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CRANIOFACIAL PHENOTYPIC VARIATION BETWEEN TWO LABORATORY MOUSE STRAINS, THE B6 AND A/J

by

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DEDICATION

To God for the incredible blessings I have in my life, more than any person could deserve.

To my in-laws, Bill and Marsha Ryan. Thanks for all you have done for us and for giving me your daughter. Mom Ryan, we miss you.

To my parents Dave and Rita Lenhart. All I can say is that you are the best parents I could have ever asked for, thank you for everything Mom and Dad.

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Most of all to my incredible wife of over fourteen years, Coleen. The sacrifices that you have endured over the last three years have been more than I should have ever asked of you, and you did it selflessly. Thank you so much. I love you more than you will ever know!

differences in skull height, whereas males showed interstrain differences in maxillary tooth row length. When combining males and females of each strain, the most appropriate measures to quantify interstrain variation were length-width ratio, premaxillary length (adjusted by femur length), and mandibular length (adjusted by femur length).

These findings suggest that craniofacial phenotypic differences between B6 and A/J mice are large enough to allow genetic dissection of craniofacial phenotypes.

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INTRODUCTION

Orthodontists are challenged by the fact that the vast majority of their patients undergo significant growth during treatment. In fact, the need for orthodontic treatment can be due to problems related to jaw growth. Therefore, it would be helpful if clinicians knew what mechanisms controlled and/or influenced dentofacial development. At the present time, a complete understanding of this process still eludes researchers (Proffit).

Based on the similar dental and skeletal patterns seen within families, one could surmise that genetics plays a significant role in the etiology of malocclusion. However, differences within families suggest that local or environmental factors play a part as well. It also appears that craniofacial size and shape are influenced by complex interactions between genes and environment. The ultimate question to an orthodontist is: To what extent are maxillary and mandibular growth, as well as dental malocclusion, influenced by environmental intervention? That is, what are the limits of orthodontic therapy? Obviously, the extent of the environmental effects exerted by the orthodontist is related to the influence of genetics. Therefore, improved understanding of this issue is important for orthodontics practice.

strains of mice derived from the B6 and A/J (parental strains). Consomic strains are bred to have single chromosome substitutions, with one of the strains providing the genetic background and the other strain donating one of its chromosomes to this background. A "panel" consists of twenty-one new strains, each with a different donated chromosome. By analyzing the phenotypic differences within this panel, the specific chromosomes that influence the particular phenotype (craniofacial morphology in this case) can be discovered.

The advantage to the specialty of orthodontics in understanding chromosomal influences on craniofacial growth is not known at this time. Perhaps an improved understanding of genetics will enable clinicians to more accurately predict growth in their patients. This would certainly modify the treatment planning process in growing individuals. It would also give a better understanding to the development of malocclusion and craniofacial deformity and help orthodontists develop better, more predictable, and more stable results.

1. An improved understanding of genetic influences on craniofacial morphological development could provide a scientific basis for orthodontic treatment decisions. This, in turn, would improve the understanding of the influences of specific treatment modalities on craniofacial morphology. A first step in understanding the genetic basis for craniofacial skeletal ontogenesis is the

identification of an appropriate animal model. That was the goal of this study

REVIEW OF THE LITERATURE

The science of genetics involves the inheritance of traits as well as the complex interactions between genes and environment. The future may hold promise for influencing genes directly, but presently, orthodontic intervention appears is solely environmental. However, a better understanding of genetic input is paramount in order to partition out the environmental contribution for that particular trait.

Expression of a trait involves the relationship between genotype and phenotype. Genotype is defined as the genetic constitution of an organism and may refer to a specific gene locus or to all gene loci in general. Phenotype is the expression of a particular genotype and usually involves a combination of both genetic and environmental influences. The part of phenotypic variance that is due solely to genotype is referred to as heritability (Mossey).

Traits can be further divided into qualitative and quantitative types. Qualitative traits may be attributable to a single allele and Mendelian analysis can be applied. It is a trait that is either present or absent without varying degrees. Quantitative traits are measurable characteristics, such as height, weight, serum cholesterol, or blood pressure. That is, a measurable quantity that differs among different individuals, often following a normal distribution of the

population. It is the genetic variability of quantitative traits that is central to understanding how genes contribute to various phenotypes (Nussbaum et al.). Craniofacial morphology likely involves quantitative genetic influences and is determined by multiple gene loci as well as environmental effects. Therefore, it is subject to multifactorial inheritance patterns. That is, morphological characterization of dentofacial structures are the end result of a number of interacting biochemical and developmental processes (Mossey). To what degree does genetics alone control these events? A starting point for answering this question would be to locate the gene loci that contribute to this particular phenotype.

Van Limborgh, in 1972, defined the two controlling factors influencing craniofacial morphogenesis as the genome and the environment. He further broke down genetic influences into intrinsic and epigenetic factors. Intrinsic factors are those that exert their influence inside the cells from which they come. Epigenetic factors, on the other hand, do not exert their action within the cells from which they come, but in the responses of the tissues they act upon. An example is the brain, which has intrinsic genetic potential but exerts an epigenetic influence on the overall growth of the calvaria. He further described endochondral bone formation as being controlled primarily by intrinsic genetic factors, whereas, intramembranous bone formation is controlled mostly via local and epigenetic factors. A more contemporary view of epigenetic factors

describes them as any factor that can affect the phenotype without change in the genotype (Nussbaum et al.).

Melvin Moss' functional matrix hypothesis claims epigenetic control of craniofacial morphogenesis. This is contrary to genetic theories that suggest it is the genome that ultimately determines phenotypic features. Moss feels that ontogenesis involves developmental cascades with multiple branching points where decisions are constantly made between alternate developmental pathways. These decisions are influenced not by genetic information but instead are responses to some epigenetic stimulus. He does, however, acknowledge that both genomic and epigenetic factors, and their interactions with each other, are needed for growth and development (Moss).

Heritability studies of facial morphogenesis are evident throughout the field of dentistry and, in particular, orthodontics. Hunter et al., in 1970, did a study to determine if the size of certain facial dimensions in adults can be predicted from the size of those same dimensions in their parents. They looked at five different facial measurements (Ar-Go, Go-Me, Na-Me, S-Na, Ar-Gn, Ar-Na) from lateral cephalometric radiographs and then calculated correlation coefficients between parents and their offspring. Their findings suggested that facial dimensions were largely inherited. There were higher correlations between fathers and their offspring than between mothers and their offspring, and the relationship between fathers and their children for mandibular dimensions was particularly strong.

In a similar study Harris et al., in 1973, examined nine different craniofacial contours in both related and nonrelated individuals. The nine contours included the posterior border of the ramus, gonial angle, antegonial notch, subsymphyseal contour, lingual symphysis, chin button, anterior nasal floor, palate, and posterior nasal floor. The contours were compared through superimposition of the structures on tracings of lateral cephalometric films. They found that the proportion of concordant measurements was always higher for siblings than for nonrelated controls for each of the structures considered. The palate and posterior border of the ramus showed relatively weak familial patterns but all others showed a definite tendency toward familial aggregation. A weak correlation for the palate might suggest that functional (environmental) mechanisms have more of an influence on the shape of the palate than do genetic factors. However, one could also argue that functional influences may also have a genetic component affecting tooth size, eruption timing, and tongue size which can, in turn, alter palatal shape.

Other studies (Harris and Johnson; Saunders et al.; Harris and Kowalski; Nakata et al.; Cleall et al.; Byard et al.) demonstrated varied degrees of familial correlation with craniofacial skeletal and occlusal measures. However, the use of familial information in diagnosis and treatment planning in orthodontic practice has not been effectively utilized. This could be due to the complexity of the interplay between genetics and environment and how each plays into the development of malocclusion.

It is generally accepted that orthodontists can influence the position of teeth in alveolar bone. However, the extent to which growth in areas more remote from the teeth, such as the mandibular condyle, can be modified remains controversial. Presently, the controversy is not that growth modification is possible with orthopedic forces, but rather that the amount of change, as well as the optimal timing for treatment, remain as issues (Kluemper and Spalding).

The randomized clinical trial is generally considered to be the gold standard for clinical research. Three such studies, supported by the National Institutes of Health, looked at the question of the timing and the effects of growth modification procedures in orthodontic patients. These studies were conducted at the University of Florida, the University of North Carolina, and the University of Pennsylvania. Their findings are certainly worth summarizing.

Researchers at the University of Florida looked at anteroposterior skeletal and dental changes after early class II treatment with bionators and headgear (with biteplane). Patients were randomly assigned to either a control or one of the treatment groups. Relapse after active treatment was completed and was also evaluated. Their findings were as follows:

1. Both headgear and bionator enhanced mandibular growth and did not significantly affect maxillary growth;

2. The achieved skeletal change was stable;

3. Retention was not needed to prevent skeletal relapse, but was needed to prevent dental relapse (Keeling et al.).

The University of North Carolina study analyzed the benefits of early class II treatment as a two phase treatment as opposed to treatment solely in a single phase. Their class II sample was randomly assigned to either a headgear, a bionator, or a nontreatment control group for the first early phase of treatment. Then after fifteen months of phase one treatment, an eventual second phase of comprehensive orthodontic treatment was done with full fixed appliances. This design addressed the question, "Does early growth modification make a difference in subsequent treatment?" They concluded that the first phase of treatment did result in significant growth modification; however, early treatment followed by a second phase of comprehensive orthodontic treatment did not, on average, lead to major differences in skeletal or dental occlusion outcome when compared to a later single phase of treatment (Tulloch et al.).

The University of Pennsylvania studied the treatment of class II, division 1 malocclusion via treatment with either headgear or a Frankel FR-2 appliance. This study did not employ a control group and had a smaller sample than the previous two randomized clinical trials. Their findings included the following:

1. Both headgear and FR-2 were effective in correcting the class II malocclusion in prepubertal children;
2. On average, both appliances restricted maxillary growth and had a similar effect on enhanced mandibular growth;
3. Treatment in late childhood can be as effective as done in an earlier phase.

Ghafari et al. concluded that a more practical single phase of treatment could be achieved if coordinated with dental eruption stage.

The above studies clearly show that skeletal changes can be imparted via orthodontic (environmental) intervention. However, it appears to be a short-term gain for growth that may already be genetically programmed and occur later, nonetheless (Liviertos and Johnston; Johnston). Based on the best available science to date, it seems that growth modification via functional appliances, in an early treatment phase, provides no better orthodontic outcomes than treatment in later childhood in a single phase of treatment (Tulloch et al.; Ghafari et al.; Livieratos and Johnston).

It is difficult to use humans in genetic studies. Therefore, to determine the contribution of genes to either facial growth or orthodontic treatment, an animal model is preferred. Mice have often been used for genetic experimentation. The mouse is an ideal organism for such studies since they are physiologically similar to humans. They reproduce rapidly (gestation eighteen to twenty days), develop quickly (adulthood in sixty days), and have a short life span.

The mouse genome is contained in 20 chromosome pairs and is about 2.7 billion base pairs in size (15% smaller than the human genome), whereas the human genome is 23 chromosome pairs and is about 3.1 billion base pairs in size (NIH news release). However, humans have the same number of genes (30,009) as mice with 90% homology between the two species (Slavkin). This

remarkable genetic similarity certainly suggests that researchers can gain insight into the function of many human genes by using the mouse as a model for laboratory research.

The first inbred mouse strain was developed early in the twentieth century for cancer research. Inbred strains are produced by at least twenty consecutive generations of brother-sister mating. In order to enhance the study of human diseases with the mouse model, the value of moving genomic sections from one strain to another became evident; thus, congenic inbred strains were developed. Congenic strains are produced by transferring a chromosome segment, carrying a particular phenotypic trait or gene loci, from one strain to another by ten or more successive backcross matings selecting for the donor chromosome segment at each generation (Bedell). According to mendelian concepts, it is expected that half of the unrelated genomic material will be transmitted to a subsequent backcross generation. Ten generations of backcross matings result in mice heterozygous for the locus of interest. They are also approximately 99.9% recipient genome and carry the new region of interest as well. This chromosome segment is then made homozygous by intercrossing. The resultant congenic strain is primarily the recipient inbred strain plus the donor segment. This process takes two and a half to three years to complete (Markel).

Due to the extensive time period required to produce congenic strains, a new technique was developed. Using microsatellite markers, the inheritance

pattern of the gene of interest can be followed. This technological advancement reduces the number of generations necessary to construct a congenic strain. Therefore, a new strain can be created in fifteen to eighteen months rather than three years (Markel).

These animal models have been valuable in genetic research. Complex traits are determined by the combination of several genes and input of environmental factors. In humans, it is difficult to map individual genes due to genetic complexity and difficulty controlling environmental factors. The inbred mouse, including congenic strains, is a good animal model to use to analyze complex traits since genetic factors can be more easily dissected and environmental factors controlled (Fortin).

Bailey, in 1956, illustrated the importance of separating environmental and genetic influences when analyzing morphogenesis in mice. He compared genetic and environmental components that affected the development of the mouse mandible. Using skeletons from twenty-four inbred strains (two males and two females of each strain), he performed a principal components analysis to determine specific factors that influenced morphogenesis of the mandible. Utilizing vectorial representations based on the mandibular measurements, he came to the following conclusions. Anterior components of the mandible seemed to be more closely related to variations in the teeth, while posterior components were associated with variations in muscle attachments. He also found that similarities among the arrangements of the vectors were found in the

genetic and environmental influences of the mandible. This suggested that both environmental and genetic factors were affecting the same pathways. Also, this interaction between genetic and environmental factors may have advantages since this would give credence to the idea of anatomic compensation for hereditary deficiencies and anomalies through environmental changes during development. He stressed the importance of separating environmental from genetic sources of phenotypic variance. Animal models, such as inbred mouse strains, provide a good model for studying morphogenetic variation.

In order to analyze genetic factors using animal models, the animals must have high genetic reliability. Festing, in 1972, identified different mouse strains by classifying them based on measurements of the mandible. He was able to correctly classify both inbred and non-inbred strains 98% of the time with his measurement techniques. In 1974b, Festing sampled mouse colonies from ten different sources to determine the genetic reliability of commercially-bred laboratory mice. He determined that some of the strains and stocks supplied were not authentic. Festing's mandibular analysis technique has made it possible to monitor the quality of commercially available mice. Later, Lovell and Johnson, in 1983, suggested using other bones in addition to the mandible to improve the resolution in the identification and quality control of mouse stocks. They discussed using such bones as the femur, scapula, humerus, and others for this purpose. Therefore, strains poorly distinguished by one bone may be properly separated by another bone.

The Laboratory Animals Center then started to monitor accredited mouse colonies, and if the center was satisfied that the colony was authentic, it gave it a "genetically monitored" status and that could be advertised by the breeder. They sample the colonies several times per year to verify consistency. Before being eligible for genetic testing, the colony must consist of at least one hundred breeding females, and the strains and stocks must be correctly named according to internationally agreed systems of nomenclature. The breeder must also agree to publish the exact history of the colony in question as well as supply samples for genetic analysis (Festing, 1974a).

The A/J and B6 are two of the most commonly used inbred strains of mice. Both of these have over 150 generations of inbreeding in their history. They are also two of the best characterized mice as marked differences in their anatomical and physiological traits have been demonstrated over the past forty years. And because they have such significant phenotypic differences with corresponding differences in their genome composition, they are particularly good animal models to create congenic strains to study the genetic component of these differences (Fortin).

Physiological phenotypic variation between the A/J and B6 strains is significant. Surwit et al., in 1995, showed that B6 mice were highly susceptible to obesity and diabetes when fed a high-fat diet, whereas, the A/J strain was resistant to both obesity and diabetes when fed the same diet. They suggested that the B6 mice carry a genetic trait that predisposes them to store fat when

dietary fat intake is high. Gill et al., in 1998, demonstrated physiological differences in alcohol preference with the B6 having higher preference levels than the A/J. In addition, Nesbitt and Skamene, in 1984, showed that these strains differed strikingly host resistance to infection and malignancy.

Airway response is a physiological trait that shows phenotypic variation between these two strains. These differences may have craniofacial implications. Han et al., in 2001, exposed mice to either 8% oxygen or 3% carbon dioxide-10% oxygen mixtures for five minutes and then reoxygenated with either 100% oxygen or ambient air. Their ventilatory behavior was observed to determine if periodic breathing (waxing and waning of ventilation) occurred. The B6 exhibited periodic breathing, whereas the A/J did not, suggesting significant differences in posthypoxic ventilatory behavior. DeSanctis et al., in 1995 confirmed the previous studies that suggested that the A/J displays an asthma-like phenotype, where the B6 showed significantly less hyperresponsiveness.

In addition to physiologic phenotypic variations in respiratory behavior, these two strains exhibit significant anatomical differences. Hoit et al., in 2002, showed distinctions in complex cardiovascular traits. The B6 had more of an "athletic heart" with greater left ventricular mass, a slower heart rate, greater exercise times on a treadmill, and simply higher activity in general than that of the A/J. Ironically, the B6 tends to be anatomically more obese as well as being at higher risk for atherosclerosis (Nadeau et al., 1995).

Two other studies looked at anatomical variations between the A/J and B6 that could have direct relationship to the craniofacial complex. Jepsen et al., in 2001, analyzed whether bone quality varied with the genetic background of the A/J and B6 strains. Mechanical, morphological, and compositional tests of long bones were conducted to examine mechanical properties of the bone (femurs were utilized). Stiffness of A/J femurs was 17% greater than the B6 femurs; however, the A/J bones were more brittle compared with that of the B6. Liu and Erickson, in 1986, looked at the susceptibility of these two strains to steroid-induced cleft palate. Mouse strains that have higher levels of glucocorticoid receptors have higher levels of risk of cortisone-induced cleft palate. The A/J strain has twice as many specific steroid receptors as the B6 strain, resulting in a significant greater susceptibility.

The above studies characterize significant variation between the A/J and B6 mouse strains. Given that these mice are raised under similar environmental conditions, it suggests that genetics has a significant influence on the development of these trait differences. However, a complete understanding of the genetic influences on these phenotypes is still unknown and requires further analysis.

As a supplement to congenic mouse strains for the analysis of genetic influence on phenotypic traits, another technique, utilizing chromosome substitution strains (also referred to as consomic strains), has been developed. Nadeau et al., in 2000, described this technique as a more efficient method to

study quantitative trait loci (QTL) since developing congenic mouse strains is both expensive and time-consuming (up to five years). Nadeau stated verbally that, "chromosome substitution strains result from individual substitution of each chromosome in the host strain by the homologous chromosome from the donor strain." With this technique, a panel can be developed with a collection of single chromosome substitutions on a uniform genetic background. It does. however, require two to three years to complete the consomic panel of twenty-one new strains. Once completed, genetic analysis of a particular phenotypic trait can be more rapidly accomplished. In order to construct a panel of this nature, the parent strains must first be selected. It is best to select parent strains that are relatively divergent so that there are significant phenotypic differences. Then the decision as to which will be the host and which will be the donor strain must be made. Typically, the parent strain that breeds best will be the host strain. Once the panel is completed, there will be a neatly partitioned genome into a series of strains with single chromosome substitutions on a defined and uniform genetic background (Nadeau et al., 2000).

The phenotype of interest is then assayed in each of the chromosome substitution strains, as well as the host strain. If the phenotype is reproducibly and significantly different from that of the host strain, at least one quantitative trait loci is located on that chromosome. Because multiple genetically identical individuals are available, the sample size can be increased to whatever is needed for statistical significance (Nadeau et al., 2000).

However, there are two main limitations in the use of consomic strains. First, quantitative trait loci are located on an entire chromosome rather than on a more specific region of approximately 20-cM that you would see with congenic strain development. Secondly, chromosome substitution strains do not distinguish multiple quantitative trait loci on the substituted chromosome. However, once the chromosome of interest is located, one can immediately backcross the newly formed consomic strain to a host strain. Then the recombinant progeny can be tested to determined if more than one QTL accounts for the phenotypic difference and localize each QTL. Ultimately this process is much less time-consuming and expensive than the previously mentioned congenic strain development (Nadeau and Frankel).

Dr. Nadeau's genetics laboratory at Case Western Reserve University has developed a chromosome substitution panel utilizing the B6 and A/J inbred mouse strains as the parent strains, with the B6 as the background (host strain) and the A/J as the chromosome donor. This panel can be utilized to study any number of phenotypic differences between these two strains, such as craniofacial form and shape, to help locate quantitative trait loci which influence these differences.

Once quantitative trait loci have been identified, continued analysis is required to identify actual genes that lead to various phenotypes. QTL analysis involves detection, map estimation, fine mapping, and the ultimate goal of eliciting the gene itself. Since many phenotypes are complex traits, that is a trait

that a one-to-one relationship between genotype and phenotype does not exist, the identification of the genes underlying complex traits is a major challenge in the field of genetics (Darvasi).

Four major approaches have been used to dissect complex genetic traits They include the following: linkage analysis, allele-sharing methods, association studies, and polygenic analysis of experimental crosses. Linkage analysis involves development of a model to explain inheritance patterns of traits observed in a pedigree. This can be challenging with complex traits because it can be difficult to find a model that appropriately explains the inheritance pattern. Allele-sharing methods attempt to prove that relatives inherit a trait more often than one would expect by chance. Association studies are actually case-control studies involving population correlation. That is, an allele is associated with a trait if it occurs at a higher frequency in affected individuals than in controls for a given population. Experimental crosses involve animal models. Successive crosses in animals provide a more homogenous genetic model enabling easier genetic dissection of complex interactions than could be done in human families (Lander and Schork).

When using recombinant inbred mouse strains for QTL mapping studies, there is risk for type I and type II statistical errors. Type I error is a false positive result, whereas type II error involves false negatives. When more stringent alpha levels are chosen to reduce type I error, the probability for false negatives increases. Because recombinant inbred strains are homozygous, the accuracy

of predicting phenotype from genotype is much greater. The key to increasing accuracy of mapping studies is to find the appropriate balance between type I and type II errors (Belknap et al.).

The concepts of "incomplete penetrance" and "phenocopy" may also complicate genetic mapping. Incomplete penetrance occurs when an individual inherits a predisposing allele but does not show the subsequent phenotype. Phenocopy results when an individual does not inherit the predisposing allele but manifests the trait (or disease) as a result of random causes or environmental influences. This confuses matters as a direct cause and effect relationship does not always occur (Lander and Schork).

Mutagenesis (or induced variation) is another method that can be used in animal studies to discover the genetic basis for complex traits or other physiologic processes. This technique involves the inducement of single- gene mutations in the gene that is suspected to be responsible for the phenotype of interest. These highly specific mutations will then have phenotypic effects in offspring of the mutagenized individuals, and observations are made to determine the phenotypic variation that occurs as a result of the mutation. If trait expression is altered in the offspring as a result of the induced mutation, the relationship between phenotype and genotype is established (Nadeau and Frankel).

Bailey, in 1985, did two studies to analyze the genes that affect the shape of the mandible in laboratory mice. The first study was a congenic strain

analysis based on strains derived from the B6 and BALB. A chromosomal segment from the BALB strain was introduced onto the B6 genetic background. The gene segments that were introduced differed in content among the congenic strains that were developed. The mandibular shape was analyzed and compared among the various strains to determine which genes contribute to this phenotype. Mandibles were obtained from euthanized mice, and soft tissues were cleared via papain digestion and a detergent wash. Twelve landmarks were chosen for shape analysis, and interlandmark distances (ILDs) were calculated from twenty congenic strains (fifteen to twenty-four mice per strain). The BALB strain as well as all the congenic strains were tested for statistical differences in the ILDs compared to the B6 strain. They also looked at mean measurements of three longbones, the femur, scapula, and innominate. Strains that differed from the B6 in regard to ILDs do not necessarily differ in regard to longbones. This suggests that the genes that affected the mandible were not the same as the genes that affected the longbones. Data presented in this study indicated that individual genes altered localized regions of the mandible and that such genes were estimated to be present in large numbers. Genes from seven different chromosomes including 3, 4, 5, 7, 8, 9, and 17 contributed to phenotypic variation in the mandibles of these mice.

A similar study by Bailey, in 1986a, utilized similar methodology but included a set of recombinant inbred (RI) strains that were derived from the B6 and BALB and had homozygous genomic mixtures of these two strains. These

recombinant inbred strains were derived from the F2 generation of a cross between the B6 and BALB parent strains and then inbred (brother-sister mating) for over fifty generations. Seven different RI strains resulted. The genes of interest were a set of alleles for which the B6 and BALB differ. The genome of each RI strain contained differing mixtures of the parental alleles. Phenotypic differences between the RI strains and parental strains provided a means to determine the genetic contribution to these differences. The conclusions from this study supported the findings of the above-mentioned paper. A large reservoir of genes exist that have size effects in localized regions of the mandible and that greater heritabilities usually apply to distances between adjacent landmarks.

Linking specific genes to specific phenotypic traits is needed to gain a clear understanding of the genetic influence on morphogenesis. Bailey, in 1986b, illustrated a technique using the same congenic strains from a previous study (Bailey) to more specifically identify the location of genes which elicit a specific change mandibular shape in mice. The congenic strains were derived from the B6 and BALB strains with a segment of chromosome 17 from the BALB strain introduced on the B6 genetic background. Measurement methodology utilizing the twelve mandibular landmarks and interlandmark distances was used in this study. The specific phenotypic trait analyzed was the elongation of the dorsal reach of the condylar process. One congenic strain carries a passenger gene that leads to lengthening of the dorsal reach of the condylar process as

does the H-2 congenic strain derived from it. A passenger gene is one that is carried on an introduced chromosomal segment along with other genes that affect other phenotypes. Two other independently created H-2 congenic strains do not carry the passenger gene as the previous strains did carry. This enables pinpointing of the location of the effective gene on the centromeric side of the H-2 complex on chromosome 17.

Another interesting study was done by Dohmoto et al. in 2002. It involved quantitative trait loci mapping for genes influencing mandibular size and used recombinant inbred mouse strains. The study focused on the identification of chromosome regions involved in regulation of anteroposterior length of the mandible as measured from menton and gonion. As mentioned earlier, it has been difficult to find relationships between craniofacial phenotypes and genotypes. The polygenic nature of inheritance in this area requires homozygous animal models, such as inbred mouse strains, as well as the ability to control environmental factors. The animal model used by Dohmoto et al. involved two parental strains, the SM/J and A/J. These strains were chosen due to the well-characterized differences in a variety of phenotypes, such as body weight. From these parental strains, twenty-one recombinant inbred strains were developed and used to analyze the genetic influence on mandibular length. A total of 230 mice, all adults, were used. Each animal was sacrificed, the soft tissue removed from the mandibles and measurement of mandibular length was taken. Mandibles were comparably large in the A/J parent strain and

small in the SM/J strain. The difference was significant, making these strains appropriate for cross-development and further QTL analysis. Measurements of all recombinant strain mandibles were done as well. These data were subjected to appropriate quantitative trait analysis. The results indicated that the major genes responsible for mandibular length were located on chromosomes 10 and 11 with some weak influence found in other chromosomes that could not be identified. The findings could then be applied to a heterozygous model (humans) where analysis would be focused on these two chromosomes when looking at the trait of mandibular length as described above. The ultimate goal is a prediction of mandibular size in a patient before the termination of growth of maxillofacial bones, thus influencing orthodontic or orthognathic treatment planning (Dohmoto et al.).

Before genetic analysis can be accomplished using different mouse strains, phenotypic differences must be demonstrated. Researchers at The Jackson Laboratory (Craniofacial Resource) studied the craniofacial characteristics of four inbred strains of mice. This was a dry skull study of the following strains: B6, C3H/HeSn, BALB/cByJ, and DBA/2J. Six males and six females aged eight weeks were used. Prior to skull preparation, the ear pinna length was measured. The soft tissue was removed using incomplete maceration in potassium hydroxide, stained with alizarin red and stored in undiluted glycerin. Using digital calipers, the following seven measurements were obtained from the dry skulls:

1. Total skull length (tip of nasal bone to back of occipital bone);
2. Nose length (nasal bone to coronal suture);
3. Skull height (lowest point of angular process of lower jaw to frontal bone);
4. Skull width (measured at the widest points of the parietal bone);
5. Inner canthal distance;
6. Upper jaw length;
7. Lower jaw length.

Their findings showed that only skull width significantly different among all four strains. The B6 and the C3H/HeSnJ were the most alike, differing in only half of their measurements. These two strains were the most different from the DBA/2J. When separated by sex, the B6 males and females were significantly different only in the ear pinna length.

Klingenberg et al., in 2000, analyzed mandibular shape in mice utilizing the procrustes superimposition technique. The sample size was 476 mice that were F2 progeny of a cross between the LG/J (large) and SM/J (small). The mice were sacrificed at seventy days of age and the skeletons were prepared by exposure to dermestid beetles, which resulted in defleshing. The mandibles were sectioned into right and left sides at the mandibular symphysis, and coordinates of the following five landmarks were used for shape analysis: (1) coronoid process, (2) molar alveolus, (3) incisor alveolus, (4) alveolar process,

and (5) angular process. The features of the mandibles that were evaluated were size, shape, and symmetry between right and left halves. After QTL analysis, it was determined that twelve QTL were located for size, twenty-five QTL for shape, and five QTL for symmetry. These gene loci were found on multiple chromosomes with varying degrees of influence. Therefore, when looking at overall size, shape, and symmetry of the mandible, polygenic patterns are apparent.

Using consomic mouse strains in genetic studies is a newer concept and little is present in the literature to illustrate this technique. However, Matin et al., in 1999, did a study analyzing the susceptibility to testicular germ-cell tumors (TGCTs) using a chromosome substitution strain. It was suspected that the development of bilateral TGCTs involved alleles on chromosome 19 from MOLF/Ei strains (Collin et al.). In order to obtain independent evidence for this linkage, a consomic strain in which chromosome 19 of 129/Sv+/+ strain was replaced by the homologous chromosome 19 from the MOLF/Ei strain. The resultant strain showed an unusually high frequency of TGCTs. With the confirmation of genetic contribution to increased susceptibility to TGCTs of chromosome 19 from the MOLF strain, further genetic mapping can be done to more specifically identify the genes involved from this chromosome. Youngren et al., in 2003, created a panel of congenic mouse strains derived from the 129.MOLF-CHr19 consomic strain and found that there were several genes located at distant sites on chromosome 19 that controlled the susceptibility to

these tumors. The above studies illustrate the power of chromosome substitution mouse strains in complex trait analysis.

In order to analyze phenotypic differences to ultimately determine genetic influences upon these differences, an appropriate animal model and method to quantify differences must be implemented. Consomic mouse strains, derived from parent strains with defined phenotypic differences, would provide a suitable animal model. Linear measurements, ratios involving linear measurements, and the procrustes superimposition technique are methods to determine such differences. One must select the appropriate method for quantification of trait differences for the given model utilized. The A/J and B6 mouse strains, along with a suitable measurement technique, may provide the first step in determining genetic influence on craniofacial morphology in this animal model and, ultimately, in humans.

STATEMENT OF PURPOSE

The purpose of this investigation is to determine if interstrain craniofacial shape differences are greater than intrastrain variations in the A/J and B6 mouse strains.

MATERIALS AND METHODS

Study Samples

Two strains of inbred mice were utilized in the present study, the A/J and B6. These strains were selected because chromosome substitution strains, developed from these two parental strains, were available from the genetics laboratory of Dr Joseph Nadeau at Case Western Reserve University. If craniofacial phenotypic differences could be demonstrated between these two strains, the chromosome substitution panel could then be used to determine which chromosomes contribute to the phenotype.

Gross visual inspection of a sample mouse from each strain suggested anatomical differences in headform. Therefore, the possibility of demonstrating additional craniofacial differences between the two strains was likely.

The mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) where they were bred according to standards for genetic purity. Strict environmental controls (diet, temperature, hours of daylight) were used to provide a standard environment. A total of twenty-four animals, six males and six females of each strain, were tested. Their ages ranged from forty-one to fifty-five days and were considered by the vendor to be skeletally mature. Prior

to experimentation or data collection, the protocol was submitted to, and approved by the Institutional Animal Care and Use Committee (Appendix A).

Upon arrival of the mice from The Jackson Laboratory, the animals were housed in room EB109 at the Animal Resource Center at the Case School of Medicine for approximately twenty-four hours. Here the animals were provided with food and water *ad libitum* in this controlled, uniform environment before sacrifice. The animals were then euthanized according to the recommendations of the Report of the American Veterinary Medical Association Panel on Euthanasia (Beaver) with carbon dioxide inhalation. Each animal was assigned an identification label and then placed into an individual container to insure that mice could be properly identified. Immediately after the animals were sacrificed, they were weighed using a digital scale (Denver Instrument Co., model TR-402, Denver, CO). The author was versed in all handling techniques via training from qualified veterinary technicians from the Animal Resource Center as well as the appropriate recommended online training.

Cephalometric Technique

A protocol described by Moullas was utilized for roetgensgraphic cephalometry. This offers a simple method to characterize craniofacial phenotypic variation.

Both lateral and dorsoventral cephalograms were obtained of each specimen. These films were taken using a modified cephalostat designed by Palmer (1978), the Broadbent-Bolton cephalometer (Series 1k550, Bolton Fund,

Cleveland, Ohio), and with standard occlusal sized ektaspeed Kodak radiographic film (CAT 1169143, size 4, Rochester, NY 14650). This technique insured reproducible object-film and source-film distances. The radiographic settings that proved to provide the highest quality images were 70 Kvp, 15ma, with two second exposure time. All films were processed using a DENT-X film processor (model 900WD, Elmsford, NY). The processing speed was set at six minutes.

In addition to the above radiographs, each animal had an additional film taken of both the right and left femurs. For this film, the animal had to be repositioned on the cephalostat to allow exposure of the femurs as flat as possible against the film. An elastic band was used to hold the femurs tightly against the film limiting any distortion of the image. The object-film and source-film distances were strictly maintained. Femur radiopgraphs were obtained as controls for animal size to be used in subsequent statistical analysis.

Prior to any data collection, the Baumrind method of registering four-corner fiducials was used on all of the radiographs (Baumrind and Frantz). A template designed by Moullas was used to punch the fiducial landmarks into the films. The fiducial points provided objective landmarks to compare accuracy of measurement procedures for multiple operators analyzing the same radiograph.

Data Collection

Each film was scanned using an Epson Expression 1600 scanner (Epson Corp., Tokyo, Japan) at 1600 dpi and 12 bit gray scale. All images were saved

as uncompressed (TIFF) files. This avoided any loss of image information during a compression process. The digitized films were displayed on a Dell Inspiron 8000 computer (Dell Computer Corp., Roundrock, TX) for landmark identification and recording of measurements.

Various anatomical landmarks were selected based on anatomic relevance and ease of identification on the digitized images. Six linear measurements and one ratio of two linear measurements were used to quantify dentofacial phenotypes for the A/J and B6 strains. Table 1 delineates the landmarks and measurements on dorsoventral (PA) cephalomentric radiographs.

TABLE 1.–PA Cephalometric Measures

Measurement	Measurement Defined
Zygomatic width	Most lateral point on the zygomatic process on the right to that on the left
Skull width	Most lateral point on the cranium on the right to that on the left.
Length-width ratio	Total skull length/zygomatic width. (Total skull length = occipital protuberance to most anterior point of maxillary incisors.)

Table 2 delineates the landmarks and measurement used on lateral cephalograms.

TABLE 2--Lateral Cephalometric Measures

Measurement	Measurement Defined
Premaxillary length	Junction of anterior molar and palate to supradentale (most inferior point on the alveolar process between the maxillary incisors).
Skull height	SH-point to junction of most distal molar and palate. (SH-point = clearly identifiable point on the superior aspect of the skull.)
Mandibular length	Most posterior point of angular process to infradentale (most superior point of the alveolar process between the lower incisors.
Maxillary tooth row length	Junction of most distal molar and palate to junction of most anterior molar and palate.

Figure 1 illustrates the configuration of the fiducial landmarks and how they were measured.

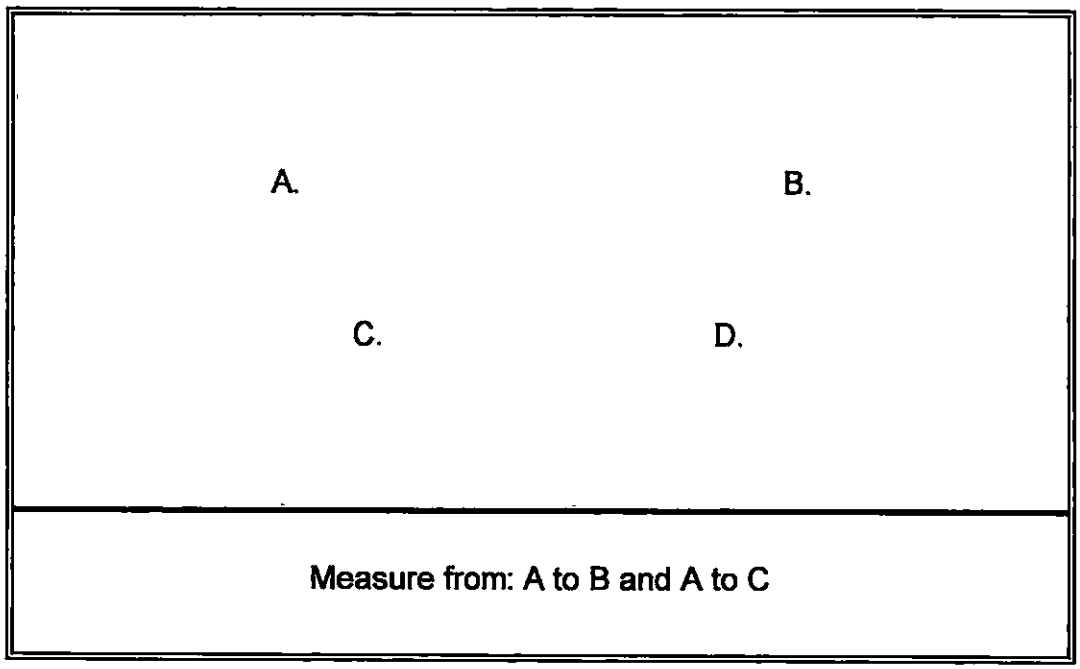


Figure 1. Configuration of fiducial landmarks.

All measurements were taken by one operator, the author, using Adobe Photoshop 7.0 image-handling software (Adobe Systems Incorporated, San Jose, CA) in a darkened environment. Typically, the images were enlarged on the computer monitor to the actual pixel size of the scanned image to insure accurate landmark identification. Also, the image brightness and/or contrast was adjusted as needed. All measurements were then duplicated one week later by the same operator under identical conditions.

Statistical Design

The study sample comprises four groups. Table 3 defines the sample.

TABLE 3.--Study Sample Design.

Group	Strain/Sex	Sample Size
1	A/J Female	6
2	B6 Female	6
3	A/J Male	6
4	B6 Male	6

Means and standard deviations were calculated for both anatomical and fiducial landmark measurements for each of the four groups for both data sets (T1 and T2 measurements). Calculation of the correlation coefficient was used to estimate intraoperator error. A high degree of correlation between the two separate measurements would indicate minimal random error, and thus, acceptable measurement technique.

The next step involved determining if statistically significant differences were present between the groups for the measured variables. Analysis of Variance (ANOVA) was used since four groups were tested. If the ANOVA was significant, a student's *t*-test was used to estimate differences between groups.

In order to control for animal size, all initial measurement data were normalized by dividing each value by the weight of the animal. A second

normalization was done by dividing all values by femur length. These normalized data sets were analyzed in the same manner as described above.

RESULTS

The results have been organized and will be presented in the following manner. First the initial raw data from both T1 and T2 data collections will be presented. Intraoperator error will be analyzed and presented based on the T1 and T2 data sets. Sample means and standard deviations along with statistical analysis via ANOVA, will then be presented. This will be followed by the appropriate paired *t*-test results based on the measurements taken at T1. The statistical tests will then be repeated with data normalized for both animal weight and femur length in order to control for animal size.

Craniofacial measurements were taken from digitized cephalograms and can be referred to in Appendix B. Each measurement was taken twice at two separate times to insure consistent landmark identification and measurement technique. Included with this data are measurements of fiducial landmarks also as a control for technique accuracy.

From this collection of data, the T1 and T2 measurements were compared to verify consistent measurement technique. Correlation coefficients (r) and coefficients of determination (r^2) were calculated from the two different time point data sets. Table 4 illustrates these results. Since the two data sets had such strong correlation, the remaining data analysis was based solely on

the T1 measurements. The results of this intraoperator error analysis show consistent technique in data collection with the methods utilized.

TABLE 4.--Intraoperator Error Determination.

INTRAOPERATOR ERROR		
Measurement	r	r^2
Skull width	0.986109	0.9724
Zygomatic width	0.972744	0.9462
Length-width ratio	0.993971	0.9880
Premaxillary length	0.986123	0.9724
Skull height	0.948457	0.8996
Mandibular length	0.965029	0.9313
Maxillary tooth row length	0.91740	0.8416
Femur length	0.968428	0.9379
Lateral ceph fiducial A-B	0.975794	0.9522
Lateral ceph fiducial A-C	0.903892	0.8170
PA ceph fiducial A-B	0.895553	0.8020
PA ceph fiducial A-C	0.961357	0.9242

The first step in data analysis involved the calculation of means and standard deviations for all of the T1 measurements. Table 5 provides this information for the four different study groups. Also included in this table are initial comparisons of these mean values for each of the variables. Because there are four means (four study groups) for each of the measurements to be compared, analysis of variance (ANOVA) was utilized to elicit the presence of any significant differences between any of the four groups. This analysis does not, however, specifically point out where the differences lie, but only that at least two of the groups are significantly different. Table 6 also provides ANOVA results for all measurements for the four test groups.

Six of the seven anatomical cephalometric measurements displayed significant differences at some level within the four groups. The only measurement that did not show statistical significance was for that of premaxillary length. The comparative significance levels for the measures that were indeed different were as follows: skull width > zygomatic width > skull height > maxillary tooth row length > mandibular length > length-width ratio.

The two size control measurements, weight and femur length, also showed significant differences within the four study groups with ANOVA. The fiducial landmark measurements, however, showed no significant differences on either lateral or dorsoventral cephalometric radiographs.

TABLE 5.--Raw Data Means, Standard Deviations, ANOVA Results

UNADJUSTED DATA					
Variable	Mean-SD				ANOVA
	A/J Females	B6 Females	A/J Males	B6 Males	
Skull width	10.08 ± 0.10	10.67 ± 0.15	10.23 ± 0.08	10.85 ± 0.08	1.22E-10*
Zygomatic width	11 80 ± 0.11	11.98 ± 0.08	12.10 ± 0.13	12.40 ± 0.06	1.12E-08*
Skull length	20.92 ± 0.35	20.03 ± 0.67	21.38 ± 0.30	20.80 ± 1.00	N/A
Length-width ratio	1.77 ± 0.02	1.67 ± 0.06	1.77 ± 0.01	1.68 ± 0 10	0.008*
Premaxillary length	5.45 ± 0.14	5.15 ± 0.19	5.63 ± 0.12	5.32 ± 0.60	0.107
Skull height	6.28 ± 0.10	6.63 ± 0.10	6.47 ± 0.05	6.80 ± 0.19	3.11E-06*
Mandibular length	10.3 ± 0.24	9.90 ± 0.33	10.67 ± 0.32	10.67 ± 0.41	0.002*
Maxillary tooth row length	3.30 ± 0.06	3.62 ± 0.25	3.35 ± 0.05	3.23 ± 0.12	0.001*
Femur length	11.3 ± 0.15	11.32 ± 0.30	12.03 ± 0.33	12.35 ± 0.29	1.73E-06*
Weight	17.93 ± 2.07	14.79 ± 0.45	22 63 ± 1.55	21.58 ± 2 60	1.28E-06*
Lat ceph fiducial A-B	60.12 ± 0.13	60.22 ± 0.12	59.97 ± 0.16	60.05 ± 0.26	0.126
Lat ceph fiducial C-D	40.93 ± 0.10	40.95 ± 0 12	40.90 ± 0.19	40.80 ± 0 11	0.253
PA ceph fiducial A-B	59.92 ± 0.16	59.88 ± 0 13	60.02 ± 0.13	59.90 ± 0.09	0 319
PA ceph fiducial C-D	40.93 ± 0.16	40.95 ± 0 08	40 93 ± 0.16	40 85 ± 0 18	0.663

* Statistically signficant difference

Then appropriate *t*-tests were done to discover whether mean differences were present intrastrain, interstrain, or both. Only the measurements that proved to have significant differences with ANOVA were subjected to *t*-tests. For those measurements, intrastrain (males vs. females of the same strain) and interstrain (same sex of one strain vs. same sex of the other strain) comparisons were carried out. The following tables show the results of these *t*-tests. It is apparent that both intrastrain (gender variation) and interstrain (strain variation) *t*-tests showed significant differences. Each measurement will now be presented comparing gender and strain variation.

The results for cephalometric measurements showed varied gender and strain differences. In terms of skull width, both gender and strain variation exist, with strain differences being more significant. Whereas for zygomatic width, gender and strain differences were also present; however, in this case, the gender variation was more significant than the strain variation. Skull height measurements showed greater strain variation than gender variation. Maxillary tooth row length showed some minor significant gender differences only with the B6 strain, as well as some minor strain variation only with the females of the two strains. Mandibular length measurement showed minor gender variation only with the B6 strain with no significant strain variation. The length-width ratio interestingly showed no significant gender variation but did reveal strain variation between the females.

The size control measurements of femur length and weight revealed some interesting differences as well. The femur length measurements showed only intrastrain gender variation to be significantly different with no strain variation. Weight measurements showed significant gender variation within the strains with only significant interstrain differences with the females. These size controls were then used to normalize the cephalometric measurement data separately. Tables 6 and 7 provide the results of the gender comparison for the A.J and B6 strains.

TABLE 6.– Unadjusted Gender Variation, A/J Strain.

UNADJUSTED INTRASTRAIN (GENDER) A/J STRAIN			
	Mean-SD		
Variable	A/J Females	A/J Males	*t*-test
Skull width	10.08 ± 0.10	10.23 ± 0.08	0.017*
Zygomatic width	11.80 ± 0.11	12.10 ± 0.13	0.001*
Length-width ratio	1 77 ± 0.02	1.77 ± 0.01	0.644
Premaxillary length	5.45 ± 0.14	5.63 ± 0.12	N/A
Skull height	6.28 ± 0.10	6.47 ± 0.05	0.012*
Mandibular length	10.30 ± 0.24	10.67 ± 0.32	0.063
Maxillary tooth row length	3.30 ± 0.06	3.35 ± 0.05	0.076
Femur length	11.30 ± 0 15	12.03 ± 0 33	0.003*
Weight	17.93 ± 2.07	22.63 ±1.55	0.003*

* Statistically significant difference.

TABLE 7.--Unadjusted Gender Variation, B6 Strain.

UNADJUSTED INTRASTRAIN (GENDER) B6 STRAIN			
	Mean-SD		
Variable	B6 Females	B6 Males	*t*-test
Skull width	10.67 ± 0.15	10.85 ± 0.08	0.0280*
Zygomatic width	11.98 ± 0.08	12.40 ± 0.06	0.0003*
Length-width ratio	1.67 ± 0.06	1.68 ± 0.10	0.9240
Premaxillary length	5.15 ± 0.19	5.32 ± 0.60	N/A
Skull height	6.63 ± 0.10	6.80 ± 0.19	0.1410
Mandibular length	9.90 ± 0.33	10.67 ± 0.41	0.0360*
Maxillary tooth row length	3.62 ± 0.25	3.23 ± 0.12	0.0280*
Femur length	11.32 ± 0.30	12.35 ± 0.29	0.0050*
Weight	14.79 ± 0.45	21.58 ± 2.60	0.0020*

* Statistically significant difference

Tables 8 and 9 present interstrain comparisons of the unadjusted data.

TABLE 8.--Interstrain Variation, Females A/J and B6.

UNADJUSTED INTERSTRAIN - A/J FEMALES VS. B6 FEMALES			
	Mean-SD		
Variable	A/J Females	B6 Females	t-test
Skull width	10.08 ± 0.10	10.67 ± 0.15	0.0002*
Zygomatic width	11 80 ± 0 11	11 98 ± 0 08	0 0280*
Premaxillary length	5.45 ± 0.14	5.15 ± 0.19	N/A
Skull height	6.28 ± 0 10	6 63 ± 0 10	0 0020*
Mandibular length	10.30 ± 0.24	9.90 ± 0.33	0.0540
Maxillary tooth row length	3 30 ± 0.06	3 62 ± 0.25	0 0390*
Femur length	11.30 ± 0.15	11.32 ± 0.30	0.1690
Weight	17 93 ± 2.07	14 79 ± 0 45	0 3860

* Statistically significant difference

TABLE 9.--Interstrain Variation, Males A/J and B6.

UNADJUSTED INTERSTRAIN - A/J MALES VS. B6 MALES			
	Mean-SD		
Variable	A/J Males	B6 Males	t-test
Skull width	10.23 ± 0.08	10.85 ± 0.08	9.3E-05*
Zygomatic width	12 10 ± 0 13	12 40 ± 0 06	0 003*
Premaxillary length	5.63 ± 0.12	5.32 ± 0.60	N/A
Skull height	6 47 ± 0.05	6 60 ± 0 19	0 009*
Mandibular length	10.67 ± 0.32	10.67 ± 0.41	1
Maxillary tooth row length	3 35 ± 0.05	3.23 ± 0 12	0 084
Femur length	12.03 ± 0.33	12.35 ± 0.29	0.913
Weight	22 83 ± 1 55	21 58 ± 2 60	0 013*

* Statistically significant difference.

A data was normalized by weight measurement, and all statistical analysis was completed with the normalized data. Individual weight measurements for the entire sample of both strains can be found in Appendix B. The weights for the individual animals shows some obvious variance, particularly for the A/J females. The data normalized by weight was then subjected to analysis of variance. These results are found in Table 10. All measurements showed significant differences within the four groups. The comparative significance levels are as follows: pre-maxillary length > maxillary tooth row length > skull height > skull width > mandibular length > zygomatic width.

TABLE 10.--ANOVA Results for Weight Normalized Data.

DATA ADJUSTED BY WEIGHT					
	Mean-SD				
Variable	A/J Females	B6 Females	A/J Males	B6 Males	ANOVA
Skull width	0.568 ± 0.058	0.722 ± 0.021	0.454 ± 0.03	0.510 ± 0.07	4.43E-08*
Zygomatic width	0.665 ± 0.070	0 810 ± 0.020	0.540 ± 0.03	0.580 ± 0.08	2.24E-07*
Premaxillary length	0.310 ± 0.030	0.350 ± 0.010	0.250 ± 0.01	0.250 ± 0.01	2.73E-09*
Skull height	0.350 ± 0.040	0.450 ± 0.009	0.290 ± 0.02	0.320 ± 0 04	2.56E-08*
Mandibular length	0.580 ± 0.060	0.670 ± 0.020	0.470 ± 0.02	0.500 ± 0.05	1.92E-07*
Maxillary tooth row length	0 190 ± 0 020	0.240 ± 0.020	0.140 ± 0.01	0.150 ± 0.02	1.08E-08*

* Statistically significant difference

The appropriate *t*-tests were run from the ANOVA results. Tables 11 through 14 provide this information. The weight normalized data reveals a different picture than the results of the *t*-tests from the data that was not normalized. The intrastrain gender differences were significant for all anatomical cephalometric measurements, whereas the interstrain differences were significant for six of the seven measurements for the females and none for the males. The *t*-test results for these measurements will now be presented.

The statistical differences with the weight normalized data were relatively consistent for the various measurements with strong gender variation and strain differences with the females only. Skull width, zygomatic width, premaxillary length, skull height, mandibular length, and maxillary tooth row measures all showed gender differences for both strains and strain variation with females only.

TABLE 11.–Weight Normalized Gender Variation, A/J Strain.

ADJUSTED BY WEIGHT INTRASTRAIN - A/J STRAIN			
	Mean-SD		
Variable	A/J Females	A/J Males	*t*-test
Skull width	0.568 ± 0.058	0.454 ± 0.030	0.004*
Zygomatic width	0 665 ± 0.070	0 537 ± 0 032	0 006
Premaxillary length	0.307 ± 0.028	0.250 ± 0.013	0.003*
Skull height	0 354 ± 0.036	0 287 ± 0 019	0 005*
Mandibular length	0.580 ± 0.057	0.472 ± 0.025	0.005*
Maxillary tooth row length	0 186 ± 0 018	0 149 ± 0 009	0 004*

* Statistically significant difference

TABLE 12.--Weight Normalized Gender Variation, B6 Strain.

ADJUSTED BY WEIGHT INTRASTRAIN - B6 STRAIN			
	Mean-SD		
Variable	B6 Females	B6 Males	*t*-test
Skull width	0.721 ± 0.021	0.510 ± 0.068	0.0002*
Zygomatic width	0.811 ± 0.021	0.582 ± 0.077	0.0002*
Premaxillary length	0.348 ± 0.010	0.247 ± 0.012	0.0001*
Skull height	0.449 ± 0.009	0.319 ± 0.038	0.0007*
Mandibular length	0.669 ± 0.019	0.499 ± 0.048	0.0005*
Maxillary tooth row length	0.245 ± 0.021	0.152 ± 0.018	0.0005*

* Statistcally significant difference

TABLE 13.--Weight Normalized Strain Variation, Females.

ADJUSTED BY WEIGHT INTERSTRAIN - FEMALES			
	Mean-SD		
Variable	A/J Females	B6 Females	*t*-test
Skull width	0.568 ± 0.058	0.722 ± 0.021	0.002*
Zygomatic width	0 665 ± 0.070	0.811 ± 0.021	0 004*
Premaxillary length	0.307 ± 0.028	0.348 ± 0.010	0.032*
Skull height	0.354 ± 0.036	0 449 ± 0.009	0.002*
Mandibular length	0.580 ± 0.057	0.669 ± 0.019	0.024*
Maxillary tooth row length	0.186 ± 0.018	0 245 ± 0.021	0.003*

* Statistically significant difference.

TABLE 14.-- Weight Normalized Strain Variation, Males.

ADJUSTED BY WEIGHT INTERSTRAIN - MALES			
	Mean-SD		
Variable	A/J Males	B6 Males	*t*-test
Skull width	0.454 ± 0.030	0.510 ± 0.068	0.078
Zygomatic width	0.537 ± 0.032	0.582 ± 0.077	0.173
Premaxillary length	0.250 ± 0.013	0.247 ± 0.012	0.758
Skull height	0.287 ± 0.019	0.319 ± 0.038	0.084
Mandibular length	0.473 ± 0.025	0.499 ± 0.048	0.306
Maxillary tooth row length	0.149 ± 0.009	0.152 ± 0.018	0.661

* Statistically significant difference

The data normalized by femur length was also subjected to the same statistical analysis as the above data sets. The results for the analysis of variance test for this data are presented in Table 15. This data shows significant differences somewhere within the four groups with all six of the individual adjusted measurements (skull length was not analyzed as it is considered in the length-width ratio). The comparative significance levels are as follows: maxillary tooth row length > skull width > skull height > zygomatic width > pre-maxillary length > mandibular length.

TABLE 15.– ANOVA Results for Femur Length Normalized Data.

DATA ADJUSTED BY FEMUR LENGTH					
Variable	Mean-SD				ANOVA
	A/J Females	B6 Females	A/J Males	B6 Males	
Skull width	0.89 ± 0.010	0.94 ± 0.030	0.85 ± 0.020	0.88 ± 0.020	5.74E-06*
Zygomatic width	1.04 ± 0.010	1.06 ± 0.030	1 01 ± 0.020	1.00 ± 0.020	0.000635*
Premaxillary length	0.48 ± 0.008	0.46 ± 0.010	0.47 ± 0.007	0.43 ± 0 .040	0.006600*
Skull height	0.56 ± 0.007	0.59 ± 0.013	0 54 ± 0.013	0.55 ± 0 .020	3 7E-05*
Mandibular length	0.91 ± 0.014	0.87 ± 0.020	0.89 ± 0.310	0.86 ± 0.023	0.011600*
Maxillary tooth row length	0.29 ± 0.003	0.32 ± 0.017	0/28 ± 0.007	0.26 ± 0.007	3 43E-08*

* Statistically significant difference

Intrastrain gender and interstrain *t*-tests were then done to determine the specific areas of difference within the four test groups. Tables 16 through 20 provide the intrastrain and interstrain *t*-test results for the femur length normalized data. These results proved to be quite varied with respect to intrastrain and interstrain differences.

The maxillary tooth row length measurement shows both gender and strain variation. The most significant difference was the strain variation with the male animals, and the least significant was with the females of different strains.

Only one measurement resulted in gender variation alone and no significant differences between strains. The zygomatic width proved to have the

most significant intrastrain gender variation with the A/J strain when compared to the B6 strain. The skull width and skull height measurements illustrate significant gender variation with both strains and interstrain differences with the females of the two strains only.

TABLE 16.–Femur Normalized Gender Variation, A/J Strain.

ADJUSTED BY FEMUR LENGTH INTRASTRAIN - A/J STRAIN			
	Mean-SD		
Variable	A/J Females	A/J Males	*t*-test
Skull width	0.892 ± 0.011	0.851 ± 0.020	0.014*
Zygomatic width	1.044 ± 0.013	1.006 ± 0.019	0.009*
Premaxillary length	0.482 ± 0.008	0.468 ± 0.007	0.058
Skull height	0.556 ± 0.007	0.538 ± 0.013	0.023*
Mandibular length	0.911 ± 0.014	0.887 ± 0.031	0.173
Maxillary tooth row length	0.292 ± 0.003	0.279 ± 0.007	0.009*

* Statistically significant difference.

TABLE 17.--Femur Normalized Gender Variation, B6 Strain.

ADJUSTED BY FEMUR LENGTH INTRASTRAIN - B6 STRAIN			
	Mean-SD		
Variable	B6 Females	B6 Males	*t*-test
Skull width	0.943 ± 0.033	0.879 ± 0.019	0.0240*
Zygomatic width	1.060 ± 0.031	1.005 ± 0.024	0.0470*
Premaxillary length	0.455 ± 0.012	0.430 ± 0.044	0.2550
Skull height	0.586 ± 0.013	0.551 ± 0.018	0.0320*
Mandibular length	0.875 ± 0.021	0.864 ± 0.023	0.4120
Maxillary tooth row length	0.319 ± 0.017	0.262 ± 0.007	0 0003*

* Statistically significant difference

TABLE 18.--Femur Normalized Strain Variation, Females.

ADJUSTED BY FEMUR LENGTH INTERSTRAIN - FEMALES			
	Mean-SD		
Variable	A/J Females	B6 Females	*t*-test
Skull width	0.892 ± 0.011	0.943 ± 0.033	0.024*
Zygomatic width	1.044 ± 0.013	0 060 ± 0.031	0.395
Skull height	0.556 ± 0.007	0.586 ± 0.013	0.011*
Maxillary tooth row length	0.292 ± 0.003	0.319 ± 0.017	0.011*

* Statistically significant difference.

TABLE 19.--Femur Normalized Strain Variation, Males.

ADJUSTED BY FEMUR LENGTH INTERSTRAIN - MALES			
	Mean-SD		
Variable	A/J Males	B6 Males	*t*-test
Skull width	0.851 ± 0.020	0.879 ± 0.019	0.0530
Zygomatic width	1 006 ± 0.019	1.005 ± 0.024	0.9190
Skull height	0.538 ± 0.013	0.551 ± 0.018	0.2420
Maxillary tooth row length	0.279 ± 0.007	0.262 ± 0.007	0 0002*

* Statistically significant difference

Overall, there were only three measurements that revealed no significant gender variation and were as follows: the length-width ratio, femur length adjusted pre-maxillary length, and femur length adjusted mandibular length. Therefore, the males and females were combined for these measurements, and a combined interstrain *t*-test was done. These results are presented in Table 20. The interstrain variation was significant for all three of these measurements.

TABLE 20.--Combined Interstrain *t*-test Results.

COMBINED INTERSTRAIN *T*-TEST			
	Mean-SD		
Variable	A/J	B6	*t*-test
Length-width ratio	1.770 ± 0.019	1.675 ± 0.078	0.003*
Premaxillary length adjusted by femur length)	0 475 ± 0 010	0 443 ± 0.033	0.003*
Mandibular length (adjusted by femur length)	0.899 ± 0.026	0.869 ± 0.022	0.005*

* Statistically significant difference

DISCUSSION

This study was intended to evaluate cephalometric measures of two laboratory mouse strains as a first step in an ongoing genetic study of facial development at the Department of Orthodontics at Case Western Reserve University. Intrastrain and interstrain variations in craniofacial measurements were assessed in an effort to ascertain the appropriateness of utilizing this animal model in future studies involving genetic dissection of the phenotype, craniofacial morphology.

The A/J and B6 inbred mouse strains used in this study have many well-documented phenotypic differences in anatomical and physiological traits (DeSanctis et al.; Gill et al.; Han et al.; Hoit et al.; Jepsen et al.; Liu and Erickson; Nadeau et al.; Nesbitt and Skamene; Surwit et al.). In addition, the findings of this study illustrated differences in the anatomical phenotype of craniofacial form. From these differences, the variables that illicit greater strain (interstrain) than gender (interstrain) variation provide the best measures to analyze this phenotype in genetic studies using this model.

Data from the unadjusted results showed the following variables to have greater strain variation:

Females - skull width,
zygomatic width, and
maxillary tooth row length

Males - skull width,
zygomatic width, and
skull height

The length-width ratio showed no significant gender differences and resulted in a combined gender *t*-test between strains that proved to have a high level of statistically significant variation as well. When using unadjusted data, the above variables would be appropriate measures to use to quantify differences in subsequent genetic studies involving crosses of these two strains.

Another data set from this study involved normalization of the measures by femur length. Based on a previous study (Bailey, 1985), it was determined that genes affecting longbones, such as the femur, did not affect mandibular shape. Intuitively, this would suggest that the use of longbones for normalization of data to control for size would be appropriate, at least with regard to the mandible. However, if different genes affect different areas of the craniofacial complex, there could be some relationship between the genes that affect longbones and those that affect craniofacial structures other than the mandible. This remains to be determined. Nonetheless, an interesting dichotomy resulted from this data set. The variables that resulted in significant interstrain variation was different for males compared with females when considering both the A/J and B6. For the females, interstrain variation was significant for the skull width, skull height, and maxillary tooth row length; whereas for the males, only the

maxillary tooth row variable resulted in significant interstrain differences. Two other variables showed interstrain differences when running a combined *t*-test (both males and females), the pre-maxillary length and the mandibular length. Thus, in a protocol that utilizes femur-adjusted data, these resultant variable differences should be considered when using this animal model.

As mentioned above, the femur-length adjusted data was quite different when looking at interstrain variation. The female data showed significant differences for three of the variables, whereas the male data showed only one variable different. One possible explanation involves the femur measurements themselves. Interstrain differences were not present with respect to femur measurements. The mean femur lengths for A/J and B6 females were 11.30 and 11.32 respectively, with the males being 12.03 and 12.35 respectively. Even though there were no statistically significant differences in these femur length measurements, the male values did differ by greater than 0.3 mm on average. Consequently, with the measurement differences in all the variables being relatively small to begin with, these femur-length differences with the males seem to have affected the presence of interstrain variation more with males than with females. Females were affected less, as their mean femur lengths were much closer. The male femur lengths were not significantly different when comparing the two strains initially. This was due to a higher level of variance in the initial measures, possibly a result of radiographic technique. Although great effort was taken to insure minimal radiographic distortion with

femur films, some may have occurred and, therefore, affected the measurements. Thus, interstrain differences with femur-adjusted data that did not show up in the males could, in fact, be present to some degree.

The weight normalized data resulted in a much different picture. Only females of the A/J strain had any variables that showed more significant interstrain variation. In this study, it appears that adjusting the data by weight is less appropriate due to the tremendous variation in weight within each of the study groups. This variation within the groups would greatly affect the reliability of the data and should be used with reservation. Given that the ages within the groups were identical and the environmental conditions were identical, one would expect less variation within each group. And with skeletal maturity equivalent within each group, this would greatly affect the data when considering skeletal measures. One explanation for such weight variation could be the effect of litter size on the early development of newborn mice.

In this study, an attempt was made to identify the youngest practical age in which to do phenotypic studies with the A/J and B6 strains. The average age for the females was forty-two days and the males, fifty-three days. Skeletal maturation was expected at forty-two to fifty-six days according to the vendor (The Jackson Laboratory, Bar Harbor, Maine). The literature suggests sixty days for adulthood with some studies using seventy (Klingenberg et al.) to ninety (Dohmoto et al.) day-old mice. It is possible that comparing mice ranging in ages as those in this study (forty-two to fifty-three days) may not be

appropriate. Another phenotypic study (Craniofacial Resource) showed that when comparing B6 males and females at fifty-six days of age, there were no significant differences when taking craniometric measurements of their skulls. The present study did show gender differences with the B6 strain which contradicts the above finding. Thus, when analyzing the comparisons between mice of forty-two days of age and those of fifty-three days, caution must be exercised. Comparing mice of like ages would, in fact, be appropriate unless one determined the exact age of skeletal maturation. If this were determined, any age after skeletal maturity would be appropriate. The results of this study did not clearly determine the age of skeletal maturity, or for that matter, the ideal age for future studies. However, the mice in the present study might, in fact, be of equal skeletal maturity, but this cannot be stated unequivocally. A comprehensive study, with age as a primary variable with multiple study groups, should be done to ascertain the youngest appropriate age for future studies.

Therefore, because of the age differences between the male and female groups in this study, one must be careful in analyzing intrastrain gender differences. Unadjusted and weight adjusted data seem inappropriate when looking at gender differences in the present study. However, adjustment by femur length would seem to account for age differences by dividing each variable by an age-appropriate skeletal measurement. Also, whenever a ratio of two measures is used, this would also account for age and skeletal

maturation differences. Again, this must be considered in future studies using consomic strains.

Phenotypic difference in craniofacial form has clearly been demonstrated in the present study between the A/J and B6 mouse strains. However, the next challenge involves understanding the contribution of genetics to this phenotype. Human cephalometric studies (Harris et al. 1973; Hunter et al.) have shown definite familial aggregation with respect to craniofacial measures. These studies also hint that complex inheritance patterns involve polygenes for these traits. Bailey and Klingenberg also demonstrated polygenic inheritance with individual genes influencing localized regions of the mandible. These studies provide a look into potential findings of future studies with the A/J and B6 mouse strains and the consomic strains derived from them. When looking at the craniofacial complex in its entirety, one would certainly expect influences from several chromosomes.

Even more specific detail was uncovered in three other studies regarding specific genetic contribution that, again, foreshadows the next step in this ongoing study at Case. Bailey, in 1986b, looked specifically at genetic influence in the elongation of the dorsal reach of the condylar process in mouse mandibles. He pinpointed the location of the gene that affects this phenotype to the centromeric side of the H-2 complex present on chromosome 17. In addition to this chromosome, Bailey, in 1985, also showed influences of chromosomes 3, 4, 5, 7, 8, and 9 on mandibular-shape changes in mice.

Dohmoto et al. looked at genetic influences on length of the mouse mandible. They found the major gene influences on chromosomes 10 and 11. Based on the literature, one would expect the above chromosomes to influence specific mandibular measures in a study of consomic strains.

Another factor to consider when analyzing genetic influence in the ontogeny of the craniofacial complex in mice is that of the mode of gene action. Do the genes act directly on the bone forming cells and tissues? Or, do they act on other tissues in juxtaposition to the bones and they just respond to the growth of these surrounding tissues? Melvin Moss' functional matrix theory certainly suggests the latter. He feels that it is possible that genes affect bone shape and size, not by acting on the bones directly, but on associated muscular, nervous, or vascular tissues. He also feels that functional spaces, such as those for respiration, are fundamental to his concepts. Nonetheless, even in Moss' paradigm, genes play a significant, if indirect, role in development of the craniofacial complex, though indirect.

The methods in the present study were designed to establish a simple, cost effective, as well as accurate technique to characterize phenotypic traits in mouse craniofacial morphology. The use of cephalometrics and inbred mouse strains provides a vehicle to accomplish this but has limitations. Problems including imprecise identification of cephalometric landmarks, as well as the two dimensional nature of cephalometric measurement, is well-known (Baumrind and Franz). However, the selection of appropriate anatomical landmarks that

can be predictably and accurately identified provided an acceptable intraoperator error level in this study. Certainly craniometric measurements can also provide accurate measurements in craniofacial studies (Moullas) but are significantly more time-consuming and costly in an extensive study involving large numbers of animals. The use of inbred mice also helps to limit type I error (Belknap) so that a more stringent alpha level is not needed, as $p < 0.05$ was utilized in the present study. These principles provided an acceptable method to quantify the phenotypic differences between the A/J and B6 inbred mouse strains.

Growth modification treatments in orthodontics have been well-documented (Keeling et al.; Tulloch; Ghafari et al.; Liviertos and Johnston; Johnston) but remain controversial. This study is the first step in an ongoing genetic study to gain insight into the role genetics play in craniofacial development. By better understanding which chromosomes and genes contribute to specific growth of the maxillofacial complex, it may be possible to develop better, more efficient, and more stable treatment results for skeletal disorders. Phenotypic differences demonstrated in the A/J and B6 mouse strains can now lead to consomic strain studies to identify which chromosomes are involved in the craniofacial ontogeny. More specific gene mapping can then follow once chromosomes are identified.

Future Studies

The immediate next step following the present study involves phenotypic analysis of the twenty-one consomic mouse strains derived from the A/J and B6 strains. The presented methods would be appropriate to undertake these comparisons. Once chromosomes involved in this phenotype are uncovered, specific gene mapping can be more readily accomplished with isolation of quantitative trait loci.

Other possible studies could involve different methods to quantify phenotypic differences in the chromosome substitution panel. As mapping for more gross anatomical craniofacial phenotypes are completed, more specific studies should be done to identify local genetic influences of different regions of individual bones of the facial complex. Bailey, in 1985 and 1986b, and Klingenberg, in 2000, showed genetic influence in localized regions of mouse mandibles suggesting more detailed breakdown of genetic influence is indicated. This would likely require even more accurate methods to quantify some possibly very subtle differences. Conventional cephalometrics, as described in the present study, may be too crude to accomplish such intricate detail. One possible solution would be the utilization of micro CT scanning technology. This idea is presently being explored at Case and the Cleveland Clinic, and could provide the means to analyze these mouse strains in the near future. This would certainly require the supporting computer software to

assimilate the data derived from CT scanned images. The author is not aware of such a program at the time of this writing.

SUMMARY AND CONCLUSIONS

The primary purpose of this investigation was to analyze craniofacial phenotype between two laboratory mouse strains, A/J and B6, and to determine if craniofacial shape differences were greater between the two different strains than between mice of the same strain.

Lateral and dorsoventral cephalometric radiographs were taken of four groups made up of twenty-four total animals, twelve of each strain (six males, six females). In addition, radiographs were also taken of the femur of each animal to be used as a size control. These films were then digitized, and seven measurements were taken based on fourteen anatomical landmarks. One of these measurements was a ratio of two different linear measures. All results were subjected to statistical analysis using ANOVA and student *t*-tests to determine where significant differences lie. Analysis of the data revealed that:

1. Significant interstrain differences were present between the A/J and B6 strains for some but not all of the variables tested. They are the following:

    a. Unadjusted data:

        Females: skull width,
        zygomatic width, and
        maxillary tooth row length

Males: skull width,
zygomatic width, and
skull height

b. Femur adjusted data:

Females: skull height

Males: maxillary tooth row length.

2. When combining males and females of each strain, the most appropriate measures to quantify interstrain variation are the following:

a. Length-width ratio: total skull length/zygomatic width.

b. Premaxillary length (adjusted by femur length).

c. Mandibular length (adjusted by femur length).

3. Adjusting for weight or femur length amplified interstrain variation in females.

4. In contrast, adjusting for weight or femur length masked interstrain variation in males.

5. Adjusting for weight or femur length amplified intrastrain gender variation, perhaps due to the age difference between male and female mice in this sample.

6. The youngest appropriate animal age for future genetic studies of this phenotype could not be specifically determined based on the results of this investigation.

7. The methodology utilized in this study is appropriate and sufficiently reliable to quantify these seven phenotypic measures.

8. Consomic strains derived from the A/J and B6 mouse strains would be an appropriate animal model for further genetic studies of this phenotype.

APPENDIX A

CWRU IACUC
WG-77 School of Medicine
(216) 368-3815

Date: OCT 11 2002

Species: mice

Protocol Number: 020145

Designated or Full Review: RC /MJ

Notice Of Intent To Use Vertebrate Animals

Form Date 8/2002

I. Contact Information

Principal Investigator: **Mark G. Hans**
Project Title: **Genetic Dissection of Craniofacial Morphological Traits**
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External Funding Source:	**AAOF**		Or Internal Funding Source†:		**N/A**
New Submission?	**No**		Or Renewal of Protocol Number:		**960157**

* This individual will be contacted when animal morbidity or welfare requires immediate action.
† Internally funded projects must receive an independent review of scientific merit. A form for submission of internal scientific reviews is here.

II. Animal Use Summary

List the total number of animals of each species required for the entire protocol: **252 mice**

Type of use: x Research Teaching Other (specify):

Check all that apply to the use of animals in this submission

Breeding	Survival (Chronic) Study	Chemical Mutagenesis
Tissue Collection	Transgenic/KO Production	Infectious Agents
Genetic Mapping	Viral Therapeutics	Surgery
Nutritional Studies	Protein/DNA Therapeutics	Multiple Surgeries
Aging	Drug Therapeutics	Inducement of Disease State
Exercise Physiology	Nude Mouse Implants	Inducement of Stress
Behavioral Tests	Neuromuscular Blockade	Prolonged Restraint
Antibody Production	Ascites Production	Tumor Implants

X Primary Reviewer	Title: Prof Chair	Signature: Ron Cohn	Date: 10/23/02
Veterinary Reviewer: MARK JAMES	Title: CLINICAL VETERINARIAN	Signature: Mark James	Date: 10/23/02
IACUC Executive	Title: Chair	Signature: Vanne Lemmon	Date: 10/25/02

Terminal (Acute) Study Irradiation Cell Implants
Other (specify): **All animals will be sacrificed**

III. Investigator Assurances

- I agree to abide by the policies of the CWRU Institutional Animal Care and Use Committee (IACUC) and all applicable federal regulations
- I will adhere to the protocol as described and as modified
- I will submit any modifications of the protocol to the IACUC for review.
- I will notify the IACUC of changes in the location of the animal research
- I will assist the IACUC in verifying compliance with the regulations
- I will notify the IACUC of any unexpected results that affect the welfare of the animals I will report any unanticipated pain or distress, morbidity or mortality to the attending veterinarian and the IACUC.
- I understand and agree that emergency veterinary care including euthanasia will be administered to animals exhibiting unbearable pain, distress or illness. An effort to contact me or my representative (the animal emergency contact identified on page 1) will be made by the veterinary staff prior to any emergency treatment.
- I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All other personnel involved in animal use in this project have been or will be trained in proper procedures in animal handling, administration of anesthetics and analgesics, aseptic technique, post-operative monitoring, and euthanasia.
- I declare that the information provided in this application is accurate. If this project is to be funded by extramural source(s), I certify that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal.
- I declare that the studies described here do not unnecessarily duplicate previous work by myself or others.

Signature of Principal Investigator **Date** 10/10/02

APPENDIX B

TABLE 19.--Raw Data for B6 Females.

Measurement (mm)	**BF-1**	**BF-2**	**BF-3**	**BF-4**	**BF-5**	**BF-6**	
Skull width	10.6	10.5	10.6	10.9	10.8	10.6	T1
	10.7	10.5	10.6	10.9	10.7	10.6	T2
Zygomatic width	12.0	11.9	11.9	12.1	12.0	12.0	T1
	12.1	11.8	11.9	12.1	12.0	12.0	T2
Length-width ratio	1.7083	1.7143	1.7311	1.6364	1.5667	1.675	T1
	1.7025	1.7288	1.7311	1.6364	1.5667	1.675	T2
Premaxillary length	5.2	5.1	5.2	5.3	4.8	5.3	T1
	5 1	5.0	5.1	5.3	4.8	5.3	T2
Skull height	6.7	6.5	6.7	6.7	6.5	6.7	T1
	6.7	6.6	6.7	6.7	6.6	6.6	T2
Mandibular length	9.9	9.9	9.9	10.1	9.3	10.3	T1
	9.8	10.0	10.1	10.1	9.2	10.3	T2
Maxillary tooth row	3.4	3.9	3.9	3.4	3.4	3.7	T1
	3.4	3.9	3.9	3 4	3.4	3.6	T2
Femur length	11.1	11.3	11.7	11.3	10.9	11.6	T1
	11.2	11.3	11.9	11.4	10.9	11.6	T2
Lateral ceph fiducial A-B	60.2	60.1	60.4	60.2	60.3	60.1	T1
	60.3	60.1	60.4	60.2	60.3	60.0	T2
Lateral ceph fiducial A-C	41.0	41.1	41.0	40.8	41.0	40.8	T1
	41.0	41.1	41.0	40.9	41.0	40.9	T2
PA ceph fiducial A-B	59.8	59.8	59.8	59.8	60.1	60.0	T1
	59.7	59.8	59.9	59.8	60.1	60.1	T2
PA ceph fiducial A-C	41.1	40.9	40.9	40.9	41.0	40.9	T1
	41.1	40.9	40.9	40.9	41.0	40.8	T2

TABLE 20.--Raw Data for B6 Males.

Measurement (mm)	BM-1	BM-2	BM-3	BM-4	BM-5	BM-6	
Skull width	10.7	10.9	10.8	10.9	10.9	10.9	T1
	10.7	10.9	10.8	11	10.9	10.8	T2
Zygomatic width	12.3	12.4	12.5	12.4	12.4	12.4	T1
	12.2	12.3	12.5	12.3	12.4	12.3	T2
Length-width ratio	1.6748	1.7097	1.656	1.7661	1.7581	1.5	T1
	1.6885	1.7317	1.664	1.7805	1.7742	1.5122	T2
Premaxillary length	5.4	5.6	5.5	5.6	5.7	4.1	T1
	5.4	5.6	5.5	5.6	5.6	4.1	T2
Skull height	6.6	6.9	7.1	6.8	6.8	6.6	T1
	6.6	6.9	7.0	6.9	6.8	6.6	T2
Mandibular length	10.9	10.8	10.5	11.0	10.9	9.9	T1
	10.7	10.6	10.4	11.0	10.8	10	T2
Maxillary tooth row	3.1	3.3	3.1	3.3	3.4	3.2	T1
	3.2	3.2	3.1	3.3	3.3	3.4	T2
Femur length	12.2	12.3	12.2	12.8	12.6	12	T1
	12.2	12.2	12.3	12.9	12.6	11.9	T2
Lateral ceph fiducial A-B	60.1	59.8	60.5	60.1	59.8	60.0	T1
	60.0	59.8	60.5	60.1	59.9	60.0	T2
Lateral ceph fiducial A-C	40.8	40.8	40.9	40.9	40.8	40.6	T1
	40.7	40.7	40.9	40.8	40.8	40.7	T2
PA ceph fiducial A-B	59.9	60.0	59.8	59.8	59.9	60.0	T1
	59.9	60.1	59.8	59.9	59.9	60.0	T2
PA ceph fiducial A-C	40.6	40.8	40.8	41.0	40.8	41.1	T1
	40.6	40.8	40.7	41.0	40.8	41.1	T2

TABLE 21.--Raw Data for A/J Females.

Measurement (mm)	AF-1	AF-2	AF-3	AF-4	AF-5	AF-6	
Skull width	10.1	10.0	10.2	10.2	10.0	10.0	T1
	10.1	10.0	10.2	10.3	10.1	10.0	T2
Zygomatic width	11.7	11.9	11.9	11.9	11.7	11.7	T1
	11.7	11.8	11.9	11.9	11.6	11.6	T2
Length-width ratio	1.7863	1.7542	1.7479	1.8151	1.7607	1.7692	T1
	1.7863	1.7479	1.7479	1.8235	1.7759	1.7645	T2
Premaxillary length	5.4	5.4	5.5	5.7	5.3	5.4	T1
	5.4	5.4	5.4	5.7	5.4	5.4	T2
Skull height	6.2	6.4	6.3	6.4	6.2	6.2	T1
	6.4	6.4	6.4	6.5	6.2	6.2	T2
Mandibular length	10.4	10.0	10.2	10.7	10.3	10.2	T1
	10.2	10.2	10.1	10.7	10.2	10.3	T2
Maxillary tooth row	3.3	3.3	3.3	3.4	3.3	3.2	T1
	3.3	3.2	3.2	3.5	3.2	3.3	T2
Femur length	11.3	11.3	11.2	11.6	11.2	11.2	T1
	11.4	11.3	11.3	11.7	11.3	11.1	T2
Lateral ceph fiducial A-B	60.1	60.3	60.1	60.2	59.9	60.1	T1
	60.1	60.3	60.1	60.2	59.9	60.1	T2
Lateral ceph fiducial A-C	41.1	40.9	40.8	40.9	41.0	40.9	T1
	41.1	40.9	40.7	40.9	40.9	40.9	T2
PA ceph fiducial A-B	59.8	59.9	59.8	59.8	60.2	60.0	T1
	59.9	59.9	59.7	59.8	60.1	60.0	T2
PA ceph fiducial A-C	40.7	41.1	41.1	40.9	40.8	41.0	T1
	40.7	41.0	41.1	40.9	40.9	41.0	T2

TABLE 22.--Raw Data for A/J Males.

Measurement (mm)	**AM-1**	**AM-2**	**AM-3**	**AM-4**	**AM-5**	**AM-6**	
Skull width	10.3	10.3	10.2	10.3	10.1	10.2	T1
	10.3	10.3	10.3	10.3	10.2	10.2	T2
Zygomatic width	12.2	12.2	12.2	12.1	11.9	12.0	T1
	12.2	12.2	12.2	12.0	11.9	12.0	T2
Length-width ratio	1.7869	1.7623	1.7623	1.7603	1.7563	1.775	T1
	1.7869	1.7705	1.7623	1.775	1.7563	1.775	T2
Premaxillary length	5.8	5.7	5.7	5.6	5.5	5.5	T1
	5.9	5.7	5.7	5.6	5.6	5.6	T2
Skull height	6.5	6.5	6.5	6.4	6.4	6.5	T1
	6.6	6.5	6.5	6.4	6.3	6.5	T2
Mandibular length	10.5	10.9	10.9	10.9	10.7	10.1	T1
	10.5	10.7	10.8	10.8	10.5	10.1	T2
Maxillary tooth row	3.3	3.4	3.4	3.4	3.3	3.3	T1
	3.4	3.4	3.4	3.4	3.4	3.3	T2
Femur length	12.4	11.9	12.4	12.1	11.6	11.8	T1
	12.4	12.0	12.4	11.6	11.6	11.8	T2
Latteral ceph fiducial A-B	59.8	60.1	60.2	59.9	60.0	59.8	T1
	59.8	60.1	60.2	59.9	60.0	59.8	T2
Lateral ceph fiducial A-C	41.1	40.6	41.1	40.8	40.9	40.9	T1
	41.1	40.6	41.1	40.8	40.9	41.0	T2
PA ceph fiducial A-B	59.9	59.9	60.1	60.1	59.9	60.2	T1
	59.9	59.9	60.1	60.2	59.8	60.2	T2
PA ceph fiducial A-C	40.7	40.8	41.1	41.0	41.1	40.9	T1
	40.7	40.8	41.1	41.0	41.1	40.9	T2

TABLE 25.--Animal Weights for Sample Groups.

A/J Females		**B6-Females**		**A/J Males**		**B6-Males**	
AF-1	18.56	BF-1	15.02	AM-1	23.06	BM-1	20.44
AF-2	17.14	BF-2	14.19	AM-2	23.97	BM-2	22.23
AF-3	18.76	BF-3	14.54	AM-3	24.68	BM-3	21.52
AF-4	21.30	BF-4	15.06	AM-4	21.92	BM-4	23.64
AF-5	15.70	BF-5	14.53	AM-5	21.56	BM-5	24.48
AF-6	16.10	BF-6	15.41	AM-6	20.58	BM-6	17.16

APPENDIX C

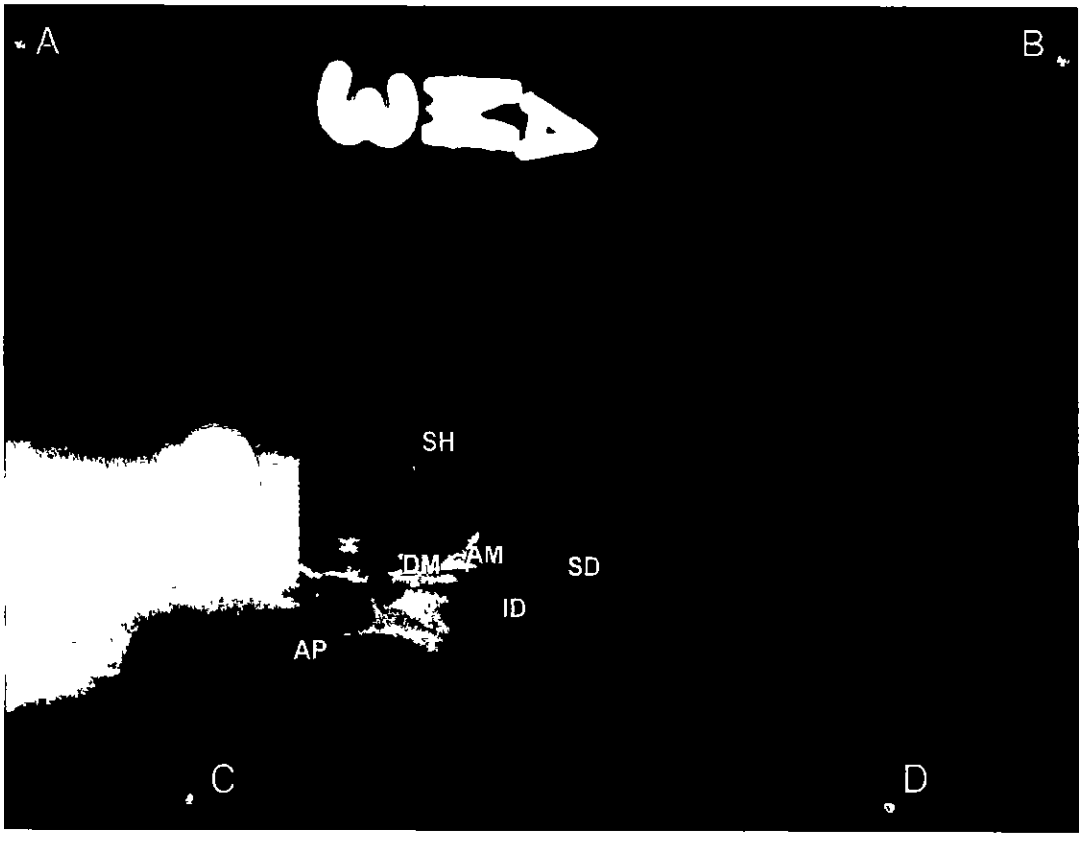


Figure 2. Landmarks for lateral cephalometric film:

SH – skull height point.
AM – junction of anterior molar and palate.
DM – junction of distal molar and palate.
SD – supradentale (most inferior point on the alveolar process between the maxillary incisors).
ID – infradentale (most superior point of the alveolar process between the mandibular incisors).
AP – most posterior point of the angular process of the mandible.
A,B,C,D – fiducial landmarks.

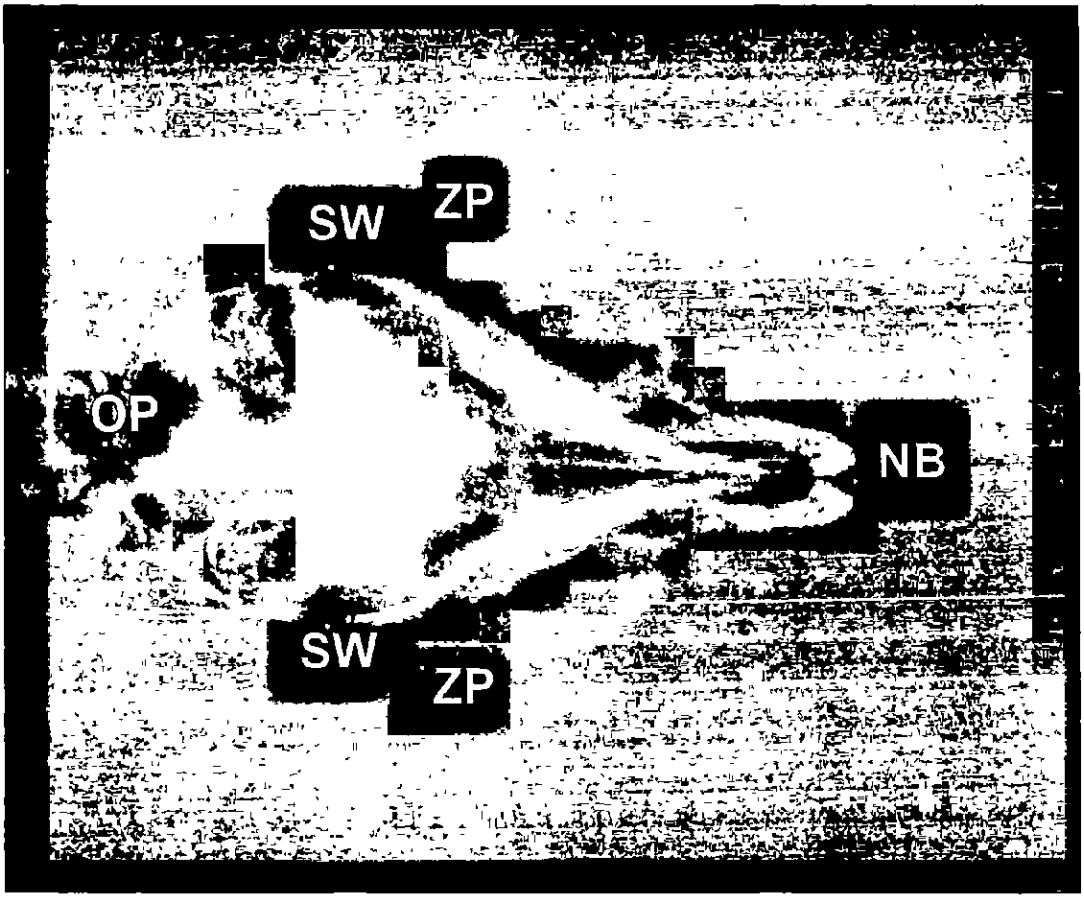


Figure 3. Landmarks for dorsoventral cephalometric film:

SW - most lateral point on the cranium on the right and left, used to measure skull width.
ZP - most lateral point on the zygomatic process on the right and left, used to measure zygomatic width.
OP - occipital process.
NB - most anterior point of maxillary incisors (landmark representing the tip of the nasal bone).

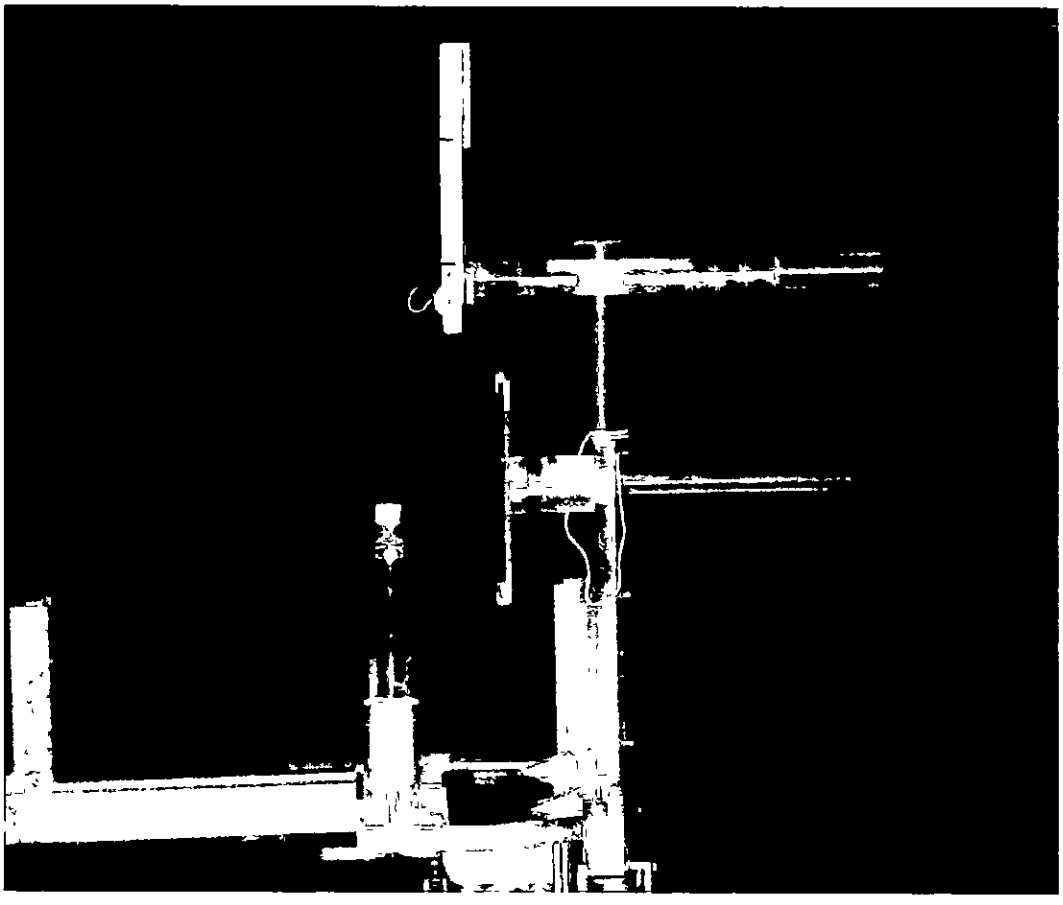


Figure 4. Cephalometer.

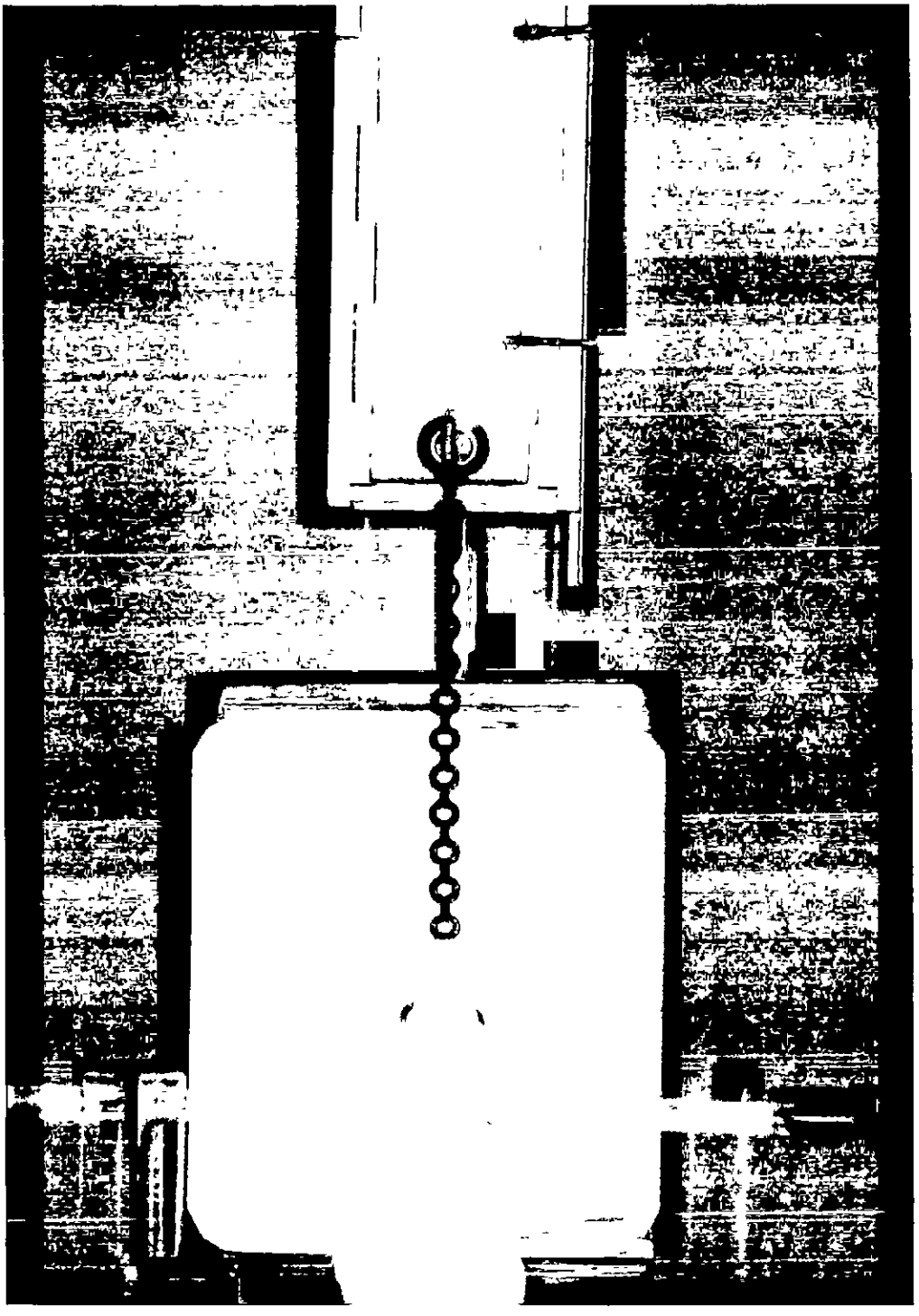


Figure 5. Cephalometric positioning.

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