MORPHOMETRIC ANALYSES OF BONE AND CARTILAGE OF THE EQUINE METACARPOPHALANGEAL JOINT

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This work is dedicated to my Father and his Mother

for my desire to succeed intellectually

and

to my Mother and her Uncle

for my love of animals

Also dedicated to the Horse, a noble and graceful yet powerful creature

who often judges Man better than Man judges himself

ABSTRACT

The studies that comprise this dissertation were developed to learn more about the nature of the bone and cartilage of the equine metacarpophalangeal joint. Joint disease is a common problem in the clinical realm of veterinary medicine, especially when dealing exclusively with horses. Although a significant amount of research has been conducted on the equine musculoskeletal system, most of it has dealt specifically with cortical bone or hyaline cartilage. It seems remarkably remiss that little has been done with the structure connecting the two anatomically, the trabecular bone. The results of these diverse studies have shed some light on the articular and subarticular environment.

In the first study, a population of horses was sought that would not have evidence of joint disease and could therefore be considered 'normal'. Although this objective was not completely realised, nevertheless a population with an excellent distribution across ages, breeds, genders and weights was the result.

In the bone histomorphometry work, classic stereological procedures of point counting (P_P) and intersect counting (P_L) were employed using a computer. The results of this work indicated that the amount of bone (B.Ar) and the size of its component parts (Tb.Wi) were highly variable within this population. Also, it was determined that the bone seems to respond to some stimulus outside the parameters tested in this study, that is age, breed, gender and weight. The most likely causative factor for the stimulus for increased bone production in this region would be mechanical stress.

In the cartilage experiments, several interesting relationships were discovered. The calcified cartilage (CC) was easily identifiable and variable in its thickness. Possibly the most important observation was how this layer seems to respond to growth and maturity. The CC layer in the young horse was much less substantial than that in a mature horse. Also of significance in this study is that the CC/TC ratio varied in relation to the location within the joint and between horses. This finding is contrary to what has been reported in the human literature.

The subchondral bone work has provided objective data about this obscure layer. It is often cited as a potential site for disease in the horse, and yet little was known about its normal properties. Although quite variable in its morphometry as well for this population, the SCB thicknesses were documented and will serve as reference values in the horse.

Finally, in the fractal study, the equine bone was considered to possess a fractal dimension within the limits of our study, i.e. digitised bone specimens. Although the fractal dimension did not relate specifically to the other bone parameters in this study, fractal geometry still holds promise for determining these relationships between healthy and diseased bone.

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MJ Martinelli

DECLARATION

I declare that this thesis describes work carried out by me, except or those matters mentioned specifically in the acknowledgements. It has not been submitted in any form for another degree or professional qualification.

MJ MARTINELLI

Parts of this thesis have been accepted for publication or presentation elsewhere.

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List of Abbreviations

B.Ar	**Bone Area**
BNF	**Buffered Neutral Formalin**
CC	**Calcified Cartilage**
DJD	**Degenerative Joint Disease**
HC	**Hyaline Cartilage (also listed as TC)**
IRU	**Increased Radiopharmaceutical Uptake**
MDP	**Methylene Diphosphonate**
MMA	**Methylmethacrylate**
NC	**Noncalcified Cartilage**
OA	**Osteoarthritis**
PMMA	**Polymethylmethacrylate**
P_L	**Points per Line**
P_P	**Points per Area**
SCB	**Subchondral Bone**
Tb.N	**Trabecular Number**
Tb.S	**Trabecular Separation**
Tb.Wi	**Trabecular Width**
TB	**Thoroughbred**
TBx	**Thoroughbred Cross**
Tc	**Technetium**
TC	**Total Cartilage (also listed as HC)**
TM	**Tidemark**

'TO MEASURE IS TO KNOW'

Lord Kelvin, University of Glasgow c1890

CHAPTER 1

INTRODUCTION

The equine industry has recognised the importance of the locomotory system when choosing topics to write about in educational journals, to investigate clinically and theoretically and even to support with funding to carry out such research. Due to this concentrated effort to uncover more information about the equine musculoskeletal system over the last 10 years, significant progress has been made in several key areas. These have been most notably on the surgical side of equine orthopaedics, with the introduction and sophistication of arthroscopic techniques as well as the advances in principles of fracture repair. The latter has been revolutionised by the development of new and superior implants, better and faster surgical techniques, and most recently, by the use of more effective antimicrobial protection, both systemically and as an invited guest to the actual fracture site in the form of antibiotic-impregnated methylmethacrylate. The introduction of therapeutic bone cement has provided more thorough control of infection locally, which, in turn, results in improved outcomes because of quicker bone healing times. **(Holcombe *et al* 1997)**

Although arthroscopy was first reported in the horse in 1949, it was a Japanese reference and the procedure was carried out by a human orthopaedic surgeon intent on visualising a large animal joint prior to attempting the same procedure in a human joint **(McIlwraith 1990)**. In the American veterinary literature, the arthroscope made its debut in 1975, mentioned by **Hall and Keeran** as a possible diagnostic tool for occult joint disease. At that time, the authors were private practitioners in Michigan who were looking for a better way to diagnose articular disorders. They cited cases when joint involvement was suspected on the basis of the physical examination, lameness examination and response to intra-articular anaesthetics, but where no corresponding radiographical lesions were present. Although those authors were able to characterise possibly one of the most important uses for the arthroscope, it still took almost 10 years for it to become accepted as a *surgical tool,* and probably at least another five after that to become well recognised as a useful *diagnostic* tool in equine surgery. The surgical uses

include such applications as removing traumatic bone chips or osteochondral fragments, repairing selected fractures, debriding cartilage lesions or debriding and lavaging septic joints. The diagnostic application of arthroscopy very closely parallels that first American publication in 1975 and is most pertinent to the subject of this dissertation, i.e. early detection of joint disease that appears radiographically silent. Over the last 5-10 years, this area seems to have shown the most growth, especially in the performance horse field **(McIlwraith 1996).**

In the diagnostic arena, there is no doubt that the recent advances in the technology of imaging have represented major improvements in the early detection of articular derangements. Radiology has advanced into the digital age with the development of computed radiography (CR) and direct radiography (DR). Both of these systems allow immediate computer enhancement of the image. Similarly, ultrasonography will produce a digital signal immediately upon entry into the console. The result of this technology is better image quality resulting in better diagnostic potential. New equipment, however, usually does result in a lag time between its introduction and a complete understanding of the technology and what it can do. Nuclear scintigraphy is an excellent example of such an evolution. Although originally introduced into the equine imaging realm in the late 70's almost simultaneously by three geographically separated individuals; Twardock in Illinois, Ueltschi in Switzerland and Attenburrow in England, it has taken almost two decades to become completely accepted and assimilated into the spectrum of equine lameness diagnostics. In view of all it has to offer in the detection of aberrant or increased bone remodelling, and even in the detection of some subtle soft tissue lesions in horses, it is incredulous that some clinicians still feel compelled to argue its pros and cons. One would hope that the aforementioned advances in radiology and ultrasound meet with more rapid acceptance because their diagnostic capabilities should revolutionise the current imaging standards. Although computed tomography (CT) has been available for use in horses for years, the veterinary community continues to gain more access to these machines through improved equipment design **(Barbee et al 1987)**. In contrast, magnetic resonance imaging (MRI) has not been feasible in live horses

until recently as coils and magnets have opened up as well as become portable **(Martinelli 1995)**.

While all of these advancements are providing more clues toward solving some of the mysteries surrounding the pathogenesis of joint disease, we are still far from having all the answers, especially in the clinical setting. Although now already over ten years old, a classic editorial by **Dyson (1987)** summarises this frustration from the clinical perspective. Joint disease is considered by her to be a common *clinical* problem in the horse, yet the *clinical* diagnosis becomes difficult for many reasons. The biggest question that still remains concerns the issue of pain. What causes the pain and from which structures does it emanate? In the human field, in spite of the sometimes-enviable ability of the clinician to be able to communicate verbally with the patient, these same questions remain unanswered **(Oddis 1996)**. Similar to Dyson's lament about the lack of correlation between radiographs and clinical signs of pain, **Dieppe (1990)** recognised this in human patients as well. He called for the cause of symptoms to be explored separately from the causes of radiographic change, historically the yardstick for determining the presence and degree of osteoarthritis (OA) in all species. Most importantly, however, it should be remembered that pain is a clinical finding, not a pathological one. Several clinicians in both the human and veterinary field have implicated 'bone pain' as a possible cause of the discomfort experienced by those afflicted with OA. **Oddis (1996)** cites ischaemia or pressure in the subchondral bone (SCB) as possible causes of pain, while **Kraus (1997)** adds subchondral microfracture as another possible cause of the bone pain. In the equine field, **Ross (1991) and Martinelli (1994)** have postulated that SCB pain may be responsible for some lamenesses in performance horses, especially in light of a positive bone scan and nondiagnostic radiographs.

The key to prevention seems to be firmly embedded within the need to make an early diagnosis . The author believes that the key to early diagnosis may lie with the determination of the causes and effects of bone remodelling and pain. Very

little has been published in regard to equine SCB, especially its relationship to the initiation and progression of OA **(Norrdin *et al* 1998, Martinelli *et al* 1996).** As a result, we felt that it was important to investigate the calcified tissues beneath the superficial layers of hyaline cartilage. Therefore, the intent of this dissertation was to carry out investigations on the calcified cartilage, SCB plate and deeper trabecular structure of the cancellous bone of the distal epiphysis of the third metacarpus in the equine forelimb.

CHAPTER 2

GENERAL BACKGROUND AND SIGNIFICANCE

Lameness is one of the most common problems leading to loss of use in the performance horse. A pre-eminent study performed on the British racing industry over 15 years ago found that lameness was the number one cause of wastage in the racehorse industry. **(Rossdale *et al* 1985)** A more recent survey carried out among practitioners in the North of England and Scotland indicated that the most common reason for veterinary treatment was also lameness. **(Mellor *et al* 1997).**

Clinical Lameness

Lameness is an all-encompassing term and should be defined more specifically. Yet over the years of private practice and referral work, the author has come to develop a very broad definition for the benefit of clients, veterinary students and referring practitioners. It is as follows: "Lameness is the inability to perform the intended task due to a musculoskeletal abnormality". The basis for this definition is that most horses are presented for veterinary examination because the owner perceives that there is some problem with the horse. In some cases, the complaint cites an obvious abnormality such as the inability to bear weight on a limb or a severely swollen tendon. In such cases, a full physical examination should still be carried out to make sure no other abnormalities exist and no systemic disturbance is the cause. More often than not nowadays, especially in the referral environment, the actual lameness is not necessarily obvious to the owner. Rather, they perceive that the horse is not performing properly. Specific questioning during the history often reveals that the abnormality has usually had an insidious onset rather than an injury-related one. All too often, the veterinary profession is very quick to dismiss subtle changes in performance, willingness or even demeanour as problems for the trainer, a better rider, or even a behavioural specialist.

It is the author's contention, however, that *any* kind of problem that can be alleviated with regional anaesthesia of some sort is more likely to have its roots in physical discomfort than improper behaviour or as the result of poor training. This is not to say that these types of problems do not exist, but it should be the duty of the performance horse veterinarian to attempt to rule out soreness as the cause of decreased or aberrant performance before abandoning the problem as one not of the veterinary domain. This should be carried out in the following manner.

History

The successful lameness diagnostician uses many methods to gather the information necessary for arriving at an accurate diagnosis. Probably the most important aspect of the examination is the history. Proper and effective history taking is more of an art than a science, and therefore is not easily learned by the veterinary student or new graduate. Again, in this situation a thorough knowledge of the horse industry and an intimate association with the horse and client is very useful. Many lamenesses can be diagnosed or nearly diagnosed by simply knowing how to ask and interpret the information properly.

Clinical Examination

Physical Examination

The next step would be the actual examination. As part of every equine lameness examination, a thorough general physical examination should be carried out. This should become routine and rehearsed for every animal seen by the veterinarian, meaning that the physical should be carried out in much the same way for every horse in order to be as consistent as possible. It is important to begin the physical examination by standing back and observing the entire horse from the front, side and back. Any significant swellings, scars, or asymmetry are noted and recorded.

Next, an in-depth palpation is carried out for each leg, first in the weight-bearing position, and then with the leg flexed and in the non-weight-bearing position. Each of the joints and soft tissue structures is palpated for heat, effusion, pain, or signs of resistance to manipulation.

Lameness Examination

Once the horse is thoroughly examined at rest, the gait analysis aspect can begin. The horse is examined at a variety of gaits, on a variety of surfaces. In many cases it is most advantageous to have the owner or trainer put the horse through its paces, especially if the proper facilities are available. In this way, the veterinarian is often able to see firsthand what the client is experiencing. The horse is most frequently walked and trotted both in the straightaway, and on the lunge line in a circle if possible. It may be cantered on the lunge line as well if the environment allows. Following the initial lameness examination, flexion tests of the joints may be carried out to try an exacerbate a more subtle lameness. Routinely the flexion tests are carried out as a generic full leg flexion, carpal flexion, hock/stifle flexion, and/or and digit flexion.

Regional Anaesthesia

After the lameness has been identified in a limb or multiple limbs, it is usually appropriate to proceed with regional anaesthesia in order to localise the lesion within that limb. In the case of a suspected fracture, however, such a clinical progression is contra-indicated. When performed though, regional anaesthesia can be carried out in one of two ways: In the first approach the nerves can be desensitised in a systematic manner commencing with the most distal nerves (palmar digital) and proceeding proximally until the lameness is abolished. The other approach would be to proceed directly to intra-articular anaesthesia. In this way, local anaesthetic is deposited directly into the joint suspected of

causing the lameness. The difference between the two techniques is in their specificity. Intra-articular anaesthesia is considered very specific in that if the lameness resolves directly after depositing the local anaesthetic into the joint, then the clinician can be confident that the lameness definitely has an articular component. To the level of our current knowledge, this usually means lesions affecting the synovium or subchondral bone (**Keegan *et al* 1996, Bowker *et al*. 1993).** Also with intra-articular anaesthesia, the clinician has more freedom to proceed in any order since the joints do not have to be desensitised in any particular sequence. Nerve blocks, on the other hand, must be carried out in a systematic fashion in order to be as exclusive as possible. This approach tends to be more time consuming in situations other than foot lamenesses, however, and there also tends to be a greater inherent risk to the horse with intra-articular anaesthesia since a joint infection can be catastrophic. If the cause of the lameness is thought to be articular in nature, especially if there is joint effusion or pain associated with flexion, then it is often best to proceed directly to intra-articular anaesthesia.

Diagnostic Imaging

Once the lameness is localised via regional anaesthesia, then it is usually appropriate to carry out diagnostic imaging. Radiology is still the most common procedure. It is an anatomical imaging modality that is used primarily to diagnose bone and joint pathology. It is most commonly used in the equine clinical setting to document OA, chip fractures and long bone fractures. Ultrasonography is another anatomical imaging modality that is helpful in determining the presence and severity of soft tissue injuries. These injuries would most often include damage to the tendons and ligaments. Fluoroscopy is also an anatomically based technology that is similar to radiology in that it is used to image bone and joints, however, it portrays the tissues in real-time. Fluoroscopy is most effective at evaluating subtle changes to the articular environment. As a general rule, the anatomical imaging modalities tend to be specific in conveying a diagnosis, but not quite as sensitive at detecting pathology. In fact, many clinicians in both the

equine and human medical fields consider radiology to be insensitive when attempting to diagnose articular disease, Often because there is a disparity between radiographic signs of OA and patient reported symptoms **(Oddis 1996, Krause 1997, Radin 1995). Watt (1987)** states that much of the problem lies with the fact that radiology is a 2D modality attempting to image a 3D object such as bone, while **Dieppe (1990)** dismisses radiographs as a 'historical anatomical record of what has happened to the joint'. In the veterinary field, **Dyson (1987)** has made some of the same observations regarding the clinical insensitivity of radiology. When lesions are detected radiographically, however, especially in young horses, they were found to have an adverse effect on racing performance **(Grondahl and Engeland 1995, Gaustad *et al* 1996).** It is also worth reiterating that the advent and acceptance of computer-enhanced radiography is likely to remedy much of these shortcomings.

Nuclear Scintigraphy

Nuclear scintigraphy, on the other hand, is a metabolic imaging, much different than radiology. For this reason, nuclear scintigraphy is considered to be much more sensitive but not as specific **(Watt 1987)**. It relies on blood flow to soft tissues and on the inherent remodelling of bone to produce an image. For most musculoskeletal imaging, a radiopharmaceutical with bone seeking properties is employed which works in the following way. A camera is used to detect gamma radiation. It consists of a number of photomultiplier tubes, 55 in the Omega 500, housed in the head of the camera. Gamma radiation enters the face of the camera and passes through a focusing grid that will reject much of the incident and randomly oriented particles. The rest will pass through to strike the NaI crystal below. The interaction of the gamma particles with the NaI crystal produces photons that are then captured by the photomultiplier tubes located beneath the crystal. The photons are multiplied in the tubes, as the name would imply, and an electrical signal is generated. The signal is passed through electronic boards that initially amplify it even further, and then assign it spacial properties. From the head of the camera, the signal is transmitted to the console where it takes on

further spacial properties before exiting the console in x, y and z leads. The x and y characteristics deal with orientation while the z component represents signal intensity. The leads are attached to a recording device that will form a picture of the imaged area. Originally, special formatting cameras were used to print the images on film, but recent innovations in computer technology allow the signal to feed directly into a computer. This, of course, produces superior images while also allowing for easier post-acquisition image manipulation.

Radiopharmaceutical Preparation. Technetium 99m is a metastable breakdown product of Molybdenum (Mo). The half-life of Tc99m is relatively short, lasting only 6 hours. This makes it ideal for medical imaging applications in that it is rapidly eliminated from the system. To create a suitable compound for musculoskeletal imaging, the radionuclide is labelled with a bone-seeking agent, usually methylene diphosphonate (MDP), to produce the radiopharmaceutical agent, Tc99m-MDP.

Imaging Process. The radiopharmaceutical agent is injected intravenously and the horse is placed in front of the gamma camera for registration of the radionuclide. There are three distinct phases of equine nuclear scintigraphy, all separated by time as well as by the physiological principle being imaged (**Chambers *et al* 1995**).

The first phase is the vascular phase. This may not be used very commonly, but it highlights vascular activity to the tissues. For this phase, the horse is usually placed in front of the camera prior to injecting the radiopharmaceutical agent. The region of interest is positioned in the middle of the camera, usually attempting to get the opposite limb in the same field. A dynamic image is obtained once the radiopharmaceutical is injected. In this phase, the radiopharmaceutical is still present in the blood vessels, so vascularity is imaged.

The second phase is the soft tissue or pool phase. It is so named because the radiopharmaceutical agent pools in the extra-cellular spaces and soft tissues. A recent publication in the human literature states that an increase in radiopharmaceutical uptake in the extracellular fluid spaces may be caused as it traverses blood vessels with increased endothelial spaces due to angiogenesis or inflammation **(McCarthy 1997)**. This phase is usually carried out up to 30 minutes post-injection.

The final phase of equine musculoskeletal scintigraphy, and probably the most useful, is the bone phase. It is performed 2-3 hours after injection. This is the amount of time it usually takes for the radiopharmaceutical agent to clear from the soft tissues and only be present in bone. By definition, bone uptake of the MDP-labelled Tc99m is due to MDP binding to exposed hydroxyapatite crystals of remodelling bone. With increased remodelling, a greater amount of Tc99m-MDP is bound to exposed hydroxyapatite crystals, leading to the formation of a "hotspot" **(Christensen 1985)**.

There are many possible reasons that a region will show up with a hotspot in the bone phase of equine musculoskeletal scintigraphy. For ease of discussion, these regions have been divided in the following way: incidental, enthesis uptake and strict bone remodelling

Incidental- there are some areas of uptake in the equine skeleton that do not appear to be associated with a lesion although it most certainly is associated with an alteration in bone remodelling. One such example involves growth plate uptake in young animals. The bone remodelling associated with growth in these animals would be considered normal **(Christensen 1981)**. Another example of an increased radiopharmaceutical uptake (IRU) that might be considered normal would be associated with a region of bone that is more superficial than the

surrounding skeleton. An example is sometimes found in the lumbar spine, where one of the vertebrae may be more superficial than the surrounding ones and therefore "hotter". There are several other regions and patterns of uptake that are often considered normal and incidental, but may merit more clinical investigation before their significance is dismissed.

Enthesis uptake- although this is a relatively new concept to the equine field, there are several patterns of IRU that appear to be associated with the attachment of soft tissue structures to bone. The most classic example is high suspensory disease, but includes other conditions such as avulsion of the muscles of the third trochanter of the femur and a similar lesion at the site of attachment of the biceps to the radius. Most importantly there appears to be a condition in the foot that mimics navicular disease in its presentation and diagnosis, but seems to be related to the attachment of the deep digital flexor tendon (**Martinelli 1998).** Of greatest significance in regard to these syndromes is that diagnosing them without scintigraphy would be difficult or impossible.

Strict Bone Remodelling- this category is undoubtedly the reason that scintigraphy is so popular as an imaging modality for horses. The classic example would be a stress fracture in the long bone of a racehorse. Such a lesion may be difficult to detect in the average case, especially when few clinical signs are present after a race. It is not uncommon that a trainer or groom has noticed just a few bad steps after a race and presents the horse for examination solely for that reason. Detection with scintigraphy will often lead to appropriate rest or surgery prior to catastrophic fracture, this saving the horse's career and possibly its life **(Martinelli and Chambers 1995)**.

It is articular uptake in this category that ties the discussion back to the subject of the dissertation. None of the radiopharmaceutical agents currently employed in the clinical setting will bind to and, therefore image, articular cartilage (**Grennan 1987**). Yet a high percentage of the lesions detected by scintigraphy involve an

IRU in close vicinity to an articulation. Radiographical findings are non-existent more often than not, therefore leaving the clinician with a somewhat abstract diagnosis of 'lameness characterised by increased radiopharmaceutical uptake in the subchondral region and peri-articular tissues'. Although a relatively frequent occurrence in the performance horse environment, nonetheless, it is an unsettling diagnostic thought process to relate back to a client. Although radiographs have not highlighted an obvious lesion associated with the IRU, a bone lesion is the most likely suspect due to the physiology of the MDP and the time of image acquisition. It is this author's contention and clinical impression after 7 years of clinical experience with equine nuclear scintigraphy, that such IRU is likely to be related to subchondral bone damage that otherwise goes undetected. Once a lameness has been identified in a limb through the physical examination, lameness examination and response to regional anaesthesia, it is not uncommon to see the IRU in the absence of radiographic signs of disease **(Fig 2.1 and 2.2).**

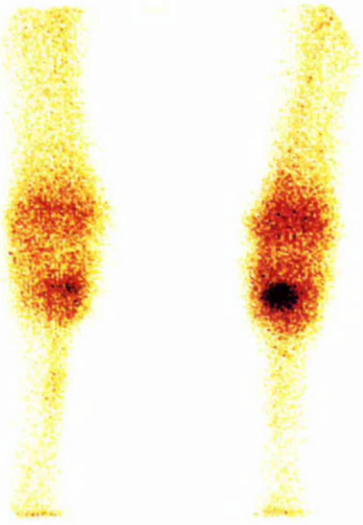


Fig 2.1 Focal and intense IRU associated with the radiocarpal/third carpal bone in a horse. Dorsal view.

Fig 2.2 Corresponding radiograph with no visible lesion. Lateral view.

Advances in the Human Field

OA is a common ailment among human patients with approximately 20 million symptomatic cases in the US **(Altman 1997)**. It is well recognised that OA is difficult to measure, researchers and clinicians often relying on clinical signs to determine the presence and severity of the disease **(Huskisson 1987)**. Only about half of those with symptoms will have radiographic signs, although imaging is becoming more sophisticated **(Altman 1997).** However, most osteoarthritic changes observed radiographically are qualitative, making them very difficult to quantify **(Watt 1987).** Furthermore, radiographic changes are considered to be simply an historical anatomical record of what has happened to the joint at some previous point in time. For this reason, some clinicians believe that radiology has become an insensitive assessment of subtle changes associated with OA, such as focal osteophytosis and bone sclerosis **(Dieppe 1990).** Some authors believe that bone scintigraphy provides the single most sensitive means of assessing degenerative arthritis in the knee because it can provide information about the early course of OA **(Watt 1987, Grennan 1987).** Increased radiopharmaceutical uptake (IRU) in the flow phase of the imaging study corresponds to differentiated vascular space diseases. IRU in the blood pool phase is caused by neovascularity, a feature of reactive granulation tissue. In the bone phase, IRU is attributable to osteoid production which is the result of increased osteoblastic activity **(McCarthy 1997).** It is thought that with most orthopaedic injuries IRU results from abnormal biomechanical stress on tendons, ligaments or bone. Although stress fractures provide the hallmark diagnostic situation for nuclear scintigraphy, bone bruises are becoming more commonly sought by the clinician. The patient may experience pain, often following direct trauma to the region, without a corresponding radiographic lesion **(Holder 1993).** In a clinical study, IRU was a good predictor of impending radiographic changes, leading the clinician to conclude that 'physiologic' imaging of bone may become the most useful method for detecting early OA **(Dieppe 1990).**

Researchers have linked early IRU to bone changes in the subchondral region. One of the most classic studies was carried out by **Radin *et al* (1984)** using a rabbit model. Unmitigated insult to the hindlimb resulted initially in an IRU associated with the periarticular tissues of the stifle joint . These changes preceded those to the articular cartilage by weeks, leading the researchers to speculate on the role of the subchondral bone in the progression of OA. Another study tested the theory that IRU was associated with binding to the surface of the hydroxyapatite crystal. The results of this research did support the theory when the radiopharmaceutical agent was almost completely eliminated by decalcifying the sections. The same group, in an investigation of human osteoarthritic femoral heads, documented that IRU was associated with remodelling subchondral bone and growth of osteophytes **(Christensen 1985).**

Further down the clinical path of physiologic imaging is magnetic resonance imaging (MRI). While Computed Tomography (CT) has little to offer the rheumatologist, MRI can demonstrate all tissues in one plane as well as many planes with three-dimensional technology **(Watt 1996).** MRI has proven to be the most sensitive technique for detecting effusions and soft tissue disease as well as subtle abnormalities of bone that correlate well with scintigraphy **(Dieppe 1990).** Evaluation of the musculoskeletal system has emerged as one of the most important uses of MRI **(Peterfy *et al* 1994)** because it can show extensive detail of the anatomical features of joint structures, both structural and non-structural, leading to an earlier diagnosis of joint involvement in a non-invasive manner **(Adams and Li 1986).** In an experimental model, MRI was used to document the onset of joint disease 4 weeks after the initial insult and 8 weeks prior to radiographic evidence of disease **(Sabiston *et al* 1987).** Researchers and clinicians alike hope that early detection of articular derangement through advanced imaging techniques will translate into prevention, or at least more successful treatment of the disease.

Further Observations Regarding Equine Lameness

Early detection of joint disease brings the discussion back to an important corollary to the described definition of lameness. Although seemingly obvious, the performance horse veterinarian must be diligent in their investigation of the musculoskeletal system. If all aspects of the examination lead to an obvious diagnosis, then the task would always be straightforward. As an anecdotal example, consider the middle-aged jumping horse that presents for stopping at the double oxers during top-level competition. The physical examination reveals an underslung heel with a long toe on both front feet and sensitivity over the middle third of the frog.

In the lameness examination, the horse demonstrates a left fore limb lameness when trotted in the straightaway, but a bilateral lameness in the inside limb when lunged in each direction. Desensitisation of the palmar digital nerves alleviates the lameness and radiographs of the navicular bones reveal lucencies along the flexor surface of both the upright pedal view and the flexor cortex view. If scintigraphy had been carried out, it would have shown obvious uptake associated with the navicular bone as well. While the above scenario may seem to be too clear cut a case of navicular disease, these types of cases still do present for diagnosis. Probably the most difficult aspect of such a case, however, is realising that the condition in this horse did not develop overnight and that the early signs were probably missed by the owner, trainer and possibly even the veterinarian. If the early clues had not gone undetected, a more successful outcome is the likely result. In this particular case, even if the current condition were treated with corrective farriery, medication and/or surgery, the horse does not have a very good prognosis for continuing his career in top level showjumping.

However, as stated earlier, many of today's lamenesses are subtle, making the task of the performance horse veterinarian all that much more challenging.

Some lamenesses may only show diagnostically significant findings during one part of the investigation. This includes a specific aspect of the history that may be classic for certain lamenesses. Some horses may only show abnormalities on the physical examination, some when trotting up and some only when lunging, especially on hard ground. Most importantly are those horses that only show the gait deficit while performing their intended task, i.e. while being ridden or driven. It is quite common that subtle lamenesses only manifest themselves in this way.

From a therapeutic standpoint, the subtle lameness is usually the most rewarding to treat. This is most likely due to the fact that the process has not been going on for long and is therefore less likely to have resulted in serious structural abnormalities. In fact, this introduces another controversial, yet important, concept in the discussion of musculoskeletal disorders. The term 'lameness' seems to indicate a potentially serious disorder involving bones, joints, and other soft tissues. 'Soreness', on the other hand, is a term that most clients can understand. After all, most humans, especially those involved in some form of athletics, will have experienced soreness at some stage of their lives. The difference is that soreness is considered to be a temporary affliction, one that is easily healed given proper rest or restricted activity. Soreness appears to be very common among horses as well. It is at this stage of the gait deficit that it is most desirable to see the horse presented and it is at this level of seriousness that many subtle lamenesses are presented. More importantly, it would be advantageous to determine when, or if, 'soreness' becomes more serious clinical and pathological lameness. Although a specific threshold may not be identifiable, investigations headed in that direction may hold the key to prevention of serious joint disease.

For this reason, the horse could become an excellent model for studying the clinical course of early musculoskeletal abnormalities. This is especially evident when comparing the canine and human forms of 'lameness' to that experienced by the horse. In small animal practice, the canine patient is most often presented once the disease process is well established. The complaint is often as serious as

the dog has difficulty rising from the sitting position, it can't go up stairs any longer, it has difficulty getting into the car, and, in the final stages, it cannot go out to urinate or defaecate. Once the disease progresses to this stage, it is usually well advanced and easily diagnosed. All the 'classic' symptoms of OA are often present. Clinically, the joints may be hot, swollen or enlarged, there may be pain on palpation or manipulation. Most importantly, there will be obvious radiographical evidence of joint disease such as osteophyte production, subchondral bone lysis or sclerosis, and possibly joint space narrowing **(Watt 1996)**. Just as in the horse with advanced navicular disease, it may be difficult to treat and most likely impossible to cure. In the earlier, more treatable stages, the small animal client is often reluctant to present a 'stiff' dog for examination. Quite to the contrary, many equine clients would consider such a complaint to be of significant merit. In fact, in the upper level performance horse, such an affliction could constitute an emergency call.

Similar to the canine situation is the human patient. The 'football injury' may have caused stiffness for years, but often it is not until the pain becomes more severe that the patient seeks professional help. Again, similar to the canine situation, the disease may be too far advanced at that stage to offer reliable treatment. Therapeutics may be limited to those that simply eliminate symptoms rather than those that might afford a cure **(Kraus 1997).**

Anatomy of the Equine Metacarpophalangeal Joint

In order to understand the concepts of joint disease, a brief introduction to the articular environment is necessary.

Gross Anatomy

An articulation or joint is formed by the union of two or more bones or cartilages by other tissues **(Getty 1975).** The synovial joint, also known as a diarthrodial joint, is the main joint associated with locomotion. It is composed of a joint space filled with joint fluid and a synovial membrane. It is formed by two articular surfaces which are relatively congruent to each other, and covered by cartilage which is usually hyaline **(Palmer and Bertone, 1994).** The bones are held together by tough ligamentous bands, and covered by a joint capsule composed of a fibrous outer layer and the inner synovial membrane **(McIlwraith, 1990).** The fibrous layer is composed of dense fibrous connective tissue and adds mechanical stability to the articulation. The synovial lining is responsible for joint fluid production and metabolic management of the articular environment.

The equine metacarpophalangeal joint is also known by the colloquial term 'fetlock', as is the metatarsophalangeal joint. It is composed of the distal aspect of the metacarpus, the proximal aspect of the first phalanx, and the paired proximal sesamoid bones **(Getty 1975) (Fig 2.3)**. The joint is classified as a ginglymus since movement is only possible in one plane. The first phalanx glides over the surface of the distal metacarpus in a palmar to dorsal direction during flexion and extension. The paired sesamoid bones are related to the complex stay apparatus of the foreleg and also glide over the palmar articular surface of the metacarpus. They remain in

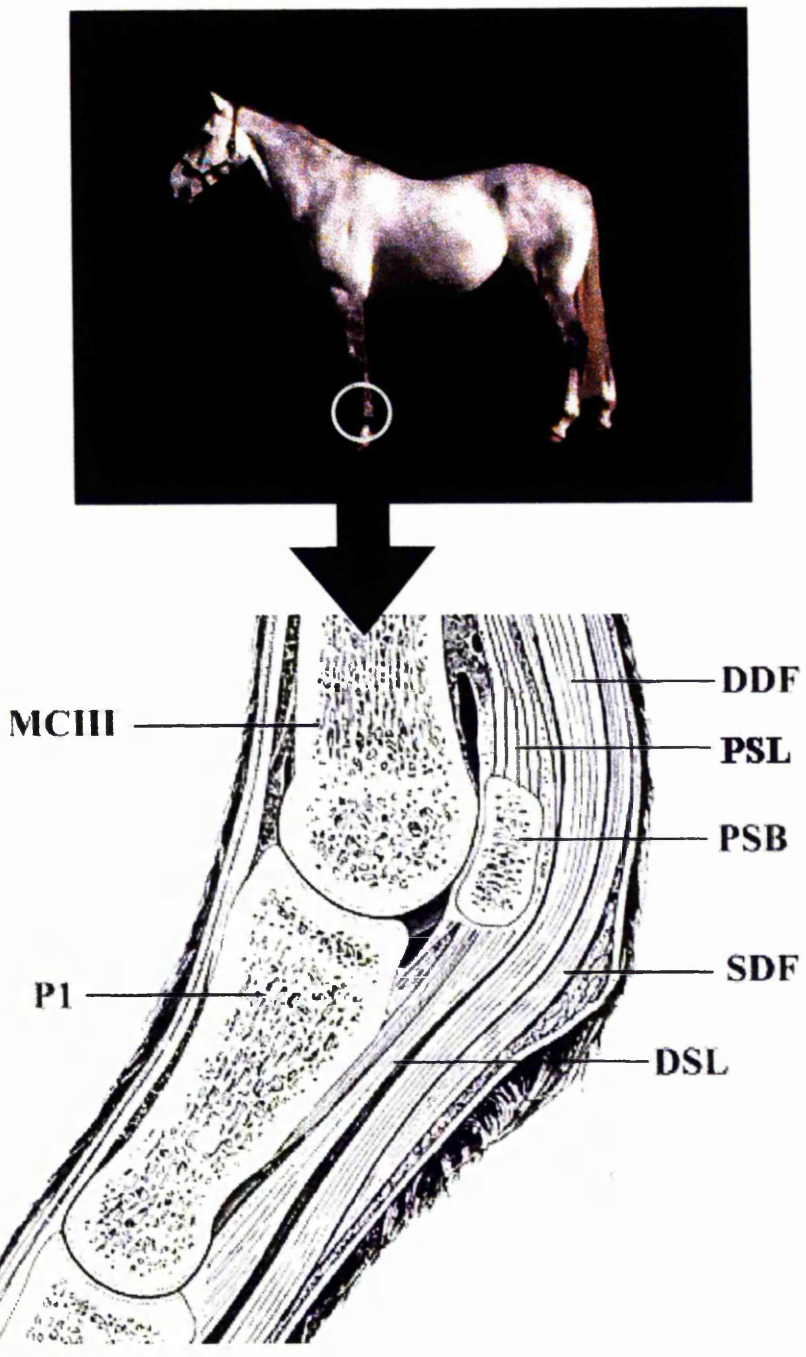


Fig 2.3 Schematic representation of the equine metacarpophalangeal joint (MCPJ). It is composed of the third metacarpus (MCIII), first phalanx (P1), proximal sesamoid bones (PSB). The soft tissues include the superficial and deep digital flexor tendons (SDF and DDF respectively), the proximal suspensory ligament (PSL) and the distal sesamoidean ligaments (DSL). (Adapted from Stashak 1990)

constant association with the palmar aspect of the first phalanx, and therefore also move in a similar and related palmar to dorsal motion. The ginglymous nature of the joint is assisted by the congruency of the articular surfaces. The distal aspect of the metacarpus is relatively cylindrical and is divided into two unequal surfaces by the sagittal ridge **(Stashak 1990).** The medial condyle is the larger of the two, presumably since more of the horse's weight is distributed toward the medial aspect of the limb. The sagittal ridge fits into the corresponding sagittal groove located on the proximal articular surface of the first phalanx. On the palmar surface, the proximal sesamoid bones are located on either side of the sagittal ridge.

The bones of the fetlock joint are joined to one another by ligaments. The collateral ligaments are located on either side of the joint and attach the first phalanx and sesamoids to the metacarpus. The superficial layer originates from the eminence of the distal metacarpus and attaches to the proximal aspect of the first phalanx. The deep layer is much stronger, and arises from the depression below the eminence, and attaches to the abaxial surface of the sesamoid, and the proximal aspect of the first phalanx **(Getty 1975).** Other structures composing the articulation but not described here include the proximal suspensory ligament, distal sesamoidean ligaments and the flexor and extensor tendons.

Microscopic Anatomy

Bone

Compact bone. Compact bone is the major supporting structure of the body. It is a mineralized form of connective tissue that possesses great strength while maintaining some degree of elasticity. Compact bone is composed of cells and an extracellular matrix. The organic components are the glycoprotein ground substance, similar to that found in articular cartilage, and a collagen fibre network. The mineralized portion of

the bone matrix is composed of inorganic salts, primarily calcium hydroxyapatite crystals **(Riggs 1990)**.

The cells of compact bone are the osteoblasts, osteocytes, and osteoclasts. The osteoblasts are derived from the osteoprogenitor cell line and produce the organic portion of the extracellular matrix. As this portion of the matrix rapidly mineralizes, the cells are trapped within it and they become responsible for maintaining the bone matrix as osteocytes. The osteoclasts are derived from the macrophage cell line, and are active in the remodelling process of bone **(Ham 1974).**

The majority of compact bone is made of parallel bony columns known as Haversian systems. The central Haversian canals contain blood vessels and nerves and are surrounded by concentric bony layers known as lamellae. The Haversian systems are held together by the interstitial system. At their outermost margin they blend with the dense cortical bone laid down by the osteoblasts of the periosteum, and at their innermost extent the Haversian systems merge with the trabecular bone of the marrow cavity **(Bloom 1972).**

Cancellous Bone. Cancellous, spongy or trabecular bone is composed of a network of bony trabeculae interspersed with communicating bone marrow spaces. Cancellous bone does not usually have Haversian systems, but rather the osteocytes have canaliculi that connect directly with the marrow spaces for transport of nutrients. The trabeculae are narrow bony struts lined by a thin connective tissue layer called the endosteum. The endosteum is responsible for the remodelling of the trabecular bone by the actions of its cell population of osteoblasts and osteoclasts **(Bloom 1972).**

While trabecular bone serves many functions to the horse including providing a region for bone marrow to reside, one of its major roles is to afford structural support to the bone. The thin trabeculae provide the support without adding significant weight to the

system and because of their interconnecting nature, spongy bone acts as a shock absorber at the ends of the long bones **(Hildebrand 1982)**.

Subchondral Bone. Embedded somewhere within the discussion of trabecular bone is usually found the term 'subchondral bone plate'. By definition, 'under the cartilage', it is often a nebulous term in the anatomy and histology references. The subchondral bone (SCB) plate is a layer of osseous tissue of variable thickness that is located just beneath the cartilage. In most joints, it consists of thin trabeculae curved under the deep layer of the cartilage. In some cases, however, the plate may thicken and resemble a type of subchondral cortex. The proximal extent of its border, however, is often an unclear concept in the literature. **Clark and Huber (1990),** however found the plate to be easily identifiable in the human specimens they examined. It is bordered distally by the interdigitating CC and proximally by the marrow spaces of the epiphysis. **Meachim and Allibone (1984)** reported that the SCB was composed of two types of lamellae, concentric layers around osteons and flat layers typical of appositional new bone formation. More about its possible role in the initiation and progression of OA will be discussed in Chapter 6.

Hyaline Cartilage

The name hyaline comes from the Greek *hyalos* meaning glass-like. This refers to the gross nature of the articular cartilage, especially when viewed in the immature state. It often appears almost translucent and of a bluish-white hue in the fresh form. Grossly, cartilage from young animals will look smooth and will feel soft, almost rubbery in nature. Articular cartilage in the human is of variable thickness, both between different bones and even in different spots on the same bone **(Buckwalter 1997).**

Composition. The hyaline cartilage of the equine articulation is composed of cells, called chondrocytes, suspended within an interstitial matrix. The matrix is

composed of collagen, proteoglycans, glycosaminoglycans, non-collagenous proteins and water in varying amounts, depending on the joint and the location within each joint.

Collagen. Thirteen different types of fibrillar and non-fibrillar collagens have been distinguished in the equine extracellular matrix of the hyaline cartilage. The majority of this has been identified as type II fibrillar collagen, which appears to represent 85-90% of all the articular collagen. Type II collagen improves the mechanical function of the cartilage by increasing tensile strength. Among the non-fibrillar collagens, type VI is located in the pericellular region, helping to anchor chondrocytes to the extra-cellular matrix, and type X is exclusive to the hypertrophic zone where it functions in the calcification process **(Palmer and Bertone 1994)**

Proteoglycans. The proteoglycan complex is known to increase the compressive stiffness of the articular cartilage. The most common one is known as 'aggrecan'. It is composed of a core protein with glycosaminoglycan side chains bound to it. The aggrecan complex is joined to a hyaluronic acid backbone at the hyaluronic acid binding region (HABR) via a link protein. The aggrecan is composed of three globular regions (G1, G2, and G3) which are connected by two linear segments (E1 and E2). These regions are susceptible to disruption via different enzymatic processes that are part of both normal catabolism and certain disease processes **(Carney and Muir 1988).**

Four smaller proteoglycans have also been identified in the extracellular matrix of the hyaline cartilage. They are known as biglycan, decorin, fibromodulin and PG-100. Decorin seems to regulate fibrillogenesis and collagen organisation, while the function of the others is unknown.

Glycosaminoglycans. The glycosaminoglycans are negatively charged, polyionic sidechains of sulphate and carboxy groups which are bound to the core protein to compose the proteoglycan complex. The most important glycosaminoglycans in hyaline cartilage are chondroitin-4-sulphate, chondroitin-6-sulphate, keratan sulphate and hyaluronic acid. The hydrophilic effect of the side-chains draws water into the extracellular matrix, expanding the matrix until resistance is generated by the unyielding collagen fibres **(Carney and Muir 1988)**. Recently, **Brown *et al.* (1996)** has documented that the chondroitin-6 to 4 ratio increases with age in the horse.

Other Proteins. These proteins include fibronectin, anchorin CII, thrombospondin and chondronectin. Their action is believed to be related to chondrocyte adhesion to the extracellular matrix.

Structure. The organisation of these components is critical to the normal function of the tissue and changes with increasing depth. The tissue composition may also change with increasing age or disease processes such as osteoarthritis **(Palmer and Bertone 1994)**. The cartilage is divided into four histologically and biochemically discrete layers known as the superficial, intermediate or transitional, the radiate or deep and the calcified layer.

The most superficial layer of adult hyaline cartilage contains the highest number of chondrocytes. They are flattened tangentially across the cartilage surface. Also of tantamount importance to this layer, are the type II collagen fibres, likewise oriented in a tangential direction relative to the surface of the cartilage. It is thought that these collagen fibres are able to provide tensile strength to the cartilage, allowing it to resist both compressive and shear forces in this manner. The proteoglycan population in this region is mainly of the chondroitin sulphate chains, with very few keratan sulfate chains. The transitional layer contains fewer, yet larger, chondrocytes, found either singly or in pairs. The proteoglycan

composition of this layer is similar to that of the superficial layer, containing a higher proportion of chondroitin sulfate than keratan sulfate glycosaminoglycans. The thin type II and VI collagen fibrils are arranged in a random manner. In the deep layer, the chondrocytes are larger again, and organised into columns with the long axis of the cell oriented perpendicular to the joint surface. The collagen fibrils radiate from between the chondrocyte columns in the same manner. The proteoglycan composition in the deep layer shifts toward the keratan sulfate group, subsequently lowering the charge density of the matrix. Because there is no concomitant decrease in chondroitin sulfate or hyaluronic acid volume with the increasing percentage of keratan sulfate sidechains, the deep layer manifests an overall increase in the proteoglycan concentration. The fourth layer of the hyaline cartilage is the calcified layer. The junction between the deep layer of non-calcified cartilage and that of the calcified layer is known as the tidemark. The border opposite to the tidemark signifies the origin of the subchondral bone. The calcified layer is considered to have little or no resilience due to the low concentration of collagen and proteoglycan. The chondrocytes there are embedded in a mineralised matrix **(Palmer and Bertone 1994).**

Articular Physiology

When considering the joint as an organ **(Radin 1995)**, the most notable absence from the hyaline cartilage is that of blood vessels, lymphatics and nerves. For most organs, nourishment is delivered via the circulatory system, while waste products are most often removed by the lymphatic system. Detection of and reaction to noxious stimuli are normally carried out by the nervous system. As a result of this evolutionary omission, these processes must be accomplished in another way, if at all.

Nourishment

It has been proposed that most of the nourishment of the chondrocytes must originate in the synovial fluid. As an ultrafiltrate of the plasma, the synovial fluid contains the metabolic provisions for the maintenance of cellular events, both anabolic and catabolic. Some of these nutrients are able to diffuse locally, however it is assumed that mechanical stimulation is needed as well, especially to reach the lower layers of chondrocytes. Along these lines, the hyaline cartilage has been likened to a sponge. During weight-bearing, waste products are 'squeezed' out of the cartilage matrix and into the synovial fluid, along with the excess water. Subsequently, when the limb assumes a non-weight-bearing configuration, the cartilage matrix expands, drawing fluid in until counter-resistance is met by the tensing collagen mesh-work. Fresh nutrients are drawn in with the water, which then diffuse into the chondrocyte cytoplasm **(Carney and Muir 1988)**.

Noxious Stimuli.

The detection of stimuli, both physiological and noxious, is not fully understood in relation to the articular environment. The complete absence of nervous tissue within the substance of the hyaline cartilage renders normal methods of sensation impossible. As a result, such functions as proprioception and nociception must be taken over by tissues juxtaposed to the cartilage itself. Recently, **Nixon and Cummings (1994)** investigated this concept in the metacarpophalangeal joint of the horse. They reported the detection of abundant sensory nerve fibres in the joint capsule, synovial membrane subintimal layers, collateral ligaments, suspensory ligament, the attachment of the distal sesamoidean ligament to the sesamoid bones and the periarticular periosteal layers. The presence of these abundant nerve fibres could explain how the joint interacts with external stimuli. However, possibly of even greater significance to the investigation of joint disease, was the detection of sparse substance P immunoreactive nerve fibres in the subchondral bone plates of the metacarpus, proximal first phalanx and dorsal surfaces of the sesamoid bones.

This unique finding of the substance P fibres in equine subchondral bone may help explain the incidence of bone pain in some cases of joint disease.

Concepts in Joint Disease

Over the years, the development of disease within the articular environment has been investigated extensively, yet far from exhaustively, resulting in numerous theories as to how the derangement develops. Although much too complicated to accurately do so, many of these concepts have been compiled into two major pathways: the mechanical and biochemical. Albeit ostensibly opposite in their aetiologies, in the clinical environment they are probably hopelessly intertwined. Nevertheless, these two pathways will be explored separately.

Mechanical.

The mechanical pathway is possibly the easier of the two to define, arguably because it seems to relate to a traumatic event **(Smith 1991). McIlwraith (1996)** describes this as type 4 degenerative joint, or that which is secondary to a previous and recognisable event, such as an intra-articular fracture or septic arthritis. Similarly, displaced fractures, even when repaired surgically, were deemed to have a lower return to racing because of the development of OA **(Foerner and McIlwraith 1990)**.

Synovitis is the disease process that often bridges the gap between mechanical and biochemical causes of joint disease. Primary synovitis is considered to induce inflammation within the synovium without gross damage to the articular cartilage or supporting structures, and with no radiographic evidence of disease. In contrast, secondary synovitis occurs as the result of ligamentous instability, articular cartilage damage or bone trauma **(Baxter 1992)**. Because of the possible severity of the primary injury, this form of synovitis is usually more serious. In

either case, the inflammation present as the hallmark of synovitis may lead to cartilage degeneration consistent with biochemical disruption **(Todhunter and Lust 1990)**.

Biochemical.

As with the syndrome of synovitis mentioned above, intra-articular inflammatory reactions result in increased production and release of enzymes **(Baxter 1992).** Some of these enzymes are considered normal within the context of conventional joint physiology and remodelling. However, they may become deleterious in higher volumes. Proteoglycan degradation may be caused by lysosomal enzymes which have been released by typical joint cells such as chondrocytes, synoviocytes, and leukocytes **(Clyne 1987).** Inflammation can cause free radical release, cytokine production and the synthesis of prostaglandins, which further degrades the proteoglycans and can suppress the synthesis of replacement tissues **(McIlwraith 1996).** Interleukin 1 was found to stimulate the production of stromelysin and collagenase, both associated with catabolism within the normal joint environment, but causative of a disease process at higher concentrations **(May *et al* 1992). (Fig 2.4 and 2.5)**

While these theories and mechanisms are well documented by now in the horse, many of the clinical observations and theories of this author are slightly at variance with much of the veterinary literature on joint disease. It is the opinion of this author that the last decade or so of equine articular research, especially that of resurfacing hyaline cartilage **(Howard *et al* 1994),** although a necessary evolution in the investigative process, may be targeting the 'innocent bystander', instead of the responsible party. The cartilage may succumb to degeneration secondary to an unhealthy supporting layer. There seems to be a significant amount of evidence in the human literature implicating the subchondral bone and calcified cartilage in the initiation and progression of joint disease, and yet few of these concepts and

theories have been studied in the horse. Hence, the aim of this dissertation was to investigate the calcified tissues of the equine metacarpophalangeal joint.

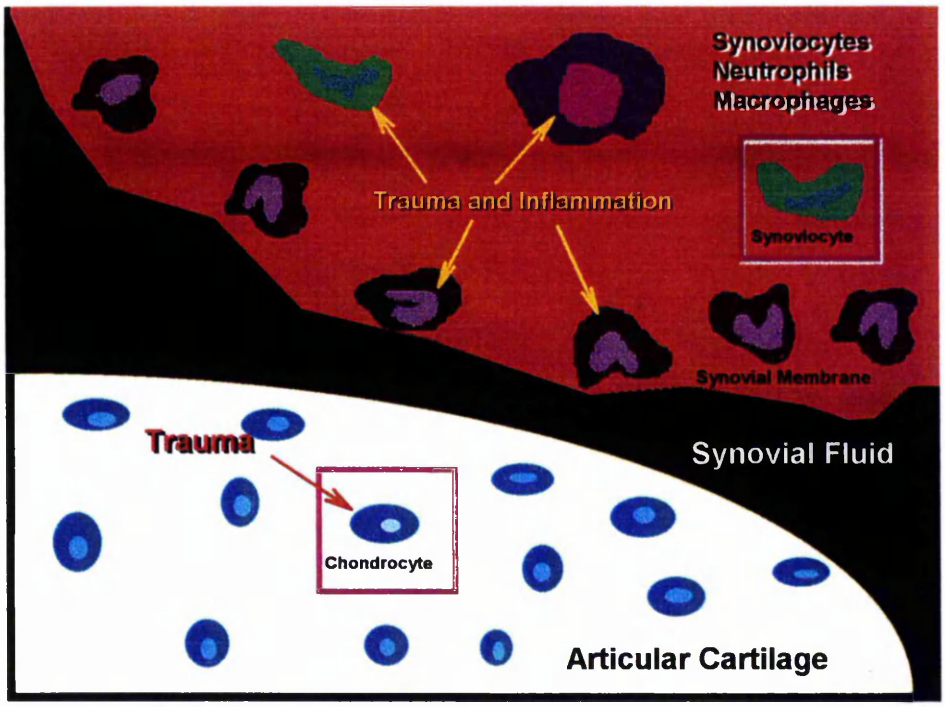


Figure 2.4 Schematic Representation of the Biochemical Pathway of OA

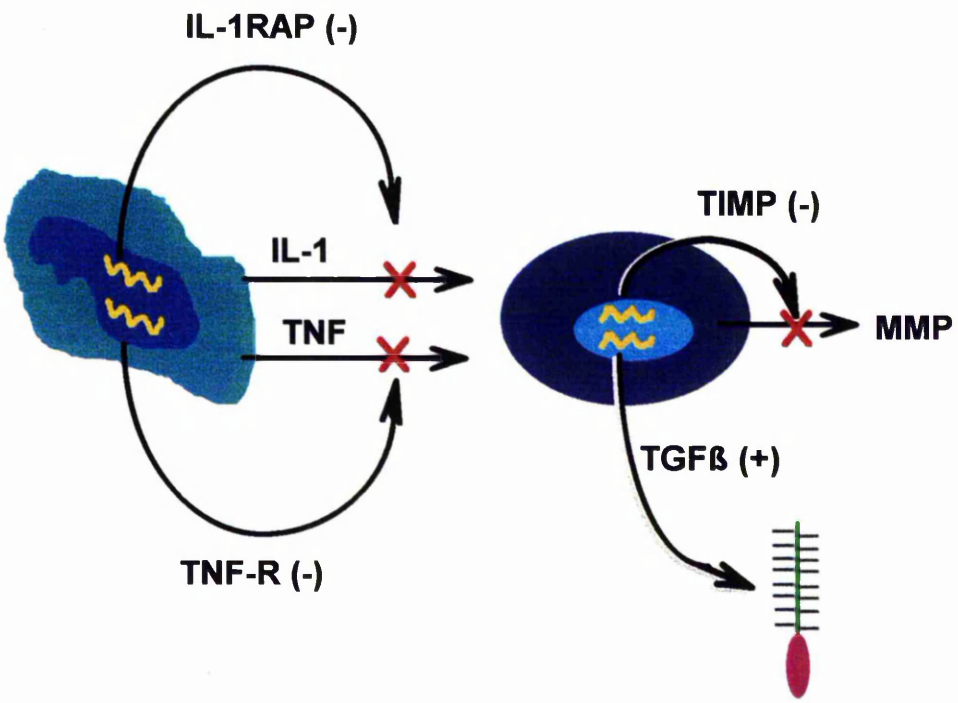


Fig 2.5 Schematic diagram of the role of the synoviocyte and chondrocyte in the biochemical pathway of OA.

CHAPTER 3

CLINICAL COMPONENT

Introduction

Studies in the equine field have generated a significant amount of information over the last 20 years or so. One of the major problems in conducting equine research involves the sample size. Because of the expense of horses, it is difficult to maintain them as research animals. Dogs, cats, and rodents are bred for research purposes and are quite cost effective to maintain during a research project. Ruminants, on the other hand, hold no sentimental value for most of the public and their bones are quite easy to acquire due to the abundance of slaughterhouses around the world dealing in beef, pork or lamb. Horses lose out on both accounts as research animals. They are large animals that eat a considerable amount of food, they require more maintenance than cattle, and they hold both the fascination and sentiments of the public. Consequently, it is often difficult to properly carry out research in the equine field resulting in two likely scenarios. One is that the work is carried out clinically, either in a retrospective or prospective manner. Unfortunately, either approach often results in abbreviated material because in the retrospective arena the researcher is limited by what information appears in the medical record, while in the prospective situation, the investigation may be curtailed by lack of funding, client non-compliance or local animal study rules such as those involving potentially invasive procedures. Some excellent publications to come out of this line of research recently include a study of the surgical outcome of 336 fetlock arthroscopies **(Kawcak and McIlwraith 1994)** and a *post-mortem* study on 496 horses that died over a 2 year period on the California racetracks **(Johnson *et al.* 1994).**

The other common situation is a *bona fide* research project, using horses as subjects. The problem with this approach is that it usually ends up using too few horses or repeated measurements are taken from the same horse. The small sample sizes result in difficulty with the statistical analyses and possibly in interpreting the data. Examples of this kind are often fraught with low numbers of animals. **Fortier *et al* (1997),** collected the chondrocytes from one 4 month old

foal and used these cells to determine the affects of TGFß on synthesis of DNA, collagen and proteoglycan. In another example, **Pleasant *et al* (1997)** used 6 horses in a study on lameness and the response to regional anaesthesia. In one of the most questionable studies of equine lameness over the last 5 years, **White et al (1994)** determined there was no effect of oral glycosaminoglycans on experimentally induced joint disease. Unfortunately, they only used 12 horses and the length of the study was a mere 26 days. Determining 'no statistical difference' in such a small population of animals is usually rampant with Type II errors, i.e. not being able to detect a significant difference when it exists **(Markel 1991)** Although such investigations are quite common in the equine field, the results, must be viewed with caution.

In this dissertation, we set out to investigate the calcified tissues of the equine metacarpophalangeal joint. Because very little objective data about these structures exists in the equine literature, especially for that of the calcified cartilage and subchondral bone, normal observations and measurements needed to be recorded. Therefore, the objective of this study was to gather a quantity of normal equine joints for analysis of the calcified tissues of the metacarpophalangeal joint.

Materials & Methods

Specimen collection

In order to collect as many specimens as possible, the acquisition was done at the local abattoir. Prior to humane destruction, the horses were inspected and the approximate weight and breed was recorded. Immediately after death, the carcasses were hung from a hindleg and bled out via jugular exsanguination. The head was then removed and the dentition was examined to determine the approximate age, which was also recorded. Each foreleg was removed by

disarticulation at the intercarpal joint and labelled with the specimen number. The palmar pouch of the metacarpophalangeal joint was punctured with a 20 gauge 1 inch needle and 5-10 ml of synovial fluid was removed with a syringe. Half of the fluid was placed in a serum tube, while the other half was deposited into an EDTA tube. The tubes were labelled with the specimen number and immediately transferred to an insulated cooler filled with ice.

The limbs were inspected grossly and the observations were recorded. Information that could be gathered macroscopically included the following: the presence of swellings, scars or obvious injuries; whether shod, and, if so, the type of shoe; evidence of foot wear or the shape or quality of the foot. The limbs were stored in a van for transport to the University of Glasgow.

Radiology

Upon arrival at the University, the limbs were unloaded for radiography. A portable radiograph machine (Xograph, UK) and high speed cassettes (Sterling, UK) loaded with rare earth film (Sterling, UK) were used to acquire the views at an exposure of 60 Kvp and 10maS. The films were developed in an automatic desktop processor (Xograph, UK).

The films were inspected for evidence of any abnormality. These observations were recorded.

Gross Pathology

The limbs were stored in a cooler overnight and dissected the following day. A sharp knife was used to incise the skin along the dorsal aspect of the lower limb extending from the proximal metacarpus to the middle aspect of the first phalanx. Care was taken not to create any iatrogenic defects in the articular cartilage surrounding the metacarpophalangeal joint. All the soft tissues were dissected free from the metacarpus, which was then disarticulated from the proximal sesamoid bones and proximal aspect of the first phalanx. The articular margins were inspected for the presence of osteophytes, erosions or chip fractures. The articular cartilage was examined for evidence of fibrillation, score lines, or full-thickness defects. Special attention was given to the region of the transverse ridge, the region on the palmar aspect of the metacarpus where the distal aspect of the sesamoid articulates with the proximal region of the first phalanx. The cartilage and subchondral bone in this region was assigned a grading system. Representative lesions were photographed. The metacarpal bones were washed, relabelled, placed in specimen bags and stored in the freezer until ready for sectioning and embedding. The rest of the limb was discarded.

Synovial Fluid

The synovial fluid was used to determine the status of the intra-articular environment at the time of death. The fluid in the serum tube was removed via a micropipetting system and 100 µl aliquots was placed in a specimen storage vial and frozen at -70°C until analysis.

Gel zymography was carried out to determine the concentration of matrix metalloproteinases 2 & 9 in the joint fluid. These analyses were carried out by Dr. P.D. Clegg at the University of Liverpool (**Clegg *et al* 1998**).

The remaining synovial fluid from the EDTA tubes was used to determine the cellularity. Both red and white blood cell counts were determined using an automatic haemocytometer.

Results

Horses

A total of 34 horses were included in the study. The distribution for age, breed, gender and approximate weight is listed in **Table 3.1.** The ages ranged from 1.5 years to 25 years. There were four general breed types represented. These consisted of Thoroughbreds (TB), Thoroughbred crosses (TBX), Ponies and cross-bred horses. Both genders were well represented. In addition, there was one young stallion that was included in the 'male' category. The weights were approximated and ranged from 500-1200 lbs.

Clinical Assessment of Horses

The clinical assessment of the horses was done *post-mortem* due to the nature of the study. Observations are included in **Table 3.2.**. Five horses were shod, all with regular plates. Three horses had contracted heels, including one of the shod horses. One each had the following observation recorded: a keratoma on the dorsal aspect of the foot, untrimmed hoof walls, an immovable fetlock joint, a bowed tendon, and emaciated body condition.

STUDY NO.	SPECIMEN	AGE (YRS)	BREED	GENDER	WT (LBS.)
1	427/97	1.5	TBX	M	600
2	429/97	1.5	X	G	700
3	2127A	2	X	F	600
4	319/97	2	X	G	700
5	2123A	3	TBX	F	800
6	378/97	4	Pony	G	600
7	314/97	6	TB	F	850
8	316/97	8	TB	G	1100
9	318/97	6	Pony	F	700
10	305/97	7	TB	F	800
11	310/97	7	TBX	G	1000
12	431/97	8	TB	F	1000
13	433/97	8	TB	G	1000
14	2119A	9	TB	F	1100
15	380/97	10	Pony	F	600
16	322/97	12	TB	F	1200
17	415/97	12	TBX	F	750
18	425/97	13	TBX	G	700
19	435/97	13	TB	F	1100
20	407/97	17	Paint	G	800
21	411/97	17	TB	G	900
22	402/97	18	TB	F	800
23	413/97	18	TB	G	1200
24	400/97	19	TB	G	1000
25	2114A	20	Pony	F	550
26	309/97	20	TBX	G	700
27	384/97	20	TB	G	1050
28	386/97	20	TB	G	1100
29	392/97	20	TB	F	1000
30	398/97	20	TB	F	1000
31	404/97	20	TB	F	1050
32	419/97	20	TB	F	800
33	423/97	20	Pony	G	600
34	388/97	25	TB	G	1000

Table 3.1 Signalment of Horses used in the Histomorphometry Studies

STUDY NO.	GROSS ARTICULAR CHANGES	RADIOGRAPHIC FINDINGS	COMMENTS
1	TR1	Enthesiophyte P1	
2	TR0	Small enthesiophyte P1	
3	TR0	WNL	
4	TR0	WNL	
5	TR0	WNL	
6	TR1	WNL	
7	TR0	WNL	Shod
8	TR3	WNL	Shod
9	TR1	WNL	
10	TR2	WNL	
11	TR2	WNL	
12	TR1, WL	WNL	
13	TR1	WNL	
14	TR0	WNL	
15	TR0	WNL	Contracted Heels
16	Medial- partial thickness ulcer	WNL	Shod
17	TR2	P1 chip	

Table 3.2 Clinical Data for the Horses in the Histomorphometry Study

STUDY NO.	GROSS ARTICULAR CHANGES	RADIOGRAPHIC FINDINGS	COMMENTS
18	TR2 palmar WL	P1 chip	
19	TR2, WL	WNL	Shod
20	TR2, WL	WNL	
21	TR1	WNL	
22	TR2	WNL	
23	TR3, many WL dorsal/palmar	P1 chip	
24	TR2	P1 ostephyte	Shod, Contracted Heels
25	TR0	WNL	
26	TR1, palmar subchondral hemmorhage	WNL	
27	TR5	P1 mild osteophyte	Bowed Tendon LF
28	TR1, significant palmar/dorsal WL	WNL	Long Feet
29	TR 2	P1 mild osteophyte	
30	TR3	Calcified suspensory ligament	Immobile Joint LF
31	TR2	P1 mild osteophyte	Keratoma LF
32	TR3 palmar subchondral hemmorhage	Lateral sesamoid fracture	Emaciated
33	TR1	WNL	Contracted Heels
34	TR2	P1 mild osteophyte	

Table 3.2 (con't) Clinical Data for the Horses in the Study

Radiology.

Further clinical assessment was carried out via radiography. These findings are summarised in **Table 3.2.** Twenty-two of the joints radiographed were considered within normal limits. Five had small osteophytes on the dorsal border of P1 and 2 had small enthesiophytes in a similar location. Three horses had a mineralised fragment on the dorsal rim of P1 and one each had a fractured lateral sesamoid and calcification of the suspensory ligament.

Gross Pathology.

Gross examination of the joint and articular surfaces revealed the presence of the previously identified chip fragments, wear lines, and osteophytes. **(Fig 3.1-3.3)**. The cartilage and subchondral bone in the region of the transverse ridge on the palmar aspect of the joint was given a special grading system as follows:

TR 0 Good cartilage, no evidence of any abnormalities

TR 1 Focal subchondral bone discolouration, overlying cartilage normal

TR 2 Overlying cartilage slightly mottled

TR 3 Overlying cartilage with a partial thickness defect

TR 4 Overlying cartilage with a full thickness defect

TR 5 Overlying cartilage has full thickness defect extending into the subchondral bone

There were 8 horses in the TR0 category, 9 in TR1, 11 in TR2, 4 in TR3, and 1 each in TR4 and TR5. In addition, 6 horses were noted to have superficial cartilage wear lines.

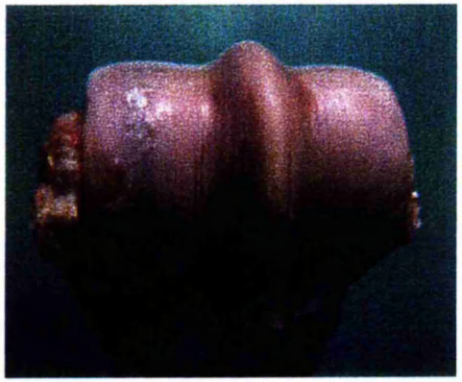


Fig 3.1 Score lines associated with OA on the distal metacarpus

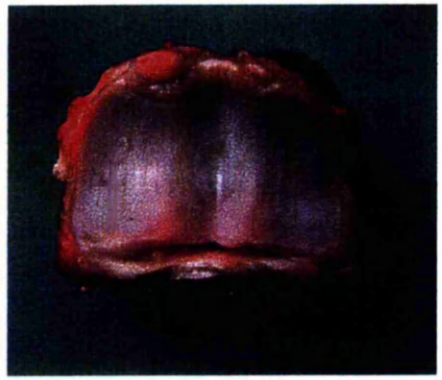


Fig 3.2 There are two dorsal P1 fragments, one medial and one lateral, present at the top of the figure.

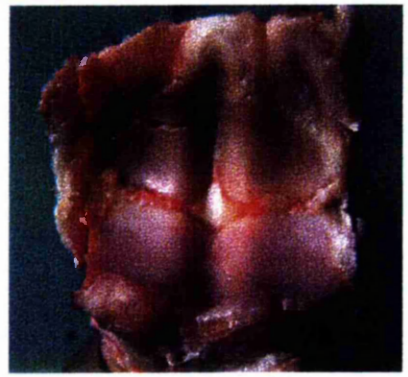


Fig 3.3 A fractured sesamoid bone is evident.

Clinicopathological Results

Cellularity.

The number of white blood cells (WBC) and red blood cells (RBC) were determined using a haemocytometer. The WBC ranged from 100 to 3300 X 10^9 cells/L. The RBC ranged from none detected to .02 X 10^{12} cells/L. The results are all summarised in **Table 3.3.** None of the horses were considered to have a significant elevation in red or white blood cells within the synovial fluid.

MMPs.

The matrix metalloproteinases 2 & 9 (MMP 2, MMP 9 respectively) were assayed to determine if MMP 9 was present at all and if MMP 2 was present, in what concentration. MMP 9 was only present in one joint and MMP 2 ranged from .02 to .63 Relative Activity Value (RAV), although only four joints were considered to have mildly elevated levels. A mild increase was considered to be $\geq$ .50 **(Clegg *et al* 1998).** These results are also summarised in **Table 3.3.**

Discussion

Normal is a difficult term to define in a biological system. For this reason, we chose to gather as large a population of horses as possible and decided that this might be best achieved at the local abattoir. As stated in the introduction, many equine studies suffer from low numbers of subjects. In this study we were able to collect limbs from 34 horses presented for slaughter in one day. While this approach may be subject to sample bias, we chose to accept this risk in acquiring our specimens **(Kaneene and Bartlett 1985)**.

STUDY NO.	SPECIMEN	WBC	RBC	MMP 2	MMP 9
		(10^9/L)	(10^{12}/L)	(RAV)	(present)
1	427/97	0.2	0	0.08	
2	429/97	0.2	0	0.08	
3	2127A	1.5	0	0.08	
4	319/97	0.5	0.01	0.11	XXXX
5	2123A	0.5	0.01	0.12	
6	378/97	0.6	0	0.36	
7	314/97	0.5	0.01	0.52	
8	316/97	1	0.02	0.04	
9	318/97	0.31	0.02	0.21	
10	305/97	0.3	0.02	0.37	
11	310/97	0.2	0.02	0.04	
12	431/97	0.5	0.02	0.08	
13	433/97	1.3	0	0.09	
14	2119A	0.3	0	0.07	
15	380/97	1.1	0	0.16	
16	322/97	0.2	0.01	0.11	
17	415/97	3.3	0.01	0.46	
18	425/97	0.5	0.01	0.15	
19	435/97	0.3	0.01	0.15	
20	407/97	0.2	0	0.14	
21	411/97	1.3	0	0.11	
22	402/97	0.5	0	0.5	
23	413/97	1.9	0.02	0.13	
24	400/97	0.4	0	0.16	
25	2114A	0.5	0	0.12	
26	309/97	1.6	0	0.11	
27	384/97	0.5	0	0.1	
28	386/97	0.1	0	0.26	
29	392/97	0.3	0	0.08	
30	398/97	0.7	0	0.39	
31	404/97	0.4	0	0.06	
32	419/97	1.2	0.02	0.12	
33	423/97	0.4	0	0.02	
34	388/97	1	0	0.63	

Table 3.3 Clinicopathological Data for the Horses in the Histomorphometry Study.

An abattoir itself is a significant risk to any study attempting to assemble a group of 'normal' horses. It could be proposed that owners would not send a normal horse to slaughter. While this may seem reasonable, it should be noted that many of the horses in this study, in particular the Thoroughbreds, seemed to be in good bodily condition, groomed and generally well-cared for. In fact, as seen from the data, there were several horses that presented wearing a full set of shoes, uncommon for horses that are not used for some degree of riding or performance.

It should also be noted that the specimen collection took place in late summer, a fact that may also have a bearing on the population of horses in that slaughterhouse. According to the owner of the slaughterhouse, there is often an increase in horses going to slaughter as winter approaches. Many owners would rather put the horse down than see it suffer through the winter, old or infirm. However, there was only one ostensibly unhealthy animal and only one old horse, in its mid-twenties. Another factor associated with the sample demographics in relation to the season, revolved around the British racing calendar. At the time of this collection, the flat racing season was finishing at some of the better tracks, while the jump-racing horses would have been in training for almost 6 weeks. Either scenario could have led to the high number of Thoroughbreds that was present in the study population. Flat horses that did not have a good season and jump-racers that did not appear to be training in a promising fashion could have been culled in this manner.

In looking at age in such a population, one would expect to encounter a bias toward the old and the young. Again, old horses are sometimes sold off in this manner, especially before winter starts. Very young horses are often present in such a population because the owners do not want the expense or responsibility of training them. It should be noted from the age distributions in this population, that neither scenario appeared to be the case and a wide range of ages was represented. In fact, especially in light of some of the study findings, it may have been more interesting if a larger number of young horses had been represented.

The gender distribution may have been the most surprising factor of the day. Although statistics would predict a 50-50 frequency between male and female, in practical experience, the geldings far outnumber the mares and stallions. This is often attributed to the mare's reproductive capabilities and to the overall higher popularity of geldings in the average riding population. Our study was successful in acquiring an almost equal distribution between the two. There was only one stallion present in the population, so the gender categories were simply assigned the title of 'male' and 'female'.

Because we set out to investigate the 'normal' situation, the joints needed to be evaluated for evidence of disease or some abnormal state. After gross examination, we chose radiology and synovial fluid assessment as clinically related parameters that could easily be measured in the *post-mortem* situation. The results of the radiology revealed the presence of visible lesions in several of the horses. We recorded these findings, but did not exclude any horses based on these observations alone.

The assessment of the articular environment via synoviocentesis is a recognised technique, but usually only employed clinically when evidence of intra-articular sepsis is suspected **(Todhunter and Lust 1990)**. In this case, none of the joints exhibited an increase in red or white blood cells indicative of inflammation at the time of slaughter. The addition of MMP 2 & 9 evaluation to the gamut of laboratory tests, searching for evidence of articular derangement, is a relatively new development **(Clegg *et al.* 1998)**. These enzymes are from the gelatinase family and, although required for normal cartilage catabolism, are considered abnormal when elevated. The results showed a marginal elevation of MMP 2 in 4 joints, while MMP 9 was only identified in one. Although this information may be pertinent to the study, no horses were excluded because of it.

Finally, the results of the gross *post-mortem* examination were included in the decision-making process. Although several 'abnormalities' were noted, none

were considered severe enough to warrant excluding the specimen from the study. Some clinicians and pathologists dismiss articular wear lines as part of the normal ageing process **(McIlwraith 1993, Pool 1996).**

Consequently, all the horses were used for data collection in the subsequent studies unless noted. In fact, 5 horses were dropped from the fractal study (Chapter 7), due to improper specimen handing during preservation and embedding.

CHAPTER 4

BONE HISTOMORPHOMETRY

Introduction

Establishing the amount of bone present within an animal or person has long occupied both researchers and clinicians in the veterinary and human medical fields. It is important to know the normal amount of bone that should be present in order to determine whether a deviation from normal is present within the patient or subject. To this end, there are many disease processes that revolve around the concept of a change in the amount of bone, be it too little *or* too much. There are several common examples of each process in the human literature. Osteoporosis, is probably the most well recognised disease of decreased bone mass. It is an oft-researched disorder, prevalent among elderly women, that frequently results in pathologic fractures because of bone loss. On the other side of the spectrum is osteopetrosis, a hereditary disorder that results in abnormally dense bone **(Radin and Rose 1986).** Within the equine field, very few quantitative studies have been published on bone histomorphometry. In one of the best experiments of its kind to date, **Young et al (1991)** determined parameters of bone histomorphometry in a group of Thoroughbreds. The investigators set out to test the effects of training regimes and track surfaces. **Norrdin *et al*** (1998) recently examined bone parameters in the MCPJ in relation to treadmill exercise in a group of horses, while **Firth *et al*** (1999) conducted a similar investigation into the results of training on the bones of the carpus.

The objectives of this study were to use stereological principles to determine the bone area (B.Ar), trabecular width (Tb.Wi), trabecular number (Tb.N) and trabecular separation (Tb.S) within several sections of a joint. Secondly, this data would be compared between sections within a horse and then between horses for the affects of age, breed, gender and weight. We hypothesised that the dorsal aspect of the joint would have different values than the palmar aspect.

Materials and Methods

Horses

The left forelimb from 34 horses was collected from abattoir specimens. The clinical details of the horses are discussed in the previous chapter (Chapter 3).

Sample Preparation

Following the clinical investigations carried out in Chapter 3, the bones were dissected free from the soft tissues of the limb, washed, and stored in the freezer. Just prior to sectioning, the bones were removed from the freezer and transported to the anatomy laboratory for sectioning. A set of callipers was used to measure the width of the distal metacarpus along the articular margin at the level of the transverse ridge. The width was recorded in millimetres and used to determine the exact location for sampling region. This was set as 1/4 of the distance axial to the medial and lateral aspect of each joint. **(see Fig 4.1 and 4.2)**.

Using a band saw, a sagittal cut was made at this location, extending from the articular surface to proximal to the physis. One cut was made both medial and lateral to the midsagittal ridge of the metacarpus. A cut was then made bisecting the midsagittal ridge to divide the distal metacarpus into four sections **(Fig 4.2)**. Finally, a transverse cut was made at approximately the region of the physis, separating the four sections of the epiphysis from the metaphysis of the metacarpus **(Fig 4.3).** The midsections from each metacarpus were labelled and packaged together for preserving and embedding. The bone ends were also packaged together but returned to the freezer for storage.

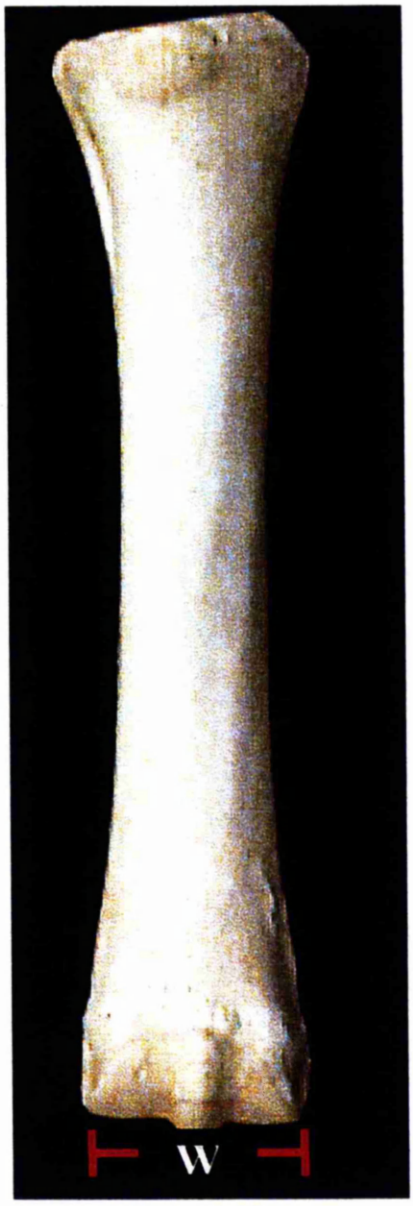


Fig 4.1 The width of the distal metacarpus was measured prior to sectioning to determine that samples were obtained from the same region of each bone.

Fig 4.2 Sample location. The sections were cut at one fourth the distance in from each side of the joint.

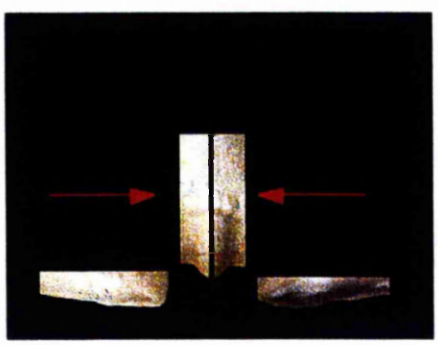


Fig 4.3 Specimen surface. The ends of the bone were discarded and the abaxial surface was embedded face down as the region of interest.

Fig 4.4 Specimen block. This is what the sample looked like after the embedding process. The region of interest is at the top of the photo

Embedding

The two mid-sagittal sections were preserved, dehydrated and embedded in methylmethacrylate to produce a block for sectioning. The protocol was adapted from **Sterchi and Eurell (1995)** and is as follows:

1. Fix in buffered neutral formalin (BNF) for 7-10 days
2. 70% alcohol - 3 days minimum
3. Defatting solution (Xylene) - 3 days on rollers
4. 100% ethanol - 2 days on rollers
5. 50% ethanol/methacrylate - 2 days on rollers
6. 70% methacrylate - 3 days on rollers
7. 90% methacrylate - 3 days on rollers
8. 100% methacrylate - 3 days on rollers
9. Washed in methylmethacrylate (MMA)/polymethylmethacrylate (PMMA)
10. Embed in MMA/PMMA and place in oven (37°C) - 3 days
11. Top up embedding moulds and place in oven (37°C) - 2 days
12. Placed in oven at 45°C - 2 days

Once the MMA had set completely, the sections were removed from the oven and broken out of the moulds **(Fig 4.4)**.

Microtome sectioning and staining

The blocks were then placed in a high-speed microtome for sectioning (Poly-cut E, Reichert-Jung). The blocks were clamped onto the movable stage and the adjustable controls were set to remove approximately 10μm per pass against the microtome blade. The blade was angled at 45 degrees to the block to reduce the cutting force applied to the block, thereby minimising the chance for damage to the specimen. The block was repeatedly sectioned until the entire surface of the embedded bone had been completely exposed. The specimen was then removed from the microtome and surface stained using toluidine blue at a pH 8.0.

Section Acquisition

Digitisation

Digitisation was carried out in two different ways depending on the intended study. The basic computer equipment was the same for each experiment. An Apple Macintosh model 7500 with a 100 MHz microprocessor, a 1 GB hard drive and 100 MB of RAM was used for this experiment. A 15 inch monitor set to a size of 640 X 480 was connected to the computer.

Digital Microscope. In this experiment, a dissecting microscope with a video camera attachment was utilised. The video was attached to the 7500 via the 'video in' port, a phono plug with a native video capture card attachment on the motherboard. The software utilised for this experiment was 'NIH Image', a freeware scientific image processing program available on the internet (http://rsb.info.nih.gov/NIH_Image). The region of interest was brought into focus under the microscope and the image was captured with the video card of the 7500. Each image was saved as a '*.tif' file for later processing.

For this study, the microscope was set at a magnification of 2X, which equated to 45X at the level of the bone surface. The microscope was calibrated by placing a stage micrometer in the field at the level of the bone surface. The photomicrograph was captured and the calibrated scale was measured manually for a distance of 1mm using NIH_Image. This equated to 0.135 pixels per μm or 1 pixel equal to 7.4 μm. All the sections for this study were digitised in 256 shades of grey for better image contrast between bone and marrow, at a resolution of 768 X 576, the limits set by NIH_Image. Therefore, the field size obtained for study was approximately 5.68mm X 4.26mm.

Sampling Methods.

The calcified cartilage layer was visualised along all aspects of the joint surface. Using an indelible ink pen, marks were made at the proximal-most aspect of the calcified cartilage layer on both the palmar (A) and dorsal (B) surfaces of the hyaline cartilage. A third mark was made at the transverse ridge of the metacarpus (C). Two imaginary chords, AC and BC were generated and the final two marks were made at the mid-point of these chords by dropping a perpendicular line back to the calcified cartilage layer (points D, E) **(Fig 4.5)**.

At a magnification of 45X, the bone was examined. Starting at Point A, the specimen was placed on the microscope stage such that the ink mark delineated the left hand border of the imaging field and the tidemark formed the bottom. The image was brought into focus and captured as a *.tif file labelled as 'Section 1'. The specimen was then moved along the microscope stage so that the right hand border of Section 1 became the left-hand border of Section 2, with the tidemark still serving as the baseline of the image. The specimen was then moved further along stage until Point D became visible as the right-hand margin of Section 3 and the left-hand margin of Section 4. Sections 5 and 6 were obtained in a similar fashion on either side of Point C at the transverse ridge. Sections 7 and 8 utilised

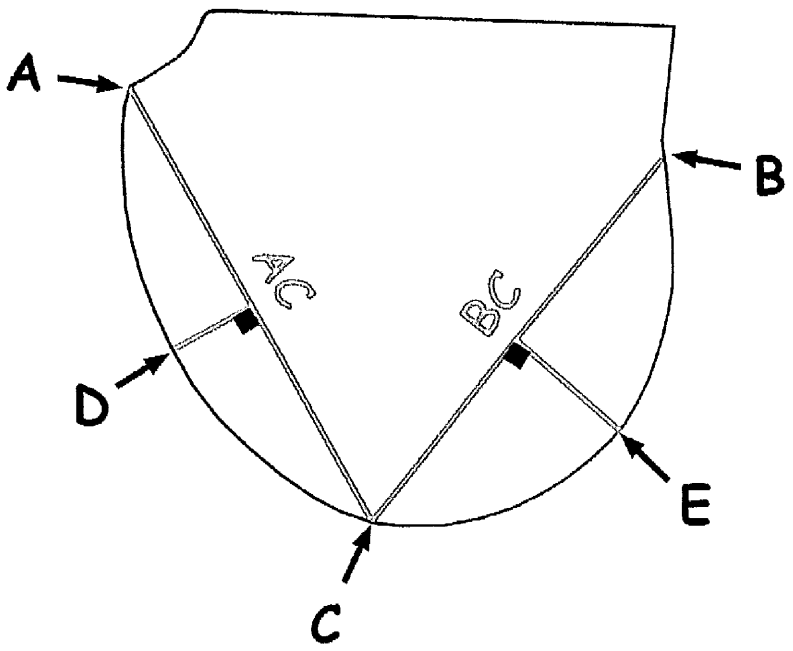


Fig 4.5 Schematic diagram of the method used to determine sample sites.

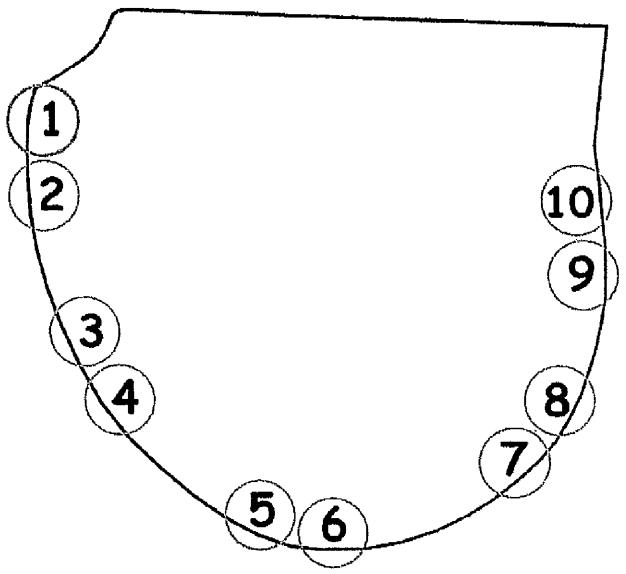


Fig 4.6 Schematic diagram illustrating the location of the sections for the bone histomorphometry studies.

Point E as their right-hand and left-hand margins respectively. Sections 9 and 10 were obtained in an opposite way to Sections 1 and 2. Section 10 was digitised before Section 9 by using Point B as the right hand margin of the field. Section 9 was subsequently obtained by transforming the left-hand margin of 10 into the right hand margin of 9 **(Fig 4.6)**. At all times the tidemark served as the baseline border of the image. Only sections 3 and 4 on the palmar aspect of the joint and sections 7 and 8 on the dorsal aspect were used for analysis. These sections are located in the regions of sesamoidean and P1 articulation respectively.

Bone Histomorphometry

Bone Area.

The percent bone area was determined by the grid counting method. In stereologic terms, this equates to estimating P_P, or the bone area divided by the total area of the field (B.Ar/T.Ar) **(Russ 1995)**. This was used instead of bone volume because the sections were effectively two dimensional. A macro was written for NIH_Image that placed grids of different sizes across the top of the image. Initially, a grid of cell size 10 pixels by 10 pixels was used and the percent bone was estimated by counting the number of times a grid intersect registered a 'hit' on bone vs. a marrow space. The same procedure was carried out for grid sizes of 20, 30, 40 and 50 pixels for validation of the technique. The results were then analysed via the coefficient of variation to determine whether each method produced comparable results. Once these results were analysed, a grid of 100 intersects and size of 50 pixels per cell was chosen for ease of counting **(Fig 4.7)**.

Estimation of P_L.

P_L is defined as the number of points or intersects per unit line **(Russ 1995)**. To estimate this, a macro was created for NIH_Image that laid a horizontal grid over the same region of bone as the bone area grid. A total was generated for each of the 10 lines on the grid and expressed as the number (P) per length of line (L).

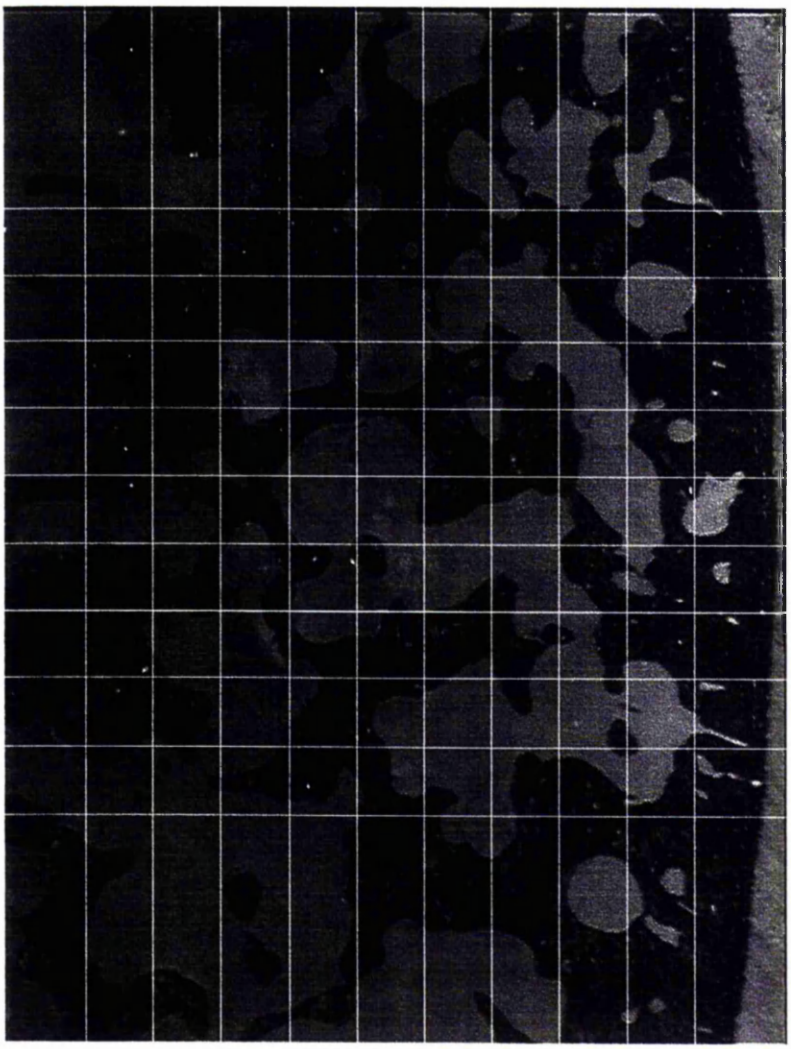


Fig 4.7 This photomicrograph shows a bone section with the grid in place for estimating B.Ar (P_p)

The P_L value for each line was determined starting at the calcified cartilage layer and proceeding proximally in the specimen. The P_L for the entire specimen was then calculated by summing the number of times the bone intersected the horizontal line over the entire specimen divided by the length of all the lines of the horizontal grid.

Calculation of Bone Histomorphometric Parameters.

Using the measured values of P_P and P_L, the standard bone histomorphometric parameters were calculated using the following formulae **(Parfitt 1987, Russ 1995)**:

Bone Area (B.Ar) = P_P

Trabecular Width (Tb.Wi) $= \frac{2 * P_P}{P_L}$

Trabecular Number (Tb.N) $= \frac{P_P}{Tb.Wi}$

Trabecular Separation (Tb.S) = (1/Tb.N) - Tb.Wi

Statistical analyses.

Statistical analyses were performed on the results to determine if there was any difference in B.Ar (P_P), P_L or Tb.Wi between the different sections within a horse using a repeated measures two-way ANOVA with one factor repetition. Then the data for each of the different sections among horses were analysed according to region using a standard two-way ANOVA with interaction to determine if there

were any age, gender or weight effects. A one way ANOVA was done to see if there were any significant affects of breed. Significance for all tests was set at $P < 0.05$. The relationship between P_P and P_L was calculated via regression analysis **(G.Gettinby, personal communication)**.

Results

Validation of Bone Area Procedure

No significant differences were noted between the different grids, therefore box size 50 was chosen for ease of counting.

Descriptive Results

The B.Ar varied greatly between sections. Morphologically, the bone displayed very obvious differences between sections. In some sections, the entire field was composed of densely packed bone with no evidence of any trabecular pattern leading to a B.Ar of almost 100%. At the other extreme, some fields appeared to be more marrow spaces than trabecular bone, producing numbers of under 50 percent bone for the section **(Fig 4.8 and 4.9)**.

The estimated B.Ar ranged from 42% to 95%. The mean B.Ar was 76 $\pm$ 12% and the median was 76%. The values were grouped and examined by age, breed, gender and weight. **(Table 4.1)**

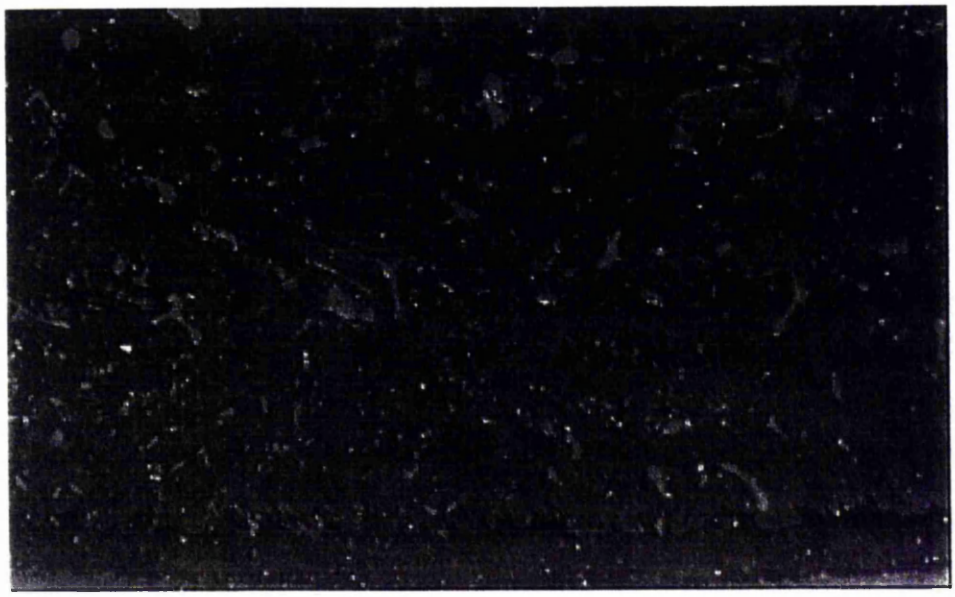


Fig 4.8 Section with high B.Ar %. Almost the entire field is bone.

Fig 4.9 Section with low B.Ar %. There are more marrow spaces than bone tissue.

	BA3	BA4	BA7	BA8
<5 Years	73 ± 9	78.33 ± 10	60.83 ± 13	63.5 ± 12
5-9 Years	79.63 ± 9	79.38 ± 10	79.88 ± 7	75.5 ± 16
10-14 Years	76.8 ± 9	77.4 ± 18	79.2 ± 11	78.4 ± 9
15-19 Years	78 ± 11	84.2 ± 9	82.4 ± 12	83.2 ± 12
20± Years	76.3 ± 15	74 ± 11	77.1 ± 14	74.33 ± 11
TB	78.53 ± 10	80.11 ± 11	78.84 ± 11	78.11 ± 11
TBx	74.83 ± 13	75 ± 11	73.5 ± 13	70 ± 18
Pony	73.6 ± 14	72 ± 15	71.6 ± 18	71.8 ± 16
X	75.75 ± 10	80.25 ± 10	71.5 ± 18	72 ± 17
Geldings	75.06 ± 11	76.59 ± 9	73.65 ± 14	72.12 ± 15
Mares	78.59 ± 11	79.47 ± 13	78.29 ± 12	77.94 ± 12
< 600 lbs	71 ± 11	70.5 ± 11	66.67 ± 16	67.17 ± 14
700 lbs	73.17 ± 13	74.83 ± 11	74.5 ± 15	74.67 ± 15
800 lbs	76.43 ± 14	79.86 ± 15	76.71 ± 16	81.14 ± 15
1000 lbs	82.11 ± 7	79.55 ± 9	80.33 ± 8	73.33 ± 15
>1000 lbs	78.83 ± 8	84.33 ± 9	79.33 ± 8	78.67 ± 3

Table 4.1 Summary of B.Ar % for sections in the Study

Comparisons for all horses

Age vs. Section

Neither age nor section independently had any significant effect on B.Ar (P = .26 and .23). The interaction between age and section, however, did have a significant effect (P = .001).

Section within Age

Section within Age Group 1. Section 4 had significantly more bone than 7 and 8, while 3 only had significantly more than 7. Sections 3 and 4 were not different from each other, nor were 7 and 8.

Section location was not significant in Age Groups 2 through 5.

Age within Section

There were no effects of age within sections 3, 4 or 8.

Age Group within Section 7. Age Groups 3 and 4 had significantly more bone than the youngest horses.

Gender vs. Section

There were no significant effects noted for gender, section or the interaction between them (P = .24, .4 and .85).

Weight vs. Section

There were no significant effects of weight, section or the interaction between them (P = .29, .44 and .58).

Comparisons within Section

Each section was examined independently for interactions between age, breed, gender and weight.

Section 3

There was no effect noted for age, breed, gender, weight or the interaction between them in Section 3. **(Graphs A1.7-10)**

Section 4

There was no effect noted for age, breed, gender, weight or the interaction between them in Section 4. **(Graphs A1.11-14)**

Section 7

There was no effect noted for breed, gender, weight or the interaction between them in Section 7. There was a trend towards significance noted for age (P = .06), with the youngest horses tending to exhibit less bone than the other four age groups. **(Graphs A1.15-18)**

Section 8

There was no effect noted for age, breed, gender, weight or the interaction between them in Section 8. **(Graphs A1.19-22)**

P_L

Descriptive

P_L, or the number of times that a straight line intersects bone, was measured for all sections in the same field used to determine P_P. P_L varied greatly across sections and within each field as well. In most cases the number of intersections increased as the distance from the CC increased, however this was not a linear relationship in any section. In other fields, most notably in young animals, the P_L was high or highest at the most basal level and decreased as the distance from the CC increased **(Fig 4.10 and 4.11, Graph A1.43 and A1.44).** As stated in the previous segment on B.Ar, the appearance of the bone in each field was very different. In those specimens with a very high B.Ar, a correspondingly small number of intersections was usually the result. In contrast, when the B.Ar decreased, there were usually more marrow spaces resulting in more, as well as narrower trabeculae.

This architecture would obviously lead to a higher P_L.

The P_L ranged from .32 to 3.92/mm. The mean P_L was 1.8 $\pm$ .69/mm and the median was 1.69/mm. The values were grouped and examined by age, breed, gender and weight.

Comparisons for All Horses

Age vs. Section

The age had a significant effect on P_L (P = .01), but the Section and the interaction between them did not (P = .1 and .32). For age, Group 1 had a significantly higher P_L than groups 2 and 4. No other differences were noted.

Gender vs. Section

There was no significant effect of gender, section or the interaction between them (P = .1, .11 and .72).

Weight vs. Section

Weight exhibited a significant effect on section (P = .025), but section itself and the interaction between weight and section was not significant (P = .1 and .14). Weight group 1 had a significantly higher P_L than groups 3 and 5.

Comparisons within Section

Each section was examined independently for interactions between age, gender and weight.

P_L3

There was no effect noted for age, breed, gender, weight or the interaction between them in Section 3.

P_L4

Age exhibited a significant effect on P_L (P = .03), with the youngest horses exhibiting the highest P_L. Weight had a significant effect (P = .03). Weight group 1 was different from both 3 and 5. Gender showed a slight trend toward significance (P = .06).

P_L7

Age had a significant effect on P_L (P = .04), with age group 1 exhibiting a higher P_L than 4. Breed, gender and weight had no effect.

P_L8

Age showed a significant effect (P = .02), with age group 1 showing a significant difference from both groups 2 and 4. Breed, gender and weight again had no effect.

Trabecular Width (Tb.Wi)

Descriptive

Visually, the thickness of the trabeculae varied dramatically between sections as can be seen in the figure relating to B.Ar **(Fig 4.8 and 4.9)**. Although this value is calculated from the bone area P_P and the surface area P_L, it was important to determine if any independent relationships existed for section, age, breed, gender or weight. Tb. Wi, Tb.S and Tb.N are reported for each of the sections **(Tables 4.3-4.5)**

Comparisons for All Horses

Age

There was no significant effect for age when examined for all horses by section. When each section was examined separately, age showed no effect when in combination with weight for any of the sections. When examined with gender, however, age showed a significant effect for gender only in Section 4 (P = .01).

	Tb.Wi (µm)		Tb.N (µm)		Tb.S (µm)	
	(Mean)	(StDev)	(Mean)	(StDev)	(Mean)	(StDev)
<5 Years	764	475	1127	366	241	57
5-9 yr	1483	957	694	300	274	50
10-14 yr	973	274	795	154	324	34
15-19 yr	1266	376	661	136	296	92
20+ yr	1019	502	884	330	251	91
TB	1194	641	773	274	271	76
TBX	1021	475	841	288	286	59
Pony	1104	752	888	445	294	92
X	820	445	1059	364	230	68
Male	965	433	897	313	275	78
Female	1248	717	773	311	270	72
600	1094	489	837	379	266	62
700	1403	494	643	176	259	99
800	1255	975	785	294	295	73
1000	861	458	1021	375	260	66
>1000	1018	410	807	185	285	86

Table 4.2 Summary of Bone Histomorphometric Values for Section 3

	Tb.Wi (µm)		Tb.S (µm)		Tb.N (µm)	
	(Mean)	(StDev)	(Mean)	(StDev)	(Mean)	(StDev)
<5 Years	788	507	1193	406	182	71
5-9 yr	1236	680	771	294	251	91
10-14 yr	864	513	992	287	245	98
15-19 yr	2043	1929	608	264	243	113
20+ yr	827	393	1032	348	251	80
TB	1313	1213	834	345	239	90
TBX	882	488	988	327	246	85
Pony	852	711	1122	467	247	114
X	933	561	1027	401	198	78
Male	874	380	1000	350	244	90
Female	1370	1320	850	380	229	90
600	545	137	1334	256	234	111
700	915	599	974	298	244	63
800	1297	703	800	438	231	107
1000	958	328	895	230	230	92
>1000	1962	1975	663	332	248	87

Table 4.3 Summary of Bone Histomorphometric Values for Section 4

	Tb.Wi (µm)		Tb.N (µm)		Tb.S (µm)	
	(Mean)	(StDev)	(Mean)	(StDev)	(Mean)	(StDev)
<5 Years	479	175	1357	337	292	79
5-9 yr	1082	381	776	180	281	54
10-14 yr	938	295	941	268	187	85
15-19 yr	1417	939	734	334	246	90
20+ yr	1203	788	843	385	269	98
TB	1213	686	770	257	270	86
TBX	791	279	999	255	263	105
Pony	965	930	1186	628	230	38
X	763	533	1128	378	235	114
Male	937	590	1010	430	263	90
Female	1160	720	824	280	255	70
600	774	860	1304	521	253	77
700	790	332	1043	291	233	90
800	1318	966	772	317	276	106
1000	1221	572	734	191	263	59
>1000	1012	257	835	259	265	118

Table 4.4 Summary of Bone Histomorphometric Values for Section 7

	Tb.Wi (µm)		Tb.N (µm)		Tb.S (µm)	
	(Mean)	(StDev)	(Mean)	(StDev)	(Mean)	(StDev)
<5 Years	519	177	1281	240	286	89
5-9 yr	1262	826	757	327	313	99
10-14 yr	1020	307	835	212	239	85
15-19 yr	1303	781	759	297	237	132
20+ yr	895	511	1003	343	240	81
TB	1152	682	815	275	267	91
TBX	787	429	1035	361	287	137
Pony	886	622	1056	454	251	55
X	694	437	1189	330	219	114
Male	819	470	1050	350	261	100
Female	1170	700	820	300	263	80
600	617	322	1209	314	269	94
700	951	593	1015	460	247	85
800	1458	958	735	341	234	105
1000	893	505	932	240	278	118
>1000	1026	234	809	227	281	87

Table 4.5 Summary of Bone Histomorphometric Values for Section 8

No such effect was noted in Sections 3, 7 or 8. In section 4, age group 4 had a higher Tb.Wi than 1 or 5. **(Graphs A1.27,31,35,39)**

Breed

There was no significant effect of breed on trabecular thickness when examined by section or within each section. **(Graphs A1.28,32,37,40)**

Gender

Gender exhibited no significant effect on trabecular thickness when examined across all sections. Like age, gender only showed a significant effect when in combination with age in Section 4 ($P = .02$). Gender had no effect in Sections 3, 7 or 8 either. In section 4, females had a higher Tb.Wi than males. **(Graphs A1.29,33,37.41)**

Weight

There was no significant effect of weight on trabecular thickness when examined by section or within each section in combination with age or gender. **(Graphs A1.30,34,38,42)**

Regression Analysis of P_P and P_L

The regression slopes were calculated for the graph of P_P vs. P_L for each section and showed that the values were correlated **(Graphs A1.45-A1.48).** Two times P_L (S_V) was also graphed against P_P for all sections and showed a high degree of correlation **(Graph A1.49).** Finally, the relationship between P_P and P_L was tested against the intra-articular blood cell counts and MMP 2 values from

Chapter 3. No significant relationship was noted for any of these pairings (P = 0.05).

Discussion

It is important to be able to measure the amount of bone present within a specimen or an animal. In this way, parameters can be set up to help with interpretation of disease processes.

It should be noted that 'measuring' bone mass in a histological specimen should more accurately be labelled 'estimating' because there is no completely precise and true way to measure it. More than one method can be employed and the results compared, but each one is still just an estimate of the actual value. Years of extensive use within the human field have led to the wide acceptance of these techniques **(Parfitt et al 1987).** Data must be collected on 2 dimensional sections and then extrapolated to a 3 dimensional object. Such translations are the hallmark of the discipline known as *stereology* **(Russ 1995).**

In this particular study, several tenets of stereology were employed in addition to the traditional methods of bone histomorphometry. First, specimen preparation and sectioning proceeded in a rigorous manner to ensure as accurately as possible that each field to be examined and compared came from approximately the same region on each horse. Although many principles of stereology rely on random sampling to establish an accurate representation of the subject, comparing regions between animals requires that the same location be sampled from each horse. After the specimen has been prepared and sectioned, fields for measurement and comparison must be established. Once again, rigorous standards were set to ensure that similar fields were examined on each specimen. We chose permanent and reproducible landmarks in order to be as consistent as possible. These were the proximal extent of the CC on both the dorsal and palmar surfaces of the joint,

the transverse ridge and the midpoint between these points. The CC was an easily defined landmark once the section was stained with toluidine blue. Similarly, the transverse ridge is a consistent finding on sagittal sections.

Sections 3 and 4 on the palmar aspect of the joint and 7 and 8 on the dorsal aspect were chosen for analysis based on the results of a loading study **(Vilar *et al* 1995).** The palmar aspect is loaded by the sesamoid bones, and the dorsal aspect by the first phalanx. These regions are also the site for disease processes commonly found in the horse **(Pool 1996).**

The results for P_P or, effectively, the amount of bone tissue present per section, were highly variable. In some sections almost the entire field was bone, resulting in a 95% composition, while other sections had more marrow spaces than bone leading to an estimated bone area of below 50%. Although the range of these results had a wide spread, very few significant differences were noted for the parameters in our study. Neither age nor section exhibited any effect independently, but the interaction between them was very significant. The most important finding here was that the location of the section was only significant in the youngest group of horses. There, the palmar section, 4, had more bone than either of the two dorsal sections, 7 and 8. The other palmar section, 3, was only different from 7. The two palmar sections had similar amounts of bone, the same way the two dorsal sections did. This would seem to suggest that some factor is different on the palmar side from that of the dorsal aspect of the joint. Because no horses younger than 18 months of age were included in the study, a congenital effect on bone formation could not be ruled out. It is possible that this pattern of bone formation, more on the palmar than dorsal aspect, occurs during foetal development as a normal phenomenon or even during early weight-bearing and ambulation. The fact that this pattern does not continue into the older horses in this study may indicate that the stress on the joint may become more distributed. Factors that were not tested in this study, such as rigorous exercise, may begin to lay down bone in a more uniform way throughout the sub-articular regions.

However, it has been well documented that the palmar surface develops significantly more bone in some horses in response to high levels of exercise **(Pool 1996).**

When age groups were examined within sections, the only one that exhibited an effect was Section 7. For that section, the youngest horses had significantly less bone than those between the ages of 10 and 19 years. While this may indicate an effect of ageing on bone production in that region, the lack of significance in the other regions may yield more important information. There was a vast degree of variation in the amount of bone present in Sections 3, 4 and 8, but no significant effect could be ascertained when the factors of age, gender and weight were examined. Therefore, it would again seem likely that some untested factor would be responsible for the differences noted. As with any biological system, these factors should originate within the realm of genetics or environment. While the genetics are an obvious component, environment could include such factors as nutrition or physical activity. We propose that the most feasible effect might be mechanical stress, such as exercise or training. Because no performance history was available for these horses, this hypothesis could not be tested any further on this data set.

P_L

Age had a significant effect on P_L such that the youngest group of horses had the highest P_L, which was significantly different from the 5-9 year olds and the 15-19 year olds. Weight was also important because the lightest horses also had the highest P_L, significantly different from groups 3 and 5. These two observations would suggest that both maturity and mechanical stress might play a role on development and reinforcement of the trabecular architecture. As the horses get older, a decreased P_L means there are fewer individual trabeculae within the section. And the same observation seems to hold true as the horses increase in

weight. **Young *et al* (1991)** obtained similar results in their study on training and postulated that it was the body's response to fortify the bone to withstand fracture.

The results of the analysis by individual section show that both age and weight affected Section 4, whereas 7 and 8 were only affected by age. The same observations were noted in that the youngest and lightest horses had the less developed trabecular patterns. A thicker trabecular strut is less likely to undergo bending **(Young *et al* 1991)**.

Another interesting observation was that P_L increased with increasing distance from the CC in all but the youngest horses. This finding is similar to a study on the growth plate **(Fazzalari *et al* 1992).** In the young horses, however, the opposite relationship was true. In that group of horses, P_L decreased as the field moved away from the CC **(Graph A1.43 and A1.44)**. This would tend to suggest that age influences trabecular architecture. Less trabecular structure near the joint surface may allow for growth at the articular margin. On the clinical side, a less developed trabecular architecture could have implications for exercise. This would support the approach of no forced exercise in foals and may help to explain how some angular limb deformities develop.

A very interesting and enlightening study into the trabecular architecture of mammalian species was carried out recently by zoologists **(Swartz *et al* 1998).** They compared cancellous bone specimens for a whole range of mammals from rodents and bats to horses and humans. They found that trabecular size showed little dependence on body mass. They maintain this suggests that some elements of trabecular architecture may be driven by the requirements of maintaining adequate surface area for calcium homeostasis instead of strictly for strength of structure. More importantly, these researchers found that connectivity among trabecular elements was qualitatively different for the small vs. large animals in the study. Small animals were discovered to have very few trabeculae that connect mainly to cortical bone, whereas the larger animals have many more

trabeculae, but they connect much more extensively to each other. It should be noted, however, that the data for the large animals came from very few specimens and may therefore be prone to bias. Nevertheless, these findings seem to refute the theory that size or weight *between* individuals may be responsible for trabecular development. Our data does support that conclusion because there was no breed or weight affect noted in our study either. It does not rule out changes in size or weight within an individual, though, as the factor responsible for different bone parameters. This, of course, is only a speculation based on the effect of age.

It is difficult to directly compare the data from this study with other equine studies because few have been carried out in a similar manner. **Young et al (1991)** determined the increased bone in their specimens to be protective against fracture. **Norrdin *et al* (1998)** examined a region of bone similar to the areas from our study. The samples were from two populations of horses; one consisted of twelve racehorses that had died on the track, and the other was 12 research horses that were run on a treadmill. They found much lower B.Ar/T.Ar values for their treadmill horses than we did in our study. Because they did not publish their exact method for the point counting technique employed, especially the size of the field analysed, it is difficult to make a direct comparison. It would appear that the fields they counted might have extended further into the trabecular bone than ours did, resulting in a decreased B.Ar %. For our study, the counting was done in the subchondral region, probably accounting for the higher values. In another recent study, **Firth *et al* 1999** found that bone in the small carpal bones of exercised horses seemed to respond to treadmill training by laying down more bone. This was examined via dual energy x-ray absorptiometry (DEXA). Although no bone histomorphometric parameters were measured, subjectively the increase in bone mass is within the subchondral region, similar to the area we studied.

In relation to trabecular thickness, number and separation parameters, to the author's knowledge, no objective data exists for the horse. However, when comparing the numbers to several studies in the human field, the horse data

exhibits a much thicker trabecula. Although not suggested by the work of **Swartz *et al* (1998)**, there could be a size or weight function. More than likely, though, the reason for the higher trabecular thicknesses may be that our samples came from closer to the dense SCB.

When examining S_V vs. P_P, another interesting relationship surfaced **(Graph A1.49)**. The high correlation between these two independent measurements demonstrates that the amount of bone tissue and the surface volume are related for this population of horses. This indicates that factors other than structure may influence trabecular architecture. A similar finding has been reported for human vertebrae **(Fyhrie *et al* 1995).**

In conclusion, this study provided us with useful data involving the trabecular bone of the equine distal metacarpus. Future studies will be able to reference the values generated here.

APPENDIX 1

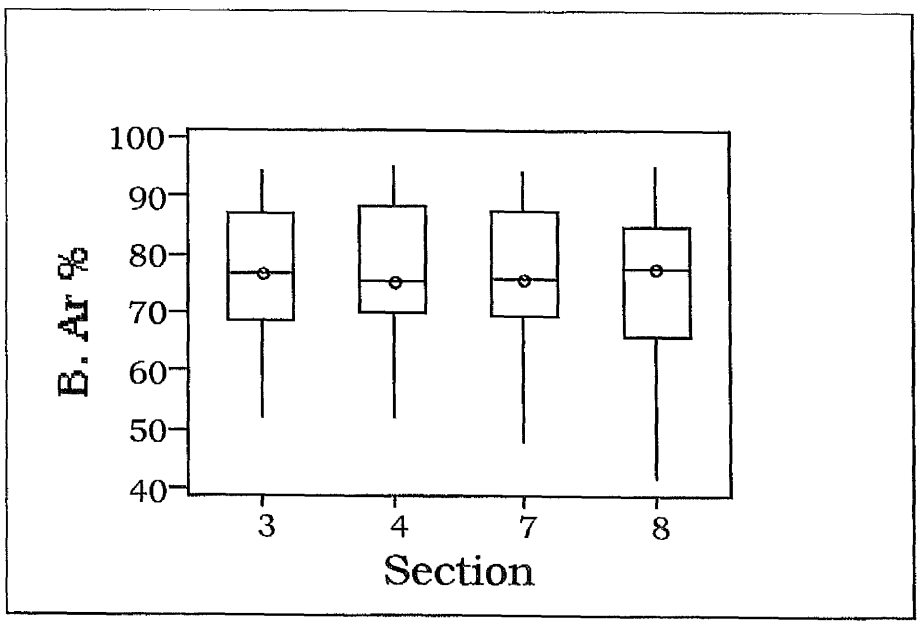


Table A1.1 Summary of B.Ar % by Section

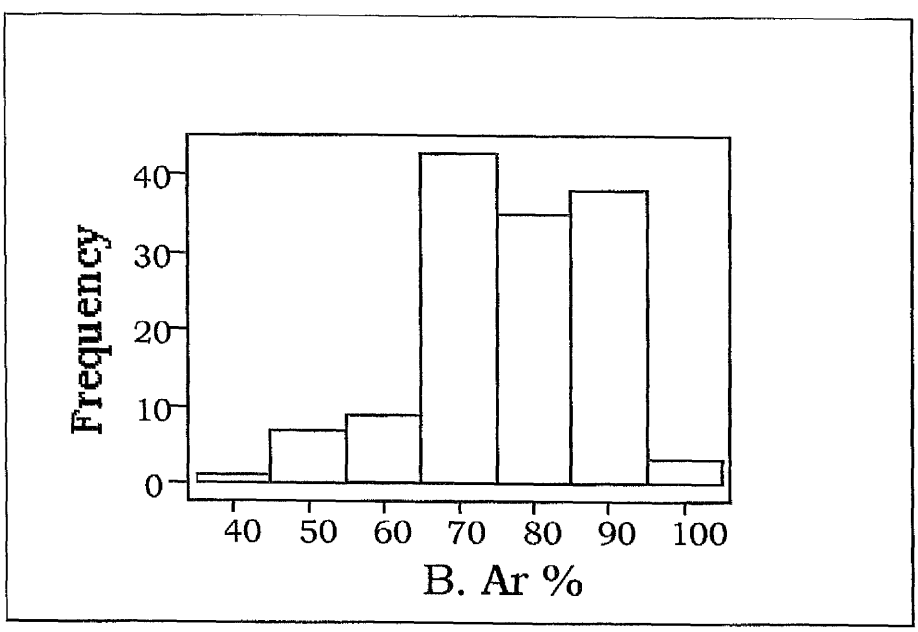
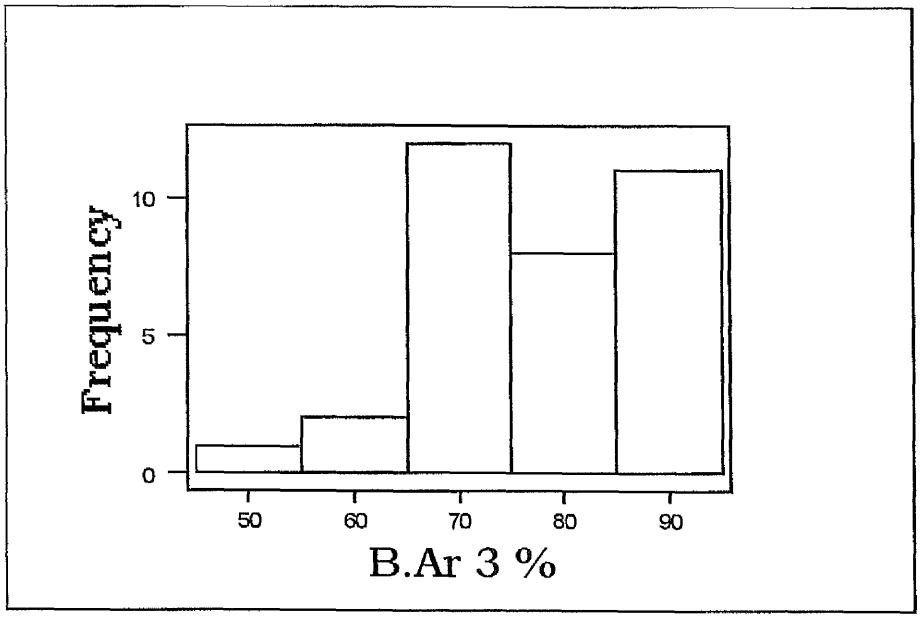
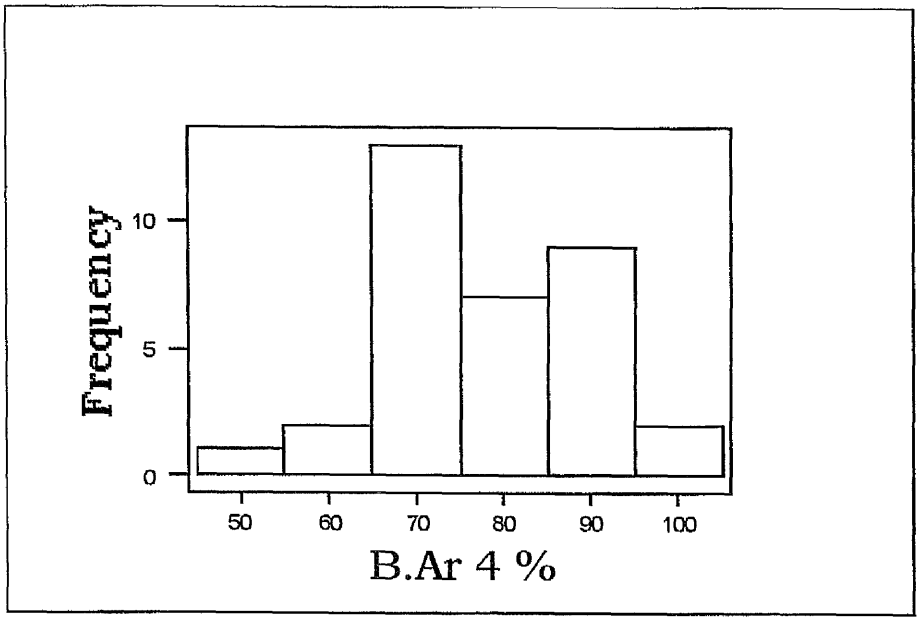


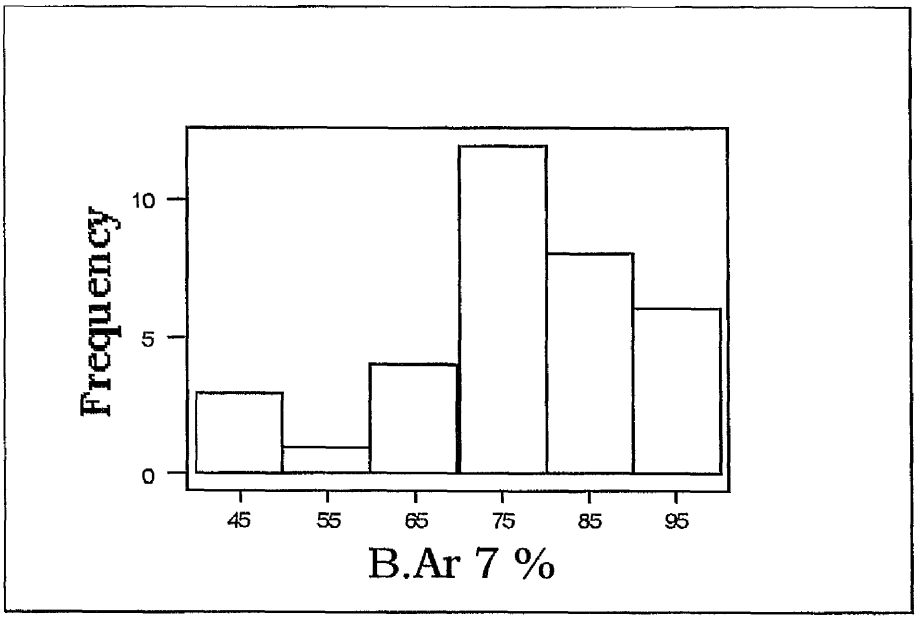
Table A1.2 Histogram of B.Ar % for all Sections



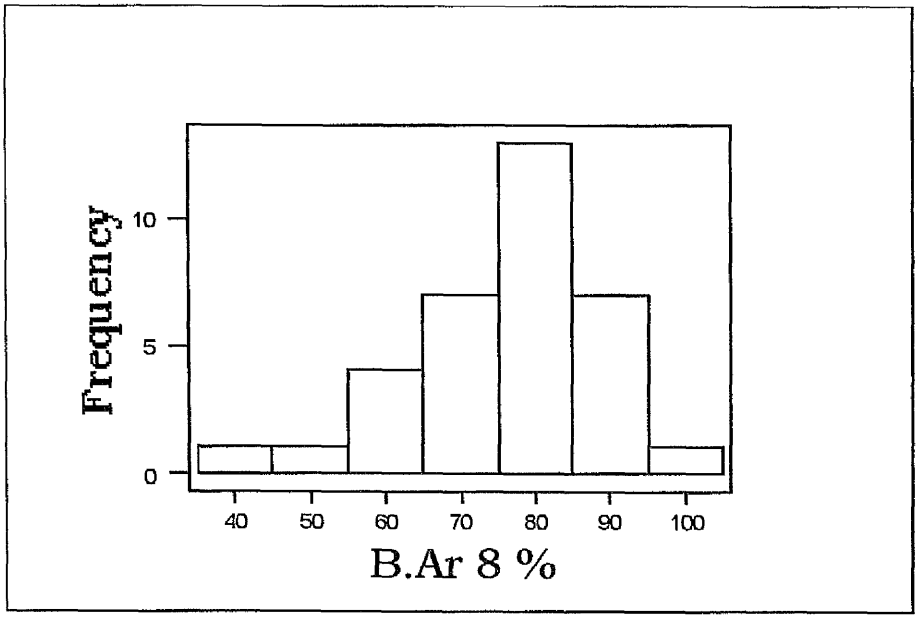
Graph A1.3 Histogram of values for B.Ar 3 %



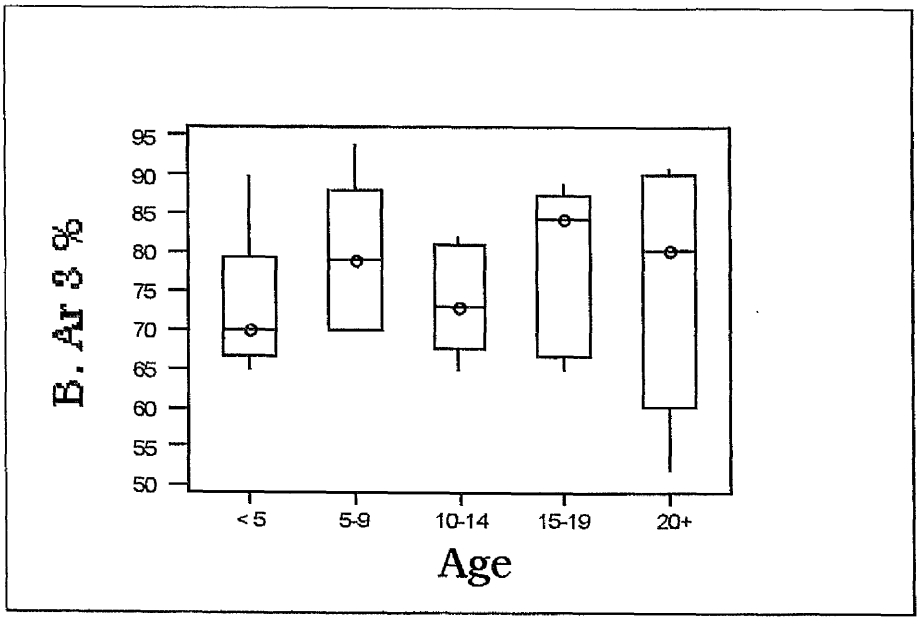
Graph A1.4 Histogram of values for B.Ar 4 %



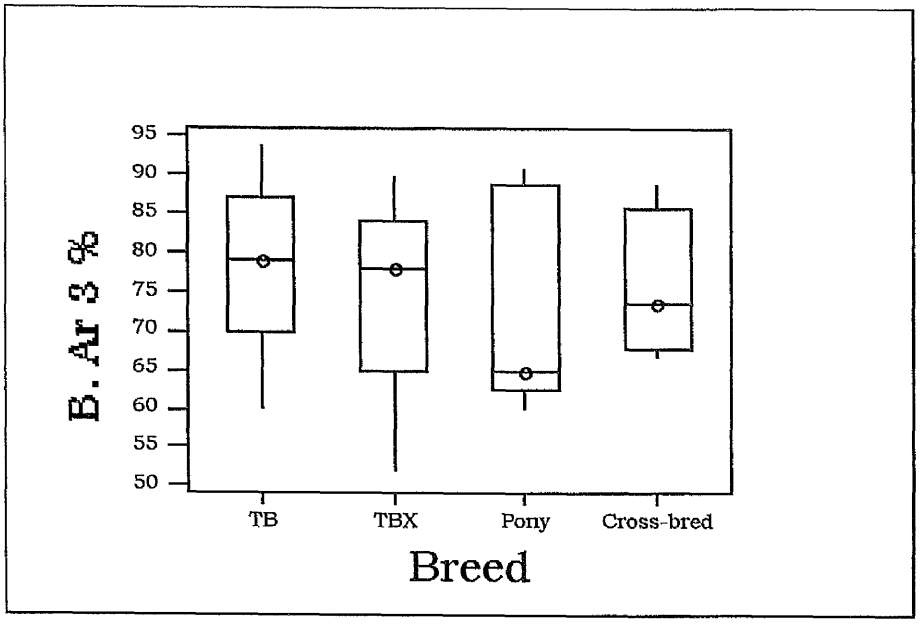
Graph A1.5 Histogram of values for B.Ar 7 %



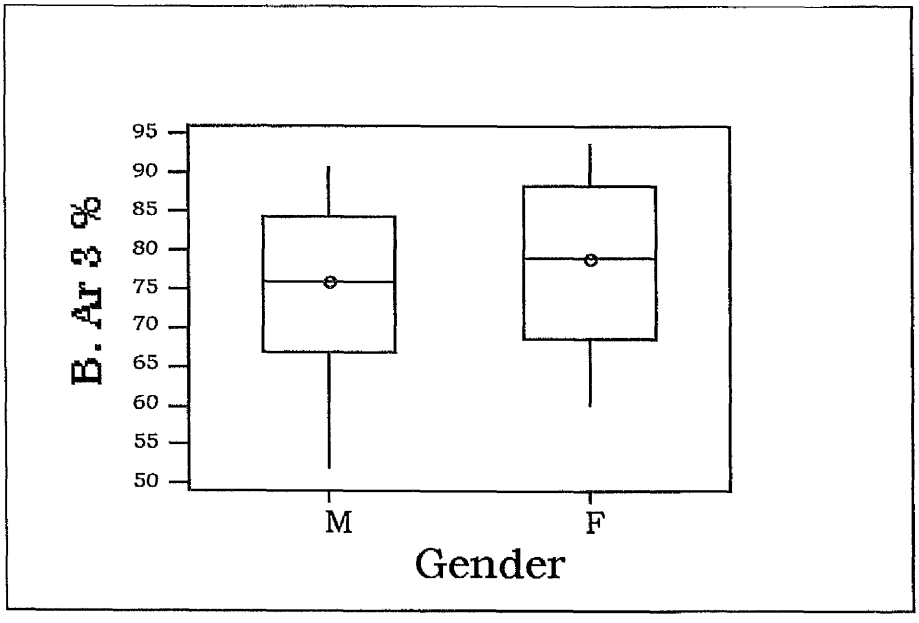
Graph A1.6 Histogram of values for B.Ar 8 %



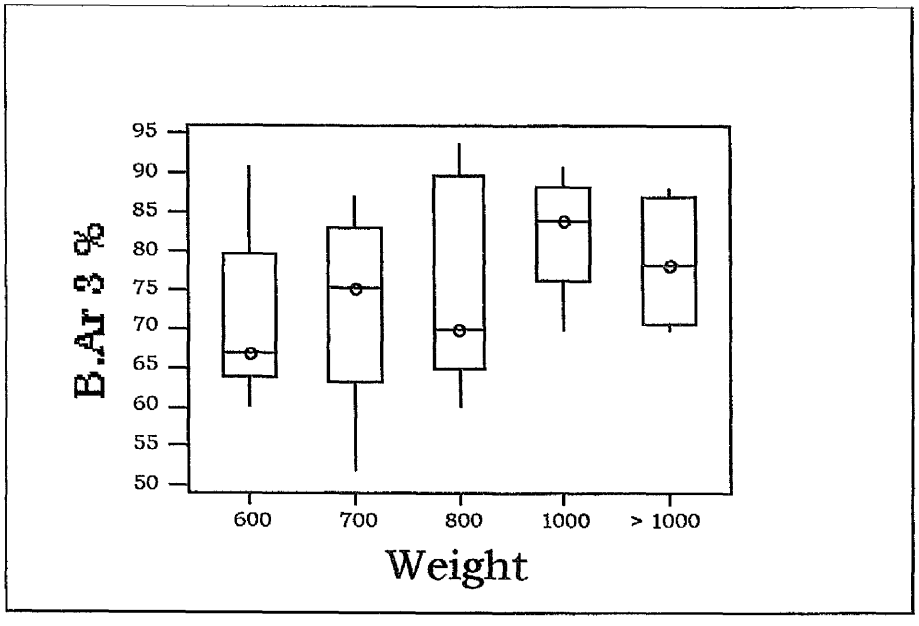
Graph A1.7 Boxplot of B.Ar 3 % by Age



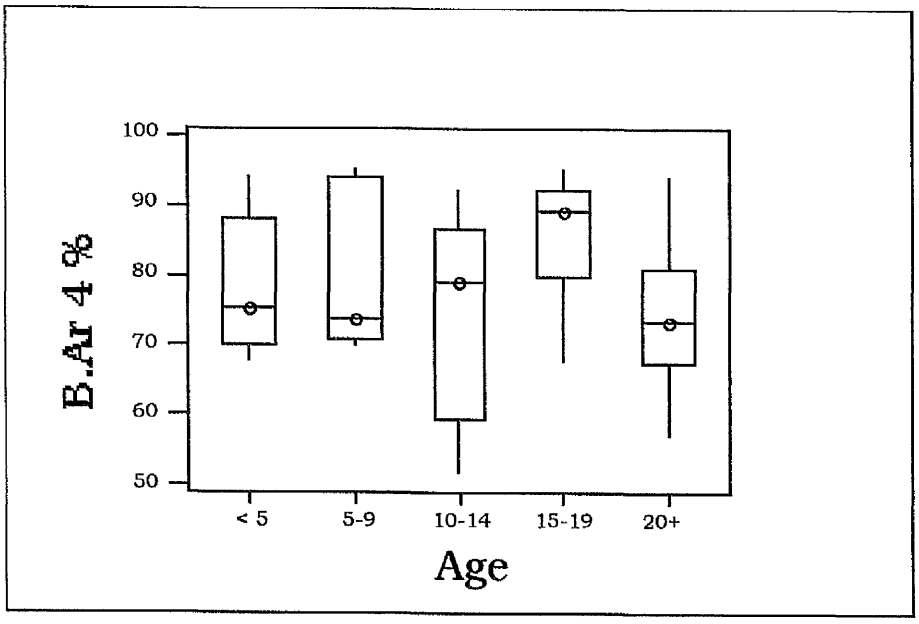
Graph A1.8 Boxplot of B.Ar 3 % by Breed



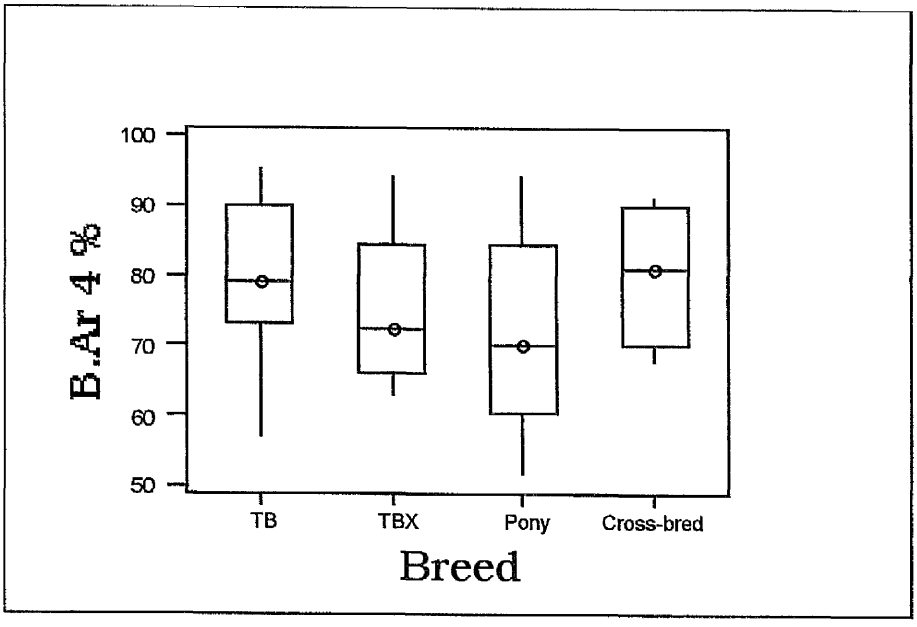
Graph A1.9 Boxplot of B.Ar 3 % by Gender



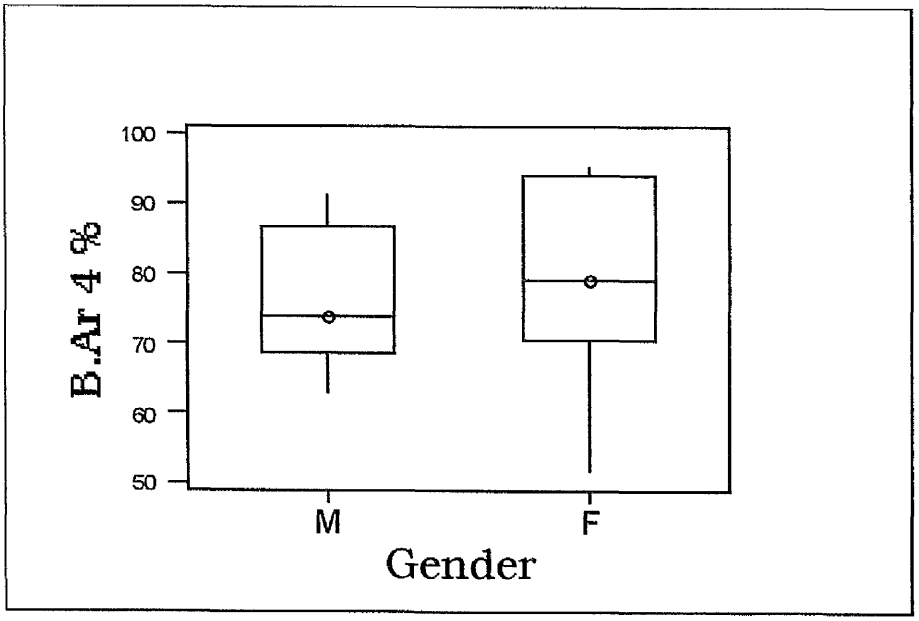
Graph A1.10 Boxplot of B.Ar 3 % by Weight



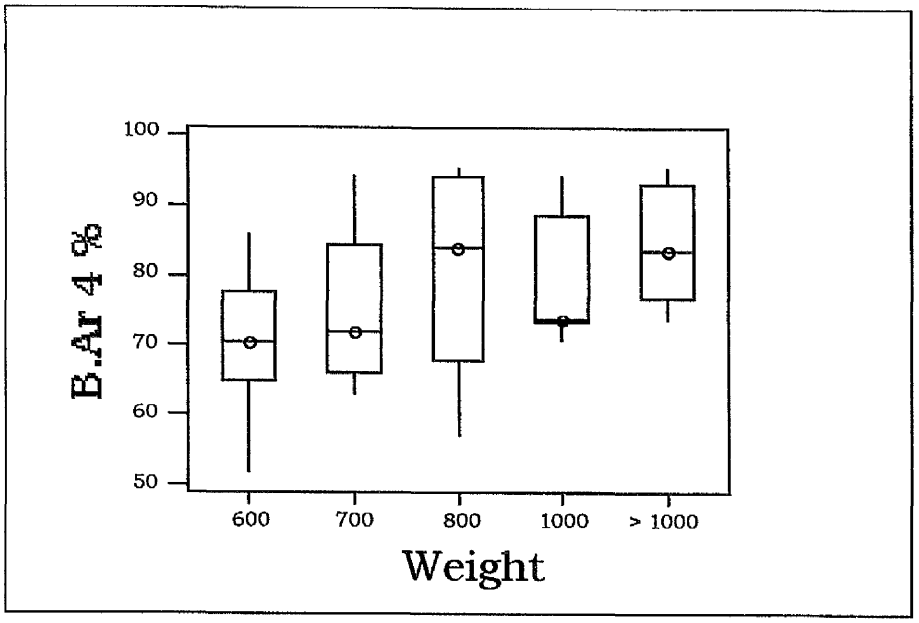
Graph A1.11 Boxplot of B.Ar 4 % by Age



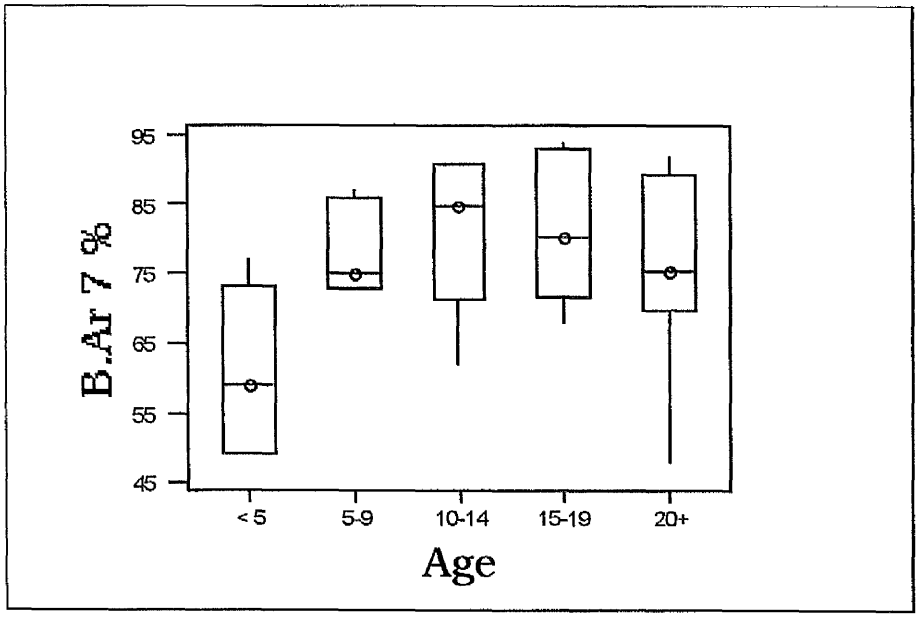
Graph A1.12 Boxplot of B.Ar 4 % by Breed



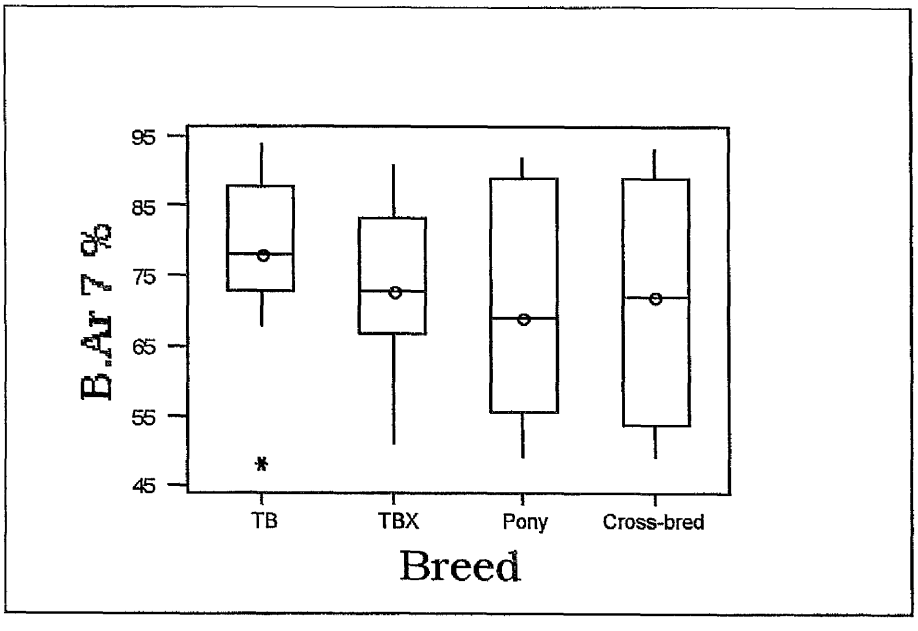
Graph A1.13 Boxplot of B.Ar 4 % by Gender



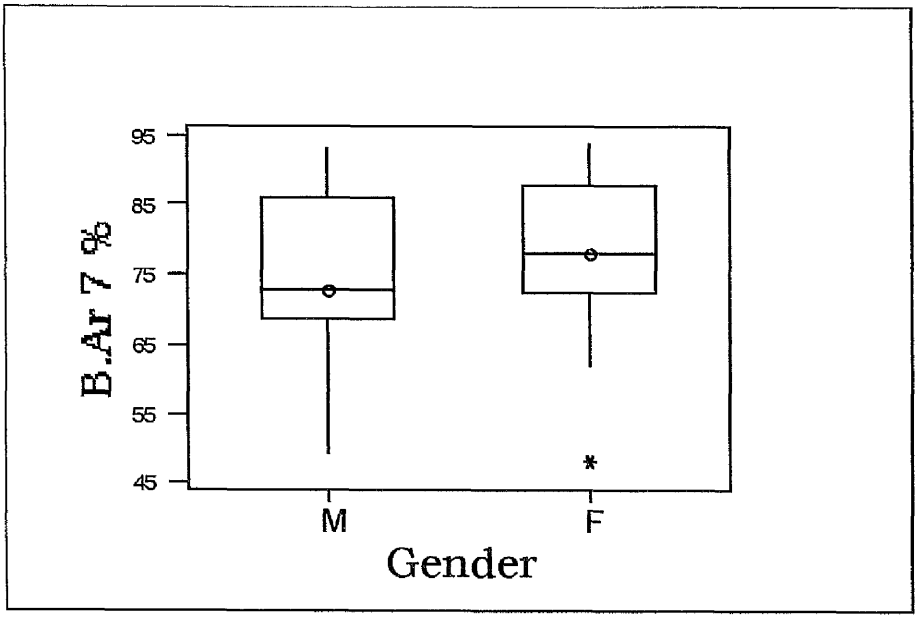
Graph A1.14 Boxplot of B.Ar 4 % by Weight



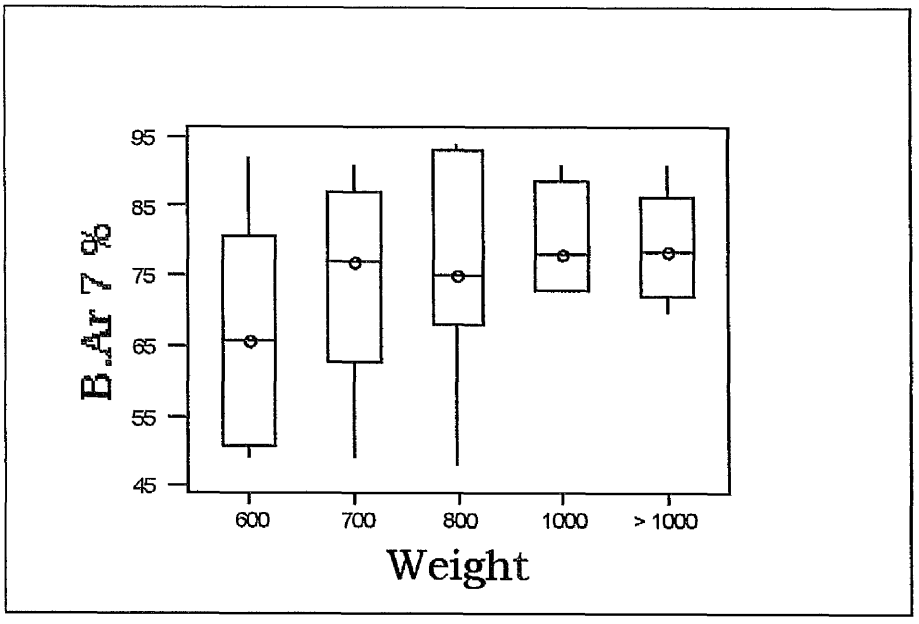
Graph A1.15 Boxplot of B.Ar 7% by Age



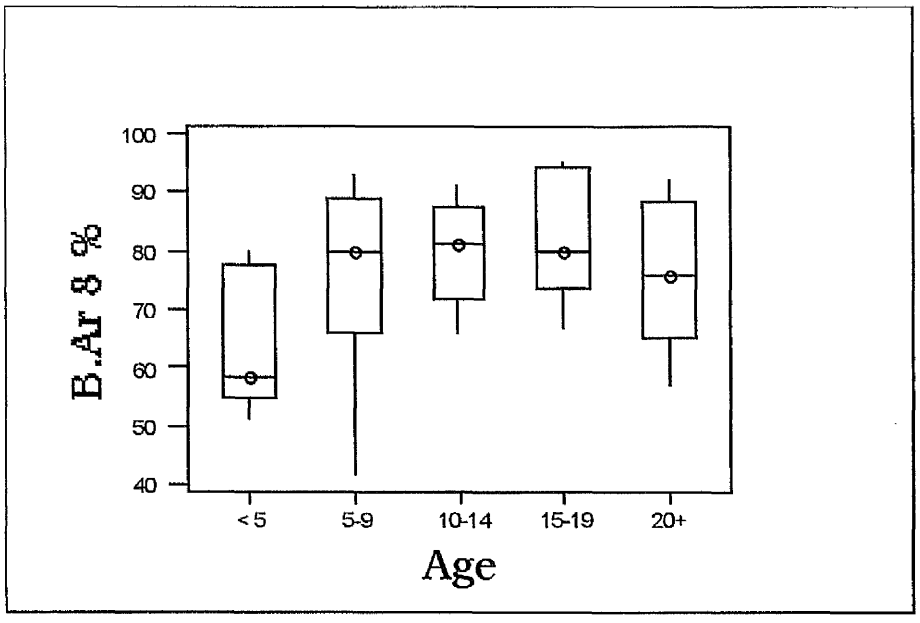
Graph A1.16 Boxplot of B.Ar 7% by Breed



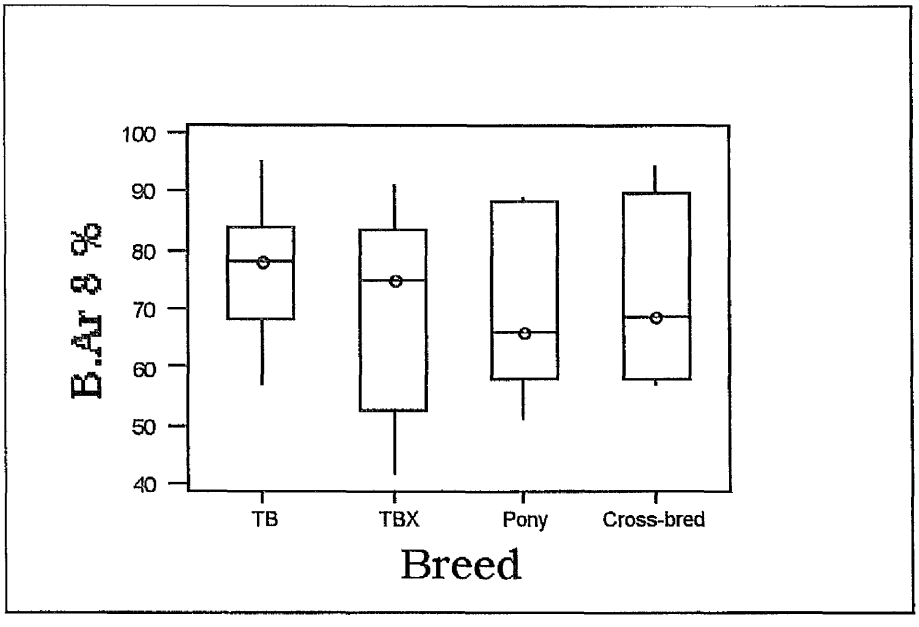
Graph A1.17 Boxplot of B.Ar 7% by Gender



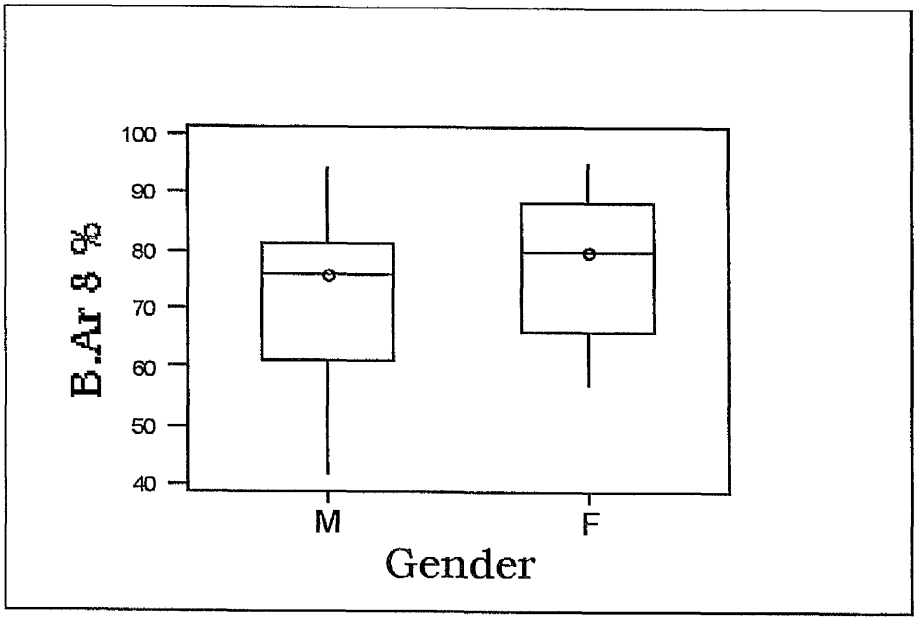
Graph A1.18 Boxplot of B.Ar 7% by Weight



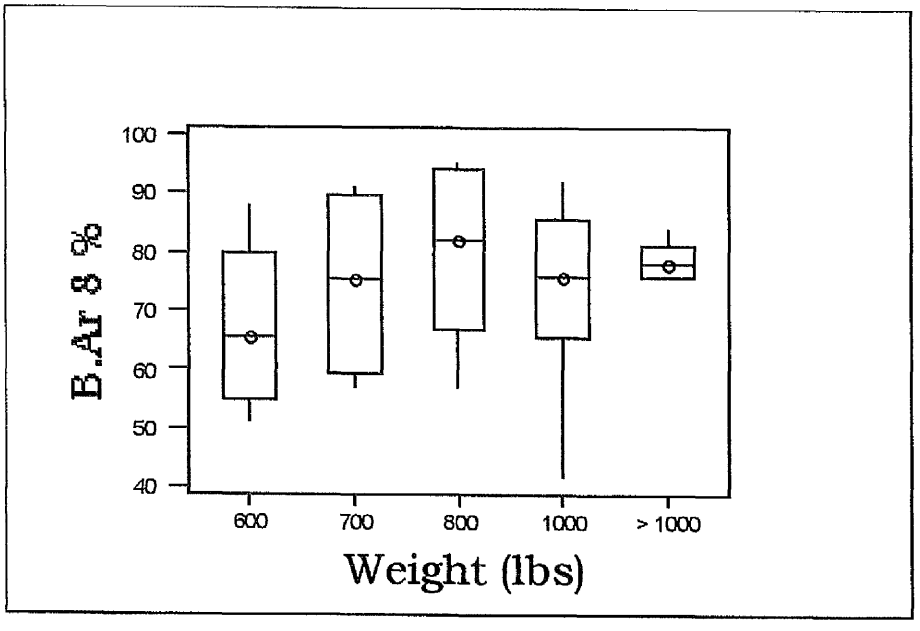
Graph A1.19 Boxplot of B.Ar 8% by Age



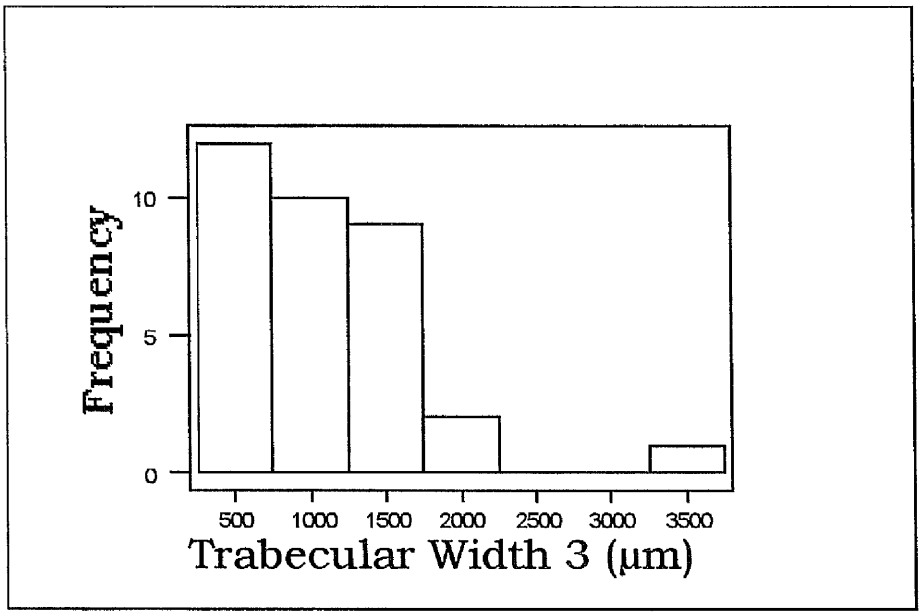
Graph A1.20 Boxplot of B.Ar 8% by Breed



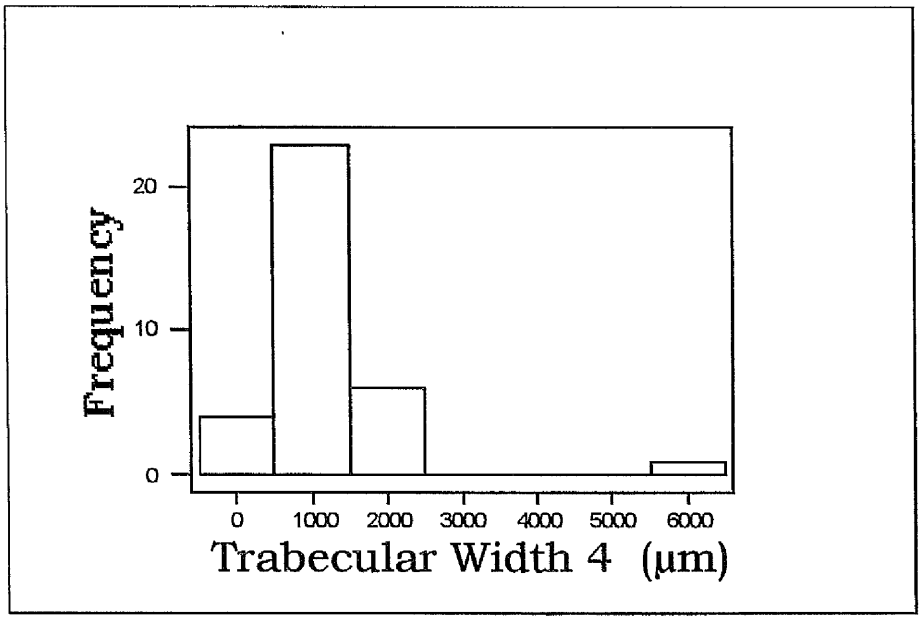
Graph A1.21 Boxplot of B.Ar 8% by Gender



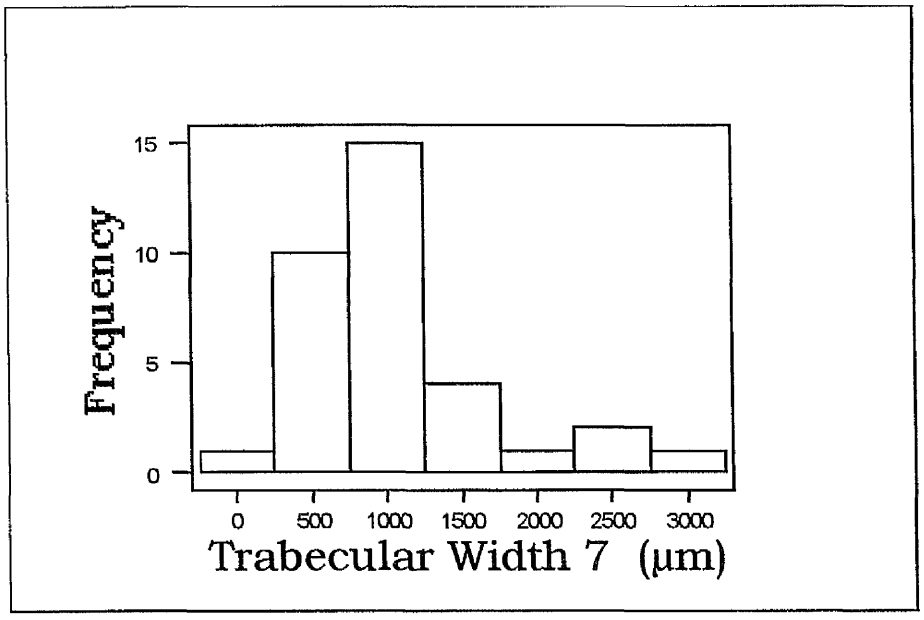
Graph A1.22 Boxplot of B.Ar 8% by Weight



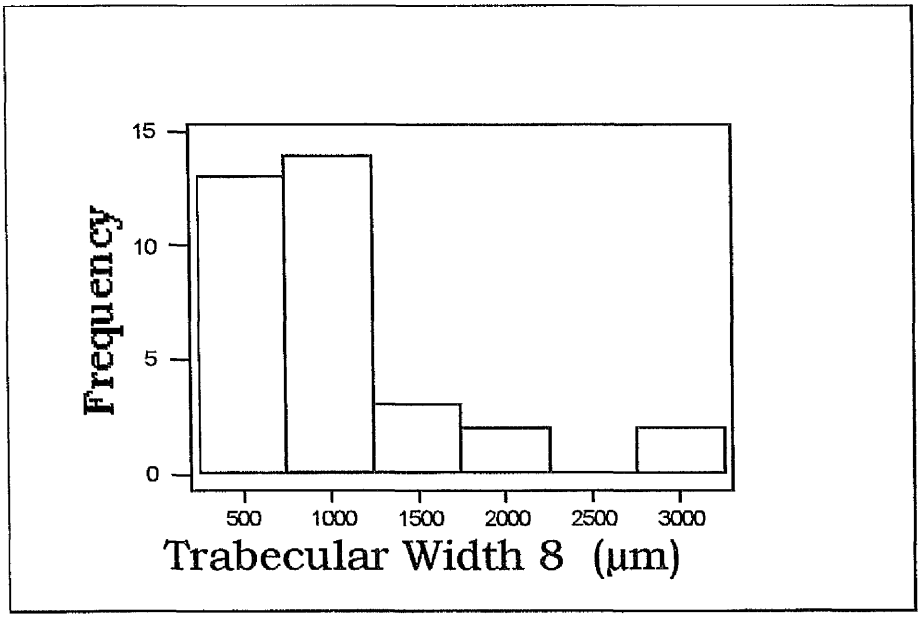
Graph A1.23 Histogram of Tb.Wi 3



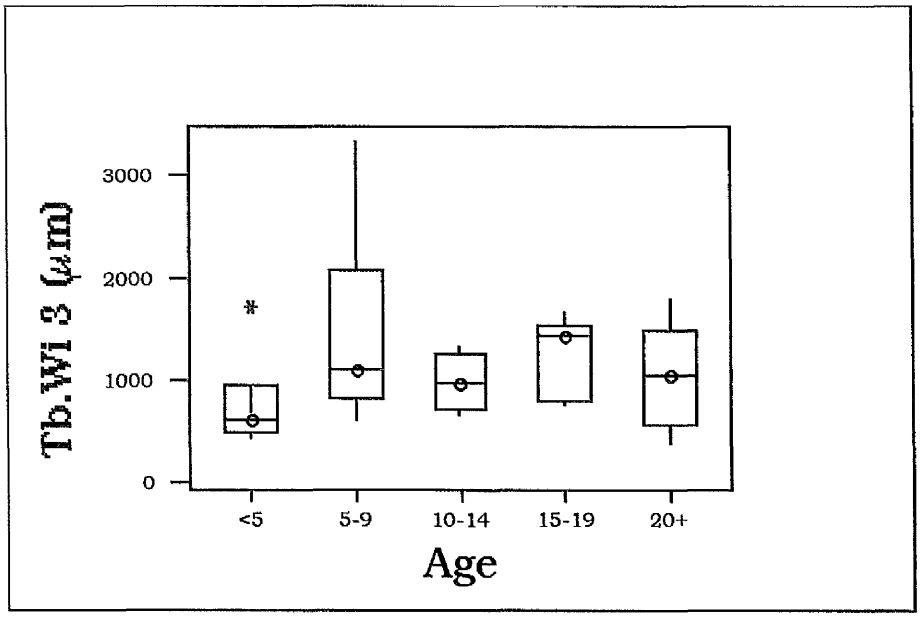
Graph A1.24 Histogram of Tb.Wi 4



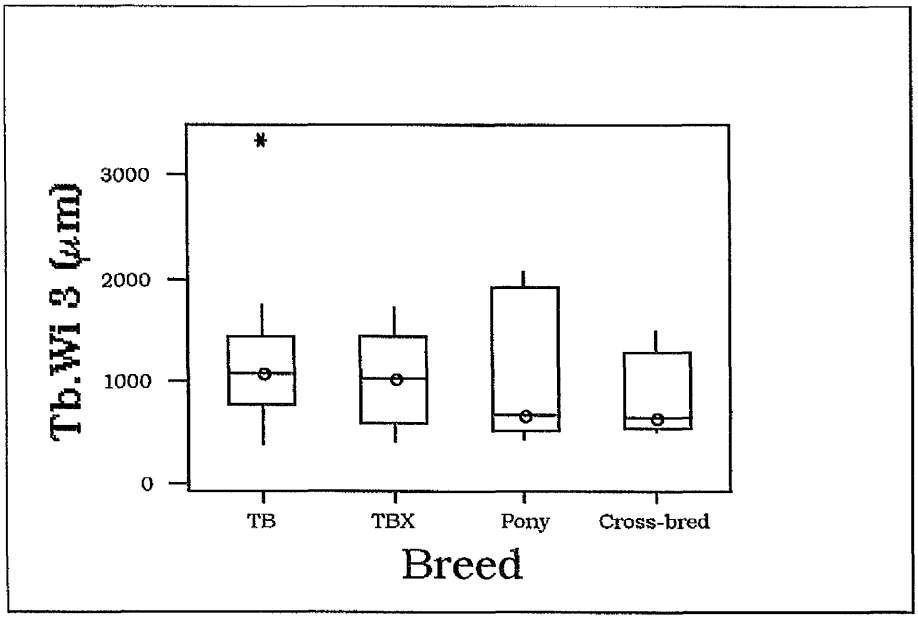
Graph A1.25 Histogram of Tb.Wi 7



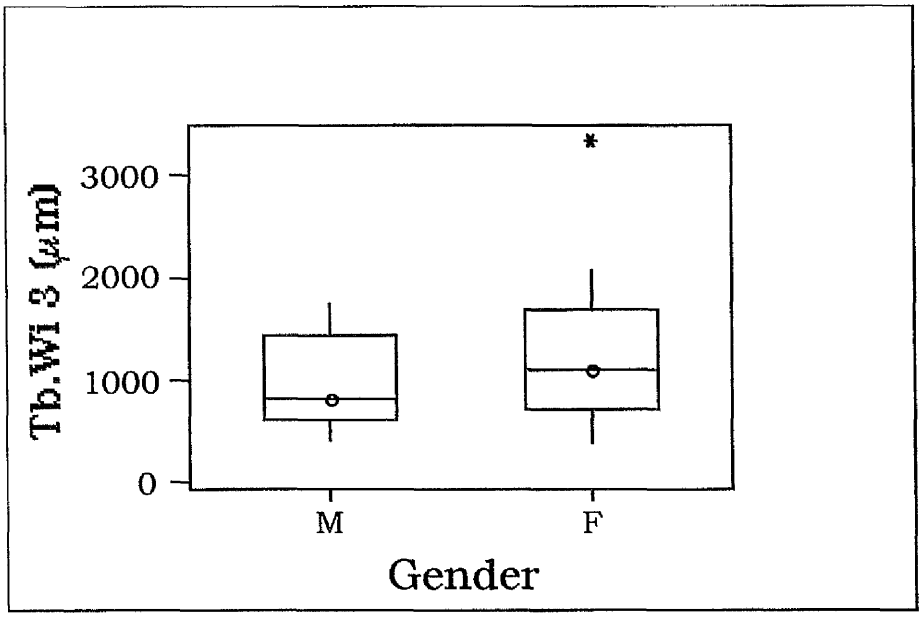
Graph A1.26 Histogram of Tb.Wi 8



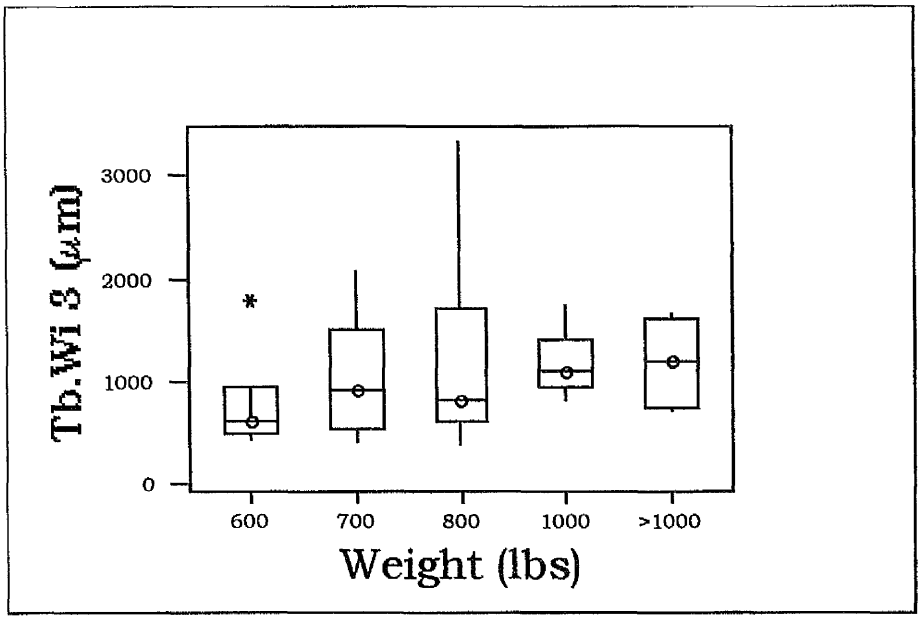
Graph A1.27 Boxplot of Tb.Wi 3 by Age



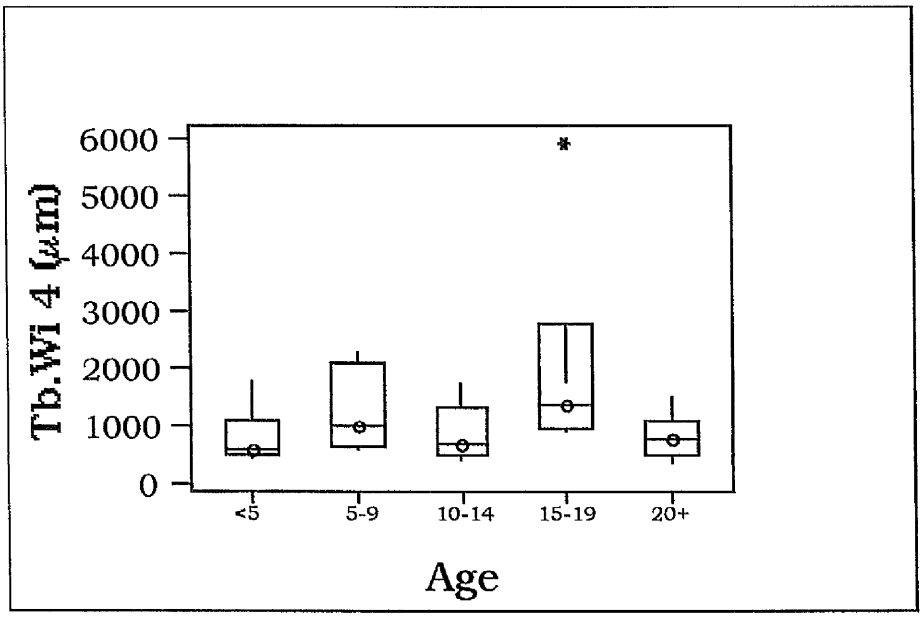
Graph A1.28 Boxplot of Tb.Wi 3 by Breed



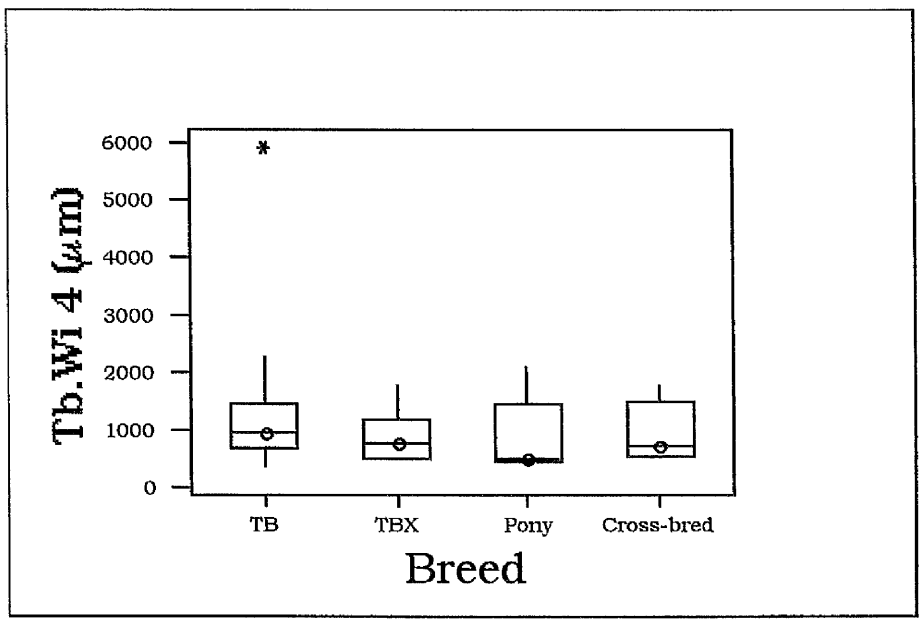
Graph A1.29 Boxplot of Tb.Wi 3 by Gender



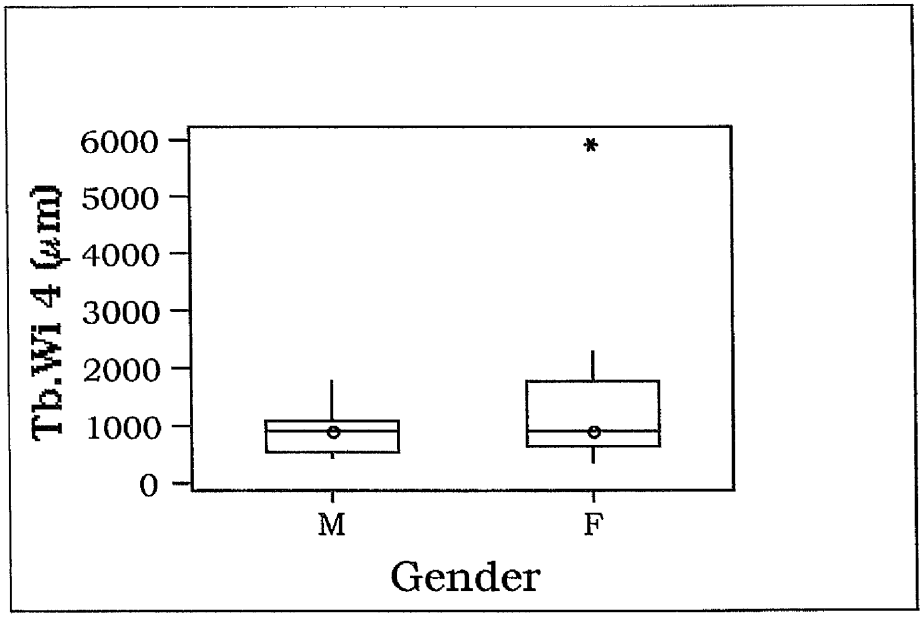
Graph A1.30 Boxplot of Tb.Wi 3 by Weight



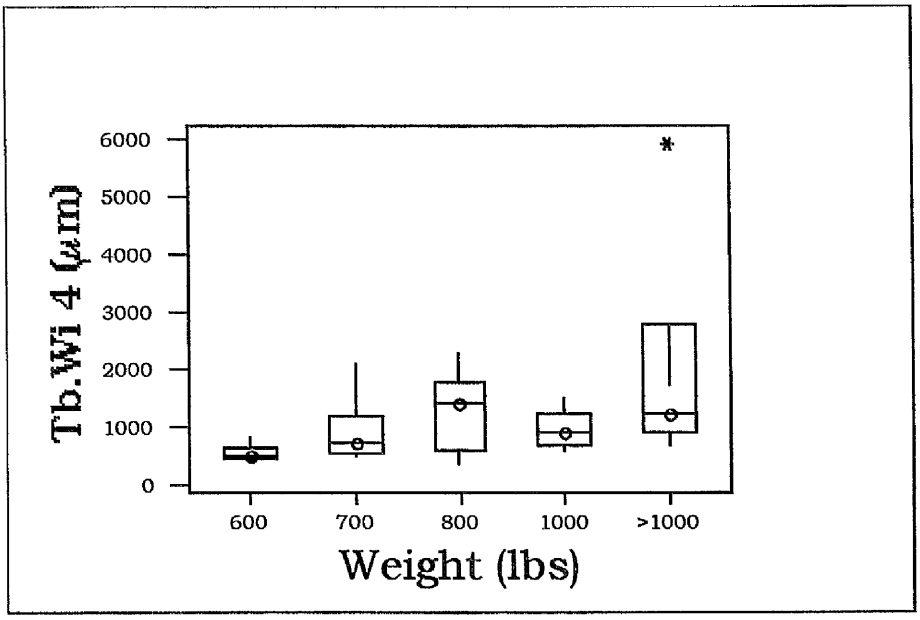
Graph A31 Boxplot of Tb.Wi 4 by Age



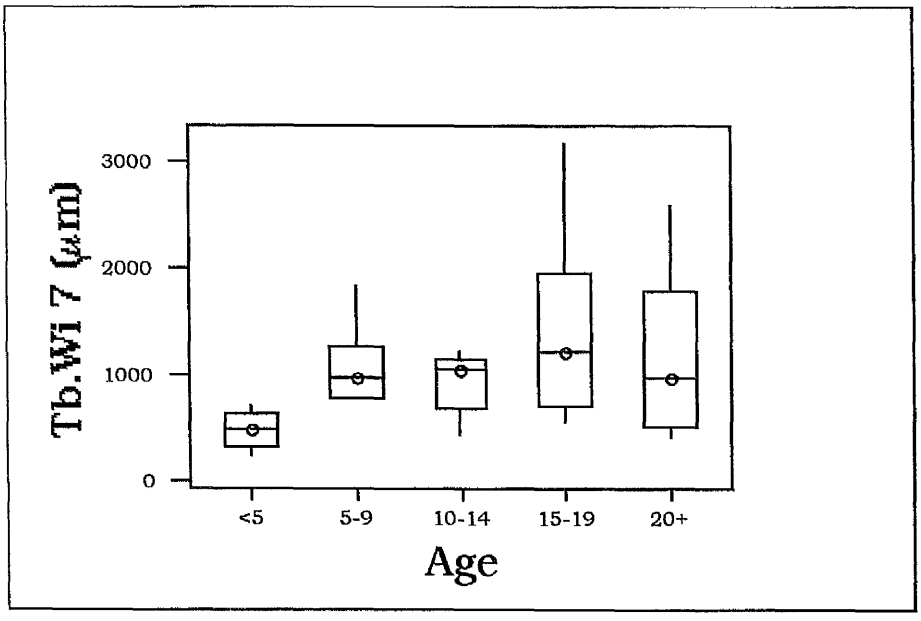
Graph A32 Boxplot of Tb.Wi 4 by Breed



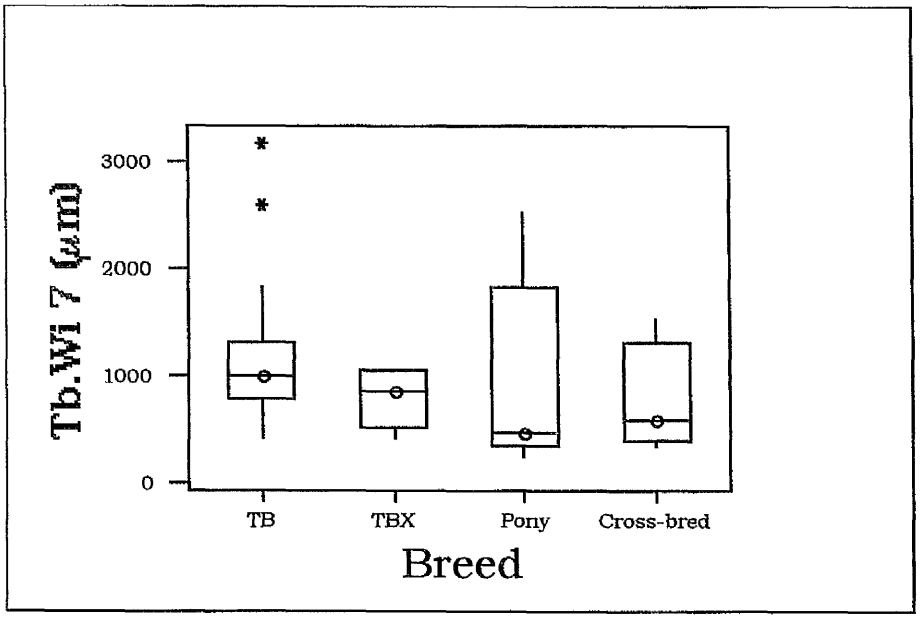
Graph A1.33 Boxplot of Tb.Wi 4 by Gender



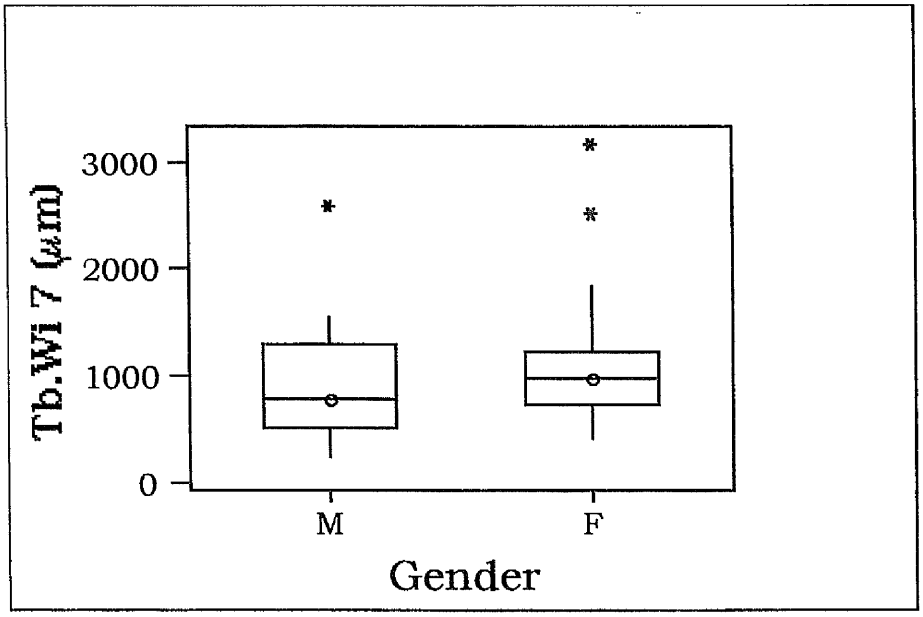
Graph A1.34 Boxplot of Tb.Wi 4 by Weight



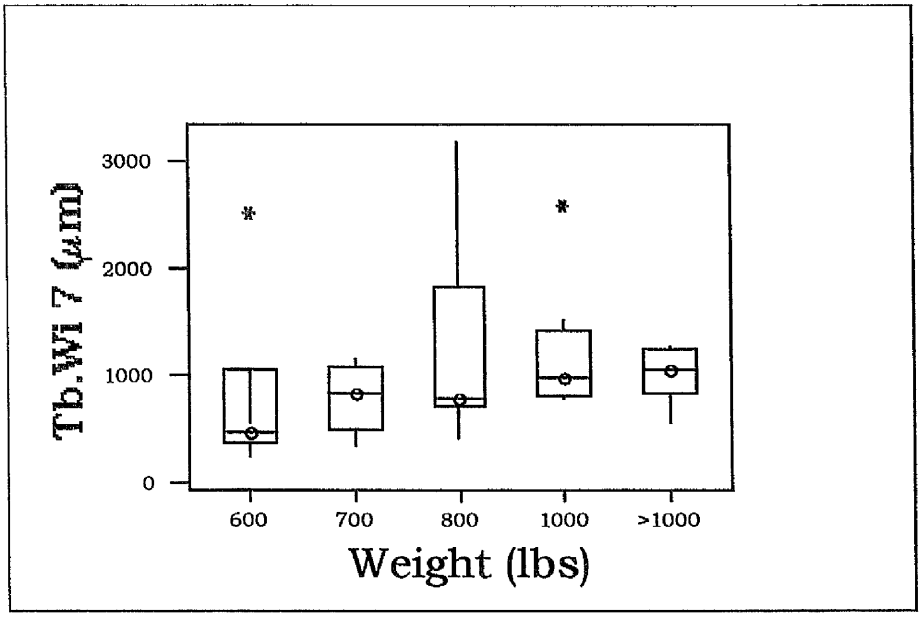
Graph A1.35 Boxplot of Tb.Wi 7 by Age



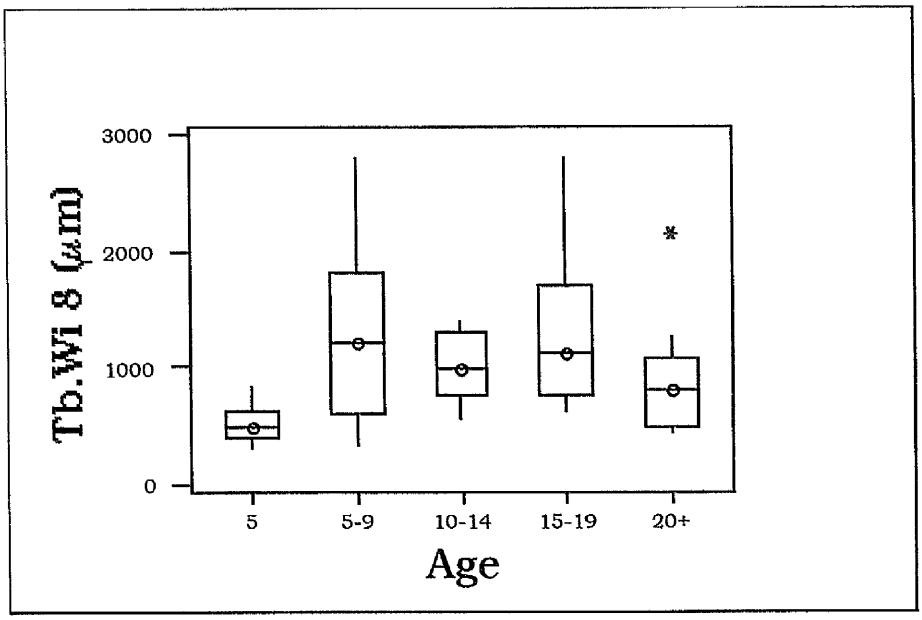
Graph A1.36 Boxplot of Tb.Wi 7 by Breed



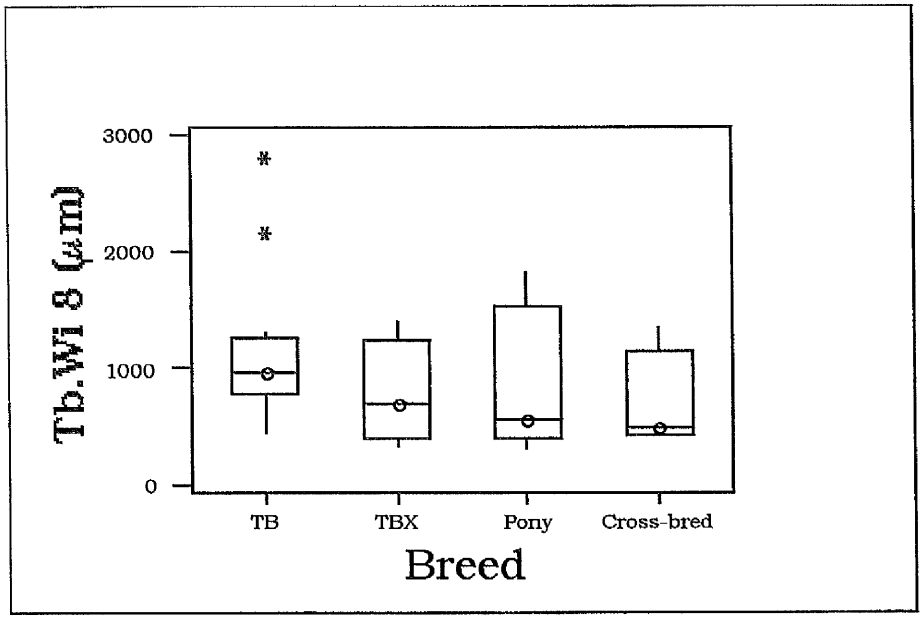
Graph A1.37 Boxplot of Tb.Wi 7 by Gender



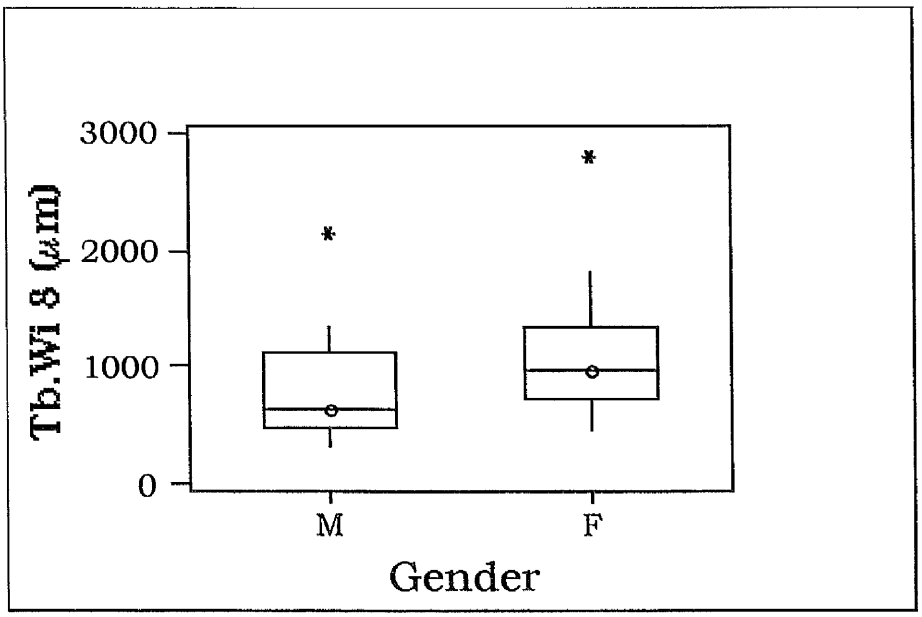
Graph A1.38 Boxplot of Tb.Wi 7 by Weight



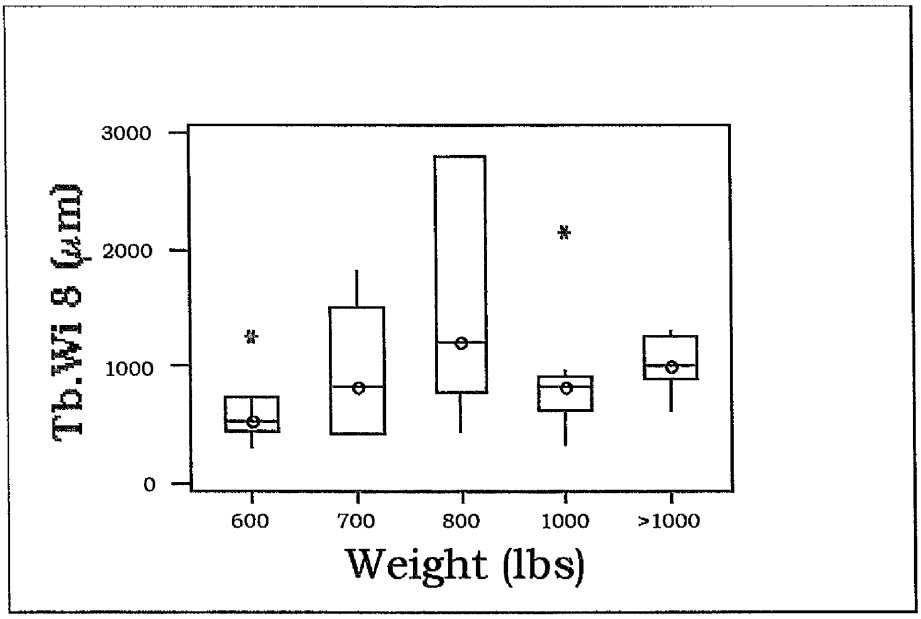
Graph A1.39 Boxplot of Tb.Wi 8 by Age



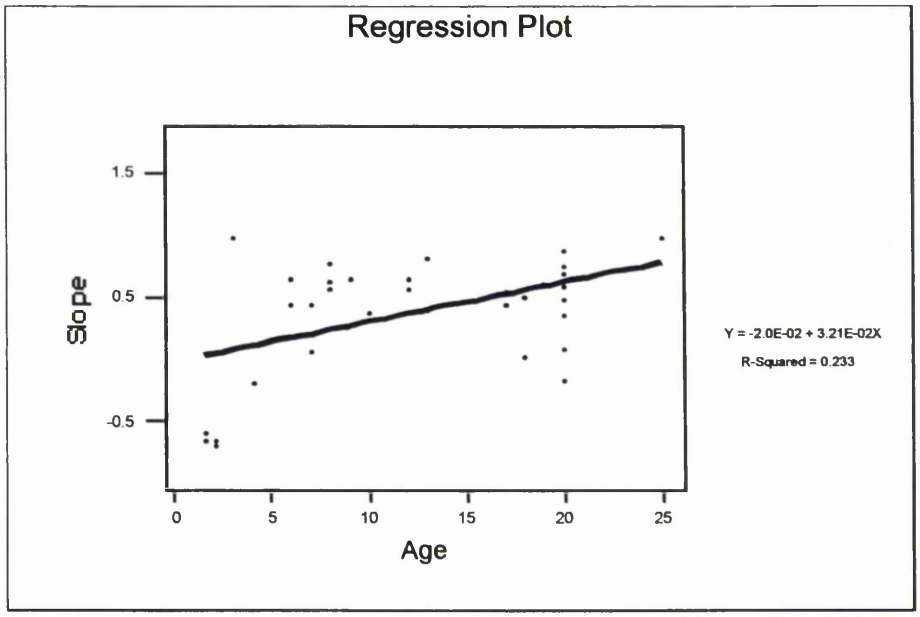
Graph A1.40 Boxplot of Tb.Wi 8 by Breed



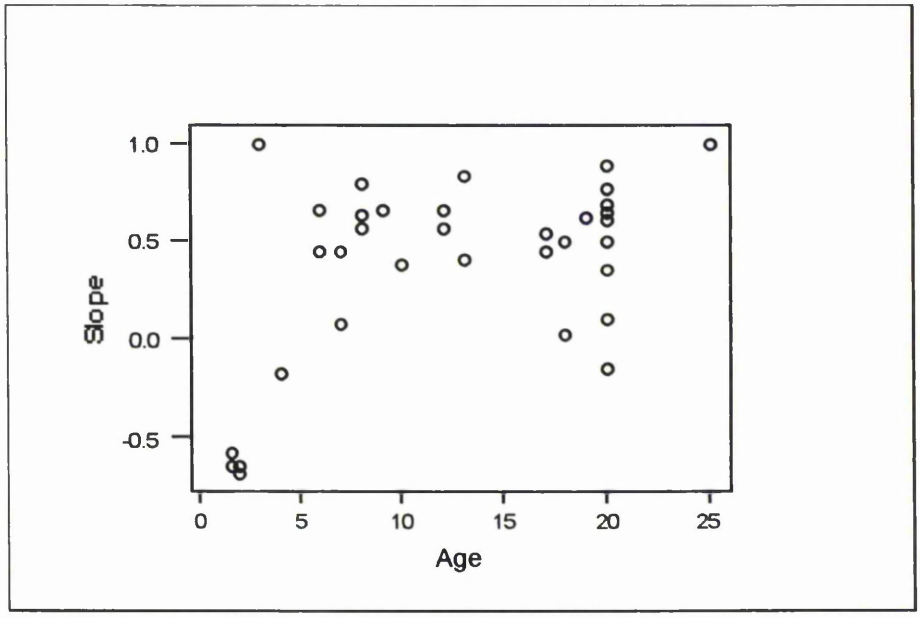
Graph A1.41 Boxplot of Tb.Wi 8 by Gender



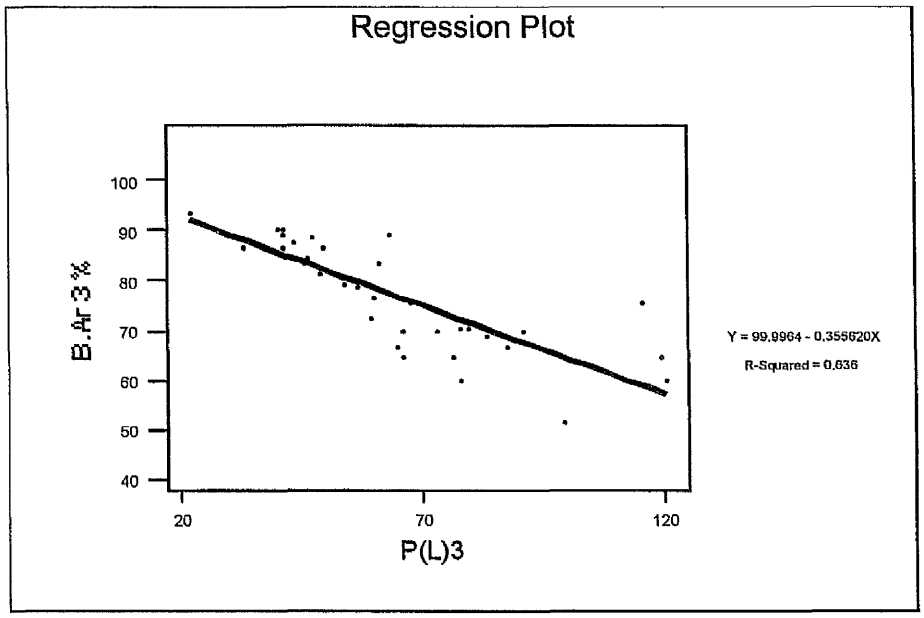
Graph A1.42 Boxplot of Tb.Wi 8 by Weight



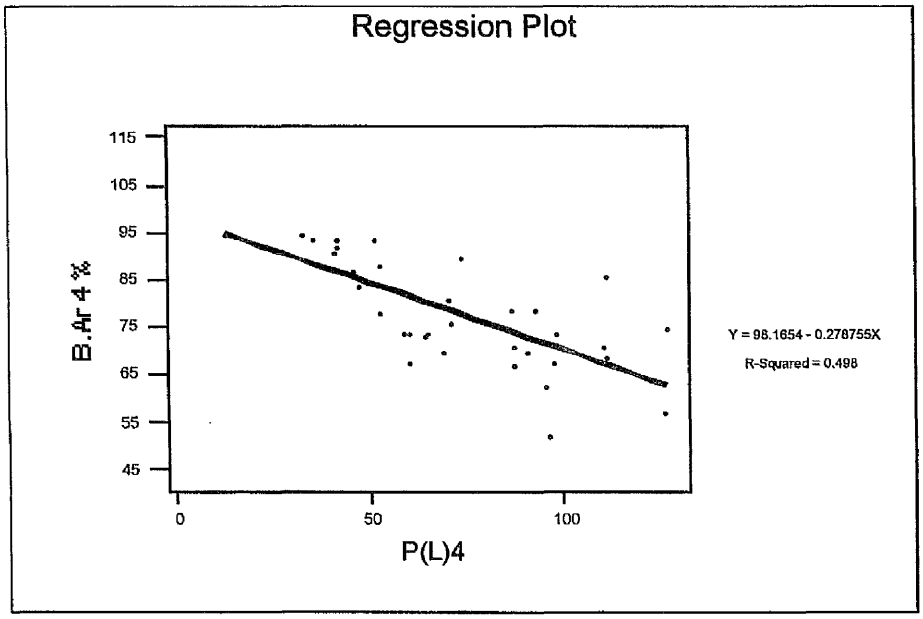
Graph A1.43 Regression plot showing the relationship between age and the slope of the line graphing the P_L with increasing distance from the CC layer. The negative slope exhibited for the graph of that line by the youngest horses on the left of this plot indicates that the P_L decreased as the distance from the CC layer increased, an inverse relationship compared to most of the older horses in the study.



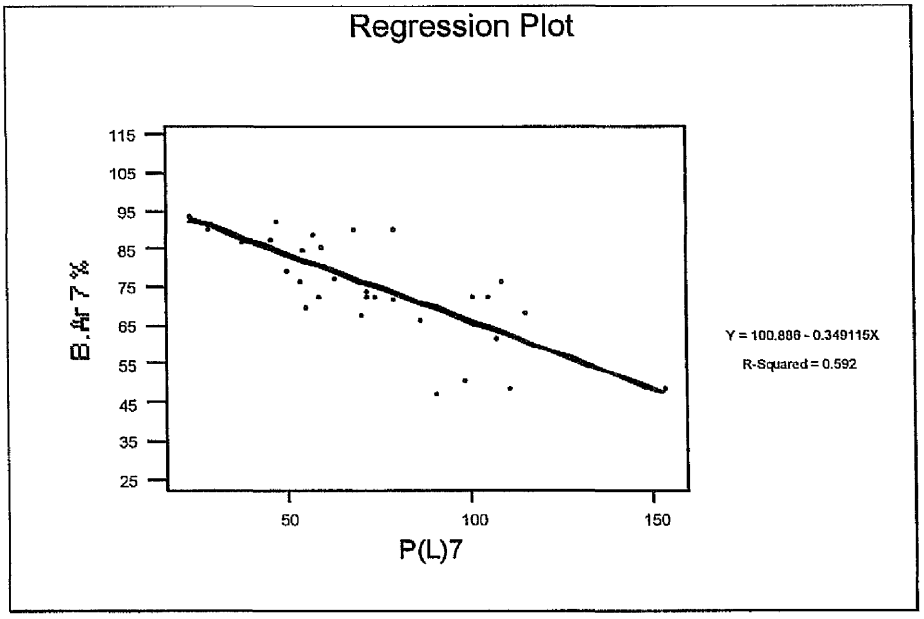
Graph A1.44 Plot of Age vs. the slope of the line graphing P_L with increasing distance from the CC layer. While the relationship increases in most horses, the P_L decreases in the youngest horses, shown by the negative slopes for the horses represented on the left of the graph.



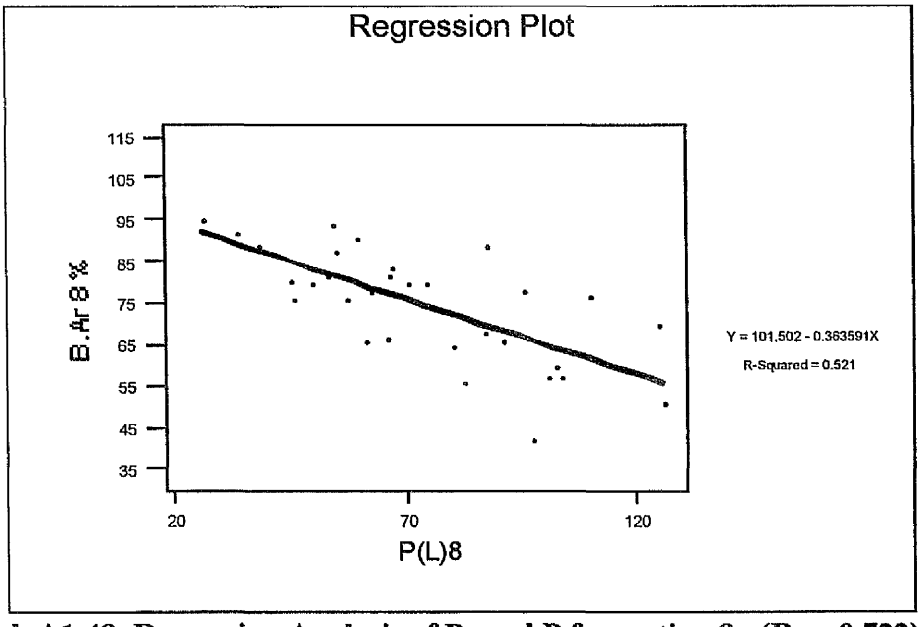
Graph A1.45 Regression Analysis of P_P and P for section 3. (R = -0.798)



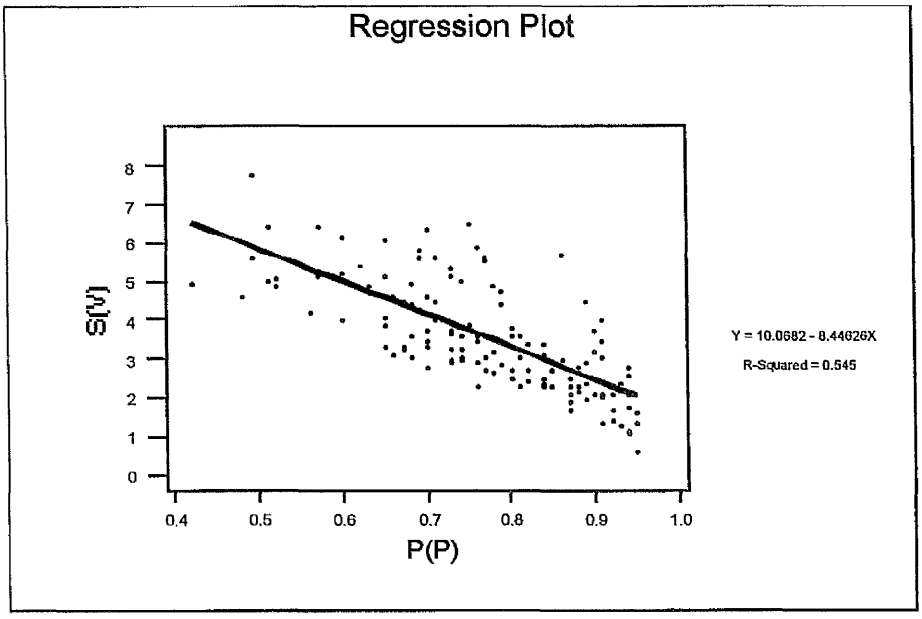
Graph A1.46 Regression Analysis of P_P and P for section 4. (R = -0.706)



Graph A1.47 Regression Analysis of P_P and P for section 7. (R = -0.769)



Graph A1.48 Regression Analysis of P_P and P for section 8. (R = -0.722)



Graph A1.49 Regression plot of S_V vs. P_P. This graph shows the high degree of correlation between these two independent measurements.

CHAPTER 5

CARTILAGE

Introduction

Much of the equine articular research over the years has concentrated on the soft tissues and non-calcified articular cartilage. The pathogenesis of articular disease has been explored most extensively as an inflammatory process originating secondary to acute trauma or more chronic wear and tear injuries. In the former situation, the inflammation is the direct result of a known insult to the articular tissues, whereas the latter condition is thought to develop over time and possibly as a response to normal training for athletic performance.

Synovitis is the generic term given to the inflammatory condition affecting the articular environment. It is considered to be inflammation of the soft tissues of the joint, which includes the joint capsule, synovium and ligaments. The inflammatory response leads to heat, pain, joint swelling, stiffness and lameness. Primary synovitis, by distinction, indicates that only the synovium is affected and that there is no articular cartilage damage **(Todhunter and Lust 1990)**. Classically, at least in the equine literature, the term *degenerative joint disease (DJD)* is reserved for those conditions where articular cartilage is affected **(Baxter 1992).** Proteoglycan depletion is often considered to be the key to development of DJD and is frequently caused by enzymatic activation and subsequent insult **(Clyne 1987).** Although many of the enzymes implicated in this deleterious process are part of the normal aspects of joint physiology, the inflammatory process may increase their production and release resulting in levels well over the normal physiological state **(Baxter 1992).** Inflammatory mediators, such as interleukin 1 (IL-1), lysozyme, collagenase and gelatinase are thought to play a role in this increased level of cartilage catabolism over the normal process of remodelling **(Palmer and Bertone 1994, May *et al.* 1992).** As a result of this line of research, treatment options have been concentrated on relieving intra-articular and systemic inflammation before it can stimulate the catabolic pathway **(McIlwraith 1996).**

These aberrant enzymatic pathways have been implicated in the depletion of proteoglycans, which is considered by some to be the hallmark condition associated with impending cartilage damage **(Clyne 1987).** However, the calcified layer of the hyaline cartilage has a very low proteoglycan level **(Palmer and Bertone 1994)** and is rarely mentioned in the disease processes associated with equine DJD or osteoarthritis (OA).

The calcified cartilage (CC) is considered to be the fourth, or deepest, layer of the hyaline cartilage (HC). Its most superficial border is known as the tidemark (TM), the junction with the deep layer of the noncalicified hyaline cartilage (NC). The opposite border interdigitates to varying degrees with its osseous neighbour, thereby firmly anchoring the CC to the underlying subchondral bone. Conversely, fibrils from the deepest portion of the NC cross the TM into the CC, serving to anchor the entire cartilaginous layer to the bone. Further functions of the calcified layer have been speculated to include forming a barrier to diffusion of water and solutes between bone and cartilage and as a component of growth and remodelling **(Anderson et al 1993)**.

In the human medical literature, there has been an increased interest in the calcified layer of the articular cartilage over the last decade, both on the clinical and research front. Several studies have attempted to measure the thickness of the CC in an effort to learn more about its role in joint dysfunction. **Flygare *et al* (1993)** looked at this region of the articular cartilage in the human temporomandibular joint of elderly individuals. They produced a set of parameters for this layer within the subjects in their study. They also found that the thickest NC was found in the same region as the thickest CC. In two separate studies, **Muller-Gerbl *et al* (1987a,b)** also measured the thickness of the CC layer at several different locations within a joint as well as for different joints. They found that the CC thickness did not vary in relation to that of the entire HC layer and that the CC was thicker in regions that encountered heavier loads and that the thickness of the CC compared to the entire HC was nearly linear. Within a joint,

the thickness of the CC and HC was not a constant, but varied depending on the location. They also concluded that pathological conditions may lead to an increase in CC thickness. **Oettmeier *et al* (1989a)** conducted several investigations into the structure and function of the CC layer. Initially, they looked at the TM region on human femoral heads under high magnification. They found that the classic TM actually consisted of three components: the granular, undulating line adjacent to the basal layer of the NC, the sublinear light-coloured region below the TM line and the demarcation line of the CC. Within the TM, they identified a balanced system of factors that promote calcification and as well as inhibit calcification. The factors that promote calcification are matrix vesicles, glycoproteins, calcium-phospholipid-phosphate complexes, alkaline phosphatase, phosphoproteins and proteolipids. Those that oppose calcification are proteoglycan, ATPase, pyrophosphate, and nucleotide triphosphate. It was proposed that the cells on either side of the TM are capable of producing either set of factors, a situation that is ultimately responsible for the physiological dynamic steady state of the TM region. In a related study, **Oettmeier and colleagues (1989b)** compared changes in the TM region seen in osteoarthritic samples with those of the normal specimen. They characterised low grade, moderate and severe changes within this region. The low grade changes were characterised by reduplication or discontinuities of the TM. Moderate changes consisted of vascular invasion and incipient calcification of the basal hyaline cartilage, while high grade changes were distinguished by the disappearance of the TM, advanced mineralisation of the basal cartilage and finally by complete loss of cartilage to the level of the TM. The thickness of the TM remained fairly constant for low and middle grade changes, however, reduplication of the TM was the most consistent TM change in early OA. It was concluded from this study that mineralisation of the articular cartilage proceeds toward the joint surface under pathological conditions, thereby effectively increasing the CC thickness while at the same time decreasing the NC thickness. Several other researchers found multiple tidemarks in regions where the calcification advance into the NC had been reactivated, most likely in response to OA **(Messner *et al* 1996, Hulth 1993, Meachim and Allibone 1984)**. This view was modified by **Oegema *et al* (1997)**

who reported the presence of multiple TM in all species, thereby not necessarily meaning they are an indication of OA. **Meachim and Allibone (1984)** reported extensive necrosis of chondrocytes within the CC layer. However, **Flygare *et al* (1993)** interpreted the empty lacunae they observed to be artefacts of preparation and not necrosis. **Sokoloff (1993)** identified microcracks in the CC layer and considered them to be real and not artefactual, leading to the hypothesis that extension of these microcracks beyond the calcified layer may mediate remodelling of the osteochondral junction in ageing and OA.

In order to take their studies further, especially into a more active and clinically oriented setting, **Oettmeier *et al* (1992)** conducted several more experiments on the CC layer of dogs subjected to a strenuous training regime. In these studies, they found that the exercise led to a thickening of the HC, CC and SCB in different regions of the joint. From this they deduced that exercise might cause a deleterious thickening of the tissues in the joint, ultimately resulting in OA. In a further study under the same conditions with canine athletes, samples from 11 different sites within the knee joint revealed thickening of both the HC and CC from the exercised group. They also determined that the CC and NC were thinner in non-weight-bearing areas than in loaded regions of normal joints.

Several other papers reported on the biomechanical function of the CC layer. **Anderson *et al* (1993)** consider the role of the CC to be multifaceted. Its purpose in childhood appears to be one of growth and modelling of epiphyseal bone. Later in life it seems to function more in a mechanical role, anchoring the NC to the bone while providing a moderate stiffness gradient between the two. **Oegema *et al* (1997)** considered the CC to be 1/10th as stiff as the underlying bone, but 10-100X stiffer than the overlying NC. Three point bending studies were carried out by **Mente and Lewis (1994)** to objectively evaluate the elastic modulus of the CC versus that of the SCB. They found that the elastic modulus of the CC was an order of magnitude lower than that of the underlying SCB, supporting the

contention that one of the functions of the CC is to serve as an intermediate layer of transition between the NC and SCB.

Currently, most of the descriptive data in regard to the CC layer are from human specimens, although several reports of experimental models involve the use of dogs **(Oettmeier *et al* 1992)** or rabbits **(Oegema et al 1997).** To the author's knowledge, there is only one published reference to morphology of the equine calcified cartilage layer, but it is in abstract form and no measurements were included **(Norrdin *et al* 1996).** Because no published data exists on this layer in the horse, the purpose of this study was to investigate the CC layer both subjectively and objectively in articular specimens from the equine distal metacarpus. We proposed that the thickness of the CC layer would be different in relation to its position within the joint. In addition, we speculated that the CC thickness in any given region would be related to the NC thickness in the same region. Finally, we hypothesised that the thickness of the CC and NC would not be affected by age, breed, gender or weight.

Materials and Methods

Horses

The horses used for this study were the same population described in the previous chapter. The left forelimb was collected from 34 horses presented to a local abattoir for slaughter. The joints were examined for normality based on the criteria previously cited in chapter 3. Sampling, specimen preparation and embedding were covered in chapter 4.

Digitisation

Digitisation was carried out with the same basic equipment as for the previous experiment, but with a slightly different methodology. The computer was an Apple Macintosh model 7500 with a 100 MHz microprocessor, a 1 GB hard drive and 100 Mb of RAM. A 15-inch monitor set to a size of 640 X 480 was connected to the computer.

Digital Microscope. As with the previous experiment, a dissecting microscope with a video camera attachment was utilised. The video was attached to the 7500 via the 'video in' port, a phono plug with a native video capture card attachment on the motherboard of the Macintosh computer. The software utilised for this experiment was 'NIH Image', a freeware scientific image processing program available on the internet (http://rsb.info.nih.gov/NIH_Image). The region of interest was brought into focus under the microscope and the image was captured with the video card of the 7500. Each image was saved as a '*.tif' file for later processing.

The microscope was set at a magnification of 4X, the maximum for this system, which equated to 90X on the absolute scale. It was calibrated by placing a stage micrometer in the field at the level of the bone surface. The photomicrograph was captured and the calibrated scale was measured manually for a distance of 1mm using NIH_Image. This equated to 0.27 pixels per μm or 1 pixel equal to 3.7 μm. For this experiment the section images were obtained at 90X magnification in RGB colour, although the resolution was still 768 x 576, the limits set by NIH_Image. Therefore, the field size obtained for study was approximately 2.84mm x 2.13mm.

Sampling Methods

For this study, the same marks were used to determine sampling regions as in the previous study. Points A and B were located at the proximal extent of the calcified cartilage layer on the palmar and dorsal surfaces respectively. Point C was made at the transverse ridge and Points D and E were at the mid point of the palmar and dorsal articulations respectively. **(Fig 5.1)**

Section 11 was brought into focus using Point A as the left hand border of the image. This time the tidemark was centred in the image, therefore digitising the entire hyaline cartilage layer and a small portion of the subchondral bone. Section 16 was obtained in a similar manner although Point B served as the right hand border of the field. Sections 12 and 15 were obtained using Points D and E, respectively, as the midpoints of their fields. Section 13 utilised Point C as the right hand border of the field, while Section 14 used the same point as its left-hand border. All images were saved as *.tif files for later analysis. **(Fig 5.2)**

Measurement

The thickness of the calcified and noncalcified cartilage layers was determined by integration according to the following formula:

$$CC = \frac{\int_{x_1}^{x_n} CC_{dx}}{250\mu m}$$

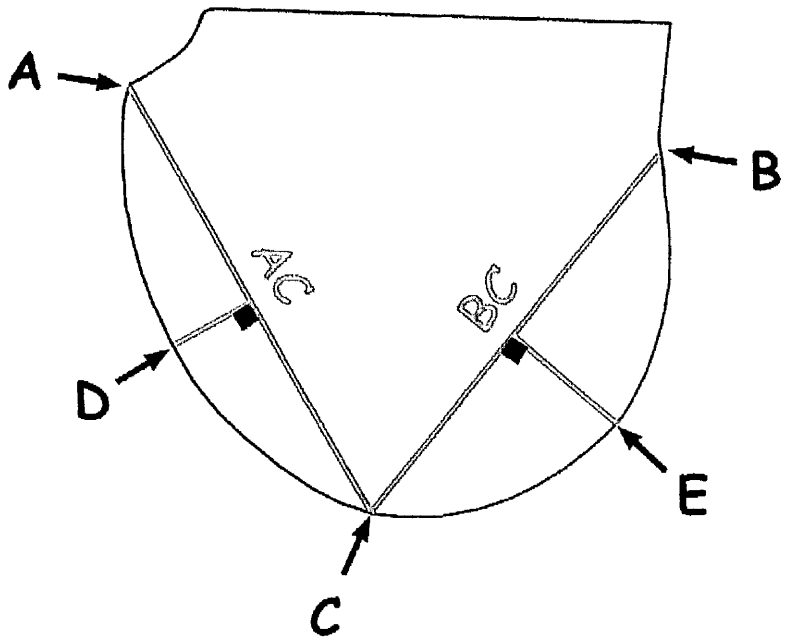


Fig 5.1 Schematic diagram of the method used to determine sample sites.

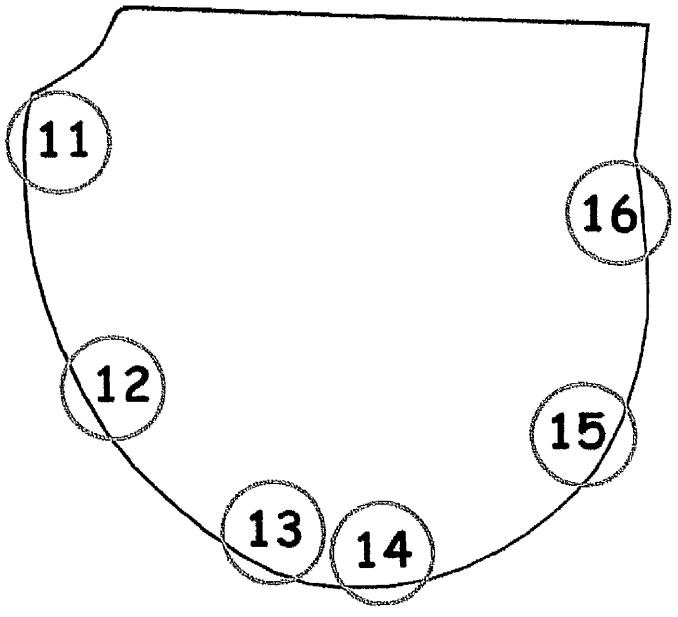


Fig 5.2 Schematic diagram illustrating the location of the sections for the calcified cartilage study.

It relies on outlining and measuring the area of the region of interest over a specified width. The area is then divided by the width to estimate the average thickness.

A macro was developed within NIH_Image that divided the image field into five 250-micron wide sections, each separated from the next by a 250 micron wide space. Utilising the 'hexagon tool', the outline of the calcified cartilage was traced and the area determined and stored using the 'measure' function **(Fig 5.3)**. The same process was carried out for the noncalcified cartilage in the same 250 micron section as well as for the CC and NC in the other four 250 micron sections. The areas of the five CC regions and their corresponding NC regions were saved as a text file.

A separate macro was recorded within Microsoft Excel to transfer the data into the spreadsheet program. The macro transferred the areas from the text file into individual data cells and then divided the area by 250 microns and recorded the estimated CC and NC thickness in another cell. Finally, the macro calculated the mean thickness and standard deviation for the five 250 micron wide sections across the entire image. The CC/Total Cartilage (TC) ratio was calculated by dividing the estimated CC thickness for the section by the TC thickness.

Statistical Analysis

Statistical analyses were carried out to determine if there was any difference in CC thickness and NC thickness between the different sections within a horse using a repeated measures two-way ANOVA with one factor repetition. Then the data for each of the different sections among horses were analysed according to region using a standard two-way ANOVA with interaction to determine if there were any age, gender or weight effects.

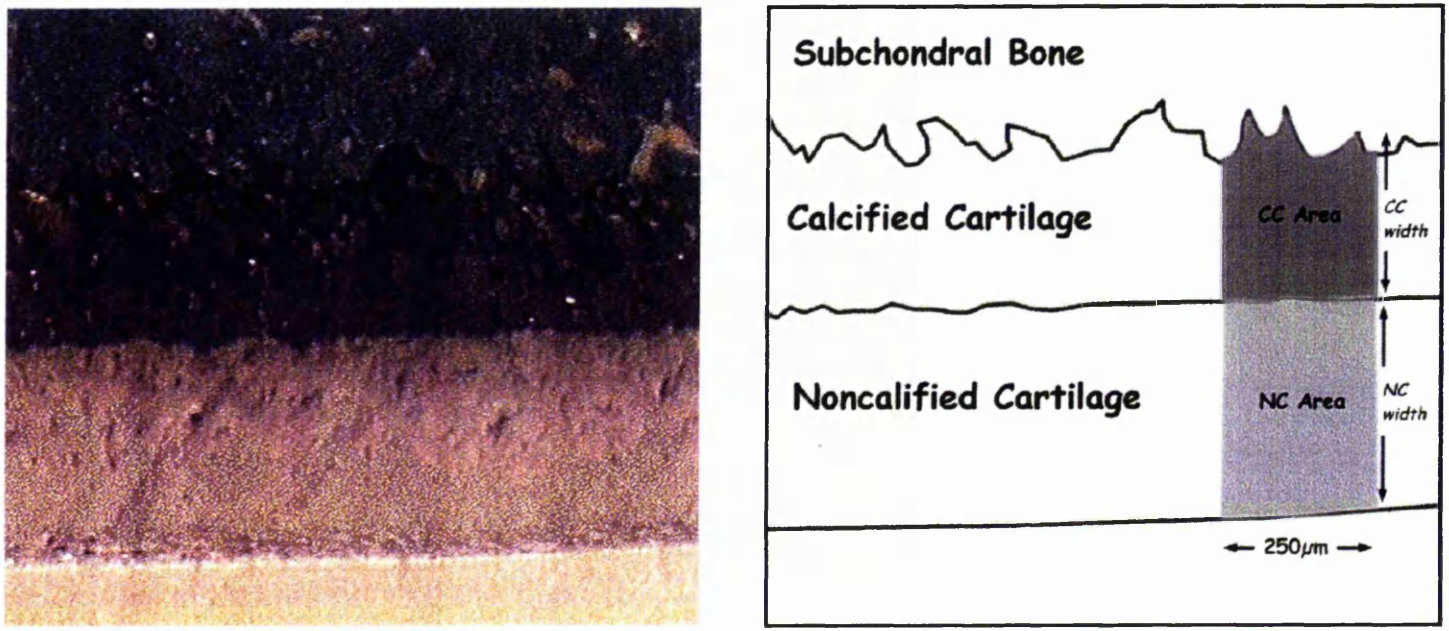


Fig 5.3 CC Measurement Methodology. The photomicrograph on the left illustrates the CC region, while the diagram on the right is a schematic representation of how the CC and NC layer were measured using NIH_Image.

A one way ANOVA was done to see if there were any significant affects of breed. The CC and NC layers were tested for correlation using polynomial regression. Significance for all tests was set at $P < 0.05$. **(G. Gettinby, personal communication)**

Results

Calcified Cartilage

Descriptive

In all sections, the CC layer was easily identified compared to the overlying NC and underlying SCB plate. It stained basophilic with the toluidine blue, resulting in a hue ranging anywhere from a dark pinkish colour to a deep purple. The superficial border of the CC was considered to be the tidemark (TM) and delineated the junction with the NC. In most specimens the TM was a gently undulating line, although in a few specimens it was more wavy than the others. It was never irregular in appearance and there were no duplicated TM noted in any of the specimens. The chondro-osseous junction marked the deep border of the CC. This border was much more irregular in its outline than that of the TM. The uneven nature of this juncture varied among specimens and between sections within a specimen. The chondro-osseous margin was most unpredictable in the horses less than 3 years of age. In these specimens, it was highly irregular in appearance. In some places the immature CC seemed to contain breaks where the SCB plate looked as though was in direct contact with the NC layer. **(Fig 5.4 and 5.5)**

The cellularity of the CC layer varied between specimens, although all sections had fewer cells than the overlying NC. In most instances, the cells were noted to

be within the mineralised matrix of the CC. In select specimens, the CC seemed to be permeated with vacuoles representative of empty lacunae where viable cells had once existed. Again, this observation was limited to the young horses, especially those under 3 years of age. This can also be seen in **Fig 5.5.**

The thickness of the CC layer varied significantly among specimens and sections. It ranged from 88μm to 426μm with a mean of 229.85μm and a median value of 224.85μm. The values were grouped and examined by age, breed, gender and weight **(Table 5.1 and 5.2).**

Comparisons for All Horses

Age vs. Section

When examined in relation to section, age had a significant effect on CC thickness (P = .003). The position of the section also had a significant effect (P = .001), while the interaction between age and section location also showed a significant effect (P = .009).

When examining age groups, 1 had a significantly thinner CC layer than 3, 4 and 5 (P = .01, .02 and .005). Sections, 15, 12 and 16 had a significantly thicker CC layer than 11, 13 and 14, but they were not different from each other. Sections 13 and 14 were also different from 11, but not from each other.

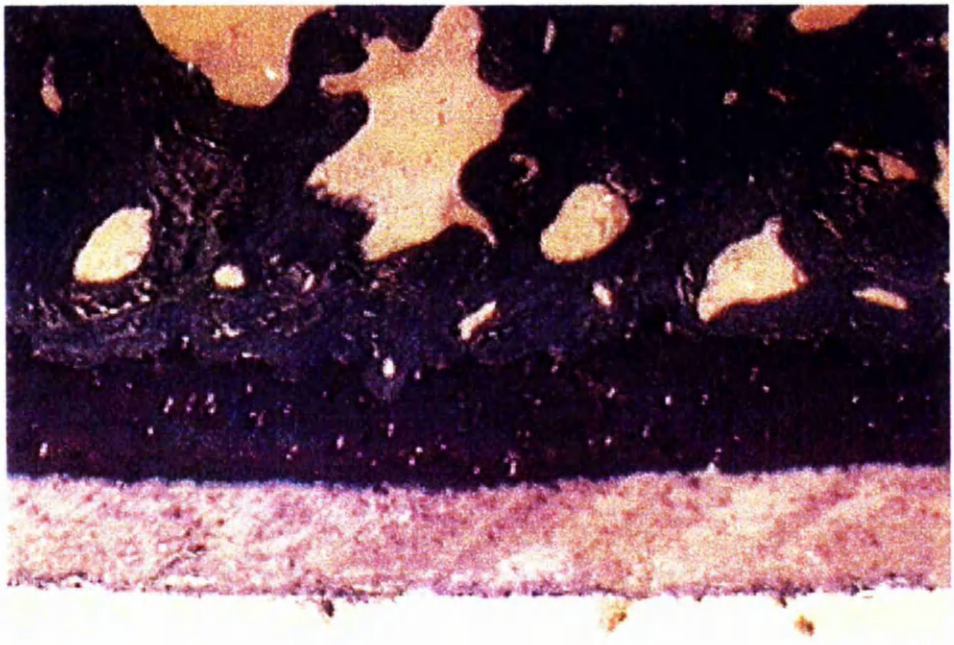


Fig 5.4 Calcified cartilage layer from a 10 year old horse.

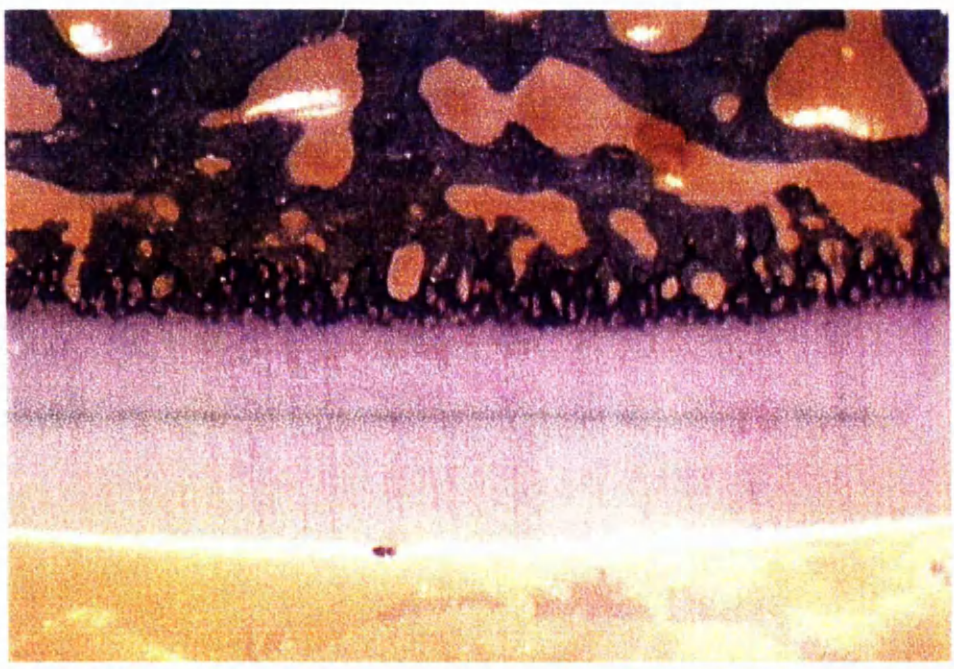


Fig 5.5 Calcified cartilage layer from an18 month old horse.

	CC11	CC12	CC13	CC14	CC15	CC16
<5 Yrs	140.55 ± 26	174.99 ± 38	170.74 ± 59	181.1 ± 62	226.28 ± 76	152.7 ± 23
5-9 Yrs	168.93 ± 45	256.98 ± 66	210.68 ± 50	204.4 ± 43	253.42 ± 59	198.69 ± 85
10-14 Yrs	207.02 ± 29	286.02 ± 43	231.99 ± 71	238.85 ± 66	292.37 ± 46	251.38 ± 42
15-19 Yrs	230.67 ± 56	289.62 ± 31	263.85 ± 42	193.15 ± 57	275.11 ± 44	281.08 ± 81
20+ Yrs	195.01 ± 49	283.33 ± 47	252.14 ± 48	203.45 ± 15	269.49 ± 53	302.56 ± 73
TB	195.25 ± 46	269.8 ± 47	244.63 ± 62	199.45 ± 39	260.68 ± 51	265.08 ± 92
TBx	166.94 ± 66	267.11 ± 103	210.47 ± 59	209.79 ± 54	271.78 ± 69	237.89 ± 90
Pony	192.61 ± 59	240.07 ± 45	209.26 ± 18	205.05 ± 69	256.03 ± 67	212.39 ± 36
X	164.73 ± 35	222.03 ± 67	188.34 ± 68	210.68 ± 65	263.37 ± 80	166.97 ± 42

Table 5.1 Summary of CC Thicknesses (μm) for Age and Breed

	CC11	CC12	CC13	CC14	CC15	CC16
Geldings	181.48 ± 59	259.75 ± 71	217.33 ± 61	183.03 ± 37	247.39 ± 53	242.99 ± 104
Mares	191.07 ± 41	258.92 ± 52	236.22 ± 58	223.81 ± 49	277.16 ± 59	238.98 ± 66
< 600 lbs	187.73 ± 57	213.92 ± 65	205.04 ± 51	215.46 ± 76	262.09 ± 84	203.42 ± 40
700 lbs	152.12 ± 52	252.67 ± 68	196.71 ± 54	199.8 ± 56	247.1 ± 58	201.82 ± 74
800 lbs	207.95 ± 61	255.54 ± 51	236.59 ± 55	220.22 ± 22	276.29 ± 62	222.12 ± 62
1000 lbs	201.14 ± 38	302.91 ± 6	244.21 ± 66	189.34 ± 35	275.46 ± 56	295.09 ± 114
>1000 lbs	171.38 ± 35	250.48 ± 23	240.98 ± 69	196.53 ± 28	241.5 ± 16	258.6 ± 78

Table 5.2 Summary of CC Thicknesses (μm) for Gender and Weight

Section within Age

Age group 1. Section 15 was significantly thicker than 16 and 11, but no differences were noted for the others.

Age group 2 Both sections 15 and 12 had a thicker CC layer than 16 and 11, but no other differences were noted.

Age group 3. Sections 12 and 15 were different only from section 11 this time.

Age group 4. Sections 12 and 15 were joined by 16, but they were significantly different from only 14.

Age group 5. Sections 12, 15, 16 and 13 had a significantly thicker CC layer than 11, but they were not different from each other. The former three were also different from 14, whereas section 13 was not.

Age within Section

Sections 12 and 16 were the only two to show a difference in respect to age. For section 12, age groups 3, 4 and 5 had a significantly thicker CC layer than age group 1. For section 16, age groups 4 and 5 were different from both 1 and 2 while age group 3 was only different from 1.

Gender vs. Section

Gender did not show a significant effect (P = .748), but the section did (P = .001). The interaction between the two was not significant (P = .491). Section 15 and 12 had a significantly thicker CC layer than 11, 13 and 14, but not 16. However, 16 was different from 11 and 14, while 13 was only different from 11.

Weight vs. Section

Weight was not significant in relation to section (P = .435), but section itself was (P = .001) and the interaction between weight and section was significant (P = .016).

Section within Weight

Weight group 1. Section 15 was significantly thicker than 11.

Weight group 2. Sections 12 and 15 were thicker than 11.

Weight group 3. Section 15 was different from 11.

Weight group 4. Section 12 was different from 14, 13 and 11, while 16 and 15 were only different from 14 and 11.

Weight group 5. All sections except 14 were different from 11, but they were not different from each other.

Comparisons by Section

Each section was examined independently for interactions between age, gender and weight.

Section 11

Within section 11, age had a significant effect (P = .005), with age groups 1 and 2 displaying a thinner CC layer than groups 3, 4 and 5. Breed and gender had no effect, while the weight groups did show a difference (P = .039). Group 2 had a thinner CC layer than group 4. There were no significant interactions noted. **(Graphs A2.17-20)**

Section 12

Age again showed a significant effect, with group 1 exhibiting a significantly thinner CC layer than all the rest of the age groups (P = .004). Weight showed a mild trend (P = .09) towards significance with group 1 horses tending to have a thinner CC layer than group 4 horses. There were no differences noted for breed or gender and there were no interactions noted. **(Graphs A2.21-24)**

Section 13

Age showed a trend towards significance (P = .06) with age groups 1 and 2 tending to have thinner CC layers than the older horses, groups 3, 4 and 5. Breed, gender and weight were not significant. **(Graphs A2.25-28)**

Section 14

There was no significant effect of age, breed, gender or weight on the thickness of the CC cartilage layer in section 14. **(Graphs A2.29-32)**

Section 15

There was no significant effect of age, breed, gender or weight on the thickness of the CC cartilage layer in section 15. **(Graphs A2.33-36)**

Section 16

Age had a significant effect on CC thickness again (P =.003) with groups 1 and 2 thinner than groups 3,4 and 5. Breed also had an affect (P = .001) with Thoroughbreds and TBX showing a significantly thicker CC layer than the cross-breds. Gender and weight did not have any affect, nor did any of the interactions. **(Graphs A2.37-40)**

Noncalcified Hyaline Cartilage

Descriptive

The NC layer was most superficial on the sections. Its deep border was distinguished by the TM at the junction with the CC layer. The NC stained a light

pink. In sections 12 to 15, the normal articular border of the NC was relatively smooth. In sections 11 and 16, however, even when considered normal, the superficial border was often irregular as the cartilage reached its most proximal limits on the joint surface, adjacent to where the joint capsule attached. In some specimens, mild surface fibrillation was present. This was most often seen in sections 13 and 14. The cellularity of the NC varied, although it was difficult to assess this parameter adequately with the chosen specimen preparation and at the selected magnification.

The thickness of the NC ranged between 54μm and 1133μm with a mean of 551μm and a median of 533μm. It showed more variability in its thickness than the CC. The values were grouped and examined by age, breed, gender and weight. **(Table 5.3 and 5.4)**

Comparisons for All Horses

Age vs. Section

When examined in relation to section, age had a significant effect (P = .02). Section also had a significant effect (P = .001), but the interaction between the two did not (P = .28).

	SECTION 11	SECTION 12	SECTION 13	SECTION 14	SECTION 15	SECTION 16
<5 Years	621.11 ± 149	549.89 ± 80	705.79 ± 253	665.88 ± 112	670.35 ± 111	876.81 ± 182
5-9 Years	578.91 ± 241	418.06 ± 60	420.54 ± 221	507.28 ± 185	554.06 ± 80	624.74 ± 246
10-14 Years	550.43 ± 174	373.49 ± 85	494.26 ± 148	657.29 ± 154	507.82 ± 85	593.63 ± 138
15-19 Years	460.81 ± 260	394.6 ± 176	592.07 ± 239	501.97 ± 217	590.24 ± 107	540.91 ± 207
20+ Years	540.59 ± 169	391.88 ± 79	556.85 ± 148	640.73 ± 140	478 ± 174	568.85 ± 160
TB	529.12 ± 203	414.95 ± 92	524.86 ± 221	561.65 ± 195	561.65 ± 195	599.85 ± 219
TBx	577 ± 232	403.49 ± 106	540.04 ± 172	593.38 ± 90	593.38 ± 90	655.69 ± 119
Pony	639.05 ± 261	445.14 ± 143	515.96 ± 123	701.88 ± 134	701.88 ± 134	543.58 ± 109
X	527.37 ± 97	468.07 ± 169	701.72 ± 306	629.02 ± 137	629.02 ± 137	892.71 ± 261

Table 5.3 Summary of NC Thicknesses (μm) for Age and Breed

	SECTION 11	SECTION 12	SECTION 13	SECTION 14	SECTION 15	SECTION 16
Geldings	477.88 ± 184	415.16 ± 131	524.73 ± 197	541.76 ± 172	568.18 ± 140	707.53 ± 220
Mares	629.17 ± 198	432.08 ± 84	569.34 ± 229	649.83 ± 150	533.28 ± 133	564.24 ± 188
< 600 lbs	566.49 ± 218	463.6 ± 134	673.54 ± 285	711.68 ± 128	623.94 ± 162	652.94 ± 198
700 lbs	612.65 ± 212	442.41 ± 135	512.93 ± 123	610.06 ± 105	543.07± 91	755.66 ± 254
800 lbs	567.26 ± 295	393.14 ± 153	603.23 ± 149	600.04 ± 95	510.84 ± 130	515.23 ± 175
1000 lbs	510.92 ± 168	417.26 ± 49	408.29 ± 240	502.42 ± 230	553.46 ± 135	604 ± 245
>1000 lbs	529.35 ± 148	409.94 ± 79	597.19 ± 135	600.76 ± 180	527.65 ± 168	687.64 ± 154

Table 5.4 Summary of NC Thicknesses (μm) for Gender and Breed

Age

Age group 1 had a significantly thicker NC layer than age group 2. No other differences were noted.

Section

Section 12 had a significantly thinner NC layer than all the other sections, which were not different from each other.

Gender vs. Section

Gender showed no effect (P = .8), but section did (P = .001) as did the interaction between them (P = .003).

Section within Males. The NC layer in sections 16, 15 and 14 was significantly thicker than in section 12, while 16 was also thicker than 13 and 11.

Section within Females. Sections 14, 11 and 16 were all thicker than section 12.

Gender within Section. There was a significant difference between genders only for sections 11 and 16, where the females had a significantly thicker NC layer in 11 and vice versa in 16.

Weight vs. Section

Weight had no effect (P = .35), but section did (P = .001). The interaction between the two did not have an effect (P = .46).

Section 12 was significantly thinner than all other sections in respect to the NC layer. The other sections were not different from each other.

Comparisons by Section

Each section was examined independently for interactions between age, gender and weight.

Section 11

Only gender showed any significant effect (P = .04) with females exhibiting a much thicker NC layer than males. Age, breed and weight had no effect, nor did the interactions between them. **(Graphs A2.41-44)**

Section 12

Age had a significant effect (P = .04), with the youngest horses possessing a much thicker NC layer than all the other groups. Breed, gender and weight were not significant, although there was a trend toward significance in both the age x gender and gender x weight interactions (P = .07 and .052 respectively). **(Graphs A2.45-48)**

Section 13

In section 13, none of the variables or interactions had any effect. **(Graphs A2.49-52)**

Section 14

Age, breed, gender and weight showed no significant affect, but the age x gender interaction did show a different response pattern (P = .04). Within age groups 4 and 5 there was a different response pattern for the males versus females. **(Graphs A2.53-56)**

Section 15

There were no significant differences noted for any of the variables or interactions, although age showed a trend toward significance (P = .065), with the youngest horses tending to have the thickest NC layer. **(Graphs A2.57-60)**

Section 16

Age is significantly different (P = .01), with group 1 showing a significantly thicker NC layer than all the rest of the age groups. The cross-bred horses also exhibited a significantly thicker NC layer than the others (P = .001). None of the other variables or the interactions were significant. **(Graphs A2.61-64)**

Calcified Cartilage/Total Cartilage Ratio

Descriptive

The CC thickness was expressed in relation to the total cartilage (TC) thickness and varied greatly between sections **(Fig 5.6 and 5.7)**. These ratios ranged from .09 to .85 with a mean of .31 and a median value of .3. The values were grouped and examined by age, breed, gender and weight. **(Table 5.5 and 5.6)**

Comparisons in All Horses

Age vs. Section.

Age and section had a significant effect on the ratio (P = .001). The interaction between the two was marginally significant (P = .049). Age group 1 had a significantly lower ratio than all the others, which were not different from each other. Section 12 had a significantly higher ratio than all the others while 15 was only significantly higher than 11. No other effects were noted.

Section within Age

Section within Age group 1. There were no significant differences noted.

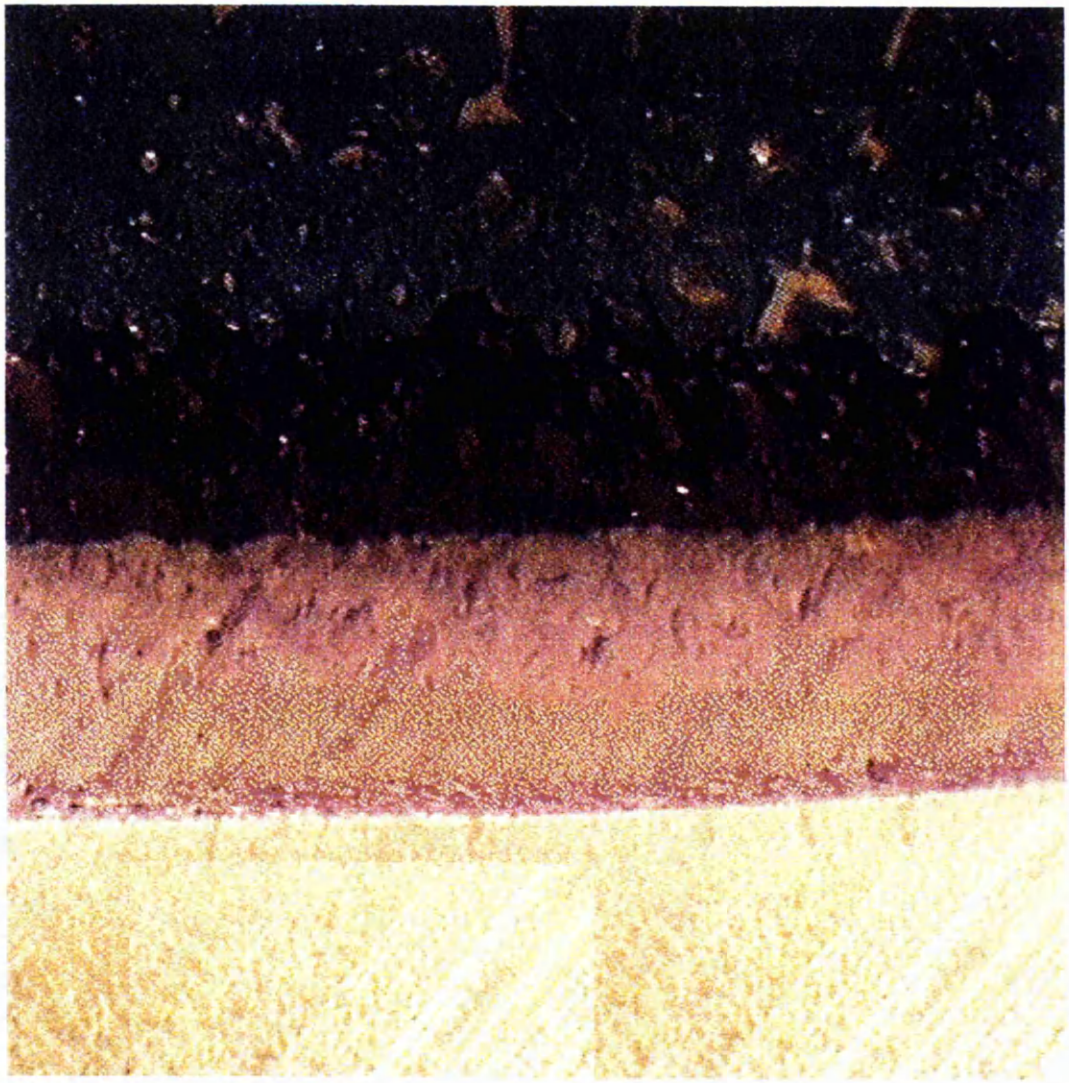


Fig 5.6 High CC/TC ratio. This photomicrograph is from a 12 year old horse.

Fig 5.7 Low CC/TC ratio. This photomicrograph is from a 2 year old horse.

	Section 11	Section 12	Section 13	Section 14	Section 15	Section 16
<5 Years	.19 ± .05	.24 ± .04	.2 ± .05	.21 ± .05	.25 ± .06	.15 ± .04
5-9 Years	.24 ± .1	.38 ± .07	.38 ± .16	.25 ± .06	.31 ± .06	.26 ± .12
10-14 Years	.28 ± .07	.44 ± .06	.33 ± .1	.27 ± .07	.38 ± .03	.3 ± .06
15-19 Years	.39 ± .26	.45 ± .14	.33 ± .09	.3 ± .08	.32 ± .07	.35 ± .08
20+ Years	.29 ± .08	.42 ± .05	.32 ± .07	.25 ± .05	.37 ± .06	.35 ± .08
TB	.3 ± .14	.4 ± .07	.35 ± .12	.28 ± .07	.33 ± .07	.32 ± .1
TBx	.24 ± .1	.4 ± .14	.29 ± .1	.26 ± .05	.34 ± .08	.27 ± .1
Pony	.26 ± .14	.36 ± .11	.29 ± .04	.23 ± .08	.31 ± .09	.29 ± .07
X	.24 ± .04	.34 ± .15	.22 ± .06	.25 ± .06	.31 ± .07	.17 ± .09

Table 5.5 Summary of CC Ratios for Age and Breed.

	Section 11	Section 12	Section 13	Section 14	Section 15	Section 16
Geldings	.3 ± .17	.4 ± .12	.32 ± .12	.27 ± .08	.31 ± .08	.27 ± .1
Mares	.25 ± .07	.38 ± .07	.32 ± .1	.26 ± .06	.35 ± .06	.31 ± .1
< 600 lbs	.27 ± .12	.33 ± .13	.25 ± .09	.23 ± .08	.3 ± .09	.25 ± .09
700 lbs	.21 ± .06	.37 ± .1	.28 ± .08	.25 ± .05	.32 ± .07	.23 ± .1
800 lbs	.33 ± .24	.41 ± .13	.28 ± .02	.27 ± .05	.36 ± .05	.31 ± .1
1000 lbs	.3 ± .06	.42 ± .07	.43 ± .13	.31 ± .09	.33 ± .08	.34 ± .12
>1000 lbs	.25 ± .07	.38 ± .05	.29 ± .09	.25 ± .05	.33 ± .09	.28 ± .09

Table 5.6 Summary of CC Ratios for Gender and Weight

Section within Age group 2. Sections 13 and 12 were significantly higher than 16 and 11.

Section within Age group 3. The ratio in section 12 was significantly higher than in sections 14 and 11.

Section within Age group 4. Section 12 was higher than 14 for this group.

Section within Age group 5. Sections 12, 15 and 16 had a significantly higher ratio than 14, while section 12 alone was also significantly different from 13 and 11.

Age within Section

Age within Section 11. Only age group 4 was significantly different from 1, possessing a higher ratio.

Age within Section 12. Age group 1 had a significantly lower ratio than all the other groups, which were not different from each other.

Age within Section 13. Age groups 2 and 3 were higher than 1, but not different from each other.

Age within Section 14. There were no differences between age groups.

Age within Section 15. There were no differences between age groups.

Age within Section 16. Age groups 5, 4 and 3 had higher ratios than 1, but were not different from each other.

Gender vs. Section

Section again had a significant effect (P = .001), but gender and the interaction between them were not significant (P = .83 and .1). Section 12 was different from all other sections with a higher ratio and 15 was higher than only 11 and 14.

Weight vs. Section

Section was still significant (P = .001), but weight and the interaction between them were not (P = .07 and .58). Section 12 was significantly different from all of the other sections except 15, while 15 was still different from 11 and 14.

Comparisons by Section

Each section was examined independently for interactions between age, gender and weight.

Section 11

None of the variables or interactions were significant, although there was a trend toward significance in gender, weight and the interaction between them (P = .07, .1 and .09 respectively). **(Graphs A2.65-68)**

Section 12

Age had a significant effect (P = .001), with the young horses showing a significantly lower ratio than the older horses. Breed and weight had no significant effect, while gender showed a trend toward significance (P = .09). The interaction between gender and weight was very significant (P = .007). The weight for females showed no differences, while with the males, weight groups 3 and 4 showed a different response pattern from 1. The genders had a different response pattern in weight groups 1 and 3. **(Graphs A2.69-72)**

Section 13

Age had a significant effect (P = .01), with age group 2 and 3 showing a higher ratio than 1, while 3 was also higher than 5. Weight showed a significant effect (P =. 006). Weight group 4 had a higher ratio than the rest, which were not different from each other. **(Graphs A2.73-76)**

Section 14

None of the variables or interactions had a significant effect on the cartilage ratios. **(Graphs A2.77-80)**

Section 15

Age had an effect (P = .01), with group 5 showing a much higher ratio than 1. No other differences were noted. **(Graphs A2.81-84)**

Section 16

Age had a significant effect (P = .002), with age groups 5, 4 and 3 showing a significantly higher ratio than 1. None of the other variables or interactions had a significant affect on the cartilage ratio. **(Graphs A2.85-88)**

The relationship between the Calcified and Noncalcified Cartilage

Regression indicated that the only relationship between the CC and NC existed as a 2nd order polynomial when CC was analysed in terms of NC (P = .007). The formula for this relationship is expressed as follows:

$$CC = 182.8 + 0.2304*NC - 0.000236*NC^2$$

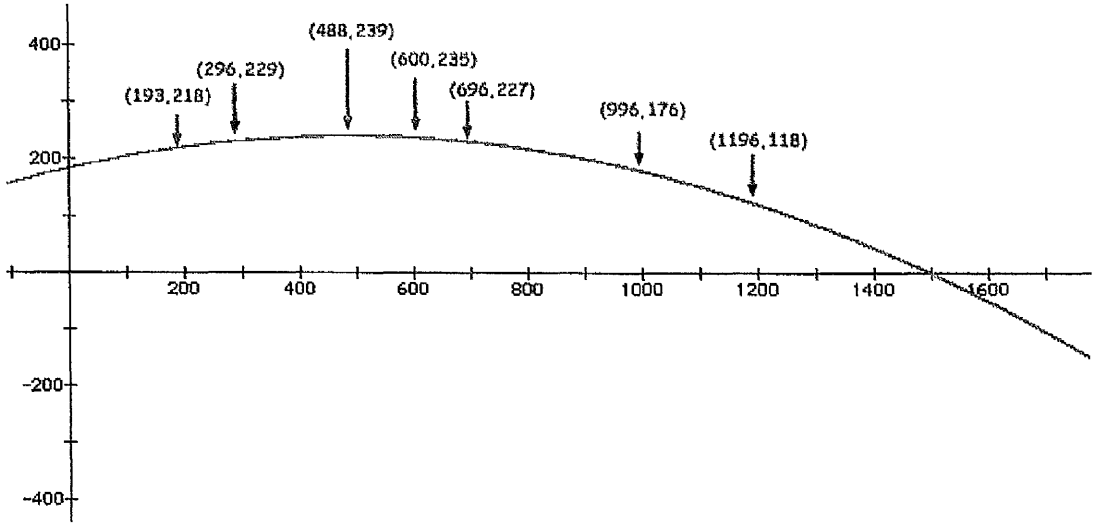


Fig 5.8 Graph of Polynomial Regression between CC and NC. The equation results in a parabola with its apex within the physiologically significant region (488, 239). This graph may indicate that the CC exhibits a range of 'optimal' thickness for the horse, corresponding to the apex, and decreasing on either side as the NC both increases and decreases.

Discussion

In this study, the thickness of the CC and NC layers were measured and then the CC layer was expressed as a ratio of the total cartilage thickness. This task was carried out for horses of different ages, breeds, genders and weights and the data was analysed accordingly. To the author's knowledge, this represents the first time that such an investigation has concentrated exclusively on the equine CC layer in such a manner. There does exist another recent reference to equine CC in the literature, but that study involved 12 young horses and no measurements are published in that abstract **(Norrdin *et al* 1996).** Another equine study established the thickness of the complete hyaline cartilage layer in a group of foals at necropsy **(Firth and Greydanus 1986)**. In the human literature, several investigators have delved into the subject of the CC layer in an effort to establish normal parameters. **Muller-Gerbl *et al.* (1987a,b)** conducted two very nice studies that measured the CC thickness in human joints. They established a very clever method for estimating the thickness of the CC, in light of the fact that the chondro-osseous junction undulates so unpredictably. They made use of a technique from calculus called 'integration' to accurately estimate the thickness. Our study used this general methodology, but, in addition, the measurements were carried out in five different 250 μm regions within one section to obtain an average thickness over the entire section. Our initial attempts to measure the thickness of the CC layer at point locations demonstrated to us that such a method would be fraught with inaccuracy due to the variability in the chondro-osseous junction. The method of integration is a mathematically sound way to solve such a practical problem.

Review of Integration

Measuring the width of a regular object, such as a rectangle, is easily carried out. Because the sides are parallel, a measurement can be taken at any position and it will accurately represent the width at another position (**Fig 5.9**).

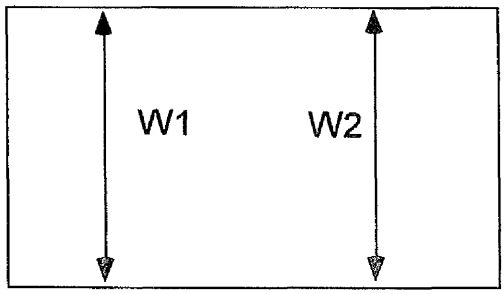


Fig 5.9 Integration 1. In this example, W1 = W2. Therefore, taking the measurement at any position within the rectangle will accurately represent the entire rectangle.

In the case of a highly irregular surface, such as the chondro-osseous border of the CC layer, taking measurements of the thickness, even in two adjacent positions, will likely produce two different answers. Therefore, in order to accurately represent the thickness of an irregular object, it should be measured as many times as possible and the values should be averaged. Theoretically, the most accurate representation would be if a defined area were measured an infinite number of times before averaging the results. This is the concept behind integration (**Fig 5.10**).

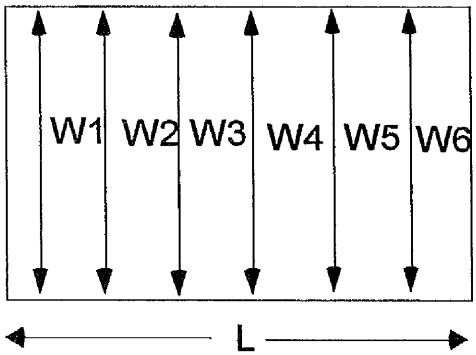


Fig 5.10 Integration 2. To carry the analogy further, the thickness of an irregular area could be expressed by adding all the measured thicknesses up and dividing by the distance over which the measurements were collected. This concept would be expressed as:

$$\frac{W1 + W2 + W3 + W4 + W5 + W6 + W(N)}{L}$$

If one measured frequently enough over an irregular region, an accurate representation of the thickness would eventually be achieved. Another simple mathematical equation is employed to complete the process. The area of an object is equal to its width multiplied by its length.

$$A = W \times L$$

Reversing the mathematics then, the width would equal the area divided by the length.

$$W = \frac{A}{L}$$

This is the concept employed in this study. The area can easily be measured and the width over which it is measured can be determined. In this way, the average thickness over this region can be determined. Expressed in terms of calculus it is as follows:

$$CC = \frac{\int_{x_1}^{x_n} CC_{dx}}{250\mu m}$$

Muller-Gerbl *et al.* (1987a) established values for the calcified cartilage thicknesses in human femoral joints. Their values ranged from 20 μm to 230 μm and they found the thickest values in regions that were under the most pressure. This observation led the investigators to conclude that the CC was thicker in regions under heavier loads. The thickness of the CC layer in our equine study ranged from 88 μm to 426 μm. According to the conclusions of the previous human study then, i.e. that mechanical stress and intended function dictate the CC thickness, it seems reasonable that the heavier horse should have a CC layer from 2-4 times as thick as that of a human.

Many interesting observations and relationships did surface in our study. When examined in relation to age, group 1, the youngest horses, were significantly different from the three oldest groups of horses. The young horses had a significantly less developed calcified cartilage than that of the older horses. This is evident when comparing **Fig 5.4 and 5.5**. The morphology of the CC layer was possibly the most interesting and important aspect of this study. The quite obvious difference noted in the CC from the young horses may hold significance for the industry. In the young horses the CC looked much less substantial, and

almost delicate in nature, compared to that of the older horses. In children, the CC is known to play a role in growth and modelling of the epiphyseal bone **(Anderson *et al* 1993)**. It is possible that this appearance of the CC in the young horses is what allows for growth and expansion of the distal end of MCIII. If so, determining the biomechanical properties of this layer, especially the way it changes with maturity, could have implications as to how young horses are trained. As can also be seen in **Fig 5.4 and 5.5**, empty lacunae are evident within the CC layer of both specimens, although the lacunae were consistently larger in the young horses. We are in agreement with **Flygare *et al* (1993)** that this appearance is an artefact of preparation and not the result of chondrocyte necrosis as suggested by **Meachim and Allibone (1984).** The larger lacunae in the younger animals however, do possibly indicate a more metabolically active layer in these animals.

When examined by Section, area 12, the region of sesamoidean articulation, had a significantly thinner CC layer in the youngest horses when compared to the three groups of oldest horses. The mean CC thickness increased by over 100μm between the horses under 5 years old and those 15 to 19 years old (Group 4). This finding suggests that the CC layer is subject to growth and maturity, just as the rest of the musculoskeletal system is. Contrary to this, in a human study, a decrease in the CC thickness was noted with increasing age **(Lane and Bullough 1980).** Alternative reasons postulated to cause CC thickening are exercise and disease **(Oettmeier *et al*. 1992)**

When section was examined within age, another pattern of maturity can be seen. In the youngest group of horses, Section 15 was significantly thicker than 11 and 16, the two proximal most sections. Section 15 is located on the dorsal aspect of the articular surface, in contact with P1. As the horses move into Age Group 2, Section 12 joins 15 in being significantly thicker than 16 and 11. In this group and the next, 12 and 15 have similar values, but in Age Groups 4 and 5, Section 12 becomes thicker than 15. There is some thought that the sesamoidean

articulation is one of the regions of the distal metacarpus that is under the most stress **(Vilar *et al* 1995).** This stress has been postulated to increase with training, causing a proliferation of bone in that region **(Pool and Meagher 1990).** The pattern of thickening of the CC that was seen in this study, especially in Section 12, could be the result of a normal increase in mechanical stress seen with ageing or possibly the result of increased athleticism in the older horses.

Weight and section exhibited a significant interaction as well. Section 11 had the thinnest CC for all weights, but only 15 was different from it in Weight Groups 1 and 3. Section 12 was also significantly different in Weight Groups 2, 4 and 5, where it exhibited a thicker CC layer than 15, although not significantly different. The thickening of the CC in Section 12 as the horses become heavier is similar to the effect seen with ageing. It may relate to changes in the stress experienced by the joint or it may be due to different, i.e. more intense, training regimes seen in the larger horses. Because the performance history of these horses was unknown, this could not be determined in this study.

When the sections were examined independently for effects of age, gender and weight, very little new information emerged. Again, Section 12 showed a significant effect of age, becoming increasing thicker with advancing age. Sections 11 and 16 were also significantly affected by age.

A second objective to this study was to examine the noncalcified region of the hyaline cartilage (NC) and to determine if any relationship exists between it and the CC. The same method of measurement was employed as was used for the CC. The deep border of the NC was considered to be the tidemark (TM), a relatively straight line between the CC and the NC. The superficial border at the articular surface was relatively straight as well, so the thickness could have been measured via conventional techniques. However, in order to maintain a standard between the two measurements, the method of integration was used.

The thickness of the NC layer ranged from 54μm to 876μm. In the only study known to the author in which cartilage thicknesses of the equine species were published, only foals were used as subjects **(Firth and Greydanus 1986).** In that study, the thicknesses obtained for the total hyaline cartilage over the distal metacarpus ranged from 1900μm to 3100μm. Even when the CC values from the first part of the study are added to our NC values, Firth's values are much greater. The total cartilage layer in our study ranged from 235μm to 1399μm with a mean and median of 781μm. It should be noted, however, that all the subjects in Firth's study were foals aged 0 to 150 days, whereas the youngest horse in our study was 18 months old. As discussed in the next paragraph, the NC thicknesses decrease with age in our study, so this may be an explanation for the much thicker cartilage measurements seen in the foals. Also, although the Firth study was an attempt to quantify a structure and establish baseline data, the method they employed for obtaining the measurements was crude. Callipers in the *post-mortem* room may have been the state of art method at that time, but the advent of digital imaging and computerised histomorphometry has provided our study with a more technically precise and repeatable methodology.

Similar to the CC, the NC experienced a significant effect on thickness by both age and section. When examined by age, Group 1 had significantly thicker NC than Group 2 horses. When the section effect was considered, only 12 showed a significant difference. The NC in the region of the sesamoidean articulation was thinner among all age groups. These two findings seem to suggest that the NC thickness may also be related to stress encountered within the articular environment. The NC becomes thinner as the horse matures and it is always thinner in a region of high compressive forces (Section 12). Although the finding of decreased cartilage thickness in relation to increasing age is in agreement with another study **(Firth and Greydanus 1986),** decreased thickness in relation to mechanical stress is not. **Oettmeier *et al* (1992)** reported an *increase* in the CC and NC thickness in response to exercise and **Muller-Gerbl *et al* (1987a)** found

that both the CC and NC were thicker in regions that bear heavy loads. Because our data are so contrary, an explanation must be sought. There would appear to be one of three scenarios. It is possible that the biomechanical work that has been done in the equine MCPJ has not properly identified the region under most stress **(Vilar *et al* 1995).** This is unlikely to be the case, especially since clinical syndromes often occur in the palmar region of the fetlock **(Pool and Meagher 1990, Martinelli *et al* 1994, Norrdin *et al* 1998).** Alternatively, the equine articular tissues may respond differently to loading conditions than other species. One final possibility is that all the samples in our study could be diseased. A thickening of the CC layer with consequential thinning of the NC layer has been reported as a change consistent with OA **(Oettmeier *et al* 1989b).** This is unlikely due to the nature of the population.

Examined by itself, gender was not significant, but in relation to section, it showed a significant difference. In males, 12 was different from 16, 15 and 14; whereas in females, 12 was different from 16, 14 and 11. The reasons for this difference are unclear. It could be related to hormonal differences during development or may not hold any physiological significance.

When documenting the effect of age, gender and weight on section, the most notable effect on NC was that of age in section 12. As noted earlier, it decreased in thickness with age from the youngest horses until it levelled off in the middle age group, 10-14 years old.

The CC/Total Cartilage ratios revealed similar information as the other parts of the study. Firstly, the meaning of the ratio should be explored. When expressed as CC/Total Cartilage, an increase in the ratio indicates that there has been a relative increase in the thickness of the CC in relation to that of the complete cartilage layer. Alternatively, this could also mean that there has been an effective decrease in the thickness of the NC layer within that section.

The age and section location did have a significant effect on the ratio. When section was examined in relation to age, the youngest group of horses did not experience any effect. Section 12, however, became significantly different from some of the other sections in the 5 to 9 year old horses and remained that way for the other Age Groups. In the oldest group of horses, Section 12 was significantly different from all the other Age Groups. When the influence of age was examined by section, the most important information was that Sections 14 and 15 were completely unaffected by age, whereas Section 12 was heavily influenced by age ($P = .001$) such that the ratios were much higher in horses over the age of 5 years. This can be appreciated visually when comparing **Fig 5.6 and 5.7.**

This information is very different from what has been determined in the human literature over the years, i.e. that the ratio does not change in relation to location within a joint (**Muller-Gerbl *et al* 1987b).** They found that the volume of the CC expressed as a percentage of the total cartilage was a constant within a joint as well as for all the joints of an individual subject. It should be noted that their study included material from only five individuals and involved taking repeated measures from that subject in addition to taking multiple samples from the same joint. Although they do not report it, the Power of their statistical tests is probably quite low, meaning that it would be difficult to detect a significant difference between measurements and samples even if one existed **(Markel 1991).** The Power for our tests was also quite low even though there were 34 horses in the study. Because a significant difference was noted, however, the low Power becomes inconsequential.

The ratios were examined further by section to determine whether age, gender or weight had an effect on the value. Section 12 was influenced by age such that Age Group 1 had a significantly lower ratio than the others. It also showed a different response pattern when analysed in relation to weight and gender even though neither value was significant on its own. The females were unaffected, but

the heavier males had a higher ratio, indicating either more CC, less NC or both in relation to the amount of TC present at that site. Although this difference could be genetic, metabolic or nutritional in nature, it may also be due to training regimes; males are often trained harder as performance animals. Because no athletic history was available for these horses, this could not be investigated further. Sections 15 and 16 were also influenced by age, experiencing significantly higher ratios in the older horses. This could be the result of less mechanical stress experienced at that site.

Within Section 13, weight showed a significant effect. The heavier horses had less NC cartilage at this site than the lighter ones. This would seem to support a mechanical influence present at the palmar aspect of the transverse ridge. Interestingly enough, no significant effects of age, gender or weight were recognised in Section 14, located a few hundred micrometers away, but on the dorsal aspect of the transverse ridge. This finding supports the theory of **Pool and Meagher (1990)** that a high degree of pressure on the palmar aspect of the transverse ridge can lead to a disease process they call 'traumatic osteochondrosis'. Pool hypothesises that these lesions develop as a result of shearing stress just palmar to the transverse ridge. That explanation seems to be in agreement with our data. In contrast to this, **Vilar *et al* (1995)** examined the forces encountered within the metacarpophalangeal joint at different gaits. They found that the sesamoid came into almost complete contact with the palmar aspect of the metacarpus, supporting the theory of stress-induced changes in Section 12. However, they also reported that the basilar region of the sesamoid bone, near the transverse ridge, became a non-contact region at a simulated gallop angulation of 240 degrees of extension. While useful information has been gained from their study, it is possible that their model of limb placement was flawed. For obvious reasons, it was a cadaveric study, meaning that all limb postures and angles of extension were extrapolated from measured fetlock angles in kinematic studies. Although 240 degrees may be the measured angle of fetlock extension during the gallop, the metacarpus was placed perpendicular to the ground surface. Close examination of the galloping horse indicates that the metacarpus is far forward of

perpendicular just before limb take-off and that this may be the period of highest stress encountered by the region of sesamoidean articulation. If this assumption is correct, then at that angle the basilar region of the sesamoid bone could come into contact with the palmar aspect of the transverse ridge because of the pull of the distal sesamoidean ligaments, creating enough stress to decrease the amount of NC present, thereby altering the cartilage ratios. **(Fig 5.11)**

Because no data exists for the CC layer of the horse, all of the comparisons have been made with other species. There may be differences between species, much the same as there are in relation to the trabecular architecture **(Swartz *et al* 1998).** For this reason, much of this discussion revolves around theories and speculation. The results of this study do seem to indicate that there are some significant differences between horses in this population as well as between species when these results are compared to those of humans **(Muller-Gerbl *et al* 1987a,b).** It does seem reasonable to speculate that the CC layer may have a yet unknown role in the pathogenesis of articular derangements in the horse, such as OA. More investigations need to be carried out on this fascinating layer.

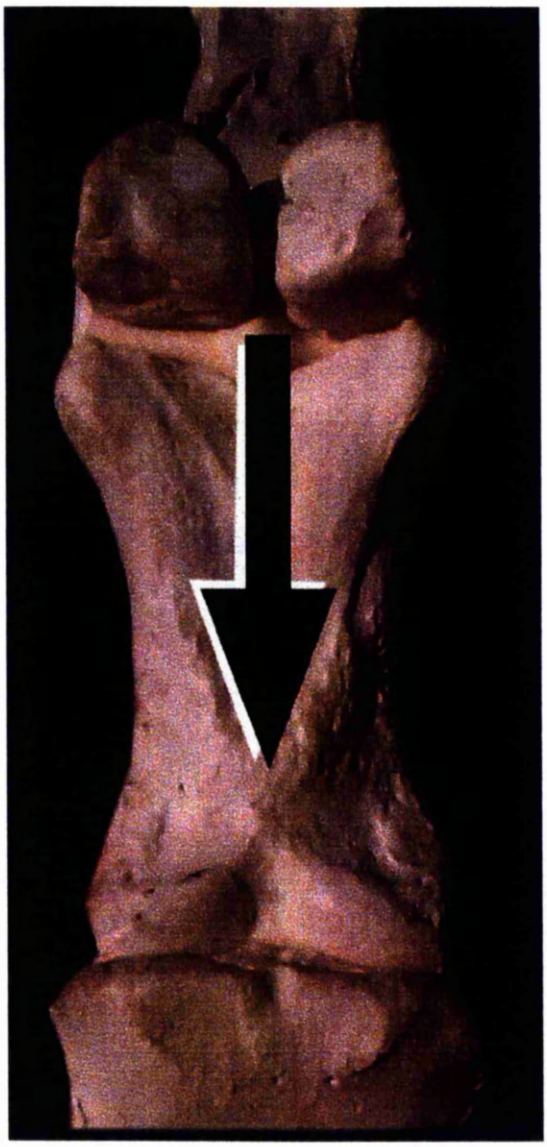
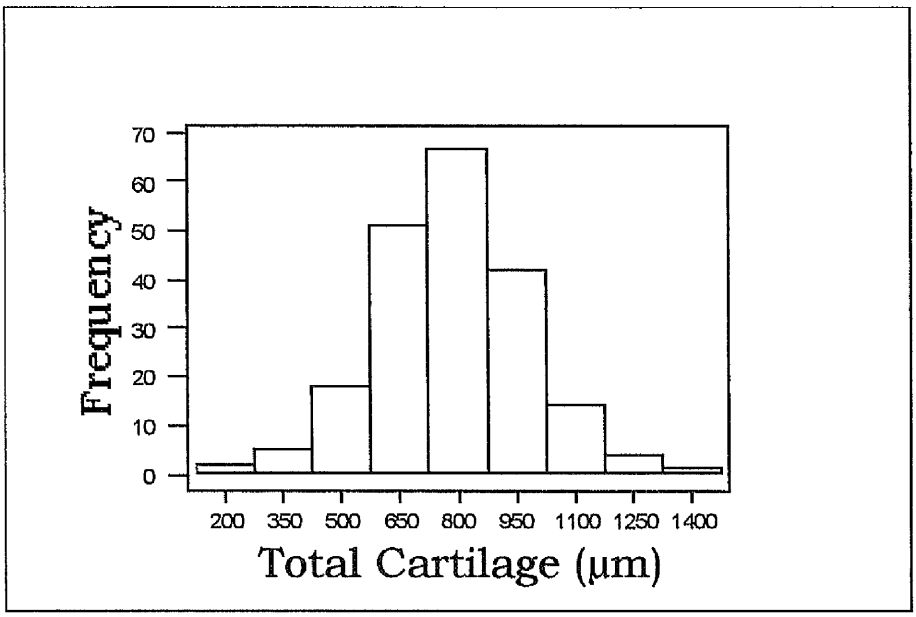
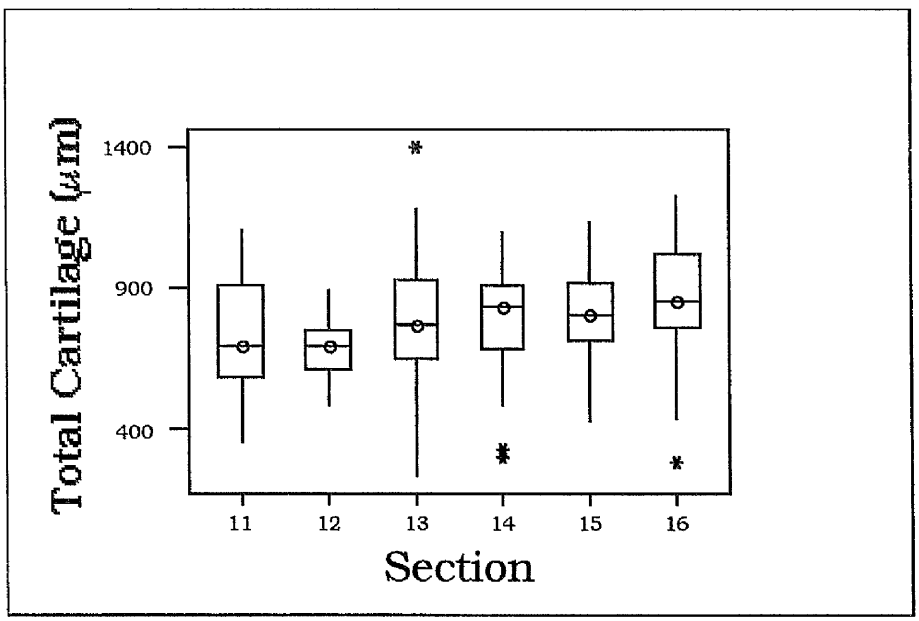


Fig 5.11 Anatomical representation of the pull of the distal sesamoidean ligaments across the palmar aspect of the fetlock. It is postulated that this biomechanical situation may be responsible for many of the effects seen in this study and in the clinical situation.

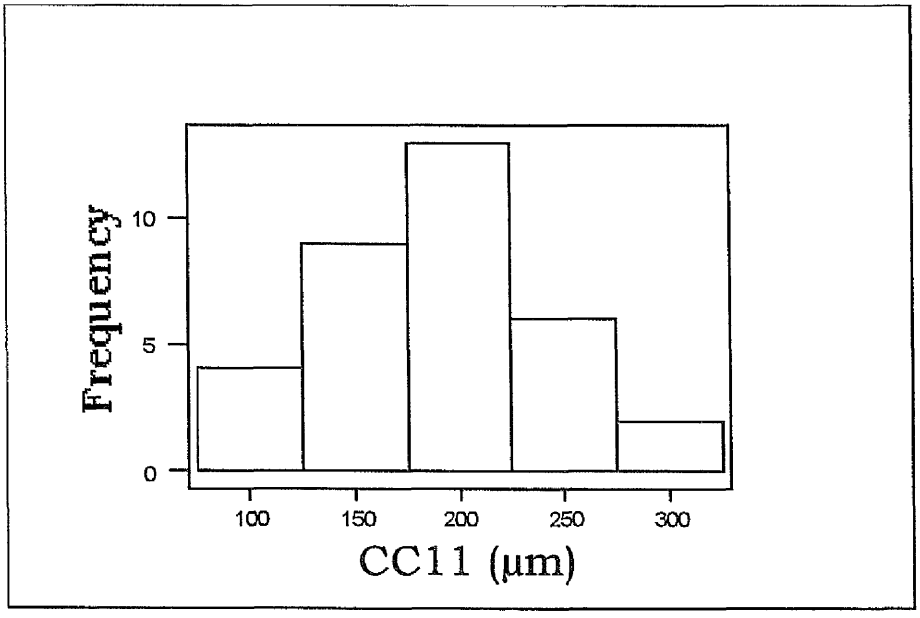
APPENDIX 2



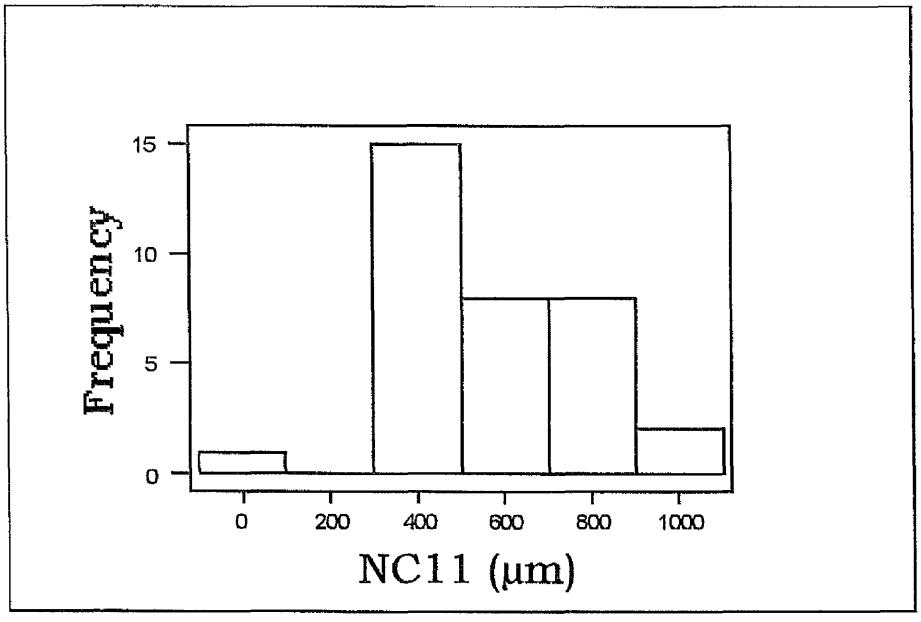
Graph A2.1 Histogram of Total Cartilage Thickness for all Sections



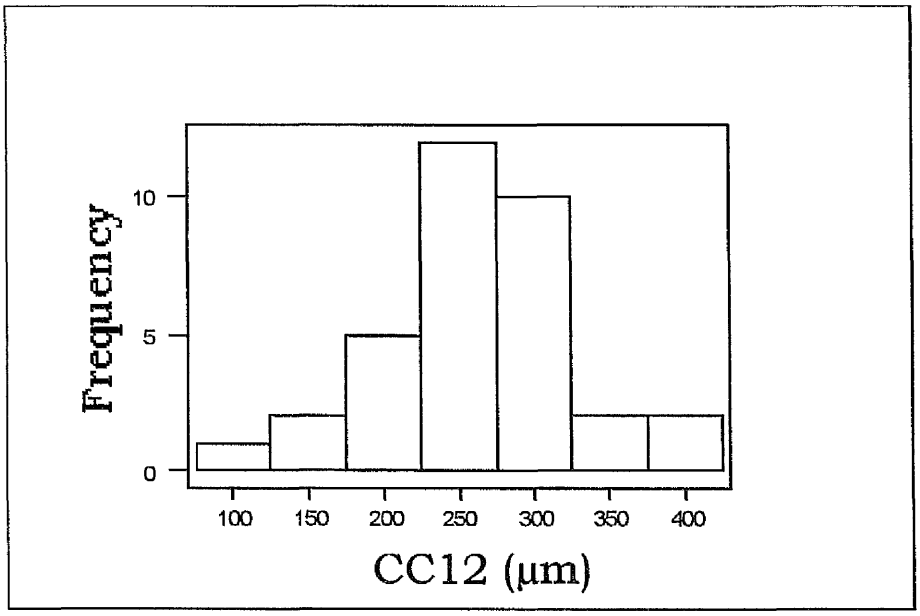
Graph A2.2 Boxplot of Total Cartilage Thickness by Section



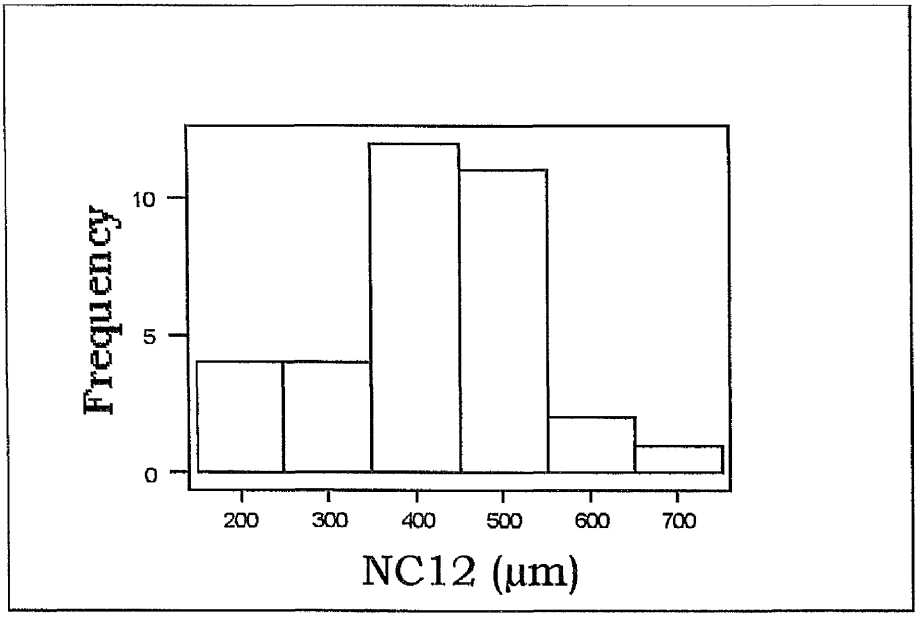
Graph A2.5 Histogram of CC11



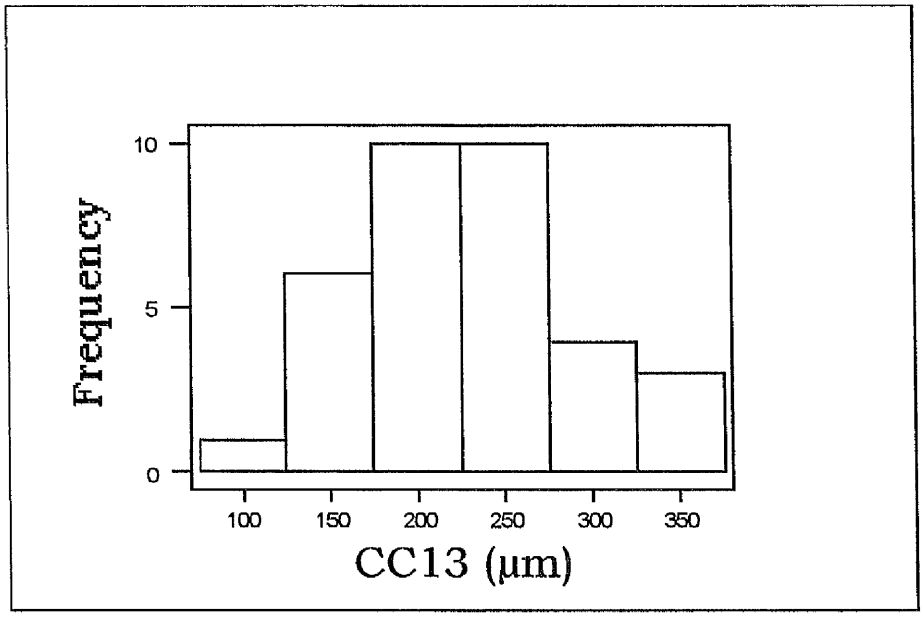
Graph A2.6 Histogram of NC11



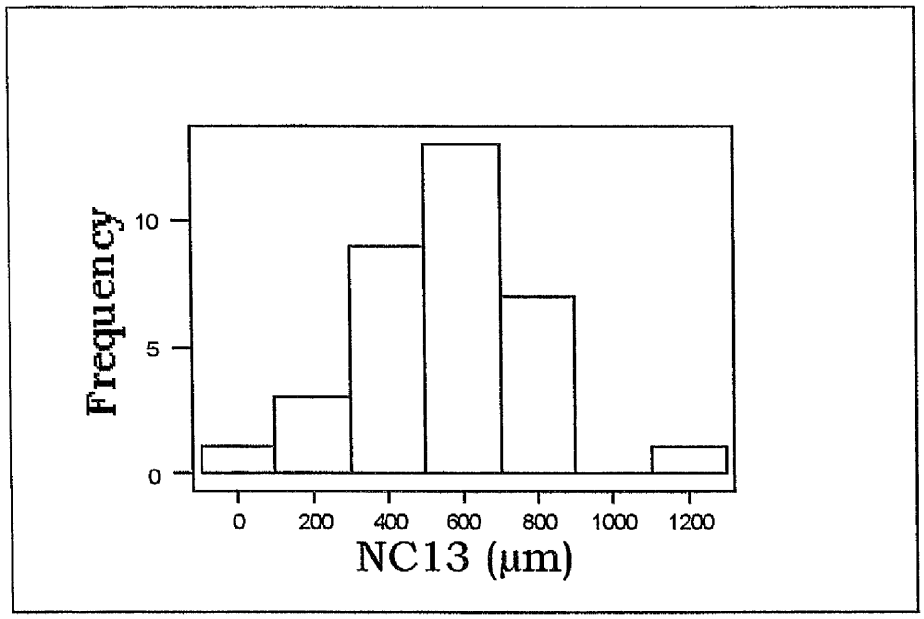
Graph A2.7 Histogram of CC12



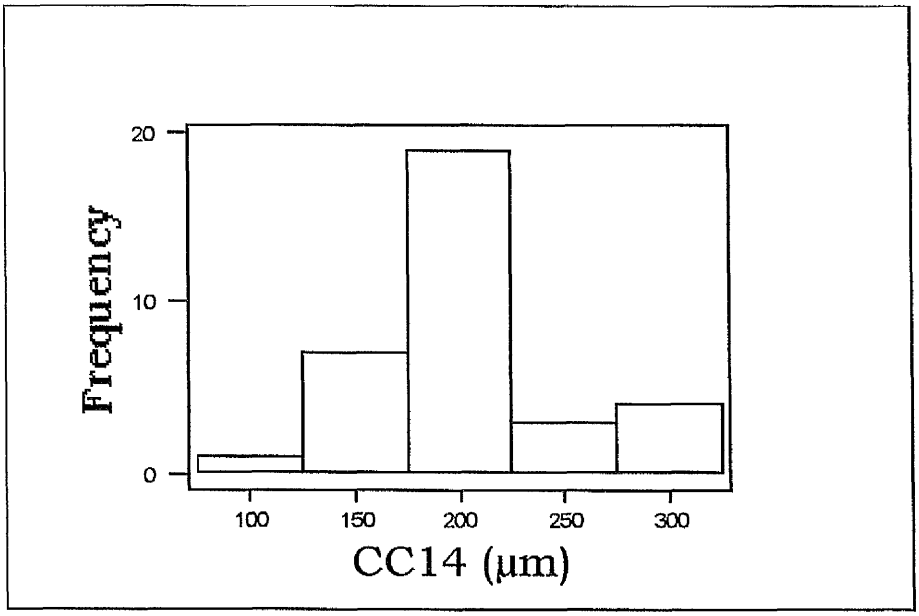
Graph A2.8 Histogram of NC12



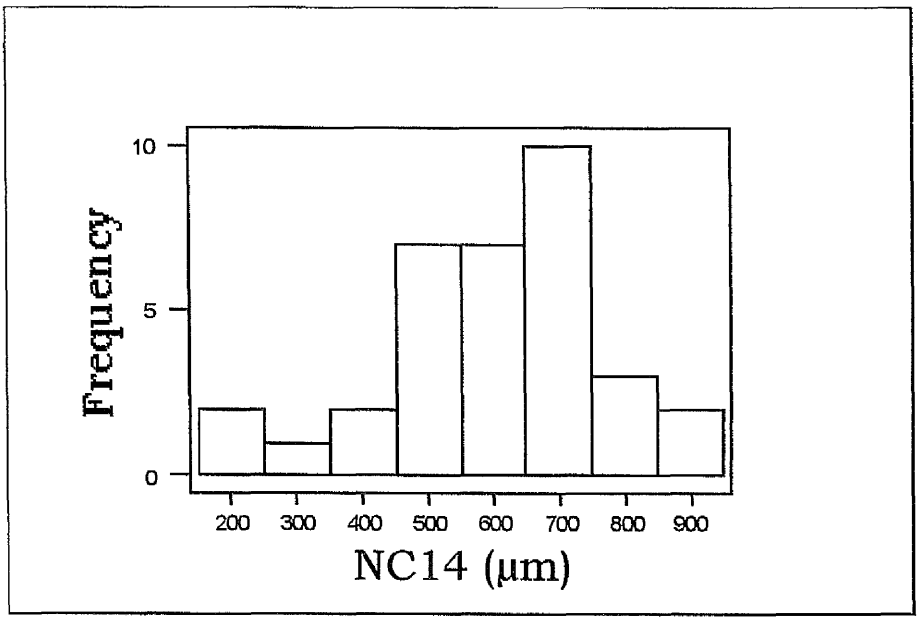
Graph A2.9 Histogram of CC13



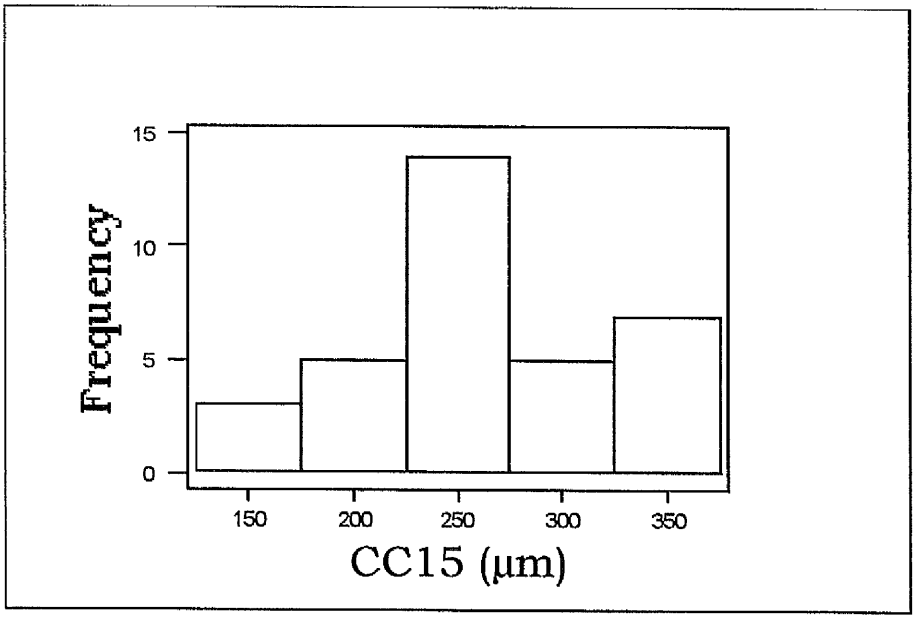
Graph A2.10 Histogram of NC13



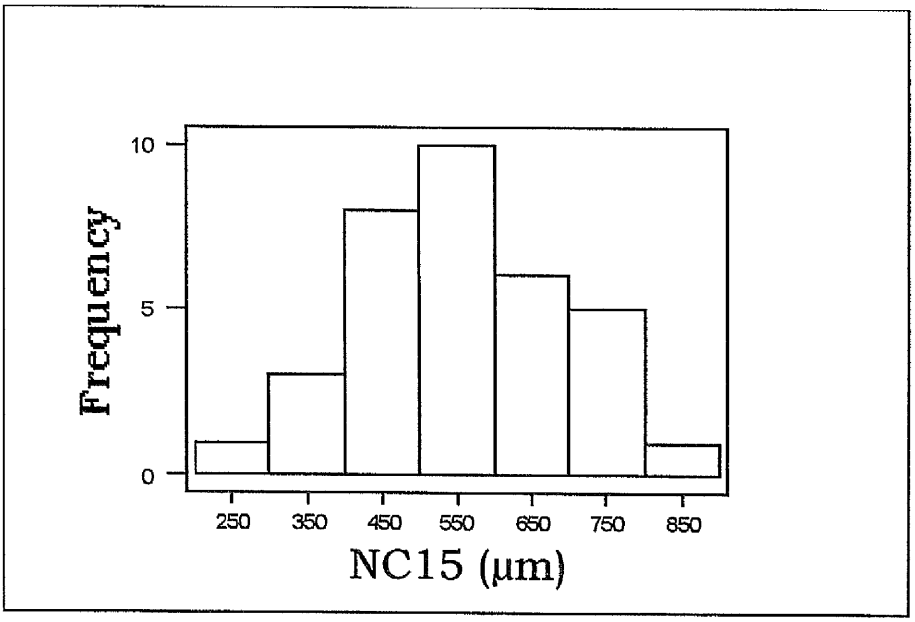
Graph A2.11 Histogram of CC14



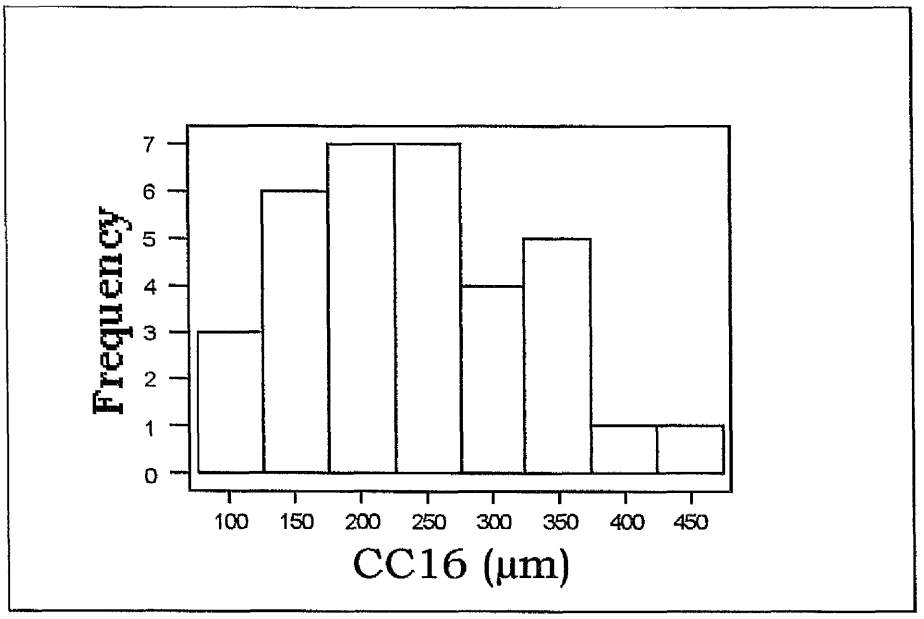
Graph A2.12 Histogram of NC14



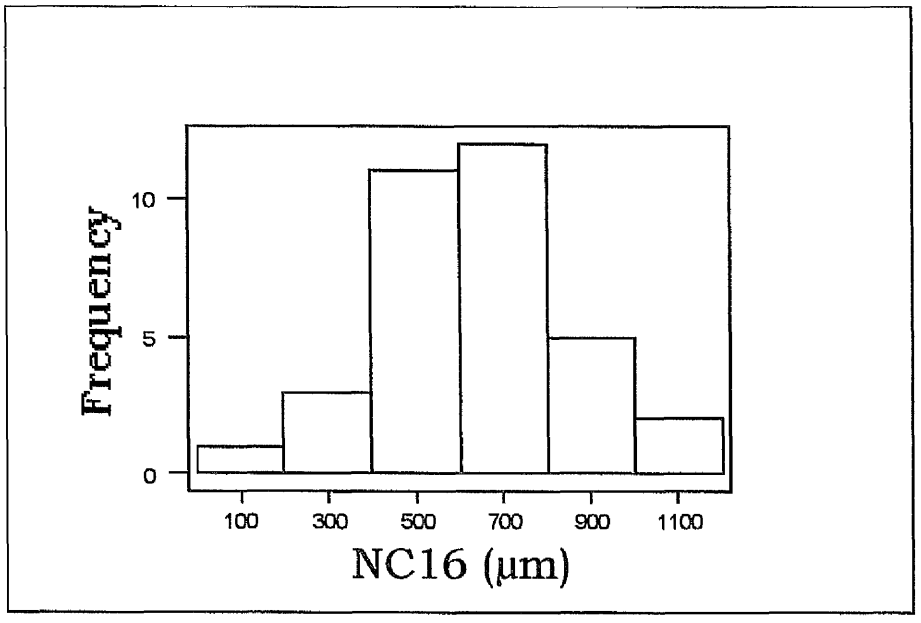
Graph A2.13 Histogram of CC15



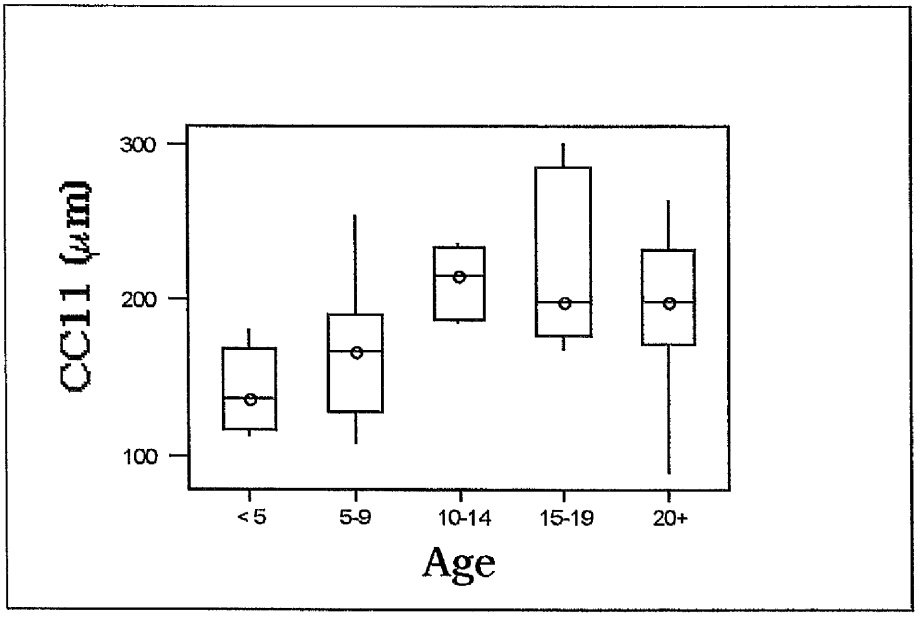
Graph A2.14 Histogram of NC15



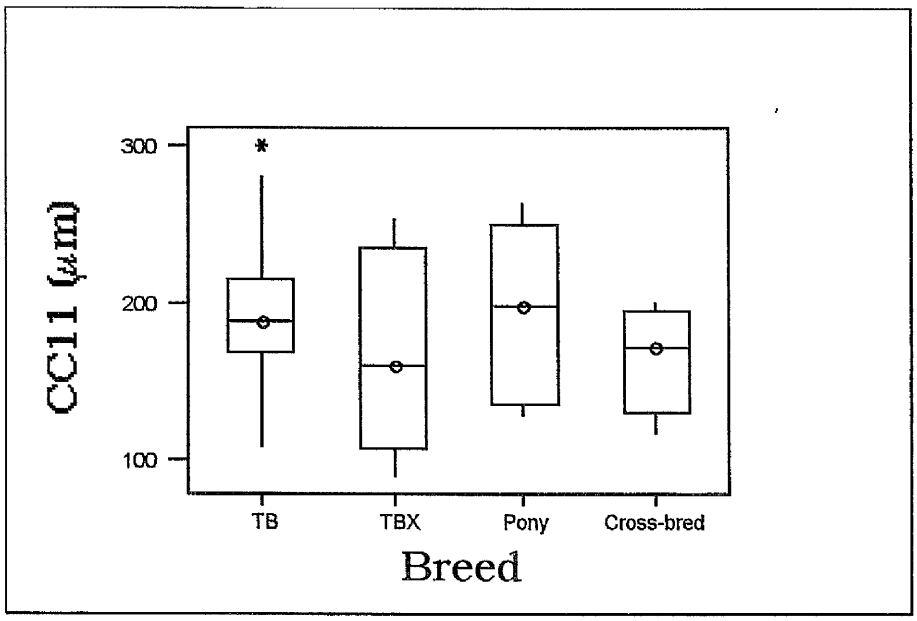
Graph A2.15 Histogram of CC16



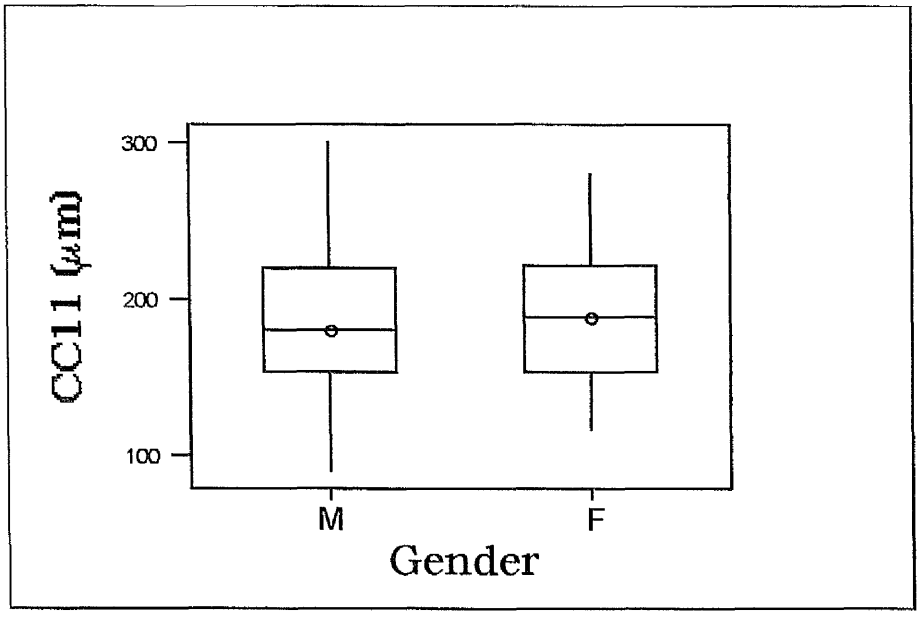
Graph A2.16 Histogram of NC16



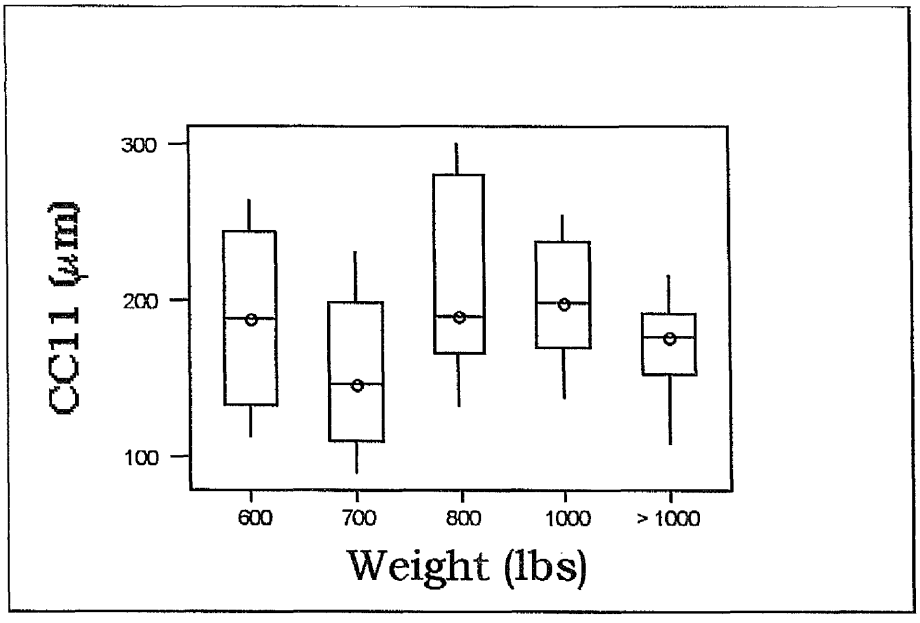
Graph A2.17 Boxplot of CC11 Thickness by Age



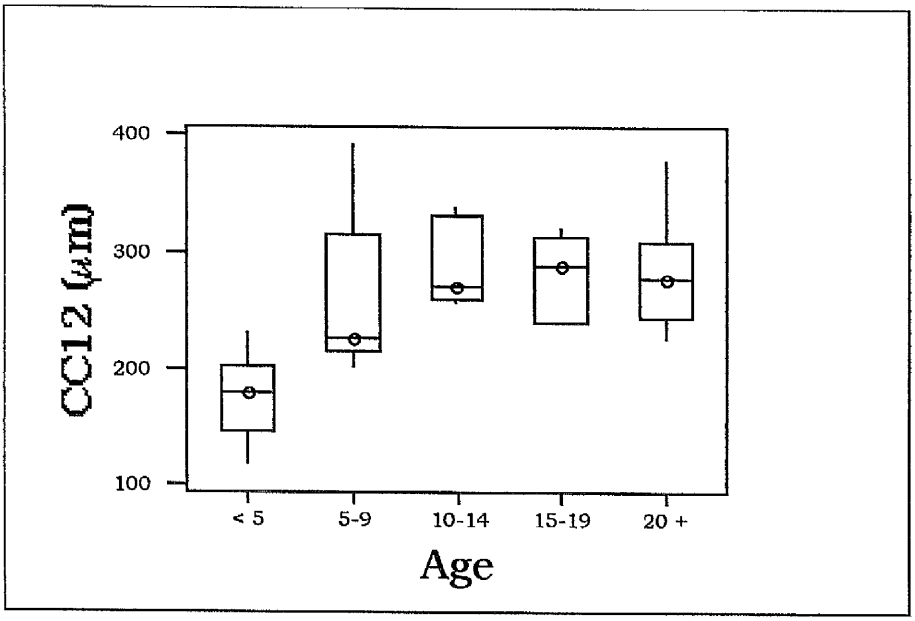
Graph A2.18 Boxplot of CC11 Thickness by Breed



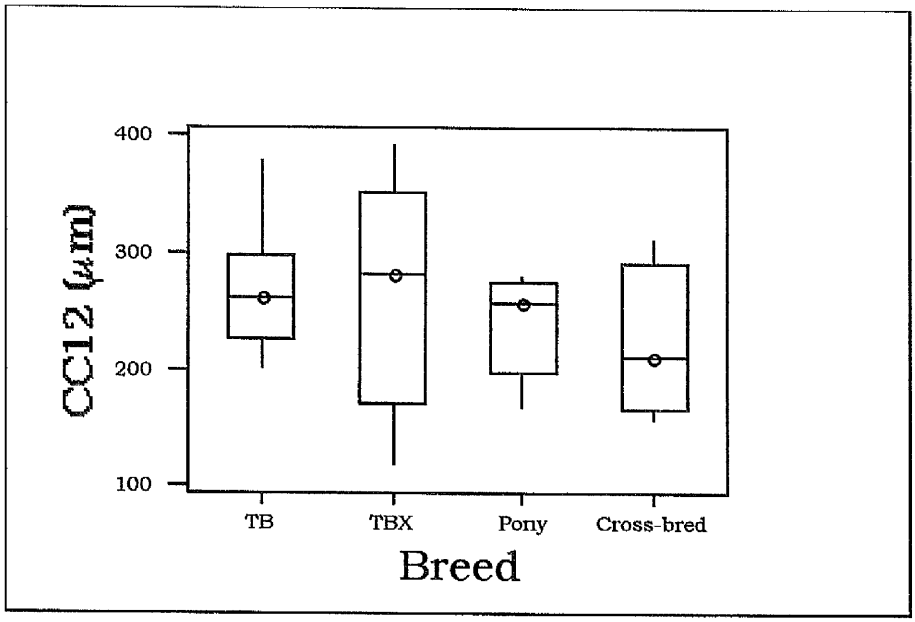
Graph A2.19 Boxplot of CC11 Thickness by Gender



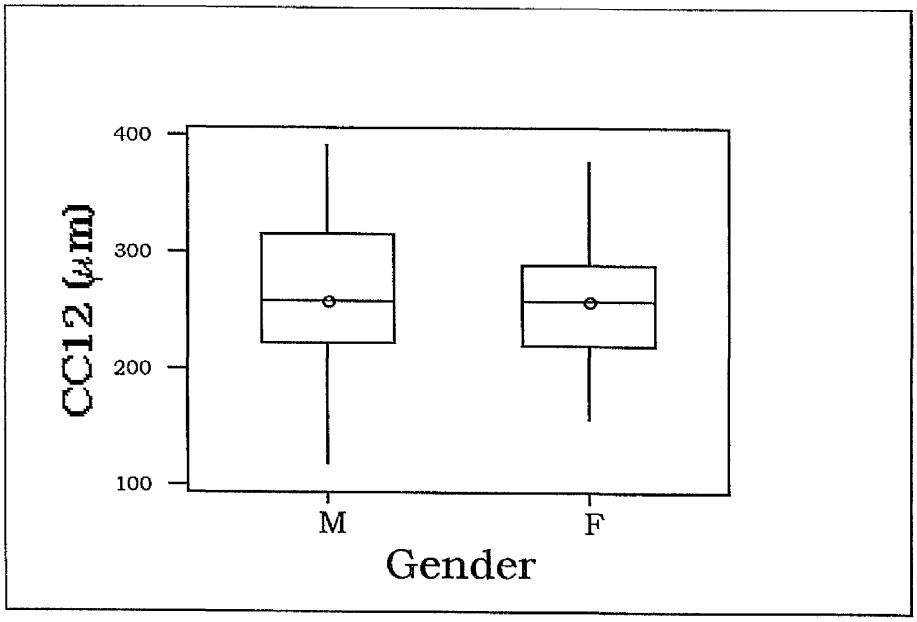
Graph A2.20 Boxplot of CC11 Thickness by Weight



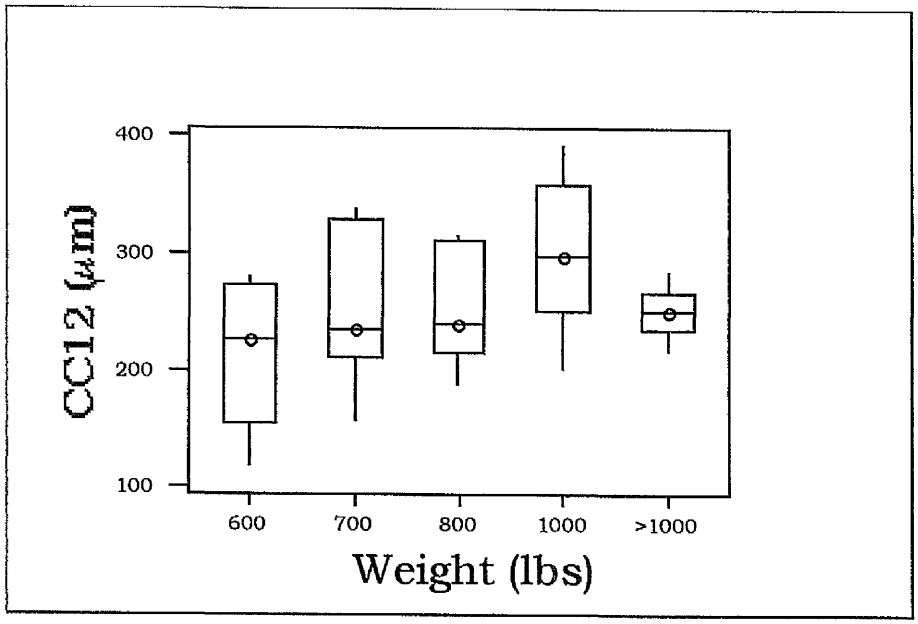
Graph A2.21 Boxplot of CC12 Thickness by Age



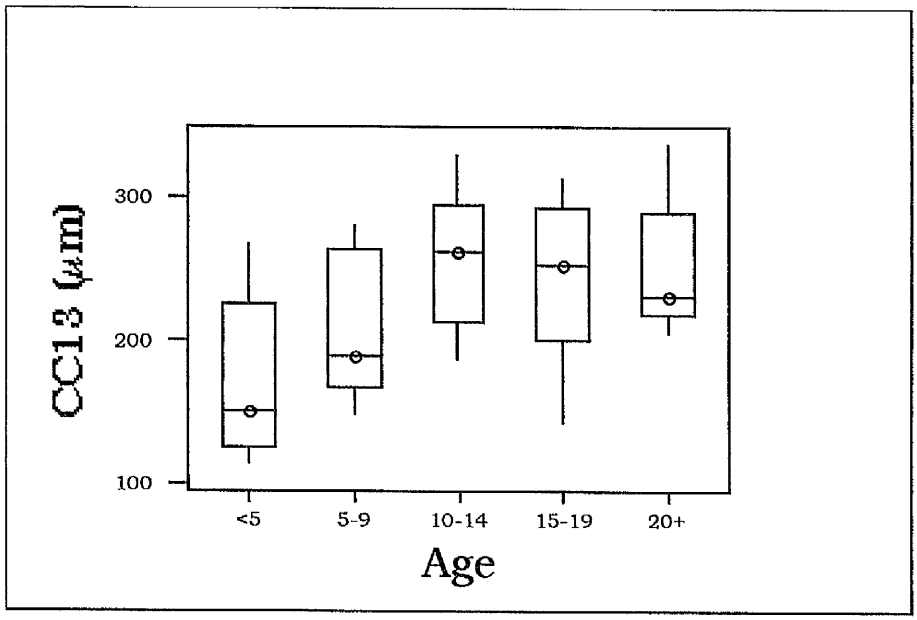
Graph A2.22 Boxplot of CC12 Thickness by Breed



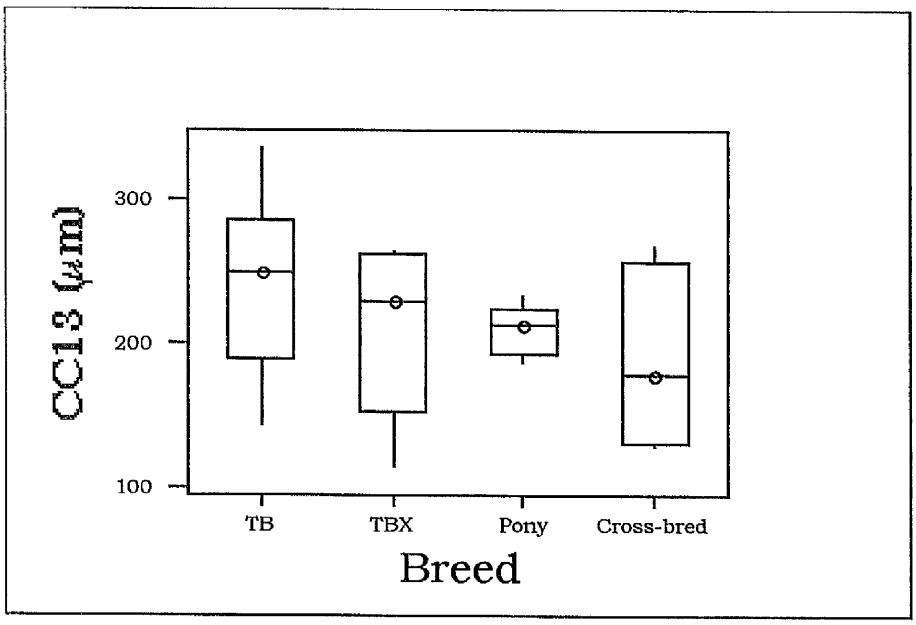
Graph A2.23 Boxplot of CC12 Thickness by Gender



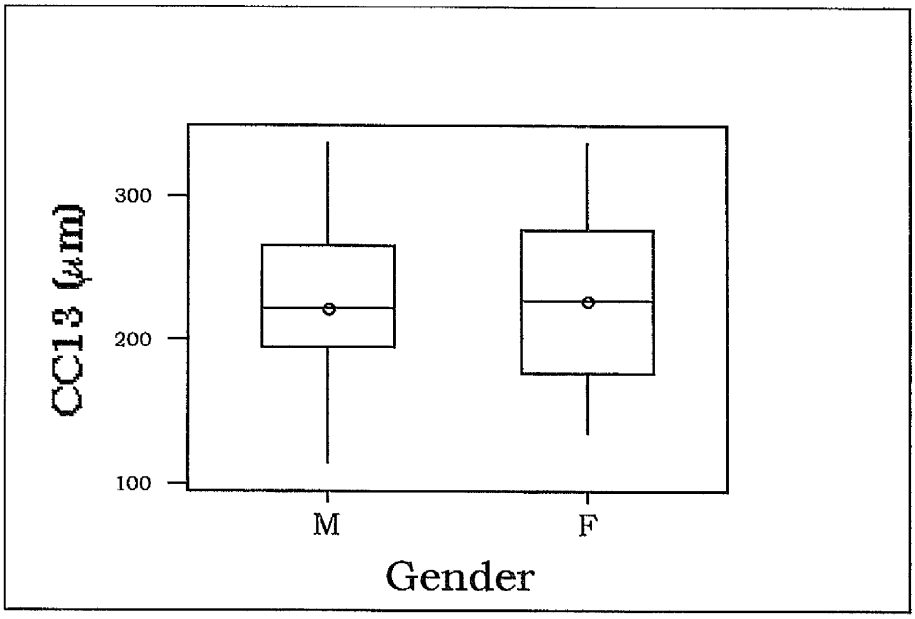
Graph A2.24 Boxplot of CC12 Thickness by Weight



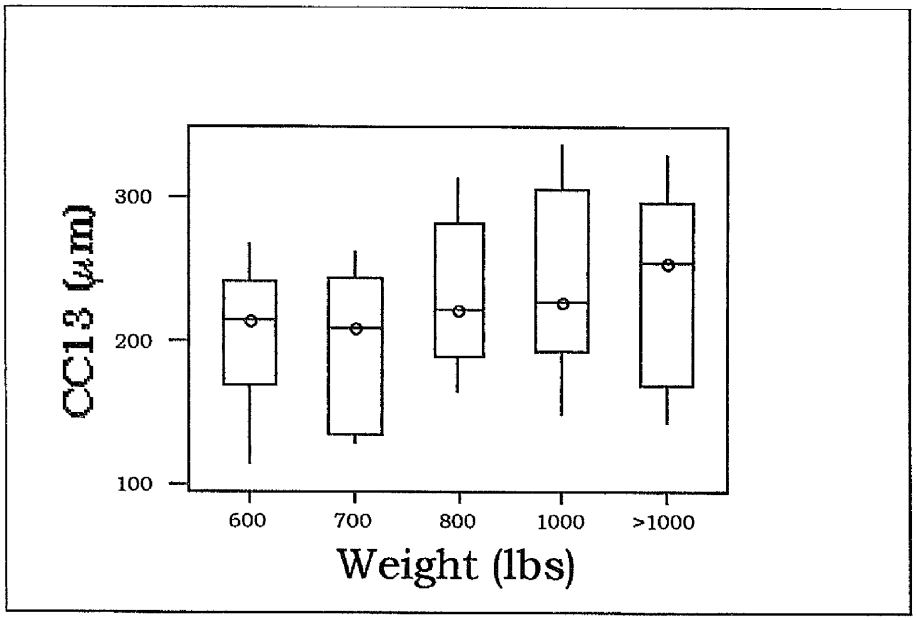
Graph A2.25 Boxplot of CC13 Thickness by Age



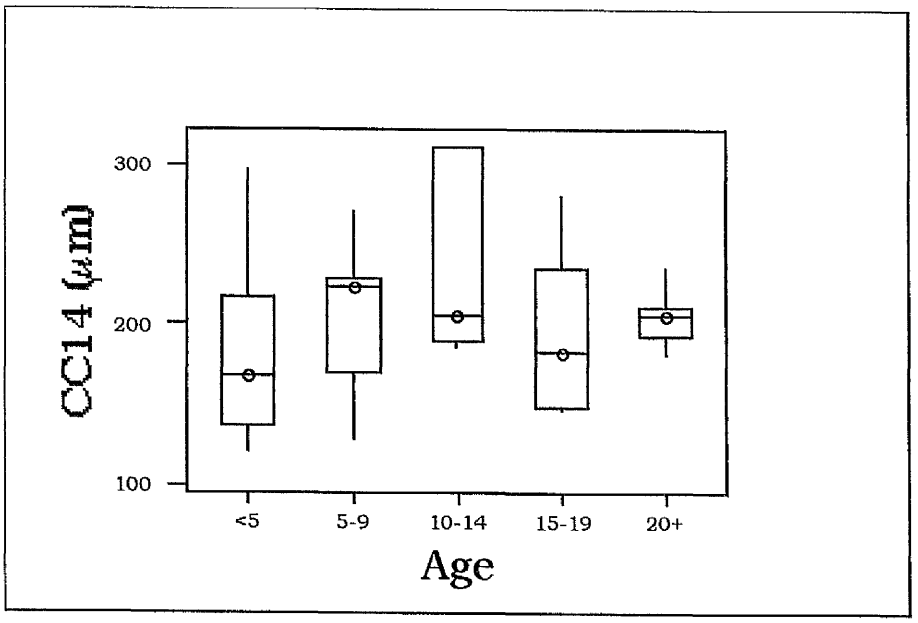
Graph A2.26 Boxplot of CC13 Thickness by Breed



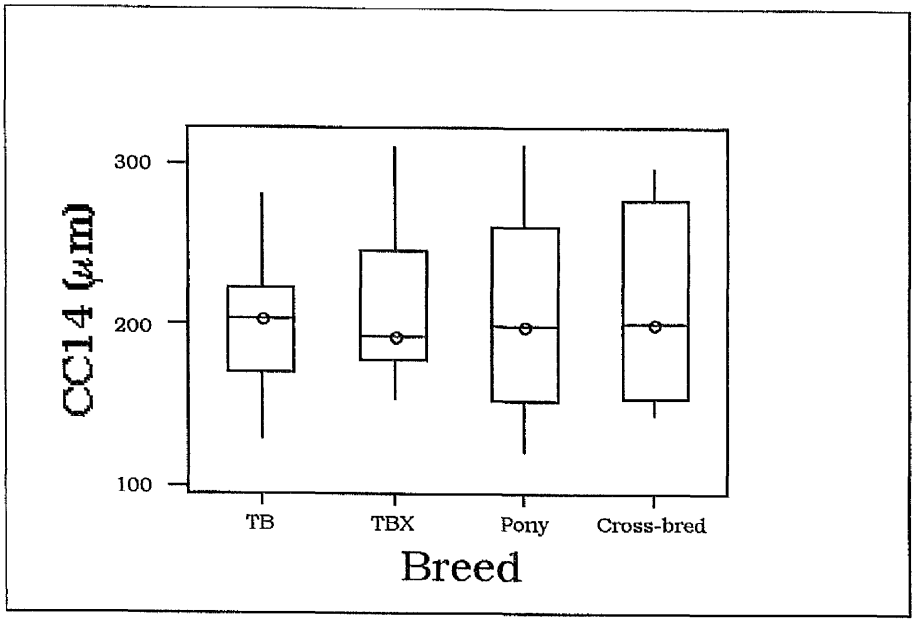
Graph A2.27 Boxplot of CC13 Thickness by Gender



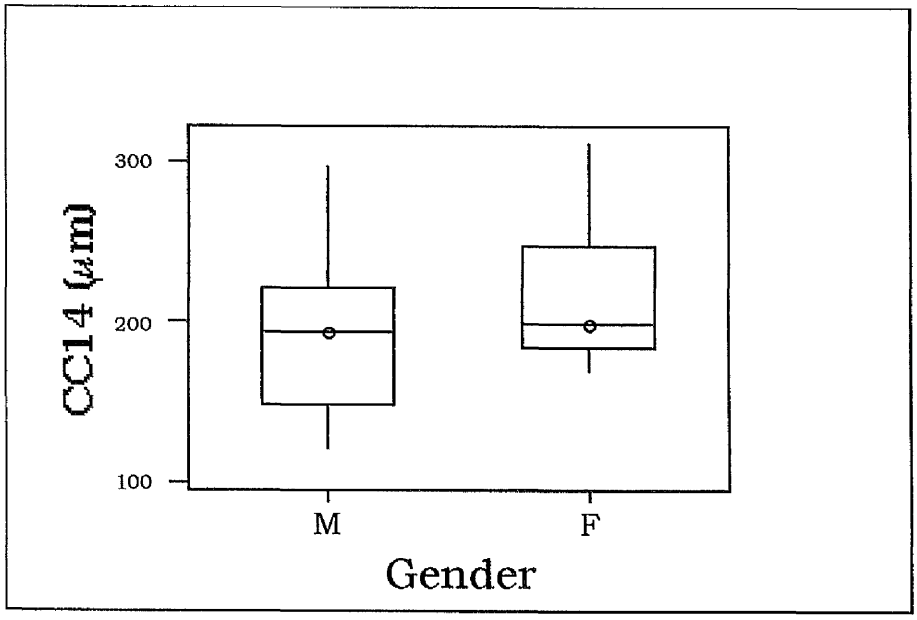
Graph A2.28 Boxplot of CC13 Thickness by Weight



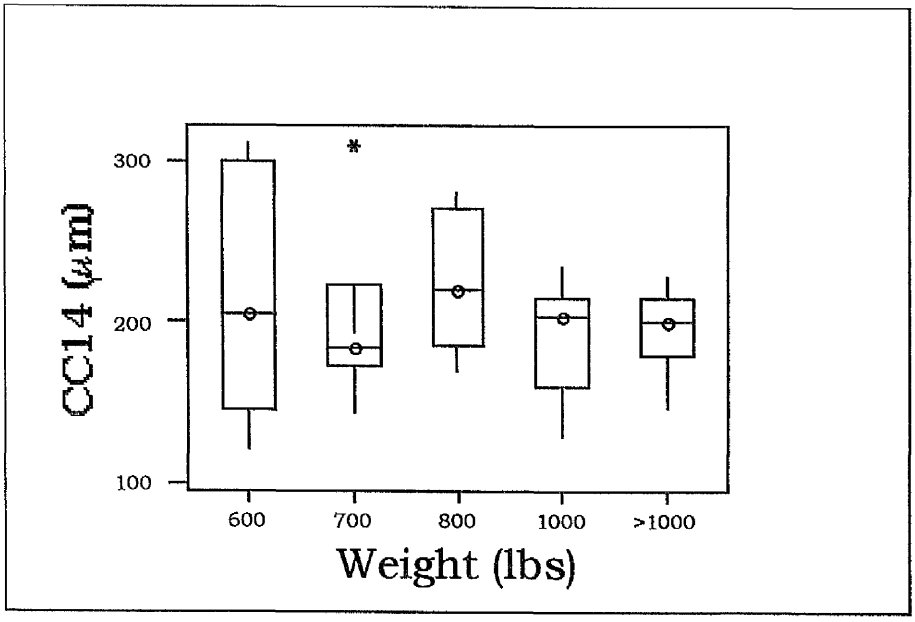
Graph A2.29 Boxplot of CC14 Thickness by Age



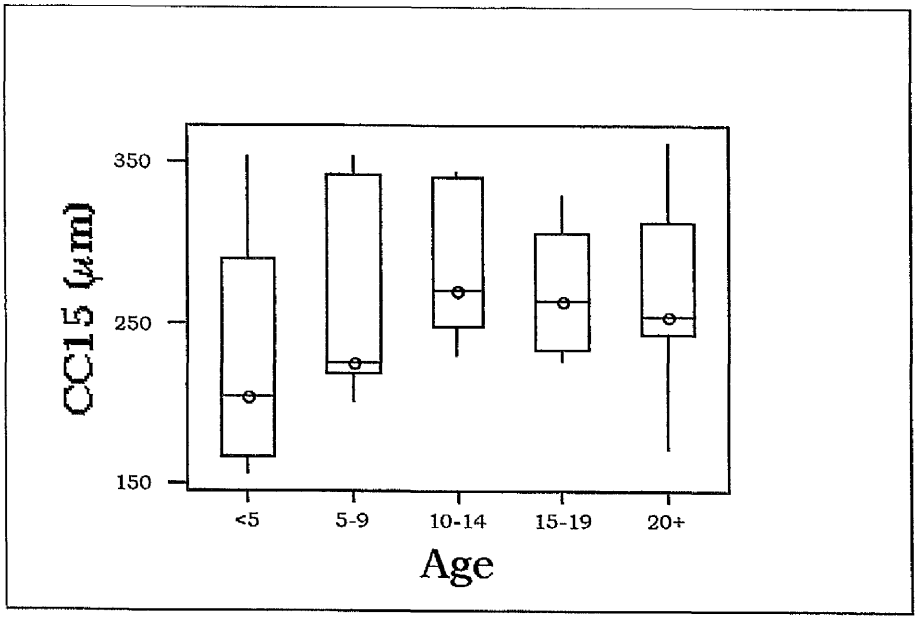
Graph A2.30 Boxplot of CC14 Thickness by Breed



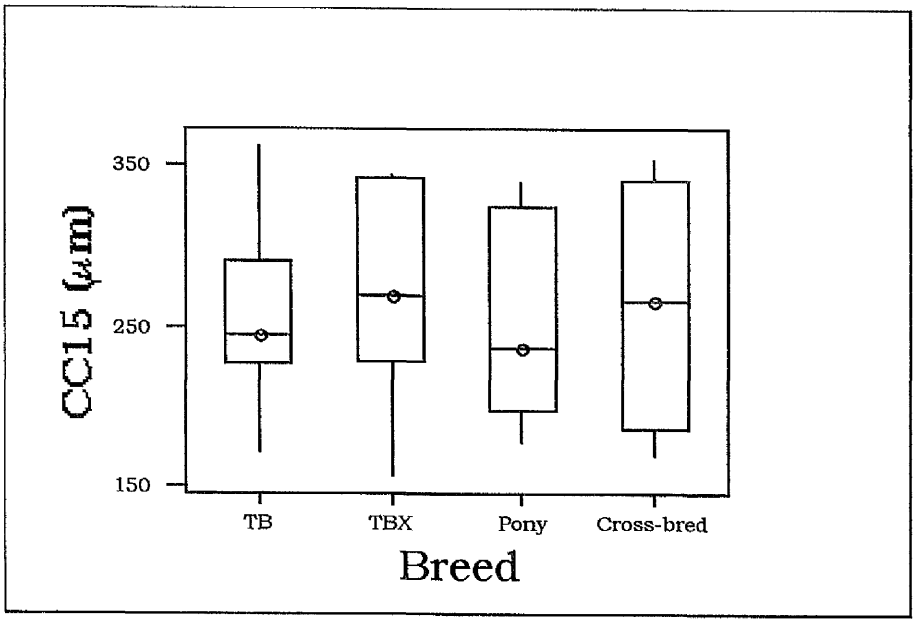
Graph A2.31 Boxplot of CC14 Thickness by Gender



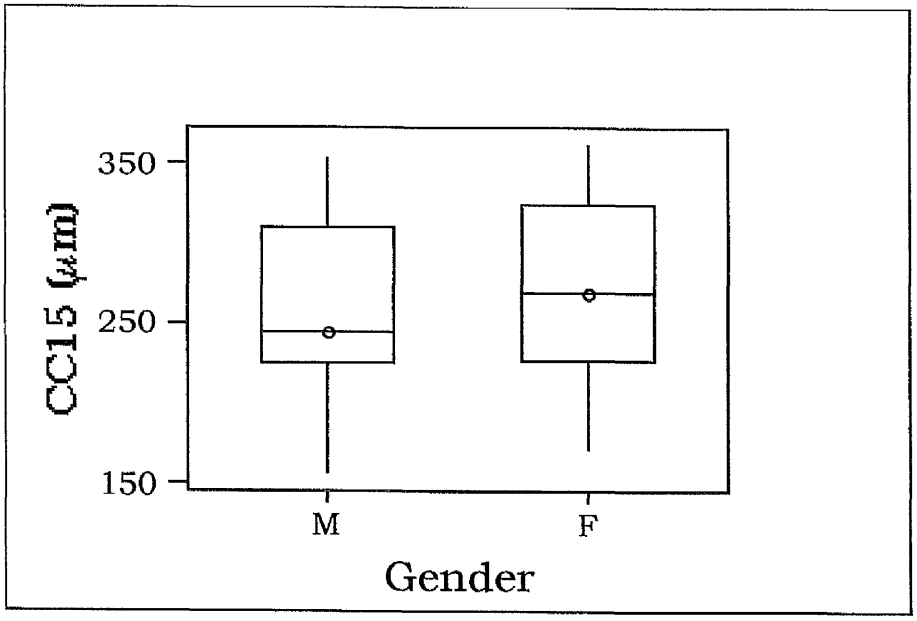
Graph A2.32 Boxplot of CC14 Thickness by Weight



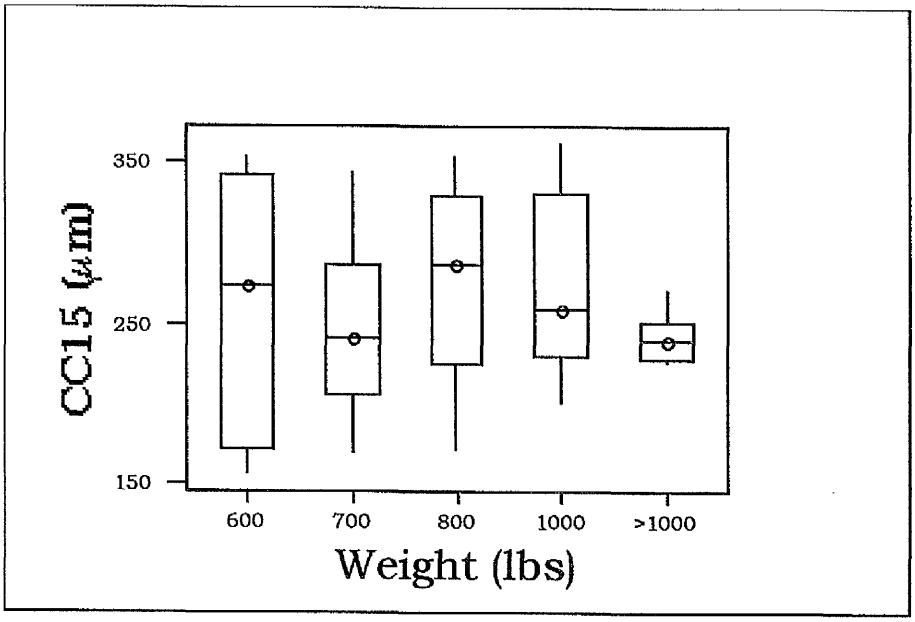
Graph A2.33 Boxplot of CC15 Thickness by Age



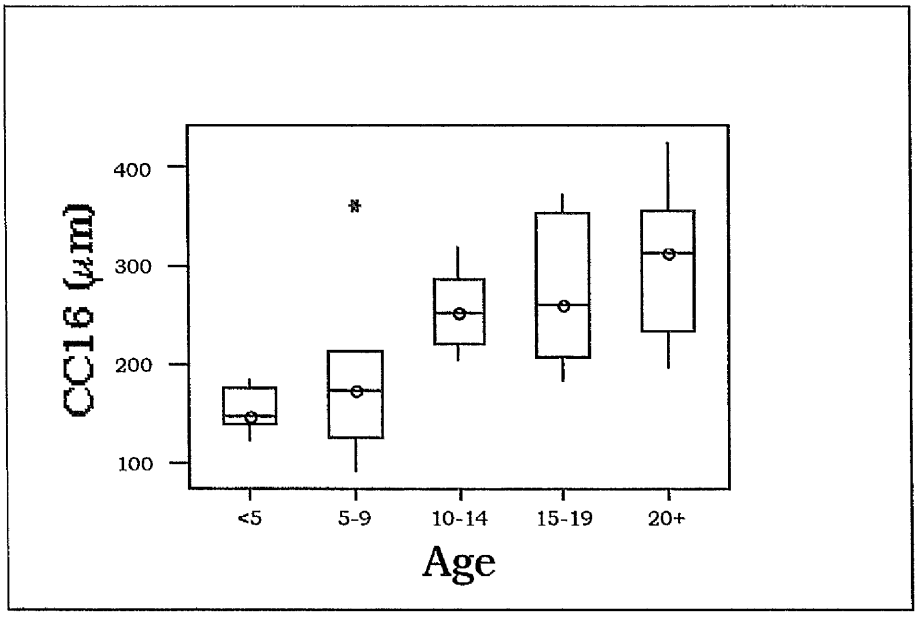
Graph A2.34 Boxplot of CC15 Thickness by Breed



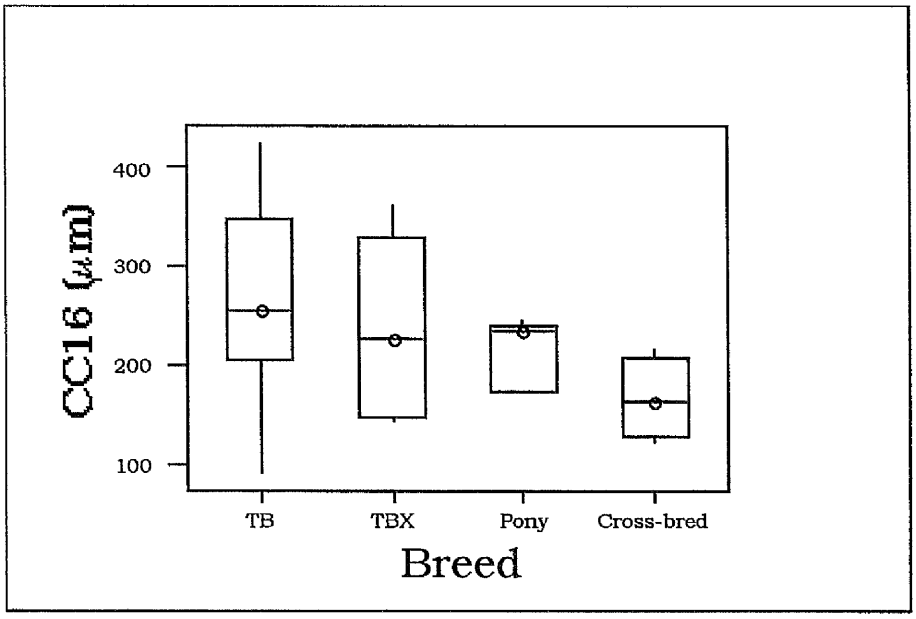
Graph A2.35 Boxplot of CC15 Thickness by Gender



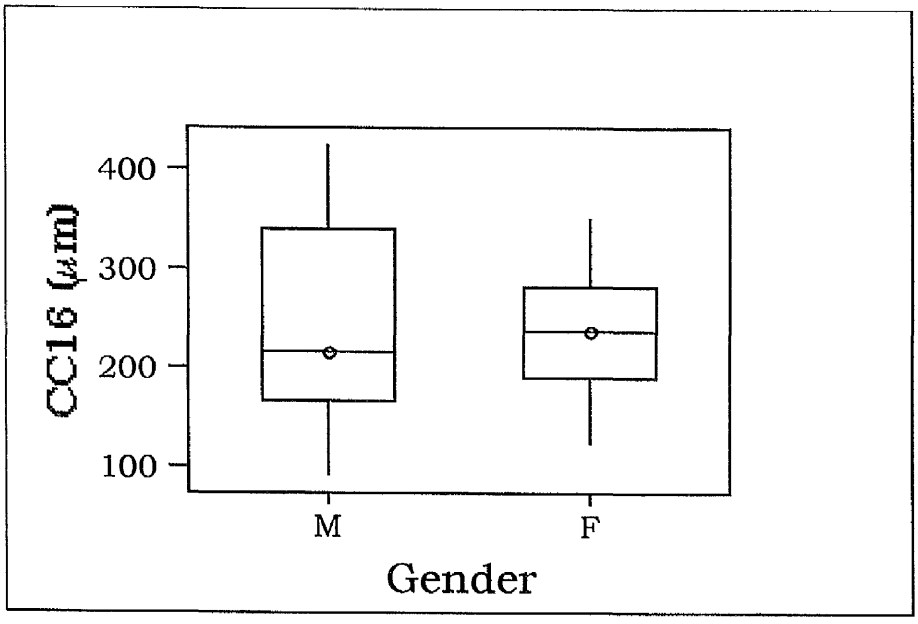
Graph A2.36 Boxplot of CC15 Thickness by Weight



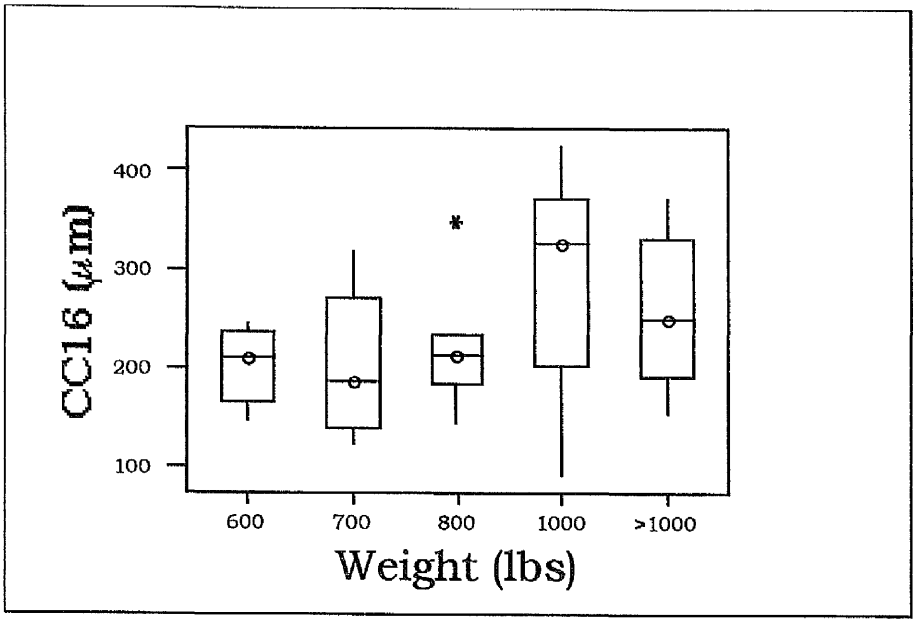
Graph A2.37 Boxplot of CC16 Thickness by Age



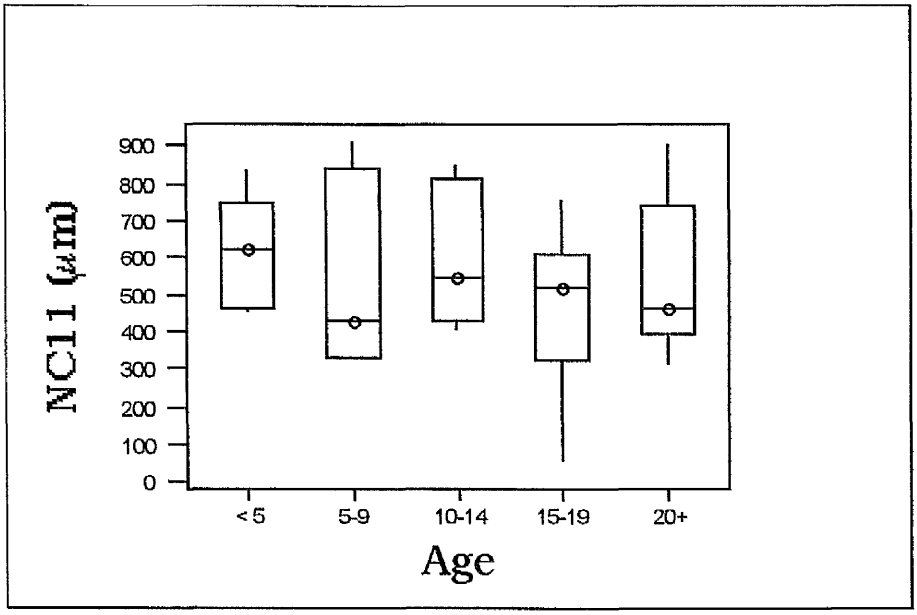
Graph A2.38 Boxplot of CC16 Thickness by Breed



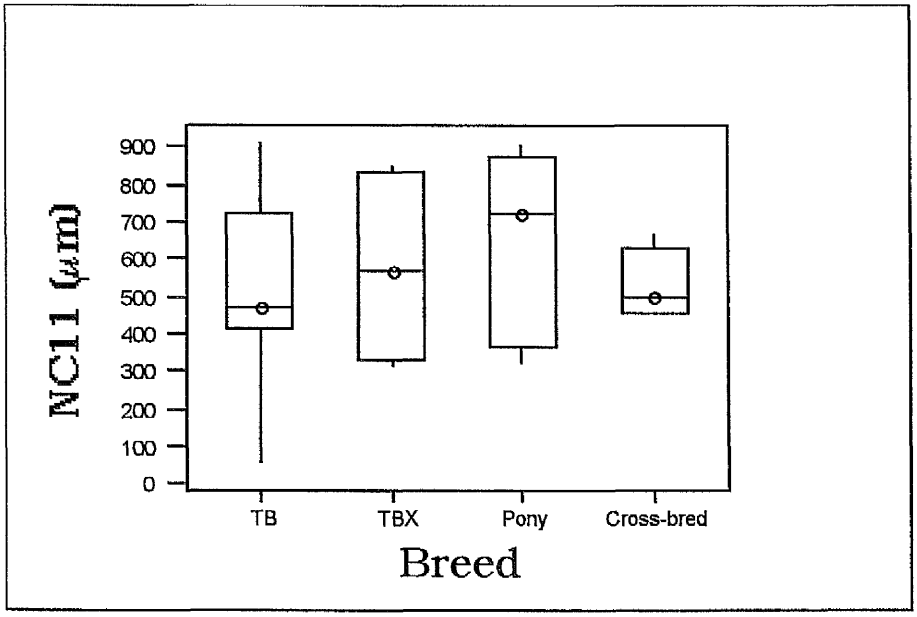
Graph A2.39 Boxplot of CC16 Thickness by Gender



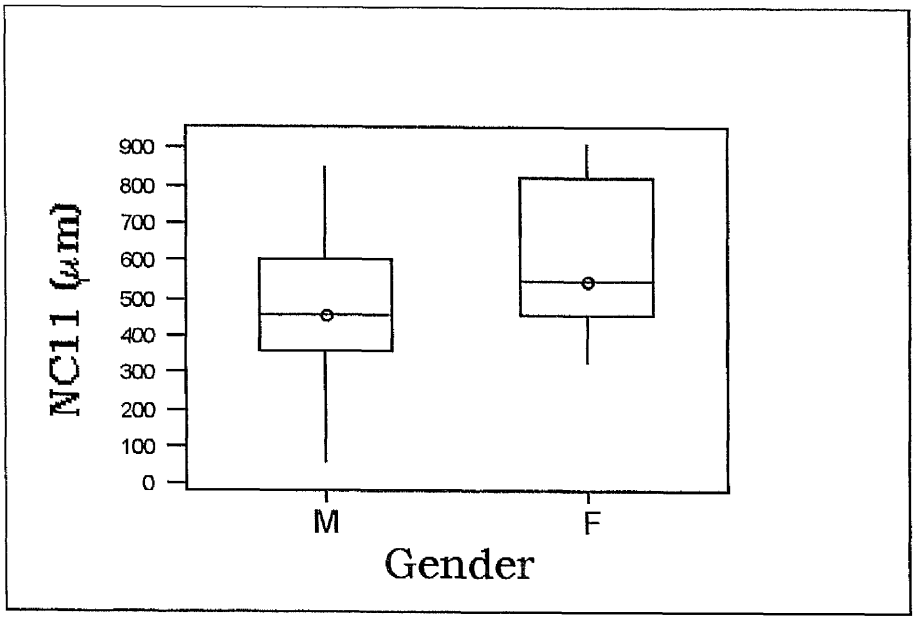
Graph A2.40 Boxplot of CC16 Thickness by Weight



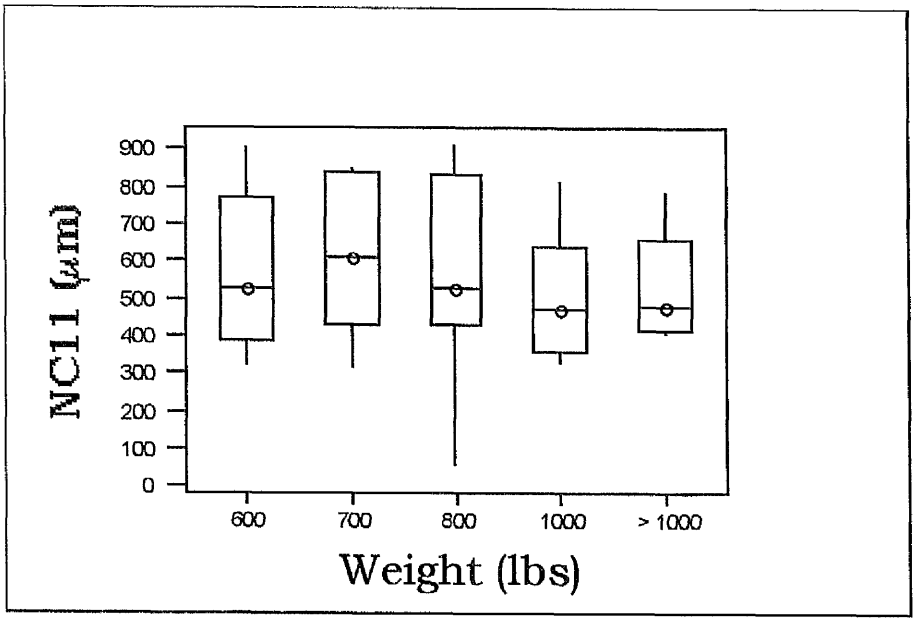
Graph A2.41 Boxplot of NC11 Thickness by Age



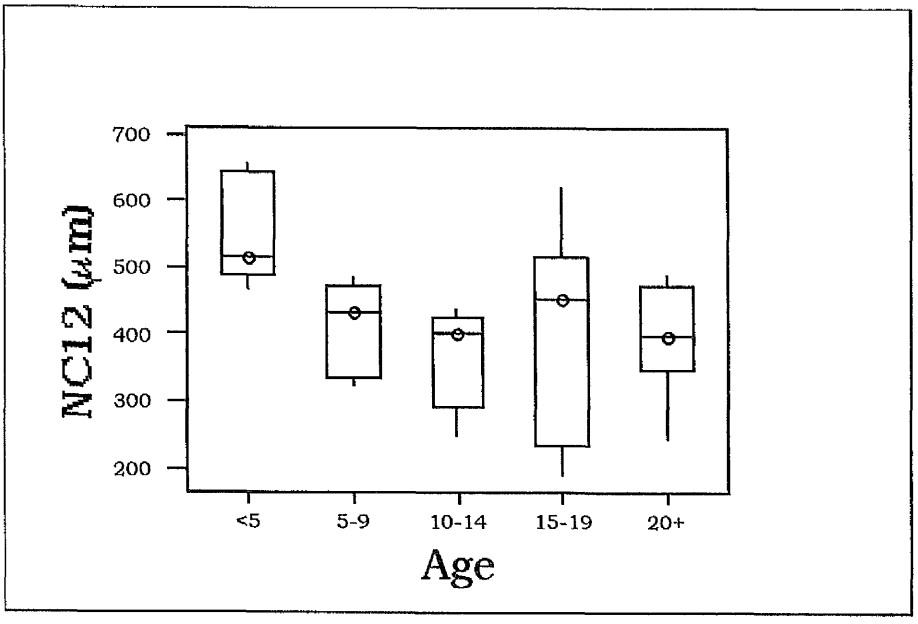
Graph A2.42 Boxplot of NC11 Thickness by Breed



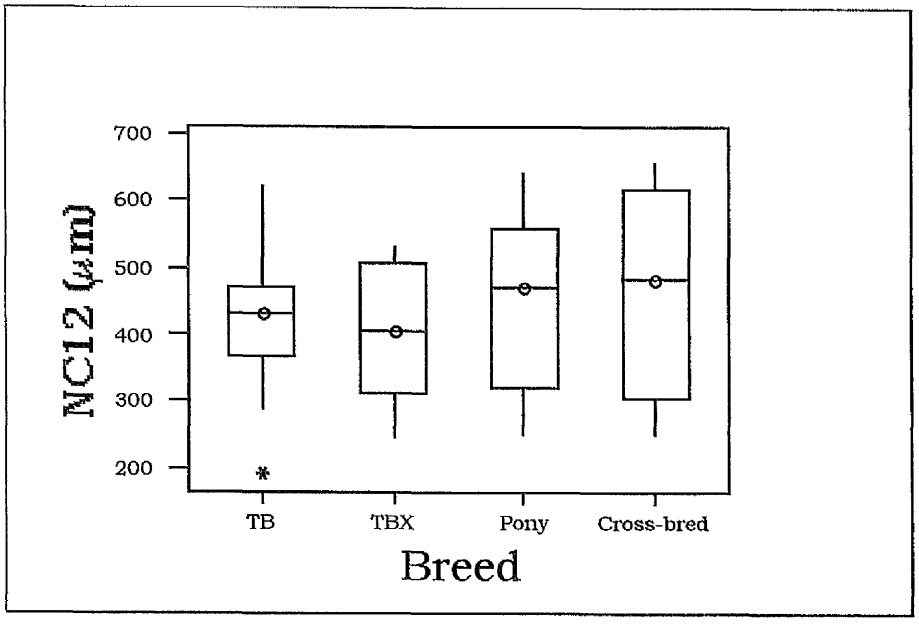
Graph A2.43 Boxplot of NC11 Thickness by Gender



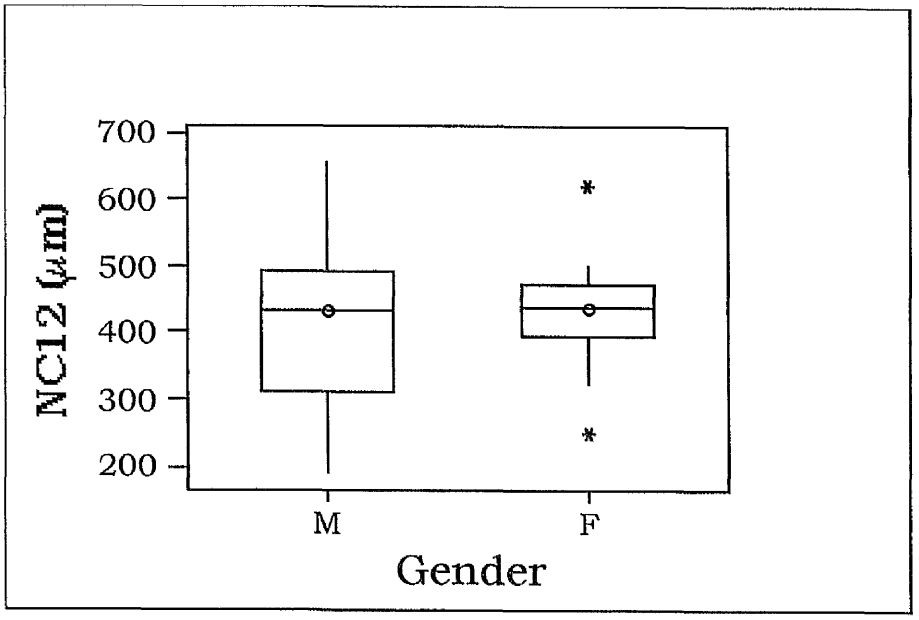
Graph A2.44 Boxplot of NC11 Thickness by Weight



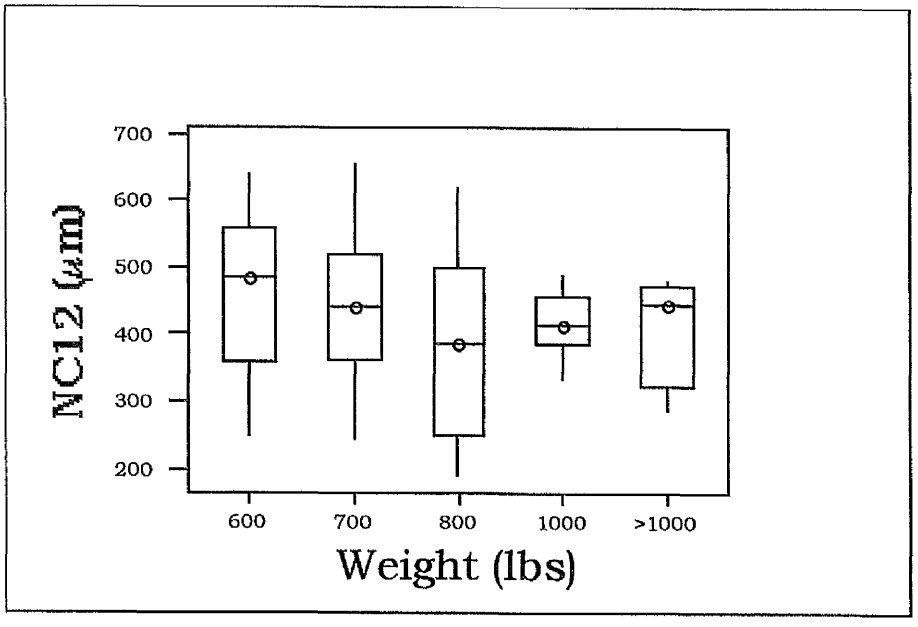
Graph A2.45 Boxplot of NC12 Thickness by Age



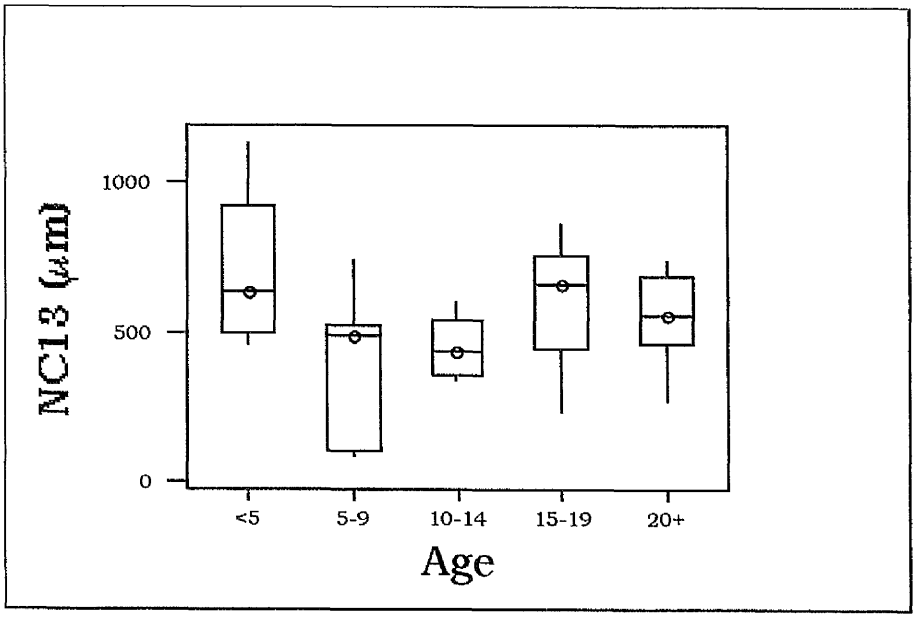
Graph A2.46 Boxplot of NC12 Thickness by Breed



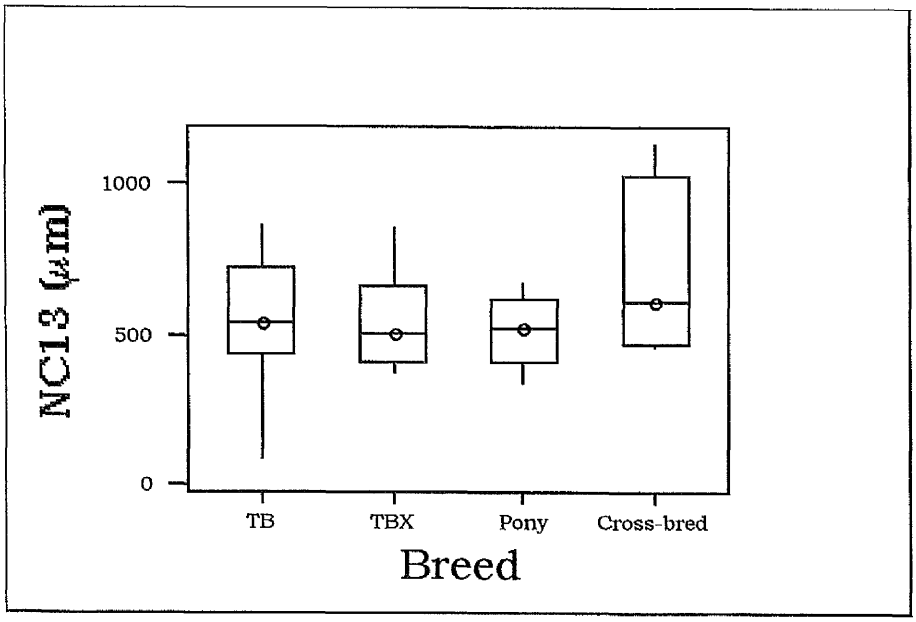
Graph A2.47 Boxplot of NC12 Thickness by Gender



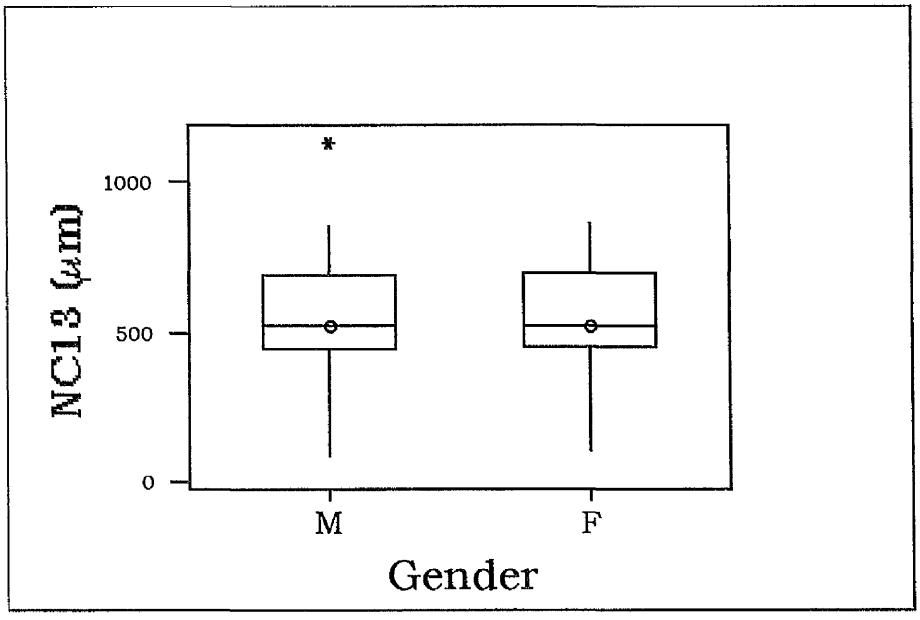
Graph A2.48 Boxplot of NC12 Thickness by Weight



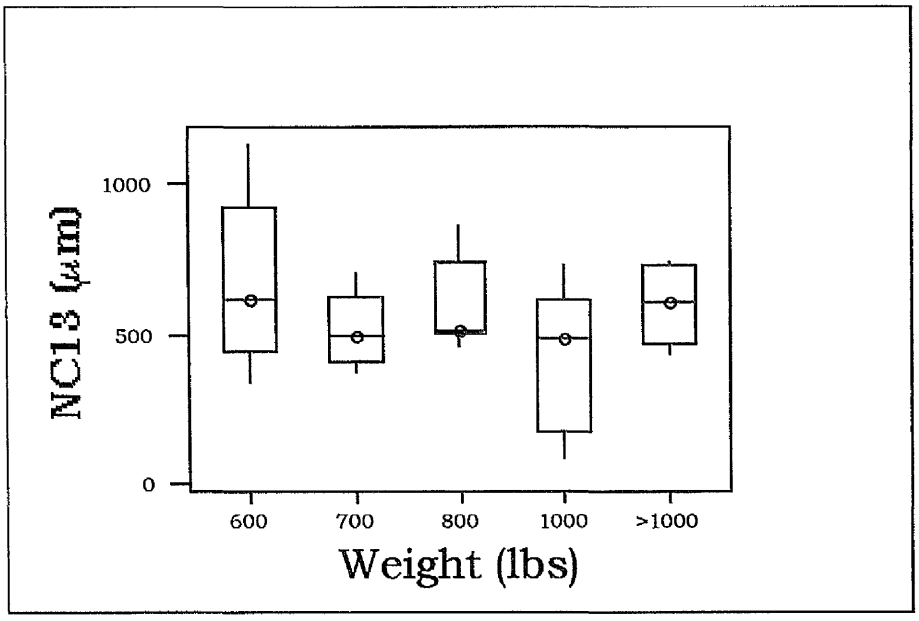
Graph A2.49 Boxplot of NC13 Thickness by Age



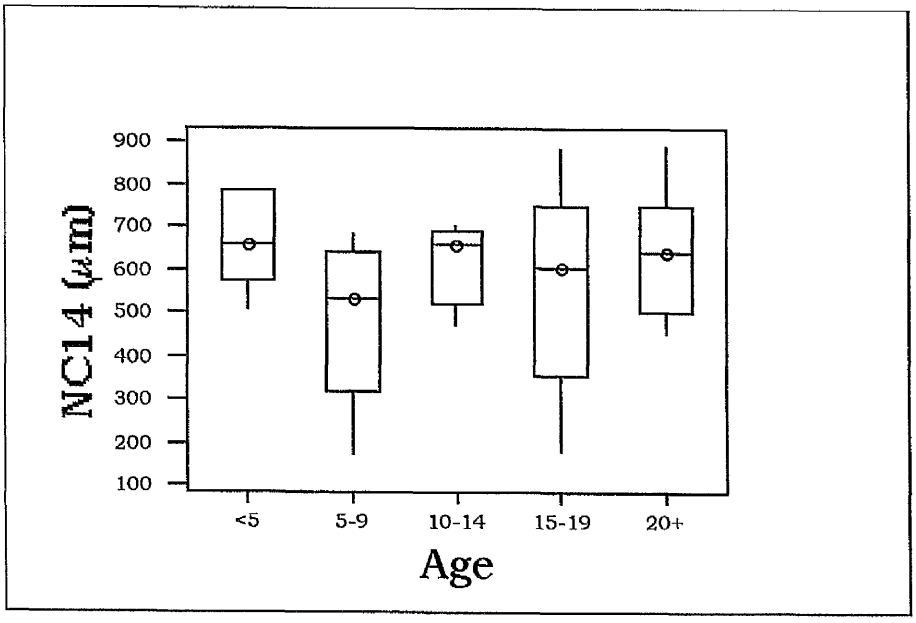
Graph A2.50 Boxplot of NC13 Thickness by Breed



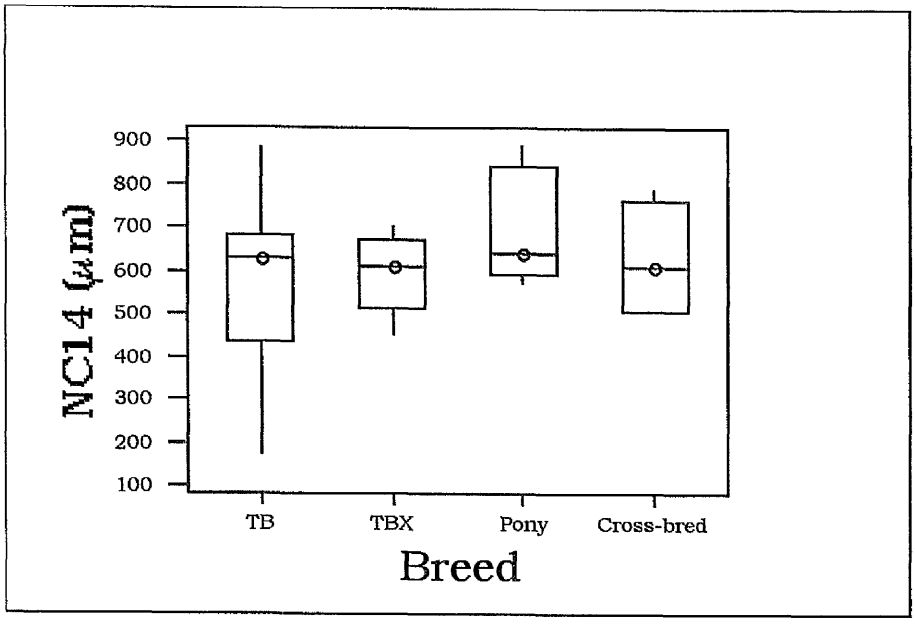
Graph A2.51 Boxplot of NC13 Thickness by Gender



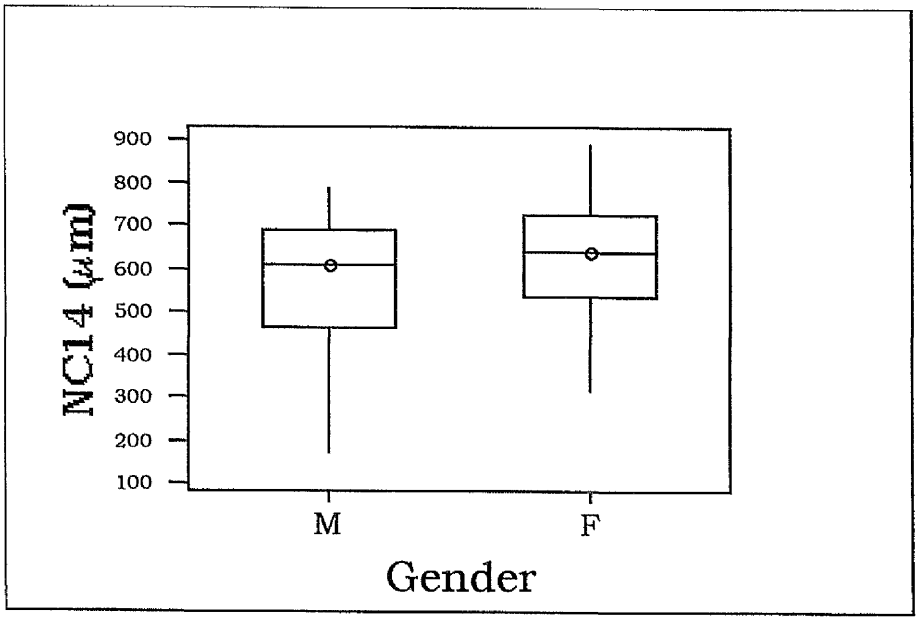
Graph A2.52 Boxplot of NC13 Thickness by Weight



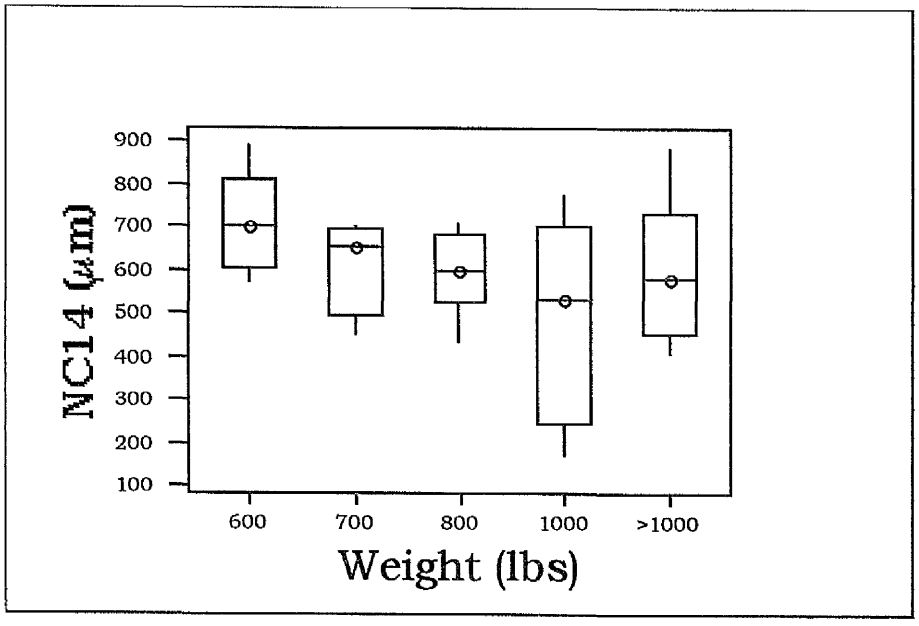
Graph A2.53 Boxplot of NC14 Thickness by Age



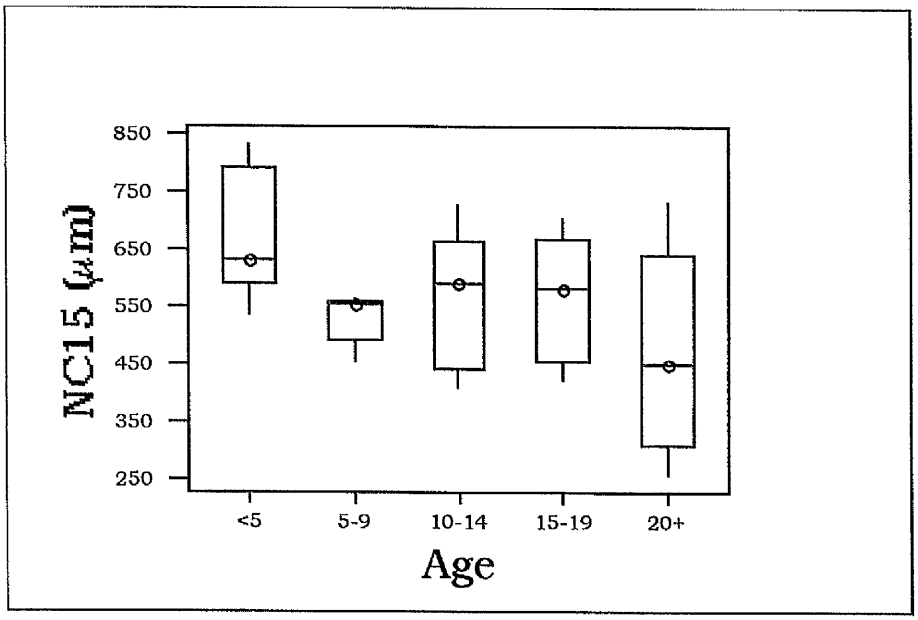
Graph A2.54 Boxplot of NC14 Thickness by Breed



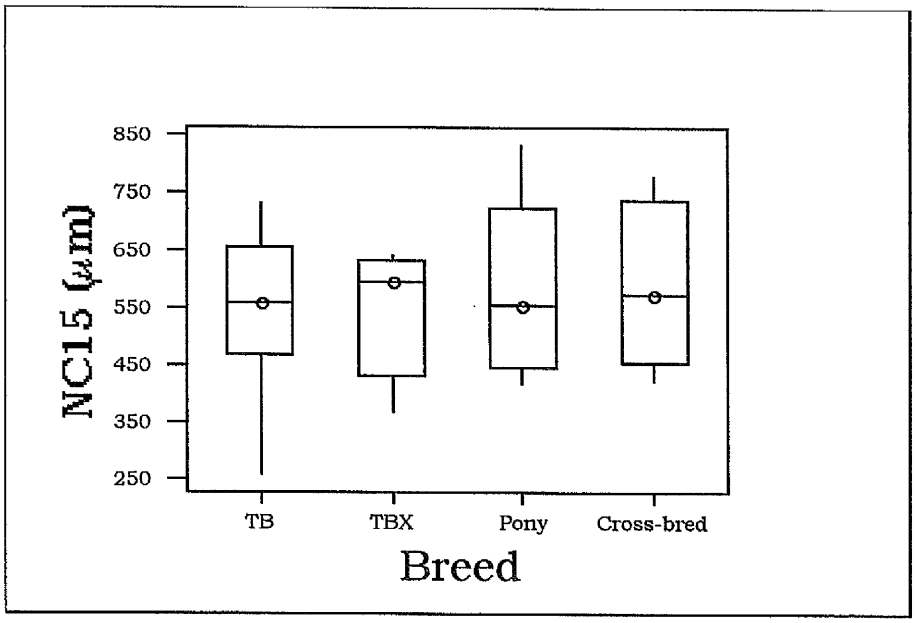
Graph A2.55 Boxplot of NC14 Thickness by Gender



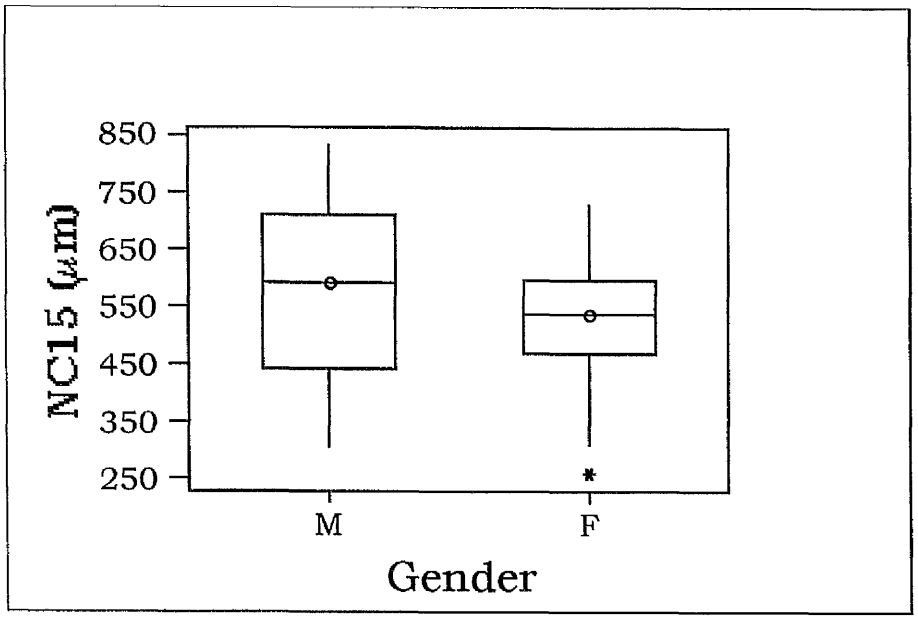
Graph A2.56 Boxplot of NC14 Thickness by Weight



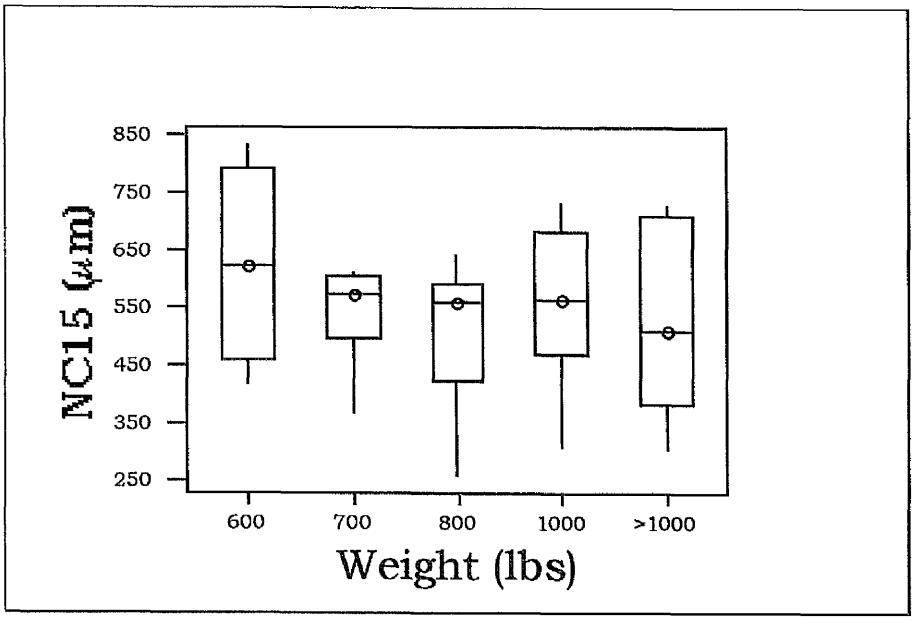
Graph A2.57 Boxplot of NC15 Thickness by Age



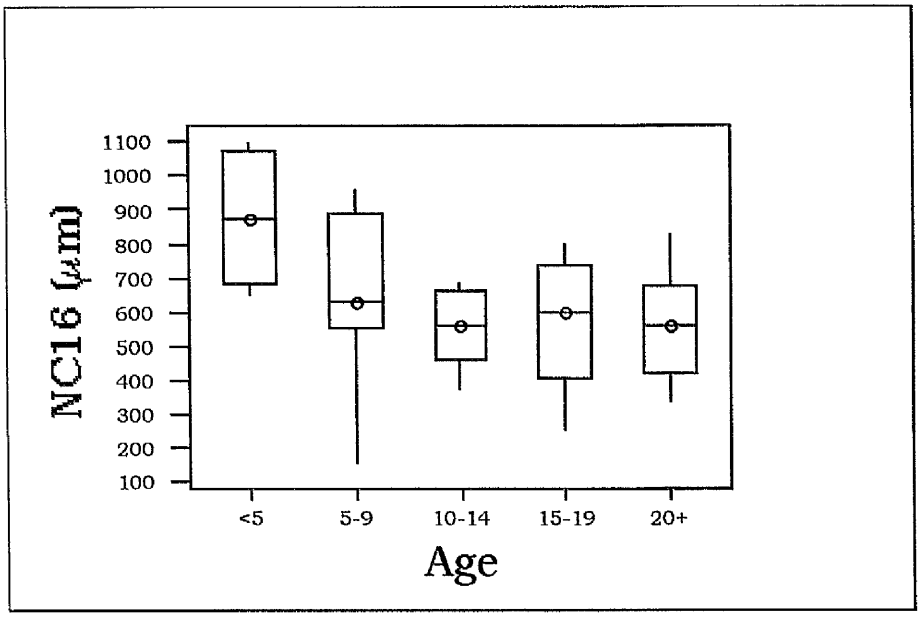
Graph A2.58 Boxplot of NC15 Thickness by Breed



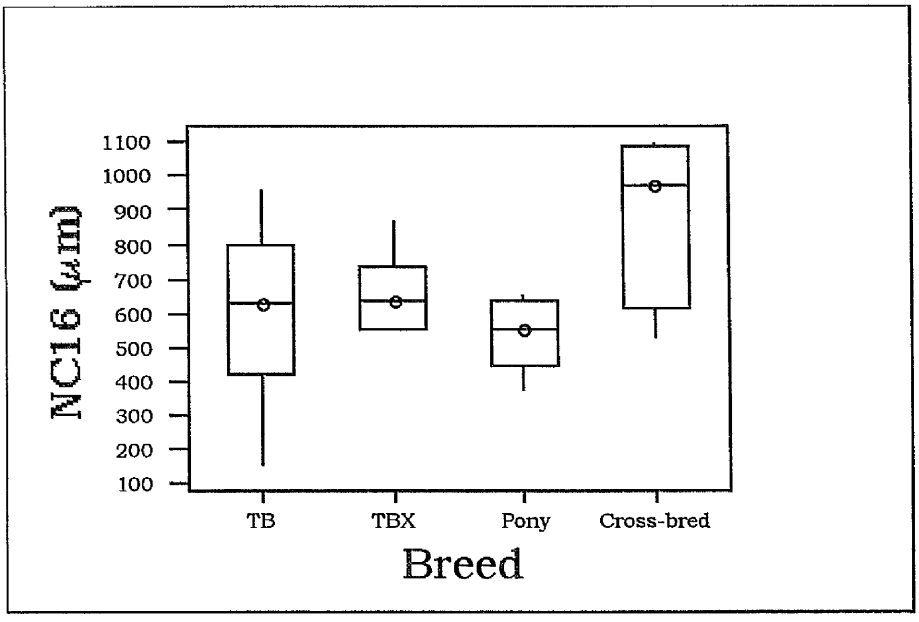
Graph A2.59 Boxplot of NC15 Thickness by Gender



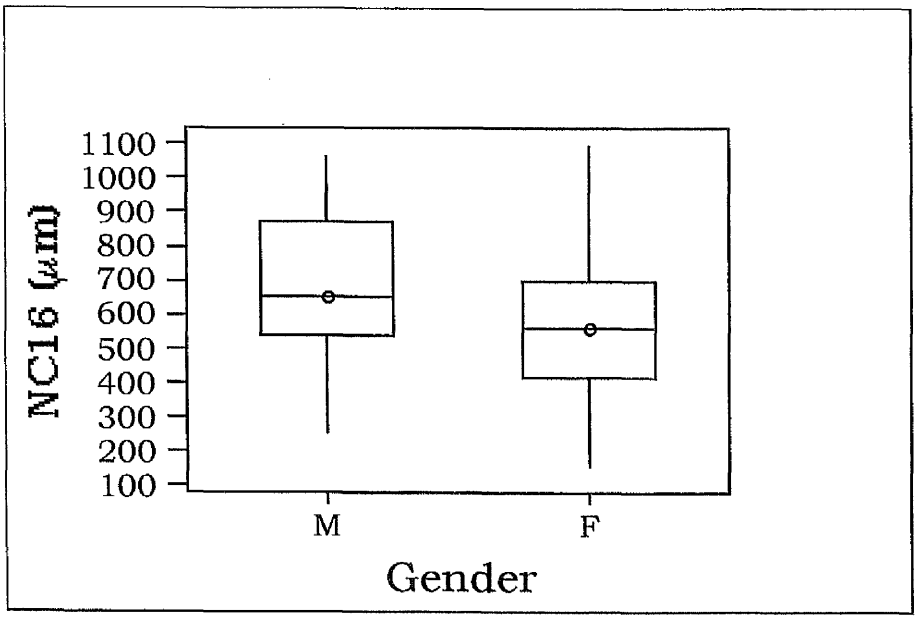
Graph A2.60 Boxplot of NC15 Thickness by Weight



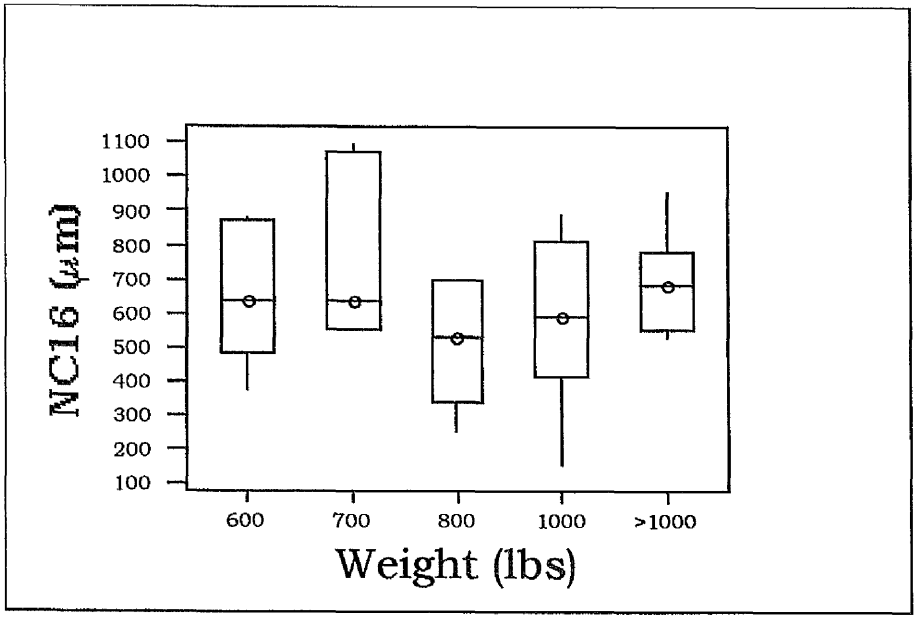
Graph A2.61 Boxplot of NC16 Thickness by Age



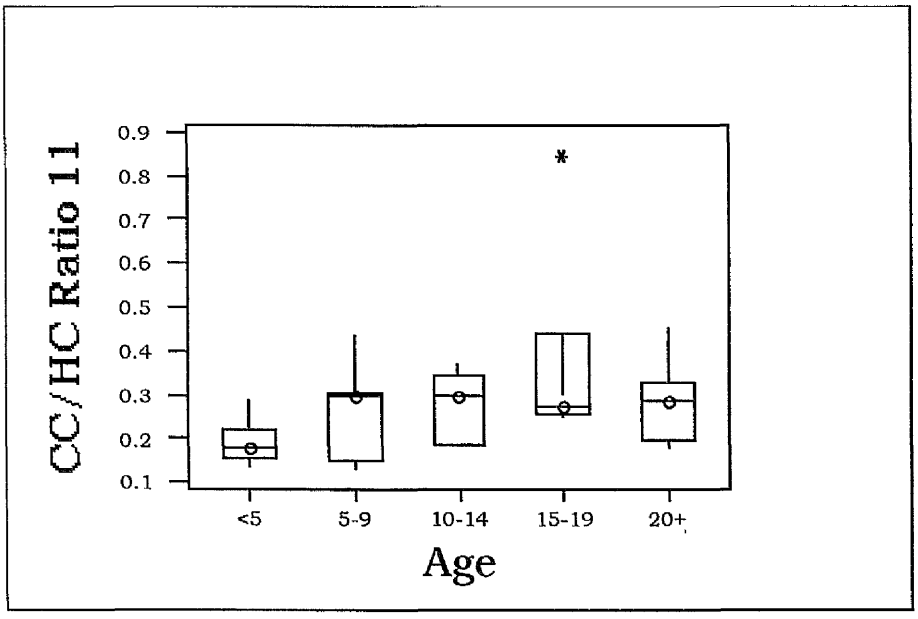
Graph A2.62 Boxplot of NC16 Thickness by Breed



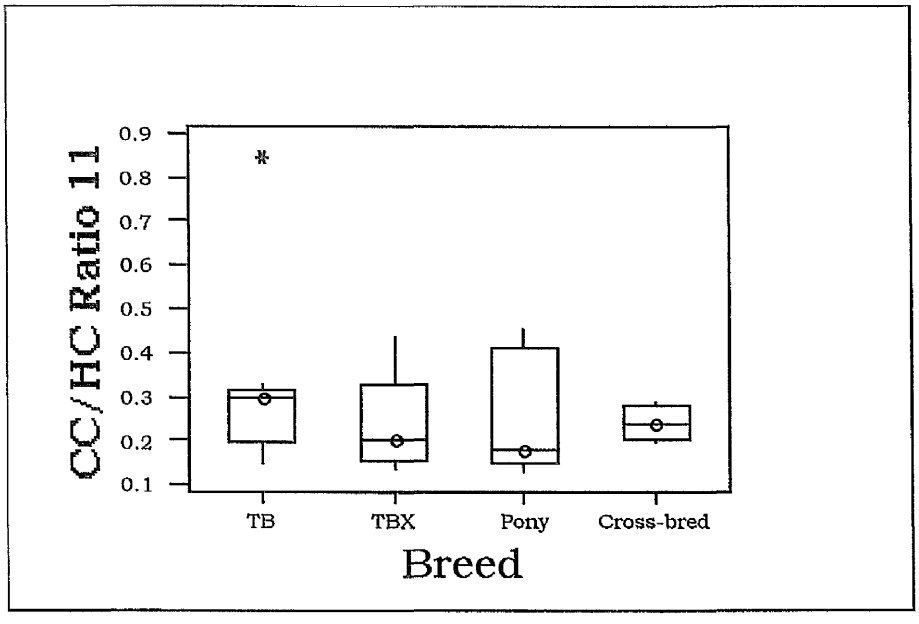
Graph A2.63 Boxplot of NC16 Thickness by Gender



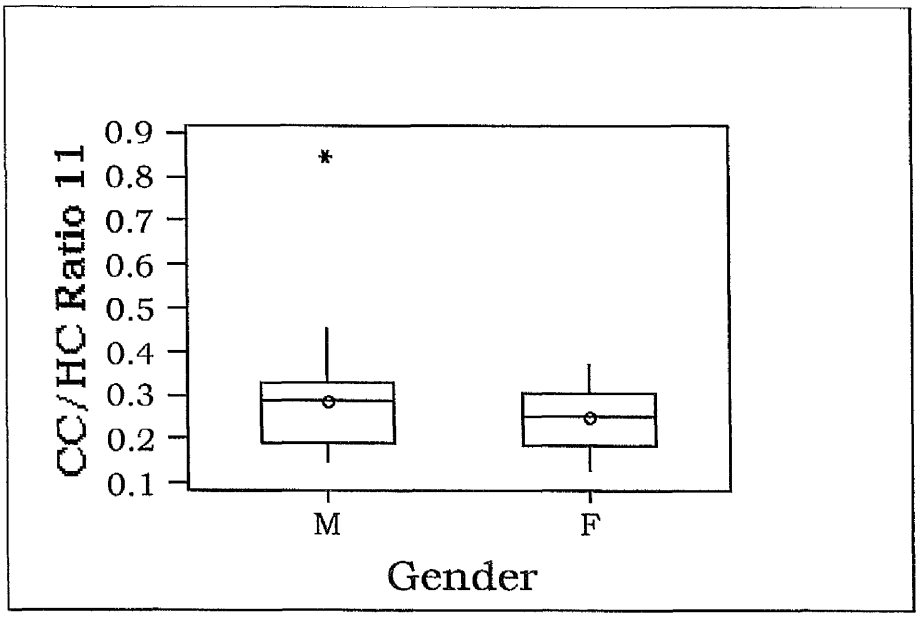
Graph A2.64 Boxplot of NC16 Thickness by Weight



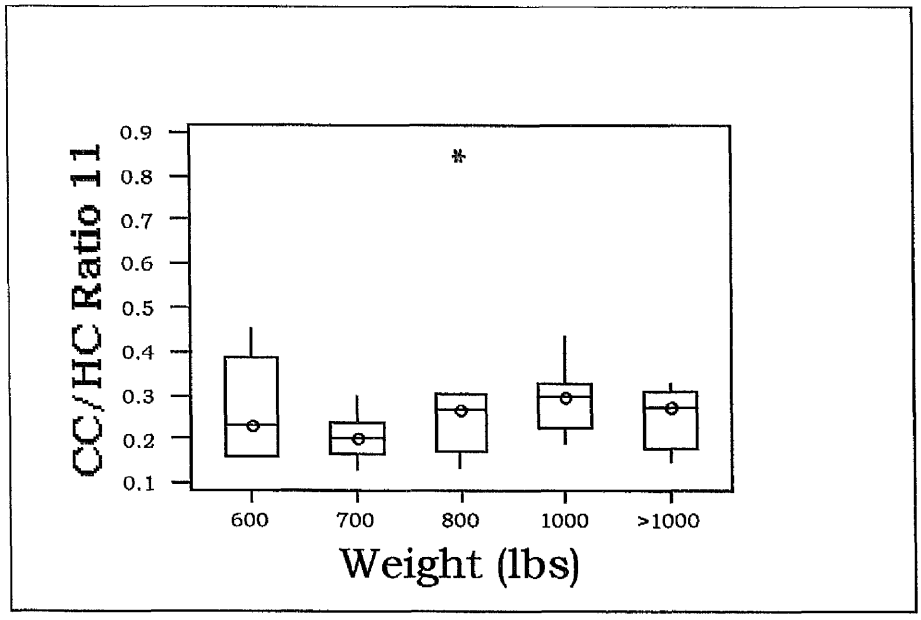
Graph A2.65 Boxplot of CC/NC11 Ratio by Age



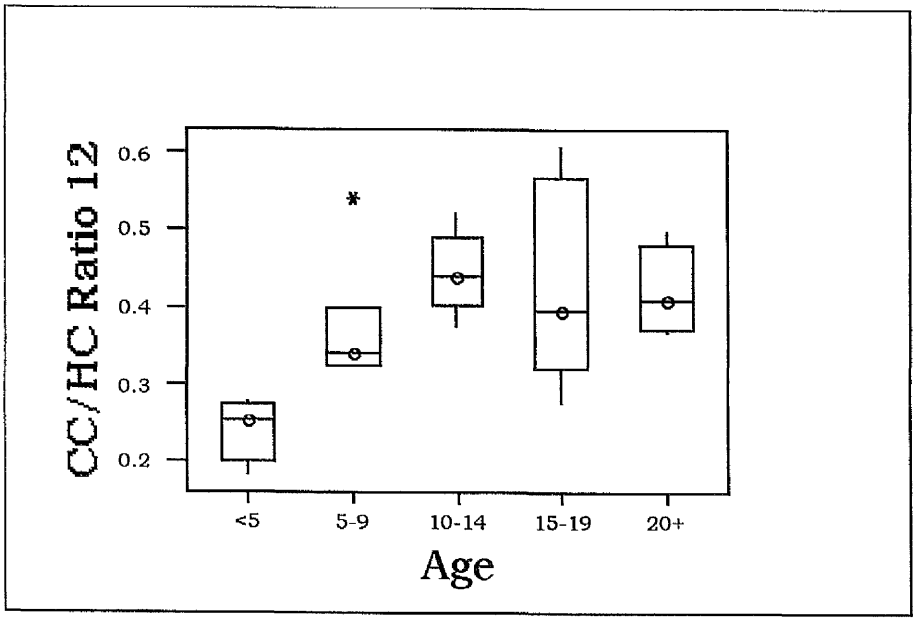
Graph A2.66 Boxplot of CC/NC11 Ratio by Breed



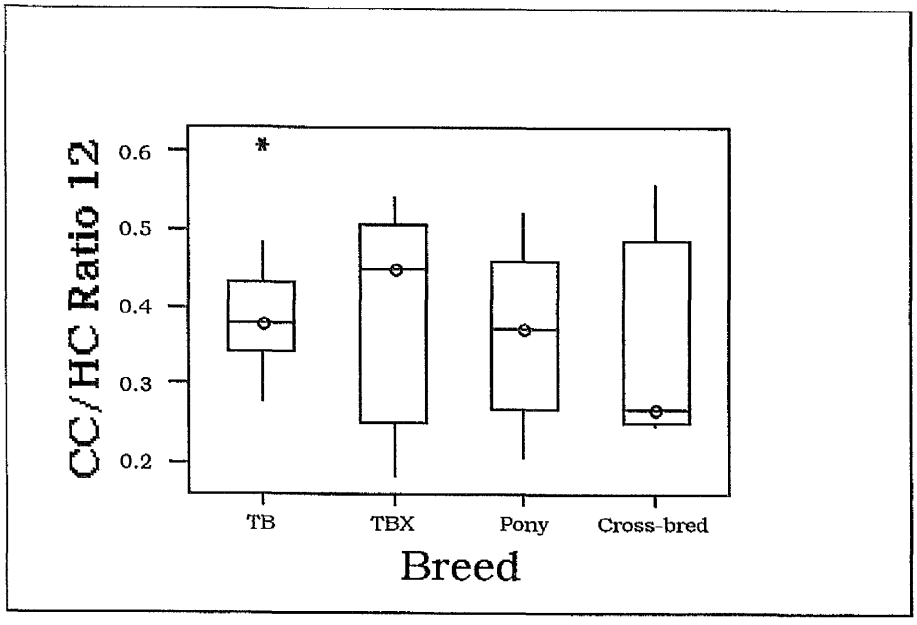
Graph A2.67 Boxplot of CC/NC11 Ratio by Gender



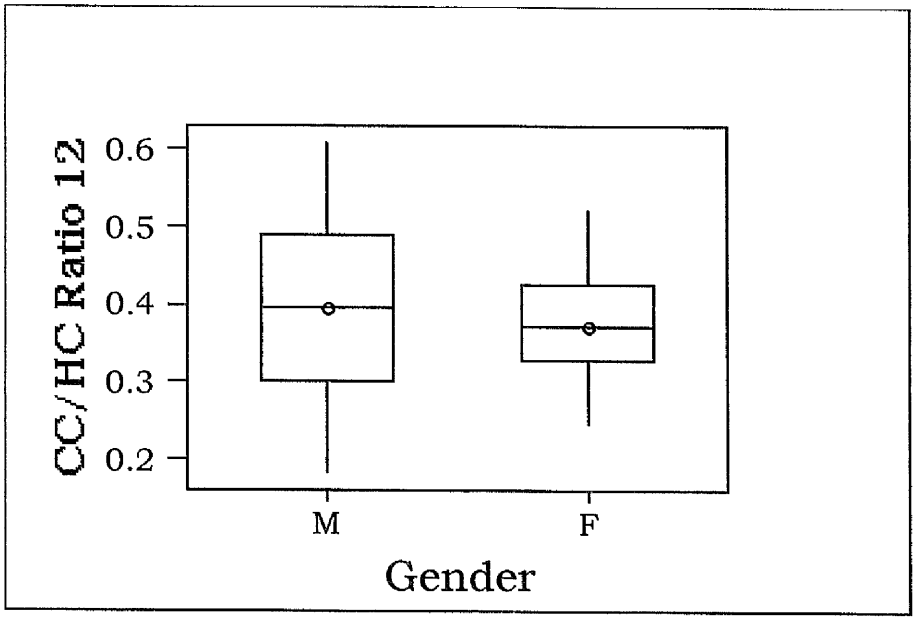
Graph A2.68 Boxplot of CC/NC11 Ratio by Weight



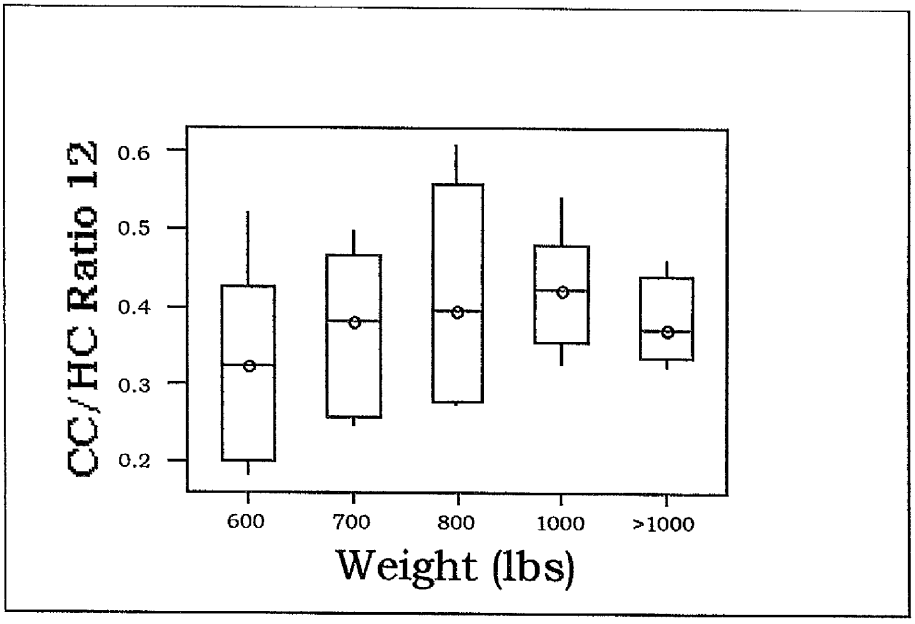
Graph A2.69 Boxplot of CC/NC12 Ratio by Age



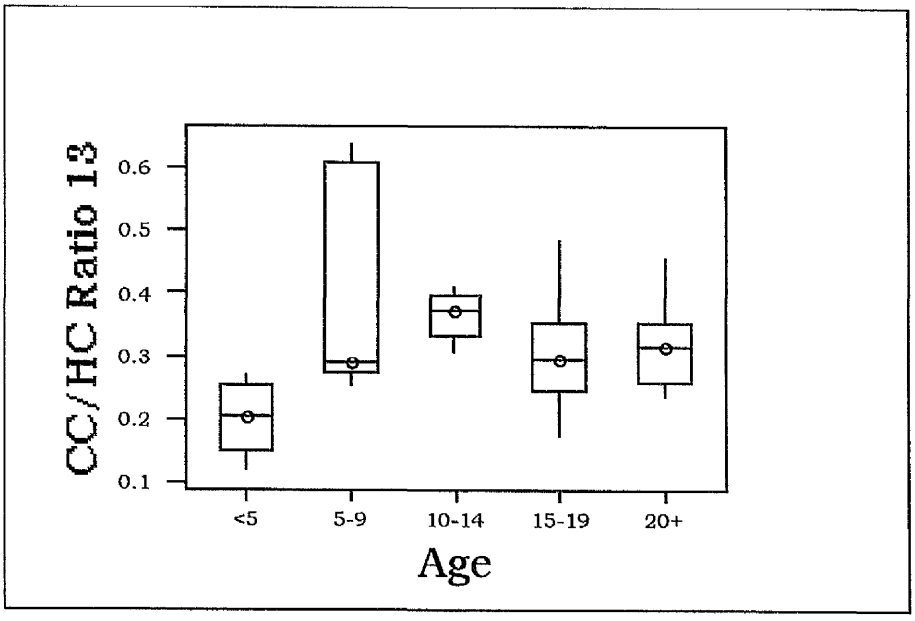
Graph A2.70 Boxplot of CC/NC12 Ratio by Breed



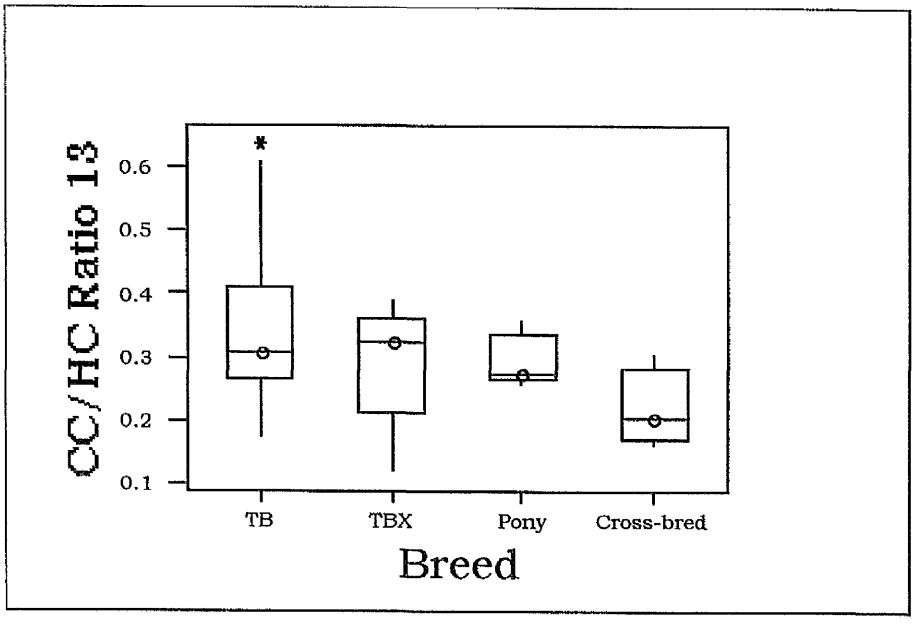
Graph A2.71 Boxplot of CC/NC12 Ratio by Gender



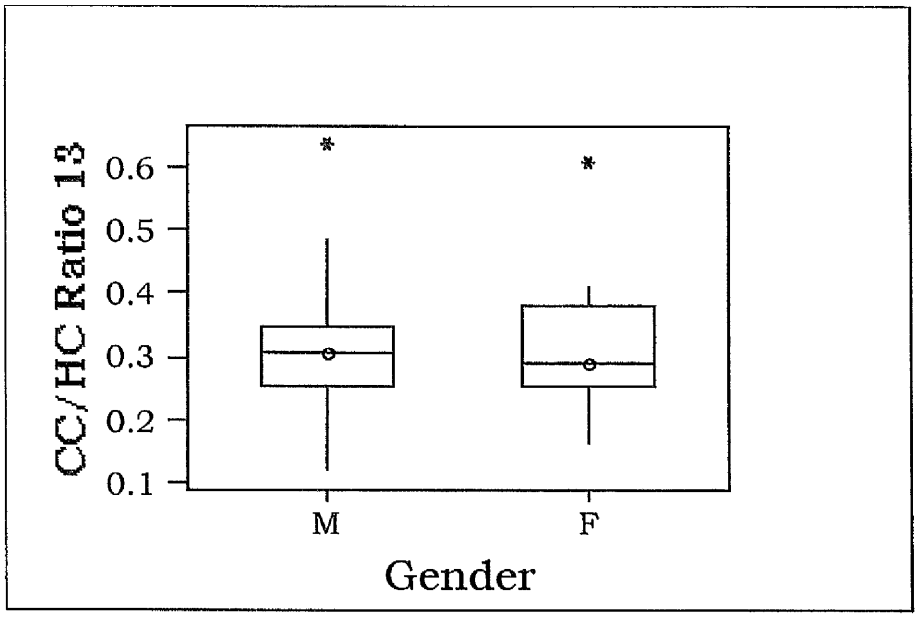
Graph A2.72 Boxplot of CC/NC12 Ratio by Weight



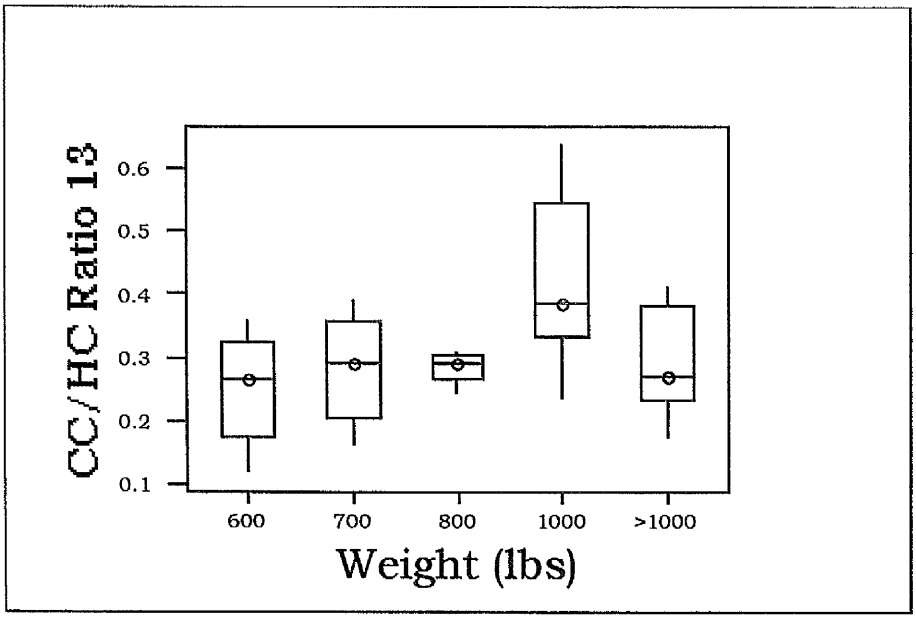
Graph A2.73 Boxplot of CC/NC13 Ratio by Age



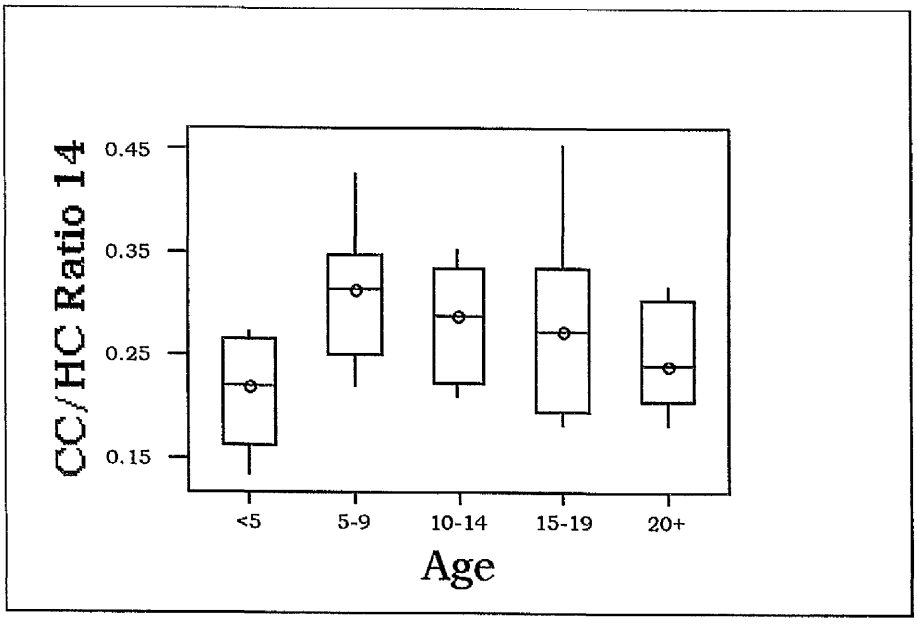
Graph A2.74 Boxplot of CC/NC13 Ratio by Breed



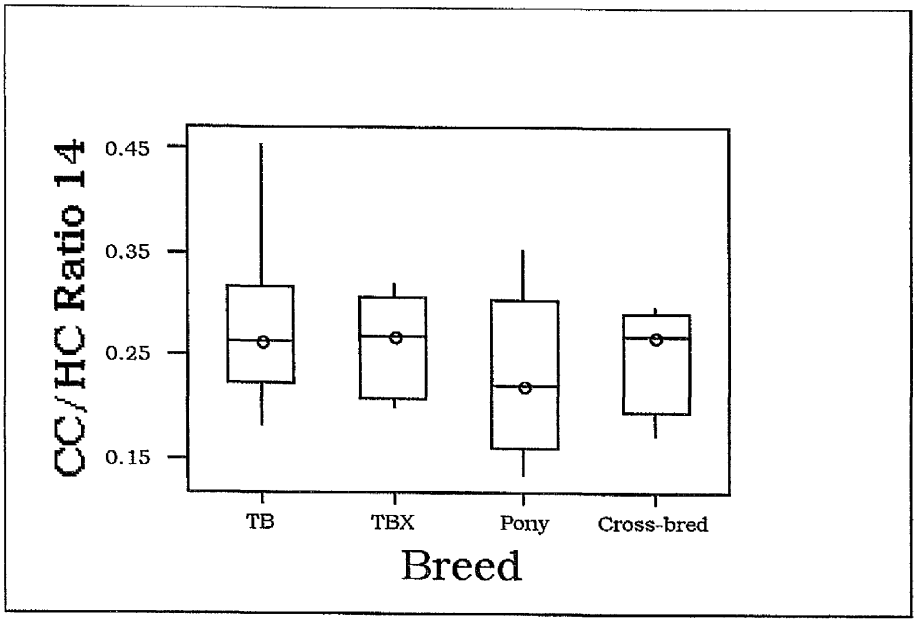
Graph A2.75 Boxplot of CC/NC13 Ratio by Gender



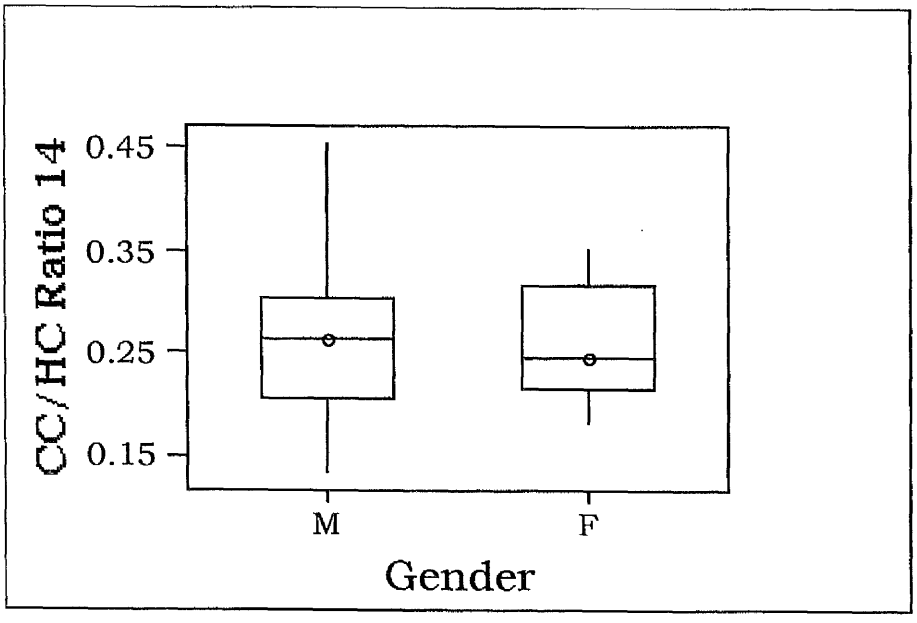
Graph A2.76 Boxplot of CC/NC13 Ratio by Weight



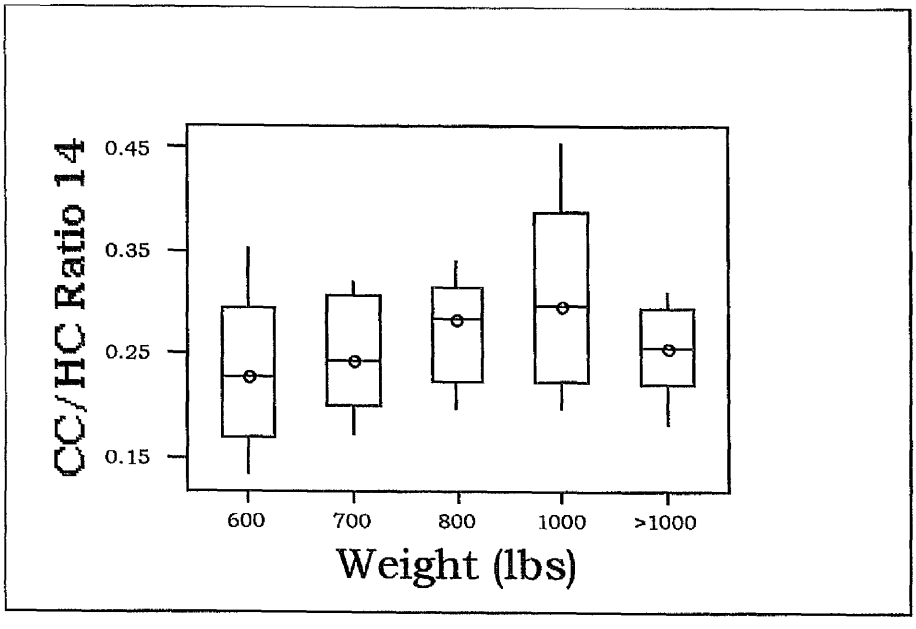
Graph A2.77 Boxplot of CC/NC14 Ratio by Age



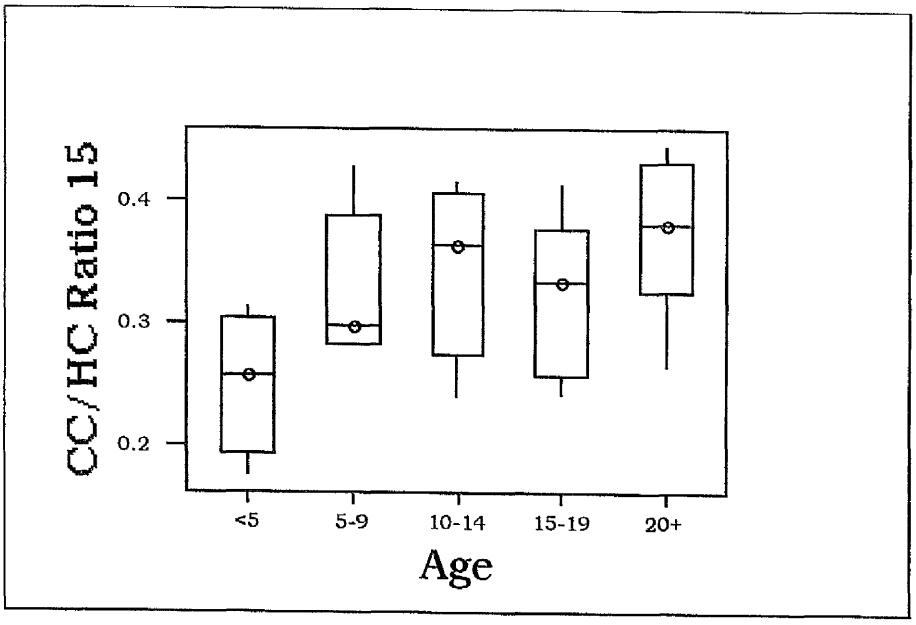
Graph A2.78 Boxplot of CC/NC14 Ratio by Breed



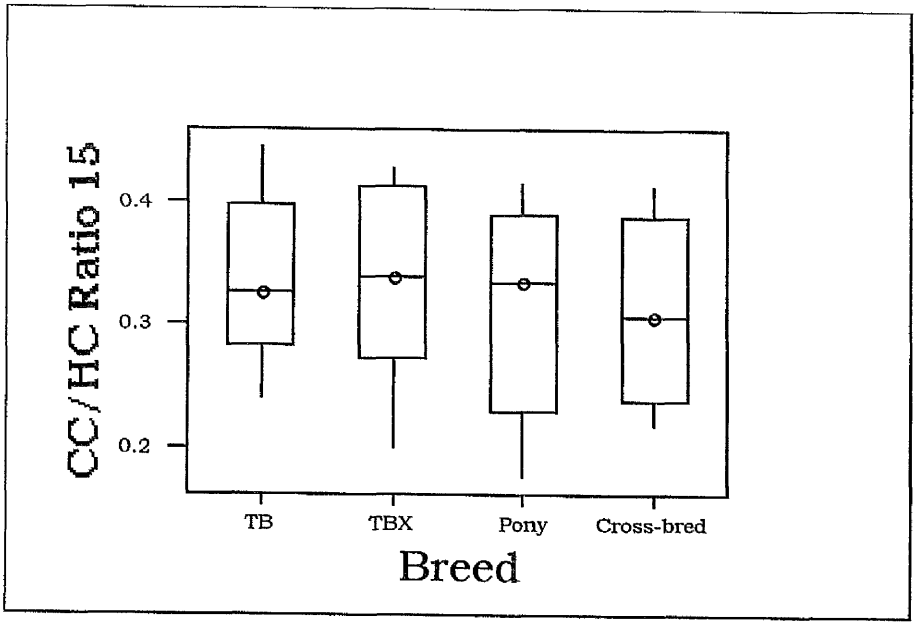
Graph A2.79 Boxplot of CC/NC14 Ratio by Gender



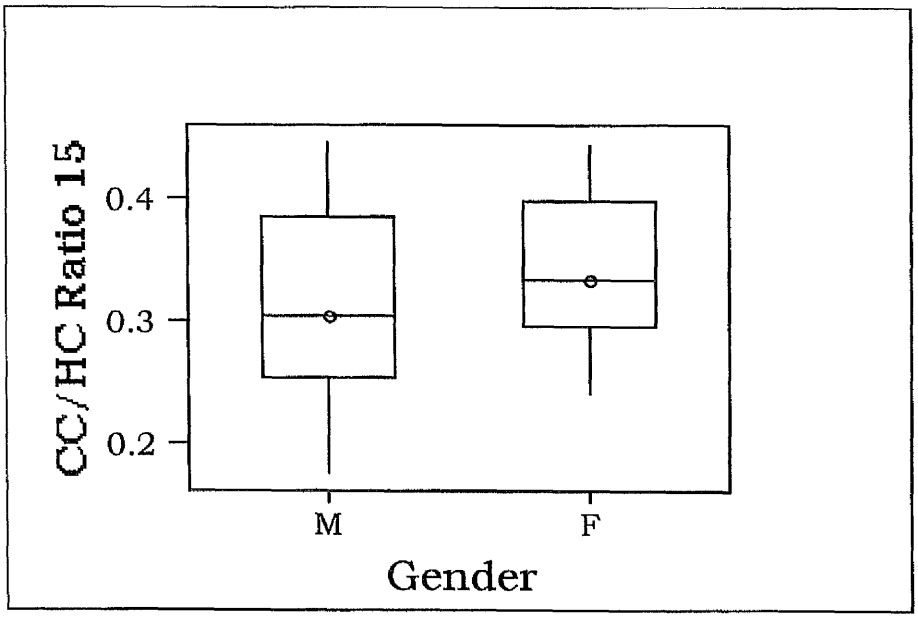
Graph A2.80 Boxplot of CC/NC14 Ratio by Weight



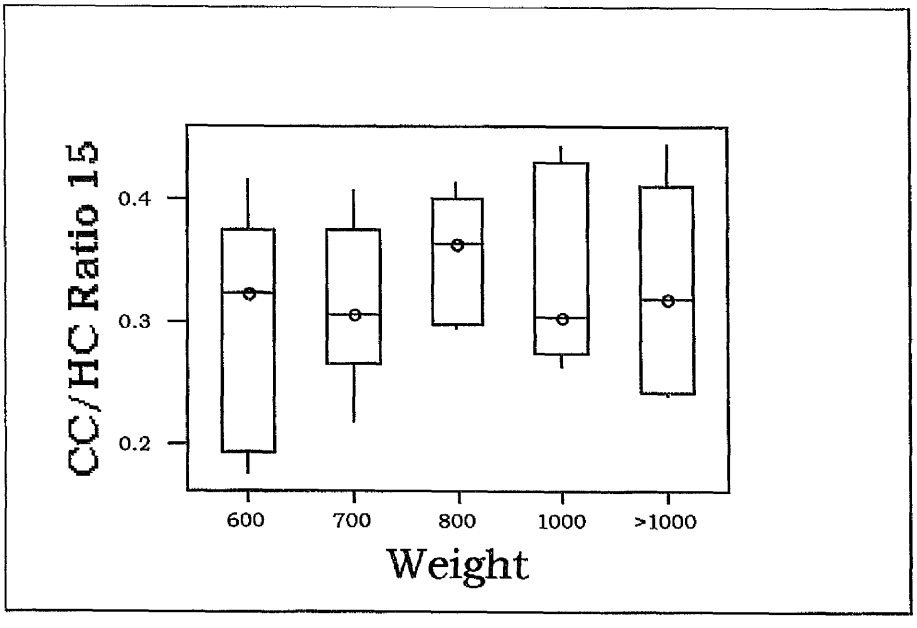
Graph A2.81 Boxplot of CC/NC15 Ratio by Age



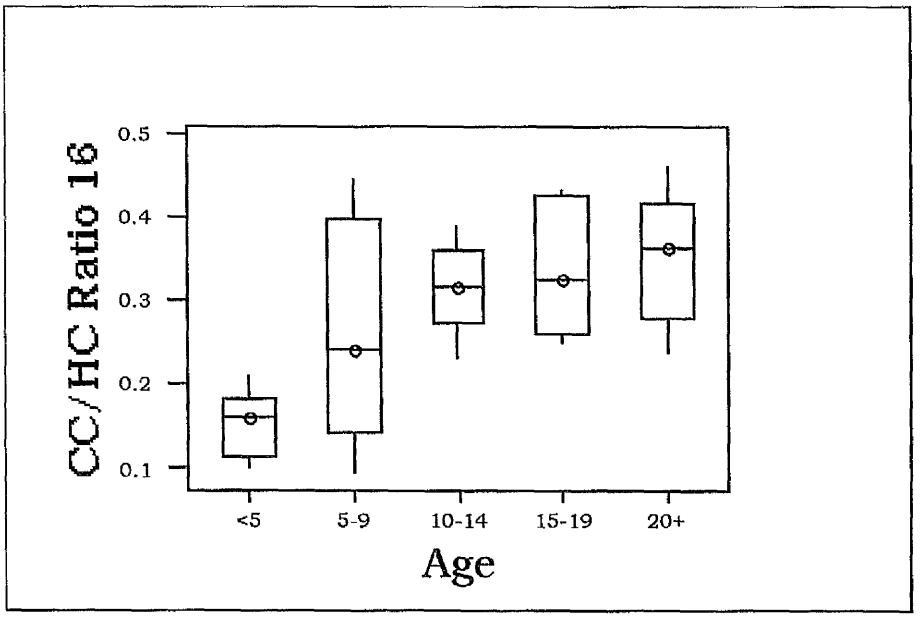
Graph A2.82 Boxplot of CC/NC15 Ratio by Breed



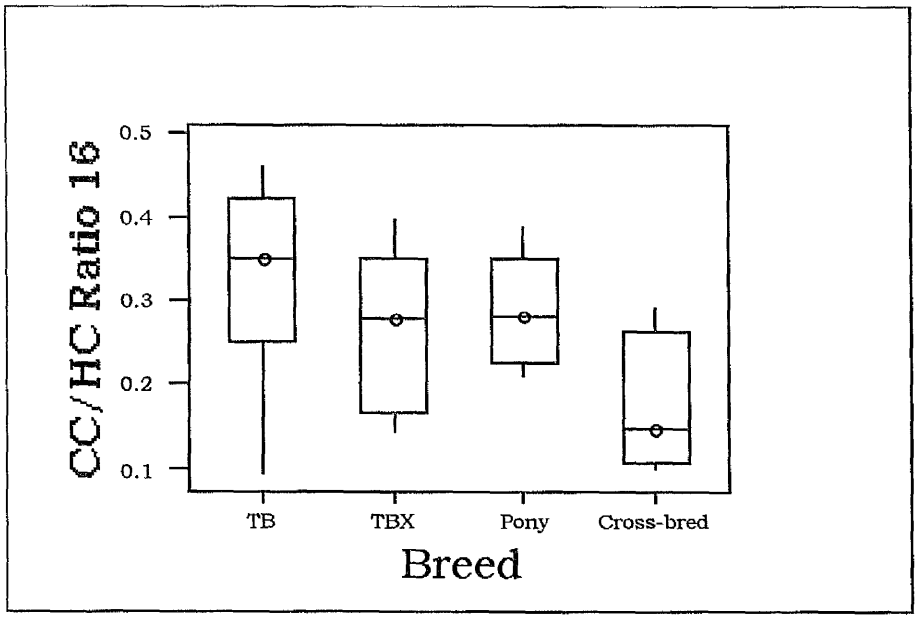
Graph A2.83 Boxplot of CC/NC15 Ratio by Gender



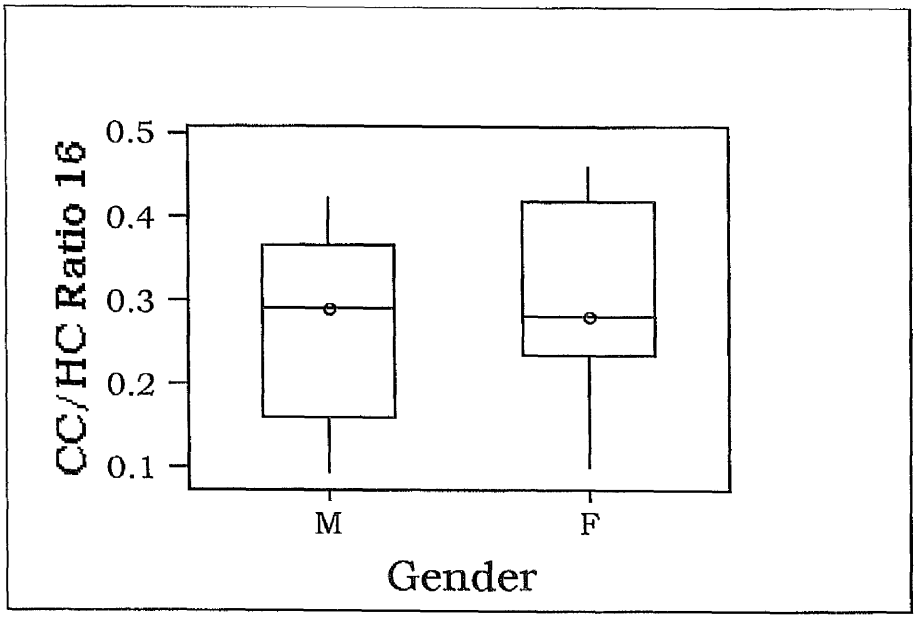
Graph A2.84 Boxplot of CC/NC15 Ratio by Weight



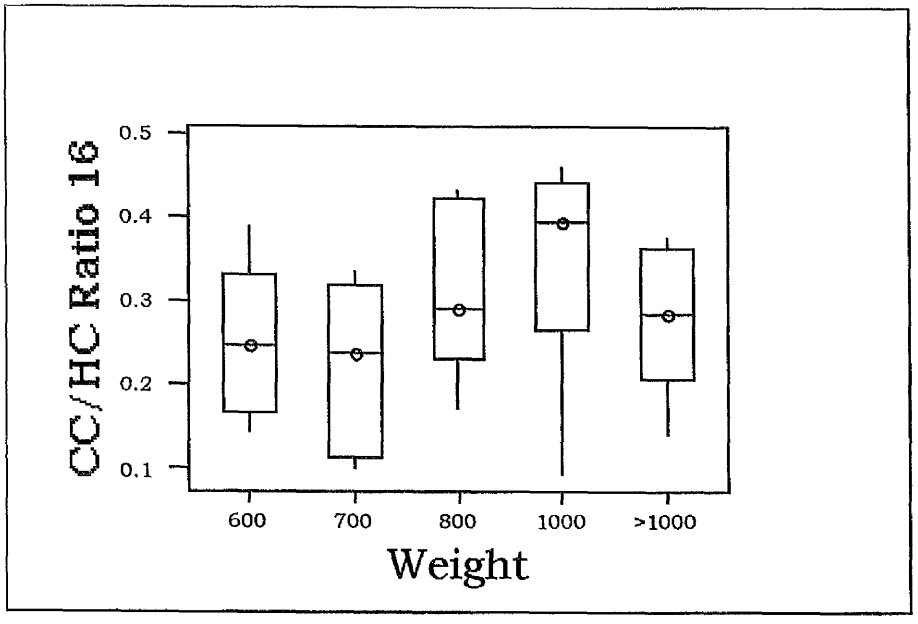
Graph A2.85 Boxplot of CC/NC16 Ratio by Age



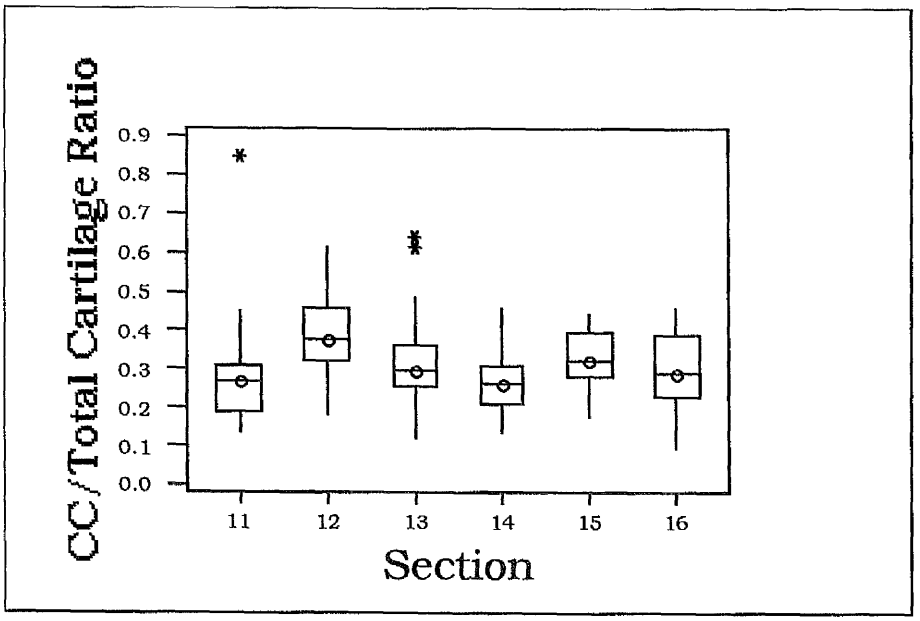
Graph A2.86 Boxplot of CC/NC16 Ratio by Breed



Graph A2.87 Boxplot of CC/NC16 Ratio by Gender



Graph A2.88 Boxplot of CC/NC16 Ratio by Weight



Graph A2.89 Boxplot of CC/NC Ratio for all Sections

CHAPTER 6

SUBCHONDRAL BONE

Introduction

As with the CC layer, the subchondral bone (SCB) has received a significant amount of attention in the human medical literature over the last decade. Speculation over its role in the initiation and progression of OA has gone on for much longer though. In what is now considered a classic experiment on the subject, **Radin and coworkers (1984)** investigated this hypothesis using a rabbit model. By splinting the hind legs, the hock joint could not dampen the effect of a load applied to the bottom of the foot. Consequently, the effect of the load was concentrated completely in the rabbit's stifle joint. The first articular change noted was an increased radiopharmaceutical uptake (IRU) associated with the periarticular tissues of the stifle joint several weeks after the initiation of the daily insults. The next changes were associated with increased bone formation and decreased porosity, an indication of bone deposition and stiffening. Mechanical damage to the articular cartilage followed the bone changes, while the biochemical and metabolic changes in the cartilage were the last to be recognised. The investigators felt that this study indicated a possible role for hard tissue changes prior to articular cartilage changes in the initiation of OA. **Radin and Rose (1986)** expounded on this concept, claiming that the health of the articular cartilage depends on the SCB. They argued that the articular cartilage can not be the sole shock absorber due to its relative thickness. As further evidence of the involvement of the SCB in the progression of OA, they noted that osteoporosis tends to spare the joints, while osteopetrosis leads to OA.

While it is accepted in the veterinary literature that cartilage damage is the hallmark of DJD and OA **(Baxter 1992)**, SCB changes may precipitate them **(Bailey and Mansell 1997).** These investigators report that it does not appear to be a higher bone mass (i.e. Blacks with higher bone mass are no more susceptible to OA than Caucasians), but a higher bone metabolism that could be responsible for OA. **Bailey and Mansell** also stated that a thickening of the SCB plate was considered more important than total bone mass in determining the mechanical

stress on the joint. The earliest changes in the bone appeared at the ligament/bone insertion site. They proposed that a change in the ligament alters the tension on the bone, thereby producing the change in the bone density. The effect of tension may somehow lead to an increased bone metabolism.

Carlson *et al* (1996) investigated a population of macaques and found that, although cartilage changes were present, thickened SCB seemed to be the inciting factor in this population of animals. SCB thickening was more severe in mild to moderate OA whereas the articular cartilage lesions only matched or exceeded the SCB thickening in the most severe cases. They further speculated that the controversy about a relationship between OA and SCB might have arisen because the SCB plate is very difficult to distinguish from the rest of the epiphyseal bone. In a guinea pig model of OA, changes in the SCB appeared to occur simultaneously with changes in the cartilage, although the increased bone thickness seemed to exacerbate the articular cartilage changes and could not be ruled out definitively as an inciting cause of it **(De Bri *et al* 1995).**

Matsui *et al* (1997) found there was a clear correlation between the grade of the cartilage degeneration and the bone volume as well as bone formation activity in SCB. Another paper cites bone sclerosis as an accepted facet of OA, but it is not agreed whether the bone changes precede, occur simultaneously or follow the articular cartilage degeneration **(Burr and Schaffler 1997)**. Changes in SCB sclerosis were not needed to develop cartilage fibrillation, but it was proposed that they might be necessary for progression. They stated that only those changes in SCB and CC close to the joint (< 3mm) seemed significant to the development of OA. These investigators felt that both bone and CC should be considered part of the SCB bed. **Grynpas *et al* (1991)** found that the SCB in OA was abnormally thickened, both in weight-bearing and non-weight-bearing regions, supporting a theory associated with a generalised increase in bone production.

In the equine literature, there are several references to the SCB plate and possible relationship to disease **(Pool and Meagher 1990, Ross 1991, Martinelli 1996).** To the author's knowledge, however, there are no objective studies that attempt to quantify any aspect of the SCB plate specifically. Therefore, the purpose of this study was to develop a method for measuring the SCB plate thickness and to determine whether age, breed, gender, weight or location had any effect on it.

Materials and Methods

Specimens

For the determination of SCB plate thickness, the greyscale images from the bone histomorphometry study (Chapter 4), digitised at 45X magnification, were used. The sections included in this study were 3 and 4, centred around Point D on the palmar articular surface; and 7 and 8, located on either side of Point E on the dorsal articular surface **(Fig 6.1)**.

Measurement

An estimation of the thickness of the SCB was generated via a similar method of integration as utilised in the CC study (Chapter 5). For this study, a macro was developed which drew lines, dividing the field into eight equal sections, each 500 microns in width. Therefore, the total distance over which the SCB plate thickness was measured, was 4 mm. The first line was placed on the image 50 pixels from

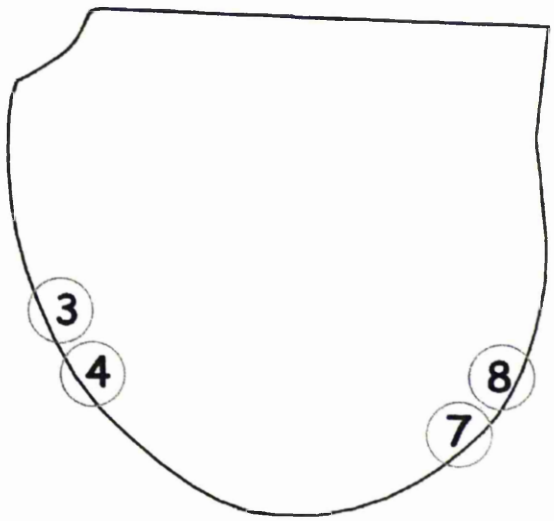


Fig 6.1 Sample sites used for the SCB study.

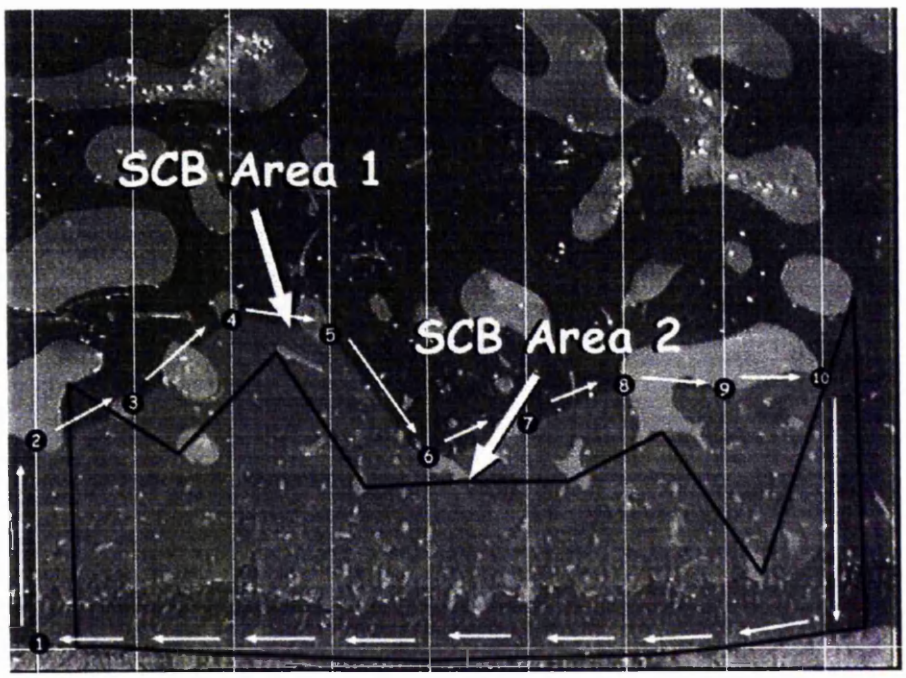


Fig 6.2 Schematic representation of the method used to estimate the SCB plate thickness. The shaded area represents the first area measured, using points on the grid as markers by which to determine sampling points. The black line represents the second sampling region, shifted to the right approximately 167μm in an effort to take a representative SCB sample.

the left-hand border of the image and subsequent lines were placed at 500 micron intervals proceeding toward the right hand border. The 'hexagon tool' was invoked to outline the region to be measured. The beginning point was located where the first line on the left-hand border of the image crossed the tidemark. After depositing a point at that junction, the cursor was dragged proximally, along the dividing line, until the first intersection with a marrow space was encountered. At that juncture, a second point was dropped. Under manual mouse control, the cursor was dragged to the next dividing line to the right on the image and another point was dropped at the distal most intersection of that dividing line with a marrow space. This procedure was continued until contiguous points had been dropped on each successive dividing line along the 4 mm length of field. Once the last dividing line was encountered at the right hand border of the field, the cursor was returned to the tidemark and another anchoring point was dropped. Finally, the cursor traced out the tidemark by dropping points where each dividing line intersected it until the original starting point was encountered at the junction of the tidemark with the dividing line located on the left hand side of the image. This action closed the polygon effectively tracing out the estimated region of the SCB plate. The 'measure' function was invoked which determined the area in square microns and recorded it in the 'results' window. The image was then returned to its original state and the entire process was repeated twice more after moving the initial starting point approximately 167 microns to the right each time **(Fig 6.2)**.

Another macro within Microsoft Excel was generated to import the data into spreadsheet format and calculate the mean and standard deviation of subchondral bone plate thickness for each section.

Statistical Analysis

Statistical analyses were initially carried out to compare subchondral bone thicknesses between sections within each horse using two way repeated measures ANOVA. Then standard two way ANOVA was used to determine if there was any effect of age, gender or weight on the same section among horses. A one way ANOVA was done on the breed category to determine if there were any significant differences. Significance for all tests was set at $P < 0.05$ **(G.Gettinby, personal communication)**.

Results

Descriptive

The subchondral bone (SCB) plate was measured for all specimens. For ease of identification, it was determined to extend from the tidemark (TM) of the hyaline cartilage (HC) proximally to the first visible marrow space. Morphologically, the SCB displayed very obvious differences between sections. In some sections, the entire field was composed of densely packed bone with no evidence of any trabecular pattern. On the other extreme, some fields appeared to be more marrow spaces than trabecular bone. For most sections, however, there was some degree of dense SCB plate before leading into a more open trabecular architecture **(Fig 6.3-6.5)**.

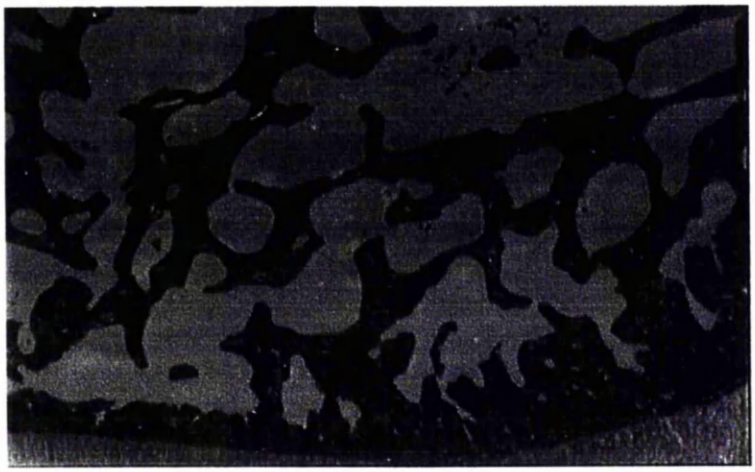


Fig 6.3 Thin SCB plate.

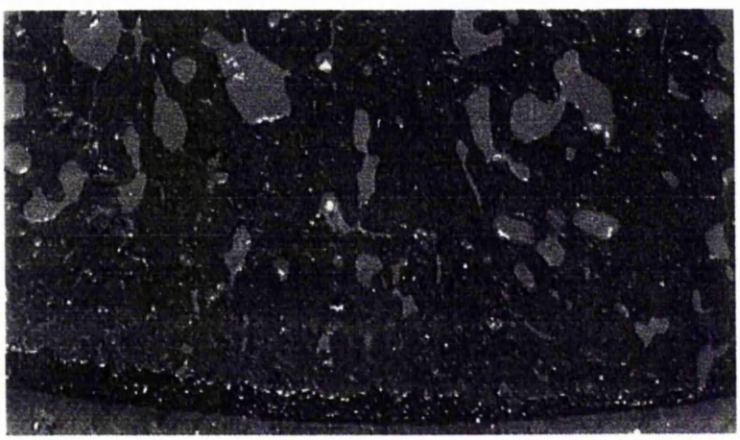


Fig 6.4 Moderately thickened SCB plate.

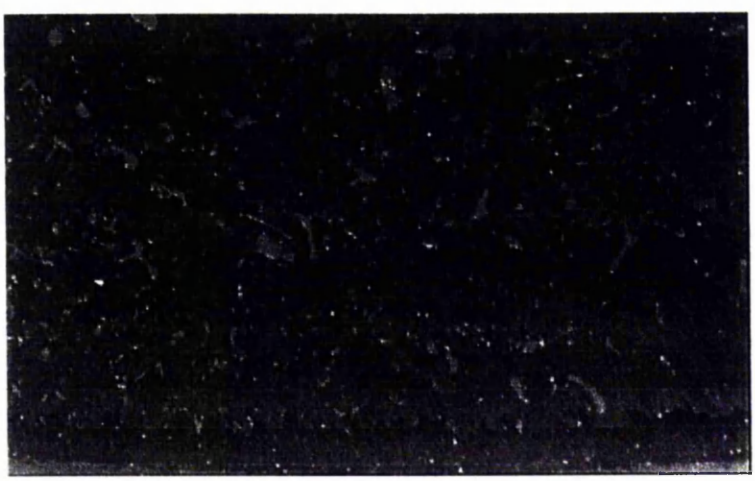


Fig 6.5 Very dense SCB plate.

The most morphologically diverse region for most sections tended to be on the palmar aspect of the condyle in the region of the sesamoidean articulation. In this area, the trabecular patterns varied considerably. Again, the most radical observation was of complete obliteration of the trabeculae, resulting in no identifiable pattern of architecture. Contrary to this condition and usually present in the specimens with the least amount of bone, were the sections that tended toward isotropy, with no organisational pattern at all in its trabecular architecture **(Fig 6.6 & 6.7)**. In these sections, the bony struts seemed to be placed in almost random configurations. In the intermediary state, some recognisable pattern of trabecular orientation was present. In some of these cases, the trabeculae seemed anisotropic, aligning the trabeculae perpendicular to the CC, in opposition to the sesamoidean contact region.

The thickness of the SCB plate varied significantly among specimens and sections. It ranged from 52μm to 754μm with a mean of 330μm and a median value of 331μm. The values were grouped and examined by age, breed, gender and weight. **(Table 6.1 and 6.2)**

Comparisons for All Horses

Age vs. Section

Age had a significant effect on SCB thickness (P = .01), whereas section and the interaction between age and section did not (P = .15 and .76). Age groups 4 and 2 had a significantly thicker SCB plate than 1.

Fig 6.6 Open trabecular architecture. Some organisation to the trabeculae at the palmar aspect of the joint, but still open in its architecture.

Fig 6.7 Dense palmar trabecular architecture. The trabecular struts appear to coalesce on the palmar aspect of this joint.

	SECTION 3	SECTION 4	SECTION 5	SECTION 6	SECTION 7	SECTION 8
<5 Years	128.38 ±103	202 ± 246	226.11 ± 194	139.19 ± 127	155.43 ± 160	135.58 ± 137
5-9 Years	366.56 ± 141	376.99 ± 149	451.16 ± 133	254.19 ± 89	376.86 ± 88	366.14 ± 101
10-14 Years	395.21 ± 150	432.1 ± 216	537.84 ± 169	362.05 ± 46	456.44 ± 85	409.41 ± 122
15-19 Years	318.28 ± 132	422.88 ± 104	409.63 ± 66	217.65 ± 105	423.03 ± 225	382.09 ± 234
20+ Years	319.65 ± 138	300.28 ± 126	337.25 ± 104	267.98 ± 56	364.58 ± 163	328.05 ± 132
TB	350.36 ± 132	387.55 ± 153	434.82 ± 138	260.48 ± 81	404.05 ± 150	373.13 ± 153
TBx	279.73 ± 173	311.94 ± 220	410.02 ± 140	344.81 ± 64	330.47 ± 165	299.75 ± 157
Pony	295.56 ± 193	265.95 ± 172	322.55 ± 164	211.42 ± 121	283.35 ± 147	270.08 ± 148
X	163.4 ± 124	235.2 ± 219	161.77 ± 130	111.68 ± 92	228.54 ± 263	185.56 ± 193

Table 6.1 Summary of SCB Thicknesses (μm) for Age and Breed

	SECTION 3	SECTION 4	SECTION 5	SECTION 6	SECTION 7	SECTION 8
Geldings	268.65 ± 157	282.83 ± 153	309.6 ± 135	204.18 ± 113	304.74 ± 184	265.31 ± 160
Mares	347.03 ± 144	393.97 ± 188	451.91 ± 153	287.6 ± 75	400.59 ± 148	380.61 ± 150
< 600 lbs	199.96 ± 155	171.26 ± 95	270.61 ± 91	215.15 ± 120	212.93 ± 157	182.54 ± 114
700 lbs	259.15 ± 209	253.55 ± 170	289.96 ± 228	187.76 ± 128	262.03 ± 167	282.61 ± 192
800 lbs	284.14 ± 151	418.49 ± 222	450.56 ± 180	252.68 ± 84	447.8 ± 201	434.43 ± 178
1000 lbs	375.13 ± 95	378.92 ± 88	386.63 ± 48	291.49 ± 73	411.5 ± 114	343.7 ± 115
>1000 lbs	391.14 ± 113	436.18 ± 185	494.28 ± 150	266.27 ± 114	383.79 ± 138	342.57 ± 152

Table 6.2 Summary of SCB Thicknesses (μm) for Gender and Weight

Gender vs. Section

There were no effects of section or the interaction between gender and section (P =.17 and .76) Gender independently showed a trend towards significance.(P = .07), the females tending to have a thicker SCB plate than males across all sections.

Weight vs. Section

Weight had a significant effect on SCB plate thickness (P = .038), whereas section did not (P = .19). The interaction between weight and section showed a trend towards significance (P = .08). The lighter horses had a thinner SCB plate than the heavier ones.

Comparisons by Section

Each section was examined independently for interactions between age, gender and weight.

Section 3

Breed showed a significant effect (P = .001), with Thoroughbreds having a thicker SCB plate than cross-breds. There were no significant effects of gender weight or the interactions in Section 3. Age showed a tendency towards significance (P = .08), with the youngest group of horses possessing the thinnest SCB plate. **(Graphs A3.7-10)**

Section 4

There were no significant effects of age, breed, or interactions for section 4. Gender and weight showed trends towards significance (P = .06 respectively). For gender, the females tended to have a thicker SCB plate than the males. In the weight category, the lightest horses tended to have the thinnest SCB plates, while the heaviest ones had the thickest. **(Graphs A3.11-14)**

Section 7

Age had a significant effect upon the SCB plate thickness in Section 7 (P = .04), with age group 4 possessing a significantly thicker SCB plate than the youngest horses. Breed, weight and the interactions had no affect. Gender was not significant (P = .07), but showed a trend with the females tending to have a thicker SCB plate than males. **(Graphs A3.15-18)**

Section 8

Age had a very significant effect on SCB plate thickness in Section 8 (P = .004), with both Age groups 4 and 2 possessing a significantly thicker SCB plate than the youngest horses. Gender and the interaction between age and gender also showed significance (P = .015 and .012). Gender only showed its affect in Age group 4, where the females had a thicker SCB plate than males. **(Graphs A3.19-22)**

A different response pattern was noted for gender in relation to age. For male horses, Age Group 5 was different from 1, whereas for females, Age Group 4 was different from 1, 5 and 3.

Neither breed nor weight had any effect on SCB plate thickness in Section 8.

Discussion

To the author's knowledge, this study represents the first attempt to quantify the thickness of the SCB plate in horses. Several studies in the literature have made reference to sclerosis of the SCB, determining this via everything from post-mortem examination, to clinical radiology to dual energy x-ray absorptiometry **(Pool 1996, Ross 1991, Firth *et al* 1999).**

The difficulty in measuring this 'structure' is that there are very few good descriptions of its extent. Most of the descriptions in the literature are of rodents, dogs or humans, and the SCB plate is an obvious layer supported by trabecular struts. It is therefore relatively easy to determine whether it is thick or thin. In our study, however, it became quite obvious that the bone beneath the CC layer was quite diverse and seldom possessed a definable border as it seems to in other species. Rarely did the equine SCB plate resemble such morphology. For the purposes of this study, we defined the SCB plate to extend proximal from the CC layer until a marrow space was encountered. It seemed reasonable to assume that solid bone extending deep to the CC layer, whether normal or not, would constitute the supporting structure for the overlying cartilage. Examination of the sections, however, revealed that the distribution of the marrow spaces were highly variable both between sections as well as within sections. Randomly measuring from the identifiable TM to the first marrow space encountered, regardless of how often it was repeated, seemed fraught with inaccuracy.

In our previous study, the undulating CC layer was measured interactively on the computer screen using the mathematical concept of integration to estimate the average thickness over a specified region. Because the appearance of the SCB plate had similar variability, it was decided to employ a similar technique in this study. We felt this method provided an accurate representation of the

SCB plate thickness. In addition, the measurements were repeated 3 different times starting in a slightly different place each time.

Age and weight both had a significant affect on the thickness of the SCB plate. Both the younger and the lighter horses had a thinner SCB plate. Like the results of the CC study, these findings would suggest that growth and maturity influence the SCB. Increased stress from a heavier body mass may also lead to a thickening of the bone just below the cartilage.

When analysed by section, there were some different findings. For section 3, breed became a significant factor with TB showing a thicker SCB plate than the cross-breds. Whenever breed becomes a factor, genetic influence can not be ruled out. It might be possible that TBs are simply born with more bone at that site or lay down more bone down in response to external stimuli. While other factors are possibly responsible for the disparity in SCB plate thickness in the TBs, exercise and training regimes can not be overlooked. Section 3 is particularly appropriate to this line of thought because it is the more proximal of the two palmar sections examined in this study. According to biomechanical studies on the equine forelimb, full sesamoidean contact is achieved at the gallop, thus significantly increasing the stress localised on the palmar aspect of the distal metacarpus **(Vilar *et al* 1995).** Although no histories were obtained for these horses, several of the TBs were in quite good condition and could easily have been racehorses. In a similar vein, **Young *et al* (1991)** conducted a study on the proximal sesamoid bones of racehorses subjected to different training regimes and surfaces. They found that both the surface and the type of exercise influenced morphometric parameters of the proximal sesamoid at *post-mortem* examination. Conversely, however, the objectives of their study were to investigate the aetiology of sesamoid fractures as opposed to the occurrence of joint disease. **Young *et al* (1991)** concluded that there is a substantial stress response to training where new bone is laid down to withstand some of the stresses that might cause the bone to fracture.

However, their findings of lower bone porosity and increased trabecular width, which are extolled in their discussion as a necessary remodelling response to prevent fractures, may actually lead to articular problems on the other extreme. An increase in SCB plate thickness has been postulated to cause a decreased shock absorbing capacity to the joint **(Radin and Rose 1986). Shimizu *et al* (1993)** found that there was a parallel correlation between cartilage degeneration and the bone volume and increased bone formation in the subchondral region of the proximal tibia in patients with OA of the knee. They noted that the highest bone volume was seen most superficially. In a similar study, bone samples from the tibia of patients with OA had a higher bone volume fraction and thicker than normal trabeculae **(Kamibayashi 1995a).** These are the same findings that **Young *et al* (1991)** report as beneficial to preventing fractures in their study of TBs. In yet another twist of bone remodelling, **Crane *et al* (1990)** determined that young healthy controls had a higher bone mass than older, healthy controls. However, older patients that developed OA had significantly more bone than older controls. **Eckstein *et al*(1993)** found that cartilage damage not only occurred in regions with a denser SCB plate, but also in regions overlying a transition from moderate to slight mineralisation, indicating that both too much support and unequal support could both be detrimental to the overlying cartilage. This hypothesis had been metaphorically illustrated by **Radin and Rose (1986)** with the cushion analogy. A cushion placed on a hard wooden chair will not degenerate as quickly as one on a chair seat that has a centre section replaced with a foam insert. The results of the interface between hard and soft will create shear forces in the basal levels of the overlying structure. Thus, support seems to be a major function of the SCB plate.

Numerous theories related to SCB plate thickening have been tested in the laboratory. In order to study the effects of a thickened SCB plate, researchers implanted metal cylinders in the subchondral region and measured stress in the juxta-positioned tissues. Using finite element analysis, they found that the stress level was increased in all the articular tissues, leading them to conclude

that SCB stiffening may create abnormal joint stresses **(Brown *et al* 1984).** In an animal model of joint stress, beagle dogs were exercised prior to sacrifice. The exercised groups showed an increased SCB plate thickness compared to the non-exercised ones **(Oettmeier *et al* 1992).** In a similar study in dogs, the anterior cruciate ligament (ACL) was removed and the subjects were forced to exercise. At 3 and 18 months after ACL transection the articular cartilage showed signs of early OA. There was a loss of trabecular bone but an increase in SCB plate thickness, significantly by 54 months. This study showed SCB changes do not need to be present to initiate articular cartilage changes in OA, but thickening of the SCB plate may lead to progression of OA. The SCB plate seems to act more like cortical than cancellous bone at this stage, which could explain these findings **(Dedrick *et al* 1993)**. This study is particularly interesting in that it seems to implicate a relatively superficial layer of SCB in the progression of OA. The trabecular bone in these dogs actually decreased, but this also could have been a response to disuse from pain associated with the severed ACL. In the bone sections from our experiment, there was a variable amount of SCB thickening observed, but the deeper trabecular bone never appeared less dense in those sections. However, no morphometric data was acquired for the deeper layers of trabecular bone in our study.

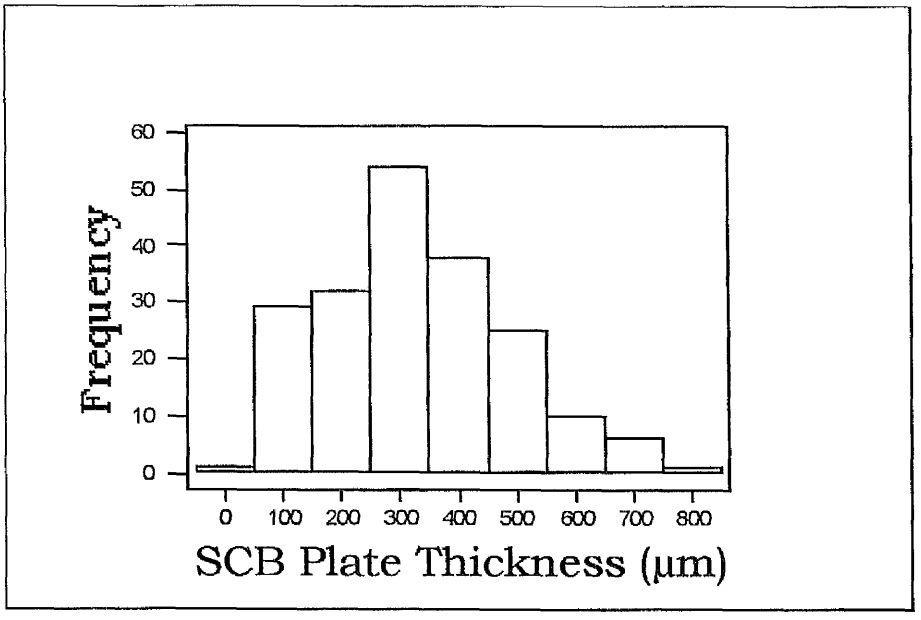
Further studies into the pathogenesis of SCB thickening and resultant OA have implicated trauma as an inciting cause. In another animal model of joint disease, removing the medial collateral ligament from a rat's knee resulted in a significant increase in SCB growth **(Messner *et al* 1996)**. In an experiment designed to explore the pathogenesis of OA, blunt trauma was delivered to the femoropatellar joint of rabbits. Although the impact produced surface lesions on the retropatellar cartilage, more importantly, the thickness of the SCB tended to increase with time after the insult and the bone exhibited significant thickening at 12 months. The overlying cartilage showed signs of degeneration, however its mechanical stiffness did not change until 12 months after insult **(Newberry *et al* 1997)**. This study supports the concept that OA may develop secondary to bone trauma and thickening of the SCB plate.

When examining the data from our study further, it became evident that both weight and gender showed trends towards an influence on the SCB plate thickness. The heavier animals and females, respectively, tended to have thicker SCB. Weight could obviously play a biomechanical role by increasing the stress on the joint, but it is difficult to explain the affects of gender. In women it is the decrease in circulating oestrogen levels that are known to cause osteoporosis **(Alden 1989).** It is possible that circulating hormones, such as oestrogen, may influence equine bone formation as well. Alternatively, there may be some other environmental factor that affects only the females and is responsible for the differences. One such possibility involves calcium homeostasis. It may be that the mare has a higher bone level so she is more prepared for gestation and lactation. **Glade (1993)** found that mares experienced a significant decrease in metacarpal breaking strength when estimated via ultrasonics during the first 12 weeks of lactation. Although the study does not report this, it would seem feasible that mares may carry more bone normally as a kind of protective measure.

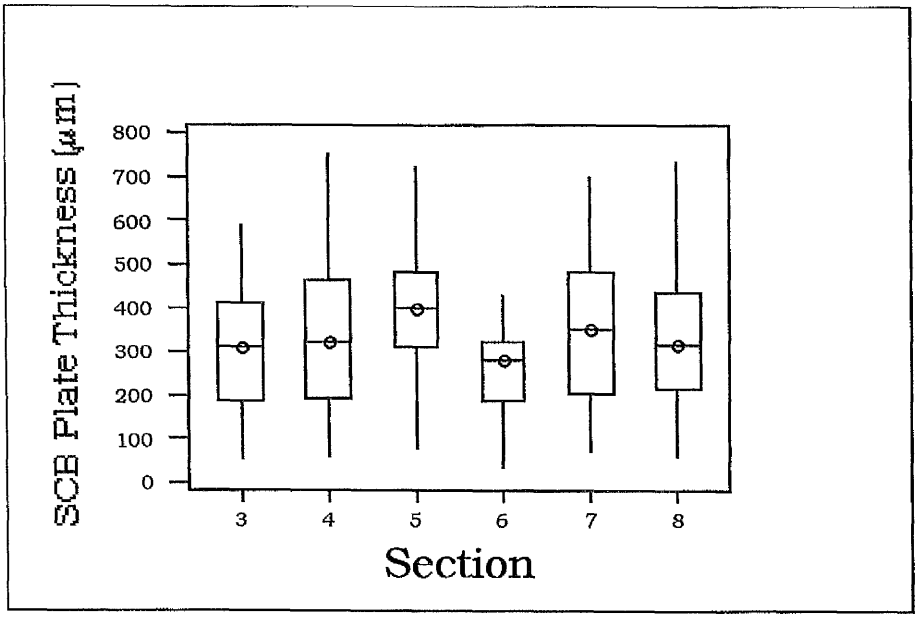
In a study with many similarities to our own, **Carlson *et al* (1996)** investigated OA in cynomolgus macaques. They found that the SCB plate was thickened in cases of OA, leading to cartilage fibrillation. In their population, both gender and weight significantly influenced the thickness of the SCB. Similar to our findings, the heavier macaques and the female macaques had a thicker SCB plate. Interestingly enough, they found that the mean SCB plate thickness was 555μm and that thicknesses of under 400μm resulted in no cartilage damage. In our study, the mean SCB plate thickness was 330μm, well below their threshold of 400μm. We attempted to gather unaffected animals in our population, so this parameter would need to be investigated further in animals affected with OA.

While there is overwhelming evidence of SCB involvement in other species, especially humans, little has been done in the horse. We consider this investigation to only begin to unravel the answers about the SCB layer to shed light on its role in the progression of OA.

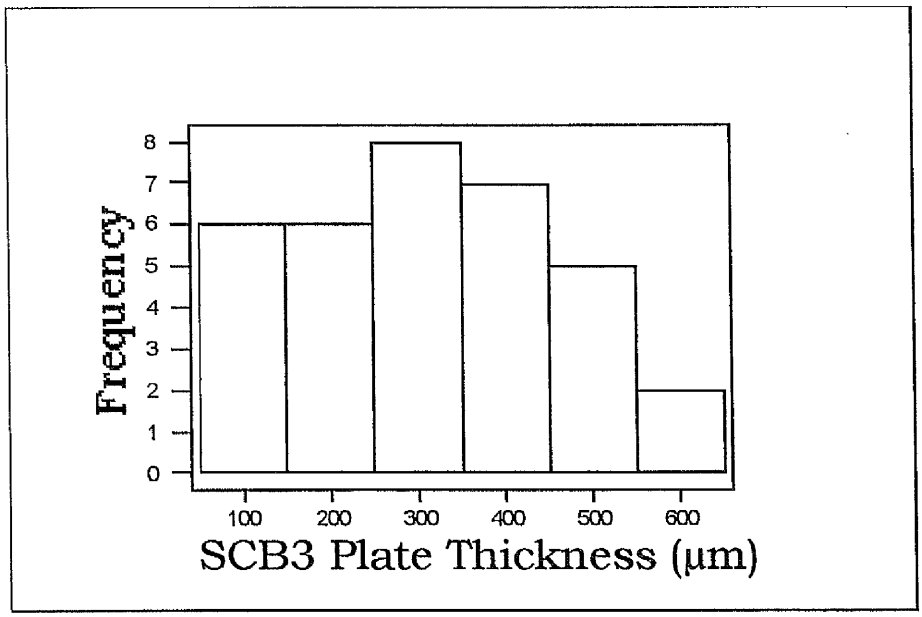
APPENDIX 3



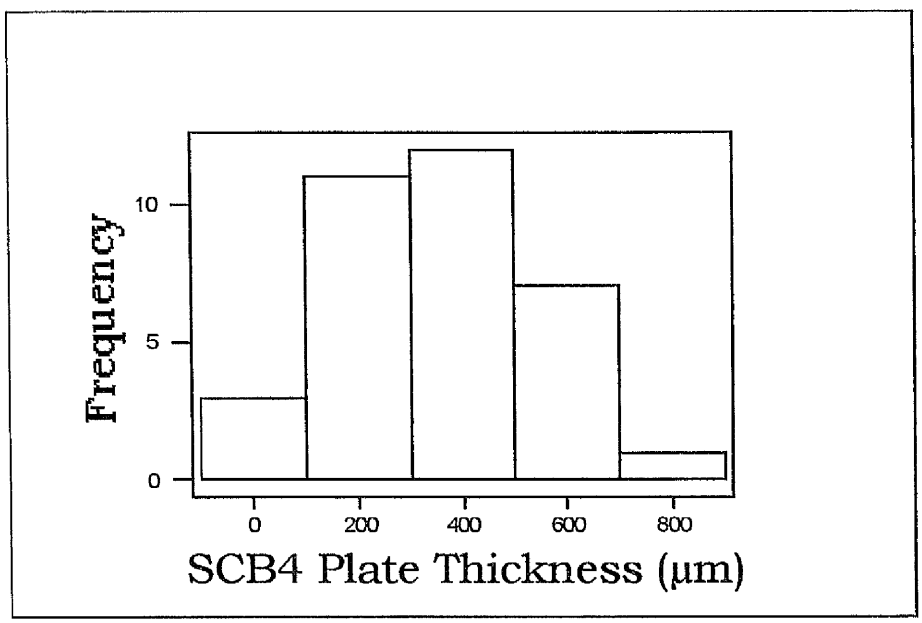
Graph A3.1 Histogram of SCB Plate Thickness for all Sections



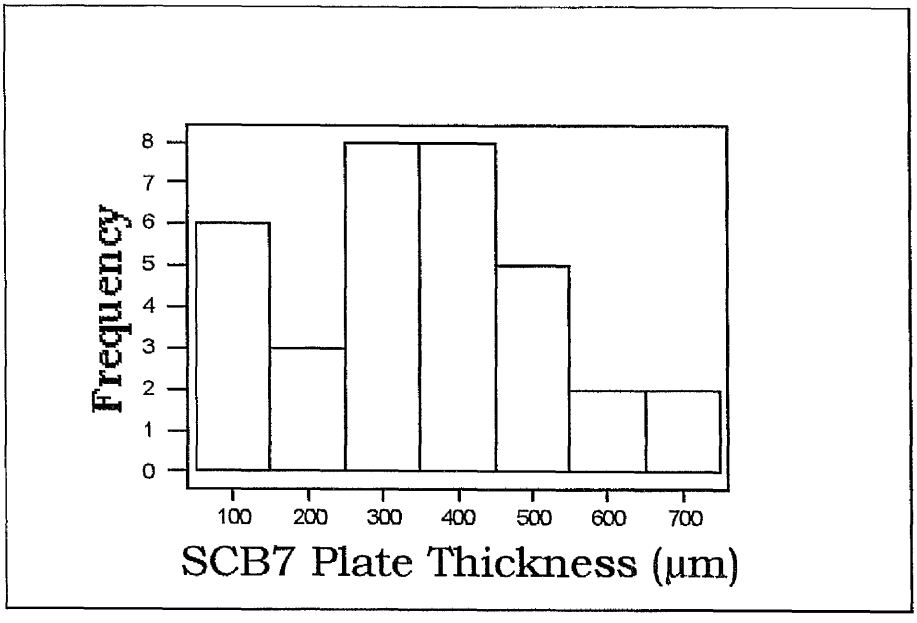
Graph A3.2 Boxplot of SCB Plate Thicknesses by Section



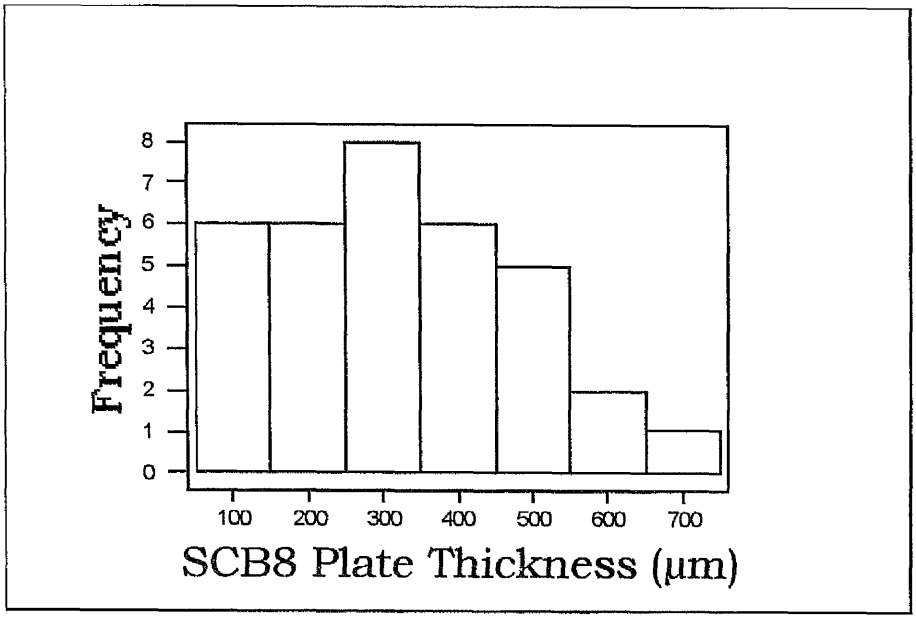
Graph A3.3 Histogram of SCB Thickness in Section 3



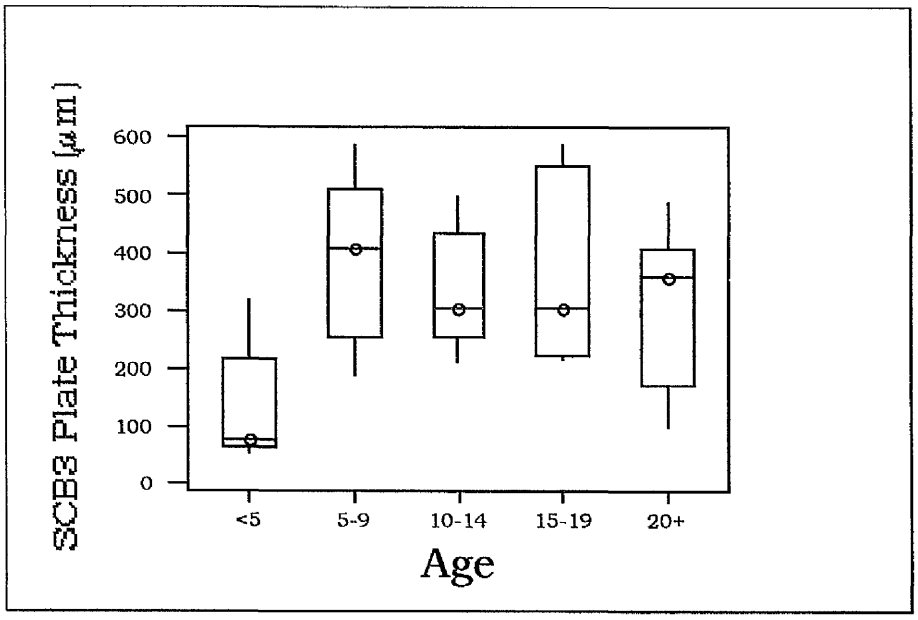
Graph A3.4 Histogram of SCB Thickness in Section 4



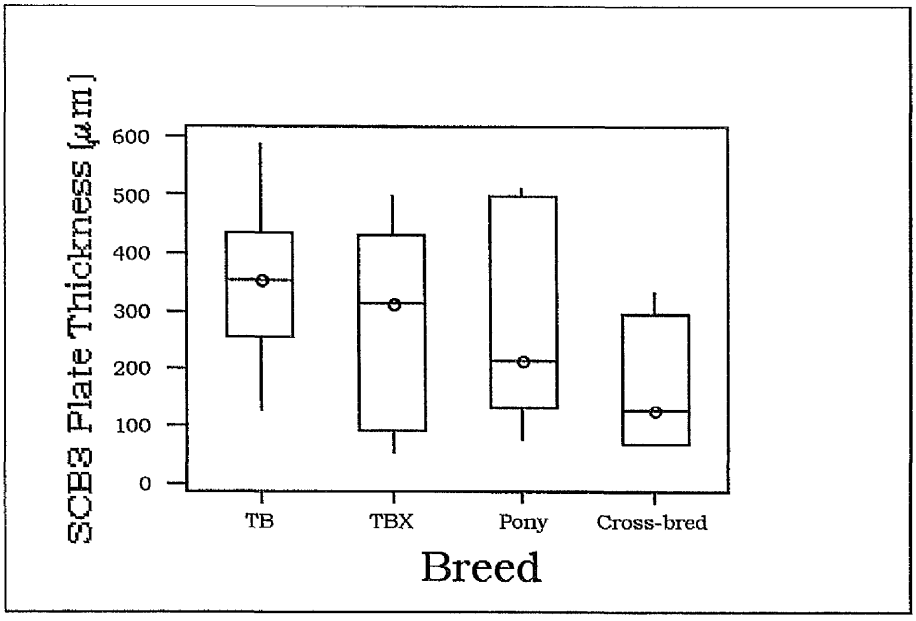
Graph A3.5 Histogram of SCB Thickness in Section 7



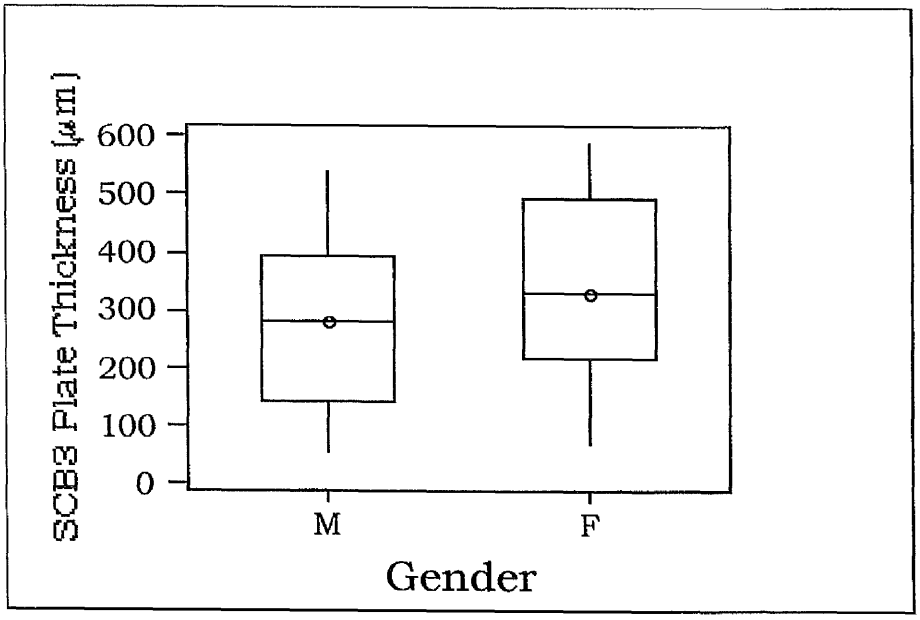
Graph A3.6 Histogram of SCB Thickness in Section 8



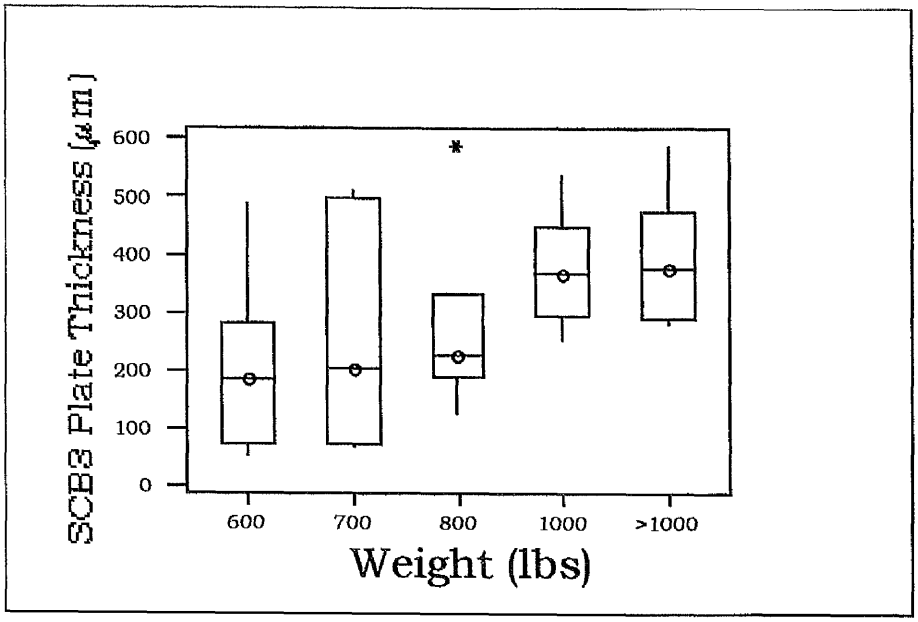
Graph A3.7 Boxplot of SCB3 Thickness by Age



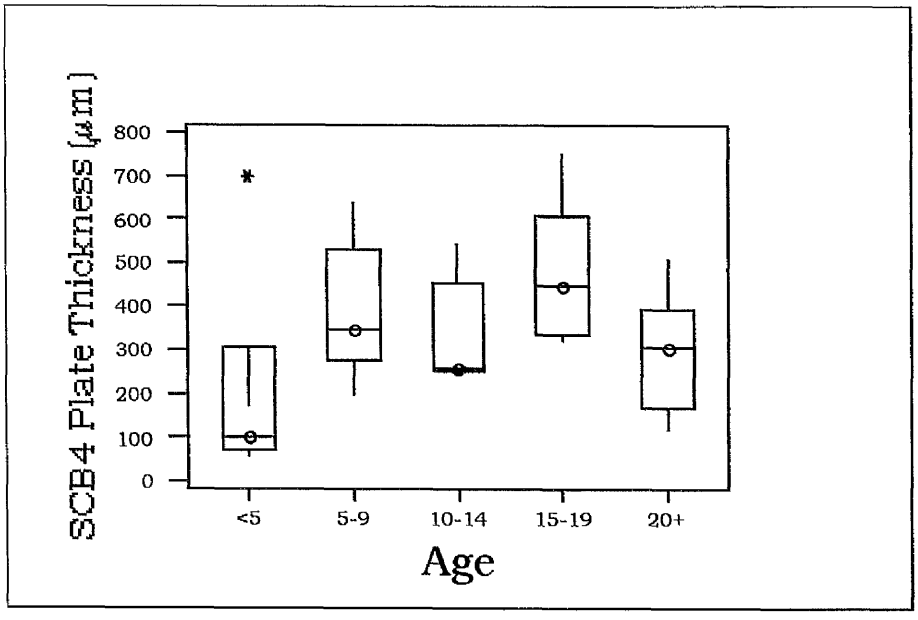
Graph A3.8 Boxplot of SCB3 Thickness by Breed



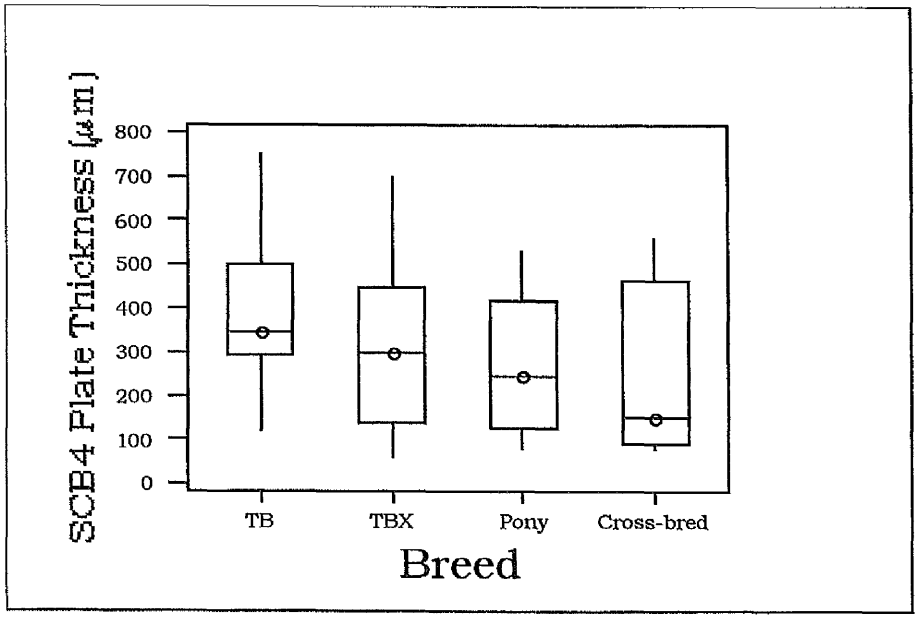
Graph A3.9 Boxplot of SCB3 Thickness by Gender



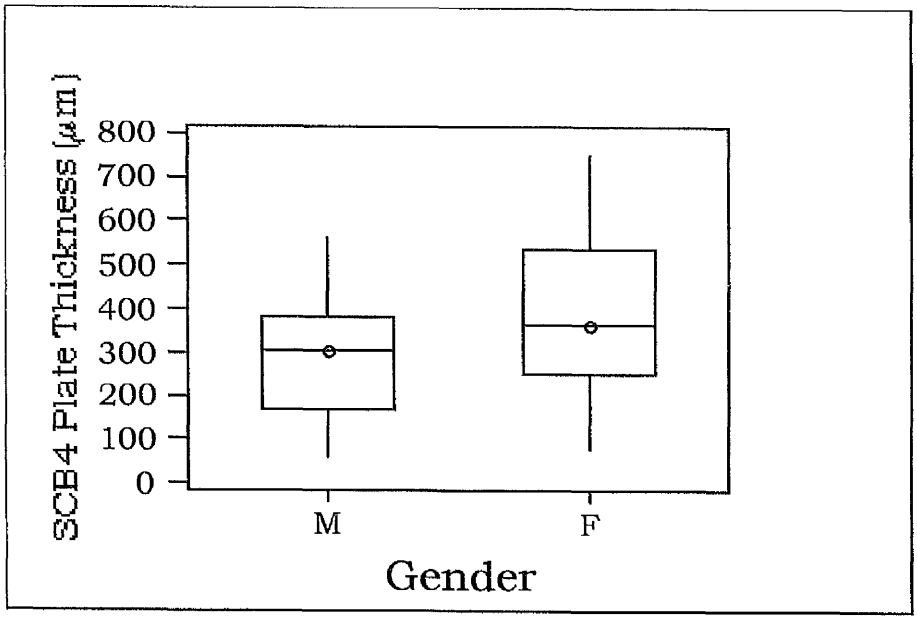
Graph A3.10 Boxplot of SCB3 Thickness by Weight



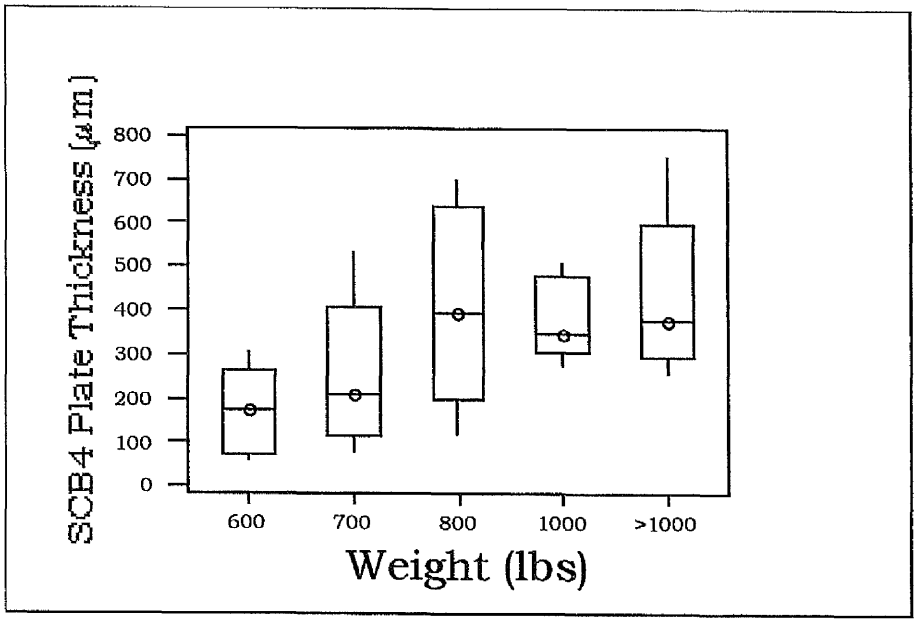
Graph A3.11 Boxplot of SCB4 Thickness by Age



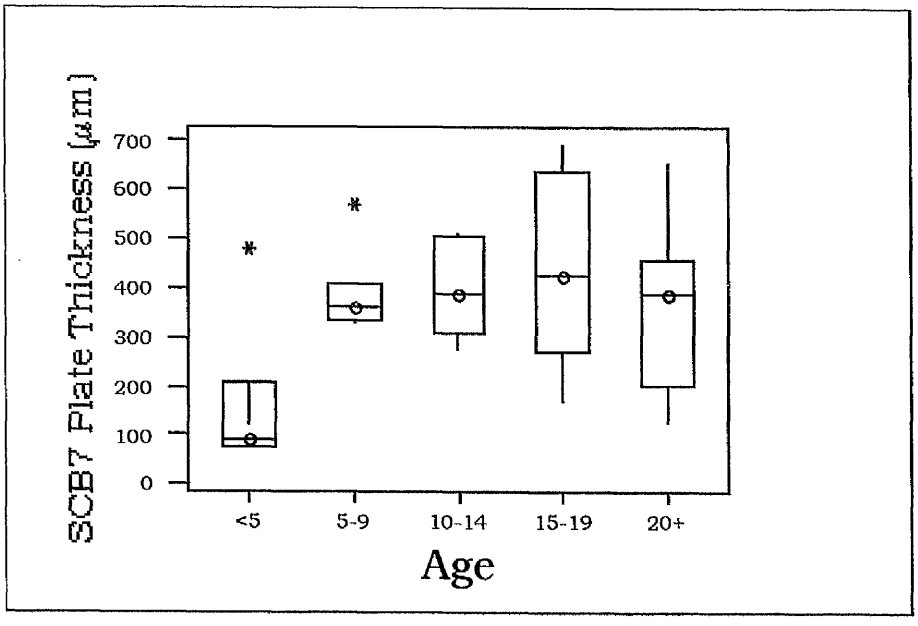
Graph A3.12 Boxplot of SCB4 Thickness by Breed



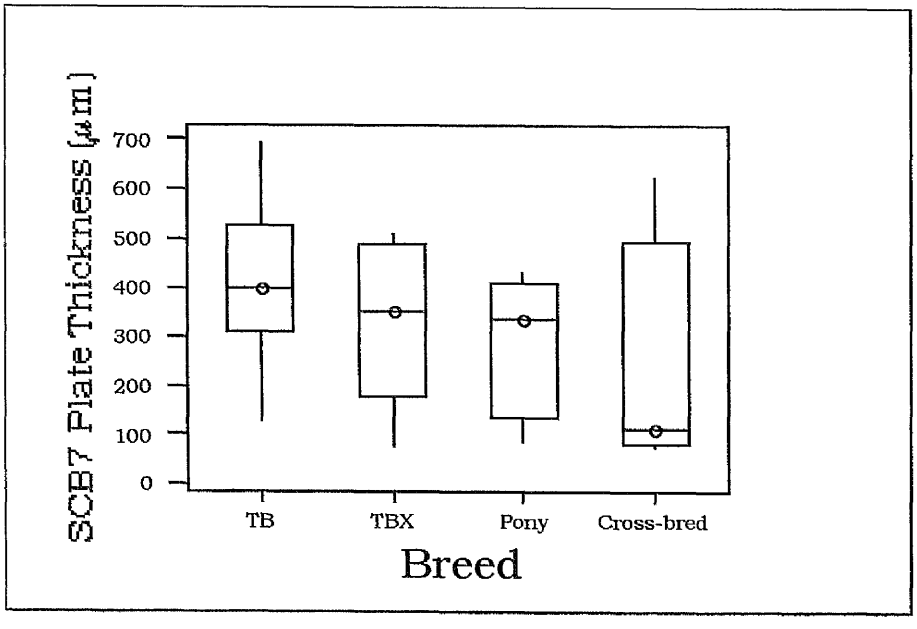
Graph A3.13 Boxplot of SCB4 Thickness by Gender



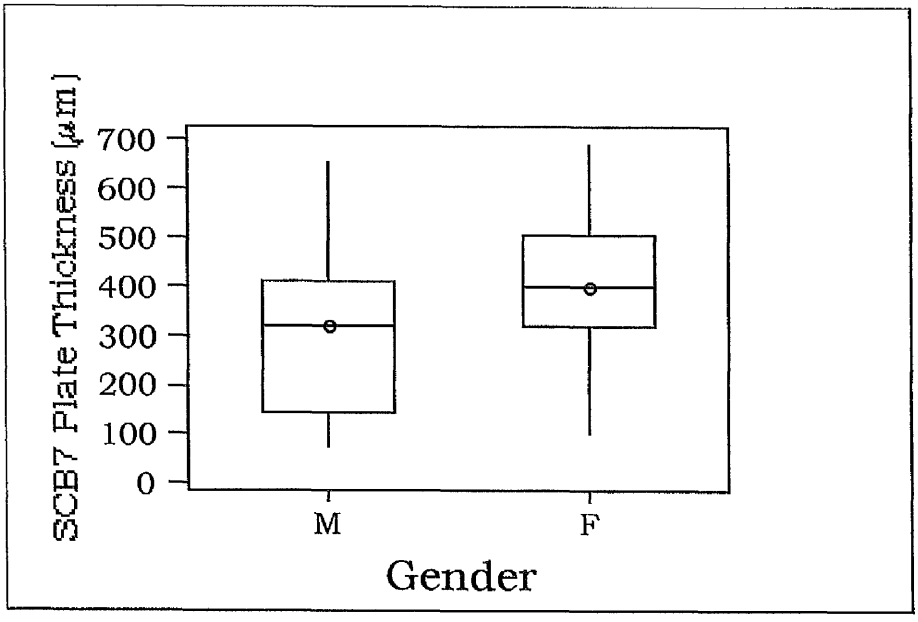
Graph A3.14 Boxplot of SCB4 Thickness by Weight



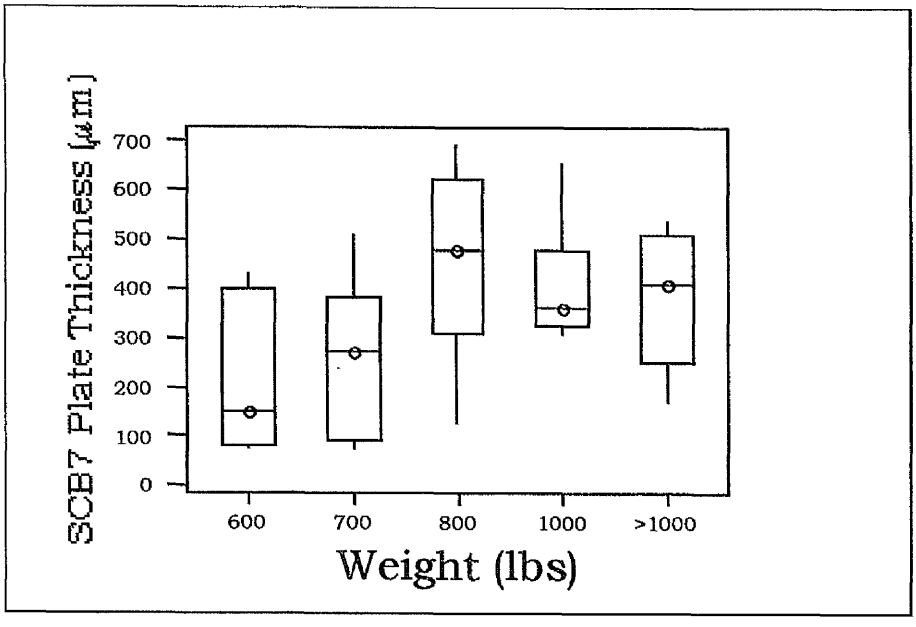
Graph A3.15 Boxplot of SCB7 Thickness by Age



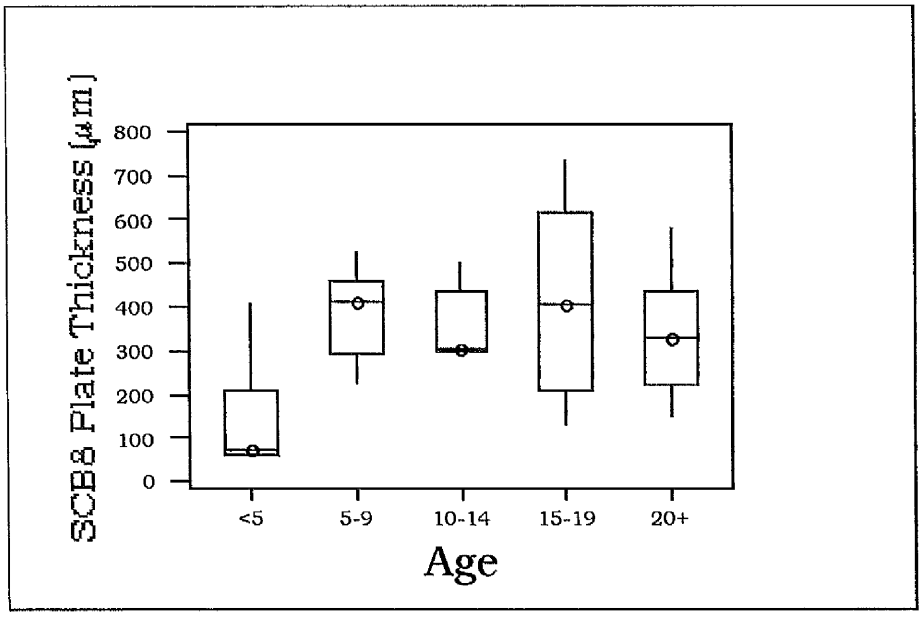
Graph A3.16 Boxplot of SCB7 Thickness by Breed



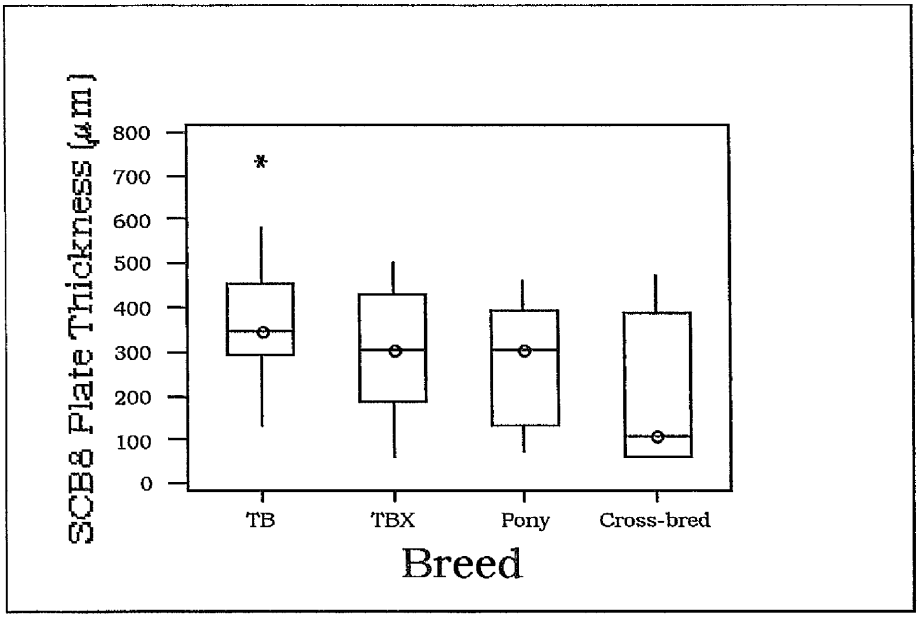
Graph A3.17 Boxplot of SCB7 Thickness by Gender



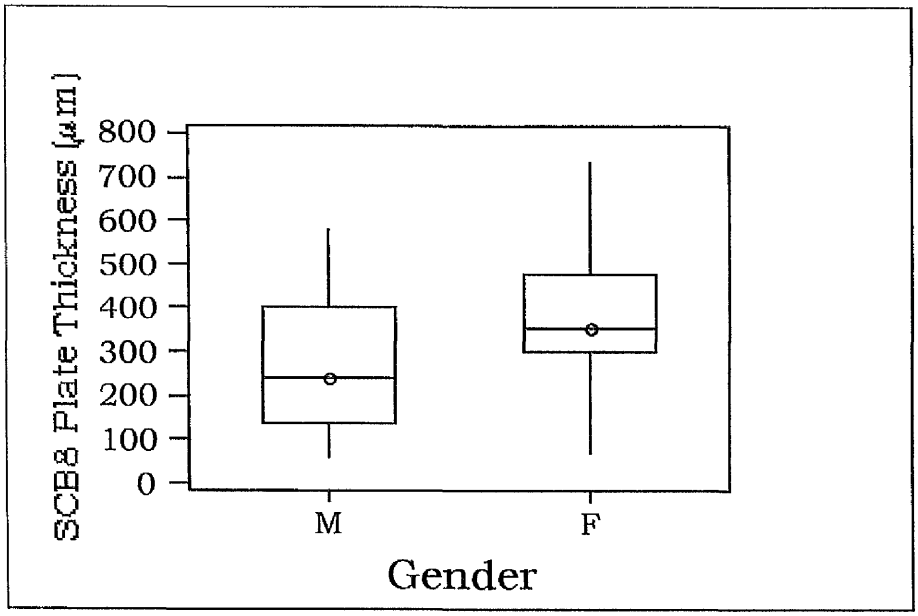
Graph A3.18 Boxplot of SCB7 Thickness by Weight



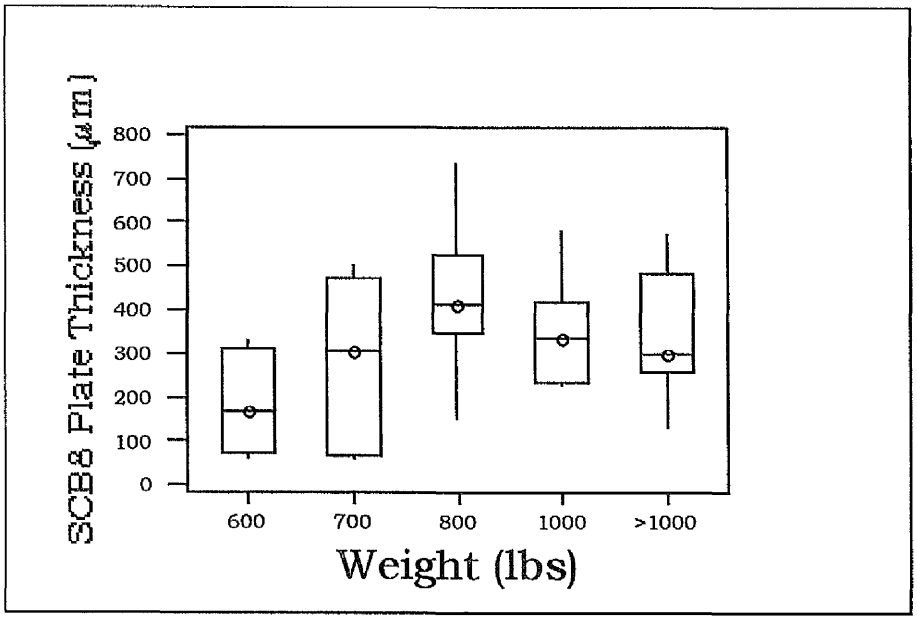
Graph A3.19 Boxplot of SCB8 Thickness by Age



Graph A3.20 Boxplot of SCB8 Thickness by Breed



Graph A3.21 Boxplot of SCB8 Thickness by Gender



Graph A3.22 Boxplot of SCB8 Thickness by Weight

CHAPTER 7

FRACTAL ANALYSIS

Introduction

Bone histomorphometry is the process of measuring the amount and structure of bone as discussed in Chapter 4. It has evolved over the years as a means of providing useful data about the bone that can be used to compare differences between normal and abnormal within a subject as well as between subjects. The practice of bone histomorphometry relies on the principles of Euclidean geometry to define the properties of the calcified tissue. Typically, this means that routine shapes such as circles, squares and straight lines are employed to gather data. While this has been the convention for decades, fractal geometry delves into a completely different realm, that of examining structures over a different range of magnifications to see if some kind of pattern exists **(Kaye 1989).** If an object, such as trabecular bone, can be described as possessing characteristics of fractal geometry, a completely new and more powerful analysis of the structure will result.

Fractal Geometry

The Theory.

The ***fractal dimension*** is a mathematical set of principles that describes an object in unconventional, yet compelling, terms. The name was coined recently by a mathematician named **Benoit Mandelbrot (1983)**. He observed the natural world is not well described by standard forms from Euclidean geometry such as circles and straight lines, and proposed a class of shapes he named *fractals*, derived from the Latin *fractus*, meaning broken or irregular. Such shapes are represented in Euclidean space as sets of a non-integer dimension.

A more theoretical definition of a fractal, as presented by **Mandelbrot**, is an object that maintains some property or pattern over different magnifications. A simple example is the Gasket, originally created by **Sierpinski (1916)** to solve a problem in topology (**Fig 7.1**). The shape is created by starting with a triangular set and recursively removing, from any filled-in triangle remaining, another triangle joining the midpoints of the edges. The result is a mathematical abstraction containing arbitrarily small structures not visible to the unassisted eye. Rather it must be viewed across a range of scales allowed by the magnification in force. Researchers have found that this concept is the same for many objects in nature **(Mandelbrot 1983)**. A property or observation may apply across some range of scales, but the same property will not pertain to all scales.

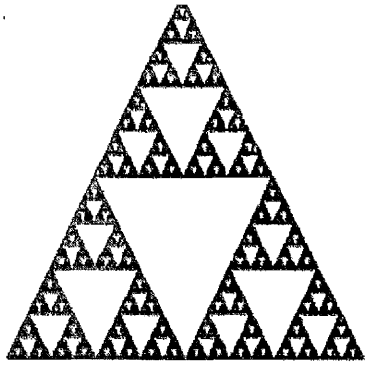


Figure 7.1 A representation of the Sierpinski gasket.

The Practice.

The first recorded observation of this scaling phenomenon seems to have been made by **L F Richardson (1951)** in his study of coastline lengths. He found that the measured length (L) of the coastline increased as the ruler length (w) used to measure it was reduced and took in more detail. What is remarkable is that in this example, a linear relationship exists between the log L and the log w which

effectively represents a Power Law **(Fig 7.2)**. This is written in terms of the number (N) of ruler lengths used to measure the coastline, as

$$N = K w^{-D}$$

where K denotes a constant. If this were to hold across arbitrarily small scales then D would equal the Hausdorff dimension **(Falconer 1990)**. If which conditions are met, by definition then, the object would be described as fractal. Because this linear relationship exists for the coastline example, it suggests that it be described as fractal. It should be noted, however, that there may be situations where a best straight line is drawn through a set of data points (log N, log w) which, regardless of how their relationships may reasonably be modelled, are manifestly not linearly related. In such situations, to allocate a fractal dimension is meaningless, because a power law simply does not hold. The first question is not what is the fractal dimension, but does one exist.

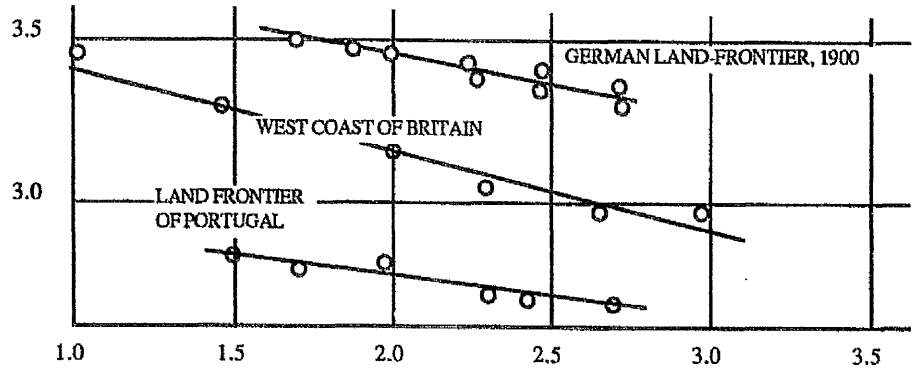


Figure 7.2 Some of Richardson's graphs of log(length) against log(ruler size) for coastlines. Here logs are to base 10 and the fractal dimension is 1 minus the graph slope **(Richardson 1951).**

In less esoteric terms, if the coastline of Great Britain is viewed from the moon, it takes on a certain outline or shape. However, if it is viewed from an aeroplane, the coast will take on a new outline as more information is gathered because one is closer to the subject. Finally, when walking along the beach itself, an even more detailed and jagged perception is the result. If the information gathered at each of these scales, graphed in log format, is linearly associated then a very powerful relationship has been established. The information has been linked to each other even though it has been collected across vastly different magnifications.

The application of fractal geometry to the study of bone has been explored over the last 5 years in several different arenas **(Fazzalari and Parkinson 1996, 1997; Cross 1993, Majumdar 1995)**. Considering bone, the existence of a fractal dimension, if present, could provide further information about the nature of the bone than currently amassed via conventional techniques of bone histomorphometry. This would especially hold true for the size and distribution of the trabecular struts within it. The objectives of this study were 1) to determine whether equine trabecular bone of the distal metacarpus behaves as a fractal and, if so, 2) whether differences in the fractal dimension could be found between age, breed, gender, and weight for the horses in this study.

Materials and Methods

Horses

For this investigation, the same horses were used as for the previous studies. There were complications with sample preparation from 5 horses which resulted in improper tissue staining and poor bone/marrow contrasting. Because this affected the accuracy of the digitisation procedure, these 5 specimens were

excluded from this study. Four of these specimens were from horses in age group 5 and one from age group 3.

Digitisation

Digitisation of the specimens was carried out using the same basic computer equipment. For this experiment, an Apple Macintosh model 6200 with a 100 MHz microprocessor, a 1 GB hard drive and 60 MB of RAM was used. A 15-inch monitor set to a size of 640 X 480 was connected to the computer. A flatbed scanner was connected to the 6200 as a Small Computer Systems Interface (SCSI) device. The acquisition software utilised was a plug-in for Adobe PhotoShop®.

Flatbed scanning. The embedded bone specimens were placed face down on a flatbed scanner. A preview was obtained over a 15 cm X 10 cm area containing the bone specimen. From this temporary image, the view box was manually shaped to the bone borders to define the eventual scan area. Digitisation of this small bone area was carried out at 800ppi in RGB colour as a reflective surface. Each bone image was saved as a '*.tif' file for later image manipulation **(Fig 7.3)**.

Image Manipulations

Once the images were acquired, they were converted from the 24 bit RGB files to 1-bit black and white files to facilitate calculations. The conversions were all carried out using Adobe PhotoShop®. The first step was to use the 'paths' function to manually trace out the epiphysis of the bone. Starting at the proximal most extent of the CC layer on the palmar aspect of the joint, the CC layer was traced out as the distal most border of the image, extending all the way to the

proximal endpoint of the CC on the dorsal aspect of the bone. The most proximal extent of the path on both the dorsal and palmar aspect of the image was connected to finish the proximal aspect of the image. The 'path' was saved and then selected with a 'zero' tolerance on the pixel level. The 'inverse' function was invoked to select only the background, leaving the desired bone region in place. The background was set to white on the colour palette. Using the 'delete' function, the unwanted portions of the image surrounding the bone were removed, leaving the bone section in 24 bit RGB colour on a white background **(Fig 7.4)**.

The next step was to change the colour mode to 'Lab' from RGB. The Lab colour mode is device independent and utilises the 'L' channel for Luminance, 'a' for the Red to Green channel and 'b' for the Blue to Yellow channel. The 'L' channel takes on values from 0 to 100, while the 'a' and 'b' channels function from -128 to 128, where zero is the neutral value on the scale. The 'L' and 'a' channels were deleted, leaving only the Blue to Yellow channel. A filter function, known as 'median' was used to remove some of the debris from the image. 'Median' will examine the pixels in the image and remove those that are more than a threshold away from the selected value. In this case, the threshold was set at one pixel to remove only very obvious noise from the image **(Fig 7.5)**.

Fig 7.3 Bone specimen after flatbed scanning. The block surface was digitised at a resolution of 800 pixels per inch (ppi) using a flatbed scanner connected to a Macintosh computer resulting in the above image.

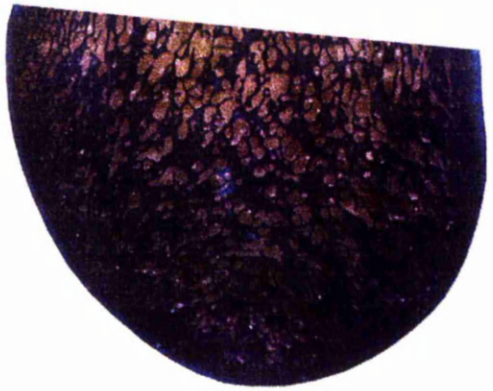


Fig 7.4 Cut out section of bone. Once the sections are digitised, they are loaded into Adobe Photoshop® for processing. The first step is to use the 'path' function to trace out the calcified cartilage all along the articular surface. The path is selected and, by using the inverse function, the unwanted background is deleted resulting in the above image of subchondral and trabecular bone.

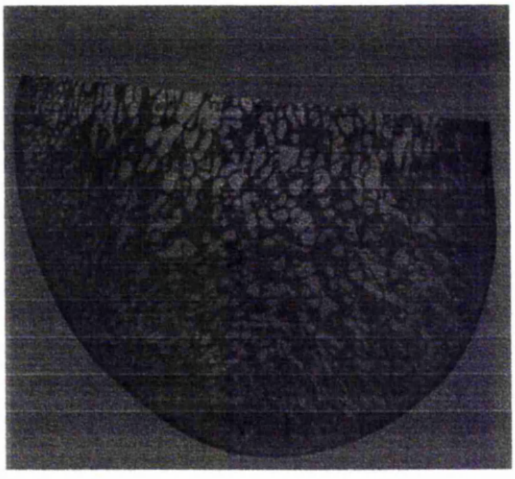


Fig 7.5 Grayscale bone image. The next step in image processing is to convert from RGB to Lab colours. By deleting the 'L' and 'a' channels, the image is left with only yellow and blue pixels although it appears grey at this stage.

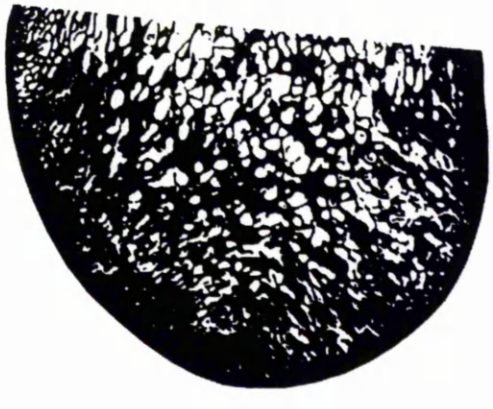


Fig 7.6 Bitmap Image. Finally, the contrast is increased to the end of the scale, taking all the blue pixels to black and the yellow ones to white. This image is then converted to a bitmap format for analysis where black pixels represent bone as above.

Finally, the contrast in the Blue to Yellow channel was increased to 100 percent, taking the Blue values all the way to 128. This manipulation converted all the Blue in the image to black and all the yellow in the image to white. For any images with remaining artefacts of processing, the debris was removed interactively under magnification of the region. Once the contrast was increased to 100 percent, the image was converted to a flat bitmap and saved as a '*.pict' file for analysis within NIH_Image. **(Fig 7.6)**.

Fractal Analysis

For this procedure, Richardson's original ruler was replaced by a more practical box counting method. A grid of squares or 'boxes' of size w was laid over the bone image, and N was considered to be the number of boxes that intersected the boundaries of the trabecular bone. Considering 3-dimensional space in Euclidean terms, the number of regular 'boxes' required to fill a fixed volume varies as the inverse cube of its edge size and in two dimensions the corresponding relation is the inverse square. By analogy, in fractal geometry, if a *punctured* area, such as trabecular bone, has the number N of squares ('boxes') intersecting the trabecular boundaries varying as the inverse D'th power of the (edge) size w of the box, a fractal dimension D is attributed to this punctured area. Thus D is a measure of the way the irregularity of the internal boundaries varies with changing scales.

A special macro was developed for NIH Image to calculate the fractal dimension of the bone specimen. In theory, the macro generates boxes of edge size w, which is measured in pixels. The starting box size was set by the macro and the finishing box size was determined by the operator. The macro then set about placing 'boxes' of size 'w1' across the bone image contiguously, but only in locations where there was a discernible trabecular strut/marrow interface. In regions of solid bone or large marrow spaces, no boxes were placed. Once the

entire bone image had been filled with boxes, the macro then calculated the number of boxes (N) of edge size w1 that were needed to do so. A 'result box' was automatically created which stored the 'w' value and N in table format and then calculated the log w and log N values to store along side their corresponding real number values. The macro carried out this procedure for box sizes of w1 + 1 pixel until the ending w value was reached, storing the results of each calculation and log transformation in the 'results window' **(Fig 7.7 and 7.8)**.

The second macro plotted out (x,y) for the log w vs log N from the 'results window' for each bone section. A third macro determined the 'best-fit' line over a range of box sizes by altering m and c from the linear equation $y = mx + c$ to minimise the squared deviations of the data points from that line. The negative of the slope of the 'best-fit' line (-m or D) was displayed as the fractal dimension for the sample and range of box sizes. Finally, a fourth macro produced a graph of the sum of squared deviations over both 'best-fit' lines against pivot points, establishing the location of the most appropriate 'break' between the two straight line segments **(Fig 7.10)** All the data for the graphs and the plots were saved as *.tif files and the fractal dimensions were recorded.

Validation. In the initial instance, the analyses were carried out on 18 bones and the fractal dimension for three to ten different sections from the same bone sample was calculated. Each bone was embedded, undecalcified, in methylmethacrylate (see Chapter 4 for details) and the bone surface was exposed using a high-speed microtome (Poly-cut E, Reichert-Jung). Care was taken to only remove enough bone so that the bone of the entire distal condyle was exposed. The surface was then stained with toluidine blue and digitised on a flatbed scanner (see above). Once the exposed bone surface was saved on the computer, the

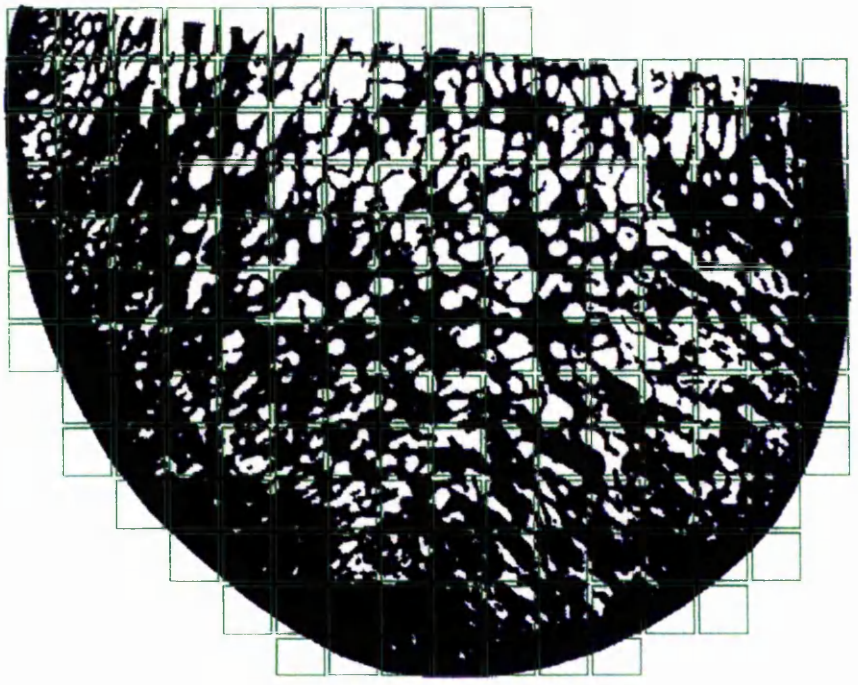


Fig 7.7 Fractal Determination- large box size. Using a dedicated macro, small boxes are placed wherever an edge of bone is encountered. The macro automatically counts the number of intersecting boxes and calculates the box size.

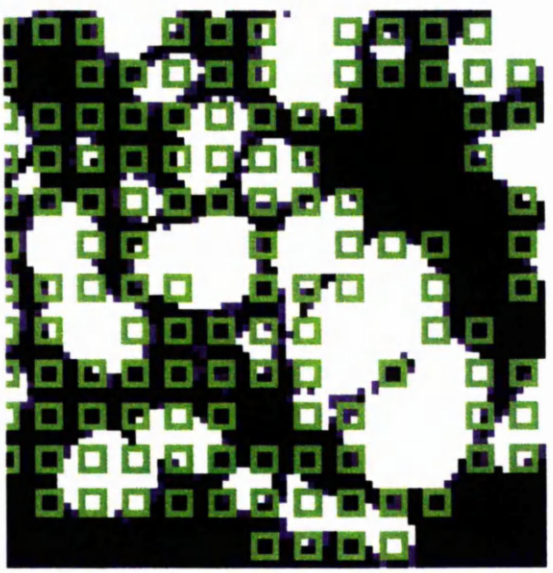


Fig 7.8 Fractal Determination- small box size. This process is carried out several times for different size boxes. This magnified view shows a much smaller box size being used. For illustrative purposes, the displayed boxes have been reduced by 1 pixel so they are not contiguous.

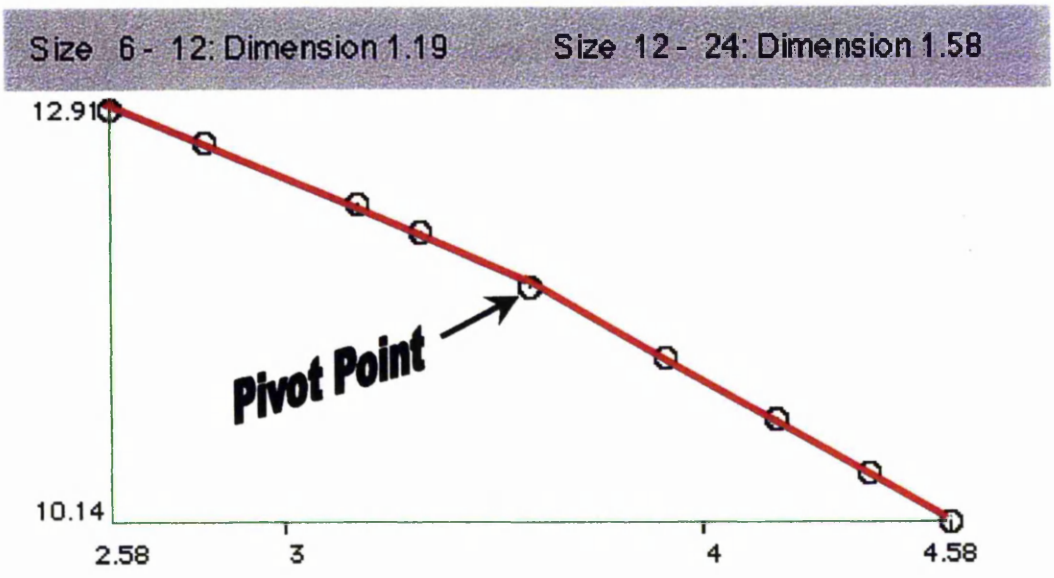


Fig 7.9 Richardson Plot. The fractal dimension is calculated by graphing the log of the box size versus the number of boxes that intersect a bone edge. If these values result in a straight line, the sample is considered to behave as a fractal. The negative slope of that straight line is the fractal dimension. Over this range of box sizes, the trabecular bone demonstrates two distinct fractal dimensions.

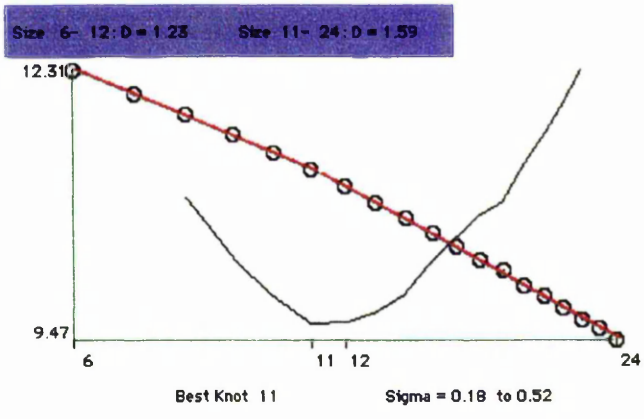


Fig 7.10 Example of a least squares curve placed across the top of a Richardson plot to determine the location of the pivot point.

methylmethacrylate block was again placed in the high-speed microtome and at least another 100μm of bone was removed from the surface. The surface staining and digitising was performed in the same way each time. This procedure was carried out from 3 to 10 times per bone. The image manipulation and fractal analysis was done on each bone image and the mean and standard deviation was calculated for each normal specimen.

Comparisons.

For the second aspect of the study, the bone specimens were collected from the medial aspect of the left forelimb and preserved and embedded in the same manner as described. Once the surface of the bone was exposed and stained, it was digitised at 800 ppi and underwent image manipulation as for the first set of specimens. In this part of the study, the fractal dimension was calculated only once over two scales for each bone specimen.

Statistical Analysis

The coefficient of variation was used to determine if the fractal values obtained for the different sections from each bone in the validation aspect of the study were similar to each other. For the second part of the study, two-way ANOVA was used to test for the effects of age, breed, gender, weight, and presence of radiographical or gross pathology. Significance was set at $P < 0.05$.

Results

The distribution of horses is shown in **Table 7.1.**

Validation

The first objective was to establish whether bone possesses a fractal dimension. Graphing the log of the box size by the log of the number of boxes needed to cover the bone/marrow interface, results in a data set that possesses two straight-line ranges. The least squares macro was invoked to establish the 'best-fit' line for the data, resulting in a convincing straight-line model, but further to suggest that two lines produce an even better fit. A minimum value was achieved for the sum of the squared deviations for both lines, at which location the pivot point for the two lines was established. This was always between box size 11 and 13 for our data set. **(Fig 7.9 and 7.10)**

Secondly, the fractal determination was carried out from three to ten times on a single bone specimen after exposing a new surface at least 100μm from the previous surface used to calculate the fractal dimension. The coefficient of variation was below 10% for all of these data sets indicating that the measure of the spread of these different fractal dimensions was within an acceptable margin to assert that no difference existed between them **(Table 7.2 and 7.3)**

The fractals were compared for differences caused by age, breed, gender, weight, by the presence of radiographical and gross pathology and the interaction between them.

STUDY NUMBER	SPECIMEN	AGE (YEARS)	BREED	GENDER	WEIGHT
1	427/97	1.5	TBX	M	600
2	429/97	1.5	X	G	700
3	2127A	2	X	F	600
4	319/97	2	X	G	700
5	2123A	3	TBX	F	800
6	378/97	4	Pony	G	600
7	314/97	6	TB	F	850
8	316/97	8	TB	G	1100
9	318/97	6	Pony	F	700
10	305/97	7	TB	F	800
11	310/97	7	TBX	G	1000
12	431/97	8	TB	F	1000
13	2119A	9	TB	F	1100
14	380/97	10	Pony	F	600
15	322/97	12	TB	F	1200
16	415/97	12	TBX	F	750
17	425/97	13	TBX	G	700
18	435/97	13	TB	F	1100
19	407/97	17	Paint	G	800
20	411/97	17	TB	G	900
21	402/97	18	TB	F	800
22	413/97	18	TB	G	1200
23	400/97	19	TB	G	1000
24	2114A	20	Pony	F	550
25	309/97	20	TBX	G	700
26	392/97	20	TB	F	1000
27	398/97	20	TB	F	1000
28	423/97	20	Pony	G	600
29	388/97	25	TB	G	1000

Table 7.1 Signalment of Horses used in the Fractal Dimension Study

MEAN	STDEV	COEF VAR	TIMES DONE
1.13667	0.005774	0.50793	3
1.29667	0.024495	1.88907	9
1.263	0.018886	1.4953	10
1.20444	0.019437	1.61373	9
1.24667	0.014142	1.1344	9
1.2325	0.012817	1.03995	8
1.17111	0.026194	2.23666	9
1.22667	0.024495	1.99687	9
1.2325	0.020529	1.66562	8
1.24333	0.045	3.6193	9
1.33625	0.0563	4.21325	8
1.25286	0.030938	2.46937	7
1.33	0.04899	3.68344	7
1.27444	0.063268	4.96432	9
1.23143	0.052099	4.23076	7
1.22857	0.025448	2.07138	4
1.1025	0.009574	0.86841	4

Table 7.2 Results of the Validation Study for Fractal 1

MEAN	STDEV	COEF VAR	TIMES DONE
1.45333	0.056862	3.91255	3
1.69333	0.03	1.77165	9
1.659	0.062796	3.78517	10
1.57889	0.034075	2.15817	9
1.62667	0.023979	1.47413	9
1.615	0.027255	1.68764	8
1.53333	0.049749	3.24452	9
1.59222	0.031929	2.00529	9
1.62	0.035431	2.1871	8
1.62333	0.055227	3.40206	9
1.74	0.034226	1.96702	8
1.65714	0.0243	1.46636	7
1.72	0.056862	3.30595	7
1.63889	0.119629	7.2994	9
1.57	0.067082	4.27274	7
1.59857	0.050803	3.17803	4
1.355	0.023805	1.75681	4

Table 7.3 Results of the Validation Study for Fractal 2

	FRACTAL 1	FRACTAL 2
<5 Years	1.29 ± .13	1.65 ± .1
5-9 Years	1.18 ± .05	1.46 ± .06
10-14 Years	1.16 ± .05	1.47 ± .1
15-19 Years	1.18 ± .03	1.53 ± .08
20+ Years	1.24 ± .04	1.61 ± .08
TB	1.18 ± .04	1.5 ± .07
TBx	1.25 ± .15	1.58 ± .16
Pony	1.25 ± .06	1.63 ± .1
X	1.2 ± .05	1.55 ± .08
Geldings	1.21 ± .05	1.56 ± .09
Mares	1.19 ± .12	1.5 ± .16
< 600 lbs	1.26 ± .05	1.67 ± .04
700 lbs	1.17 ± .08	1.48 ± .15
800 lbs	1.23 ± .16	1.53 ± .18
1000 lbs	1.19 ± .03	1.5 ± .06
>1000 lbs	1.15 ± .08	1.45 ± .12
RadPath	1.19 ± .02	1.54 ± .07
No RadPath	1.22 ± .1	1.55 ± .12
ArtPath	1.17 ± .04	1.48 ± .08
No ArtPath	1.22 ± .09	1.56 ± .11

Table 7.4 Results of Fractal Study by Parameter

Comparisons

Fractal 1

Age (Graphs A4.3)

Age vs. Breed

Age had a significant effect on Fractal 1 (P = .03), with the youngest horses exhibiting the highest fractal dimension (1.29 $\pm$ .13). Breed had no effect (P = .164).

Age vs. Gender

Age had a significant effect on Fractal 1 (P = .04), but Gender and the interaction between them did not (P = .82 and .58).

Age vs. Weight

Neither age nor weight had a significant effect on Fractal 1 (P = .057 and .31).

Age vs. Presence of Radiographical Lesions

Age had a significant effect (P = .01), but the presence of radiographical pathology showed a trend towards significance (P = .08). Age group 1 had a significantly higher fractal than only Age group 2 and no others.

Age vs. Presence of Gross Articular Pathology

Age showed a trend towards significance (P = .09) while the presence of gross articular pathology had no significant effect on Fractal 1 (P = .83).

Breed **(Graphs A4.4)**

Breed vs. Gender

Neither breed, gender nor the interaction between them had any significant effect on Fractal 1 (P = .138, .918 and .14).

Breed vs. Weight

Neither breed nor weight had a significant effect on Fractal 1 (P = .44 and .46).

Breed vs. Presence of Radiographical Lesions

Neither breed nor evidence of radiographical pathology had an effect on Fractal 1 (P = .29 and .39).

Breed vs. Presence of Gross Articular Pathology

Neither breed nor the presence of gross articular pathology had a significant effect on Fractal 1 (P = .38 and .32).

Gender **(Graphs A4.5)**

Gender vs. Weight

Neither gender, weight nor the interaction between the two had a significant effect on Fractal 1 (P = .8, .47 and .51).

Gender vs. Presence of Radiographical Lesions

Neither gender, presence of radiographical pathology or the interaction between the two had a significant effect on Fractal 1 (P = .61, .29 and .97).

Gender vs. Presence of Gross Articular Pathology

Neither gender, the presence of gross articular pathology nor the interaction between the two resulted in a significant effect on Fractal 1 (P = .74, .17 and .92).

Pathology

Presence of Radiographical Lesions vs. Presence of Gross Articular Pathology

Neither the presence of radiographical nor gross pathology or the interaction between the two had a significant effect on Fractal 1 (P = .82, .33 and .3).

Weight **(Graphs A4.6)**

There were no effects for weight.

Fractal 2

Age **(Graphs A4.7)**

Age vs. Breed

Both age and breed exhibited a significant effect on Fractal 2 (P = .001 and .036 respectively). Age group 1 had a significantly higher Fractal 2 than groups 4, 3 and 2, but not from Age group 5. Age group 5 is also different from 2, but not from the others. For breed, 3 was significantly higher than 4, but no different from the others.

Age vs. Gender

Age had a very significant effect on Fractal 2 (P = .007), while gender and the interaction between the two did not (P = .53 and .87). Both Age groups 1 and 5 were significantly higher in Fractal 2 than group 2. Age group 1 was also different from 4.

Age vs. Weight

Age again showed a significant effect (P = .02), but weight did not (P = .12). This time Age groups 1 and 5 were only significantly different from 2.

Age vs. Presence of Radiographical Lesion

Age had a very significant effect on Fractal 2 (P = .001), whereas the presence of radiographical pathology did not (P = .14). Similar to the Age vs. Gender crossing, both Age groups 1 and 5 were significantly different from 2. Age group 1 was also different from 4.

Age vs. Presence of Gross Articular Pathology

Age had a very significant effect (P = .006), but the presence of gross articular pathology did not (P = .57). Age group 1 was only different from 2, but not from the others.

Breed **(Graphs A4.8)**

Breed vs. Gender

Neither breed, gender nor the interaction between the two had a significant effect on Fractal 2 (P = .13, .45 and .61).

Breed vs. Weight

Neither breed nor weight had an effect on Fractal 2 (P = .79 and .17).

Breed vs. Presence of Radiographical Lesions

Neither breed nor the presence of radiographical pathology had a significant effect on Fractal 2 (P = .11 and .73).

Breed vs. Presence of Gross Articular Pathology

Neither breed nor the presence of gross articular pathology had any effect on Fractal 2 (P = .2 and .26).

Gender **(Graphs A4.9)**

Gender vs. Weight

Neither gender nor the interaction between them had a significant effect (P = .4 and .51), however, weight was marginally significant (P = .051).

Weight **(Graphs A4.10)**

Both Age groups 1 and 5 were significantly different from 2. Age group 1 was also different from 4.

Weight vs. Presence of Radiographical Lesions

Weight exhibited a significant effect on Fractal 2 (P = .01), whereas the presence of radiographical pathology did not (P = .44). Weight group 1 had a significantly higher Fractal 2 than both 5 and 4.

Weight vs Presence of Gross Articular Pathology

Weight was significant (P = .048), but the presence of gross articular pathology was not (P = .48).

Pathology

Presence of Radiographical Lesions vs. Presence of Gross Articular Pathology

Neither the presence of radiographical or gross pathology or the interaction between the two had a significant effect on Fractal 2 (P = .43, .29 and .14).

Discussion

Classically, bone sections are examined by histomorphometric methods (**Parfitt *et al* 1987**). These methods rely on basic techniques and formulae from Euclidean geometry to analyse the complex nature of the 3 dimensional structure of trabecular bone. Euclidean geometry, of course, is based on the premise that the objects being measured conform to regular shapes such as circles and squares. In contrast, fractal geometry is a non-integer-dimensional system capable of describing irregular borders more accurately (**Cross 1997**).

It should be noted that the term *fractal* takes on two distinct meanings in the medical literature. First and foremost is the theoretical definition of a fractal. This has been proposed by several mathematicians, most notably **Mandelbrot (1983)**. Simply put, the theoretical fractal involves the concept of 'self-similarity' over different scales of magnification. The classic example of a theoretical fractal is the **Sierpinski (1916)** gasket (**Fig 7.2**). In this example, a repeating pattern is easily distinguished as one gets closer and closer to the subject. Another classic and oft-cited example is the 'Coastline of Great Britain'. Although more practical in its application, this mathematician's dream still remains too esoteric for many to accept. **Richardson's (1951)** initial observation was that the coastline appeared to grow longer as it was viewed at a smaller and more magnified scale. Thus, measuring the length of the coastline as it is viewed from an aeroplane would result in a shorter distance than if the same border were measured while walking along the shore. Richardson concluded that as the measuring device became infinitely smaller, the distance measured should become infinitely larger. He also found that a graph of the log of the distance measured versus the log of the measuring device resulted in a straight-line relationship. Hence, these log plots were later renamed by **Mandelbrot to 'Richardson Plots' (1983).**

When applying the technique of fractal geometry to the study of bone, it should be noted that the context relates specifically to the nature of the trabecular structure of the cancellous bone as it is viewed or analysed across different scales. This is

somewhat different to the concept of self-similarity or infinite replication across different scales. It is this misunderstanding that has lead to the rejection of the concept that trabecular bone may possess fractal properties **(Cross *et al* 1993, Chung *et al* 1994).**

The human literature has several references over the last 10 years that have reported the possibility that trabecular bone behaves in a fractal manner. As a result of the diverse situations in which it has been utilised, differing opinions as to the feasibility and applicability of fractal geometry have emerged. In one of the first studies of its kind, **Majumdar *et al* (1993)** applied fractal geometry to the study of bone. Initially, they used digitised photomicrographs of transiliac crest biopsies and then used quantitative computed tomography (QCT) of dried excised human vertebral bodies to test whether the bone behaves as a fractal object. They found that the fractal dimension changed with the trabecular bone content and therefore suggested that fractal analysis may be useful in distinguishing osteoporotic bone structure from normal.

Several subsequent studies have concluded that bone does not possess the characteristics required to call it fractal. Two of these studies, however, rely on digital imaging techniques for their material. **Chen *et al* (1994)** concluded that their model for analysing the fractal dimension of trabecular bone was inadequate because system noise significantly affected the signal and therefore, the calculated fractal dimension of the bone. Furthermore, they stated that before conventional radiographs are used for fractal analysis in the clinical environment, many of the technical problems associated with this methodology must be addressed. **Chung *et al.*** investigated the same concept in a similar way, using nuclear magnetic resonance (NMR) images instead. Although they employed a high-resolution system with pixel sizes on the order of 50μm and low signal to noise ratios, they also concluded that the fractal dimension of the trabecular structure is undefined using NMR and can vary significantly as a function of image signal-to-noise ratio. Advances in the imaging fields, even in the 5 years since these studies were

published, have significantly improved the resolution acquired. Higher resolution images may have generated a different outcome for these studies.

The primary objective of this investigation was to determine whether equine trabecular bone of the distal metacarpus behaves as a fractal object utilising histological sections. For the first aspect of the study, we chose normal joints from 6 different horses for analysis. A box counting algorithm, purpose-written for NIH_Image, was used to 'examine' the bone at different magnifications. A box was placed at the junction of bone tissue and marrow space as a method of outlining the trabecular architecture. By increasing the size of the box by one pixel each time, the different scales were simulated. At each box size, beginning with a 6 pixel by 6 pixel square and extending to a 24 X 24, the number of boxes needed to fill the entire bone was counted. The macro then graphed the log of box size by the number of boxes needed to fill the entire bone area. By minimising the squared deviations, we found that this graph produced an acceptable straight line through these points. By definition then, the bone in our specimens can be said to behave as a fractal object. More importantly, by applying the second least squares macro, we were able to determine that the data points are more accurately described by two straight line segments than one, thus implying that the bone more accurately behaves as a fractal across two different scales. The use of the two straight-line segments provides even more evidence that the bone does possess fractal qualities because each becomes a better fit for the data points.

Cross *et al* (1993) carried out a light microscopic study of the fractal nature of a histological specimen of human bone. They concluded that the bone did not possess fractal characteristics because a straight-line relationship did not exist for their data. We would contend, however, that their conclusions might have been too rigorous in that they attempted to fit just a single straight line to their points, rather than explore the possibility of two straight-line segments. Careful analysis of their data points indicates that just such a relationship may exist. Based on our

results with two straight-line segments, it is premature to dismiss the possibility of fractal behaviour without exploring more than one straight-line segment.

The validation procedure in our study involved exposing a new surface of bone at least 100μm more axial than the original section and repeating the entire process of staining, digitising, image processing and determining the fractal dimension. The results here were surprisingly similar, leading us to two conclusions. First and foremost, the repeatability of the fractal calculation suggests more emphatically that a fractal dimension does actually exist for our bone specimens. Secondly, however, it demonstrates that the precise location of the section does not appear to be important to the value for the fractal dimension. Although we carried out the entire investigation utilising rigorous sampling methods to ensure a valid comparison between horses, the above finding indicates that $\pm$ a few hundred microns from the intended sampling region should not affect the calculated fractal dimension.

In the second part of our study, Fractal 1 and 2 were compared independently. More significant results appeared for Fractal 2.

Fractal 1

Age was a significant variable when Fractal 1 was analysed in relation to gender and the presence of radiographical lesions. In the latter comparison, only Age Group 1 was different from 2. No other significant differences were noted when analysing Fractal 1 for the effects of age, breed, gender, weight, or the presence of radiographical and gross pathology.

Fractal 2

Both age and breed had a significant effect on Fractal 2. The youngest horses in the study had the highest Fractal values, which were significantly different from those horses in Age Groups 2, 3 and 4. The horses in group 5, the oldest ones, exhibited a value for Fractal 2 almost as high as that for Age Group 1, but it was only significantly different from Age Group 2. When analysing for Breed effect, it was noted that Group 3, the ponies, had a significantly higher Fractal value than for that of the cross-breds or TBs.

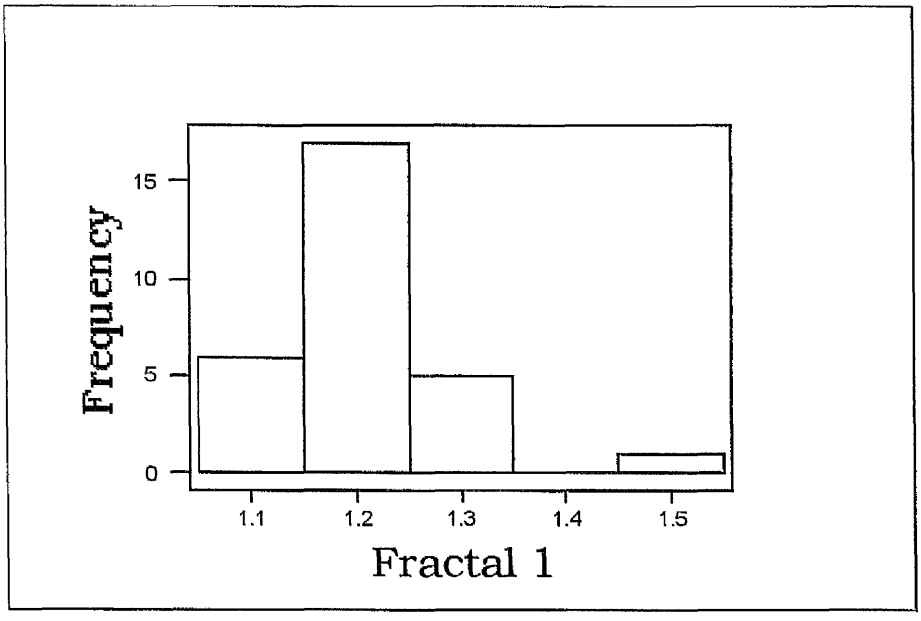
While it is significant to conclude that the bone samples in our study behave as fractals, interpreting the rest of the data may be more difficult. Primarily, it should be reiterated that the definition of fractal that is subscribed to in this study is not the theoretical one of self-similarity across infinite scales. Rather, it is that a Power Law exists for the bone across different scales. This finding implies that some characteristic of the trabecular bone is related when the bone is analysed at different magnifications. **Fazzalari and Parkinson (1995 and 1997)** have authored two of the most accurate and perceptive publications on the relevance of fractals to the study of trabecular bone. In their original report, they found that the bone from femoral heads did behave as a fractal object. Moreover, they found that the fractal dimension was 1.19, a similar result to many of the horses in our study. It should be noted, however, that they only attempted to define a single fractal dimension for their specimens. In the more recent study, **Fazzalari and Parkinson (1997)** compared the calculated fractal dimension for control bone with that of bone from patients with OA. In that study, they defined multiple fractal dimensions and were able to assign clinical significance to the 'pivot points' or points between straight-line segments. In their study, these points related to the histomorphometric data, most closely with the trabecular thickness and separation. In analysing their data carefully, it seems feasible that two scales of significance are present for their specimens. However, the smallest scale they used resulted in a fractal dimension of 1.06, very similar to the topological

dimension of a simple line. It seems likely that this conclusion may represent an over-interpretation of their data set. We propose that a fractal dimension calculated at such a small scale, i.e. less than 6 pixels as a starting point for our data, will actually be testing the resolution at which the image is digitised instead of the structure of the specimen itself.

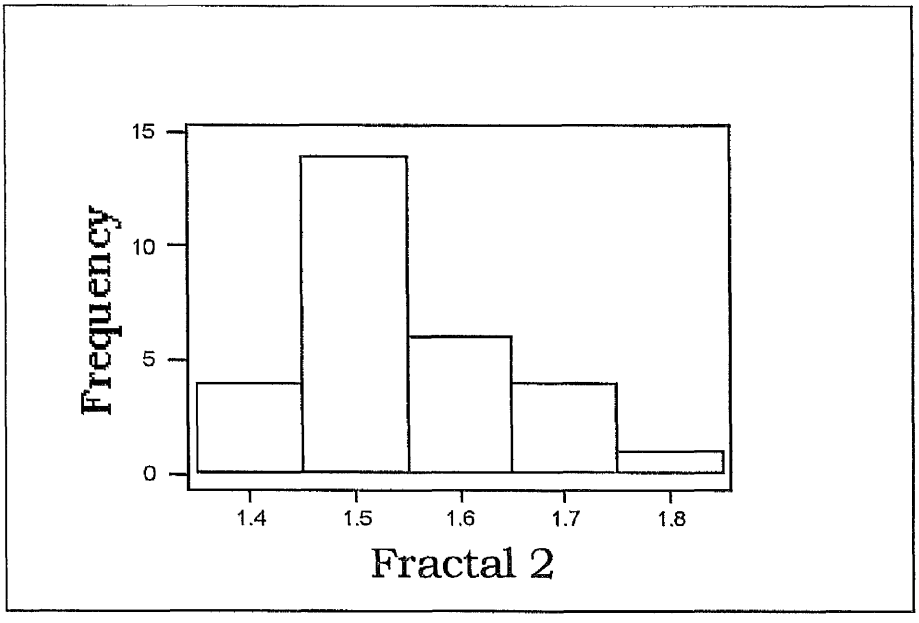
In our data, the fractals were calculated over a scale from 6 pixels to 24 pixels. The digitising scale we employed for this study was 800 pixels per inch. This equates to .031 pixels per micron or each pixel equal to 32μm. Therefore, the scale over which the bones were analysed was 192 to 768μm with the pivot point at roughly 350-380μm. **Fazzalari and Parkinson (1997)** were able to show that these values corresponded to those from their classic bone histomorphometry data for trabecular thickness (Tb.Th) and trabecular separation (Tb.S). For our data, we were not able to establish such a correlation. The trabecular measurements in our study (**Chapter 4- Table 4.3**) were almost twice that thick. This is most likely due to that fact that the fractal dimension was taken across the entire distal end of the bone, whereas the bone histomorphometry concentrated almost exclusively on the bone just proximal to the articular surface. In this region, the bone appeared to be denser than in the centre of the epiphysis.

Future studies on the usefulness of fractals should revolve around imaging modalities. Although two studies have already questioned the existence of a fractal dimension when applying such techniques, the resolution of the system appears to be the limiting factor **(Chung *et al* 1994, Chen *et al* 1994)**. As imaging modalities continue to evolve and refine the resolution of their system, then the application of fractal dimensions may become less esoteric.

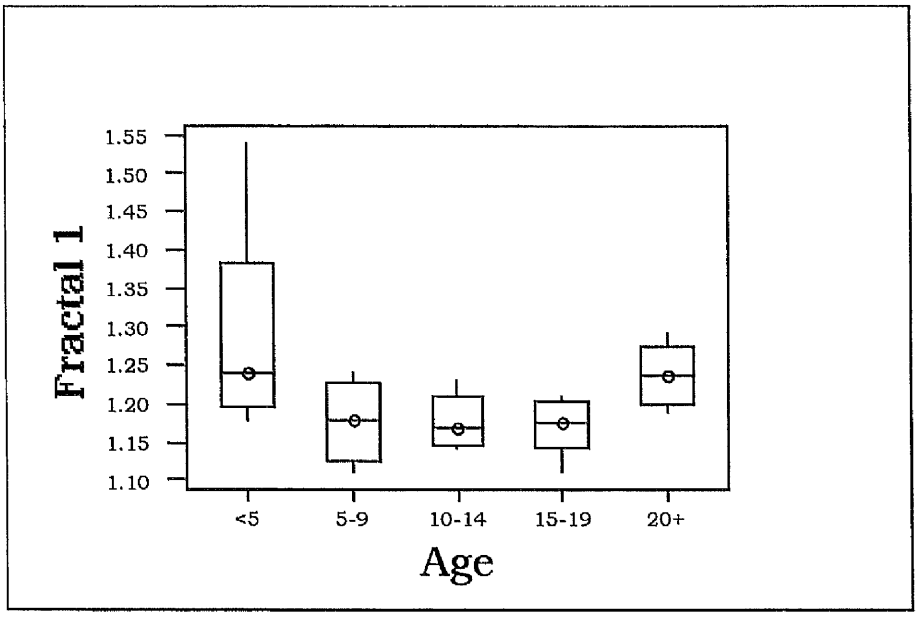
APPENDIX 4



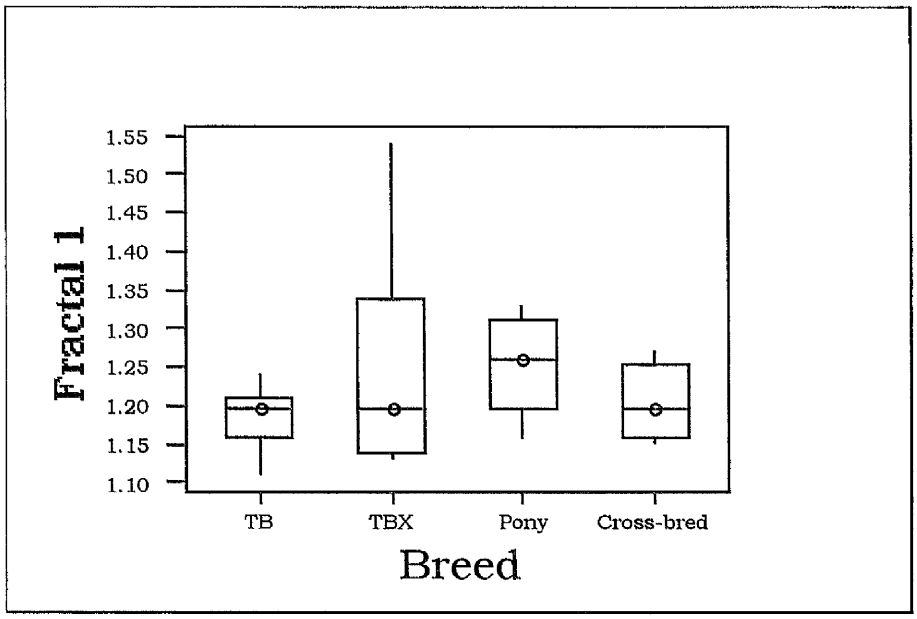
Graph A4.1 Histogram of the values for Fractal 1



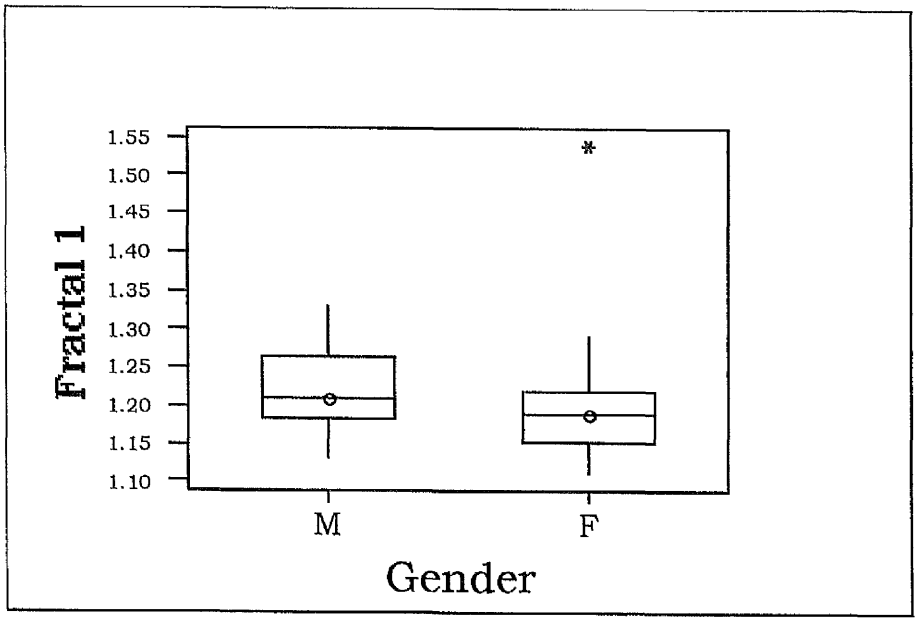
Graph A4.2 Histogram of the values for Fractal 2



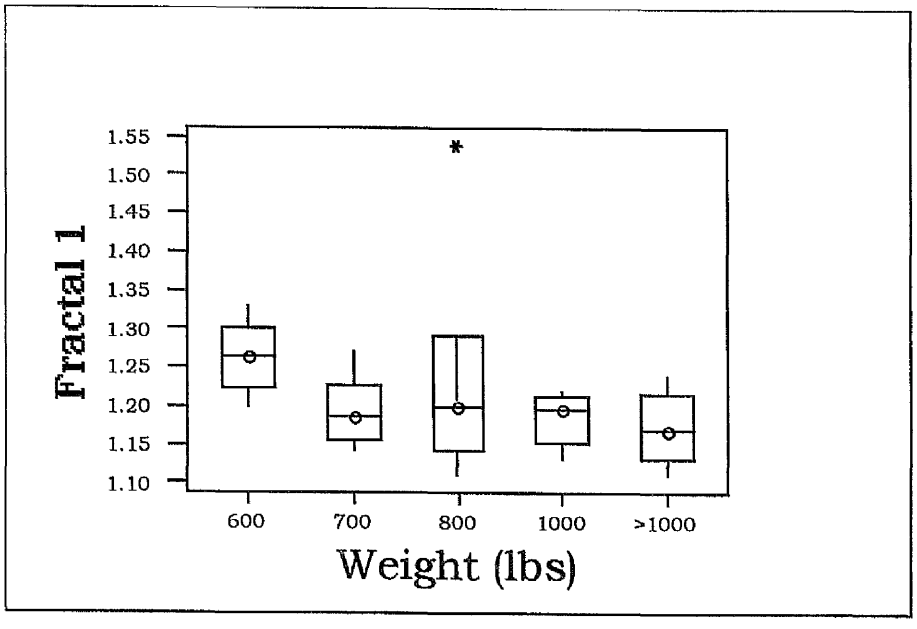
Graph A4.3 Boxplot of Fractal 1 by Age



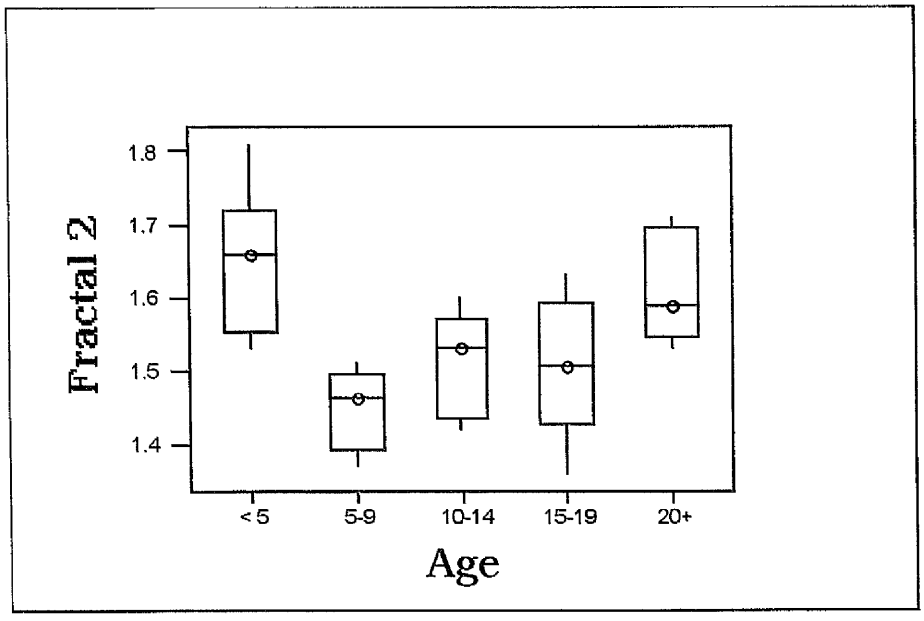
Graph A4.4 Boxplot of Fractal 1 by Breed



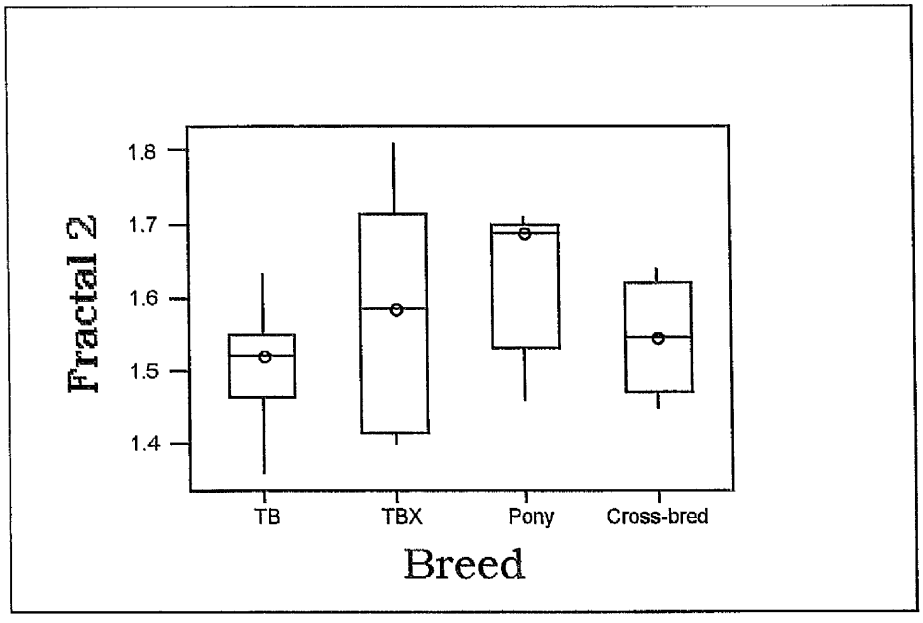
Graph A4.5 Boxplot of Fractal 1 by Gender



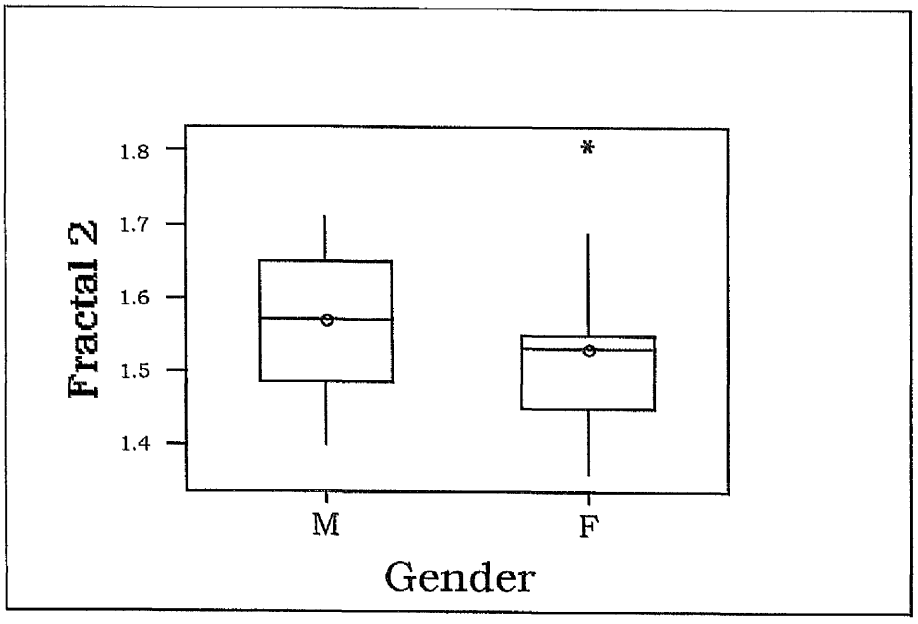
Graph A4.6 Boxplot of Fractal 1 by Weight



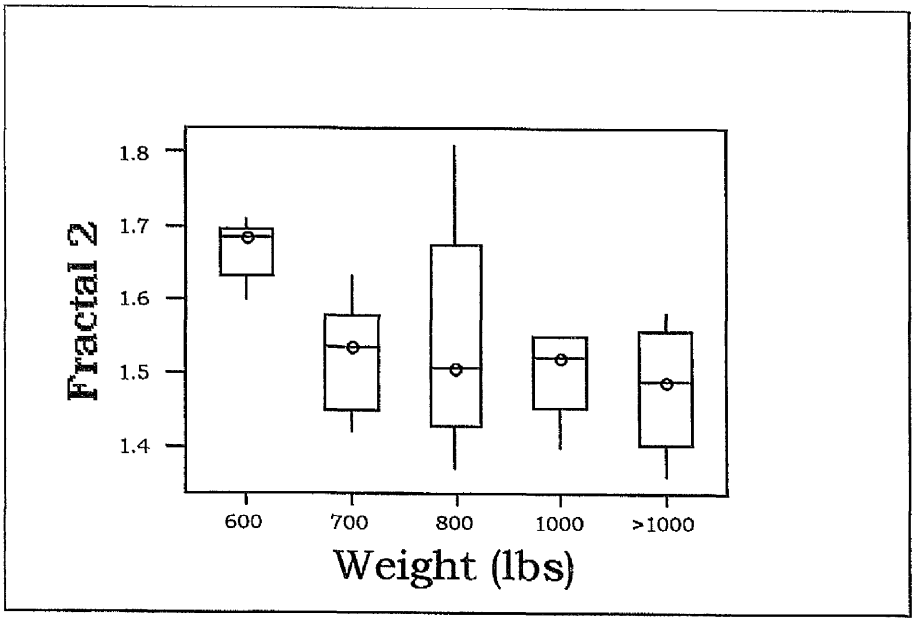
Graph A4.7 Boxplot of Fractal 2 by Age



Graph A4.8 Boxplot of Fractal 2 by Breed



Graph A4.9 Boxplot of Fractal 2 by Gender



Graph A4.10 Boxplot of Fractal 2 by Weight

CHAPTER 8

CONCLUSIONS

This series of studies was conducted on the articular and subarticular tissues of the distal metacarpus of the horse in an effort to learn more about their role in both health and disease of the joint. Many interesting relationships became evident during the complex data analysis. Because it was a *post-mortem* study, only certain parameters could be analysed. These included age, breed, gender and weight.

In deciding what tissues to investigate, we chose both the superficial as well as the deeper structures of the joint. The noncalcified cartilage (NC) and calcified cartilage (CC) constitute the most superficial layer in the articular environment. Although a vast amount of equine musculoskeletal research is aimed at the hyaline cartilage in general, the CC gets little mention independently. Therefore, we specifically chose to isolate this layer from its overbearing partner, the NC. The use of the toluidine blue stain facilitated this distinction because the stain binds to the proteins and lipids of the TM, producing very nice contrast between it and the structures both superficial and deep to it **(Oegema *et al* 1997)**. We chose to conduct most of the investigations in a quantitative manner. In this light, the CC layer presented the biggest challenge initially, simply because its irregular nature did not allow for simple point to point measurements of accurate thickness. Instead, we adopted a mathematical solution to the problem, modelled after that which **Muller-Gerbl (1987a)** had developed. It involved measuring the area of the CC over a known distance to calculate the mean width. Based on the theory of integration, this method provided a reasonable estimate of the thickness. Consequently, when attempting to measure the SCB plate, some of the same logistical problems arose. Therefore, a similar technique was developed to estimate the SCB plate thickness over a distance. The biggest obstacle to measuring the SCB plate was determining what constituted its deepest border in each section. Textbooks tend to avoid a formal definition of this region. However, in viewing slides of the subchondral region of humans, rats and rabbits, it is quite clear that the SCB plate in horses is significantly different. Rarely did it have an easily identifiable proximal margin. In several cases, though, it looked similar to the other species mentioned. In these uncommon cases from our study,

obtaining data was uncomplicated. Because easily identifiable SCB plates were rarely found in this study, we developed the second method of integration for determining the average thickness over the section. We defined the SCB plate as beginning at the tidemark (TM) of the HC layer. This was readily visible and was previously cited in the literature **(Burr and Schaffler 1997).** No obvious landmark flagged its proximal extent, so we defined it as ending at the encounter with the first marrow space when proceeding proximal from the TM. Furthermore, though, because these encounters could even be sporadic at times, it was decided to repeat the measurement three times, moving the area of measure slightly to the right each time so a representative bone outline was covered for each section. The technique worked well, producing repeatable measurements each time **(Fig 6.2, Tables 6.1 and 6.2)**. Finally, we chose to measure more traditional parameters of bone resulting in values for B.Ar. and Tb.Wi. These two parameters provide information about the composition and structure of the bone using well-established methods of stereology **(Parfitt *et al* 1987, Russ 1995)**

As for regions from where the measurements were obtained, we chose landmarks on the bone that were not only identifiable , such as the CC and transverse ridge, but were also the site of reported lesions. Traumatic osteochondrosis is one of the most cited disease processes in the fetlock joint, occurring just palmar to the transverse ridge **(Pool and Meagher 1990, Pool 1996, Norrdin *et al* 1998, Riggs *et al* 1999)**. Quite obviously, the cartilage measurements were the most superficial ones taken on the section. The subchondral bone measurements also tended to be rather superficial in its extent. The bone information was collected from the same similar region, but it extended further into the trabecular architecture than the previous mentioned measurements. This detail may lead to some important conclusions in regard to this data set. Finally, the fractal work relied quite extensively on the deeper trabecular architecture to produce its analysis. This, too, could have significant bearing on the numbers generated in this study.

When examining all parameters in the study, it became evident that different relationships and significances surfaced. Overall, the two most noteworthy observations dealt with age and section. The young horses consistently exhibited different parameters than the older horses. Morphologically, this becomes even more important because of the defined age subsets. The 'young' horses were grouped together out of statistical necessity into an 'under 5 years' category. While that group was able to show some significant differences from the rest of the horses, subjectively comparing the two horses under the age of 2 years with those between 2 and 5 years also seemed to indicate striking differences. The intent of this dissertation was initially to include some material from younger horses and even foetal tissue if possible. In this way, a more emphatic statement could be made about genetics and congenital development versus response to external stimuli such as nutrition or exercise. Unfortunately, the foetal and young foal specimens did not become available during this study. Again, though, because significant differences showed up in the young group under the current sampling constraints, these differences must be present.

When generalising across all studies included here, the young horses definitely showed differences in the development of the musculoskeletal system. In general, the CC was thinner while the NC was thicker than in other age groups. As a result, the CC/NC ratio was noticeably lower, although this finding was more evident in some sections than others. The young horses had a thinner SCB plate and a higher P_L, indicative of greater surface area of trabeculae exposed because the trabeculae were thinner **(Fig 4.8 and 4.9)**. Because no horses under the age of 18 months were included in these studies, it is difficult to say exactly why these observations are present. The most likely explanation, of course, centres around growth and immaturity. The thinner CC and SCB plates will more readily allow for the expansion of epiphyseal growth. Alternatively, these significant differences could be due to lack of stimuli needed to induce the development of the bone and cartilage in a 'Wolffian' manner. Although the foal is ambulatory immediately after birth, it may require more formal exercise and training to development these tissues. While a study of foetal and young horse specimens

should provide the answers to these questions, the former hypothesis seems more likely, i.e. growth and proper maturity are important factors in the articular tissues of younger animals.

If, indeed, significant growth must continue to take place in the cartilage and bone of young horses for some specific period of time, this might have important implications for the equine industry. Consider, for instance, the CC layer in Section 12. Between the age groups of less than 5 and the 5-10 year olds, the CC gains almost 100μm in thickness, or approximately one third of its ultimate thickness for that section. If it becomes evident through this study and subsequent ones that the CC layer is important in the overall health of the joint, then many training practices may need to be re-examined. It might become important to limit the articular stress until this zone of cartilage matures. **Nunamaker *et al* (1987)** has had a massive impact on the racing industry with studies of cortical bone remodelling and subsequent training of the bone to race conditions. The conclusions of his orthopaedic research group were that bone needed to be trained for a racing environment and not a training environment. In the situation defined here in regard to the joint, it may be that early training practices encroach on the physiological immaturity of the articular structures. Some horses and joints may respond to such stimuli by laying down more bone in the subchondral region in addition to thickening the CC layer. Indeed, this very situation has been shown experimentally. **Oettmeir *et al* 1992** found that trained beagles developed a thicker CC, NC and SCB in response to treadmill exercise. **Dedrick *et al* (1993)** determined that the SCB thickened while the deeper trabecular bone decreased in an ACL resection model. Conversely, there will be some of these animals that cannot withstand the articular stress and they may develop lameness, with or without obvious diagnostic signs. As mentioned in Chapter 2, the absence of radiographic signs should be considered favourable, yet not completely liberating, with reference to impending joint dysfunction. Inflammation and 'bone pain' may develop in the immature articular tissues necessitating rest and healing. Further training on a compromised joint could lead to more serious derangements such as chip fractures, superficial hyaline cartilage damage, or, in the end stage, complete

cartilage and SCB plate collapse **(Pool 1996).** The economics of the racing industry indicates that the most prize money in U.S. racing is often available to the 2 and 3 year olds **(Martinelli *et al* 1996).** Therefore, it is not feasible that all young horses should necessarily be held out of training and competition. However, evidence of 'soreness' should be treated seriously by owners, trainers and veterinarians. Future research avenues could explore the non-invasive use of MRI to evaluate the health and maturity of the CC layer. It has already been shown in a cadaveric study that the CC layer can be visualised with an MR image **(Martinelli 1995)**. If such a technique were easily achievable as a diagnostic tool, it could be possible to evaluate the relative maturity of this layer in young horses. Sound advice could be given in regard to the proper age to enter training, the type and intensity of the training best suited to each individual animal and even when racing could commence. The development of non-invasive techniques for evaluating the joint could provide the useful information needed to pre-empt articular disease. Initially though, more needs to be investigated in regard to the CC layer, especially in young horses.

Another very interesting observation in regard to maturity revolved around the level of tissue at which ageing processes were manifest. Traversing from the joint surface inward, the tissues behaved in different ways in respect to function. The NC experienced a decrease in thickness with age, while the CC layer exhibited an opposite effect, or increase in thickness, as stated earlier. The SCB plate tended to follow the lead of the CC layer in becoming thicker with age. The number of intersections measured (P_L) were highest in the region closest to the CC layer in the young horses, again seeming to indicate a need for growth. The only tissue parameter not to experience any kind of age effect in our study was the B.Ar. As stated earlier, this parameter was measured to a much deeper level than all the other values, except the P_L. This observation may become important because it seems to present evidence that change mainly occurs superficially in the joint. **Burr and Schaffler (1997)** make a similar observation when they note that only the SCB plate to a level of 3mm is significant in the development of OA. Furthermore, **Oettmeier *et al* (1992)** noted an overall decrease in the bone mass

of beagles in an ACL resection model, but an increase in SCB plate thickness as a result. These changes were thought to lead to OA. Therefore, the results of the studies within this dissertation seem to concur with the concept that a more 'superficial maturation' is the key to articular competence.

Perhaps the next most important discovery was the locational bias recognised at Section 12. This is the region of sesamoidean articulation **(Vilar *et al* 1995).** Again, the most notable changes were observed most superficially. The NC layer decreased significantly with age in Section 12, while the CC layer increased in thickness with age. These two conditions led to a resultant significant increase in the CC/TC ratio. The SCB plate thickened significantly with age and somewhat with weight. The sesamoidean articulation has been postulated to be under substantial stress **(Vilar *et al* 1995, Pool 1996).** If the changes noted in Section 12 are stress related, then it would appear that the calcified tissues, the CC and SCB, develop in response to the increased loads applied. The NC, on the other hand, seems to decrease in thickness as its response to articular loading. This finding is in direct opposition to the findings in the human and animal model literature. Several studies report an increase in both CC and NC in response to exercise or in areas under greater stress **(Muller-Gerbl 1989a, Flygare *et al* 1993 and Oettmeir *et al* 1992).** This observation would seem to imply that the horse tissues behaved differently *in vivo* compared to human and canine.

Several other observations should be discussed. Trabecular microfracture is a term frequently mentioned in the human literature **(Todd *et al* 1972, Radin and Rose 1986)** and in the equine literature **(Norrdin *et al* 1998).** It represents one of the hypotheses regarding how bone in the subarticular environment remodels in response to stress. Special equipment and stains are required to see evidence of this microdamage. While we did not specifically look for any microfractures because of the limitations of our equipment and sections, there was no evidence of microdamage such as callous formation. Conversely, **Pool (1996)** hypothesises that bone deposition is appositional in the subchondral region of the palmar

metacarpal bone and due to microbending of the trabecular struts. Bending stress will alter the charge of the calcium hydroxyappatite crystals leading to a concentration of positive ions along the concave aspect of the bone surface. This, in turn, will attract the osteoblasts to this surface, allowing them to lay down precursors of new mineral materials in the form of osteoid. At the same time, the negative charge on the opposite or convex side, will attract the osteoclasts resulting in production of acid phosphatase and bone resorption. In the case of athletic training, more bone is laid down than that that is resorbed. This, in turn, will lead to a generalised increase in bone mass. While this process seems imperative to withstanding the athletic stresses enumerated above, there is evidence that overproduction of bone may be detrimental as well. One such theory is that the osteocytes of the subchondral regions must rely on either intra-marrow blood vessels or diffusion of nutrients from the synovial fluid for nourishment. As the subchondral bone becomes more dense with remodelling, the marrow spaces become filled in, obliterating the blood vessels and preventing that route of nutrition **(Norrdin *et al* 1998).** In an interesting contrast to our studies here, **Young *et al* (1987)** recognised changes in bone porosity and structural anisotropy with increased athletic training. They report on the favourable effects such reinforcement of the trabecular bone will have on the prevention of fractures. However, the converse must be considered as well, i.e. the development of an abnormal loading environment due to a stiffened SCB plate **(Radin and Rose 1986, Brown *et al* 1987, Burr and Schaffler 1997).** Therefore, it would seem reasonable to hypothesise that a very delicate balance must be achieved between training to produce enough bone mass to withstand fracture without causing stiffening of the SCB plate. Similar to recommendations of **Nunamaker *et al* 1987** in regard to training cortical bone in Thoroughbreds for the stress of racing, it would appear to be beneficial to establish a preferred technique for training the SCB. Histologic studies such as this one and that of **Norrdin *et al* (1998)** should provide the basis for such extrapolations to the clinical realm.

Finally, a few more observations about the concept of fractal dimensions. Although a seemingly complex and esoteric mathematical abstraction, fractal geometry is based on concrete observations of object structure. It is the interpretations of these results that present the challenge. We have identified two straight line segments in our data based on the application of a least squares algorithm **(Fig 7.10).** The first fractal corresponds to a magnification scale of 192 to 360μm. That means the bone possesses some recognisable property within this magnification scale. The second fractal is graphed over a magnification of 360 to 768μm. Because Fractal 2 is different, these results imply that another characteristic of the bone is responsible for the fractal dimension at this magnification. **Fazzalari and Parkinson (1997)** were able to show that the fractal dimension of human iliac crest biopsies correlates well with the bone histomorphometric data from the same section. Although we cannot make the same assertion based on our data, it does not invalidate the results.

In our study, it is quite clear that young horses possess a higher fractal dimension across both scales. Similar to the results of the cartilage and SCB experiments then, it would appear that some property of the bone is different in the young horses of this study. When examining all the possibilities, it would appear that P_L may be most likely to be responsible for this difference. The cartilage and SCB were at the borders of what was being measured by the fractal dimension and the B.Ar was not significantly different for the horses in this study. The P_L was significantly higher in the young horses, especially close to the SCB plate. As a measure of surface area, this indicated a more open trabecular pattern. Evaluations of the fractal dimensions individually also revealed that a less dense trabecular pattern resulted in a higher value. Subsequent work will have to be done on this concept before useful information can be gleaned. More importantly, some method of determining the fractal dimension of bone non-invasively could render this technique clinically useful. Until then, it remains an interesting mathematical abstraction.

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