	0.033	0.017	16.556	11.617	2.425	885	5.0
34	0.375	4.444	0.161	0.027	0.013	13.429	2.161	0.365	133	4.8
35	0.561	3.101	0.160	0.031	0.015	15.265	2.439	0.411	150	2.9
36	2.893	1.555	0.441	0.030	0.015	15.171	6.695	1.291	471	7.9
37	0.535	2.864	0.136	0.028	0.014	14.054	1.915	0.309	113	4.3
38	0.397	15.748	0.474	0.034	0.017	17.088	8.106	1.103	403	5.1
39	0.533	15.704	0.958	0.040	0.020	19.917	19.074	3.799	1387	5.7
40	0.736	0.396	0.035	0.027	0.014	13.556	0.471	0.107	39	4.9
41	0.343	2.599	0.146	0.028	0.014	14.043	2.055	0.556	203	6.1
42	0.497	2.704	0.117	0.030	0.015	15.169	1.781	0.283	103	3.9
43	0.353	0.562	0.032	0.027	0.013	13.346	0.426	0.120	44	4.9
44	0.563	3.021	0.272	0.027	0.014	13.625	3.701	1.083	395	4.6

Table 1 (cont.)

ID	clLeOcbs	clOcNoBA	clOcNoTA	gml/bw%	gmr/bw%	cmarr	lmarr
1	0.345	240.9	23.7	0.28	0.309	0.237	0.212
2	0.255	352.5	25.0	0.295	0.29	0.256	*
3	0.107	69.6	12.3	0.261	0.255	0.210	0.209
4	0.611	302.9	24.5	0.269	0.287	0.258	0.232
5	0.383	199.1	9.8	0.266	0.285	0.237	0.271
6	0.158	113.7	19.1	0.27	0.27	0.217	0.250
7	0.198	110.6	13.0	0.272	0.28	0.208	0.213
8	0.406	227.9	31.1	0.278	0.297	0.230	0.218
9	0.237	119.9	21.2	0.288	0.296	0.233	0.230
10	0.258	145.1	14.1	0.279	0.284	0.236	0.234
11	0.491	184.7	15.3	0.262	0.255	0.242	0.298
12	0.676	229.9	27.0	0.277	0.287	0.207	0.289
13	0.349	249.3	22.0	0.25	0.244	0.286	0.273
14	0.185	134.6	11.0	0.279	0.259	0.228	0.293
15	0.155	85.1	7.8	0.252	0.273	0.198	0.330
16	0.372	195.7	21.5	0.263	0.272	0.235	0.245
17	0.085	87.4	7.6	0.261	0.312	0.242	0.287
18	0.203	202.8	6.1	0.265	0.276	0.256	0.269
19	0.197	95.1	15.1	0.227	0.233	0.278	0.249
20	0.184	72.0	24.1	0.204	0.228	0.284	0.314
21	0.153	100.4	13.1	0.229	0.251	0.235	0.308
22	0.285	245.8	10.3	0.232	0.219	0.234	0.263
23	0.377	149.3	32.2	0.318	0.298	0.328	0.294
24	0.616	631.1	34.8	0.291	0.282	0.321	0.292
25	0.563	156.2	37.7	0.31	0.297	0.269	0.200
26	0.582	188.7	22.8	0.299	0.304	0.348	0.302
27	0.283	183.0	17.4	0.313	0.343	0.218	0.234
28	0.547	288.0	18.3	0.299	0.29	0.251	0.267
29	0.211	74.0	17.3	0.312	0.311	0.294	0.303
30	0.320	162.3	5.5	0.326	0.316	0.281	0.304
31	0.570	213.5	13.4	0.36	0.346	0.390	0.348
32	0.366	276.4	18.9	0.309	0.286	0.307	0.341
33	0.277	149.8	15.0	0.251	0.24	0.282	0.289
34	0.384	180.9	22.5	0.257	0.248	0.273	0.260
35	0.238	116.3	9.6	0.264	0.234	0.236	0.280
36	0.784	310.6	5.4	0.273	0.252	0.295	0.341
37	0.260	158.0	14.5	0.277	0.255	0.224	0.275
38	0.464	222.4	22.9	0.296	0.298	0.268	0.270
39	0.633	144.2	18.7	0.302	0.294	0.244	0.242
40	0.433	267.3	12.7	0.276	0.293	0.311	0.266
41	0.604	167.3	29.2	0.269	0.259	0.405	0.279
42	0.261	158.7	14.3	0.283	0.294	0.252	0.262
43	0.307	194.8	24.4	0.316	0.307	0.280	0.288
44	0.622	178.7	14.9	0.291	0.276	0.276	0.273

Table 2. Additional Data in 2% Study

ID	ID2	rFN	rMPa	rGPa	lFN	lMPa	lGPa	vBV/TV%	vBPm/TA	vderSv
23	1	101.19	87.03	0.35	106.75	94.97	0.32	19.01	6.919	8.808
24	2	98.97	93.38	0.41	105.64	83.11	0.31	24.58	6.894	8.777
25	3	92.3	101.63	0.58	97.86	74.45	0.3	20.72	5.5	7.001
26	4	93.41	116.89	0.55	95.63	84.75	0.39	27.38	7.642	9.728
27	5	95.63	100.01	0.5	93.41	75.75	0.3	22.69	6.9	8.783
28	6	88.96	103.47	0.54	102.3	115.56	0.46	23.43	6.043	7.692
29	7	106.75	91.02	0.41	106.75	78.75	0.28	20.11	5.827	7.418
30	8	97.86	119.72	0.61	102.3	96.2	0.34	30.29	5.959	7.586
31	9	92.3	93.85	0.48	101.19	88.11	0.38	36.17	6.851	8.722
32	11	82.29	84.73	0.42	85.62	110.97	0.47	24.75	6.061	7.715
33	13	95.63	100.95	0.54	100.08	120.13	0.45	22.93	6.142	7.819
34	14	95.63	126.77	0.67	98.97	121.1	0.61	32.71	9.675	12.317
35	15	102.3	101.75	0.58	95.63	105.02	0.39	28.94	7.607	9.684
36	16	88.96	157.84	1.05	100.08	138.39	0.62	18.15	6.128	7.801
37	17	87.85	95.67	0.5	96.74	125.21	0.58	40.89	8.958	11.404
38	18	93.41	139.02	0.9	100.08	108.69	0.47	23.50	7.469	9.509
39	19	94.52	123.16	0.69	95.63	111.93	0.45	18.87	6.604	8.407
40	20	91.18	105.51	0.58	91.18	113.72	0.58	29.45	9.061	11.534
41	21	96.74	148.49	0.89	98.97	126.2	0.63	14.08	5.287	6.73
42	22	90.07	110.93	0.57	88.96	117.96	0.41	25.62	7.236	9.211
43	23	70.06	87.51	0.74	98.97	129.88	0.64	35.59	8.54	10.872
44	24	87.85	102.63	0.64	91.18	109.43	0.47	30.30	8.44	10.745

Table 2 (cont.)

ID2	vBV	vTV	vBS	vTbTh	vTbN	vTbSp	vNOc	vNoOc/BS	vLeOc	vARS	vOcNo/TA
1	1.80	9.474	65.55	42	4.4	184	288	4.39	5.99	0.09	30.4
2	1.98	8.053	55.52	56	4.4	174	287	5.17	7.19	0.13	35.64
3	1.15	5.558	30.57	46	3.5	240	92	3.01	2.05	0.07	16.55
4	2.59	9.458	72.28	57	5.1	139	168	2.32	3.02	0.04	17.76
5	1.34	5.897	40.69	54	4.3	179	48	1.18	0.85	0.02	8.14
6	1.95	8.329	50.33	66	3.8	194	167	3.32	4.22	0.08	20.05
7	1.06	5.282	30.78	53	3.9	205	238	7.73	6.75	0.22	45.06
8	2.35	7.753	46.20	82	3.9	176	266	5.76	8.61	0.19	34.31
9	2.40	6.647	45.54	84	4.8	124	95	2.09	2.11	0.05	14.29
11	1.66	6.726	40.77	62	4	188	183	4.49	4.78	0.12	27.21
13	1.33	5.795	35.59	78	3	257	82	2.3	2.07	0.06	14.15
14	1.68	5.124	49.57	63	5.6	115	122	2.46	3.4	0.07	23.81
15	1.27	4.374	33.27	56	5.2	135	73	2.19	1.59	0.05	16.69
16	1.22	6.742	41.32	47	4	201	301	7.29	7.97	0.19	44.64
17	2.35	5.739	51.41	71	5.8	101	52	1.01	0.94	0.02	9.06
18	1.62	6.884	51.42	52	4.3	179	233	4.53	4.61	0.09	33.85
19	1.02	5.432	35.87	41	4.7	172	131	3.65	3.46	0.1	24.12
20	1.94	6.592	59.73	49	6	118	329	5.51	8.35	0.14	49.91
21	0.84	6	31.72	41	2.7	328	155	4.89	3.8	0.12	25.83
22	1.59	6.221	45.01	63	4.5	160	254	5.64	6.23	0.14	40.83
23	2.58	7.239	61.83	68	5.4	118	219	3.54	6.63	0.11	30.25
24	1.96	6.482	54.71	52	6.6	99	265	4.84	7.52	0.14	40.89

Table 2 (cont.)

ID2	VLength	cvMS	cvMS/BS	cvIrLTh	cvum/d	cvMar	cvBFR	cvBFR/TV	cv%/Yr
1	5.81	19.70	0.301	0.039	0.019	19.50	5.861	0.619	226
2	5.99	8.79	0.158	0.027	0.014	13.69	2.168	0.269	98
3	5.79	11.31	0.370	0.031	0.015	15.44	5.712	1.028	375
4	5.93	17.82	0.246	0.029	0.015	14.60	3.598	0.380	139
5	5.9	1.22	0.030	0.025	0.013	12.56	0.377	0.064	23
6	6.33	9.67	0.192	0.029	0.015	14.63	2.810	0.337	123
7	6.38	5.92	0.192	0.028	0.014	14.21	2.735	0.518	189
8	6.36	10.20	0.221	0.032	0.016	15.97	3.526	0.455	166
9	5.96	10.08	0.221	0.030	0.015	14.94	3.306	0.497	182
11	5.73	17.99	0.441	0.033	0.016	16.40	7.236	1.076	393
13	6.39	6.03	0.169	0.030	0.015	15.14	2.567	0.443	162
14	5.47	11.74	0.237	0.030	0.015	14.84	3.514	0.686	250
15	5.97	4.64	0.139	0.029	0.015	14.53	2.024	0.463	169
16	5.48	6.61	0.160	0.028	0.014	13.77	2.204	0.327	119
17	5.07	17.99	0.350	0.038	0.019	18.83	6.589	1.148	419
18	5.46	15.48	0.301	0.032	0.016	15.82	4.763	0.692	253
19	6.26	5.99	0.167	0.035	0.017	17.48	2.918	0.537	196
20	4.89	16.91	0.283	0.030	0.015	15.19	4.302	0.653	238
21	6.09	8.46	0.267	0.031	0.015	15.50	4.134	0.689	251
22	5.11	10.70	0.238	0.029	0.014	14.42	3.427	0.551	201
23	6.26	12.49	0.202	0.031	0.016	15.55	3.143	0.434	158
24	5.69	6.91	0.126	0.017	0.008	8.42	1.064	0.164	60

BIBLIOGRAPHY

Albright, J.A. and H.C.W. Skinner. 1987. Bone: Structural organization and remodeling dynamics. In J.A. Albright and R. A. Brand (Eds.), *The Scientific Basis of Orthopaedics* (p. 161-198). Norwalk, Connecticut: Appleton and Lange.

American Society of Agricultural Engineers (ASAE). 1995. Shear and three-point bending test of animal bone. *ASAE Standards* p. 479-481.

Albertson, A.M., R.C. Tobalmann, A. Engstrom, and E.H. Asp. 1992. Nutrients intakes of 2- to 10-year-old American children: 10-year trends. *Journal of American Dietary Association* 92:1492-1496.

Antonios, T.F. and G.A. MacGregor. 1995. Deleterious effects of salt intake other than effects on blood pressure. *Clin. Exp. Pharmacol. Physiol.* 22:180-184.

Bagi, C.M., S.C. Miller, B.M. Bowman, G.L. Blomstrom, and E.P. France. 1992. Differences in cortical bone in overloaded and underloaded femurs from ovariectomized rats: Comparison of bone morphometry with tortional testing. *Bone* 13:35-40.

Bindels, R.J.M., L.A.M. Van den Broek, M.J.M. Jongen, W.H.L. Hackeng, C.W.G.M. Lowik, and C.H. Van Os. 1987. Increased plasma calcitonin levels in young spontaneously hypertensive rats: role in disturbed phosphate homeostatis. *Pflugers Archives* 408:395-400.

Block, M.H., L. Trenner, P. Ruegg, and M. Karr. 1982. Glycol methacrylate embedding technique emphasizing cost containment, ultrarapid processing, and adaptability to a variety of staining techniques. *Lab. Med.* 13:290-298.

Box, G.E.P., W.G. Hunter, and J.S. Hunter, 1978. *Statistics for Experimenters; an Introduction to Design, Data Analysis and Model Building*. (653 pp). New York, N.Y: John Wiley and Sons.

Chan, E.L. and R. Swaminathan. 1993. Effect of different amounts of sodium intake for 4 months on calcium metabolism in normal and oophorectomized rats. *J. Bone Miner. Res.* 8:1185-1189.

Chan, E.L. and R. Swaminathan. 1998. Calcium metabolism and bone calcium content in normal and oophorectomized rats consuming various levels of saline for 12 months. *J. Nutr.* 128:633-639.

Chan, E.L.P., C.S. Ho, D. MacDonald, S.C. Ho, T.Y.K. Chan, and R. Swaminathan. 1992. Interrelationships between urinary sodium, calcium, hydroxyproline and serum PTH in healthy subjects. *Acta. Endocrinologica.* 127:242-245.

Cohen, A.J. and F.J. Roe. 2000. Review of risk factors for osteoporosis with particular reference to a possible aetiological role of dietary salt. *Food Chem. Toxicol.* 38:237-253.

Crews J.K., J.G. Murphy, and R.A. Khalil. 1999. Gender differences in Ca(2+) entry mechanisms of vasoconstriction in Wistar-Kyoto and spontaneously hypertensive rats. *Hypertension* 34:931-936.

Cuff, Y.H. 1996. Essential chemistry. In *Ceramic Technology for Potters and Sculptors.* (pp. 5-22) Philadelphia, Pennsylvania: University of Pennsylvania Press.

Currey, J., 1984. *The Mechanical Adaptations of Bones.* (294 pp) Princeton, NJ: Princeton University Press.

Dahl, L.K., M. Heine, and L. Tassinari. 1962a. Effects of chronic salt ingestion. Evidence that genetic factors play an important role in the susceptibility to experimental hypertension. *J. Exp. Med.* 115:1173-1190.

Dahl, L.K., M. Heine, and L. Tassinari. 1962b. Role of genetic factors in susceptibility to experimental hypertension due to chronic excess salt ingestion. *Nature* 194:480-482.

Dahl, L.K., M. Heine, and L. Tassinari. 1965. Effects of chronic excess salt ingestion. *J. Exp. Med.* 122:533-545.

Delmas, P.D., A. Schlemmer, E. Gineyts, B. Riis, and C. Christiansen. 1991. Urinary excretion of Pyridinoline crosslinks correlates with bone turnover measured on iliac crest biopsy in patients with vertebral osteoporosis. *J. Bone Miner. Res.* 6: 639-643.

DeWardener, H.E. 2001. The Hypothalamus and Hypertension. *Physiological Reviews* 81: 1599-1658.

Drozdzowska, B., W. Pluskiewicz, and J. Przedlacki. 2002. Prediction of the biomechanical properties of cancellous bone using ultrasound velocity and bone mineral density--an in vitro study. *Med. Sci. Monit.* 8:15-20.

Dyer, A.R., R. Stamler, P. Elliott, and J. Stamler. 1995. Dietary salt and blood pressure. *Nature Medicine* 1:994-996.

Eberle, J., S. Schmidmayer, R.G. Erben, M. Stangassinger, and H.P. Roth. 1999. Skeletal effects of zinc deficiency in growing rats. *J. Trace Elem. Med. Biol.* 13:21-26.

Evans, G., H.U. Bryant, D. Magee, M. Sato and R.T. Turner. 1994. The effects of Raloxifene on tibia histomorphometry in ovariectomized rats. *Endocrinology* 134:2283-2288.

Ferrell, F. and S.D. Gray. 1985. Longitudinal study of salt preferences in normotensive and hypertensive rats. *Hypertension* 7:326-332.

Fisher, J.E., M. Gentile, S.B. Rodan, D. Jenkins, and G.A. Rodan. 1995. Urinary deoxypyridinoline measurements, by enzyme-linked immunosorbent assay, correlate with bone loss caused by ovariectomy in 6 month old rats. *J. Bone Miner. Res.* 10: Suppl. 1, S242.

Frost, H.M. Bone histomorphometry, choice of marking agents and labeling schedule. In R.R. Recker (Ed.), *Bone Histomorphometry* (pp. 39-61). Boca Raton, FL: CRC Press, 1983.

Furuse, M., M. Okumura, T. Watanabe, and J.I. Okumura. 1992. Mineral balance and bone histology in spontaneously hypertensive rats. *Physiol. Behav.* 52:731-736.

Gluer, C.-C., C.Y. Wu, M. Jergas, S.A. Goldstein, and H.K. Genant. 1994. Three quantitative ultrasound parameters reflect bone structure. *Calcif. Tissue Int.* 55:46-52.

Goulding, A. and D. Campbell. 1983. Dietary NaCl loads promote calciuria and bone loss in adult oophorectomized rats consuming a low calcium diet. *J. Nutr.* 113:1409-1414.

Goulding, A. and D.R. Campbell. 1984. Effects of oral loads of sodium chloride on bone composition in growing rats consuming ample dietary calcium. *Miner. Electrolyte Metab.* 10:58-62.

Goulding, A., and B.E. McParland 1990. Fasting and 24-h urinary sodium/creatine values in young and elderly women on low-salt and salt-supplemented regimens. *J. Cardiovascular Pharmacol.* 6:s47-s49.

Grisk, O. and R. Rettig. 2001. Renal transplantation studies in genetic hypertension. *News Physiol .Sci.* 16:262-265.

Hallback, M. 1975. Consequence of social isolation on blood pressure, cardiovascular reactivity and design in spontaneously hypertensive rats. *Acta Physiol Scand.* 93:455-465.

Hsu, C.H., P.S. Chen, D.E. Smith, and C.S Yang. 1986. Pathogenesis of hypercalciuria in spontaneously hypertensive rats. *Miner. Electrolyte Metab.* 12:130-141.

Huffer, W.E. and R.B. Lepoff. 1992. An indirect method of measuring widths suitable for automated bone histomorphometry. *J. Bone Miner. Res.* 7:1417-1427.

Huffer, W.E., P. Ruegg, J.M. Zhu and R.B. Lepoff. 1994. Semiautomated methods for cancellous bone histomorphometry using a general -purpose video image analysis system. *Journal of Microscopy* 173:53-66.

Inoue, T., A. Moriya, K. Goto, T. Tanaka, and M. Inazu. 1995. What is the difference of bone growth in SHR and SD rats? *Clin. Exp. Pharmacol. Physiol.* Suppl 1:S242-S243.

Iwamoto, J., J.K. Yeh, and J.F. Aloia. 1999. Differential effect of treadmill exercise on three cancellous bone sites in the young growing rat. *Bone* 24:163-169.

Izawa, Y., K. Sagara, T. Kadota, and T. Makita. 1985. Bone disorders in spontaneously hypertensive rat. *Calcif. Tissue Int.* 37:605-607.

Johnson, R.J., J. Herrera-Acosta, G.F. Schreiner, B. Rodriguez-Iturbe. Subtle acquired renal injury as a mechanism of salt–sensitive hypertension. *N. Engl. J. Med.* 346:913-923.

Kiiskinen, A. 1977. Physical training and connective tissues in young mice--physical properties of achilles tendons and long bones. *Growth* 41:123-137.

Krari, N. and P. Allain. 1991. Altered element concentrations in tissues of Dahl salt sensitive rats. *Biol. Trace Elem. Res.* 31:199-207.

Lau, K., D. Zikos, J. Spirnak, and B. Eby. 1984. Evidence for an intestinal mechanism in hypercalciuria of spontaneously hypertensive rats. *Am. J. Physiol.* 247:E625-E633.

Law, M.R., C.D. Frost, and N.J. Wald. 1991. By how much does dietary salt reduction lower blood pressure? *Br. Med. J.* 302:811-815.

Lawler, J.E., M.M. Abel, and S.K. Naylor. 1993. Effects of salt intake on blood pressure and heart rate responses to footshock stress in SHR, BHR, and WKY rats. *Physiol. Behav.* 53:97-102.

Leblondel, G. and P. Allain. 1988. Altered element concentrations in tissues of spontaneously hypertensive rats. *Biomed. Pharmacother.* 42:121-129.

Liang, H, Y. Ma, S. Pun, M. Stimpel, and W.S. Jee. 1997. Aging- and ovariectomy-related skeletal changes in spontaneously hypertensive rats. *Anat. Rec.* 249:173-180.

Li, X.J., S.S.J. Webster, C. Sien-Yao, and D.M. Woodbury. 1990. Adaptation of cancellous bone to aging and immobilization in the rat: A single photon absorptiometry and histomorphometry study. *Anat. Rec.* 227:12-24.

Liu, C., R. Sanghvi, J.M. Burnell, and G.A. Howard. 1987. Simultaneous demonstration of bone alkaline and acid phosphatase activities in plastic-embedded sections and differential inhibition of the activites. *Histochemistry* 86:559-565.

Lucas, P.A., R.C. Brown, D.B. Lacour, J.A. Metz, and D.A. McCarron. 1986. Abnormal vitamin D metabolism, intestinal calcium transport, and bone calcium status in the spontaneously hypertensive rat compared with its genetic control. *J. Clin. Inv.* 78:221-227.

MacGregor, G. A., and F.P. Cappuccio. 1993. The kidney and essential hypertension: a link to osteoporosis. *J Hypertens.* 11:781-785.

MacGregor, G.A., N.D. Markandu, F.E. Best, D.M. Elder, J. MCam, G.A. Sagnella, and M. Squires. 1982. Double-blind randomized crossover trial of moderate sodium restriction in essential hypertension. *The Lancet* 1:351-355.

McCarron, D.A., P.A. Pingee, R.J. Rubin, S.M. Gaucher, M. Molitch, and S. Krutzik. 1980. Enhanced parathyroid function in essential hypertension: a homeostatic response to a urinary calcium leak. *Hypertension* 2:162-168.

McParland, B.E., A. Goulding, and A.J. Campbell. 1989. Dietary salt affects biochemical markers of resorption and formation of bone in elderly women. *Br. Med. J.* 299:834-835.

Malluche, H.H., D. Sherman, W. Meyer, and S.G. Massry. 1982. A new semiautomatic method for quantative static and dynamic bone histology. *Calcif. Tissue Int.* 34:439-448.

Maede, H., D.B. Kimmel, D.M. Raab, and N.E. Lane. 1993. Musculoskeletal recovery following hindlimb immobilization in adult female rats. *Bone* 14: 153-159.

Matsuda, J.J., R.F. Zernicke, A.C. Vailas, V.A. Pedrini, A. Pedrini-Mille, and J.A. Maynard. 1986. Structural and mechanical adaptation of immature bone to strenuous exercise. *J. Appl. Physiol.* 60:2028-2034.

Metz, J. A., N. Karanja, E.W. Young, C.D. Morris, and D.A. McCarron. 1990. Bone mineral density in spontaneous hypertension: differential effects of dietary calcium and sodium. *Am. J. Med. Sci.* 300:225-230.

Muntzel, M., and T. Drueke. 1992. A comprehensive review of the salt and blood pressure relationship. *Am. J. Hypertens.* 5:1s-42s.

Navidi, M., I. Wolinsky, P. Fung, and S.A. Arnaud. 1995. Effect of excess dietary salt on calcium metabolism and bone mineral in a spaceflight rat model. *J. Appl. Physiol.* 78:70-75.

Need, A.G., H.A. Morris, D.B. Gleghorn, D. De Nichilo, M. Horowitz, and B.E.C. Nordin. 1991. Effect of salt restriction on urine hydroxyproline excretion in postmenopausal women. *Arch. Int. Med.* 151:757-759.

Neuman, W.F. and M.W. Neuman. 1958. *The Chemical Dynamics of Bone Mineral.* (209 pp) Chicago, IL. The University of Chicago Press .

Nieman, D.C. 1990. *Fitness and Sports Medicine: An Introduction.* (583 pp) Palo Alto, CA. Bull Publishing Co.

Ovesen, J., B. Moller-Madsen, J.S. Thomsen, G. Danscher, and L. Mosekilde. 2001. The positive effects of zinc on skeletal strength in growing rats. *Bone* 29:565-570.

Parfitt, A.M. 1992. The physiologic and pathogenetic significance of bone histomorphometric data. In F.L. Coe and M.J. Favus (Eds.), *Disorders of Bone and Mineral Metabolism.* (pp. 475-489) New York, NY: Raven Press.

Parfitt, A.M., M.K. Drezner, F.H. Glorieux, J.A. Kanis, H. Malluche, P.J. Meunier, S.M. Ott, and R.R. Recker. 1987. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res.* 2:595-610.

Pelker, R.R., Friedlaender, G.E. Markham, T.C., M.M. Punjabi, and C.J. Moem. 1984. Effects of freezing and freeze-drying on the biomechanical properties of rat bone. *Journal of Orthopaedic Research* 1:405-411.

Pfeffer, J.M., M.A. Pfeffer, and E.D. Frohlich. 1971. Validity of an indirect tail-cuff method for determining systolic arterial pressure in unanesthetized normotensive and spontaneously hypertensive rats. *J. Lab. Clin. Med.* 75:957-962.

Rapp, J.P. 1987. Development of inbred Dahl salt-sensitive and inbred salt-resistant rats. *Hypertension Suppl. 1*, 9:1-21 – 1-23.

Rapp, J.P. and H. Dene. 1985. Development and characteristics of inbred strains of Dahl salt-sensitive and Dahl salt-resistant rats. *Hypertension* 7:340-349.

Reckelhoff, J.F., H. Zhang, and K. Srivastava. 2000. Gender differences in development of hypertension in spontaneously hypertensive rats role of the renin-angiotensin system. *Hypertension* 35:480-483.

Recker, R.R. 1992. Embryology, Anatomy, and Microstructure of Bone. In Coe, F.L. and M.J. Favus. (Eds.) *Disorders of Bone and Mineral Metabolism.* (1118 pp) New York, NY: Raven Press.

Reeves, P.G., F.H. Neilsen, and G.C. Fahey. 1993. AIN purified diets for laboratory rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformation of AIN - 76A Rodent Diet. *J. Nutr.* 123:1939-1951.

Rizzoli, R., J.P. Bonjour, and S.L. Ferrari. 2001. Osteoporosis, genetics and hormones. *J. Mol. Endocrinol.* 26:79-94.

Roudebush, R.E., D.E. Magee, D.N. Benslay, A.M. Benedele , and H.U. Bryant. 1993. Effect of weight manipulation on bone loss due to ovariectomy and the protective effects of estrogen in the rat. *Calcif. Tissue Int.* 53:61-64.

Rude R.K., M.E. Kirchen, H.E. Gruber, M.H. Meyer, J.S. Luck, and D.L. Crawford. 1999. Magnesium deficiency-induced osteoporosis in the rat: uncoupling of bone formation and bone resorption. *Magnes Res.* 12:257-267.

Ruegg, P. and L. Smith. 1996. *Histotechniques for sectioning mineralized bone.* National Society for Histotechnology (NSH). September, 1996. *Workshop* #46.

Russ, J.C. 1999. *The Image Processing Handbook.* (771 pp) Boca Raton, FL. CRC Press.

Seldin, E.D. 1965. A rheologic model for cortical bone. A study in the physical properties of femoral samples. *Acta. Orthop. Scand, Suppl.* 93:20-43.

Shaw, S. R., A.C. Vailas, R.E. Grindeland, and R.F. Zerniche. 1988. Effects of a 1-week spaceflight on morphological and mechanical properties of growing bone. *Am. J. Physiol.* 254:R78-R83.

Shen, V., R. Birthman, R. Xu, R. Lindsey, and D.W. Dempster. 1995. Short term changes to histomorphormetric and biochemical turnover markers and bone density in estrogen and/or calcium deficient rats. *Bone* 16:149-156.

Shortt, C., and A. Flynn. 1990. Sodium-calcium inter-relationships with specific reference to osteoporosis. *Nutr. Res. Rev.* 3:101-115.

Tabeling, J. 1997. DDK (Delaware Diamond Knives) on-line guide to plastic sectioning and embedding. Retreived 1997 from http:www.ddk.com.

Umemura, Y., T. Ishiko, K. Aoki, and A. Gunji. 1992. Effects of voluntary exercise on bone growth and calcium metabolism in spontaneously hypertensive rats. *Int. J. Sport Med.* 13:476-480.

United States Environmental Protection Agency. 1996. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods. *SW-846.* 3050B.

Wallach, S. and R.L. Verch. 1986. Tissue magnesium in spontaneously hypertensive rats. *Magnesium* 5:33-38.

Wallach, S. 1990. Effects of magnesium on skeletal metabolism. *Magnes. Trace Elem.* 9:1-14.

Williams, J.R., T.R. Harrison, and A. Grollman. 1939. A simple method for determining the systolic blood in the unanesthetized rat. *J. Clin. Inv.* 18:373-376.

Wilson, D.K., L. Bayer, and D.A. Sica. 1996. Variability in salt sensitivity classifications in black male versus female adolescents. *Hypertension* 28: 250-255.

Wronski, T.J., P.A. Schenck, M. Clintron, and C.C. Walsh. 1987. Effect of body weight on osteopenia in ovariectomized rats. *Calcif. Tissue Int.* 40:155-159.

Yamakawa, H., H. Suzuki, M. Nakamura, Y. Ohno, and T. Saruta. 1992. Disturbed calcium metabolism in the offspring of hypertensive parents. *Hypertension* 19:528-534.

Yamori, Y., M. Matsumoto, H. Yamabe, and K. Okamoto. 1969. Augmentation of spontaneous hypertension by chronic stress in rats. *Jpn. Circ. J.* 33:399-409.

Yamori, Y., S. Fukuda, S. Tsuchikura, K. Ikeda, Y. Nara, and R. Horie. 1991. Stroke-prone SHR (SHRSP) as a model for osteoporosis. *Clin. Exp. Hypertens. A* 13:755-762

Young, E.W., R.D. Bukoski, and D.A. McCarron. 1988. Calcium metabolism in experimental hypertension. *Proc. Soc. Exp. Bio. Med.* 187:123-141.

Yu, H.C.M., L. M. Burrell, M.J. Black, L.L. Wu, R.J. Dilley, M.E. Cooper, and C.I. Johnston. 1998. Salt induces myocardial and renal fibrosis in normotensive and hypertensive rats. *Circulation* 98:2621-2628.