Avian articular cartilage
- effects of age, genotype and disease.

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Abstract of thesis

The degradation of articular cartilage, causing degenerative joint disease (DJD), is a documented cause of lameness in broiler strain fowl, which is a major welfare problem. Broiler strain fowl are both heavier and more susceptible to DJD than laying strain fowl. In this thesis the biochemical and morphological basis for this susceptibility has been investigated, particular attention has been paid to the effects of body weight and genotype on avian articular cartilage.

Articular cartilage from three distinct sites was analysed. Samples from mature broiler strain females, susceptible to DJD, had higher hydration and uronic acid content than age matched, non-susceptible, laying strain fowl. In addition to these biochemical features (also seen in mammalian DJD), broiler strain fowl exhibited DJD histopathology including cartilage thinning and chondrocyte cluster formation. In general, both strains at one day old showed no significant biochemical differences between the articular cartilage sites sampled. However the cartilage from the broiler strain distal tibiotarsus (DTT) at one day old appeared to be biochemically and morphologically distinct from the cartilage of the layer strain DTT and from the other broiler strain joint surface of the same age.

Production of an infectious arthritis by inoculation of mycoplasma into the tibiotarsal joint of broiler strain fowl resulted in variable biochemistry of articular cartilage form this joint. However in the non-injected, contralateral joint, sampled from the DTT, there was an increase in hydration and uronic acid content which is dependent upon the degree of lameness. This indicates the importance of *in vivo* loading in the biochemical composition of avian articular cartilage.

Broiler strain birds fed *ad libitum,* feed restricted and J-line (wild type) were surveyed over the course of one year. Only the *ad libitum* fed birds developed overt DJD, which suggests that the mass of the bird, and not an overriding genetic element, is the major cause of the susceptibility of broiler strain fowl to DJD. The joint surface which presented the first and most severe signs indicative of early cartilage degeneration was the DTT. The biochemical results obtained from articular cartilage samples of the three groups include hydration, uronic acid, DNA and hydroxyproline content. Histology of the samples was assessed using haematoxylin and eosin stained sections. Proteoglycan content was investigated further by using selected samples for sulphated glycosaminoglycan assays and staining selected sections with Toludine blue and Safranin O. Samples from diseased and non-diseased groups were assayed for pyridinium in order to investigate the role of mature collagen crosslinking in DJD.

The exact role of load and the metabolic consequences of body weight in the development of DJD is controversial. A method for artificially loading non-obese birds has been developed. Loading feed restricted broiler strain fowl with an additional 10% of their body weight over a three week period initiated a response including significant biochemical changes in the cartilage of the DTT. These results again emphasise the importance of load on the articular cartilage and the susceptibility of the DTT to DJD.

Declaration

I declare that this thesis was composed by me, and the work of which it was a record was performed by me except where stated.

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Dedication

This work is dedicated as J.S. Bach dedicated his works "soli Deo gloria". Also in grateful thanks to my mother who taught me that if you can read, you can do anything, then taught me to read, and to Ian who has been everything which his name means to me.

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List of abbreviations

64 OP	90% alcohol w/w methylated spirit
74OP	99% alcohol w/w methylated spirit
AT	antitrochanter
BAPN	β-Aminoproprionitrile
BSA	bovine serum albumin
CNP 30	kem-sol from Kem-serv, Crammlington
COMP	cartilage oligomeric matrix protein
COOH	carboxyl
CS	chondroitin sulphate
DJD	degenerative joint disease
DMB	1,9-dimethylene blue
DNA	deoxyribonucleic acid
DPX	mountant for microscopy, Merck.
DTT	distal tibiotarsus
EDTA	ethylenediaminetetraacetic acid
GAG	glycosaminoglycan
gly	glycine
KS	keratan sulphate
MG	*Mycoplasma gallisepticum*
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MS	*Mycoplasma synoviae*
OA	osteoarthritis
PBS	phosphate buffered saline
PH	proximal humerus
PTM	proximal tarsometarsus
RA	rheumatoid arthritis
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacylamide gel electrophoresis
SEM	standard error of mean
SGAG	sulphated glycosaminoglycan
TBS	TRIS-buffered saline
TCA	tri-chloroacetic acid
TIMP	tissue inhibitor of metalloproteinase
TRIS	TRIZMA base (Tris[hydroxymethyl]aminomethane)
Tween	Polyoxyethylenesorbitan

1. Introduction and Background

1.1 Aim of the project

The aim of this project was to investigate the effects of age, genotype and disease on avian articular cartilage. The degradation of articular cartilage, causing degenerative joint disease (DJD), is a documented cause of lameness in broiler strain fowl (Duff, 1990) and is a major welfare problem. Broiler strain fowl are both heavier and more susceptible to DJD than laying strain fowl. In this thesis the biochemical and morphological basis for this susceptibility has been investigated, particular attention was paid to the effects of body weight and genotype on avian articular cartilage.

1.2 What is cartilage?

Cartilage is a connective tissue which is relatively resistant to compressive, tensile and shearing forces (reviewed by Stockwell, 1979). Cartilage is sufficiently rigid to provide support for structures such as the ear, respiratory system and parts of the rib cage where elastic deformability is required. However, cartilage is also deformable enough to distribute the load on bones in articulating joints.

Cartilage consists of a network of collagen fibres in which proteoglycan molecules and cells are enmeshed. The three types of cartilage in the body are distinguished by their fibre content. In hyaline cartilage, the proportions of proteoglycan and collagen in the tissue are such that the cartilage has a glossy appearance and no fibres can be seen macroscopically or with routine histological techniques. Fibrocartilage has a high collagen content and a low proteoglycan content. The fibres are visible to the

naked eye and accessible to routine histological stains. Elastic cartilage is characterised by the presence of elastic fibres, in addition to collagen and proteoglycan. A fibrous membrane, the perichondrium, surrounds all cartilaginous tissues, except articular cartilage.

During development much of the skeleton is initially cartilaginous. During growth chondrocyte proliferation and hypertrophy in the epiphyseal growth plates permits increase in the length of a bone without disturbing organisation of the joint (Archer, 1994) .

1.3 The function of articular cartilage

Articular cartilage, an example of hyaline cartilage, is a layer of white tissue which is found at the articulating surface of all diarthrodial joints. The tissue is aneural, avascular and alymphatic. Articular cartilage functions to (i) provide a covering material that protects the bone from abrasion and other damage (ii) transmit and distribute high compressive loads and shearing forces to the subchondral bone (iii) provide joint congruity and maintenance of low contact stress between opposing bones and (iv) provide a smooth lubricated surface that facilitates movement with little friction between articular surfaces (Poole, 1993). Compressive forces are transient within the joint cartilage, but can be very high. In humans the pressures rise from 1-2 atmospheres when unloaded (Grushko *et al.*, 1989) to 100 to 200 atmospheres on standing, and when walking, cycle between 40-50 atmospheres (Afoke *et al.*, 1987).

Articular cartilage consists of chondrocytes embedded in a large volume of extra-cellular matrix, which the chondrocytes produce and maintain. It contains collagen II (and some collagen XI) molecules within fibrils, which are networked together with collagen IX to provide a meshlike framework (Wu *et al.*, 1992). Collagen accounts for the major proportion (50-90%) of the dry weight of articular cartilage. Proteoglycan molecules with glycosaminoglycan side chains are found within the collagen network (Muir, 1995). Aggrecan, a large proteoglycan, which contains chondroitin sulphate and keratan sulphate, interacts with link protein and hyaluronan to form high molecular weight aggregates (Hardingham and Fosang, 1995). Non aggregating proteoglycans (which contain dermatan sulphate), collagen VI and other glycoproteins are also present in cartilage (Ayad *et al.*, 1994). The meshwork of articular cartilage is illustrated in Figure 1.1.

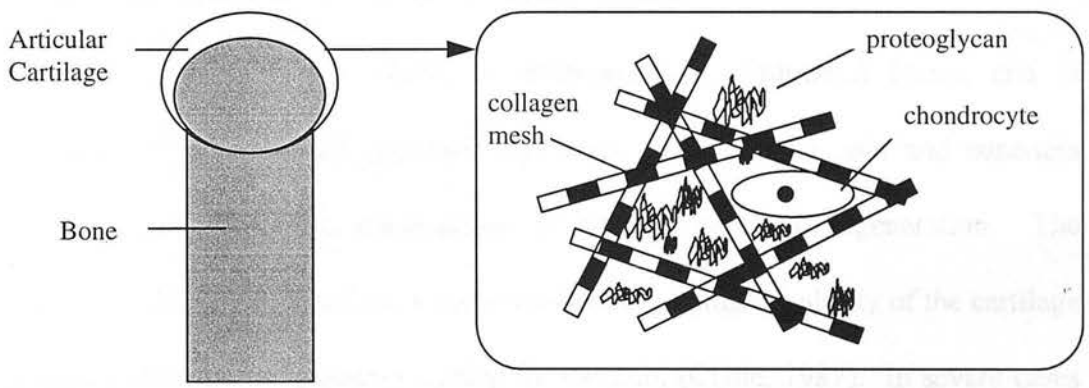


Figure 1.1 - Articular cartilage covers the end of long bones and consists of a meshwork of collagen II fibrils interspersed with proteoglycan molecules.

Articular cartilage is 70-80% water (Muir, 1995). The water content of the tissue is controlled by the collagen network and the proteoglycans. The negatively charged groups on the proteoglycans attract positive ions, the majority of which are sodium ions, and hence water by osmosis, thus

they provide a Donnan osmotic pressure. The collagen network resists the influx of water by physically constraining the proteoglycans, to about 20% of their volume in free solution (Muir, 1988). It is the water, ions and proteoglycans within the collagenous network which endow cartilage with its load bearing properties. When a joint is loaded, the water is physically forced out of the tissue until the osmotic force equals the physical force, at which point no more water can be expelled from the tissue. When the load is removed, the water will be attracted back into the tissue up to the point at which the osmotic force equals the compressive force of the collagen network, when the water content will again stabilise (Figure 1.2).

1.4 Degenerative Joint Disease

1.4.1 General features of degenerative joint disease

Degenerative joint disease (DJD) is widespread in diathrodial joints, and is particularly common in all mammals and avian species. The pain and lameness associated with DJD is a consequence of articular cartilage degeneration. The integrity of the cartilage surface is compromised, impairing the ability of the cartilage to provide a smooth articulating surface for the joint (Clyne, 1987). In severe cases the subchondral bone is exposed. The pathology of DJD includes areas of cartilage thinning, surface fibrillation and necrosis. The histopathology seen in the articular cartilage is characterised by areas of fibrillation, acellularity and the formation of chondrocyte clusters (Gardner, 1993).

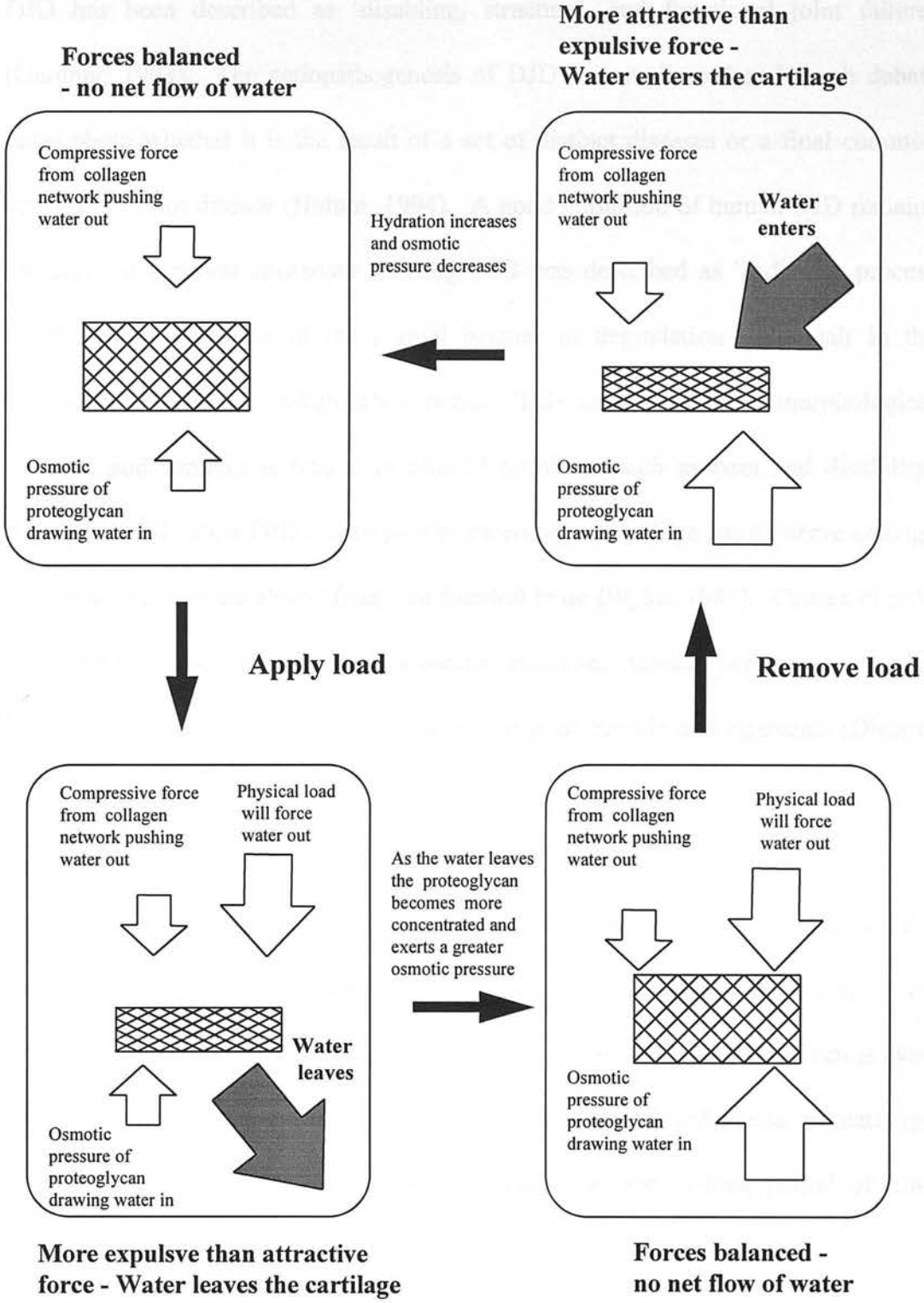


Figure 1.2 - The water content of cartilage allows two equilibrium states to be reached within the cartilage, an equilibrium on loading and an equilibrium at rest. This provides protection for the bone in both states.

DJD has been described as 'disabling, structural, and functional joint failure' (Gardner, 1993). The aetiopathogenesis of DJD is complicated and much debate rages about whether it is the result of a set of distinct diseases or a final common pathway to joint disease (Hutton, 1994). A good definition of human DJD remains elusive. At a recent consensus meeting DJD was described as 'a disease process involving a disturbance of the normal balance of degradation and repair in the articular cartilage and subchondral bone. This causes areas of morphological damage, and sometimes results in clinical problems such as pain and disability' (Dieppe, 1995). How DJD causes pain is uncertain, as cartilage has no nerve endings and pain receptors are absent from subchondral bone (Wyke, 1981). Causes of pain are possibly due to raised intra-osseus pressure, tender periarticular spots, inflammation and instability with strain on the joint capsule and ligaments (Dieppe, 1987).

The loss of cartilage matrix integrity that characterises DJD is a result of the destructive processes of cartilage breakdown being more active than the synthetic processes. In disease, where synthesis appears to be elevated, the catabolism is even more elevated and degradation occurs. Even a small imbalance in cartilage anabolism and catabolism could lead to degradation over a long period of time (Trippel, 1995).

1.4.2 Degenerative Joint Disease in fowl

DJD is observed in fowl, and is a particular problem in meat type poultry, i.e. both broiler strain fowl and turkeys (Riddell,1992). Lesions, which are manifest areas of

cartilage loss, are common in the hip joint and the distal tibiotarsus (Duff, 1990). Over 50% of male breeding broiler strain fowl show degenerative changes in the articular cartilage of the hip joint (Duff and Hocking, 1986). In female broilers the incidence of hip joint cartilage degeneration can rise up to 90% (at 60 weeks of age; Hocking, 1992) and a similar incidence has been reported on post-mortem examination of male turkey breeders (Duff, 1990). The lameness caused by this disease is a major welfare problem and may cause low fertility (Hocking and Duff, 1989).

A role for rapid growth and high body mass in the progression of DJD in fowl is suggested by the fact that restricted feeding reduces the incidence and severity of destructive cartilage loss (Hocking, 1992). The biochemistry of the progression of DJD in fowl is unknown. Elucidating the molecular mechanisms associated with DJD in fowl may suggest methods for reducing its incidence in flocks. Studying the naturally occurring disease in fowl may also improve our knowledge of DJD progression in general. Figure 1.3 shows a schematic representation of the two articular surfaces of the leg which were investigated in this thesis; the distal tibiotarsus (DTT) and proximal tarsometatarsus (PTM). These are the two opposing surfaces in the hock joint of the chicken. In addition the articular cartilage from the proximal humerus (PH) was studied and in the longitudinal study the antitrochanter (AT) was also investigated.

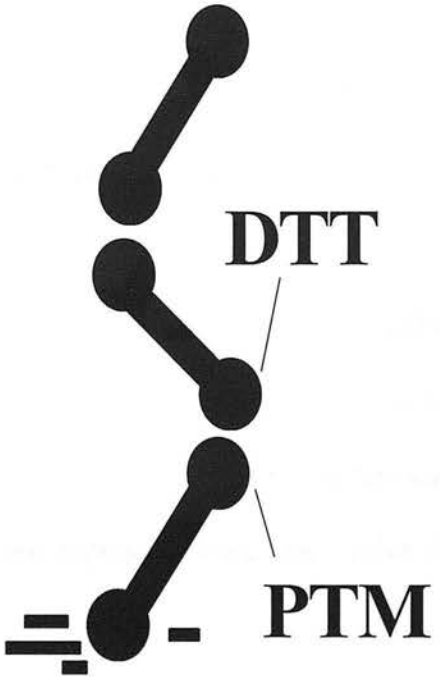


Figure 1.3 - Schematic representation of an avian leg.

1.4.3 Susceptibility to DJD

Large inter-individual variations in the biochemistry of normal articular cartilage have been observed; these are probably genetically determined and may be a factor predisposing some individuals to develop DJD (Muir, 1977).

It is not surprsing that those individuals with mutations in genes for collagenous components of articular cartilage often show symptoms of DJD. Mutations in genes for type II collagen are associated with mild forms of chondrodysplasia, which may present with precocious generalised DJD (Bleasel *et al.*, 1995), and other heritable diseases of cartilage (Williams and Jimenez, 1995). Mutations in collagen IX and XI have been associated with DJD syndromes, and transgenic murine models of these diseases have also been made (Jacenko and Olsen, 1995).

Human DJD (osteoarthritis, OA) is about three times more common in women than men. The incidence increases with age, and obesity is a major risk factor for human DJD conferring up to a 7 fold increased risk of knee OA (Dieppe *et al.*, 1992). There is an apparent association of DJD with heavy meat type poultry, so body mass may be an important factor in the aetiopathogenesis of DJD in fowl. Other associations with DJD in humans that have been described include, reproductive variables in women (e.g. hysterectomy and menopause), hypermobility, cigarette smoking (which may be protective), diabetes, hypertension and hyperuremia (Spector and Campion, 1989). Abnormal biochemical loading is a non-systemic risk factor for OA. Cruciate ligament rupture and total menisectomy are well-known factors which pre-dispose to knee OA. Joint shape is difficult to study *in vivo*, but it has been

suggested that minor abnormalities in the angulation of the femoral condyles may be an important factor in pathogenesis of knee OA (Cooke, 1985).

1.4.4 Animal models of DJD

Biochemical, cell or organ culture models can provide information and insight into the mechanisms for functional events within cartilage, bone or synovium or selected cells within these tissues. However, *ex vivo* models cannot simulate the structural changes which occur in joints over time (Burton-Wurster *et al.*, 1993). DJD models can be split into two major groups: experimentally induced models and spontaneous disease models. Induced models can be a mechanical or structural arthrosis. Mechanical arthroses are induced by altering the amount and distribution of biomechanical forces on otherwise normal joint tissues. Structural arthroses are induced by physical, chemical, endocrine, biochemical or immunological alteration of one or more joint tissues (Pritzker, 1994).

All experimentally induced mono-articular osteoarthritis models are essentially joint injury and repair models. The advantage of these models is that the time of onset of disease can be reasonably estimated from the type of injury (Pritzker, 1994). Mono-articular, biomechanical arthroses are of two types: joint instability models and displaced biomechanical load models. Methods for inducing joint instability include patella dislocation (Walton, 1979), patellectomy (Garr *et al.*, 1973) and transection of the anterior cruciate ligament of the canine knee joint (Pond and Nuki, 1973). Partial menisectomy has also been used to produce joint instability (Colombo *et al.*, 1983).

Joint instability models which are produced by intrusion into the joint capsule first induce inflammation and repair responses, then erosive and proliferative changes in cartilage and subchondral bone, similar to those in spontaneous arthritis, are observed.

Displaced biomechanical load models are those in which degenerative changes in joints are induced by altering load distribution without decreasing biomechanical stability. Myectomy and tenectomy (Arsever and Bole, 1986), compression (Ginsberg *et al.*, 1969) and immobilisation (Helminen *et al.*, 1983) are examples of procedures which can redistribute load in the joint. These models appear to produce varying degrees of cartilage degeneration in different animals (Pritzker, 1994). Repeated impact loading of joints *in vivo* can also induce articular cartilage and subchondral bone degenerative changes (Radin *et al.*, 1991).

In experimental structural arthroses, tissue composition of one or more structural components of joints is disrupted. This can adversely affect tissue mechanics resulting in increased susceptibility to injury and varying incapacity to complete the repair process. Small surgical defects or abrasion (Meachim, 1963), contusion and freezing (Simon *et al.*, 1981) allow induction of a cartilage lesion in a controlled and focal manner. Intra-articular administration of proteolytic enzymes such as papain (Murray, 1964) can be used to induce experimental arthroses.

Chemical agents can also be used to induce degenerative arthritis, either to affect the cartilage directly (for example using iodoacetate (Williams and Brandt, 1984)) or

using compounds such as talc (Gershuni *et al.*, 1981) to induce inflammation which is then followed by a post-inflammatory degenerative arthritis.

Since human DJD is a polyarticular disease, emphasis has been placed on using polyarticular models of disease. It is possible to induce a polyarticular disease using a specific agent. For example, an acute degenerative arthritis can be produced by oral administration of quinolone analogues to immature animals (Burkhardt *et al.*, 1990). Spontaneous disease can also be used as a model, and, although DJD is widespread in vertebrates, research has concentrated on spontaneous DJD in laboratory bred colonies of mice (Rataki *et al.*, 1980), dogs (Lust and Summers, 1981) and rhesus macaque monkeys (Kessler *et al.* 1986) and also on nutritionally controlled models of DJD in rats and guinea pigs (Gardner, 1992). For the purpose of this study, comparisons with models of DJD have been used in order to try and elucidate the causes and consequences of DJD in fowl.

1.5 Organisation of the articular cartilage matrix

1.5.1 Chondrocytes - cells under pressure

The chondrocyte is a cell with remarkable properties and capabilities which set it apart from other types of mesenchymal cell (Muir, 1995). The rounded undistinguished appearance disguises a highly differentiated cell that is the sole architect of cartilage. Confocal microscopy shows that the spatial organisation of the different macromolecules surrounding chondrocytes is not uniform (Wotton *et al.*, 1991). The proportion of cells to matrix is much lower in cartilage than in other

tissues: for example, in adult human femoral head cartilage there are only 10,000 cells/mm^3 (Venn and Maroudas, 1977). There is an inverse relationship between cell density and cartilage thickness which is irrespective of the size of a given animal. As cartilage is avascular, its nutrition depends on diffusion from outside and this may limit the total number of cells that can be sustained in a given volume (Stockwell, 1979). Chondrocytes can exist under very low oxygen tensions and are adapted to survive in tissue where the oxygen tension could be as low as 1% (Brighton and Happenstall, 1971). Chondrocytes are mesenchymal cells that differentiate during development, but after growth has ceased there is no detectable cell division in healthy articular cartilage (Mankin, 1963). When the integrity of the collagen network is compromised, as happens in the vicinity of lesions in osteoarthritis, cells appear to be newly divided, albeit rather slowly (Muir, 1995).

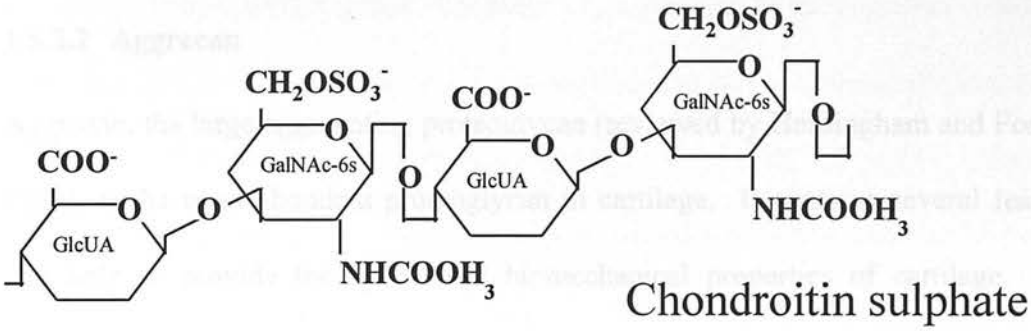
Chondrocyte metabolism is modulated to some extent by physical forces (Urban, 1994). These physical forces, and availability of nutrients to the chondrocyte, vary with the depth of the chondrocyte in the cartilage. There appear to be inherent differences between chondrocytes derived from the superficial tangential and the deep radial zones of articular cartilage; cells from the two zones exhibit metabolic differences during culture *in vitro* (Aydelotte and Keuttner, 1993). The metabolism of chondrocytes is also affected by growth factors, hormones and cytokines. For example interleukin-1 has various effects on chondrocytes isolated from different zones of the cartilage (Aydelotte *et al.*, 1988). The ability of a chondrocyte to respond to a cytokine may be affected by many factors including an alteration in chondrocyte membrane gangliosides (David *et al.*, 1995).

1.5.2 The proteoglycans

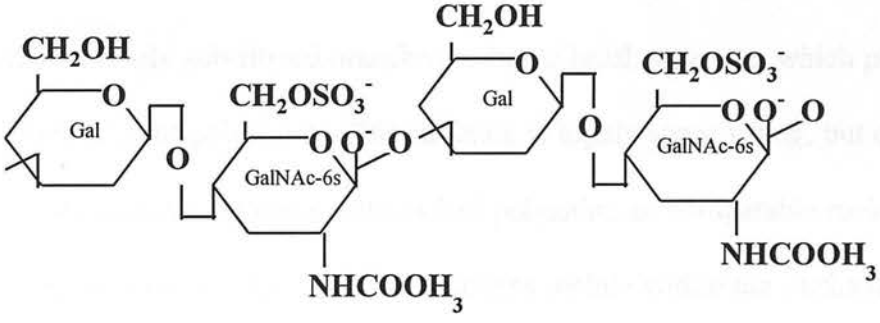
1.5.2.1 What is a proteoglycan?

Proteoglycans (reviewed by Neame, 1993) consist of one or more glycosaminoglycan (GAG) chains, covalently attached to a protein core. The GAG chains consist of a repeating disaccharide structure of variable length which is sulphated to differing degrees. The structures of the repeating disaccharides found in the cartilage matrix GAGs are illustrated in Figure 1.4. The repeating disaccharide usually consists of a uronic acid (either glucuronic acid or its epimer, iduronic acid) and an N-acetylated sugar (either N-acetyl glucosamine or N-acetyl galactosamine). Hyaluronic acid is an exception to this in that it consists of a GAG chain that is synthesised at the plasma membrane and is not sulphated. A key feature of GAG chains is their high charge density, resulting from carboxyl groups on the uronic acid and sulphated groups on the N-acetylated hexose. This causes the GAG to form an extended structure and occupy a large hydrodynamic volume.

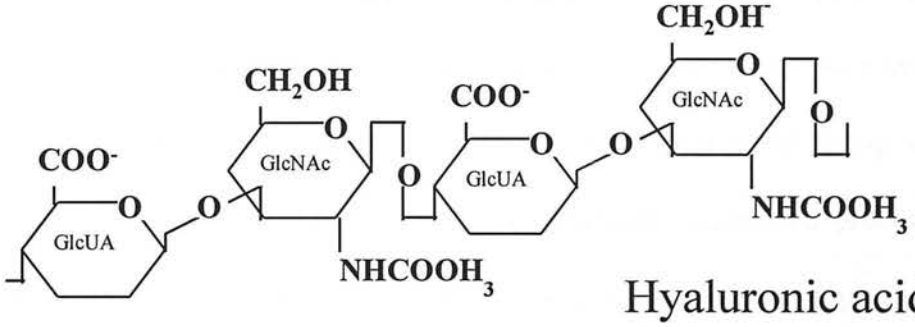
Chondroitin sulphate and dermatan sulphate are O-linked to a protein core through a serine-xylose-galactose-galactose structure. Keratan sulphate can either be N- or O-linked. When O-linked the linkage to either a serine or threonine is through a galactosamine. When N-linked, the linkage to an asparagine is through a galactosamine and has many of the characteristics of an N-linked oligosaccharide. In addition to the characteristic GAG chains, proteoglycans can also contain N- and O-linked oligosaccharides in the same way as any extracellular protein.



Chondroitin sulphate



Keratan sulphate



Hyaluronic acid

Gal = galactosamine GalNAc= N-acetyl galactosamine
GlcNAc= N-acetyl glucosamine GlcUA=glucuronic acid

Figure 1.4 - The dissacharide repeating units of glycosaminoglycans.

1.5.2.2 Aggrecan

Aggrecan, the large aggregating proteoglycan (reviewed by Hardingham and Fosang, 1995), is the most abundant proteoglycan in cartilage. It contains several features that help to provide the specialised biomechanical properties of cartilage. The structure consists of an extended protein core to which many chondroitin sulphate (CS) and keratan sulphate (KS) chains are attached (Hardingham *et al,*, 1992). This forms a densely substituted branched or bottle brush structure, which provides a high concentration of polyanion. This structure is highly space filling, but of much lower viscosity than a long chain unbranched polyanion of comparable molecular weight, such as hyaluronan. Aggrecan thus remains mobile within the cartilage matrix when newly secreted by the chondrocyte. However, its ability to aggregate, by specifically binding to hyaluronan, provides an extracellular mechanism for helping it immobilise within the matrix. Hyaluronan binding is assisted by a separate globular link protein, which binds to both aggrecan and hyaluronan. There also appears to be a mechanism that limits the immediate avidity of aggrecan for hyaluronan following secretion, which then changes over 1-2 days as some structural maturation process occurs (Bayliss *et al.*, 1984; Sandy *et al.*, 1989). The size of proteoglycan aggregates varies from being very small (2-5 aggrecan molecules), when there is a large excess of hyaluronan, to exceedingly large (400-800 proteoglycans). The N- terminus has a globular G1 and G2 domain; the C terminus has a globular G3. The substitution of CS on the protein core is entirely restricted to the regions between the G2 and G3 domains. KS is found much closer to the N terminus of the protein core, including

on the interglobular domain, as well as in a more specific KS rich region on the C-terminal side of the G2 domain. Cartilage proteoglycans of the aggrecan type show great variation in carbohydrate composition, and this was a severe handicap in the elucidation of their structure. Cartilage aggrecan contains CS, KS and O- linked and N- linked oligosaccharides. The number, size, sulphation pattern and charge density of these substituents are known to change with development and ageing and vary from site to site.

1.5.2.3 Non-aggregating proteoglycans

Decorin, biglycan and fibromodulin are leucine rich proteoglycans which contain several copies of a leucine rich repeating domain. Decorin and biglycan contain CS and dermatan sulphate GAG chains. Decorin is the smaller of the two and the GAG is attached at the N-terminal of the molecule. In mammals there is a single GAG chain (Chopra *et al.* 1985) but recent work has shown that avian decorin can contain two GAG chains (U.K. Blaschke, personal communication). Decorin binds to collagen types I and II and has been implicated in the regulation of collagen fibrillogenesis (Vogel *et al.*, 1984).

Although the structure of biglycan is known (Fisher *et al.*, 1989) , it has two GAG chains close to the N-terminal of the molecule, its function remains obscure. Fibromodulin contains up to four keratan sulphate GAG chains (Plaas *et al.*, 1990) and, in addition to the leucine rich repeats, there is also a sulphated tyrosine rich domain.

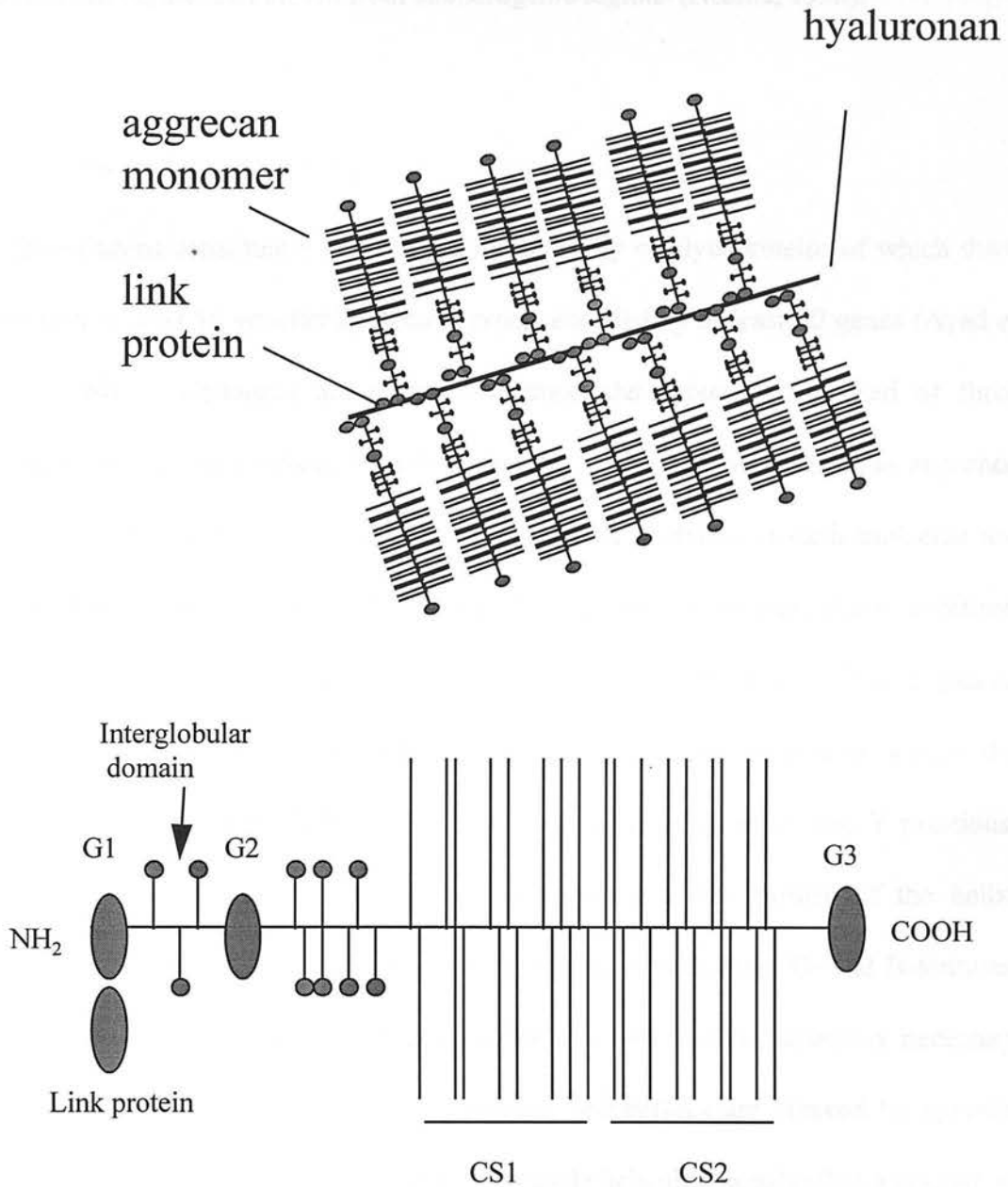


Figure 1.5- The structure of aggregates (top) and aggrecan monomers (below)

A large non-aggregating chondroitin sulphate proteoglycan is also found in cartilage. Heparin sulphate proteoglycans, although they are associated with early development of limb buds, are later absent from chondrogenic regions (Neame, 1993).

1.5.3 The collagens

The collagens constitute a highly specialised family of glycoproteins of which there are now at least 16 genetically distinct types, encoded by at least 30 genes (Ayad *et al.*, 1994). Collagens are extended extracellular proteins composed of three polypeptide chains (α-chains), each possessing a characteristic tripeptide sequence (gly-X-Y) that forms a left handed helix. Three α-chains in each molecule are twisted tightly into a right-handed helix to form a rope-like structure that is stabilised by hydrogen bonds, while peptide bonds are buried inside the helix. Glycine, placed at every third residue of the tripeptide sequence, is small enough to occupy the crowded interior of the helix. Side chains of residues at the X and Y positions, commonly proline and hydroxyproline, are located at the surface of the helix. Collagen precursors, or procollagens, are synthesised with large C- and N-terminal extensions which, among other functions, are involved in chain assembly necessary for triple helix formation. These extension propeptides are cleaved by specific procollagen peptidases after secretion. The triple helical molecules then associate to form fibrils. The fibril association occurs by a series of post-translational modifications, both intra and extracellular, that require a number of specific and non-

specific enzymes (Eyre *et al.*, 1992). The fibrils are further stabilised by crosslinks that involve lysine residues. Fibrils grow to a uniform thickness by accretion of single molecules in solution. Mutations that affect any step in fibril formation are likely to be detrimental and usually result in some sort of aberrant phenotype, illustrating the crucial importance of correct fibril formation for proper development of cartilage (Muir, 1995).

1.5.3.1 Collagens of cartilage

The major collagen of all hyaline cartilages is type II, which is assembled in a staggered array to from the fibrils commonly observed by electron microscopy (Mayne and Brewton, 1993). Type II collagen is closely related to the other interstitial collagens (types I, III, V, and XI) and its primary function is in the formation of fibrils (Miller, 1976).

Type IX collagen, about 1-2% of the total collagen in cartilage, is also considered to be a proteoglycan because of its single glycosaminoglycan chain. It is found at the surface of type II collagen fibrils and is covalently bound by hydroxypyridinium cross links to type II collagen. Type IX collagen is thought to play an important role in the integrity of the collagen network (van der Rest and Mayne, 1988). The cross-linking sites and anti-parallel orientation of crosslinked collagen II and IX molecules has been established by partial degradation and peptide analysis (Wu *et al.*, 1992). This orientation allows links, type IX bridges, to form between different fibrils, tying the fibrils into an open meshwork of fibres that permits limited, but necessary, deformation under compression, as has been observed when wet cartilage is

compressed (Broom, 1982). Proteolytic attack, by for example stromeolysin which cleaves type IX collagen near cross-linking sites (Wu *et al.,* 1992), can weaken the collagenous meshwork and allow cartilage to swell, which is an early event in osteoarthritis.

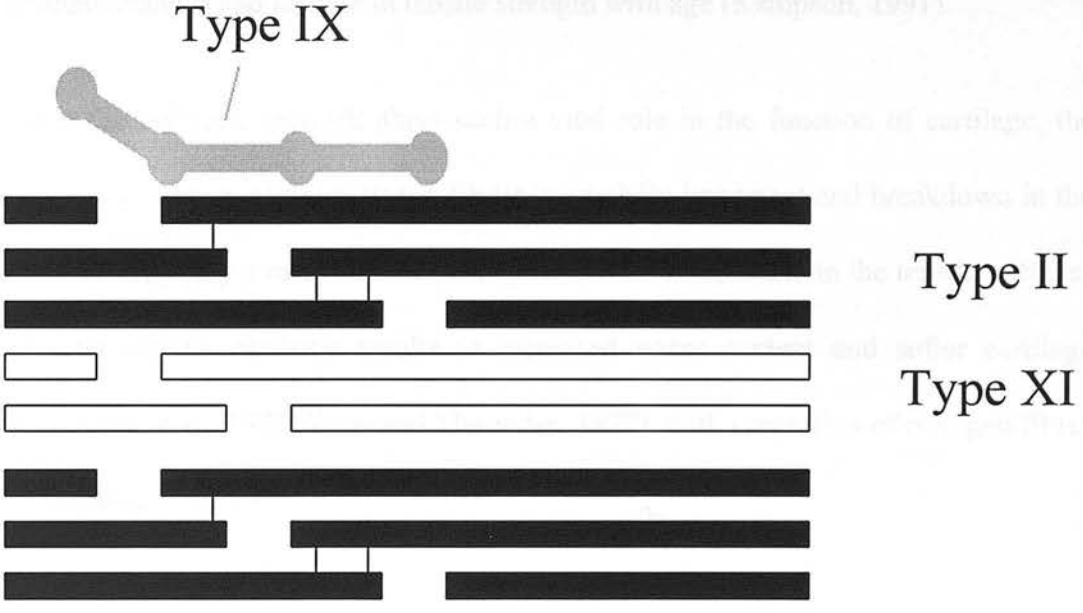


Figure 1.6 - Arrangement of collagens II, IX and XI in the collagen fibril of cartilage. (adapted from Mayne and Brewton, 1993).

Other collagens found in cartilage include: Type XI collagen which is located within the fibrils, and may partly control their lateral growth during matrix formation (Mendler *et al.*, 1989). Type X collagen is synthesised specifically by hypertrophic chondrocytes and in normal articular cartilage is localised with the collagen fibrils in noncalcified regions of hypertrophic cartilage (Schmid and Linsenmayer, 1985). Small quantities of type VI collagen have been identified in the territorial matrix of isolated chondrons (Poole *et al.*, 1988) and in osteoarthritic cartilage (McDevitt *et*

al., 1988). Avian articular cartilage also contains some type I collagen (Eyre *et al.*, 1978; Wardale and Duance, 1996). The exact distribution and function of the type I collagen in avian articular cartilage remains unclear. Collagen in articular cartilage is normally very long lived (Maroudas *et al.*, 1992) and, as a consequence, undergoes gradual changes and decline in tensile strength with age (Kempson, 1991).

Since the collagen network plays such a vital role in the function of cartilage, the physical condition of the collagen fibrils is crucially important and breakdown in the network may play a role in cartilage degeneration. A decrease in the tensile stiffness of osteoarthritic cartilage results in increased water content and softer cartilage (Kempson *et al.*, 1973, Venn and Maroudas, 1977), with separation of collagen fibres (Stockwell *et al.,* 1983).

1.5.4 Glycoproteins

Noncollagenous, non-proteoglycan glycoproteins, constitute a small, but significant portion of cartilage (Heinegard and Pimentel, 1992). Generally the functions of these proteins are unknown. The major cartilage glycoproteins are described below.

Link protein is a 41-48 kD glycoprotein involved in the stabilisation of proteoglycan-hyaluronic acid aggregates in cartilage. There are three link proteins which share a common amino-acid backbone and only appear to differ in their degrees of glycosylation (Hardingham, 1979). Chondronectin is a disulphide-bonded 180 kD glycoprotein which mediates the adhesion of chondrocytes to the fibres of type II

collagen (Hewitt *et al.,* 1980). Fibronectin is a large glycoprotein, consisting of 220-250 kD subunits, which mediates the attachment of fibroblasts to collagen. Chondrocytes synthesise small amounts of fibronectin which may play a role in chondrocyte attachment to the extracellular matrix (Brown and Jones, 1990). Cartilage matrix protein is a 148 kD trimeric glycoprotein that is selectively present in cartilage and consists of 58 kD subunits. This protein sediments with proteoglycan aggregates in caesium chloride gradients and may interact with aggregates *in vivo* (Paulsson and Heinegard, 1979). Cartilage oligomeric matrix protein (COMP) is preferentially localised in the territorial matrix surrounding chondrocytes. It consists of five disulphide-bonded subunits which give a molecular mass of 524 kD. There are four six-cysteine EGF repeats and seven thrombospondin type 3 repeats. COMP is homologous to the COOH-terminal portion of thrombospondin (Oldberg *et al.*, 1992).

1.6 Mechanisms of articular cartilage degradation

There is thought to be a role for oxygen derived free radicals in degradation of collagens, proteoglycans and hyaluronic acid. Chondrocytes as well as phagocytes can also produce reactive oxygen radicals under certain conditions (Ahmadzadeh *et al.*, 1990). However, the primary cause of the pathological destruction of cartilage is the elevated proteolytic enzymic activities, reviewed by Nagase and Woessner (1993). A number of proteinases have been identified in cartilage, synovial fluid and synovium which may participate in cartilage destruction. These include lysosomal enzymes, matrix metalloproteinases, the plasminogen activator/plasmin

system and several other serine and cysteine proteinases. Matrix metalloproteinases all share homology with tissue collagenase (MMP-1) and contain a propeptide region which is removed upon activation of the MMP zymogen (Murphy *et al.*, 1990). The catalytic domain contains a zinc binding region. All MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Pelletier *et al.*, 1990). MMPs appear to play the major role in matrix degradation although the mechanisms controlling their production, activation and activity *in vivo* are not fully understood. Interleukin-1 stimulates the release of metalloproteinases in chondrocytes, and can regulate the synthesis of extracellular matrix molecules (Arner *et al.*, 1989). Tumor necrosis factor, interferon-gamma and transforming growth factor beta may also play roles in regulating the pathological changes in cartilage degeneration (Reife *et al.*, 1993).

1.7 Summary

Articular cartilage is a specialist connective tissue which functions to protect bones and enable smooth articulation in diarthrodial joints. The molecular basis for the degradation of the tissue, leading to degenerative joint disease, is unknown. There is a malfunction of the chondrocytes and the collagen and proteoglycan filled extracellular matrix which surrounds them. By studying avian articular cartilage under varying conditions of age, genotype and disease, some of the molecular mechanisms affecting articular cartilage metabolism may be elucidated.

2. Preliminary Study

2.1 Introduction

Broiler strain fowl are more susceptible than layer strain fowl to DJD. The underlying aetiology of this difference is unknown. This chapter describes the results of a preliminary study which was undertaken to assess the biochemical and morphological differences in the articular cartilage between the two strains, differences which may mediate this variation in susceptibility to DJD. There is very little published data on mature avian articular cartilage and so this preliminary study was necessary to establish methodologies for the investigation of avian articular cartilage.

Two age groups, from two genotypes were used in this preliminary study: mature adults and day-old chicks. One aim of this study was to establish the individual variation, so to enable group size to be determined for further studies. Studying day-old chicks ascertained the feasibility of carrying out the measurements on relatively small samples, while at the same time, it was of interest to investigate whether the composition of articular cartilage varied between the day-old chicks of different genotypes. Such variations may indicate a predisposition to disease in a genotype.

Group sizes of six for the adults and five for the day-old chicks were chosen. In order to investigate the role of load on articular cartilage, samples were taken from both loaded and relatively unloaded joints. The distal tibiotarsus (DTT) and the proximal tarsometatarsus (PTM) provided representative samples of loaded articular surfaces, while the proximal humerus (PH) was considered relatively unloaded.

As described in Chapter 1, the hydration of the cartilage is an important parameter of cartilage condition, as the hydration provides the mechanism underlying tissue resiliency. The major factors controlling hydration of the articular cartilage are the collagen network and the proteoglycan content. Indications of the proteoglycan content can be obtained by the determination of uronic acid. Uronic acid is present in all glycosaminoglycans with the exception of keratan sulphate, and can be easily assayed by utilising the ability of carbazole dye to react with the unstable acid hydrolysed dehydrated derivatives of hexuronic acids (Chaplin, 1986). The cartilage was digested using the proteinase K/SDS method of Lipman (1989), a procedure that had been successfully utilised in the laboratory for the digestion of rabbit articular cartilage. This method also allowed the samples to be assayed for DNA content using a fluorimetric dye binding technique (Lipman, 1989). Additional samples were taken for histology in order to ascertain the morphology of the cartilage in the two strains.

2.2 Materials and Methods

2.2.1 Choice of birds

To provide the mature adult samples, two groups of six fowl were selected at random from female flocks of one-year old, adult, broiler breeder (commercial type) and layer strain fowl. The flocks were both considered normal and there was no evidence of debilitating disease or noteworthy lameness. Groups of five one day old chicks of each genotype were obtained commercially. All fowl were killed by an overdose of barbiturate.

2.2.2 Collection and processing of samples

Articular surfaces were analysed from the hock and shoulder joint of each bird, i.e. right and left distal tibiotarsi (DTT), right and left proximal tarsometatarsi (PTM), all of which were load bearing, and right and left proximal humeri (PH), which were relatively non-load bearing. Bone extremities were dissected out and divided in half sagitally. One half was preserved for histology in buffered neutral formalin, while the articular cartilage was removed from the other half and placed in a pre-weighed sealed container. The wet mass of the tissue was determined. After drying at 60°C until a constant mass was achieved, dry mass was determined and the hydration was calculated. The tissue was solubilised using the proteinase K/SDS method of Lipman (1989) adapted to include two rounds of digestion (see appendix). The digest was aliquoted out and uronic acid was determined using a standard carbazole assay (Chaplin, 1986) adapted for use in lockable eppendorf tubes (see appendix). The collection of the mature adult samples from commercial flocks necessitated storage for a prolonged period before dehydration, thus risking the possibility of extensive DNA degradation during storage. As a result of this possiblility, DNA analysis of these samples is not reported here. In contrast, the day-old chicks were sacrificed on site at the Roslin Institute, hence the period before dehydration was minimised. The DNA assays (Lipman 1989) on these samples are presented in this chapter.

The hydration and assay results were analysed using unpaired Student t-tests in Microsoft Excel. Except where stated otherwise, differences in results are considered significant where $p<0.05$ for these tests.

After 7 days, the formalin fixed specimens underwent decalcification in Gooding Stewarts Fluid (see appendix). Samples were washed in tap water overnight, returned to buffered neutral formalin and processed through ascending alcohol concentrations and CNP 30 to paraffin wax. The resulting sections were stained with haematoxylin and eosin (see appendix).

2.3 Results

In pursuit of clarity, the results are first presented separately for the mature adult birds and for the day-old chicks, and then comparisons are made in section 2.3.3.

2.3.1.1 Results from the mature adult birds

2.3.1.2 Hydration

The hydration results for the cartilage are shown graphically in Figure 2.1.

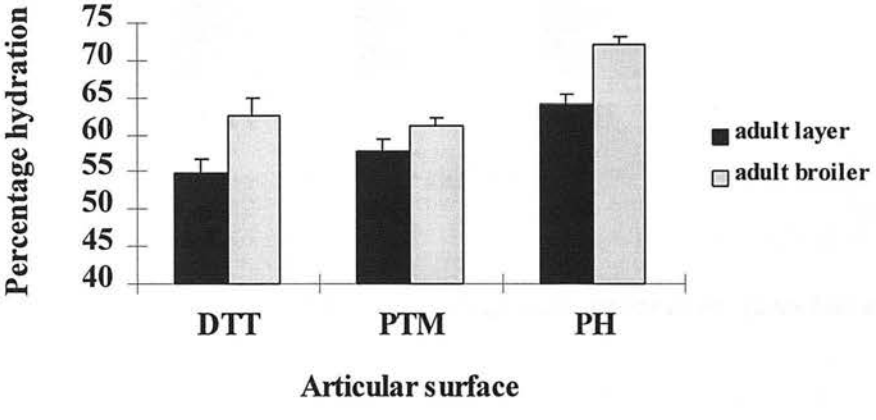


Figure 2.1 - Hydration of articular cartilage from mature birds expressed as a percentage of wet weight. Error bars are S.E.M.

Within both groups (layer and broiler) the articular cartilage from the loaded joints (DTT and PTM) was similarly hydrated, but significantly less hydrated than that from the unloaded joint (PH). Cartilage samples from both broiler DTT and broiler PH were significantly ($p<0.05$) more hydrated than those of the corresponding layer strain samples. The cartilage from the broiler strain PTM was also more hydrated than the cartilage from the layer strain PTM ($p=0.09$).

2.3.1.3 Uronic acid

The uronic acid content of the mature adult samples is shown in Figure 2.2.

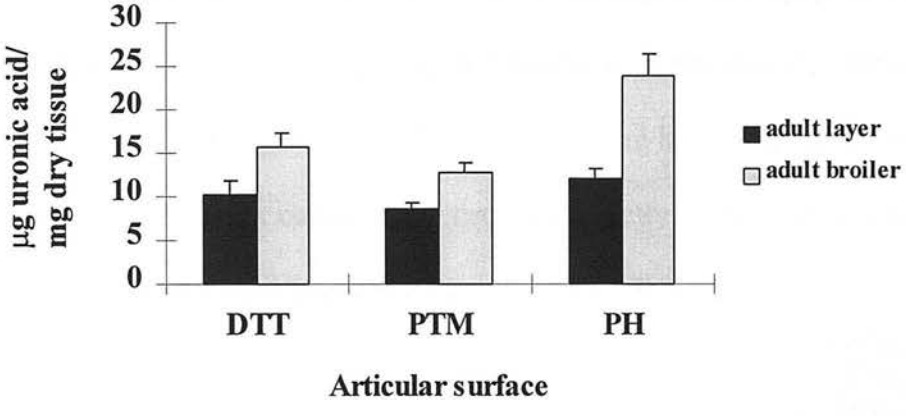


Figure 2.2 - Uronic acid content of articular cartilage from mature birds. Error bars are S.E.M.

The mature adult broiler unloaded cartilage (PH) had significantly ($p<0.05$) higher amounts of uronic acid than either of the broiler strain loaded cartilages. In addition, the cartilage from the layer strain PH had significantly higher levels of uronic acid than the cartilage from the layer strain PTM.

2.3.1.4 Histology

Tissues were processed in the Roslin Institute bone histology facility.

Tissue sections of DTT contained small areas of synovial tissue. In layer strain fowl, the synovium was 1-4 cells thick and the synoviocytes were flattened in appearance (Figure 2.3). In contrast, the synovium of the broilers showed mild hyperplasia and hypertrophy (Figure 2.4), indicative of mild synovitis, in 4 of the 12 DTT examined.

In all samples of articular cartilage, three zones could be identified. The surface zone contained flattened chondrocytes and fibres, oriented parallel to the surface. The middle zone was identified by its rounded chondrocytes and the presence of fibres oriented at various angles, including perpendicular to the articular surface, but not parallel to it. The chondrocytes of the deep zone were larger than those of the middle zone and distinct fibres could not be detected visually in the matrix which had the appearance of epiphyseal hyaline cartilage (Figure 2.5).

The articular cartilage of the DTT of layer strain fowl varied in apparent thickness both across the surface of the joint and between individuals. In 6 DTT the articular cartilage appeared thin, approximately 7-8 chondrocytes deep (Figure 2.5). The articular cartilage of the remaining six DTT was thicker, approximately 12-15 cells deep. Where present, cartilage thinning did not appear to be specific to any one zone. In one layer strain sample, the articular cartilage of the DTT showed reduced cellularity and a pale eosinophilia of the surface zone (Figure 2.6).

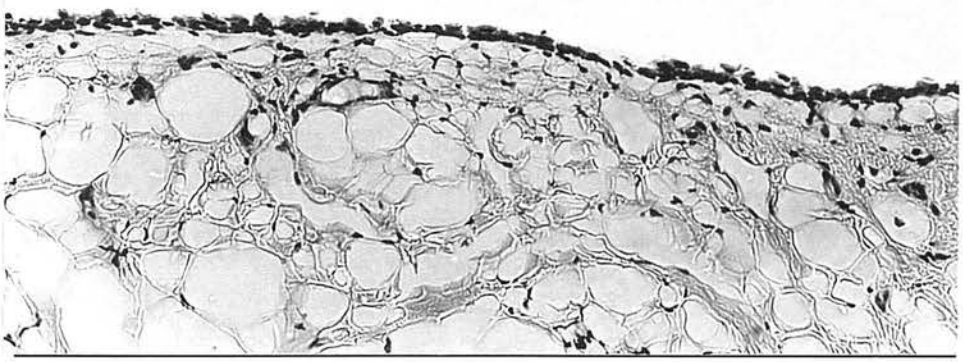


Figure 2.3 - Synovial tissue from the hock joint of the mature layer strain fowl. Haematoxylin and eosin, x70.

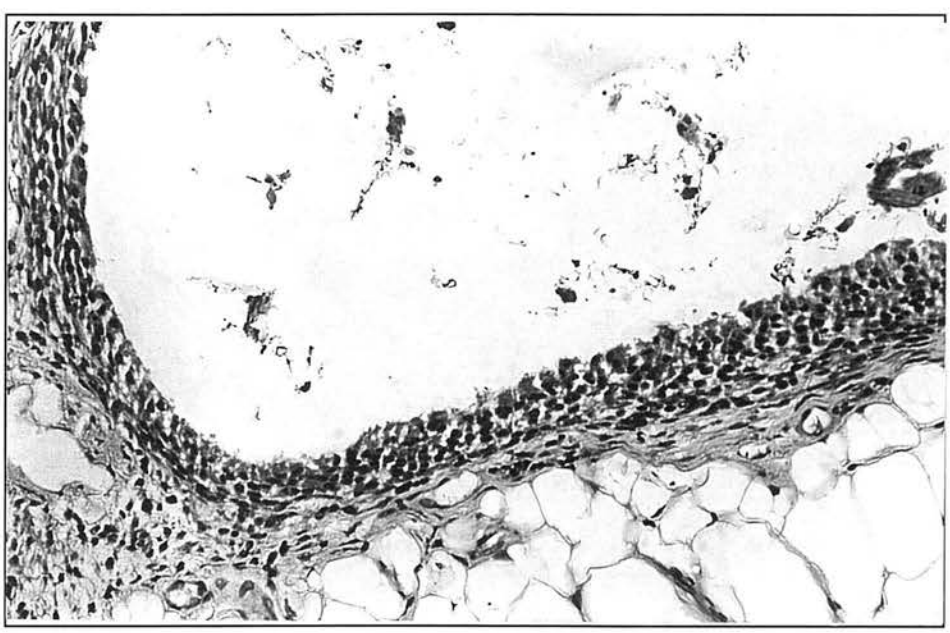


Figure 2.4 - Synovium from the hock joint of a mature broiler strain fowl. The synoviocytes show hyperplasia and hypertrophy when compared with that of the layers (Figure 2.3). Haematoxylin and eosin, x70.

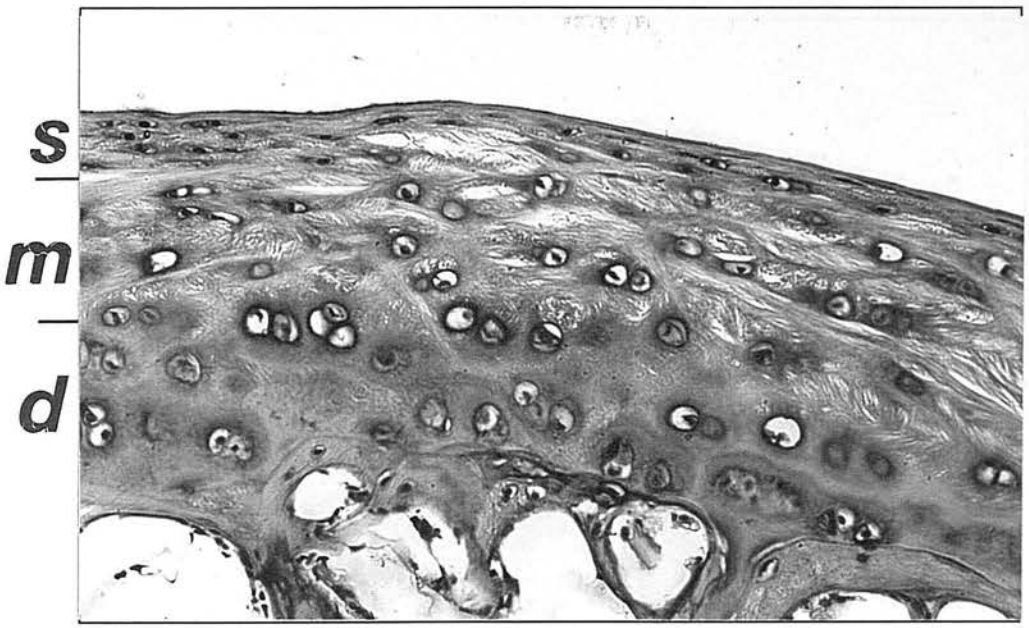


Figure 2.5 - Articular cartilage of the DTT from the mature layer strain fowl. The surface zone (s) contains flattened chondrocytes and collagen fibrils parallel to the surface. The middle zone (m) has rounded chondrocytes and the collagen fibrils are not parallel to the surface. The deep zone (d) contains no obvious collagen fibrils and the rounded chondrocytes are surrounded by a basophillic halo. Haematoxylin and eosin, x70.

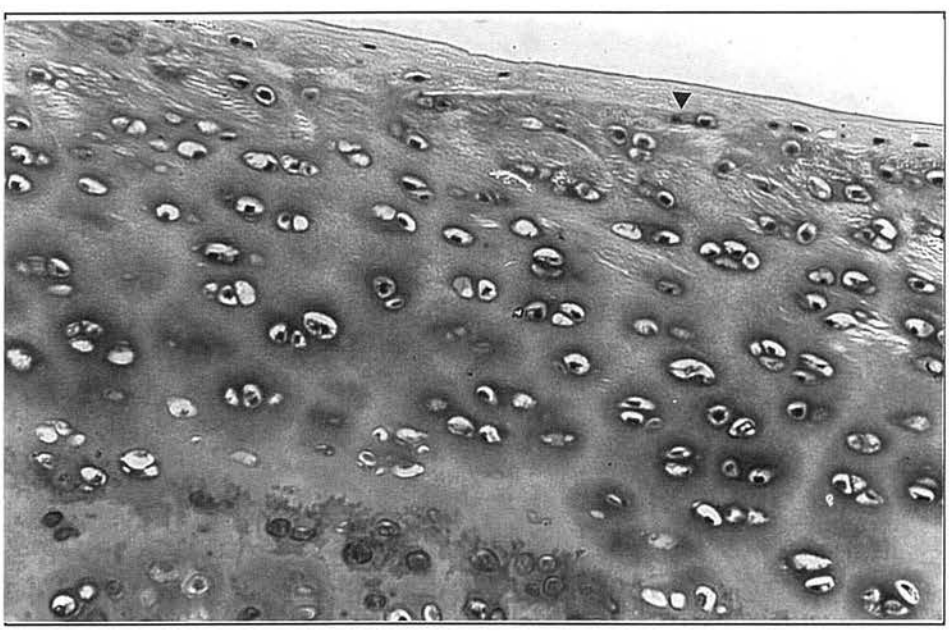


Figure 2.6 - DTT from a mature layer strain fowl. There is reduced cellularity and pale eosinophilia of the surface zone. Some of the remaining surface zone chondrocytes appear to have pyknotic nuclei (arrow). Haematoxylin and eosin x70.

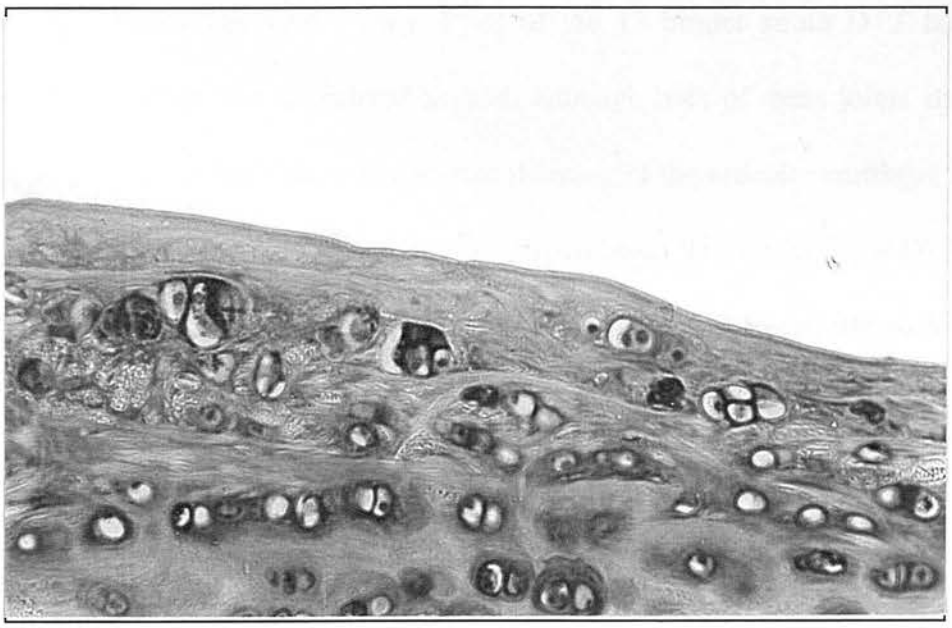


Figure 2.7 - DTT from a broiler strain fowl. There is cartilage thinning and loss of cellularity within the surface zone, which is eosinophilic. Within the middle zone there are accumulations of chondrocytes. Haematoxylin and eosin x70.

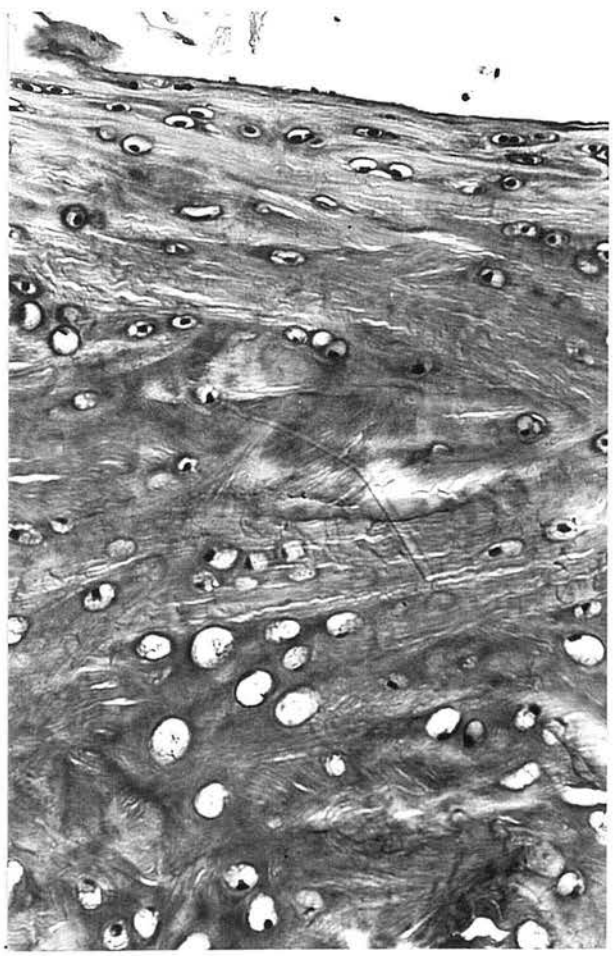


Figure 2.8 - Articular cartilage from the proximal tarsometatarsus of a layer strain fowl. Haematoxylin and eosin x70.

The articular cartilage from the DTT of the broiler strain fowl was distinct from that of the layer strain fowl. Only 2 out of the 12 broiler strain DTT had articular cartilage which was considered normal, although both of these joints showed mild synovitis. Four DTT showed apparent thinning of the articular cartilage, 2 of which also had mild synovial hypertrophy and hyperplasia. The remaining 6 DTT showed a loss of cellularity within the surface zone, which was also frequently eosinophilic. In some sections small clusters of chondrocytes were occasionally seen within the articular cartilage, beneath an area with a loss of surface zone cellularity (Figure 2.7).

The articular cartilage from the PTM was normal in 5 of the 12 layer strain samples (Figure 2.8). Within the remaining 7 layer strain PTM specimens, 2 showed focal thinning of the articular cartilage and 3 showed areas of poor cellularity within the surface zone, with one specimen showing both features. Pale eosinophilia was not seen in the surface zone within any of the layer strain tarsometatarsi. One PTM showed some fibrillation of the surface zone and the adjacent matrix contained some small clumps of chondrocytes.

In contrast to the layer strain birds, none of the 12 PTM samples from broiler strain birds were normal. Articular cartilage from all but 2 samples showed focal loss of cellularity within the surface zone, which was frequently associated with pale eosinophilia. Pyknotic nuclei were also frequently noted within surface zone chondrocytes (Figure 2.9) and minor fibrillation of the articular surface was noted in the PTM from 3 broiler strain fowl.

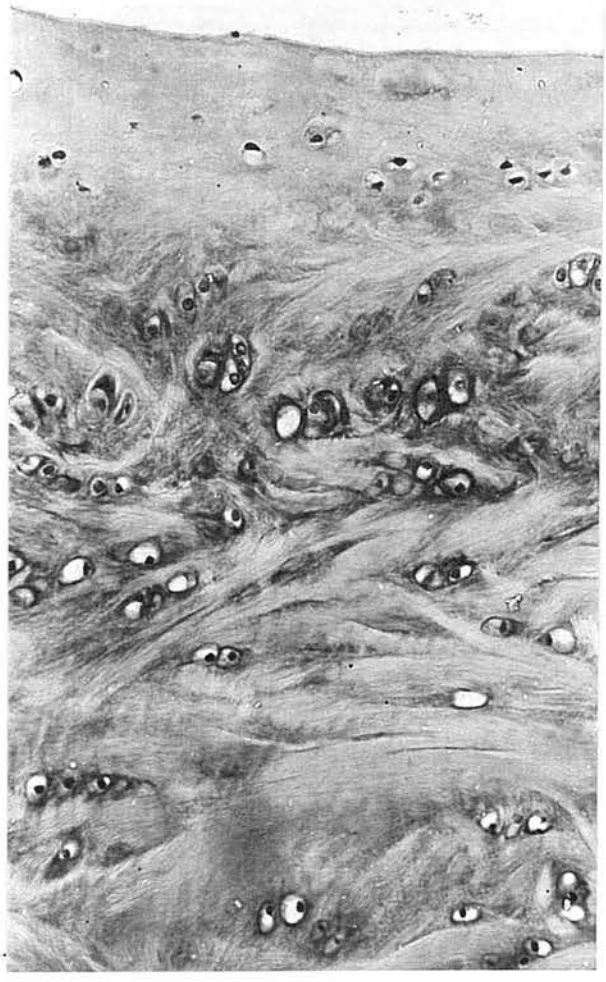


Figure 2.9 - Articular cartilage from the PTM of a broiler strain fowl. In the surface zone there is loss of cellularity, pale eosinophilia of the matrix and pyknotic nuclei. Haematoxylin and eosin, x70.

Articular cartilage from the 12 PH of the layer strain fowl appeared normal, while 9 of the 12 broiler strain joints showed evidence of degeneration. These changes varied from focal cartilage thinning with no other changes (one example) to focal loss of cellularity of the surface zone, pyknotic nuclei within remaining chondrocytes and pale eosinophilia of the surface zone. Moreover one broiler PH also showed focal, total loss of articular cartilage with exposure of the underlying subchondral bone (Figure 2.10). The adjacent cartilage contained large clusters of chondrocytes (Figure 2.11).

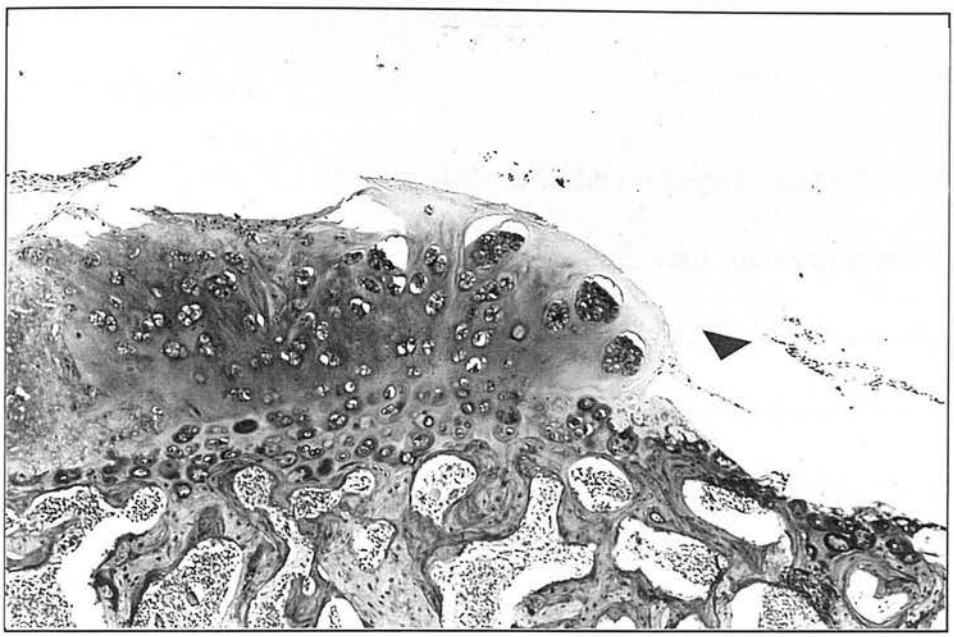


Figure 2.10 - PH from a broiler strain fowl showing focal total loss of the articular cartilage with exposure of the underlying subchondral bone (arrow). Haematoxylin and eosin, x70.

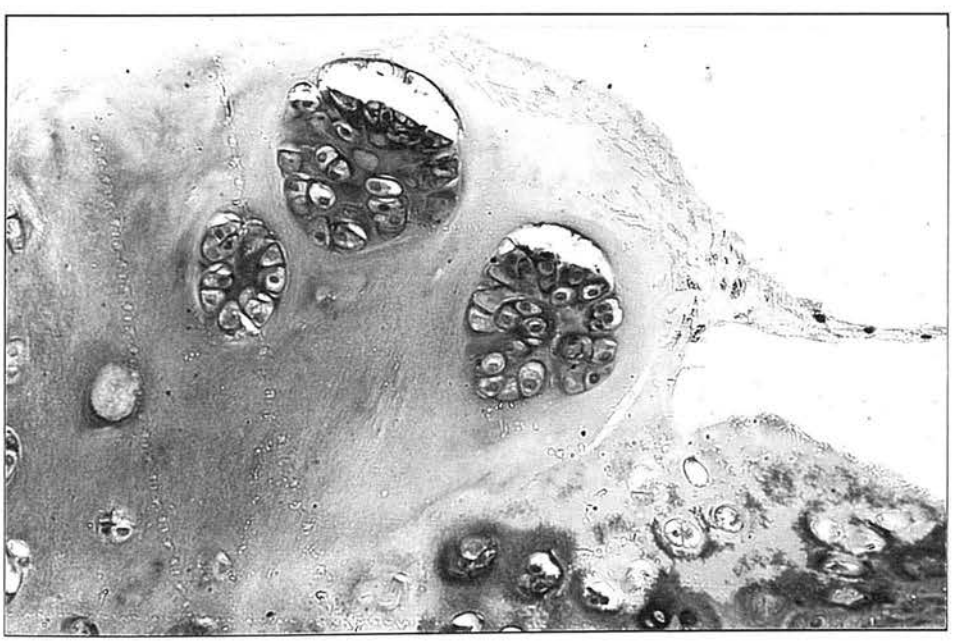


Figure 2.11 - The cartilage adjacent to the defect in Figure 2.10 contains large clusters of chondrocytes. Haematoxylin and eosin, x110.

2.3.2 Results from the day-old chicks

2.3.2.1 Hydration

Figure 2.12 shows the hydration of the articular cartilage from the day-old chicks. In general all the cartilages are similarly hydrated, with the exception of the broiler strain DTT. The pattern observed in the mature adult samples is not seen here, instead the broiler DTT cartilage is significantly more hydrated than the cartilage from the broiler strain PTM. Unlike the broiler strain PTM, the DTT is not significantly less hydrated than the broiler strain PH sample. The broiler strain DTT cartilage was significantly more hydrated than the layer strain DTT cartilage.

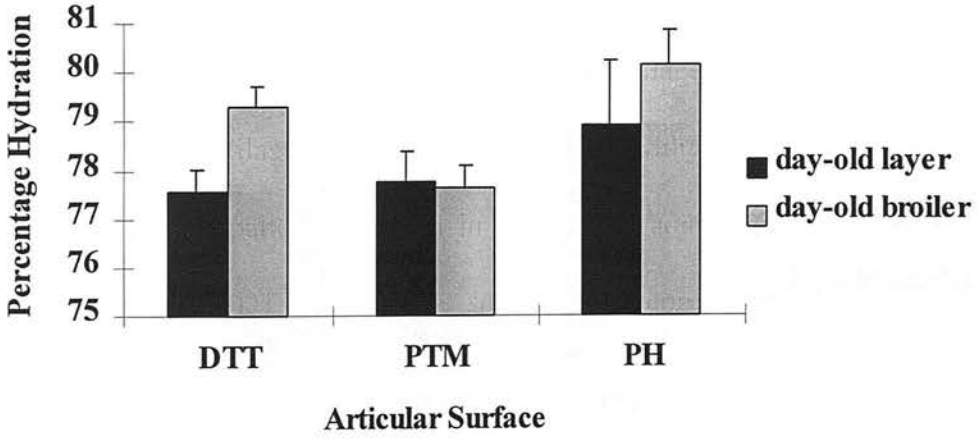


Figure 2.12 - Hydration of articular cartilage from day-old chicks, expressed as a percentage of the wet weight of the tissue. Error bars are S.E.M.

2.3.2.2 Uronic acid content

The uronic acid content of the articular cartilage from day-old chicks is shown in Figure 2.13. The broiler strain DTT sample contained significantly more uronic acid than both the layer strain DTT and broiler strain PTM samples. This corresponds to the hydration results for these samples. The layer strain PTM contained significantly less uronic acid than the layer strain DTT. The broiler strain PH contained significantly less uronic acid than the broiler strain DTT. The broiler strain PTM contained significantly more uronic acid than the layer strain PTM despite there being no significant difference in the hydration of these two samples.

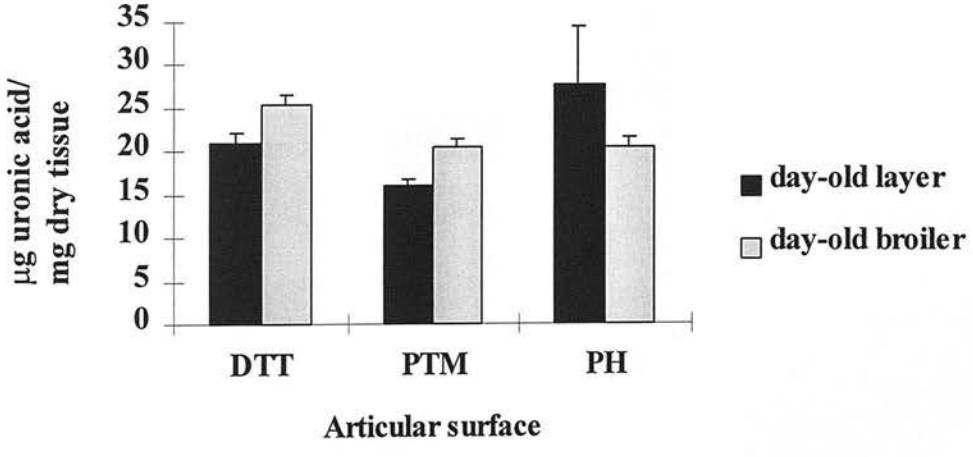


Figure 2.13 - Uronic acid content of articular cartilage from day-old chicks. Error bars are S.E.M.

2.3.2.3 DNA content

Figure 2.14 depicts the DNA content of the samples from the day-old chicks. No significant differences were seen between the loaded joints (DTT and PTM) either within the groups or between the groups. The DNA content of the broiler strain PH sample was not significantly different to the that of the broiler strain PTM or DTT samples, although the layer strain PH did contain significantly less DNA than the other layer strain samples and much less than that of the broiler strain PH.

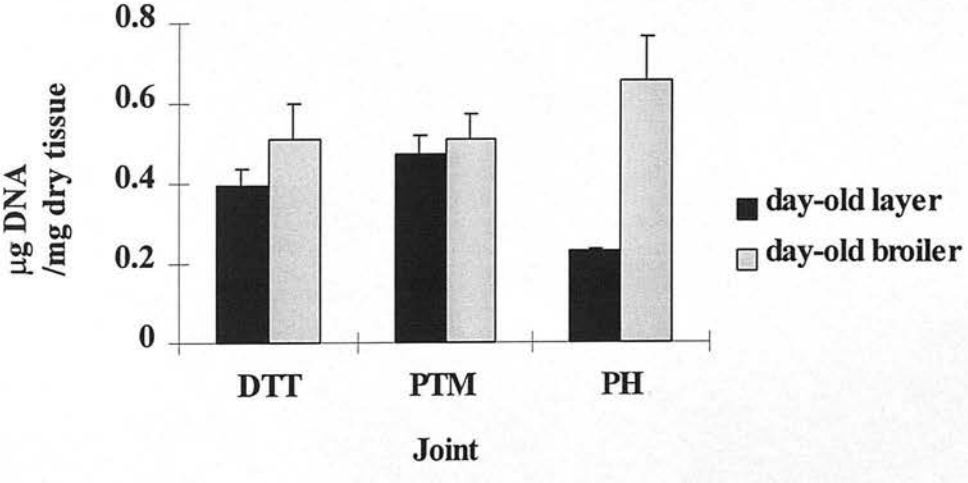


Figure 2.14 - DNA content of articular cartilage from day-old chicks. Error bars are S.E.M.

2.3.2.4 Histology

2.3.2.4.1 The proximal tarsometatarsus

As shown in Figure 2.15, the articular cartilage of the broiler and layer strain PTM appeared very similar. There was a surface layer with a high density of cells and no visible lacunae. In the middle to deep zones, the chondrocytes were aligned in linear arrays. In both strains the linear arrays were at an angle which varied from 45 degrees to parallel to the surface. Below the deep zone the epiphyseal cartilage could be seen.

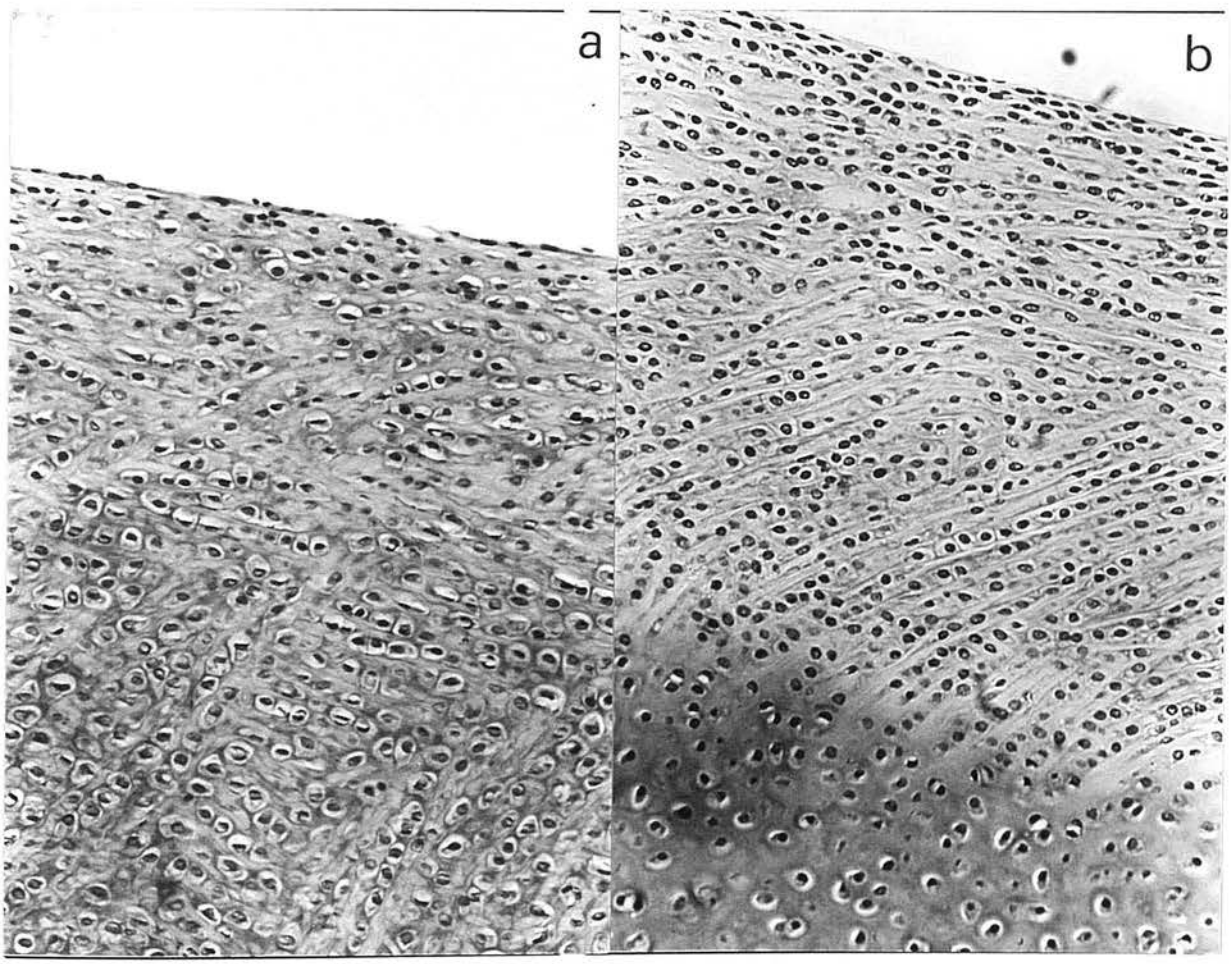


Figure 2.15 - Articular cartilage from the proximal tarsometatarsus of (a) layer strain and (b) broiler strain day-old chicks. Note the orientation of the linear arrays and the similarity between the two strains. Haematoxylin and eosin x 70

2.3.2.4.2 Proximal humerus

As found with the DTT, the articular cartilage of the PH was only a few cells thick (Figure 2.16). The morphology of the samples from the two strains was similar, although in some places the articular cartilage of the broiler strain samples was considerably thicker (2-3x) and appeared more cellular than that of the layer strain samples. In both strains the articular cartilage was eoisinophilic. No linear arrays of chondrocytes were apparent.

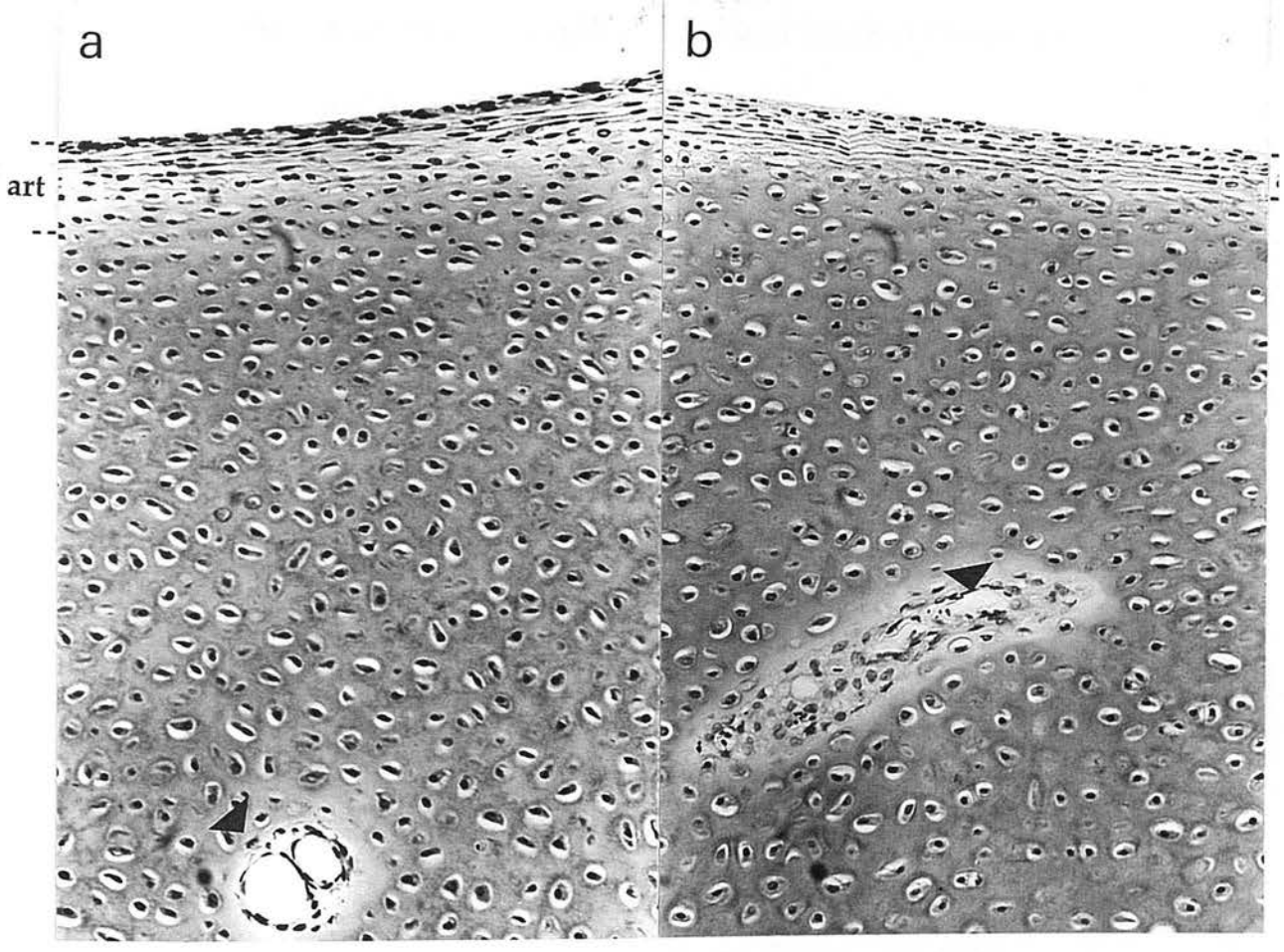


Figure 2.17 - Articular cartilage from the distal tibiotarsus of the (a) layer and (b) broiler strain day-old chicks. The broiler strain articular cartilage (art) is thicker and appears more cellular. The epiphyseal cartilage can be seen below the articular cartilage and contains cartilage canals. (arrows). Haematoxylin and eosin x 70.

2.3.2.4.3 Distal tibiotarsus

The articular cartilage from the DTT was only a few cells thick in both strains. The underlying epiphyseal hyaline cartilage could be identified by the presence of cartilage canals (Figure 2.17). A highly cellular surface layer was found in the cartilage from both strains, below which was a fibrous area two or three cells deep. As with the PH, no linear arrays of chondrocytes were visible in the cartilage from either strain. The surface zone of the layer strain samples appeared thinner and more eosinophilic than that of the broiler strain samples.

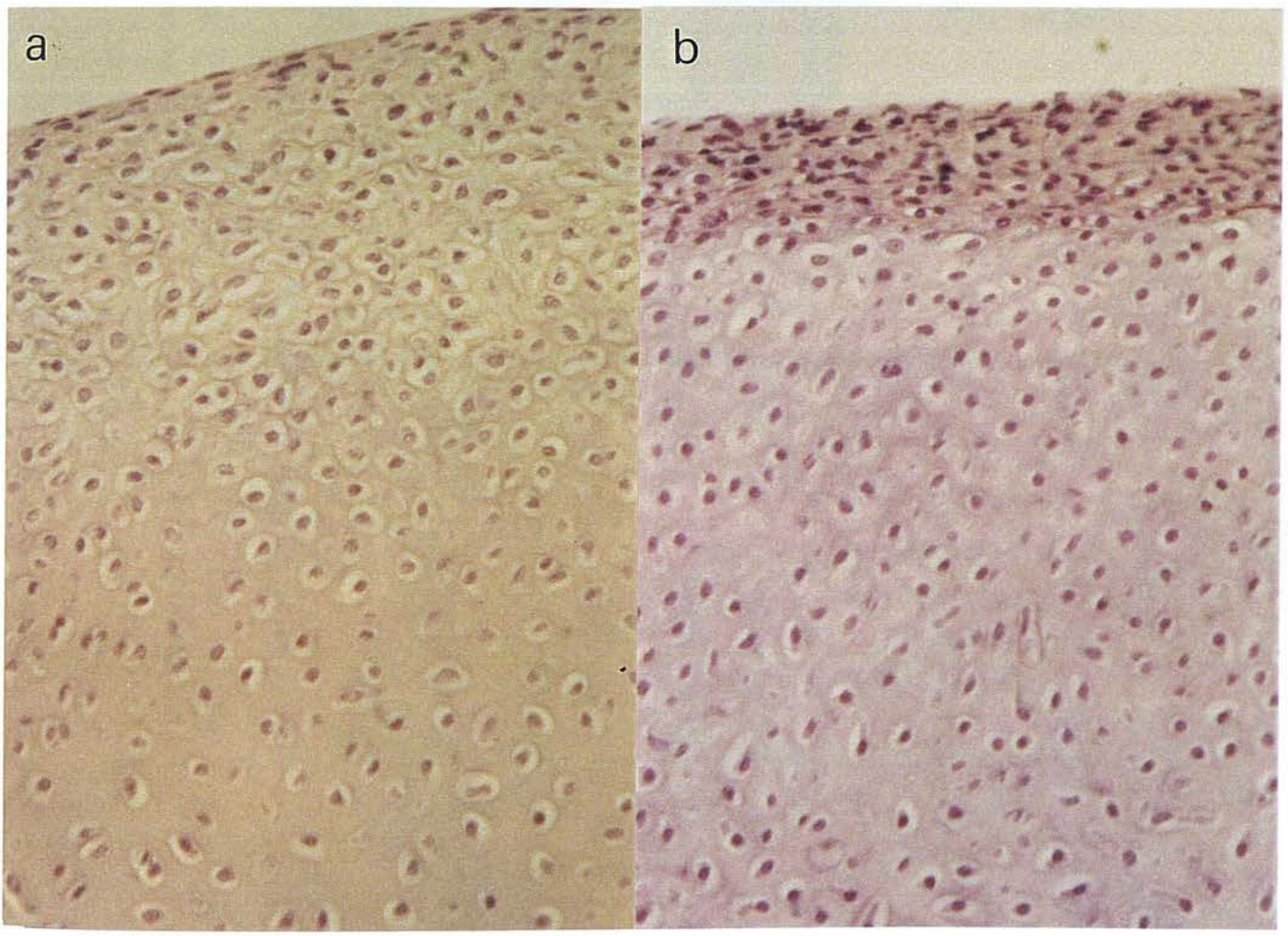


Figure 2.16 - Articular cartilage from the proximal humerus(a) layer and (b) broiler strain day-old chicks. Haematoxylin and eosin x 70.

2.3.3 Comparison between the day-old chick and mature adult data

The cartilage of the day-old chicks was consistently more hydrated than that from the corresponding mature adult joints (Figure 2.18). In general the same trend emerged with the uronic acid data (Figure 2.19) except for the proximal humerus where there was no significant difference in the uronic acid content of the mature adult and day-old samples.

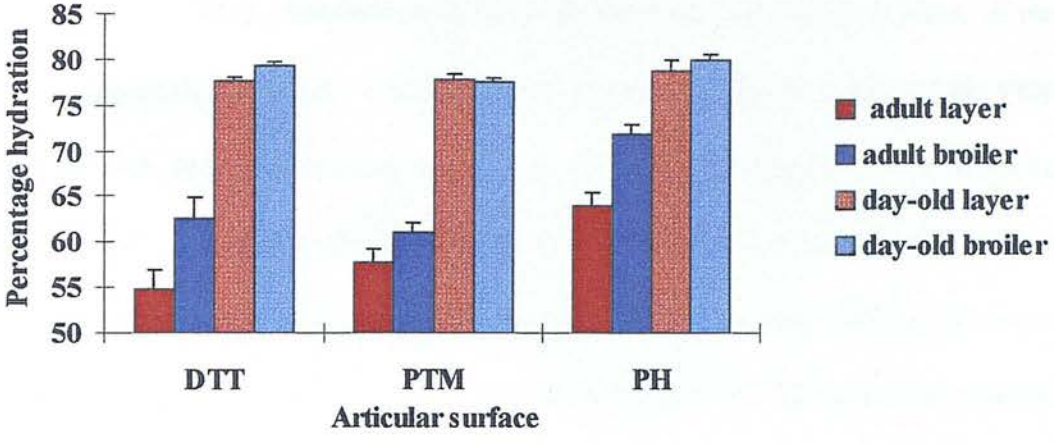


Figure 2.18 - Hydration of the mature adult and day-old chick samples. Error bars are S.E.M.

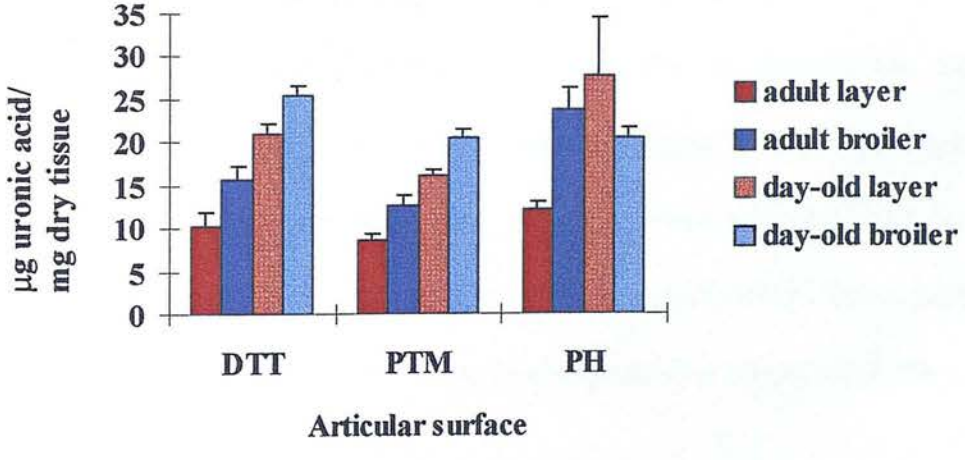


Figure 2.19 - Uronic acid content of the cartilage from day-old chicks and mature adult. Error bars are S.E.M.

2.4 Discussion

The morphology of the articular cartilage from the adult broiler strain fowl varied markedly from that previously reported for adult layer strain fowl (Gentle and Thorp, 1994). The articular cartilage of the adult broiler strain fowl showed pronounced areas of cartilage thinning, loss of surface zone cellularity, pale eosinophilia and pyknotic nuclei; all these histological features are associated with DJD in mammals (Gardner *et al.,* 1987). Extreme changes in the cartilage were noted in some of the adult broiler strain fowl; these included the presence of clumps of chondrocytes deep to the surface zone and (in one sample) an area of total loss of all the articular cartilage with exposure of the underlying bone. No severe cartilage changes were observed in the adult layer strain fowl and the frequency and severity of minor cartilage changes were far less in the adult layer strain fowl. These results confirm that broiler strain fowl have DJD at a greater incidence and severity than the layer strain fowl.

Despite the presence of type I collagen in avian articular cartilage (Eyre *et al.,* 1978), the morphology of avian cartilage appears similar to mammalian cartilage (Stockwell, 1979). The adult broiler strain cartilage in this study presented morphological signs of DJD which were similar to those seen in human DJD and mammalian models of osteoarthritis (Burton-Wurster *et al.,* 1993), this suggests that a common mechanism for articular cartilage degeneration might cross the phylum boundaries.

The biochemical differences between articular cartilage from adult broiler strain and adult layer strain fowl were similar to those observed in mammalian DJD. Increased

hydration and uronic acid content, as shown for the broiler strain fowl, have been reported previously in dog models of DJD in areas where degeneration is expected to occur (McDevitt and Muir, 1976). Increased hydration is a common feature of developing DJD (Muir, 1988) and an increase in proteoglycan synthesis is also associated with early stages of cartilage degeneration in dog models (Muir, 1977) and in other animals including Rhesus macaque monkeys (Brandt, 1993).

The morphology of the articular cartilage of the day-old chicks did not show such marked differences between strains as were observed with the adult, broiler strain and layer strain samples. This suggests that in additon to any genetic developmental factors, environmental factors, such as load on the joint, may affect the morphology of adult articular cartilage.

Analysis of the samples from the day-old chicks shows that, although the hydration results follow the same trend as the adult samples, generally the differences between the joint surfaces were not significant. The broiler strain DTT was an exception. This joint surface was more hydrated and contained more uronic acid than both the layer strain DTT and the broiler strain PTM. These results suggest that, even at one day-old, the broiler strain DTT is different in some way from the layer strain DTT and from the other broiler strain articular surfaces examined. This unique nature of the DTT is confirmed by the results reported in Chapter 4 which show that of the four joint surfaces studied the DTT presented the first and most severe signs of degenerative joint disease in broiler strain fowl.

In the day-old chicks the broiler strain PTM contained higher amounts of uronic acid than the layer strain PTM although there was no significant, difference in hydration. This implies that either there are significantly more keratan sulphate containing proteoglycans in the layer strain cartilage, or that the osmotic drawing power of the higher level of proteoglycan in the broiler cartilage is counteracted by some other factor, possibly greater restraint by the collagen network, which thus prevents the expected greater hydration.

In general, the DNA results from the day-old chick samples are all very similar. The only marked difference is the low level in the proximal humerus of the layer strain fowl. Morphologically, the broiler strain PH articular cartilage did appear much more cellular than the layer strain PH cartilage.

The cartilage samples from the day-old chicks were much more hydrated than those from the mature adults. In new-born humans the water content of articular cartilage is also higher than that of adults (Stockwell 1979). In the avian situation, up until hatching the articular cartilage has had little exposure to loading, which will expel water. That load plays a role here may also be inferred from the fact that the joint surface where the difference in hydration between mature adult and day-old birds was least is the proximal humerus, which is a relatively unloaded joint. However, since this relatively unloaded joint exhibits a difference between day-old chicks and mature adults, load is not the only factor mediating a decrease in hydration with age. Another factor is proteoglycan content, as the mature adult cartilage samples have significantly lower levels of uronic acid than the corresponding day-old samples, with the exception of the proximal humerus. This lack of significant difference

between the uronic acid content of the day-old and mature proximal humerus could be due to a variety of factors including the disease state of the mature adult samples, a high inherent variability and the unloaded nature of this joint surface. A role for load in mediating properties of the cartilage is also suggested by the high levels of hydration and uronic acid in the adult, unloaded, PH compared to those of the other, loaded, joint surfaces.

2.5 Conclusion

These results have shown that despite the previously reported differences between avian and mammal cartilage (Eyre *et al.,* 1978), the morphological and biochemical characteristics of early stage degenerative changes seen in articular cartilage from adult broiler strain fowl closely resemble those seen in mammalian joint disease and joint disease models. Further studies with these fowl should help elucidate the progression of avian degenerative joint disease and may provide a non-intrusive model for investigation of the general disease processes which lead to articular cartilage degeneration.

The DTT has been identified from the day-old chick samples as a joint surface which has a distinctive morphology and biochemical composition. This already suggests it may be vulnerable to degenerative joint disease, as is confirmed by the work presented in Chapter Four. The role of load in affecting avian articular cartilage has been examined briefly by studying the PH (unloaded) in comparison with the two loaded joint surfaces of the PTM and DTT. The role of load in affecting articular

cartilage is a common theme throughout the rest of this thesis. The initial aim of the preliminary study has been achieved in that it has established the feasibility of using samples from birds as young as one day-old for further studies and also it has determined that 5 birds (joints) is a satisfactory group size.

3. Effects on the Articular Cartilage of Mycoplasmal Infection in the Tibiotarsal Joint of Young Fowl

3.1 Introduction

3.1.1 Aim

The aim of this study was to determine the biochemical characteristics of the articular cartilage of joints in which joint degeneration had been induced by using an experimental mycoplasmal infection. This also allowed comparison of the biochemical changes produced in the cartilage by infectious, erosive arthritis with both those described in Chapter 2, which are associated with DJD, and those which are usually associated with an infectious or rheumatoid arthritis.

This study was conducted in collaboration with Dr. Chris Morrow, a visiting scientist to the Roslin Institute from the Victorian Institute of Animal Health, Melbourne, Australia and Dr. Janet Bradbury from the Department of Veterinary Pathology, University of Liverpool.

3.1.2 Mycoplasma

Mycoplasma are the smallest free-living organisms known. They differ from bacteria by lacking cell walls (Stainer *et al.*, 1988). Pathogenic mycoplasma are species and tissue specific. *Mycoplasma synoviae* infection in fowl, although primarily localised to the respiratory tract, can become systemic (Kleven *et al.*, 1991) resulting in an infectious arthritis. This is predominantly localised in the tibiotarsal joint, causing swelling, synovitis and erosive arthritis (Kawakubo *et al.*, 1980). Infection with other strains of mycoplasma, in particular *M. gallisepticum,* has also been associated with infectious arthritis (Bradbury and Garuti, 1978).

3.1.3 *Mycoplasma synoviae* and rheumatoid arthritis

The articular and peri-articular changes seen in chickens infected with *M. synoviae* have been compared with rheumatoid arthritis (RA) in man (Olson, 1959). Joint inflammation and destruction are hallmarks of both diseases (Krane, 1990). In both rheumatoid arthritis and *M. synoviae* infection, the synovium becomes inflamed, forming a pannus which is infiltrated by lymphoid cells (Zvaifler, 1994; Kleven *et al.*, 1991).

Chickens inoculated intra-articularly with mycoplasma can develop acute articular lesions, although the recovery of mycoplasma from the joint is low (Kawakubo *et al.*, 1980). This suggests that *M. synoviae* infection in chickens can cause a chronic immune disease similar to RA. Since the two diseases appear so similar, the biochemistry associated with the mycoplasmal infection might be expected to resemble that seen in RA and in models of RA.

Although adjuvant arthritis in rats is a commonly used model for RA, little is known of the associated connective tissue degradation. Most work has focused on the inflammatory and immunological aspects of the disease (Greenwald, 1993). In other models of RA diseases, biochemical studies of the affected cartilage have been more comprehensive. There is progressive loss of hexosamine content (DeSimone, 1983) and Safranin O staining (Hasty, 1990) in collagen induced arthritis. In rabbit serum immune models of RA there is a substantial loss of proteoglycans as soon as three days after antigen injection (Lowther *et al.*, 1978). This loss is mediated not only by

degradation due to inflammation but also by a reduction in proteoglycan synthesis (Sandy *et al.*, 1980; Malemud *et al.*, 1987). Direct joint injection with live bacterium such as *Escherichia coli* or *Staphylococcus aureus* or even heat treated *Mycoplasma butyricum* will also cause acute cartilage degradation which is associated with proteoglycan depletion (Smith and Schurman , 1983).

3.1.4 Choice of mycoplasmas

Four strains of mycoplasma were chosen for this experiment, 2 *mycoplasma gallisepticum* (S6 and B31/88) and 2 *mycoplasma synoviae* (B94/81 and B31/88). *Mycoplasma synoviae* (Kleven *et al.*, 1991) has been more commonly associated with infectious synovitis than *Mycoplasma gallisepticum* (Yoder *et al.*, 1991). However since Olson *et al.* (1964) isolated *Mycoplasma gallisepticum* from joints of chickens with synovitis, other studies have also shown that *Mycoplasma gallisepticum* can cause synovitis in chickens (Morrow *et al.*, 1990).

3.2 Materials and methods

The fowl used were Ross broiler, relaxed selection line (bred non-selectively for 20 years), stock free from mycoplasmal infection. At 7 days old the birds were injected into the left tibiotarsal joint with either 0.1ml of culture or control media. The mycoplasma strains were kindly provided by Dr. Janet Bradbury. The birds were wing banded in the right wing (band mean mass 0.52g) All the birds were reared together at a low stocking density, in a pen with pig decking and supplementary heat. They were fed *ad libitum* on a conventional broiler diet.

The birds were scored weekly for swelling and lameness. Lameness was scored on a scale of 0 to 3 (0 = not lame) Swelling was scored similarly (0 = no swelling compared to the contralateral joint). It became obvious during the experiment that some birds were lame due to mechanical problems in the joint.

The birds were sacrificed at 5 weeks by intravenous administration of dilute barbiturate. The tibiotarsal joint was dissected out and the distal tibiotarsus was divided in half sagitally. One half was preserved for histology in buffered neutral formalin. The articular cartilage was removed from the other half and placed in a pre-weighed sealed container. This sample was dried, digested and assayed for uronic acid. Results were analysed as described in Chapter 2, with differences being considered significant where $p<0.05$ in a Student's t-test (unpaired).

3.3 Results

Dr. Chris Morrow assessed the histopathology and bone deformity. These aspects of the work has been published elsewhere (Morrow *et al.* 1997), although some of the results are pertinent here.

3.3.1 Histopathology

The 2 *M. Gallisepticum* strains (MG/S6 and MG/B31/85) were initially associated (week 1 post injection, 2 weeks of age) with the most severe joint swelling and lameness. With MG/S6 the severity of lameness and joint swelling increased during

the experiment, while the severity declined in birds injected with MG/B31/85. Less joint swelling and lameness were seen at the start of the experiment in the MS injected birds. These 2 clinical signs tended to remain constantly low in the birds injected with MS/B31/88, but increased over time in those injected with MS/B94/81.

No gross pathology was detected in the hock joints of the controls. All the joints injected with mycoplasma showed a distinct synovitis, irrespective of the mycoplasma species or strain. The signs of articular cartilage degeneration were more variable. Most joints injected with mycoplasma showed many small indentations (pitting) in the articular surface and many showed yellowish discolouration of the synovial fluid. In some instances there was cartilage thinning and some peripheral pannus formation. The prevalence and severity of gross pathology were greatest in the group injected with MG strains, especially MG/S6.

3.3.2 Hydration

The media only group, which showed no signs of lameness, had no significant difference in hydration between the injected and contralateral joints. The groups of cartilage samples from the mycoplasma injected joints all showed significant increases in hydration over the cartilage samples from the media only group. The cartilage samples from the experimental groups also showed increases in hydration in the injected joints over the contralateral joints (Figure 3.1). This was significant ($P<0.005$) except for group S6. The hydration of the cartilage from the contralateral joints of the fowl injected with S6 was significantly higher than both that of the fowl injected with B31/88 and that from the media only group.

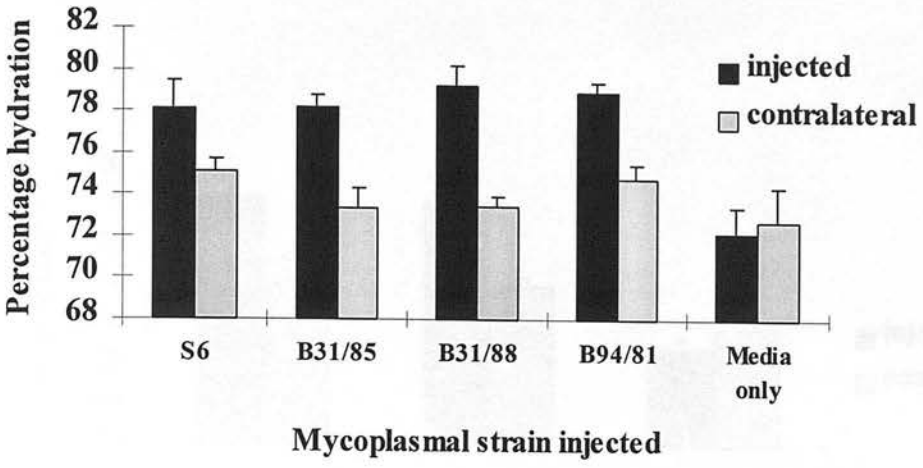


Figure 3.1 - The increase in hydration of the injected joints over the non-injected joints. Error bars are S.E.M.

When the cartilage samples from the non-injected (contralateral) joints were examined there were significant differences in hydration which correlated to the degree of lameness of the bird (Figure 3.2). Birds were designated into three groups: very lame, lame or the media only group. The media only group showed no signs of lameness. The very lame birds had a lameness score of 2 or 3, and a swelling score equal or greater than one, with synovitis and pitting of the cartilage but no fibrosis. The lame birds had a lameness score of one, showed synovitis and pitting and a swelling score greater than or equal to one but no fibrosis. Birds with valgus deformities were excluded from these groups because an aberrant gait affects loading on the joint.

The articular cartilage from the non-injected joints of the very lame birds was significantly more hydrated than the articular cartilage from the non-injected joints of the lame birds and these in turn were significantly more hydrated than the articular cartilage of the media only group.

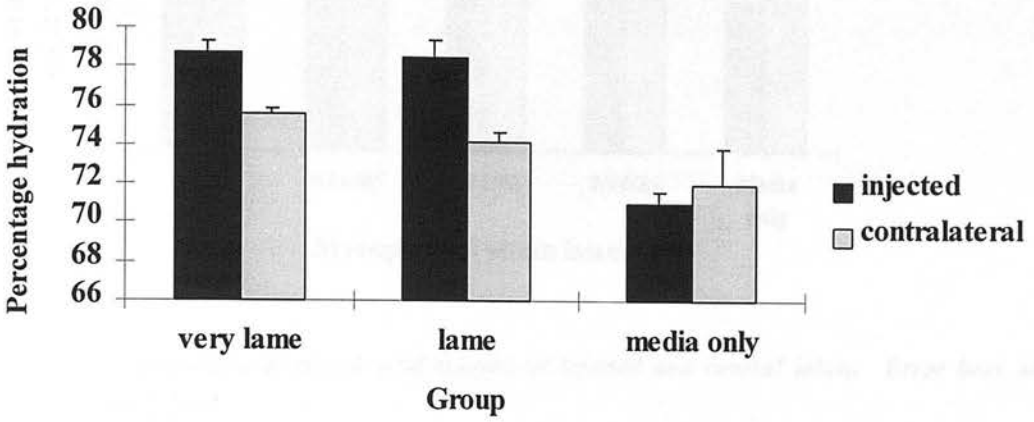


Figure 3.2 - Hydration of the articular cartilage of the samples grouped according to the lameness of the bird. Error bars are S.E.M.

3.3.3 Uronic acid content

Groups B31/85, B31/88 and B94/81 showed no significant difference in uronic acid content between the injected or contralateral legs or when compared to the media only group (Figure 3.3). The S6 group, which presented the most severe gross pathology, did however have significantly more uronic acid in the cartilage from both the injected and contralateral legs when compared to that of the media only group. In the B94/81 group there was significantly more uronic acid in the cartilage of the contralateral joint than in the cartilage from the media only group; this however was not true for the injected joint from this group.

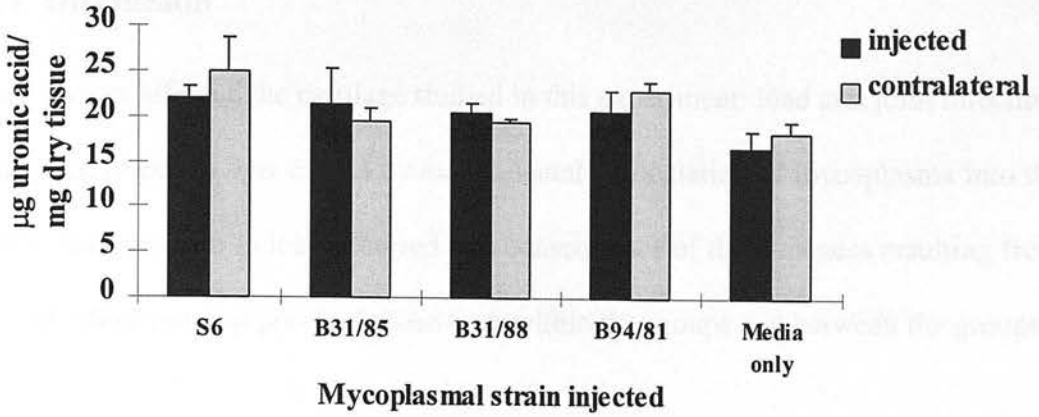


Figure 3.3 - Comparison of uronic acid content of injected and control joints. Error bars are S.E.M.

The same groupings as in section 3.3.2 were applied to the uronic acid results. The articular cartilage samples from the contralateral legs of the very lame birds contained most uronic acid (Figure 3.4), followed by those of the lame birds and in turn the articular cartilage from the birds injected with control media.

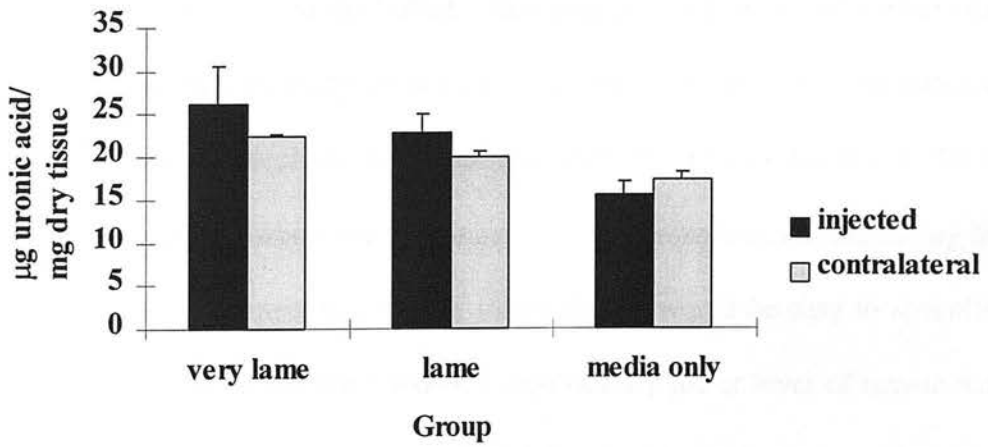


Figure 3.4 - Uronic acid content of articular cartilage from the very lame, lame and media only groups. Error bars are S.E.M.

3.4 Discussion

Two factors affected the cartilage studied in this experiment: load and joint infection. The joint infection was caused by experimental innoculation of mycoplasma into the joint. The variation in load occurred as a consequence of the lameness resulting from the infection and was poorly defined both within the groups and between the groups.

Infection of the joints caused an increase in hydration of the cartilage from all the infected joints in comparison to the cartilage from the media only group. The inflammation and synovitis, which are consequent to the mycoplasmal infection, cause swelling, which by its nature brings fluid into the joint.

As discussed in the introduction to this chapter, infectious/RA type arthritis usually decreases the proteoglycan content in cartilage. This was not observed in the results presented here. There were no significant differences in uronic acid content between the injected and contralateral legs of any group. In two cases there was an apparent (although insignificant) decrease in uronic acid content, (groups S6 and B94/81). These are the two groups where the swelling and lameness were increasing during the experiment. If the experiment had run for longer then it would be easy to speculate that these groups may indeed have shown a significantly lower level of uronic acid content in the cartilage from the injected joint in comparison to that of the contralateral joint. A decrease in proteoglycan content is evident as early as 3 days after antigen injection in serum immune models of mammalian RA (Lowther *et al.*, 1978). Even 3 weeks after injection this effect is not seen here. The severity of the arthritis may be less or just slower developing.

The biochemistry of articular cartilage is affected by load (Urban, 1994). This was also demonstrated in this study by the comparison of groups by lameness. The contralateral leg of the very lame group experienced the most loading, followed by that of the lame group, while both legs of the media only group were equally and much less loaded. An increase in hydration and uronic acid content were detected with increased loading. This concurs with the results obtained elsewhere after immobilisation of one limb (Caterson and Lowther, 1978) where an increase in proteoglycan content was detected in the cartilage from the contralateral limb. The effect of load can also be seen when the cartilage is grouped by strain of mycoplasma injected. The S6 group presented the worst gross pathology and an increasing amount of swelling and lameness during the experiment. The cartilage from the contralateral leg of this group was more hydrated than the cartilage of the media only group and also more hydrated than the cartilage of the contralateral leg of the B31/88 group. The lameness of the S6 birds resulted in the contralateral leg of the S6 birds being more loaded than the contralateral leg of the hardly lame B31/88 group. Again the effectiveness of lameness at inducing load related change in the contralateral leg is demonstrated.

3.5 Conclusions

The data presented here have shown that innoculation with *M. synoviae* or *M. gallisepticum* does not bring about the decrease in proteoglycan content in the cartilage expected in some models of RA type disease. However, this could be due to

any of a number of factors including the variation in individual response to the infection and the speed at which the disease progressed. Or the different response could be due to differences between the aetiology of avian and mammalian infectious arthritis.

By comparing the contralateral legs of the birds which have been grouped by lameness, the effect of load has been investigated. The results of this study have shown how the increased load on the contralateral joint causes increases in proteoglycan content and hydration associated with increased load and early stage DJD. So, by inducing an experimental infectious arthritis in one leg we appear to induce DJD type changes in the other.

4. Rationale and pathology of the longitudinal study

4.1 Introduction

The results of the preliminary study (Chapter 2) clearly showed that there were biochemical and morphological differences between the mature adult articular cartilage of the broiler strain fowl, which are susceptible to DJD, and that of the non-susceptible, laying strain fowl. Such marked morphological and biochemical differences between the strains were not observed at one day old, which suggests that genetically determined events during growth and ageing are the major factors in development of this disease. Previous research into muscoskeletal disease of broiler strain fowl has mainly focused on birds which were culled for lameness or examined at the end of their breeding life (Hocking and Duff, 1989) although a comparative study of development of the antitrochanter has been reported (Hocking *et al.,* 1996). The longitudinal study, described here, was undertaken in order to investigate more completely the effects of age, phenotype, genotype and disease on avian articular cartilage. Groups of fowl were reared under very similar conditions, thus lessening any variation due to environmental factors. Sequential sampling determined when a population as a whole developed DJD, with samples taken from previous time-points being, by definition, in a pre-disease state.

4.1.1 Groups of fowl

Three groups of fowl were used in this study: broiler strain fed *ad libitum,* feed-restricted broiler strain and J-line fowl, (a traditional strain of the brown leghorn type).

The role of body weight in the progression of DJD has long been controversial. In humans, population based studies consistently show that overweight people are at a higher risk of knee OA than non overweight controls (Felson, 1995). Broiler strain fowl have been selected for rapid growth and large mass; this large body weight could be the major cause of susceptibility to DJD. Alternatively, the major cause of the susceptibility to DJD could be a genetic factor or factors, co-selected with the ability to become massive, which produces an inherent predisposition to cartilage weakness. If there is such an overriding genetic factor causing susceptibility to DJD, we might expect broiler strain fowl which are fed *ad libitum* and those which are reared on a feed-restricted diet to develop DJD at the same time.

In the preliminary study (Chapter 2), laying strain birds were used as non-susceptible controls. These fowl were readily available and indeed they were found not to develop DJD. However, laying strain birds have been selected for egg production and the results of this selection process may well affect the articular cartilage in these birds. Cartilage thinning has been reported in laying strain fowl in extreme old age (Yamasaki and Itikuru, 1983) and osteoporosis is common in females of strains selected for egg production (Jordon and Pattison, 1996). In humans, an inverse relationship is seen between osteoporosis and osteoarthritis (Dequeker *et al.*, 1996) which casts some doubt on the suitability of a highly selected layer strain as a control group. A more rigorous control for this study is the J-line fowl. The J-line fowl is a strain of small bodied brown leghorns which have been maintained for many years without selection. No DJD

has been reported in these fowl (Hocking *et al.* 1996). These were therefore chosen to be the third group of fowl in this longitudinal study.

In the results reported here the 3 articular surfaces that had been used in the preliminary study (Chapter 2) and also the antitrochanter (AT) from the hip joint (Figure 4.1) were examined. The hip joint of the birds consists of a ball and socket joint formed by the acetabulum and hemispherical head of the femur, together with and additional articulation between the greater trochanter and the cartilagenous antitrochanter. The trochanter antitrochanter articulation restricts the movement of the hip joint (Nickel et al. 1977). Cartilage degeneration of the antitrochanter has been observed in some broiler strain birds (Hocking, 1992) and in turkeys (Hocking and Lynch, 1991). The degeneration of the cartilage is charactersied by cartilage flap formation and maceration, resulting in the exposure of underlying bone (Duff, 1984).

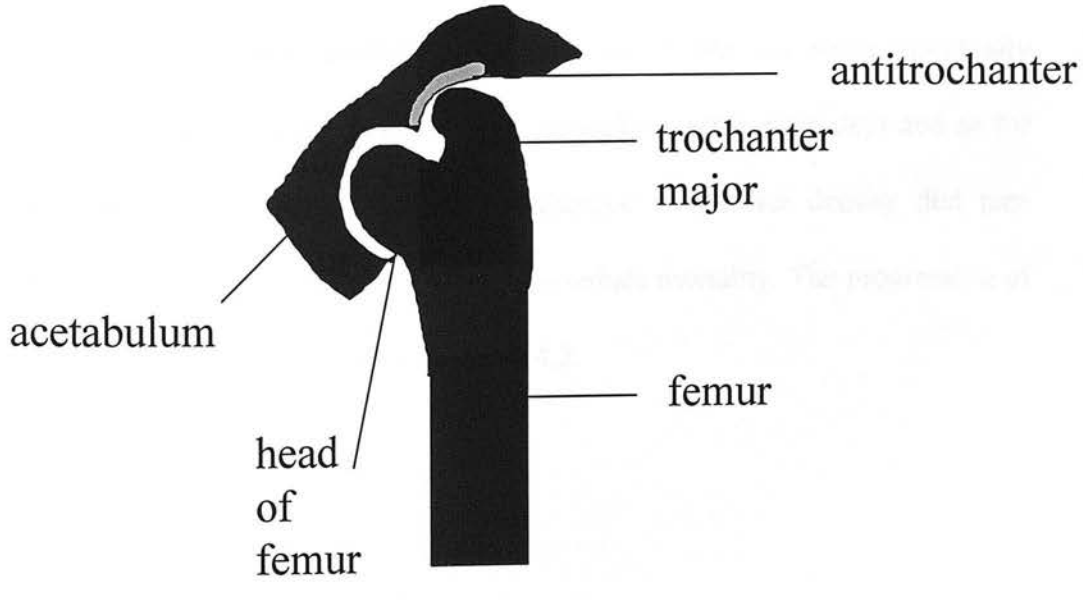


Figure 4.1 - The position of the antitrochanter within the pelvic girdle

The three groups sampled were: *ad libitum* fed broiler strain fowl; feed-restricted broiler strain fowl and J-line fowl. The articular surfaces sampled were DTT, PTM, PH and the AT. Samples from all groups were taken at 1, 19, 61, 79, 113, 180 and 279 days of age. Samples were also taken from the J-line and feed-restricted groups at 376 days of age.

4.2 Rearing the birds

The diet for the feed-restricted group was calculated from the Ross commercial restriction plan (see appendix). Feed-restriction started at 3 weeks, therefore the *ad libitum* results were used for the feed restricted group for the first 2 sample points (day 0 and day 19). The *ad libitum* fed birds suffered particularly from health problems associated with their rapid growth and there was a high mortality rate mainly due to acute cardiovascular problems. In addition, some chronically sick birds were euthanased. This became particularly acute at 56 days and so for animal welfare reasons the birds were changed to a lower density diet (see appendix) to prevent further distress and to reduce mortality. The progression of average mass with age is shown in Figure 4.2.

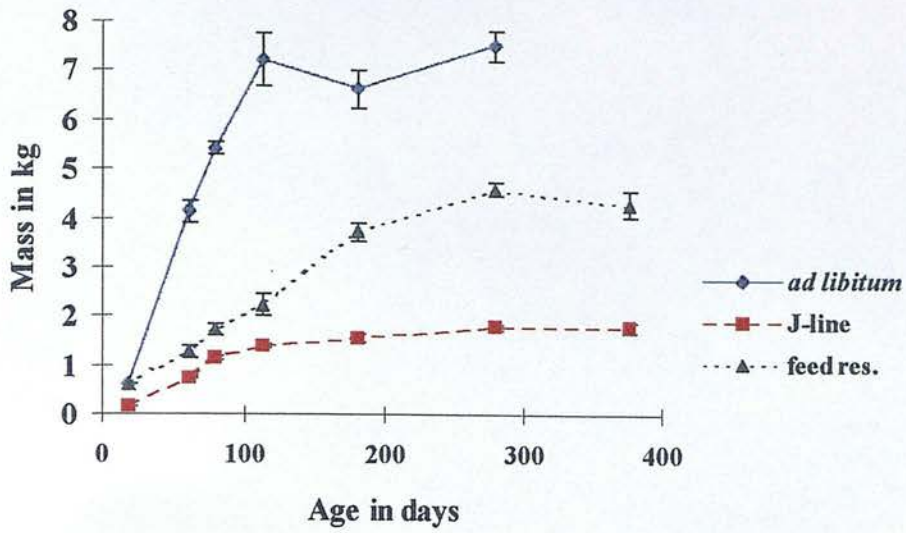


Figure 4.2 Progression of mass with age. Error bars are ± S.E.M. Some error bars are smaller than the symbols.

The J-line birds showed a slow, steady increase in mass. The *ad libitum* birds initially increased greatly in mass, gaining weight by 0.58kg per week during the 3 to 9 week period, compared to a rate of 0.1kg/week for both the feed restricted and J-line birds at the same time. After day 113 the average mass of the sampled *ad libitum* fed broilers dropped and then recovered. This was probably because the less massive *ad libitum* fed broilers survived better. By day 279 there were only 6 *ad libitum* fed birds surviving. All these were sacrificed, although, in order to maintain consistency of group size, samples for this study were only taken from the first 5 birds. The surviving feed-restricted and J-line birds were also sampled at day 376. The difference in size between *ad libitum* and feed restricted broiler strain fowl is illustrated in Figure 4.3.

Figure 4.3 Feed restricted and** ad libitum **fed birds at 20 weeks, the feed-restricted bird (left) is much smaller and has poor comb development in comparison to the** ad libitum **fed bird (right).

4.3 Gross Pathology

Gross pathology was assessed visually as the samples were being collected. Photographs were not taken since the delay involved would have jeopardised the accuracy of the hydration results. The gross pathology is summarised in tabular form in the appendix.

4.3.1 Distal tibiotarsus

From day 79, there was an apparent increase in synovial fluid in the hock joints of the *ad libitum* fed broilers. One of the *ad libitum* fed broiler samples showed signs of cartilage thinning and unevenness in the right DTT. None of the other DTT articular cartilages sampled at this time point showed any signs of gross pathology. At day 113 again one DTT from the *ad libitum* fed broiler group showed thinning and unevenness, which was in the right leg of a bird with a bad left leg valgus deformity.

At day 180 there were signs of severe gross pathology in the DTT of the *ad libitum* fed group. Only one bird in this group showed no pathological change. One bird presented small foci of cartilage thinning in one leg and cartilage thinning and pannus formation in the other leg. The remaining 3 birds (6 samples) showed severe changes in both right and left DTT, 3 of which were characterised by a circumscribed area of cartilage loss 1cm by 0.5cm on the medial condyle with associated pannus formation. Two others showed similar pathology which was slightly less severe and the remaining sample exhibited similar changes of increased severity.

At day 279 the DTT of the *ad libitum* fed broilers exhibited gross changes. Cartilage thinning was observed at the periphery of the joint surface in 6 of the 12 samples examined, though none of the serious pathological changes visible at day 180 were observed. Two of the feed restricted birds at this age also showed cartilage thinning in the DTT of both legs. Two birds of the feed restricted group

at the next time point (day 376) also showed signs of bilateral cartilage thinning in the DTT.

No DJD was observed in the DTT of the J-line birds.

4.3.2 Proximal tarsometatarsus

The first PTM which showed any signs of degeneration was from a feed restricted bird. One bird at day 113 showed cartilage thinning in one PTM. The only other PTM which showed degeneration, were in 2 of the *ad libitum* fed birds at day 279, which showed areas of visible articular cartilage loss on the palmal interchondrial area.

4.3.3 Proximal humerus

Even though the PH is a relatively unloaded joint, signs of gross pathology were visible in some samples. Bilateral cartilage thinning was visible in one bird from the *ad libitum* fed broilers at day 279. Unilateral thinning was also visible in two other birds in this group. At the same time point, one of the feed restricted broilers also had a unilateral peripheral defect with pannus formation in the right PH. At day 376 one of the feed restricted birds showed cartilage thinning in the PH. Cartilage thinning was also visible in all the J-line samples at day 376.

4.3.4 Antitrochanter

At day 279, 4 of the 6 *ad libitum* fed broilers showed degeneration of the AT. In two cases this was bilateral. The degeneration varied from complete loss of articular cartilage and exposure of the subchondral bone through various stages of cartilage loss to cartilage thinning. One sample was very uneven and irregular with many clefts running into the cartilage. At the same time point one of the feed-restricted birds showed mild degeneration of the right antitrochanter. Of the 5 feed-restricted birds sampled at day 376, one bird presented a misshapen AT and 2 other birds showed some unilateral signs of degeneration.

4.4 Histopathology

Samples were taken and processed as in Chapter 2. Sections were originally assessed blindly by Dr. Barry Thorp and the results analysed by myself.

The sample area from which the biochemical and histological sections were taken was kept constant throughout the experiment. This ensured continuity but ocassionally the area which showed severest gross pathology was not sampled providing opportunity for inconsistency between the gross and hisotpathological results. The histopathology results are summarised in the appendix.

4.4.1 Distal tibiotarsus

Four of the 5 samples (right legs sampled) from the *ad libitum* fed group at day 113 showed DJD histopathology, i.e. cartilage thinning and focal poor cellularity. The samples from the other 2 groups did not show any of these features except one J-line sample which showed a variable cellularity in the surface zone.

Of the 10 samples studied from the *ad libitum* fed group at day 180, 7 presented histopathological signs of DJD. These varied from a slight loss of surface zone cellularity and articular cartilage thinning through to complete loss of articular cartilage with pannus formation. One of the 10 DTT samples from the feed restricted group at this time point showed slight cartilage thinning whilst one showed focal matrix degeneration and chondrocyte cluster formation. No signs of cartilage degeneration were visible in the J-line samples.

At day 279, despite the fact that grossly the DTT of the *ad libitum* fed birds presented some cartilage thinning, no histological signs were seen in the sections. One of the 10 J-line DTT samples showed some focal loss of cellularity and cluster formation and one of the 10 DTT samples from the feed restricted group showed some focal loss of surface zone cellularity.

At day 376, two of the ten J-line DTT samples studied showed some focal loss of surface or middle zone cellularity. One of the 10 DTT samples from the feed restricted group showed reduced surface zone cellularity.

Examples of the histopathology of the DTT are shown in Figure 4.4 -Figure 4.6.

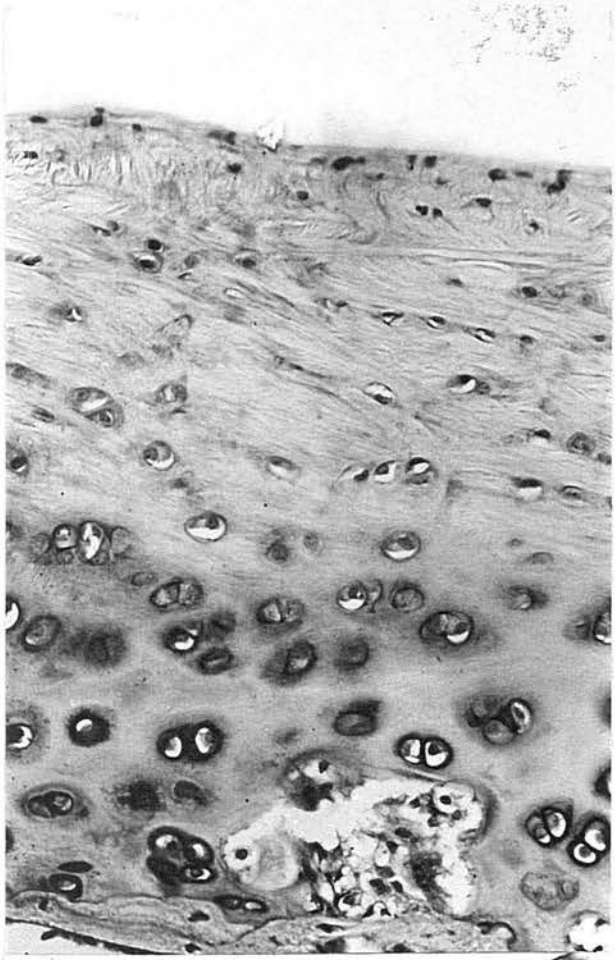
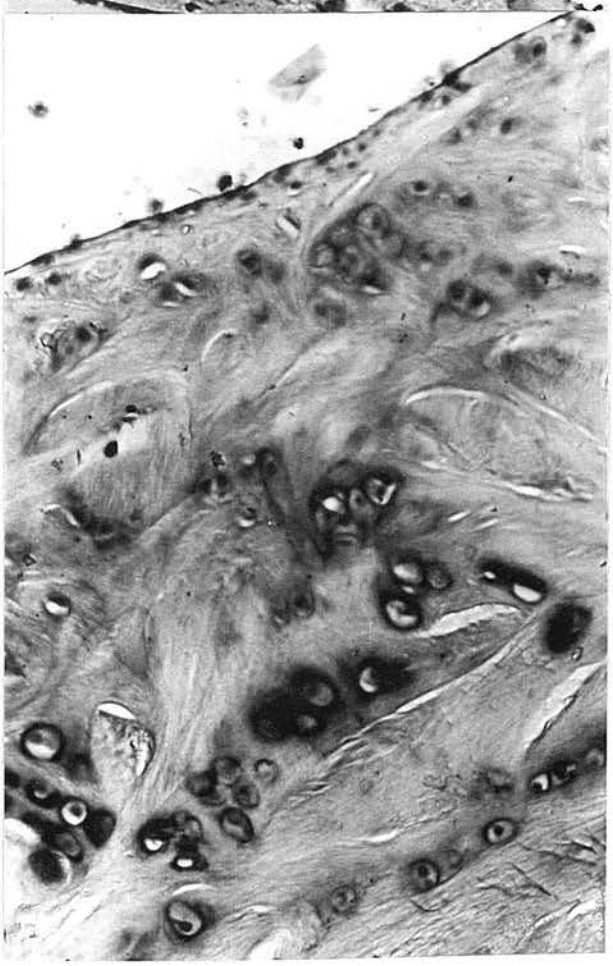


Figure 4.4 - Histopathology of the DTT. Haematoxylin and eosin x70

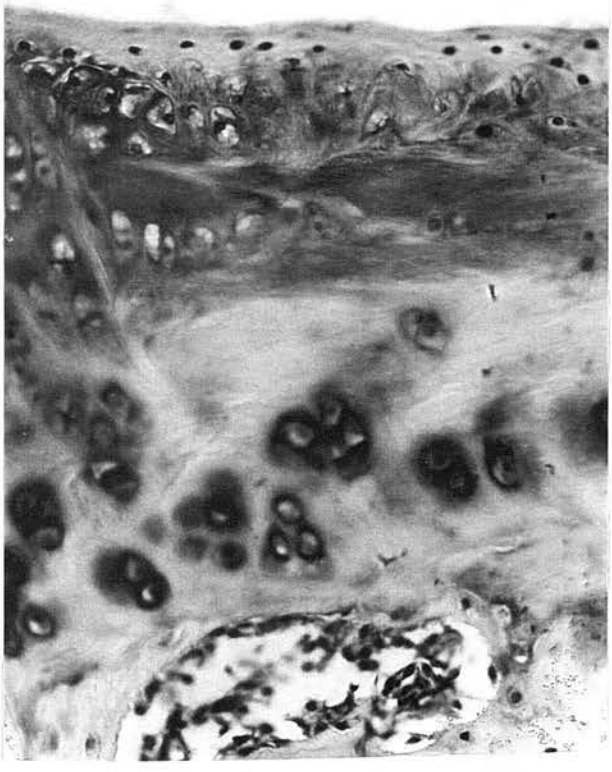
(a) normal articular cartilage



(b) disrupted cartilage showing areas of acellularity fissures in the cartilage and basophillic matrix surrounding mid-zone chondrocytes. Also there is loss of the surface zone.

Figure 4.5

(a) Acellularity of DTT surface zone and basophillic matrix surrounding deep zone chondrocytes. Haematoxylin and eosin x70



(b) Area of DTT cartilage thinning and focal acellularity. Haematoxylin and eosin x70

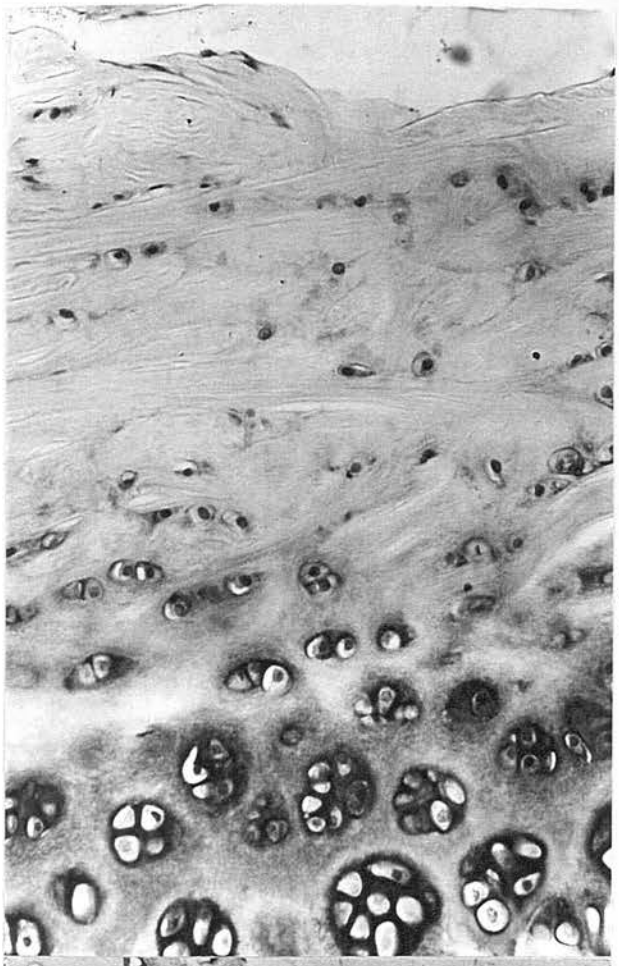
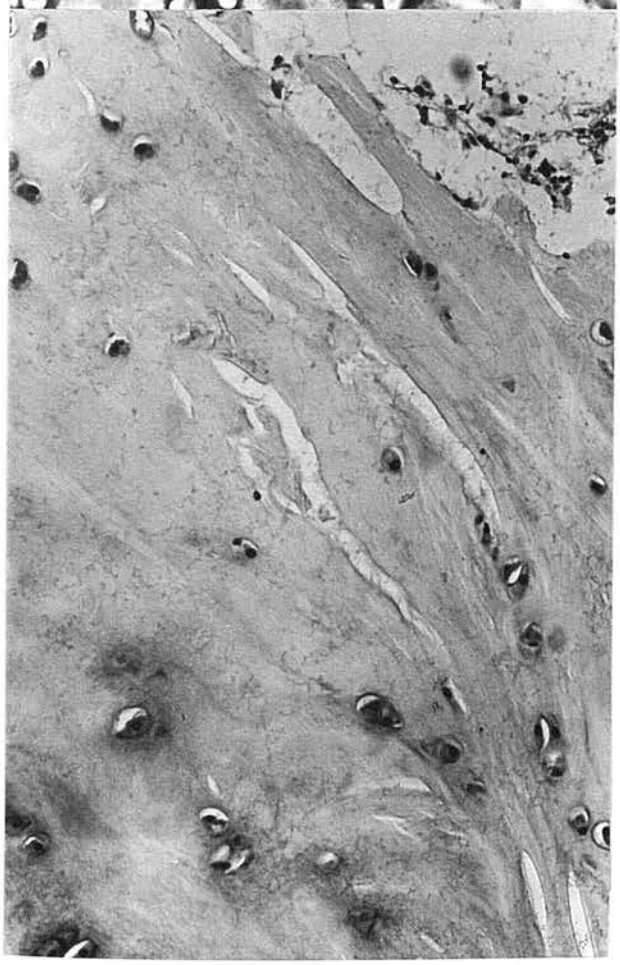


Figure 4.6 - Examples of severe histopathology seen in the DTT. Haematoxylin and eosin x70

(a) Loss of surface layer and acellularity in middle zone, the deep zone is populated with chondrocyte clusters.



(b) An area of severe acellularity which is accompanied by splits in the cartilage and loss of surface layer.

4.4.2 Proximal tarsometatarsus

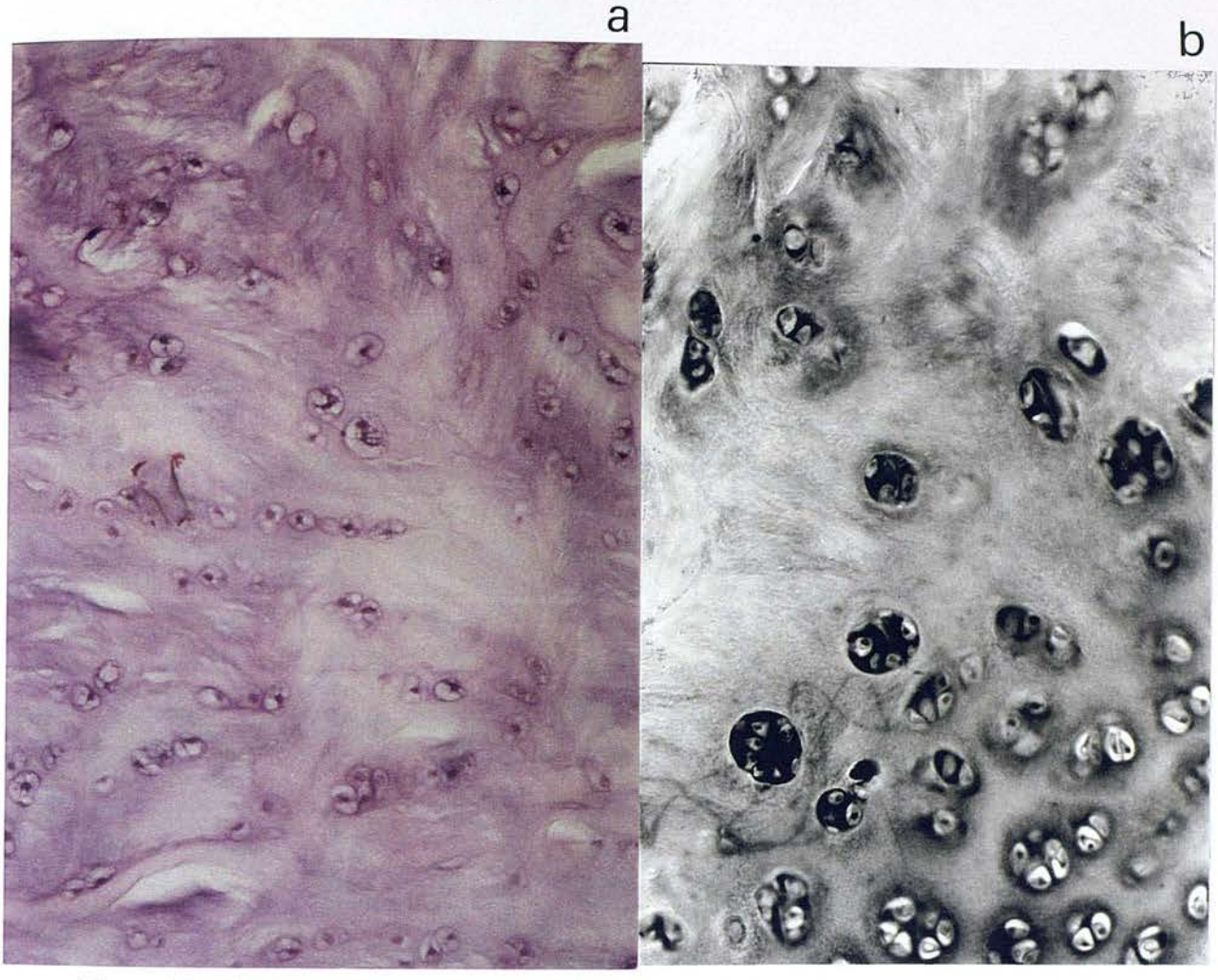


Figure 4.7 - (a) Degeneration of middle zone chondrocytes in the PTM of an ad libitum fed bird, (180 days). (b) Chondrocyte cluster formation in the one example from the PTM. Haematoxylin and eosin x70

At day 113, one of the PTM samples from the *ad libitum* fed group showed focal defects of the superficial and middle zones, which were also observed in one PTM sample from the feed restricted group. One sample from the feed restricted group showed focal chondrodegeneration in the deep zone. One PTM sample from the J-line group showed loss of the surface layer with underlying fibrillation and matrix degeneration. Many PTM samples from all 3 groups at day 180 showed some signs of chondrocyte degeneration in the middle or deep zones (Figure 4.7) including 6 out of 10 samples from the *ad libitum* fed group, 3 out

of 10 samples from the feed restricted group and 2 out of 10 samples from the J-line group.

At day 279, 2 of the 10 PTM samples from the *ad libitum* fed group and one of the 10 PTM samples from the J-line group showed some loss of surface cellularity. One PTM sample from the feed restricted group and one PTM sample from the J-line group also showed basophilic pools around many mid zone chondrocytes. At day 376, 2 PTM samples from both the J-line and feed restricted groups showed focal loss of cellularity in the surface or middle zone.

4.4.3 Proximal humerus

In general, few degenerative changes were seen in the PH. At day 113, one of the PH samples from the *ad libitum* fed broiler group showed focal developing OA with altered matrix, acellularity and fibrillation. Another PH sample from the *ad libitum* fed broiler group showed small foci of articular cartilage thinning. At day 180, 3 of the 10 PH samples from the *ad libitum* fed group and two of the ten PH samples from the feed restricted group, showed variable cellularity of the surface zone. No histopathology was seen in the PH samples from the J-line group at this time point. At day 279, one PH sample from the *ad libitum* fed group and one PH sample from the feed restricted group showed some loss of cellularity and the PH sample from the feed restricted group also showed some unmasking of fibrils in the mid zone (Figure 4.8). At day 376 no degenerative changes were seen in any of the PH samples.

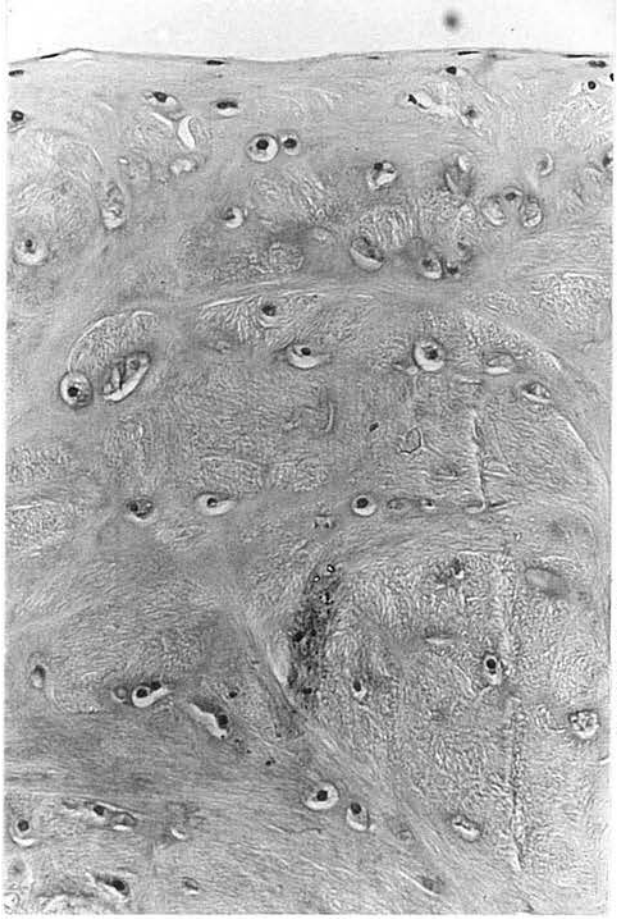
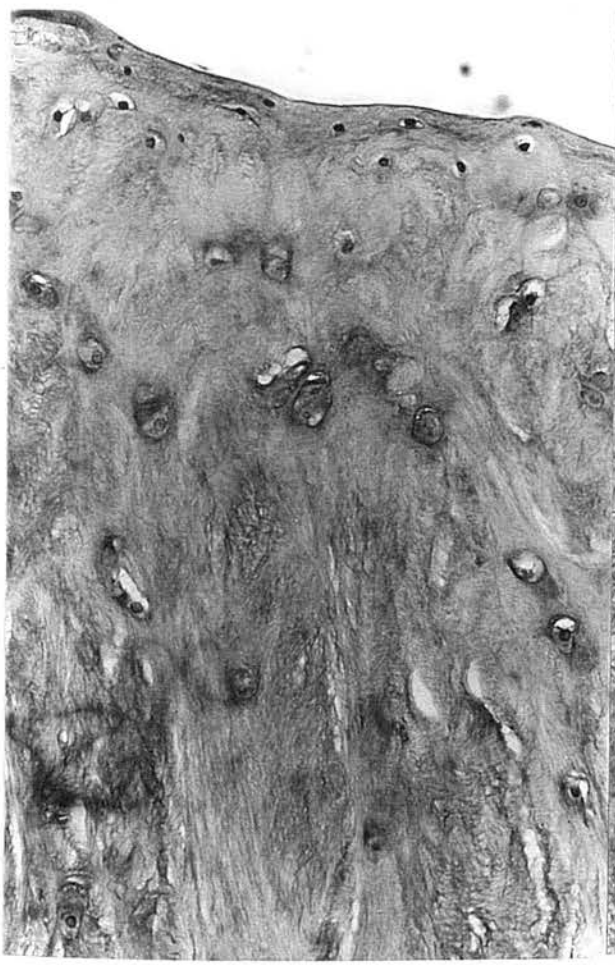


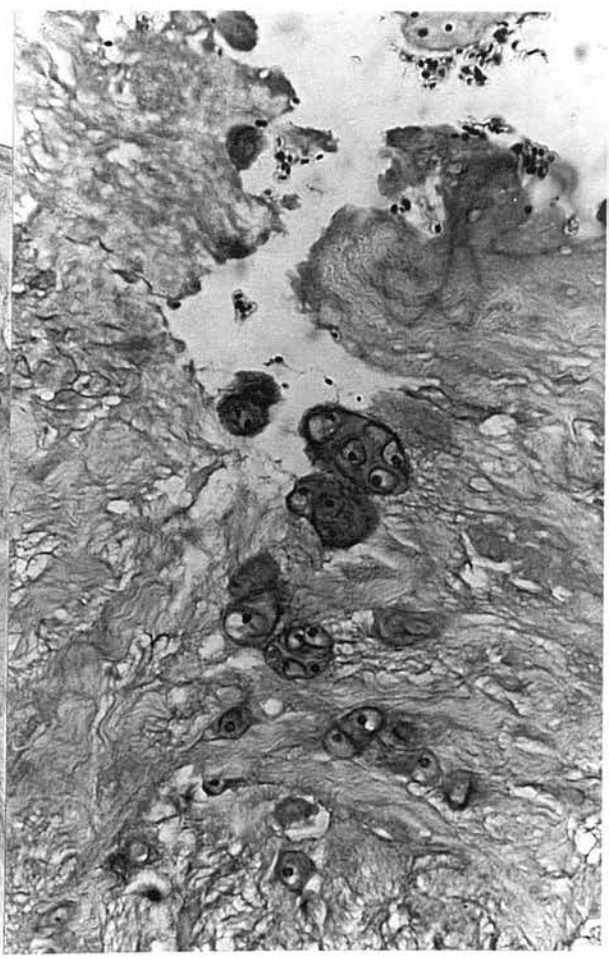
Figure 4.8 - Mid zone degeneration and acellularity in a sample from the proximal humerus Haematoxylin and eosin x70.

Figure 4.9 - (below) (a) An example of mid zone degeneration in the AT (b) Formation of chondrocyte clusters in a degenerate AT. Haematoxylin and eosin x70.

(a)

(b)





4.4.4 Antitrochanter

Obtaining representative samples for histology in addition to the samples for biochemistry from the AT was technically difficult due to the small overall size of the samples. Therefore some of the sample sets are incomplete. It was also felt that histological sample frequently may not have represented the appearance of the remainder of the antitrochanter adequately (B.H. Thorp personal communication).

At day 113 the only histopathology observed in any of the AT samples was a very slight suggestion of cartilage degeneration and altered cellularity in one of the *ad libitum* samples.

At day 180 one of the *ad libitum* fed AT samples showed chondrocyte cluster formation. Six of the 10 *ad libitum* fed AT samples showed mid-zone chondrocyte degeneration and 3 samples had basophillic lakes surrounding the chondrocytes in the middle zone (Figure 4.9). Four of the 10 *ad libitum* fed AT samples showed surface zone variability. Five out of 8 AT samples from the feed restricted group at day 180 also showed chondrocyte degeneration in the mid-zone, which was also detected in one J-line AT section at this time point.

Of the 5 samples from the *ad libitum* group at day 279, 2 showed chondrocyte cluster formation and another showed middle zone chondrocytes in pools of basophillic matrix. No histopathology was observed in the other samples from this time point.

Of the samples taken at day 376, one AT sample from the feed restricted group showed some reduced cellularity of surface zone and a tendency for the chondrocytes to be in linear arrays, however all the other samples at this time point showed no sign of cartilage degeneration.

4.5 Discussion

4.5.1 Load, body mass and obesity

The *ad libitum* fed birds grew very fast and to a much larger size than the genetically identical, feed-restricted birds. The mass of the *ad libitum* fed birds was approximately 4 times higher than that of the feed restricted group at days 61, 79 and 113. The metabolism of the *ad libitum* fed birds would have been different in many ways to that of the feed-restricted birds, however a major factor affecting the articular cartilage of these birds would have been load. Variations in loading of cartilage are known to have many physiological and sometimes pathological effects on articular cartilage (Urban, 1994). Although the feed-restricted birds are much lighter than the *ad libitum* fed birds, there is almost no difference in the surface area of the joints. For example, the average diameter of the DTT at 180 days is 18mm in both groups. This similarity in joint surface area and the large difference in mass leads to a major difference in cartilage loading between the *ad libitum* fed and feed restricted birds. There may still be a role for a systemic factor in the induction of DJD in the heavier birds, however this factor undoubtedly will act in addition to, and might even be produced as a result of, the increased load on the cartilage. Unlike most human cases where the association

between obesity and OA has been studied (Hochberg *et al.*, 1995), the heavy birds did not have large amounts of body fat. Instead the majority of the increase in weight was due to selection for large deep and superficial pectoral muscles. The role of adipose tissue in develpment of DJD is uncertain; it has been proposed that in humans it may contribute to the production of systemic factors which cause or exacerbate DJD (Hart *et al.*, 1995).

4.5.2 Mortality and morbidity

Feed restriction is generally known to increase the life span of animals (Monnier, 1990). This was certainly the case in the present study. The population from the *ad libitum* fed birds was biased to some extent in that only the surviving birds from a large number (140) were available. The *ad libitum* fed birds at day 180 show a great prevalence of DJD. These, since they are surviving must be assumed to be generally more healthy birds. Only 6 of the *ad libitum* fed birds survived to day 279, these 6 birds must have been amongst the very healthiest of the group and although the samples showed some gross signs of cartilage thinning there were no histopathological signs of DJD. These surviving, healthy birds were not suffering from DJD. Although DJD is not a fatal disease of itself, if the pain it produces has influenced the mortality of other diseases and chronic disorders, the survival of the last cohort of *ad libitum* fed birds will have been positively influenced by the absence of DJD.

4.5.3 Genotype and the development of DJD

Only a few of the J-line samples showed any histopathological signs of DJD. A few did show some signs of mid zone chondrocyte degeneration and even less showed any of the classical signs of DJD, i.e. cartilage thinning, acellularity or fibrillation. This low incidence of cartilage disruption vindicates the choice of J-line as the control group. The lack of DJD symptoms is also in agreement with the study of antitrochanteric development carried out by Hocking *et al.* (1996) The J-line proximal humeri at day 376 did show some gross peripheral cartilage thinning. However there was no histopathology was observed in the samples from these joints. This suggests that this thinning effect may not be a pathological change but could, instead be age related (Meachim, 1969). The incidence of thinning in all the J-line PH samples at this time point supports this hypothesis and emphasises the difficulties in distinguishing age and disease related change (Gardner, 1987).

The broiler strain birds did develop DJD, which confirms the susceptibility of this strain to DJD. The *ad libitum* fed birds developed DJD earlier, and much more severely than the feed-restricted group. A low incidence of DJD in feed-restricted broilers has been reported previously (Hocking 1994). This suggests that the overriding factor leading to the development of DJD in broiler strain fowl is not simply genetic but is in fact related to the mass of the bird. It could be purely a physical inability of the cartilage to withstand the size of the load, or there could be a genetic factor which weakens the cartilage and produces this inability to withstand loading. However, since the feed restricted birds develop

significantly less DJD than the *ad libitum* fed birds, a catastrophic genetic weakness in the broiler strain cartilage has been ruled out by this experiment. Together these results suggest the interaction of genotype and environment to cause DJD in broiler fowl.

4.5.4 Joints which develop DJD

The DTT was the joint surface which developed the first and most severe signs of the disease, as shown by the classical signs of DJD, including chondrocyte clustering, fissures in the cartilage, cartilage thinning and erosion. The opposing articular surface the PTM, also developed histopathological signs, but these were very different to those seen in the DTT. These changes involved apparent chondrocyte degeneration in the middle zone and the appearance of basophillic lakes. These were not restricted to the *ad libitum* fed broilers as the histopathology seen in the DTT. In general, the PTM did not pass through this stage into a full degeneration of the cartilage.

Even at one day old, the broiler strain DTT is morphologically and biochemically different from the layer strain DTT and from the other broiler strain joints. One can therefore speculate that this joint surface, which fares so badly in DJD in comparison to the other joints surveyed, is somehow predisposed to degrade (This is discussed further in Chapter 8).

The pathology of the AT appears at first to be similar to that of the PTM, with chondrocyte degeneration in the middle zone observed in all 3 groups at the middle time points. Although there is apparent mid-zone cartilage degeneration in all groups at earlier time points, this appears only to progress to classical DJD in the *ad libitum* fed broiler fowl, where chondrocyte cluster formation can be seen in some of the later samples. This again suggests that the cartilage of the *ad libitum* fed birds is more susceptible to DJD than that of the other two groups.

Some of the PH samples do show histopathological signs of DJD. This confirms the findings of the preliminary study (Chapter 2). The PH is a relatively unloaded joint and yet it develops DJD even though there is little possibility of increasing load playing a role. This is a similar situation to the development of hand OA in overweight patients (Felson, 1995) and suggests a systemic factor may be influencing the cartilage. The birds which develop PH DJD are feed-restricted and *ad libitum* fed broilers, so possibly a genetic factor is responsible. Since these birds do not fly, these PH samples may be abnormally under-loaded. The PH is designed to take the load of the whole mass of the bird in flight. Instead of suffering an excess of load, which is seen in the hock and hip joints of the *ad libitum* fed birds. These PH samples are relatively unloaded. Lack of load does affect cartilage, altering proteoglycan metabolism and in some cases causes degenerative changes (Buckwalter, 1995). It may be that the pathological changes witnessed in the PH samples are as a result of this lack of load.

4.6 Conclusions

In this study, cartilage degeneration was detected which was morphologically similar to that observed in the preliminary study and other instances of DJD. The *ad libitum* fed broilers develop DJD first and more severely than the feed restricted birds. This shows that body mass plays an important role in the development of avian DJD.

5. Biochemical analysis and histochemistry of samples from the longitudinal study

5.1 Introduction

These results describe the basic biochemical pattern of cartilage development during the longitudinal study. Sampling at various time points allowed pathological changes to be assessed by observation of gross and histopathological alterations in the morphology of the cartilage samples. Thus it was possible to determine which cartilage was degrading and to correlate this with any biochemical changes observed.

The exact nature of the relationship between alteration in biochemical parameters of articular cartilage and development of DJD is unclear. There are, however, three obvious possibilities. Observation of change in articular cartilage biochemistry could indicate that a pathological change has occurred, or it could indicate that a pathological change in the cartilage was about to occur as a result of the altered biochemistry. Also pathological change could occur concurrently with, but not as a result of, biochemical alteration in the articular cartilage.

The biochemical parameters investigated in the longitudinal study are general but provide useful information on the development of articular cartilage as a whole. Analysing such a broad base of parameters identifies areas where further, more specific analysis would be appropriate. The biochemical parameters investigated here are hydration, uronic acid, DNA and sulphated glycosaminoglycan (SGAG) content.

Hydration is important since water plays such a vital functional role in articular cartilage and increase in hydration has been widely associated with DJD (Venn and Maroudas, 1977). Uronic acid content is an important indicator of proteoglycan content. Assaying for sulphated glycosaminoglycan examines the proteoglycan further and determines how sulphation is related to proteoglycan content in the tissue. DNA content is an indication of cellularity, alterations in which may be implicated in the onset of disease or associated with a proliferative response to disease.

By investigating these parameters, in the context of the pathological changes observed, the biochemical changes in the articular cartilage which occur with normal development and those which are associated with disease can be determined.

5.2 Materials and methods

Cartilage samples were obtained as described in Chapter 4 (Section 4.1). The joint was dissected sagitally and a piece of tissue from this plane was fixed in buffered neutral formalin and processed for histology (Section 2.2.2). Cartilage samples were removed from the remaining part of the articular surface. All the samples were weighed, dried overnight at 60°C, then reweighed, and hence hydration was calculated. Samples were digested with SDS/proteinase K (Lipman 1989) as in Chapter 2. The carbazole assay was carried out on the SDS/proteinase K digests using a method adapted for microtitre plates (Riley,

1993), which were measured using a Dynatech MR7000 plate reader and RMS software. The microtitre plate method greatly improved the safety and efficiency of this assay. DNA assays were carried out on the SDS/proteinase K digests as described by Lipman (1989), using a Perkin Elmer fluorimeter, (excitation 450nm, emission 340nm). The results for this assay were calculated using Microsoft Excel.

It became apparent that the SDS in the Proteinase K digestion mixture significantly interfered with the di-methylmethylene blue dye binding assay for sulphated glycosaminoglycans (Farndale *et al.*, 1982). Therefore additional samples were taken from time points 180, 279 and 376 days, which were digested with papain (see appendix) and these samples were assayed for SGAG using a method adapted for use in a microplate reader.

The results of the assays were calculated using Microsoft Excel and analysed by using unpaired t-tests. Where $p<0.05$ when comparing 2 groups, it was considered to indicate a significant difference between the 2 groups.

5.3 Age related change in articular cartilage biochemistry

5.3.1 Hydration

The hydration data for all the samples, over the time course of the experiment are shown in Figure 5.1 and Figure 5.2. In general, hydration in all samples at one-

day old is approximately 80%, which then decreases with ageing. The hydration of the cartilage from the *ad libitum* fed broiler group remained consistently higher than that of the feed-restricted and J-line group. Only the PTM of the *ad libitum* fed broiler group fell below 68% whilst at equivalent time points the hydration of the majority of feed restricted and J-line samples had decreased to below this level.

In all groups, the PH was the sample with the highest hydration. The hydration of this cartilage showed a consistent decrease with time except in the J-line fowl for time points 276 to 376 days which saw a significant increase in hydration. This is concurrent with the development of gross cartilage thinning in these samples although no histopathology was visible.

The hydration of the AT fell broadly into the same area as that of the 2 indisputably loaded joints, the DTT and the PTM. The hydration of this joint surface also decreased with time. There was a significant increase in the hydration of the J-line AT between one day old and 19 days.

The hydration of the PTM decreased in all groups until day 279 and then increased slightly in the feed restricted and J-line groups. In the *ad libitum* fed broiler group the PTM was the least hydrated of the joint surfaces investigated. Although it converged and was similarly hydrated to the DTT at day 113 the hydration then diverged again with the hydration of the PTM decreasing further.

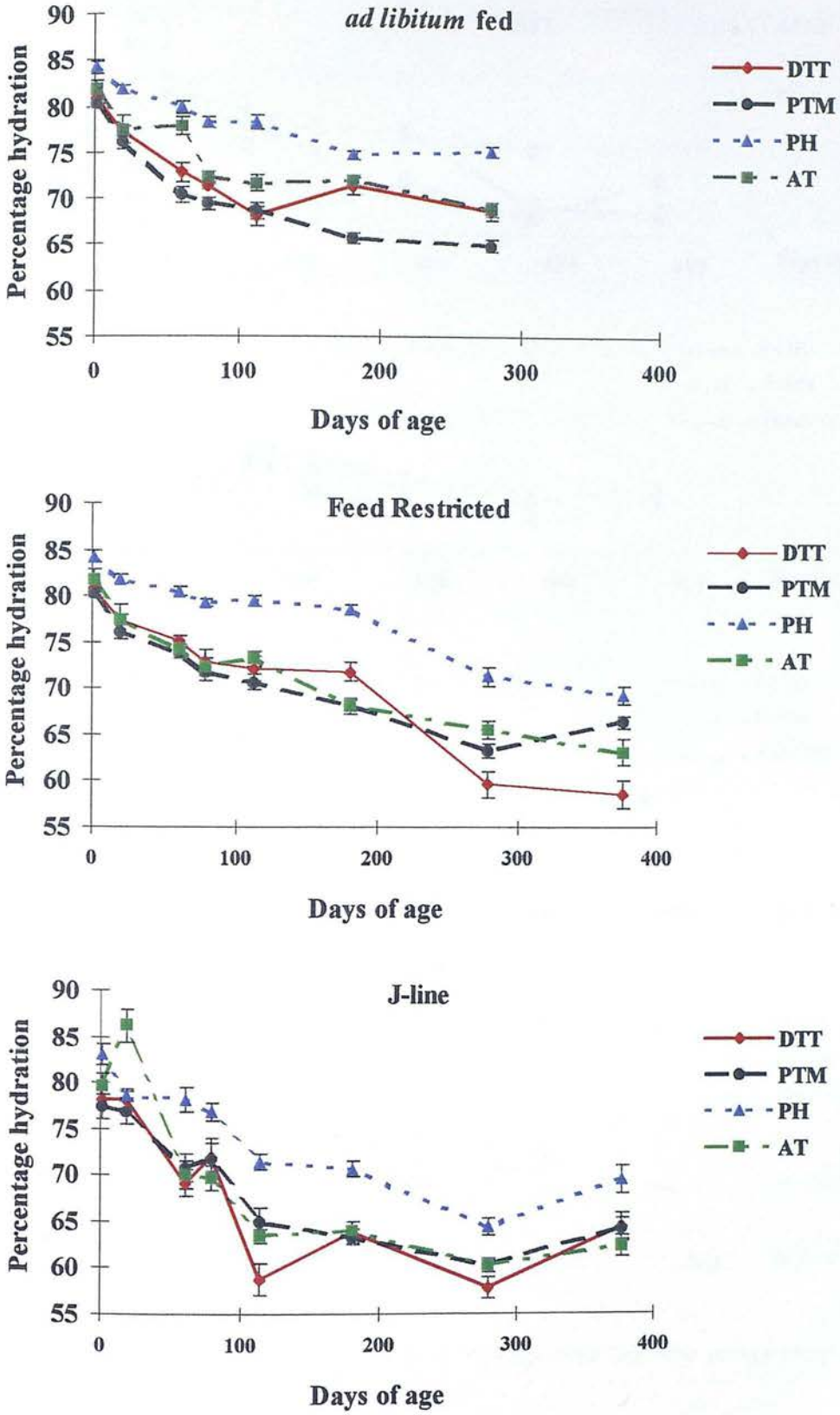


Figure 5.1- The hydration of the articular cartilage from the three groups over time. Hydration expressed as percentage of wet weight. Error bars are S.E.M.

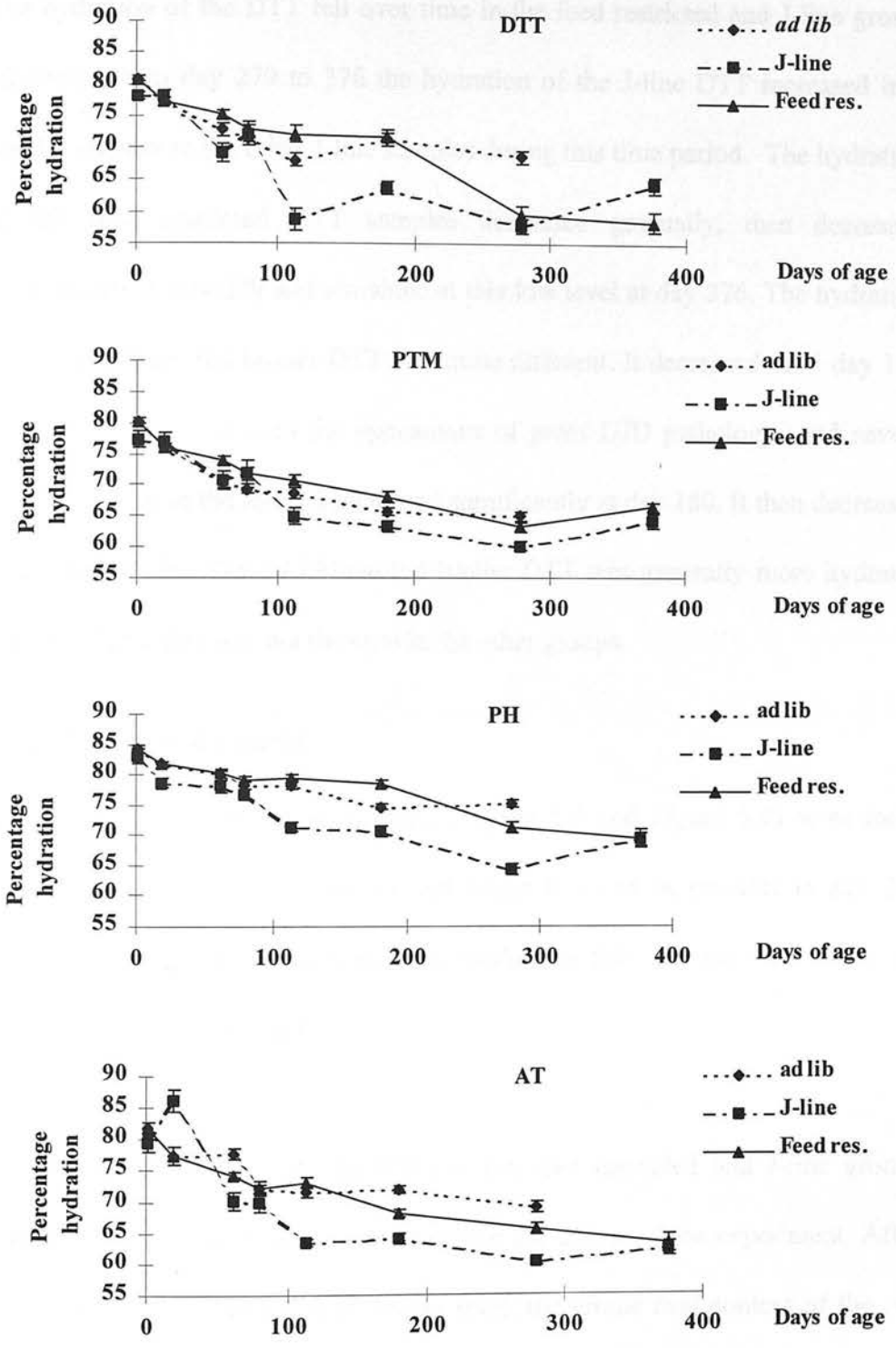


Figure 5.2 - The hydration of the articular cartilage from the three groups compared by articular surface. Error bars are S.E.M.

The hydration of the DTT fell over time in the feed restricted and J-line group. However, from day 279 to 376 the hydration of the J-line DTT increased in a similar manner to the other J-line samples during this time period. The hydration of the feed restricted DTT samples decreased gradually, then decreased significantly at day 279 and remained at this low level at day 376. The hydration of the *ad libitum* fed broiler DTT was quite different. It decreased until day 113 and then, concurrent with the appearance of gross DJD pathology and severe histopathology in the joint, it increased significantly at day 180. It then decreased again at day 279. The *ad libitum* fed broiler DTT was generally more hydrated than the PTM; this was not the case in the other groups.

5.3.2 Uronic acid content

In general the results for uronic acid (Figure 5.3 and Figure 5.4) were more variable than the hydration results and whilst it might be possible to say that overall there appears to be a decrease with time this was not true in for all articular surfaces examined.

The uronic acid content of the PTM of the feed restricted and J-line groups actually remained fairly constant throughout the course of the experiment. After an initial decrease from a much higher level, the uronic acid content of the AT became similar to that of the PTM in both these groups. In the J-lines it increases above that of the PTM at day 376. In the feed restricted group the uronic acid content of the AT decreased below that of the PTM at day 180. Apart form these

two exceptions, after 19 days the uronic acid content of the AT and PTM were not significantly different in either the feed restricted or J-line groups .

The uronic acid content of the PH in the J-line fowl was generally the highest and this was also true, to a lesser extent, for the feed-restricted group. This was not true for the *ad libitum* fed broilers where, apart from at one day old, the DTT had a similar or higher level of uronic acid than the PH. The uronic acid content in the J-line DTT was variable, after an initial increase at day 19 it then decreased and followed the variation in hydration seen in this joint. The uronic acid content in the DTT of the feed restricted group remained steady and then fell at day 180. This was again similar to the pattern observed for the hydration results, except for this sample the decrease in hydration did not occur until day 279.

The uronic acid content of the PTM in the *ad libitum* fed broiler group decreased to day 79 and then increased again. The same was true in the AT where the increase occurred at day 180. These changes were not observed in the hydration results. The uronic acid content of the DTT in the *ad libitum* fed broilers decreased slightly and then increased sharply at day 180. This increase was concurrent with the development of gross pathology and severe histopathology in this joint surface.

The uronic acid content of the articular cartilage samples from the three groups is shown in Figure 5.4.

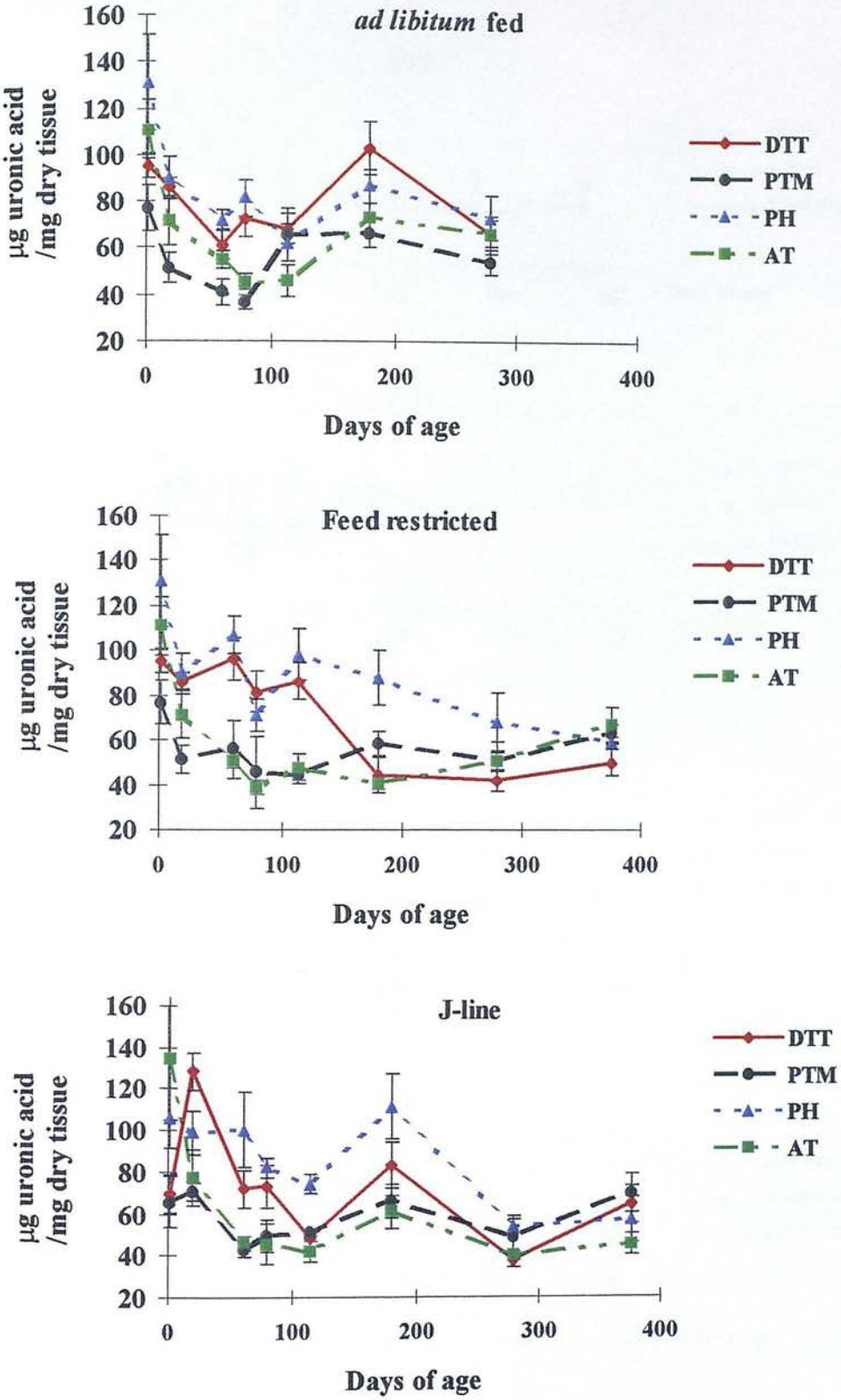


Figure 5.3 - The uronic acid content of the three groups with age, expressed as micrograms per milligram dry tissue. Error bars are S.E.M.

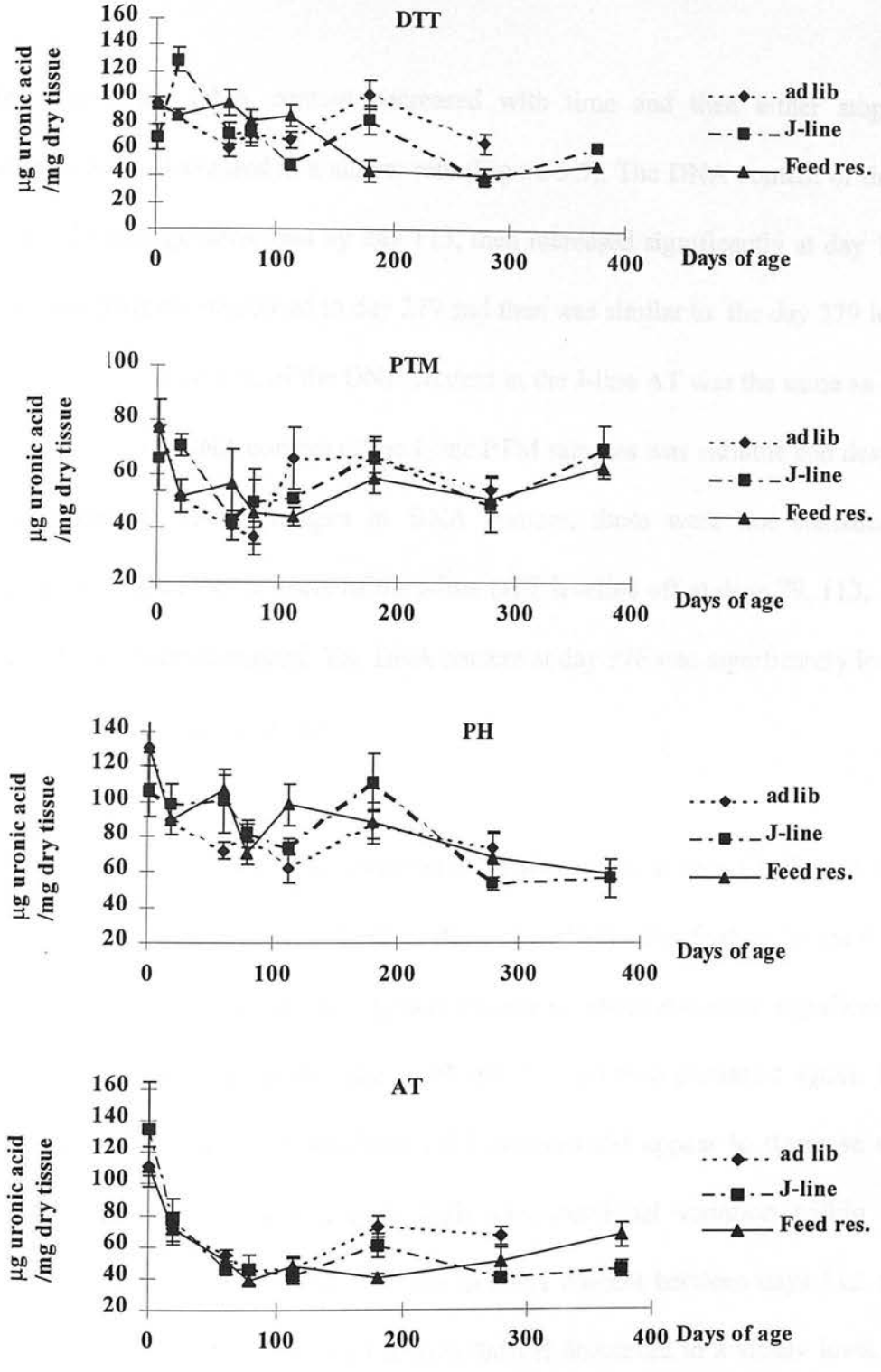
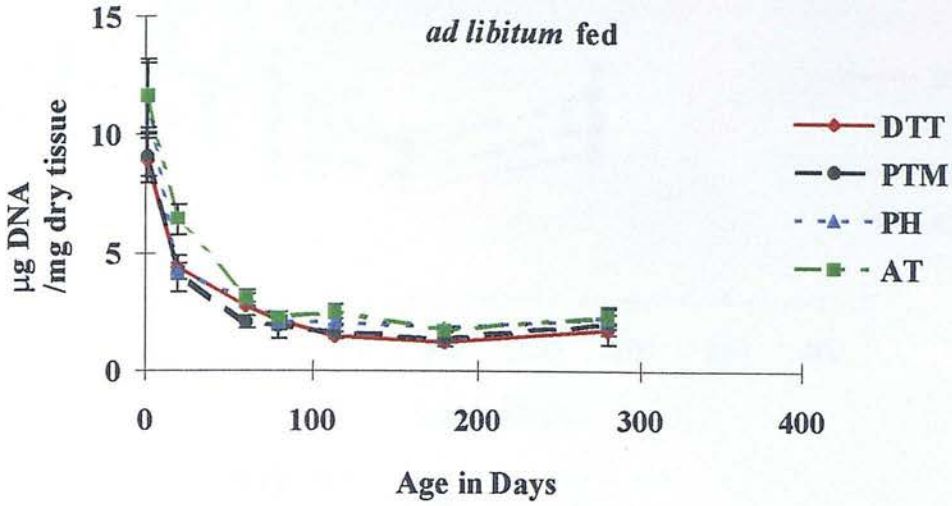


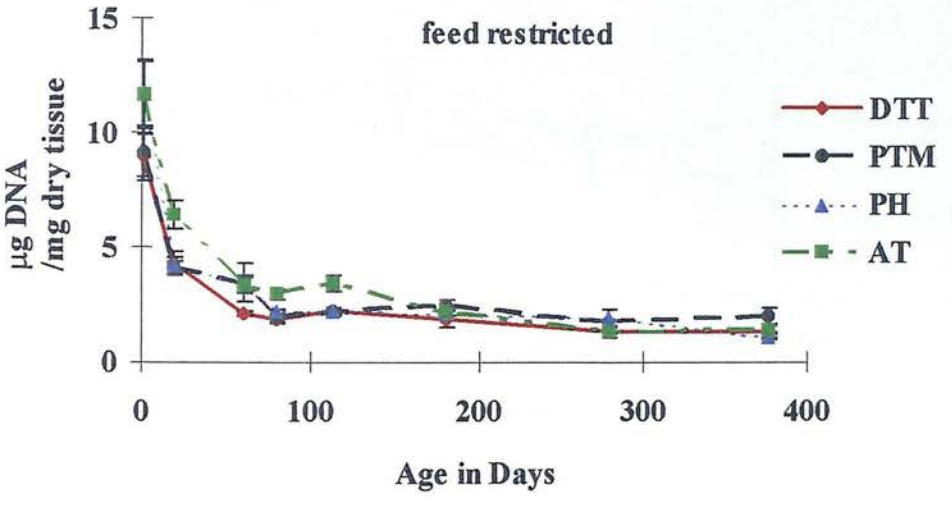
Figure 5.4 - The uronic acid content of the articular cartilage samples

5.3.3 DNA content

In general the DNA content decreased with time and then either stopped decreasing or decreased at a slower rate (Figure 5.5). The DNA content of the J-line PH cartilage decreased by day 113, then increased significantly at day 180, then significantly decreased to day 279 and then was similar to the day 279 level at day 376. The pattern of the DNA content in the J-line AT was the same as that for the PH. The DNA content of the J-line PTM samples was variable and despite the apparently large changes in DNA content, these were not statistically significant. The DNA content of the J-line DTT levelled off at days 79, 113, 180 and 279 and then decreased. The DNA content at day 376 was significantly lower than that at days 113 and 180.

The DNA content of the feed restricted AT was constant at days 61, 79 and 113, it then decreased and levelled again at day 279 and 376. The feed restricted PTM DNA content decreased at day 79, then levelled to within statistical significance, followed by a significant decrease until day 279 and then plateaued again. The DNA content of the feed restricted DTT samples did appear to decrease and although the results showed quite high inter-individual variation within the groups there was a significant decrease in DNA content between days 113 and 279. The DNA content of the *ad libitum* fed PH decreased to a steady level, as did that of the feed restricted group until day 279, then there was a significant decrease from days 279 to 376.





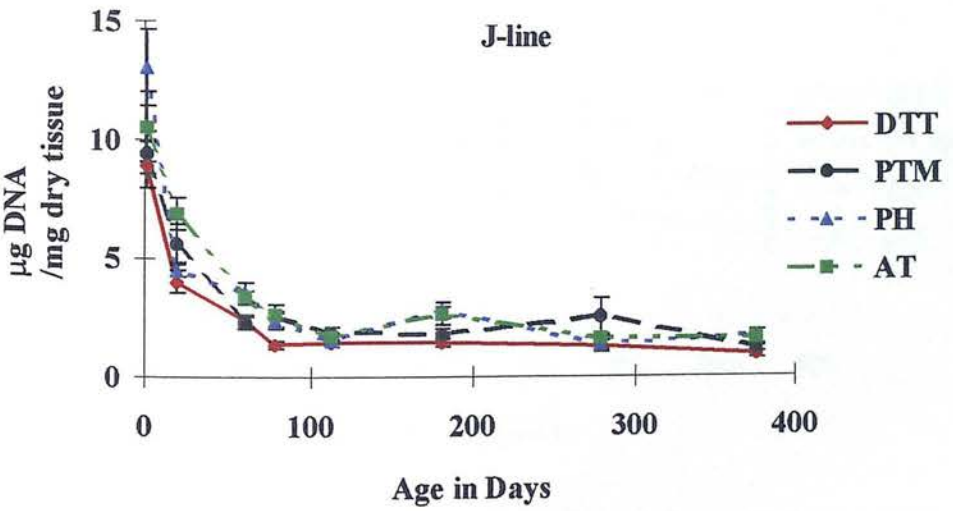


Figure 5.5 - DNA content of articular cartilage from the three groups, Error bars are ±S.E.M.

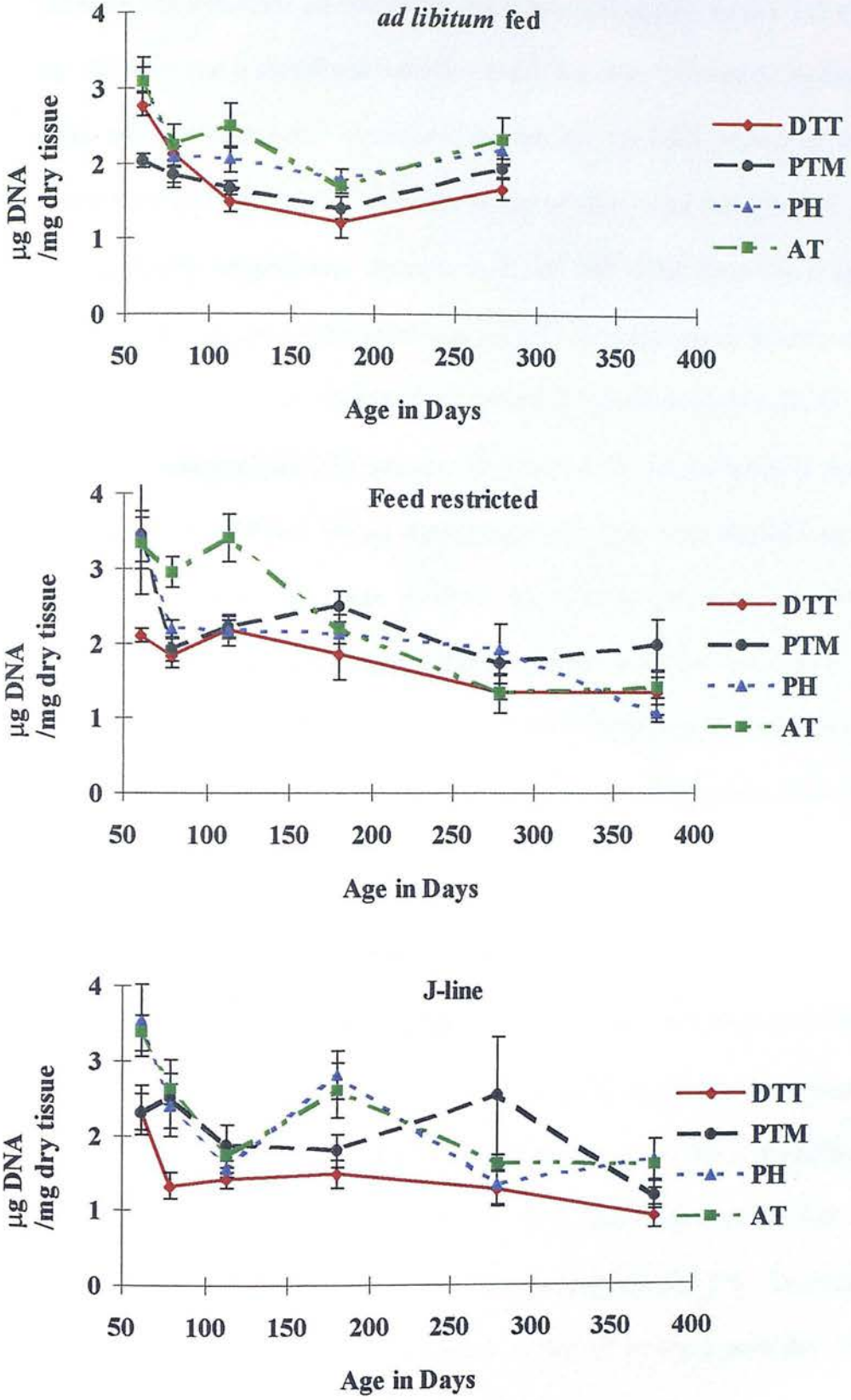


Figure 5.6 - The DNA content of the articular cartilage surfaces concentrating on the later time points.

The DNA content of the *ad libitum* fed AT appeared to steady by day 113 then at day 180 there was a significant decrease, which was then followed by an increase which was not statistically significant. By day 61, the DNA content of the *ad libitum* fed PTM samples had reached a steady level showing an apparently slow, but statistically insignificant, decrease until day 180 when there was a sudden rise. The DNA content of the *ad libitum* fed DTT samples was different in that it did not reach any sort of plateau level. Instead it fell consistently until day 180. The DNA content of the DTT samples from the *ad libitum* fed group at days 61 and 113 and days 79 and 180 are significantly different. Since the DTT samples from the *ad libitum* fed group exhibited the most severe signs of DJD this suggests that consistently falling DNA content may be associated with development of DJD. The DNA content of DTT samples of the *ad libitum* fed group were higher at day 279 than at day 180, but the *ad libitum* fed birds at day 180 were not suffering from DJD.

5.3.4 Sulphated glycosaminoglycan content

The sulphated glycosaminoglycan content of selected samples is shown in Figure 5.7. Most of the J-line sample groups showed a dip in SGAG content at day 279 followed by an increase at day 376. The only groups where this was statistically significant were in the DTT, where there was a significant decrease from 180 days to 279 days followed by a significant increase to day 376. There was a significant increase in the SGAG content of the AT samples from day 279 to 376.

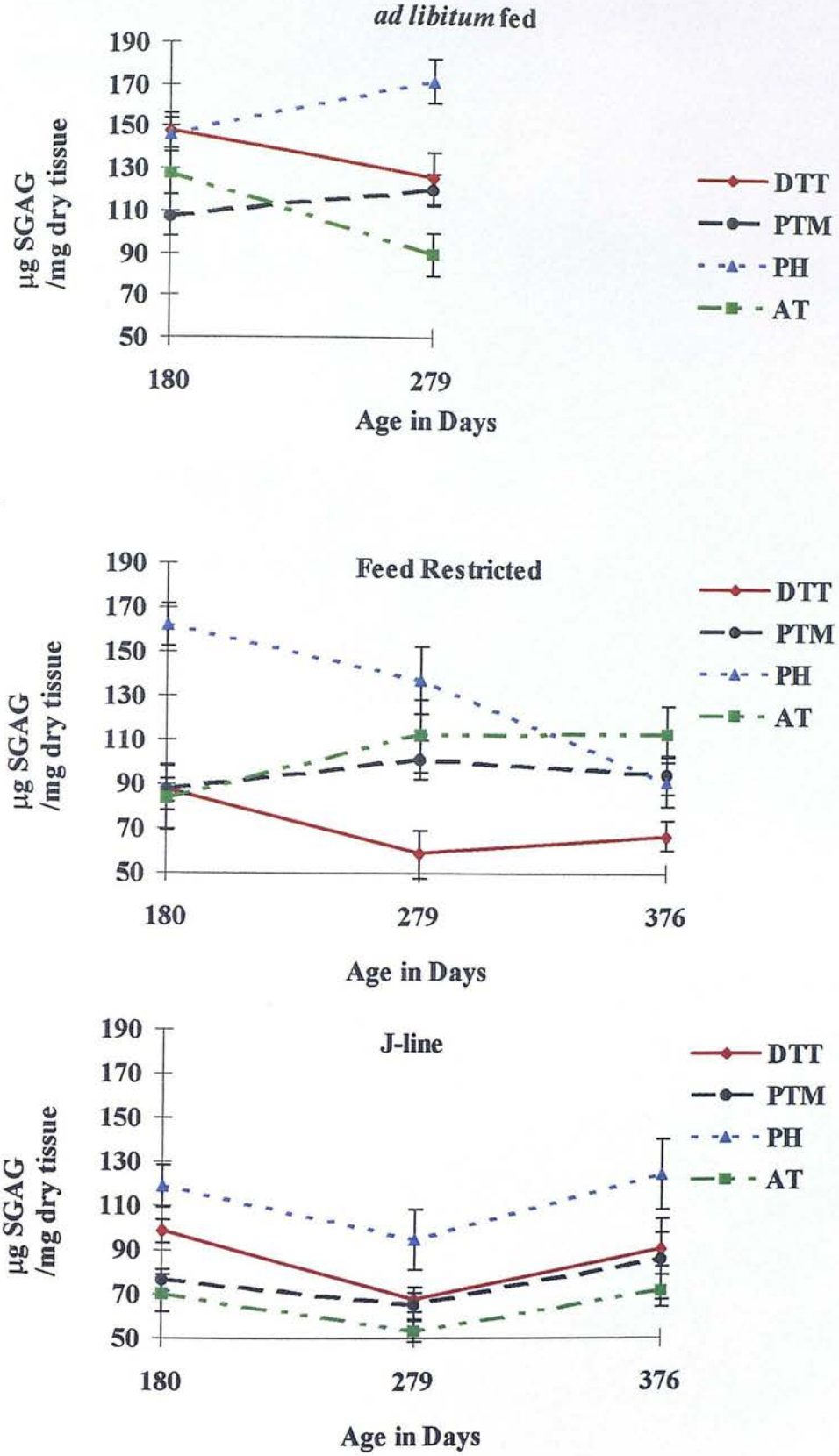


Figure 5.7- SGAG content of cartilage with age. Error bars are ±S.E.M

The only significant change in SGAG content in the *ad libitum* fed broilers between day 180 to 279 was a decrease in the AT.

The SGAG content of the DTT of the feed restricted group decreased significantly from day 180 to day 279 and remained at this lower level until day 376. The SGAG content of the PH fell significantly between days 180 and 376, (180 compared to 376 p=0.0001, 279 to 376 p=0.02).

5.4 Disease related change

In order to investigate the association between hydration and development of disease it is expedient to concentrate on those articular surfaces which developed degenerative changes. A prime example of this is the DTT of the *ad libitum* fed broiler birds.

The hydration of the *ad libitum* fed DTT samples (Figure 5.8) decreased with time as is the general trend with age. However, at day 180, there was a significant increase in hydration which was concomitant with the severe pathology shown in this joint at this time, (described in Chapter 4). The hydration of the DTT cartilage at day 279 was significantly lower than that at day 180 but not significantly different to that observed at day 113. The DTT samples at day 279 exhibited cartilage thinning but none of the severe pathological changes observed at day 180.

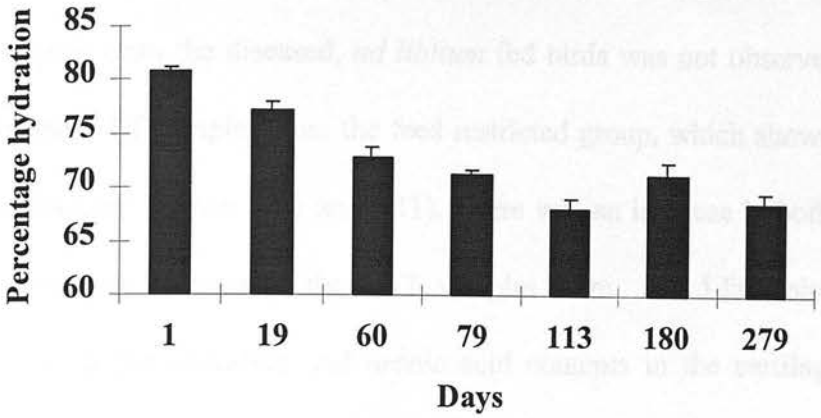


Figure 5.8 - Hydration of the DTT from the ad libitum fed birds. Error bars are S.E.M.

The pattern of uronic acid content of the *ad libitum* fed DTT samples (Figure 5.9) were remarkably similar to that of the hydration results. There was a decrease with time until day 60. The uronic acid content then ceased to decrease and there was a significant increase in uronic acid content at day 180 followed by a significant decrease at day 279. The uronic acid content at days 113 and 279 were similar.

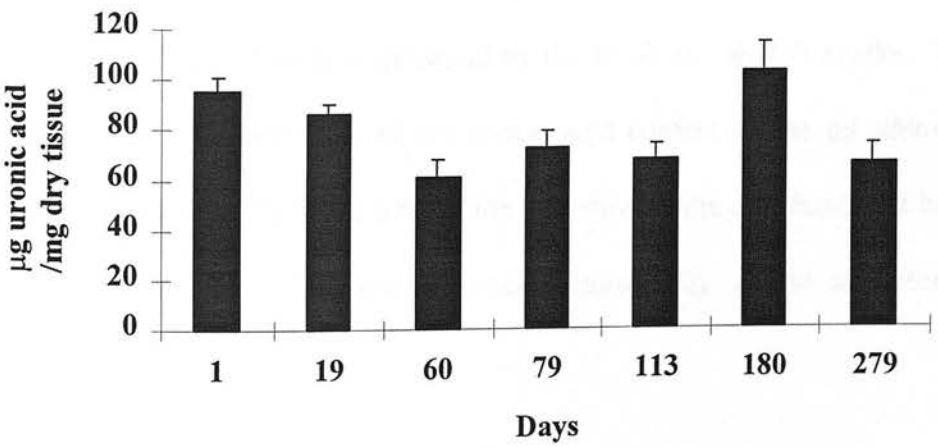


Figure 5.9 - Uronic acid content of the DTT samples from the ad libitum fed broiler strain fowl. Error bars are S.E.M.

The increase in hydration and uronic acid content at day 180 seen in the DTT samples from the diseased, *ad libitum* fed birds was not observed in the results for the DTT samples from the feed restricted group, which showed little disease at this time (Figure 5.10 and 5.11). There was an increase in both hydration and uronic acid content in the DTT samples from the J-line birds at day 180, although the hydration and uronic acid contents in the cartilage from the *ad libitum* fed birds was very different to those from the J-line. The increase in hydration in the DTT samples from the *ad libitum* fed birds was from 68.1% at day 113 to 71.4% at day 180. The hydration of the DTT samples from the J-line birds increased from 58.7% to 63.8%. The increase in uronic acid content was from 68.1 to 102.9 μg per mg dry tissue for the DTT samples from the *ad libitum* fed birds and from 48.5 to 83.1 for the DTT samples from the J line birds.

Since the *ad libitum* fed broilers were suffered from severe DJD in the DTT and the J-lines did not, a different type of response seemed to have occurred in the two groups to produce an increase in hydration and uronic acid content of the articular cartilage. This is emphasised by the DNA and SGAG results. The J-line samples contained 81% of the uronic acid content as the *ad libitum* fed broilers. In addition they had 89% of the hydration of the *ad libitum* fed broilers but only contain 66.5% as much SGAG (Figure 5.12) as the *ad libitum* fed broilers.

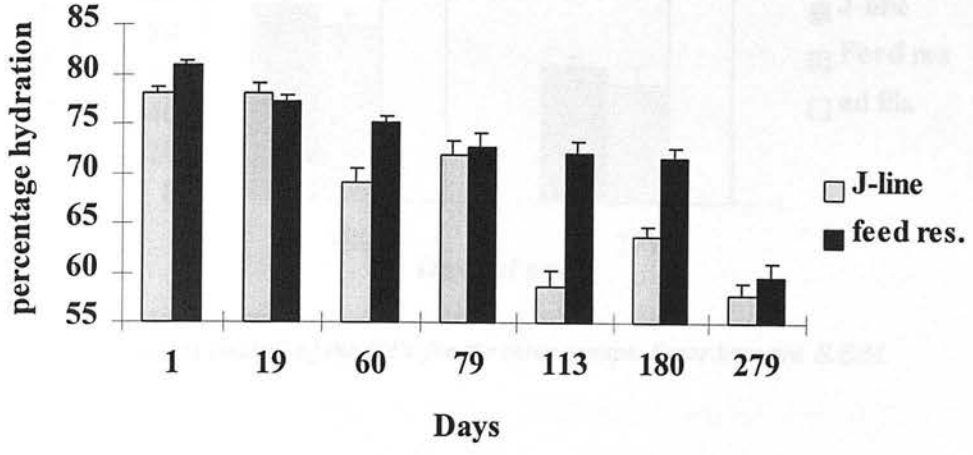


Figure 5.10 - Hydration of DTT samples from the J-line and feed restricted groups. Error bars are S.E.M.

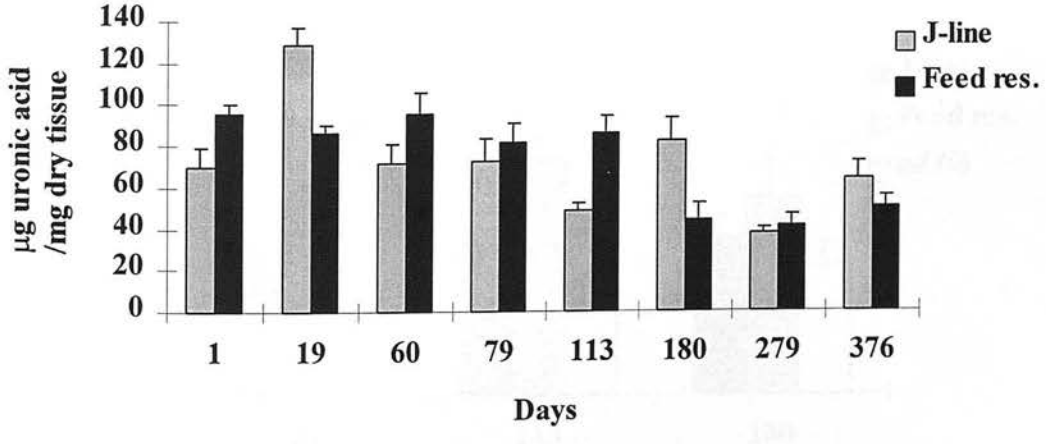


Figure 5.11 - Uronic acid content of the DTT samples from the feed restricted and J-line groups. Error bars are +S.E.M.

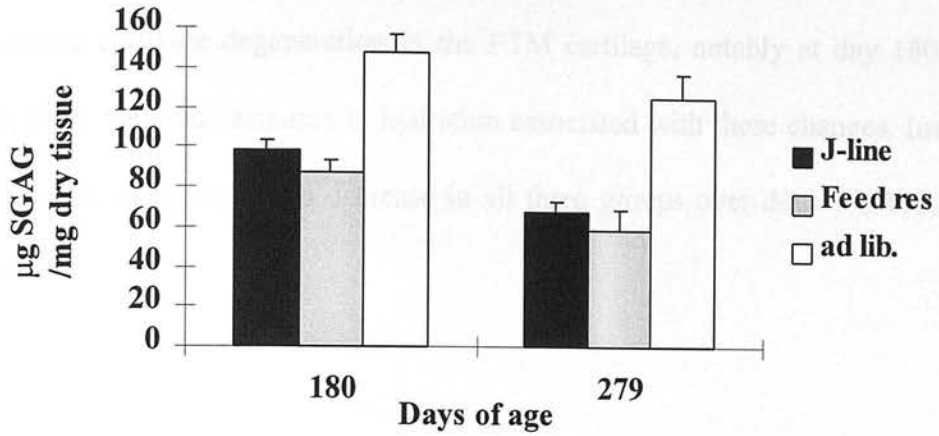


Figure 5.12 - SGAG content of the DTT for the three groups. Error bars are S.E.M.

Differences between the biochemistry of the DTT articular cartilage for the J-line and the *ad libitum* fed broilers were also apparent in the DNA content (Figure 5.13). The DTT samples from the *ad libitum* fed broilers showed a decreasing DNA content with time, there was significantly less DNA at day 180 than at day 113. This was not the case for the J-line samples which had a steady level of DNA.

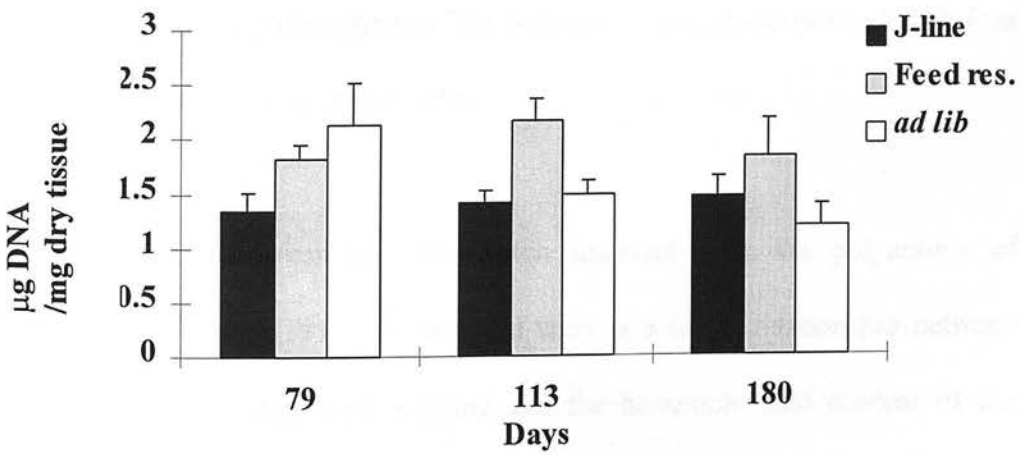


Figure 5.13 - DNA content of the DTT. Error bars are S.E.M.

Although there were a few occurrences of gross pathology and more widespread mid-zone cartilage degeneration in the PTM cartilage, notably at day 180 and 113, there were no increases in hydration associated with these changes. Instead the hydration continued to decrease in all three groups over days 79, 113, and 180.

5.5 Histochemical staining for proteoglycans

There was a large difference between the disease states of the DTT articular cartilage of the *ad libitum* fed broilers and the feed restricted birds at day 180. The DTT of the *ad libitum* fed birds showed many gross and histopathological features of DJD while only one of the feed restricted samples showed some signs of DJD histopathology, (chondrocyte clusters). There was a highly significant difference in the amount of SGAG in these samples (Figure 5.12). The difference in disease and SGAG content made these samples ideal candidates for investigation using the histochemical stains Safranin O and Toludine blue which selectively stain for proteoglycans. The Safranin O was counterstained with Fast Green to provide contrast in the slides.

Toluidine blue is a cationic dye which interacts with the polyanions of proteoglycans. Poole (1970) showed that there is a linear relationship between the density of Toludine blue staining and the hexuronic acid content of the cartilage matrix. Safranin O is a cationic dye composed of a mixture of dimethyl phenosafranin and trimethyl phenosafranin; this stain also binds to proteoglycans

in cartilage but has the advantage of being highly specific and interacting with chondrotin-6-sulphate and keratan sulphate in a stoichiometric manner. The content of dye molecules in histological sections is therefore in direct relation to the fixed negative ion content of the glycosaminoglycan (Kiviranta *et al.*, 1985).

5.5.1 Feed restricted samples

The sample from the feed-restricted group, which showed chondrocyte cluster formation in the middle zone, showed intense staining for proteoglycans around the clusters, which was contrasted by the lack of staining in the matrix around the clusters and in the surface zone of this sample (Figure 5.14).

The other feed restricted samples, which had no overt DJD, but some disruption in the structure of the cartilage, had variable Safranin O staining in the sections. In general, the cartilage towards the periphery of the joint showed less staining than that at the load bearing surface. In some areas no staining was visible, however there was abundant Safranin O staining around the deep zone chondrocytes, some around the middle zone and very little in the surface zone (Figure 5.15).

The staining pattern with Toludine blue in the feed restricted samples reflected the pattern seen in the Safranin O sections (Figure 5.16). There was a general a lack of surface zone staining, a strong staining around the chondrocytes in the deep zone and a variable amount of staining in the middle zone.

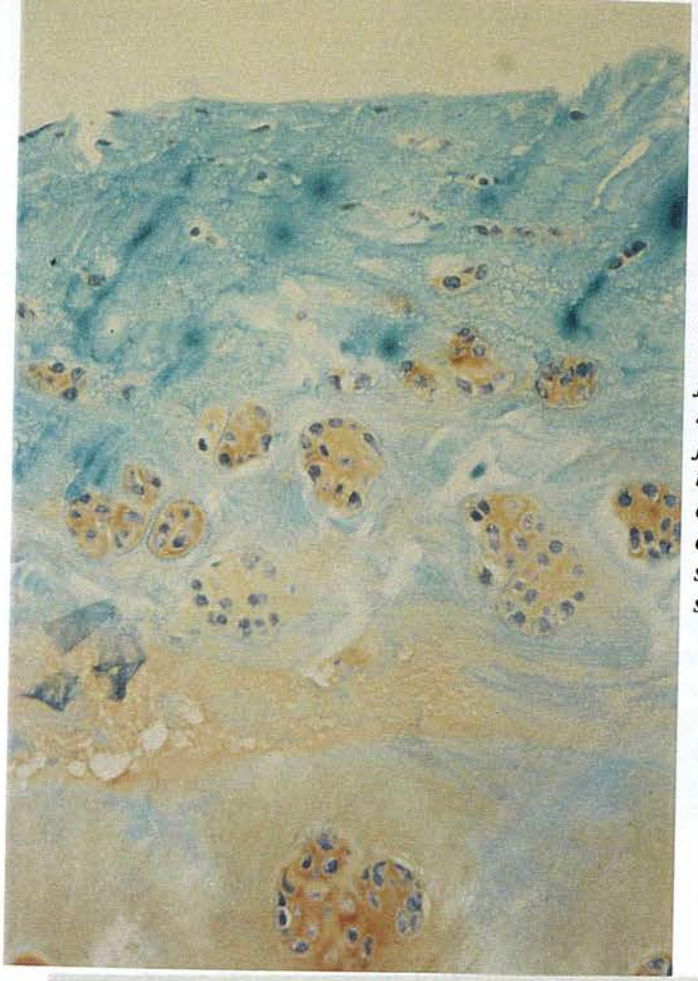


Figure 5.14 - Safranain O / Fast Green stained section from the feed restricted bird which showed chondrocyte cluster formation. The area immediately surrounding the clusters is strongly stained orange whilst the fibrillated surface zone has very little staining for proteoglycans. .x70.

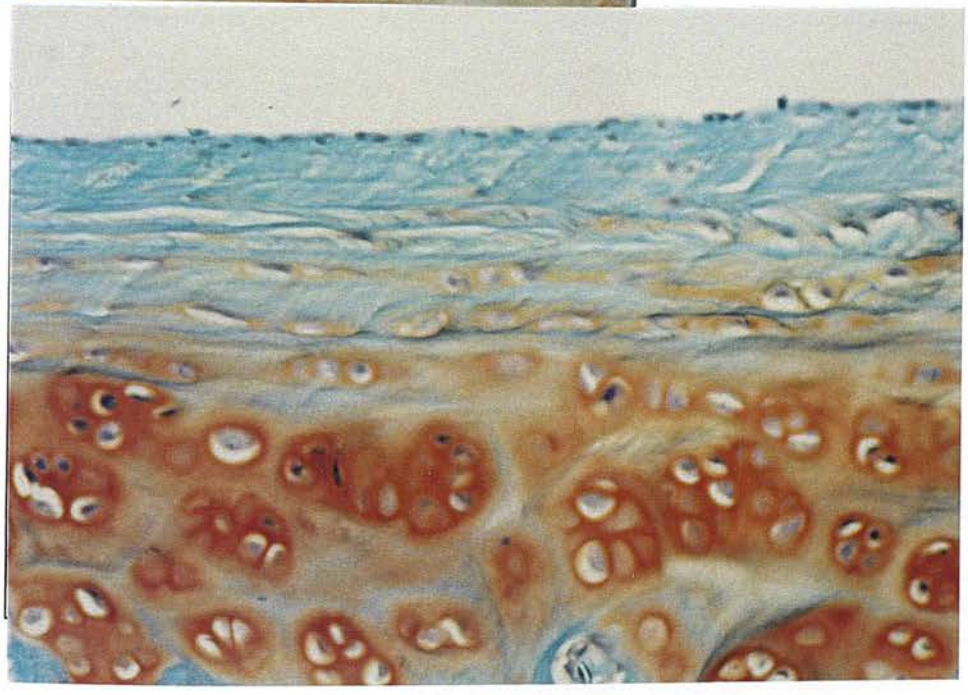


Figure 5.15 - Typical Safranin O / Fast Green section from the feed restricted samples, there is a surface zone where there is little staining , some staining in the middle zone and strong staining around the deep -zone chondrocytes which has also spread into the surrounding matrix. x70

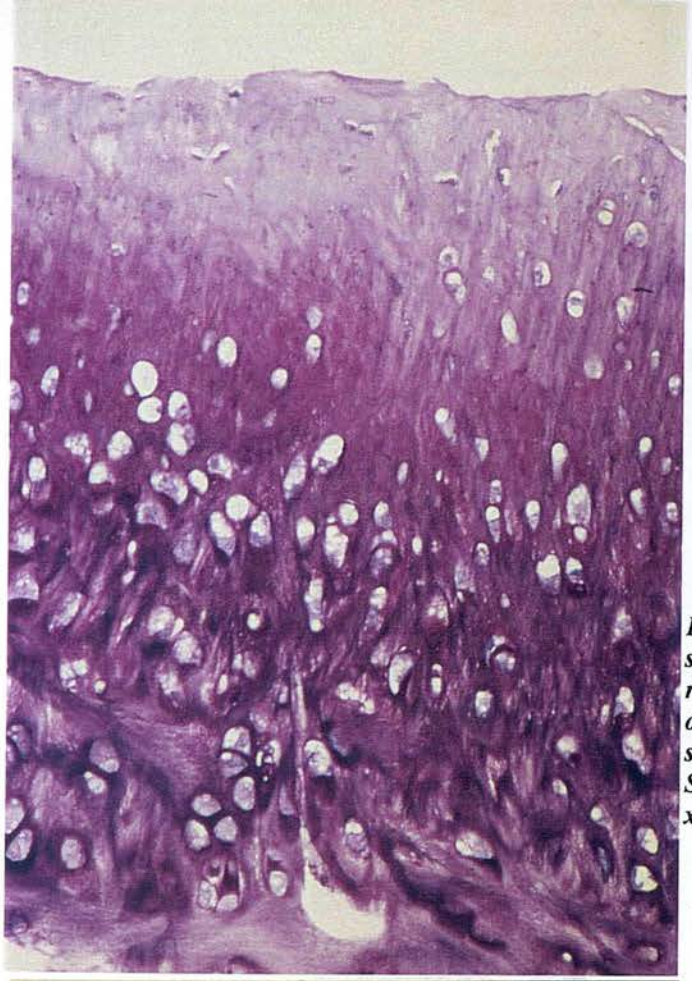


Figure 5.16 - Toludine blue stained section from the feed restricted samples. There is a lack of staining in the surface zone similar to that seen in the Safranin O/Fast Green sections. x70.

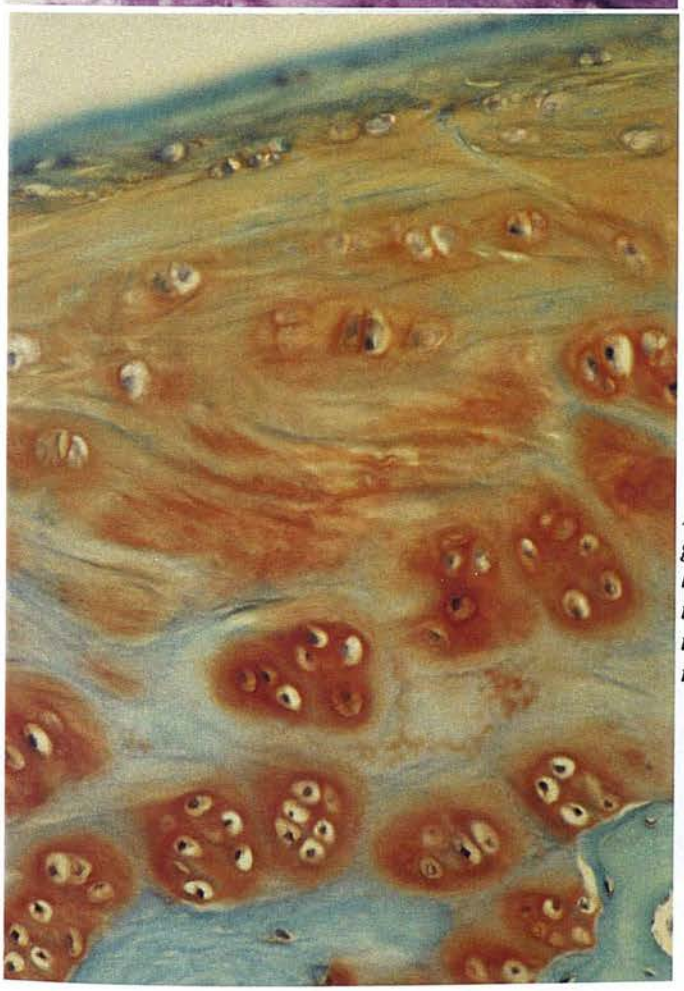


Figure 5.17 - Safranin O/ fast green stained section of ad libitum fed broiler DTT. There is staining throughout the section in contrast to that of the feed restricted samples. x70.

5.5.2 *Ad libitum* fed samples

Many of the samples from the *ad libitum* fed birds showed intense Safranin O staining throughout the zones. A much narrower surface zone than in the feed restricted samples was devoid of staining (Figure 5.17). However throughout this group there were focal areas which had no staining at all. Generally this seemed associated with areas of reduced cellularity and sometimes lack of staining was associated with areas of pannus formation. In some parts of the sections the only Safranin O staining could be seen around some isolated mid and deep zone chondrocytes (Figure 5.19). This variablility in the tissue reaction to the Safranin O stain is shown in Figure 5.18.

These results were reflected in the Toludine blue staining with areas where generally the whole section was better stained than the feed restricted samples (Figure 5.20).

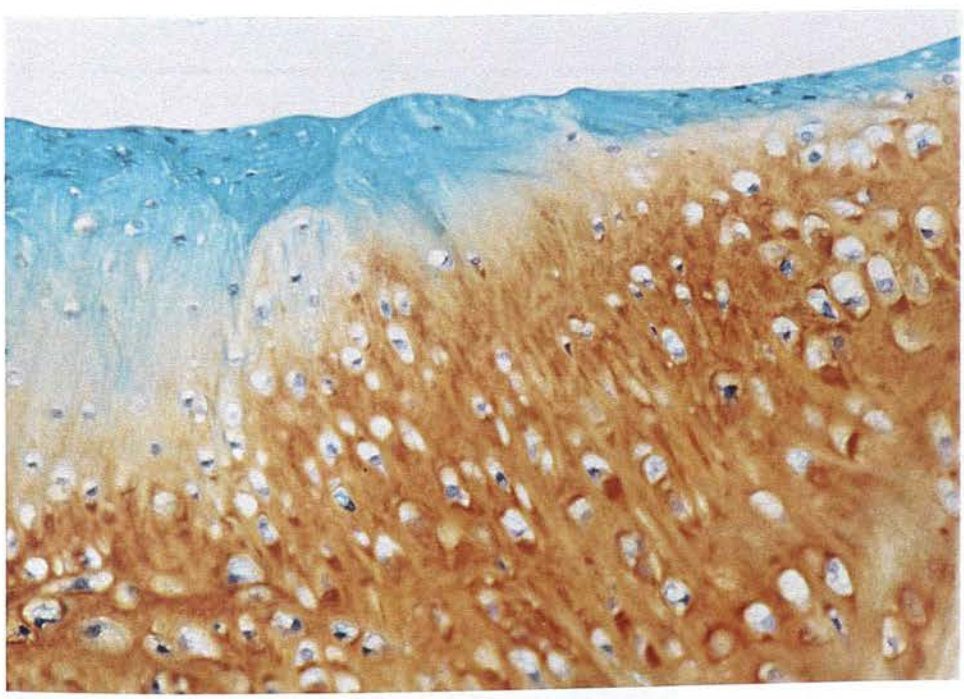


Figure 5.18 - Safranin O staining of a sample from ad libitum fed group showing the variablity of the proteoglycan staining within the tissue.x70.

Figure 5.19 - Area from ad libtum fed broiler section showing almost no staining. The only stained areas are around a few deep and mid-zone chondrocytes. x70.

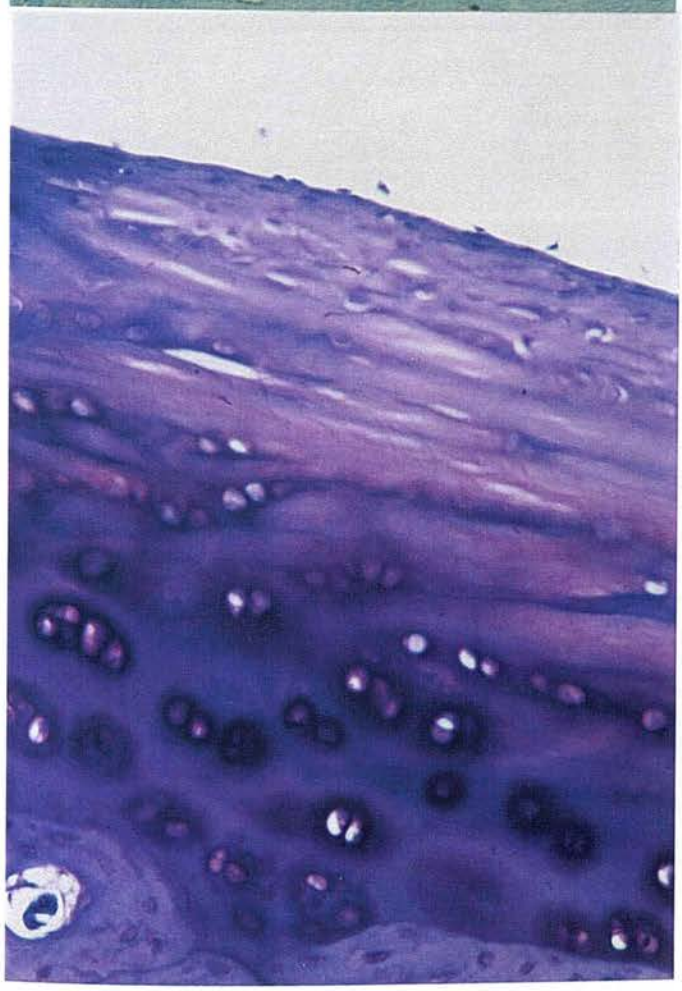


Figure 5.20 - Toludine blue staining of an ad libitum fed broiler DTT, The staining is much more widespread through the tissue than that of the feed restricted group x70.

5.6 Discussion

The general decrease in hydration with time was in agreement with the age related changes seen in other species (Muir, 1980; Stockwell, 1979). The most hydrated sample in all the bird groups was that of the proximal humerus. This was the relatively unloaded joint, which appears to implicate a role for increased loading in decreasing the hydration of the articular cartilage.

One of the most consistent observations on the biochemistry of DJD cartilage was that it has higher water content than normal cartilage (Maroudas and Venn, 1980). This was also true for avian DJD. The increase in hydration which was observed at day 180 in the *ad libitum* fed broiler DTT samples was concomitant with severe gross pathological and histopathological changes observed in these samples. The *ad libitum* fed broiler DTT samples showed only histopathological signs of DJD at day 113, but no increase in hydration was observed. This suggests that increased hydration may not be a biochemical cause of DJD pathology but rather a consequence, possibly caused by the weakening of the collagen network during the development of DJD. Studies at time points between 113 and 180 days may elucidate the relationship between the increase of hydration and the pathology of DJD more exactly.

The hydration of the cartilage from the *ad libitum* fed broiler was lower at day 279 than at day 180. Although some cartilage thinning was visible at day 279 there were no signs of severe pathology or histopathology. As discussed in

Chapter 4, the *ad libitum* fed broilers sampled at day 279 were not suffering from DJD. The hydration of the DTT cartilage samples reflected this observation since hydration was not significantly different to that at day 113 when the DTT cartilage was relatively healthy.

In the older birds sampled, from day 279-376, the hydration increased in articular cartilage samples in both the feed-restricted and J-line birds. These increases in hydration were not associated with any presentation of DJD disease pathology.

The biochemical changes associated with the development of DJD in the *ad libitum* fed broiler DTT are similar to those seen in other animal models of disease. In canine models of OA, before the phase of massive cartilage loss, the cartilage first exhibits a hypertrophic response (Adams, 1989). The increase in uronic acid content of the *ad libitum* fed broiler DTT at day 180 was in agreement with the result that aggrecan accumulates in tissue at this stage of DJD (Adams, 1994). The high SGAG content of the DJD compared to the other groups at this time was supported by observations both *in vivo* (Moskowitz *et al.*, 1981) and *in vitro* (Sandy *et al.*, 1984; Carney *et al.*, 1992) that proteoglycan synthesis is increased in early experimental OA.

There was a continuing decrease in DNA content in the *ad libitum* fed DTT when the DNA content of the other samples had steadied. Decrease in DNA content is consistent with a decline in numerical cell density which has been measured in similar early stage experimental OA when cartilage fibrillation is minimal or

absent and chondrocyte clustering is modest (Vignon *et al.*, 1983). Direct DNA measurements of similar cartilage also show a decline (Adams *et al.*, 1995).

As with the *ad libitum* fed broiler DTT samples, the J-line DTT samples also show an increase in hydration and uronic acid content at day 180. However this was not associated with disease in the J-line fowl. No increase in hydration or uronic acid content was detected in the feed restricted DTT at day 180. Both the J-lines and the *ad libitum* fed broilers were putting maximal loading on their joints whereas the size of the joint area in a feed restricted bird was relatively large in comparison to its load. The actual figures for hydration and uronic acid content for the J-line and *ad libitum* fed broilers were quite different and the actual composition of the cartilage must be distinct between the two strains. Possibly there is an age related increase in hydration which occurs naturally, but not in the feed restricted group due to their non-maximal loading. As the hydration of the cartilage is already very high in the *ad libitum* fed broilers the structure of the cartilage fails and begins to break down.

Although there were similarities in the pattern of hydration and uronic acid content around day 180, the SGAG and DNA content of the DTT were very different between the J-lines and the *ad libitum* fed broilers. There was a decrease in DNA content in the *ad libitum* fed broiler DTT samples through the time when the cartilage was degrading, a corresponding decrease was not observed in the J-lines. The decrease in DNA content was consistent with the reduced cellularity observed in the histological sections of these samples and with other observations

of similar disease states (Adams *et al.*, 1995). The disproportionately higher levels of SGAG in the *ad libitum* fed broilers compared to those in the J-line suggest that a higher proportion of the proteoglycans in the *ad libitum* fed broiler DTT were sulphated than those in the J-line samples.

The difference in proteoglycan content between the diseased, *ad libitum* fed broiler samples and the non-diseased, feed-restricted samples at day 180 was investigated using specialist staining techniques. These have showed that there was generally a more thorough distribution of proteoglycan through the depth of the tissue in the *ad libitum* fed broiler birds. The surface zone of the samples from the feed restricted birds had a low proteoglycan content. These facts concur with the uronic acid and SGAG results which show that the *ad libitum* fed samples had more proteoglycan. However within the sections from the *ad libitum* fed broilers, the distribution of proteoglycans was extremely variable. There were areas of the *ad libitum* fed broiler samples which were completely devoid of staining for proteoglycan. The majority of staining for proteoglycan in all samples was observed in the matrix surrounding the cells. The focal acellularity in some parts of the *ad libitum* fed broiler samples appeared to be associated with this lack of staining for proteoglycan. It may be that the proteoglycans cannot move sufficiently through the matrix from the areas where they are produced by healthy cells into the areas where no cells are present or where the cells are not producing proteoglycans. This hypothesis is supported with evidence from Wong *et al.* (1996) where the distribution of radioactive grains of ^{35}S-Sulphate in

cartilage explants was primarily concentrated pericellularly, suggesting that newly synthesised proteoglycan molecules are effectively inhibited from diffusing into the interterritorial matrix.

It has also been shown by *in situ* hybridisation that some chondrocytes in OA cartilage produce high levels of mRNA for type II collagen and proteoglycan whereas other chondrocytes in the same tissue produce neither (Aigner *et al.*,1992; Vertel *et al.*, 1979). This dis-coordinate regulation of proteoglycan synthesis and impairment of proteoglycan movement through the matrix appear to be a metabolic characteristic of OA and may well contribute to its pathogenesis (Adams *et al.* 1995). Certainly, the variability in proteoglycan content must present a physical difficulty for the tissue, with some areas being more hydrated than others; the shear forces which this creates could be destructive to the cartilage.

5.7 Conclusions

The degenerative changes observed in the tissue associated with the development of avian DJD are: an increase in hydration, proteoglycan content and SGAG content, accompanied by a decrease in DNA content and decreased in cellularity. The areas which experience the decrease in cellularity also show a localised decrease in proteoglycan content, whilst those areas which remain cellular appear to increase their proteoglycan content. Lack of homogeneity in proteoglycan content may provide shear forces which will physically strain the cartilage.

6. Alterations in collagen in the longitudinal study

6.1 Introduction

Collagen is a major component of articular cartilage, to which it contributes up to 90% of the dry weight of the tissue. The collagen network provides the tensile strength of the cartilage. Measurements of various aspects of the collagen content can give important insights into the condition and development of the cartilage, and the possible roles of cartilage collagen metabolism in the development of DJD. The approaches which were used in this study were measurements of total collagen content, measurement of mature collagen crosslinks, and development of polyclonal antibodies specific to collagen types II and IX.

6.2 Possible roles of collagen metabolism in DJD

The important role of the collagen network in controlling hydration of the cartilage coupled with the fact that the only true consensus opinion on the biochemistry of DJD is that an increase in cartilage hydration occurs. This has led to speculation that the collagen network is weak or degraded in DJD cartilage. There are various factors involved and one, or any combination of these, could be involved in mediating the increase in hydration. There could be less collagen, providing less tensile force and a generally weaker network more prone to degradation. Alternatively, the collagen network may be weaker due to a lack of crosslinks. Not only would this allow more swelling but the integrity of the network could also be easily compromised by proteolytic enzymes.

6.2.1 Formation of mature collagen crosslinks

Lysl oxidase catalyses the oxidative deamination of the ε-amino groups of lysl and hydroxylysl residues (Eyre *et al.*, 1984). The aldehyde formed condenses with the amino group of another lysl/ hydroxylysl residue to form a Schiff base . As collagen matures, the aldimines, formed from hydroxylysl residues, rearrange to form chemically stable ketoamines, which are the predominant forms found in collagen fibres. Depending on the pathway utilised, divalent or trivalent crosslinks are formed. One of these crosslinks, the trivalent fluorescent pyridinoline, is present in articular matrix at concentrations approaching one mole per mole of triple helical collagen. The formation of pyridinoline is shown in Figure 6.1.

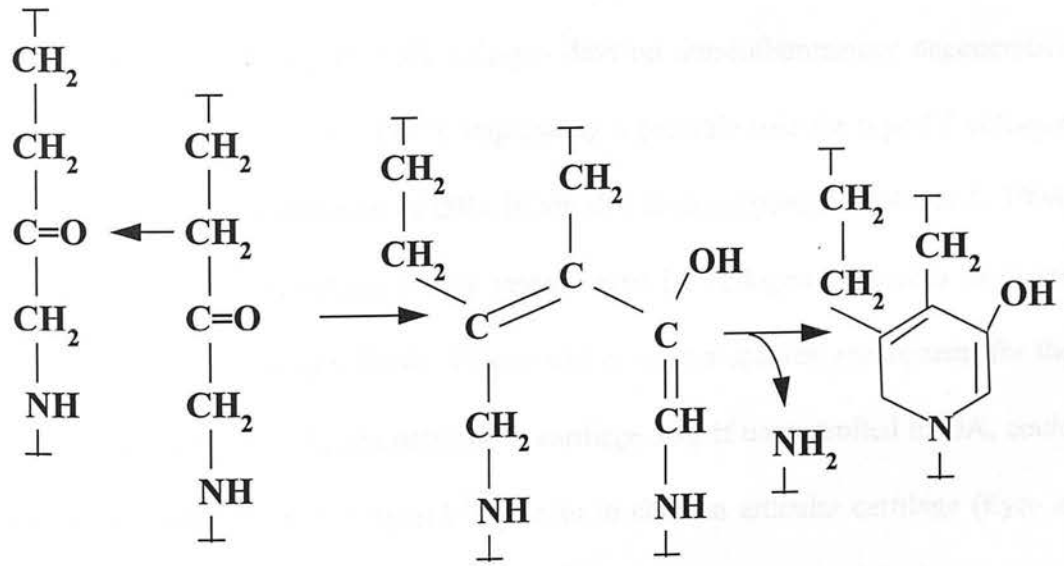


Figure 6.1 - Scheme showing how two ketoamine cross-links can interact to prodice a 3-hydroxypyridinium residue. (Eyre et al., 1984)

Since DJD occurs with greater frequency in older individuals (Hamerman, 1989), it has been thought that post-synthetic modification of collagen, especially through crosslinking, may play a role in increasing the susceptibility to OA in ageing (Pokharna *et al.*,1995). In distrophic dysplasia, which is a heritable osteochondrodysplasia, one case has been reported where there is a marked decrease in the pyridinoline content of the cartilage (Bailey *et al.*, 1995).

6.2.2 Type II and IX collagen interactions

Type II collagen crosslinks to type II collagen (Wu and Eyre, 1984) and type IX collagen (van der Rest and Mayne, 1987). Type IX collagen also crosslinks to other type IX molecules, the bridge thus formed being thought to provide an important mechanism for maintaining the integrity of the collagen network (Wu *et al.*, 1992). Transgenic mice lacking type IX collagen develop non-inflammatory degenerative joint disease (Fassler *et al.*, 1994), implicating a possible role for type IX collagen metabolism in the progression of DJD. It has also been suggested (Diab *et al.*, 1996) that the action of stromelysin-1 may remove type IX collagen molecules from the surface of type II collagen fibrils. This would provide a specific mechanism for the reorganisation of the collagen network in cartilage and, if uncontrolled in OA, could lead to network failure. Collagen I is present in chicken articular cartilage (Eyre *et al.*, 1978). Collagen I is not known to bind to collagen IX. It is possible that the presence of type I collagen in avian articular cartilage may physically compromise the functionality of the collagen network.

The potential for collagen IX in controlling hydration of a network is demonstrated during chick development. Immediately before the swelling of the primary corneal stroma, (a natural developmental stage), immunohistochemically detectable collagen IX disappears (Fitch *et al.*, 1988). The amount of collagen IX decorating the collagen II fibril in cartilage could affect the the collagen network (Bailey *et al.*, 1995), such an abnormality of type IX collagen has been reported in a single case of diastrophic dysplasia (Diab *et al.*, 1993).

6.2.3 Distinctive features of type IX collagen

The pepsin fragments of chicken collagen IX are the high molecular weight (HMW) and low molecular weight components (LMW) (Figure 6.2). After reduction, HMW migrates on SDS-PAGE as three components called C1 (Mr 87,500), C2 (Mr 51,000) and C3 (Mr 36,400). A fourth component C4 (Mr 14,000) is also present in HMW but this is not disulphide bonded and dissociates on heat denaturation. The LMW is made up of L1 (Mr 11,000), L 2A (Mr 10,600), L 2B (Mr 10,500) and L3 (Mr 10,600) (Reese and Mayne, 1981). These are often not seen when separated by reduction as they run with the dye front in a low percentage polyacrylamide gel. The C1 component probably arises from a non-reducible cross-link between C3 and either C2 or C5.

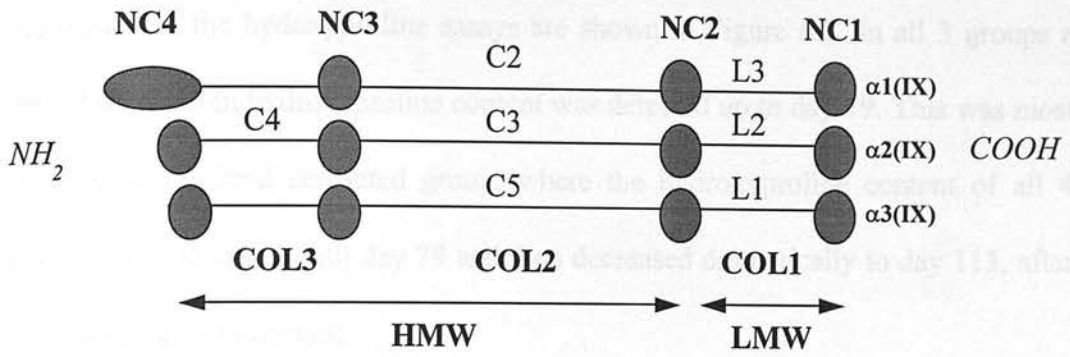


Figure 6.2- Schematic representations of intact collagen IX, and the pepsinised fragments. (from van der Rest and Mayne, 1987)

6.3 Total collagen content

The total collagen content of all samples in the longitudinal study was estimated by assaying for hydroxyproline. This provides useful information on the amount of collagen present in samples.

6.3.1 Materials and methods

The hydroxyproline assay method used was that of Firschelin and Shill (1966) an adaptation of the original method of Woesnner (1961) (see Appendix). The results were calculated and statistical tests carried out in Microsoft Excel. As in previous chapters if a t-test between two groups returned a value of $p<0.05$ the difference between the two groups was considered to be significant.

6.3.2 Hydroxyproline results.

The results of the hydoxyproline assays are shown in Figure 6.3. In all 3 groups a general increase in hydroxyproline content was detected up to day 79. This was most dramatic in the feed restricted group, where the hydroxyproline content of all 4 sample sites increased until day 79 and then decreased dramatically to day 113, after which it remained constant.

The hydroxyproline content of all the J-line samples increased significantly from day 1 to day 19, and then remained stable (within the bounds of experimental error) until day 113. Between day 113 and day 180 the hydroxyproline content of the articular cartilage increased significantly in all 4 sample sites and then significantly decreased again to day 279. In the J-line group there was no significant difference in hydroxyproline content between sites sampled at day 279 and at day 376.

The hydroxyproline content of the *ad libitum* fed broiler DTT, PTM and AT samples also increased significantly from day 1 to day 19. A general increase of hydroxyproline content was then observed until day 113 after which the hydroxyproline content then decreased slightly in the PTM, PH and AT. The hydroxyproline content of the DTT decreased more dramatically. At day 180 the hydroxyproline content of the DTT was significantly less than that of the other 3 *ad libitum* fed broiler joint surfaces although it was not significantly lower than the feed restricted DTT at this time. The hydroxyproline content of the *ad libitum* fed broiler DTT continued to decrease to day 279 where it was significantly less than the content of the PTM and the AT.

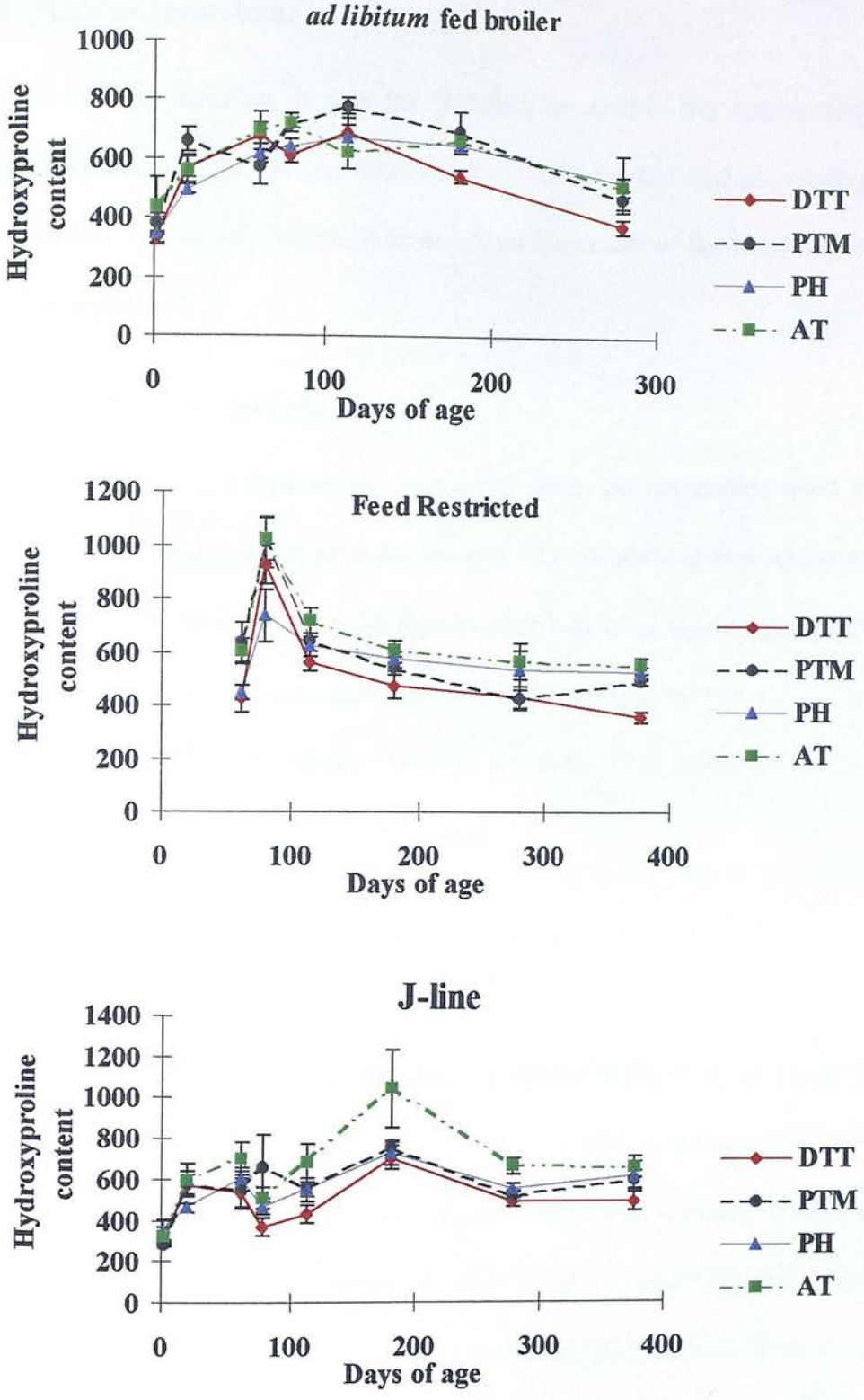


Figure 6.3 - Hydroxyproline content of the samples (nanomoles per mg dry tissue). Error bars are S.E.M.

6.4 Mature cross-links

Due to limited facilities, it was not possible to analyse the mature crosslinks (pyridinoline) in all the samples from the longitudinal study, and so certain groups were chosen. In this way questions arising from the results of the longitudinal study could be addressed.

6.4.1 Materials and methods

A portion of the acid hydrolysate was taken from the preparation used for the hydroxyproline assays and dried under vacuum. The sample was then rehydrated and analysed at the Rowett Research Institute in Aberdeen using ion-pair reversed phase HPLC directly linked to an automatic sample preparation system for solid-phase extraction on disposable columns (Pratt *et al.* 1992). The results were collected, processed and analysed using Microsoft Excel.

6.4.2 Pyridinoline results

The first hypothesis to be tested was whether broiler strain fowl have less mature collagen crosslinking in their articular cartilage, thereby accounting for the high hydration observed in the broiler strain samples and hence cartilage weakness and pre-disposition to DJD. This hypothesis was tested by analysing the amount of pyridinoline in the DTT samples from all 3 groups *ad libitum,* feed restricted and J-line) at days 180 and 279. At day 180, the DTT from the *ad libitum* fed birds were showing signs of DJD. As illustrated in Figure 6.4, there was no significant difference between the pyridinoline content of the 3 groups at either age.

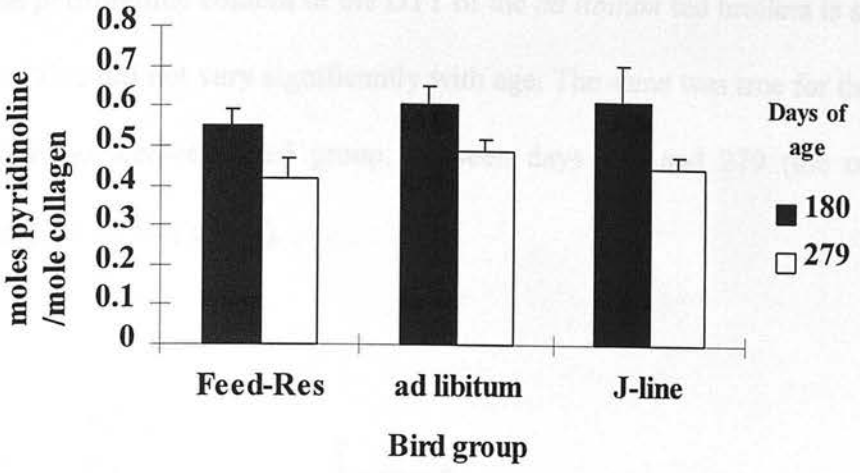


Figure 6.4 - Pyridinioline content of the DTT of the three groups at day 180 and 279. Error bars are S.E.M.

When the pyridinoline content of all sites in all groups at day 279 were examined, the DTT of the J-line and the feed restricted groups contained significantly less pyridinoline than the other sites within each group. This was not true for the *ad libitum* fed group, where the p value was between 0.09 and 0.05 for t-tests between the DTT and the other joints. This is illustrated in Figure 6.5.

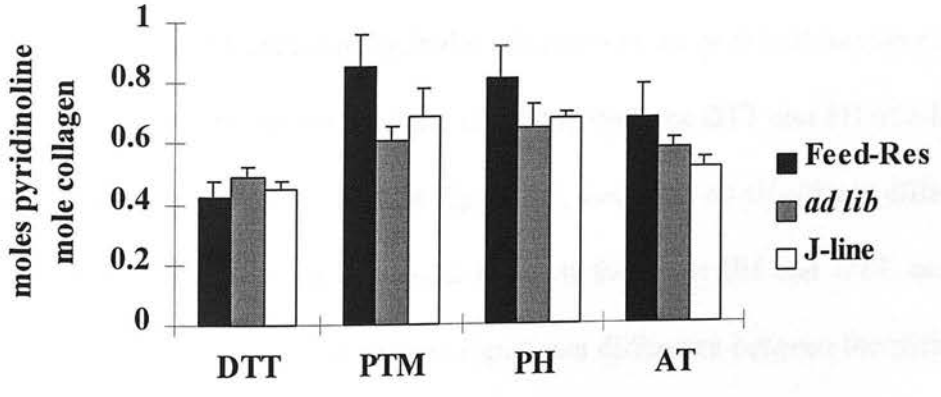


Figure 6.5 - Pyridinoline content of the samples from day 279. Error bars are S.E.M.

The pyridinoline content of the DTT of the *ad libitum* fed broilers is shown in Figure 6.6. This did not vary significantly with age. The same was true for the DTT samples from the feed-restricted group, between days 180 and 279 (the only time point sampled for this group).

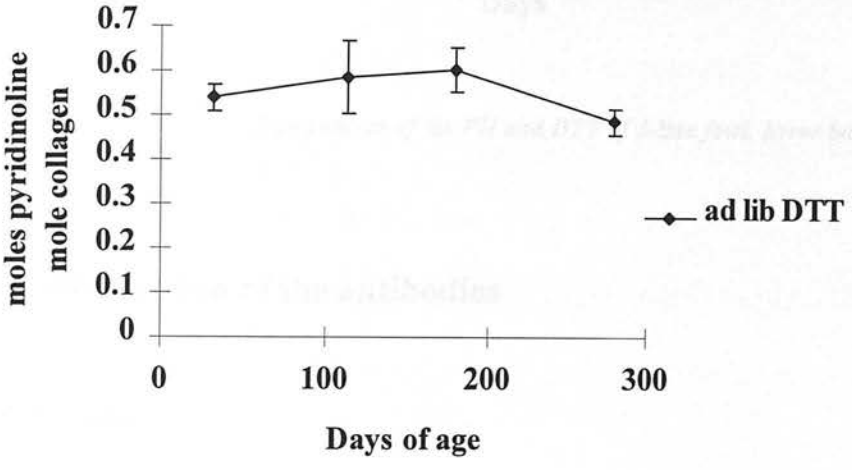


Figure 6.6 - Pyridinoline content of the DTT from the ad libitum broilers. Error bars are S.E.M.

In order to investigate the role of load in the formation of mature collagen crosslinks, and to investigate if a difference in mature crosslinking could be responsible for the increase in hydration observed in the proximal humerus of the J-line fowl at day 376, the pyridinoline content in both the DTT and PH of J-line fowl was investigated. As can be seen in Figure 6.7, there was no significant difference in the content of pyridinoline crosslinks between the J-line PH and DTT samples at days 279 and 376. There was also no significant difference between the pyridinoline content of the DTT samples at day 180 and 279. However, there was a significant increase in the pyridinoline content of both the PH and DTT between days 279 and 376 (unlike in the *ad libitum* or feed restricted groups).

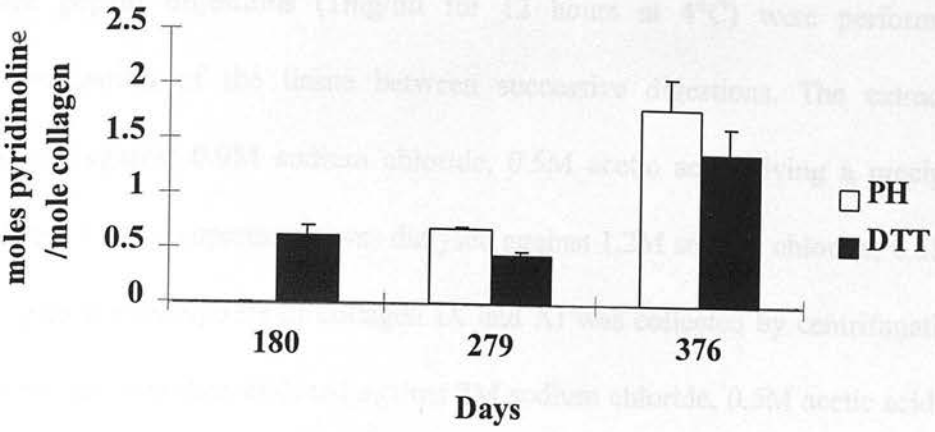


Figure 6.7- The pyridinoline content of the PH and DTT of J-line fowl. Error bars are S.E.M.

6.5 Production of the antibodies

6.5.1 Aims

This work was carried out to provide antibodies against chicken collagen II and collagen IX. These were to be used not only for immunostaining and Western blotting in this project but also for other projects within the group, in particular immuno gold labelling for electron microscopy.

6.5.2 Preparation of the antibodies

The first task was preparation of pure chicken collagen IX fragments for use in raising specific antibodies. The method of Reese and Mayne (1981) was used. The bone and perichondrial membrane were removed from 20 chicken sterna from 8 week old birds, the remaining cartilage was then chopped and pulverised after freezing in liquid nitrogen, producing approximately 65 grams of tissue. Proteoglycans were removed by extracting twice in 4M guanidium hydrochloride.

Three pepsin digestions (1mg/ml for 12 hours at 4°C) were performed with repulverisation of the tissue between successive digestions. The extracts were dialysed against 0.9M sodium chloride, 0.5M acetic acid, giving a precipitate of collagen II. The supernatant was dialysed against 1.2M sodium chloride, 0.5M acetic acid and the precipitate of collagen IX and XI was collected by centrifugation. The supernatant was then dialysed against 2M sodium chloride, 0.5M acetic acid and the precipitate of collagen IX was collected by centrifugation. The precipitates were dissolved in 0.5M acetic acid and dialysed against 0.1M acetic acid before freeze drying. The extracts were electrophoresed on 5-7.5 % gradient gels and on 6 % gels under both reducing and non-reducung conditions. The third pepsin digestion produced the highest protein yield and the precipitates from this extract are shown in Figure 6.8.

Type IX collagen (2.0M sodium chloride precipitate) from the third pepsin digest was chosen to be the antigen for raising antibodies. It clearly contained type IX collagen and had less impurities than the other extracts. Although this was not a completely pure preparation it was considered adequate since the antiserum would be affinity purified before use.

Intact collagen II from lathyritic chicken sterna was kindly provided by Mark Marsden. The collagen II and IX preparations were dissolved in 0.5M acetic acid, then dialysed against 10mM acetic acid, and frozen in aliquots.

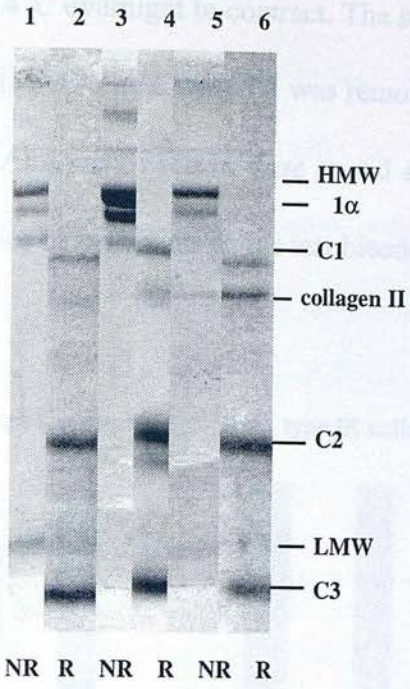


Figure 6.8 - SDS-PAGE analysis of pepsinised chicken collagen. Coomassie Blue staining.Lanes 1 and 2 contain the 2M precipitate (type IX), lanes 3 and 4 contain the 1.2 M NaCl preciptate (type XI and IX) , lanes 5 and 6 are the 0.9M preciptate (type II). Lane 1: HMW, fragments and LMW. Lane 2: C1, C2, C3. Lane 3: similar to lane 1 but high molecular weight crosslinked material is also present and LMW is not visible. Lane 4: C1, C2, and C3 as well as other proteins. Lanes 5 and 6: collagen type II band heavily contaminated with type collagen type IX (C1, C2 and C3). Lanes 1,3,5 unreduced (6% SDS-PAGE), lanes 2,4,6, reduced(5-7.5% SDS-PAGE gradient gel) .

Pre-immune bleeds were taken from the rabbits and treated as the test bleeds below. Rabbits were injected subcutaneously with either collagen II or IX (500 µg in 500ml) which had been emulsified with 500ml Freunds complete adjuvant. Freunds complete adjuvant was used for the initial immunisation since collagen has a weak immunogenicity (Allison, 1974). The rabbits were boosted 10 weeks later with 100mg of the appropriate antigen in Freunds incomplete adjuvant and after that a test bleed was carried out. After collection, the blood was allowed to clot for 30-60 min and then was separated from the side of the collection vessel using an unfolded paper clip.

The clot was left at 4°C overnight to contract. The serum was then removed from the clot and the remaining insoluble material was removed by centrifugation at 10,000g for 10 min at 4°C. Aliquots of serum were stored at -20°C and -70°C (Harlow and Lane, 1988) Examples of the results of the test bleeds are shown in Figure 6.9.

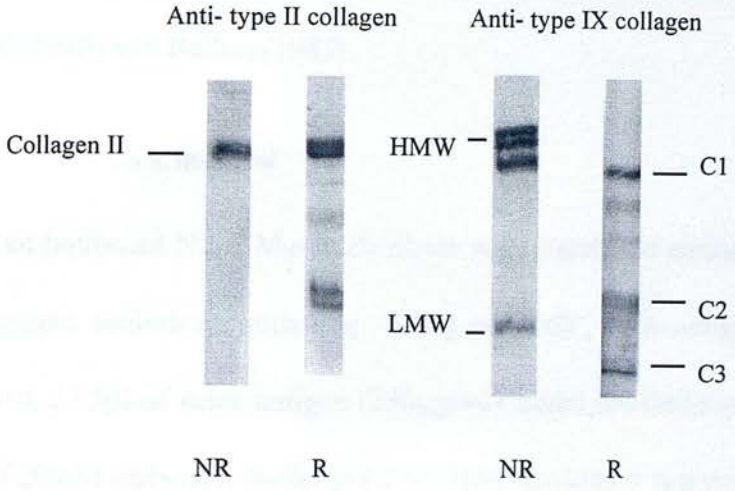


Figure 6.9 - Typical examples of the western blots of the test-bleeds Since these were test bleeds the serum was diluted 1 in 20. 6% SDS-PAGE gels were run containing samples of the collagen preparations used as antigens and then Western blotted, each serum against the appropriate antigen, using standard methods. The blots were visualised using ECL.

In the bleeds from the rabbits innolculated with type IX collagen, the presence of HMW and absence of C1, C2 and C3 in the non-reduced sample indicates that the rabbits were producing antibodies to type IX collagen. This result is typical of the other 3 rabbits immunised with type IX collagen. The pre-immune blots (not shown) were clear even at this very high serum concentration.

It was decided that a final bleed would be taken from the rabbits. This option had the advantages that the supply of antibody was not dependent on the health of the rabbits, also the antibody population was uniform throughout the final bleed and so

the serum was more reliable and easier to characterise than that produced from smaller bleeds taken at various time points. Tolerance to the antigen can develop if the animal is continually boosted and this will eventually result in low antibody titres (Sambrook *et al.*, 1989). To ensure maximum antibody production the final bleed should be carried out when antibody titres have stopped increasing with each boost. To determine the antibody titre an ELISA was developed, adapted from Rennard *et al.*(1980) and Bellon (1985).

6.5.3 ELISA method

Flat bottomed Nunc Maxisorb plates were incubated overnight at 4°C with 100µl of antigen soulution containing 0.1µg per well , (The antigen solution was prepared with 22.5µl of stock antigen (250µg/ml) stored in 10mM acetic acid diluted in 4.5ml of 20mM carbonate buffer pH 9.6).This provides 0.1µg per well. The plate was then washed 5 times with PBS-Tween. The wells were blocked for 2 hours at 4°C with 150µl PBS-Tween-BSA, then washed 5 times in PBS-Tween. 100µl of appropriate antibody preapartaion in PBS-tween was incubated in the dark at room temperature for 2 hours, the plate then washed 5 times in PBS-Tween. Then anti-rabbit antibodies conjugated with horseradish peroxidase were added (1 in 500 dilution in PBS-Tween -BSA) and incubated for 2 hours at room temperature in the dark. After 5 washes in PBS-Tween-BSA, 200µl of O-phenylenediamine substrate (dissolved in methanol (10 mg/ml) and diluted 1 in 100 into 0.03%(v/v) hydrogen peroxide in distilled water) was added and the plate was incubated for 1 hour in the dark. The reaction was stopped with 50µl of 4M sulphuric acid and the absorbance measured at 490nm using a Dynatech ELISA plate reader.

The test bleeds were used at various concentrations and graphs of absorbance versus test serum concentration were plotted. From these it was possible to determine if the antibody titre had increased from one test bleed to the next. An example is shown in Figure 6.10. Once the antibody titre had stabilised the rabbits were boosted once more and bled out. The serum was prepared as in the test bleeds.

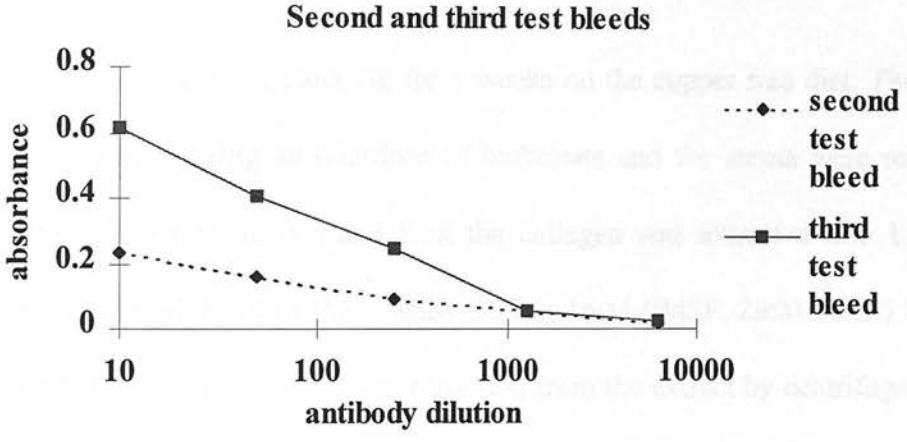


Figure 6.10 - Typical graph produced from ELISA results. Since the third test bleed obviously has a higher antibody titre the rabbit should be boosted again and the antibody titre of the fourth test bleed should be compared to that of the third.

6.5.4 Preparation of additional lathyritic type II collagen

Additional type II collagen was required for the affinity purfication of the antibodies. The original collagen had been prepared by feeding birds β-Aminoproprionitrile (BAPN) (Bannister and Sood, 1974) from hatching for three weeks, BAPN inhibits the action of lysl oxidase and prevents the collagen produced from being crosslinked, enabling the collagen to be extracted without the use of proteolytic agents such as pepsin. The molecules produced are therefore full length, with intact telopeptides.

The production of lathyrism in chicks using BAPN is potentially hazardous to staff handling the animals, and so it was decided to feed the birds on a diet which was essentially copper free (Lilburn and Leach, 1980). Since lysl-oxidase is a copper-dependent enzyme, this compromises the activity of the enzyme without the hazard risks of using BAPN. The diet was essentially based on skimmed milk and the formulation is included in the Appendix.

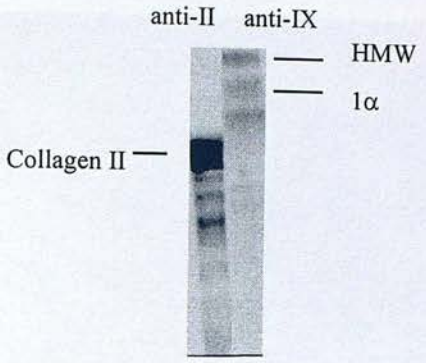
Chicks were fed from hatching for 3 weeks on the copper free diet. The birds were then sacrificed using an overdose of barbituate and the sterna were removed. The sterna were homogenised and then the collagen was extracted into 1.0M sodium chloride 50mM TRIS pH7.5, 20mM EDTA, 1mM PMSF, 2mM NEM) for 72 hours at 4°C. The remaining pulp was separated from the extract by centrifugation and the extract was dialysed against 3.0M sodium chloride (three changes 24 hours). The type I collagen precipitate was isolated by centrifugation and the remaining extract was dialysed against 4.0M sodium chloride (three changes in 24 hours). The precipitate was collected by centrifugation, and redissolved in 1.0M sodium choloride. This lathyritic type II was used for the preparation of nitrocellulose strips for affinity purification of the antibodies.

6.5.5 Affinity purification of the antibodies

The method used was adapted from that of Whitely (1990) see Appendix. Well-less 6% SDS-PAGE gels were run of the antigen preparation used to prepare the antibodies, or additional lathyritic collagen II. The collagen IX preparation was electrophoresed under non-reducing conditions. The gels were Western blotted

overnight at 200mA, then developed using Ponceau S. The collagen II or collagen IX HMW bands were excised from the blots to create strips for affinity purification. The strips were then blocked using 3%BSA in PBS-Tween, washed, then the serum was applied to the strip (as much as can be held by surface tension). The strip was incubated in humid conditions for two hours at room temperature to allow binding, then the excess serum was removed and the strip washed in PBS-Tween. The antibody was eluted from the strip under acid conditions then the solution was neutralised.

Satisfactory purification of the antibodies was obtained using this method. The extent of purification is shown in (Figure 6.11). The antibodies were tested using dot blotting and were not reactive against BSA, milk proteins and type I collagen. As the anitbodies were not raised against completely pure preparations, it was important to assess that the affinity purification had removed activity against type IX collagen in the antibody produced agianst and purified to type II collagen and vice versa. To test this, a well-less non reduced 6% SDS-PAGE gel was run containing a mixture of type II and type IX collagen. This gel was blotted and cut into strips, each of which is identical and contains HMW type IX collagen preparation and type II collagen. The strips were incubated with the affinity purified antibodies. As can be seen in Figure 6.11 there is no cross reactivity of the affinity purified antibodies.



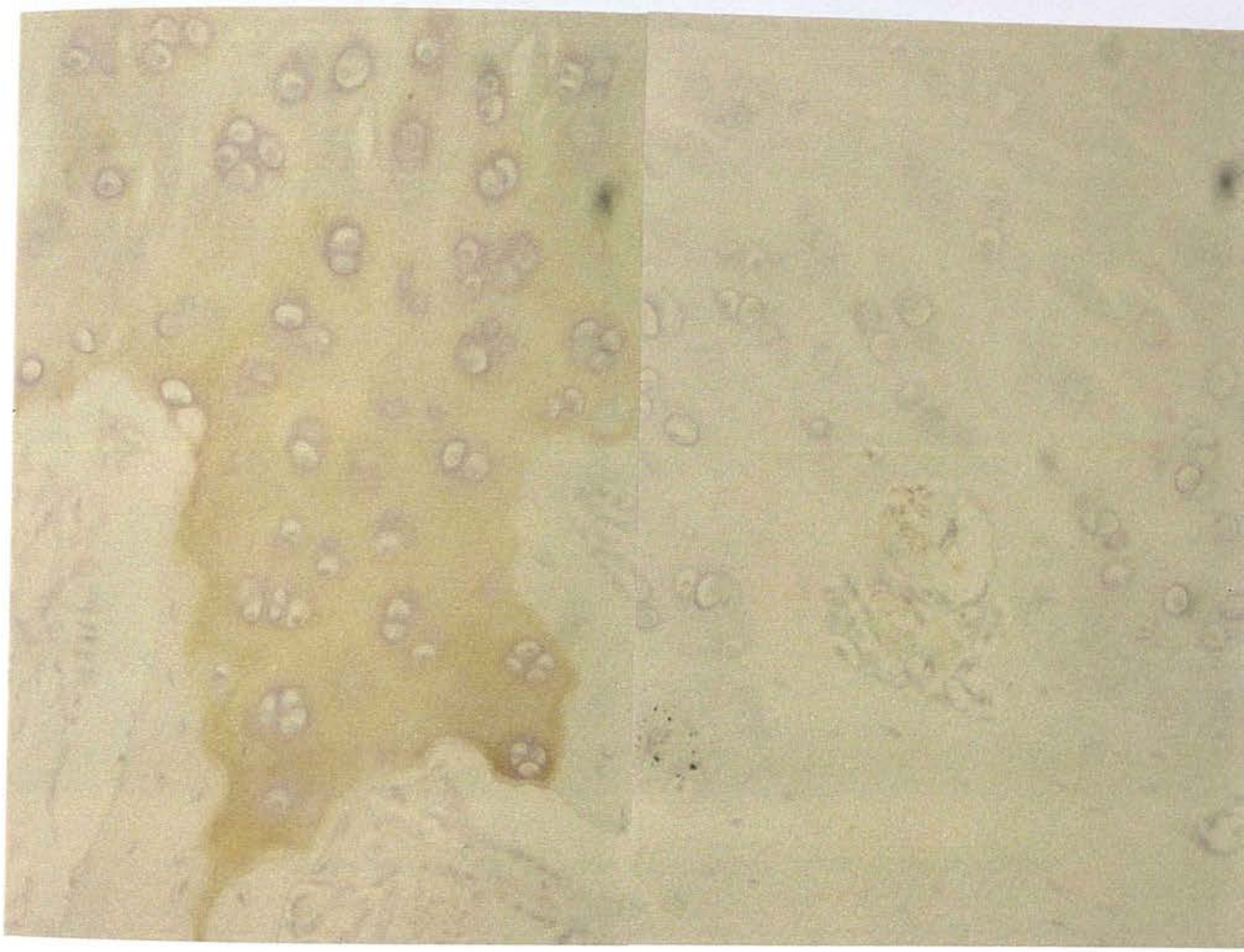
The nitrocellulose strips each contain both type II and type IX collagen

Figure 6.11 - Western blots showing the extent of the affinity purification. Although each nitrocellulose strip contains both collagen type II and type IX the antibody preparations only react with the appropriate antigen.

6.6 Immunostaining

The antibodies prepared against the HMW band of collagen IX were used for immunostaining of some sections from the longitudinal study (for method see Appendix). Preliminary results are presented here. The control serum was prepared by affinity purification of the pre-immune serum as described for the experimental serum.

As can be seen in Figure 6.12 the staining for type IX collagen was mainly localised to the deep zone of the articular cartilage. This is in accordance with the original type IX localisation studies in pig articular cartilage (Duance *et al.*, 1982), where the majority of staining was also found in the deep zone. The staining, as can be seen in



a b

Figure 6.12 (a) Staining for type IX collagen (brown) (b) control section

Figure 6.13, is not confined to the pericellular region which was the case in the original studies by Duance *et al.* (1982) .The high content of type IX collagen in the pericellular area was confirmed by Poole *et al.* (1988) where isolated chondrons from porcine articular cartilage and rat chondrosarcoma stained very intensely with antibodies to type IX collagen.

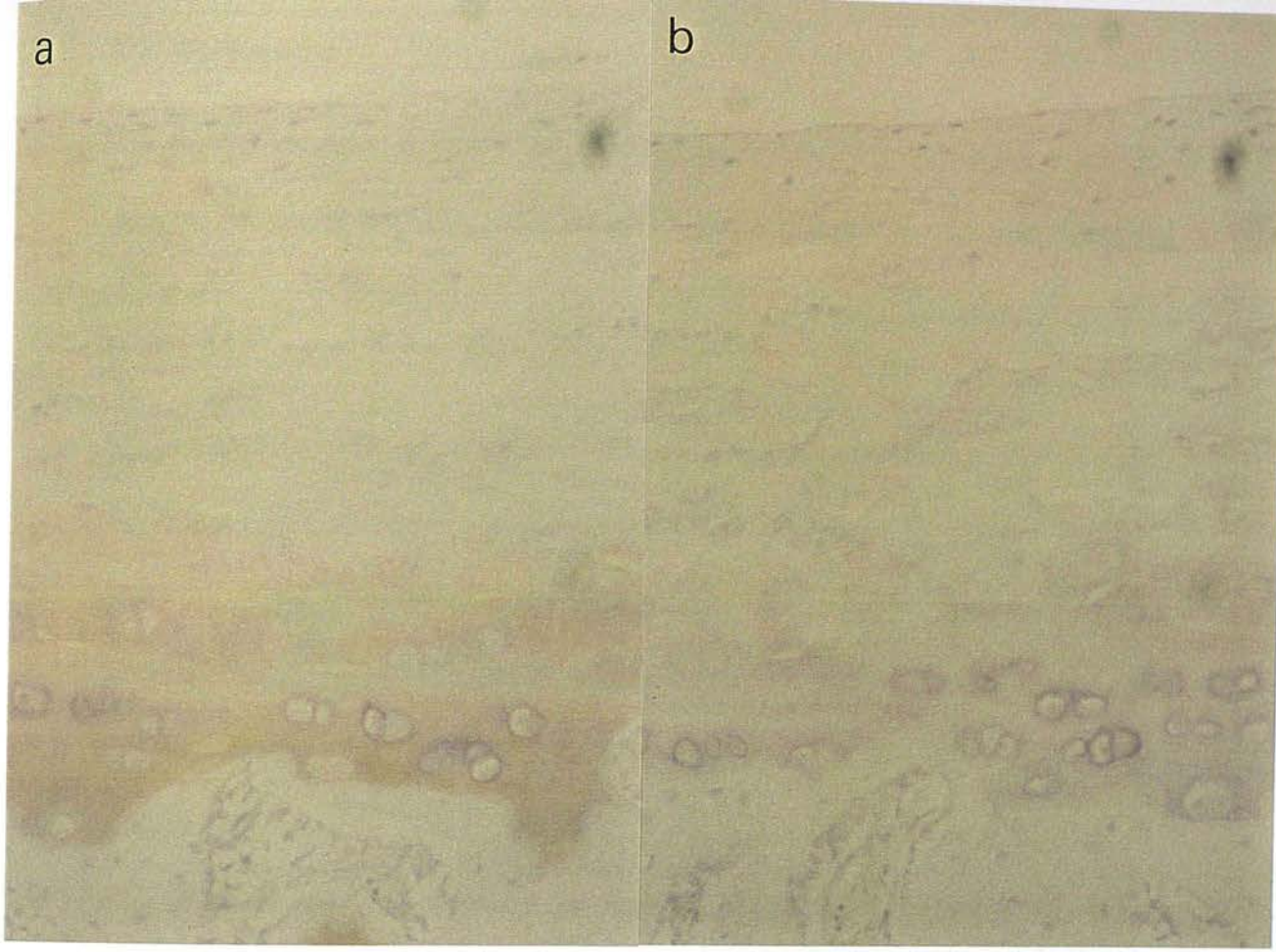


Figure 6.13 (a) - The staining for type IX collagen is spread through the matrix (b) control.

However, studies on chick sternal cartilage have revealed a more even distribution of type IX collagen through sternal tissue (Irwin *et al.* 1985) and the sections in this study show staining spread thoughout the matrix. There is some controversy over the role of hyaluronidase digestion in the exposure of type IX eptiopes (Poole *et al.*, 1985) and while it is possible that the hyaluronidase treatment of the sections on the present study does reveal epitopes, it may also be that as with the avian sterna the collagen type IX distribution in avian articular cartilage is more widespread than that in mammals.

6.7 Discussion

The general increase in hydroxyproline per mg dry tissue is compatible with the general decrease in proteoglycan content observed with time, with the most dramatic changes occuring between day 1 and 19 as with the changes in proteoglycan content.

In the J-line fowl there was a transient increase in hydroxyproline content at day 180. There was also a significant increase in uronic acid content and hydration at day 180 in the J-line fowl, suggesting that a developmental change was occuring at this time. In the *ad libitum* fed birds, which showed an increase in both uronic acid content and hydration at day 180, there was a decrease in the hydroxyproline content. This is probably related to the higher proportion of proteoglycan in the tissue at this time. It may also be a sign of some other change as well, considering that the hydroxyproline content at day 279 was lower than that at day 180, although the DTT samples from the *ad libitum* fed birds at day 279 showed no histopathological signs of disease and the uronic content had decreased again to be similar to that of the samples at day 113.

The results for the pyridinoline assays are expected mainly to show differences in the formation of these crosslinks. They will not give us information on collagen network breakdown. The broiler strain fowl do not have less mature collagen crosslinking in their articular cartilage than the J-line fowl. The increase in hydration observed in disease must be due to some other factor. The lack of association of pyridinoline content with disease is in agreement with the observation that total pyridin oline content did not change with development of OA in partially menisectomised rabbits

(Pokharna *et al.* 1995). Also in humans there is no difference in pyridinoline content between OA and normal articular cartilage samples (Takahashi *et al.* 1994).

The susceptiblity of the DTT to disease could be explained by the DTT containing less mature crosslinks in the cartilage. The DTT of the feed restricted and J-line groups certainly do contain less pyridinoline crosslinks per collagen molecule than the other articular surfaces examined within each group of birds. However, this is not detected in the *ad libitum* fed birds, although the results are very close to being significantly different. Clarification of this point is very important, it could be that lower crosslinking is one factor involved in the susceptibility to disease of the DTT. In particular an emphasis in comparing crosslinks in samples from DTT and PTM could elucidate this matter.

The progression of crosslinking with age is another matter under consideration. The *ad libitum* fed DTT did not show any increase in pyridinoline content with age, which is in agreement with Uchiyama *et al.* (1991) who observed that pyridinoline levels in cartilage did not increase with age. The DTT of the feed restricted samples at day 180 and 279 did not have a significantly different pyridinoline content and neither did the DTT samples of the J-line at days 180 and 279. However a large increase in crosslinks was observed in the J-line birds DTT and PH between days 270 and 376. This increase in crosslinking could be due to the extreme old age of these birds and requires to be investigated further.

Load, in the context of the difference between PH and DTT, does not appear to play a role in the development of pyridinoline crosslinks in the J-line fowl, since there is no significant difference in crosslinking between the J-line PH and DTT samples at day 279 and 376. These results do not clarify the increase in hydration observed in the J-line PH at day 376.

A more extensive investigation of the distribution of type IX collagen in avian articular cartilage is required, however preliminary results suggest that unlike mammalian cartilages (Duance *et al.* 1982) the type IX collagen is not restricted to the pericellular area but instead is spread throughout the matrix. This widespread staining is similar to that observed in developing avian sterna (Irwin *et al.* 1985), but appears to be confined to the deep zone. Since type IX collagen tends to be localised with type II (Muller-Glauser *et al.*, 1986) it would be interesting to investigate the distribution of collagen I and collagen II in parallel tissue sections.

6.8 Conclusions

These results have shown that the susceptibility of broiler strain fowl to DJD is not mediated by a deficiency in pyridinoline crosslinking. The susceptibilty of the DTT to DJD may be influenced by lower amounts of pyridinoline crosslinking, although this is far from clear. Preliminary results suggest that the distribution of type IX collagen in avian articular cartilage appears to be widespread throughout the matrix of the deep zone.

7. Artificial loading study

7.1 Introduction

7.1.1 Aim of the experiment

The aim of this experiment was to investigate the role of *in vivo* loading on the articular cartilage of broiler strain fowl. The work presented in chapters 4 and 5 strongly suggested that load has a vital role to play in the development of DJD since the *ad libitum* fed broilers develop DJD much more severely and earlier than feed restricted birds. It is, however, possible that a systemic factor may also be involved. In this study feed-restricted birds were artificially loaded to minimise the metabolic consequences of growing to a large body mass. The role of load thus becomes the primary variable under study.

7.1.2 Loading articular cartilage

Load has an important influence on the metabolism of cartilage. Chondrocytes and cartilage are affected if the load across the joint is altered (Urban, 1994). The focal lesions of OA in knee and hip occur at the positions of peak loading, implying that mechanical factors are involved in the formation of these lesions (Dieppe and Kirwan, 1994). *In vitro* loading of articular cartilage has become a commonplace tool in trying to ascertain the role of load in development of articular cartilage and progression of DJD. Most animal models of OA utilise an increase in load in local areas of the joint either by introducing joint instability, for example by anterior cruciate ligament section (Pond and Nuki, 1973), or altering loading across the joint such as menisectomy models (Hoch *et al.*, 1983). These large changes in load on the

joint affect cartilage structure and chondrocyte activity within days and may eventually result in complete loss of cartilage (Burton-Wurster *et al.*, 1993).

Traditionally it has been assumed that *in vivo* loading studies are too complicated to study, in detail, the mechanisms of the loading response (Urban, 1994). Growth and ageing complicate interpretation of the results. The effect of exercise, as a means of increasing load, affects circulating factors. These problems are addressed in this study by using 16 week old feed restricted fowl which have reached maturity. Ageing effects over the 3 week period should be negligible and identical for the experimental and control groups. Since the load is applied directly and not as a result of increasing exercise, the effect of circulating factors in the experimental group is minimised.

Artificial loading of chicks has been previously reported (Cook *et al.*, 1984; Patterson *et al.*, 1986). Such experiments were aimed to investigate the role of load on development of leg deformities. The loading was carried out between one and four weeks of age. The method involved looping rubber bands through steel washers and then under the wings of the birds. In the present study this method was improved by using 5mm elastic fabric instead of the rubber bands. The birds were loaded with approximately 10% of their body weight. Biewener and Bertram (1993, 1994) have used artificial loading of fowl to examine the effect of extrinsic loading on bone modelling in early postnatal growth. The birds in these studies were subjected to vigorous exercise carrying an additional 20% of their body weight in weights distributed by a cloth cumberband around their trunk. The excercise periods were restricted to 15 minutes per day and the rest of the day the birds were kept in

restrictive cages. A more natural approach to loading was required in the present study, the birds having free movement in pens whilst continually being additionally loaded, and so artificial loading devices, 'rucksacks', were designed.

7.2 Materials and methods

The fowl used in the present study were feed-restricted broiler strain, aged 16 weeks at the start of the loading. They had been kept on a commercial feed restriction programme. This age was chosen since the birds had reached maturity and so growth should not be a complicating factor in the experiments. The birds were reared in pens. In order that loaded birds did not have to compete (presumably unfairly) for food with unloaded birds, birds in each pen were subjected to the same loading regime. The experiment involved increasing the body mass of the loaded birds by 10%. The possible effect of increased energy utilization on feed intake and body weight was considered. However, the method for calculating the diet on the restriction schedule uses large error margins and, taking into account previous studies on the energy requirements of fowl (MacLeod and Jewitt 1985, MacLeod *et al.* 1982), it was not considered that the extra energy utilized for a ten percent increase in body weight would necessitate an increase in the ration for the loaded birds (personal communication Dr. Murdo MacLeod).

The rucksacks were designed so that the birds would carry the load in the centre of the back. Three internal pockets inside the rucksack prevented the weights from slipping within the rucksack and unbalancing the bird. The main body of the

rucksack was made from white cotton fabric: this matches the colour of the birds feathers to minimise rejection by the birds. The straps were made from 3/4 inch white elastic and fastened with white velcro: the back of the rucksack was also fastened shut with white velcro. The design of the rucksack is shown in Figure 7.1.

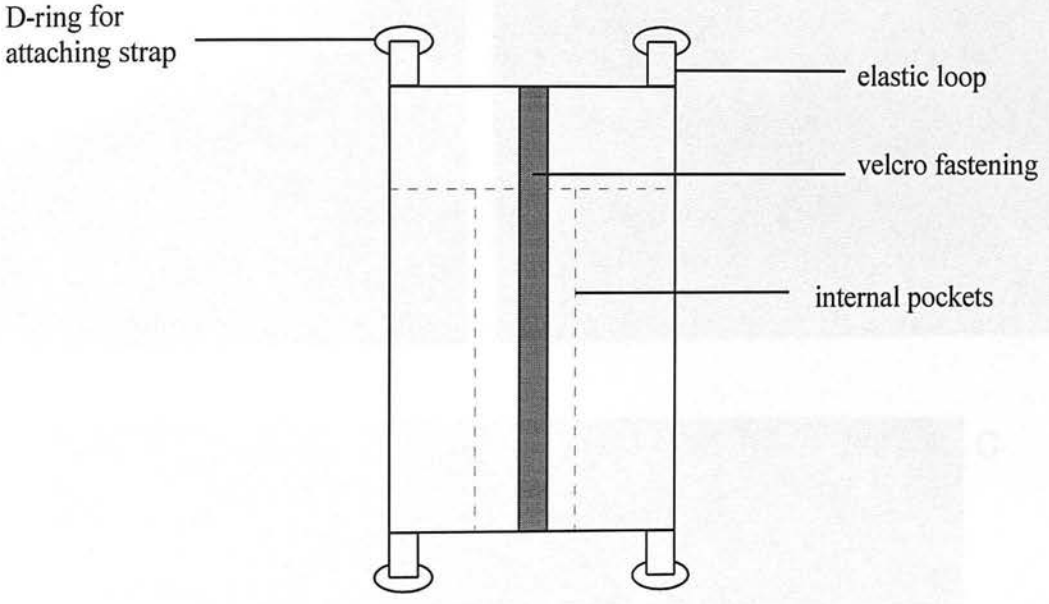


Figure 7.1 - Design of the rucksack for the chickens. Three internal pockets prevent the weights from slipping within the rucksack during use. The elastic straps were fastened with velcro and were adjusted by reducing the length by knotting to fit individual birds.

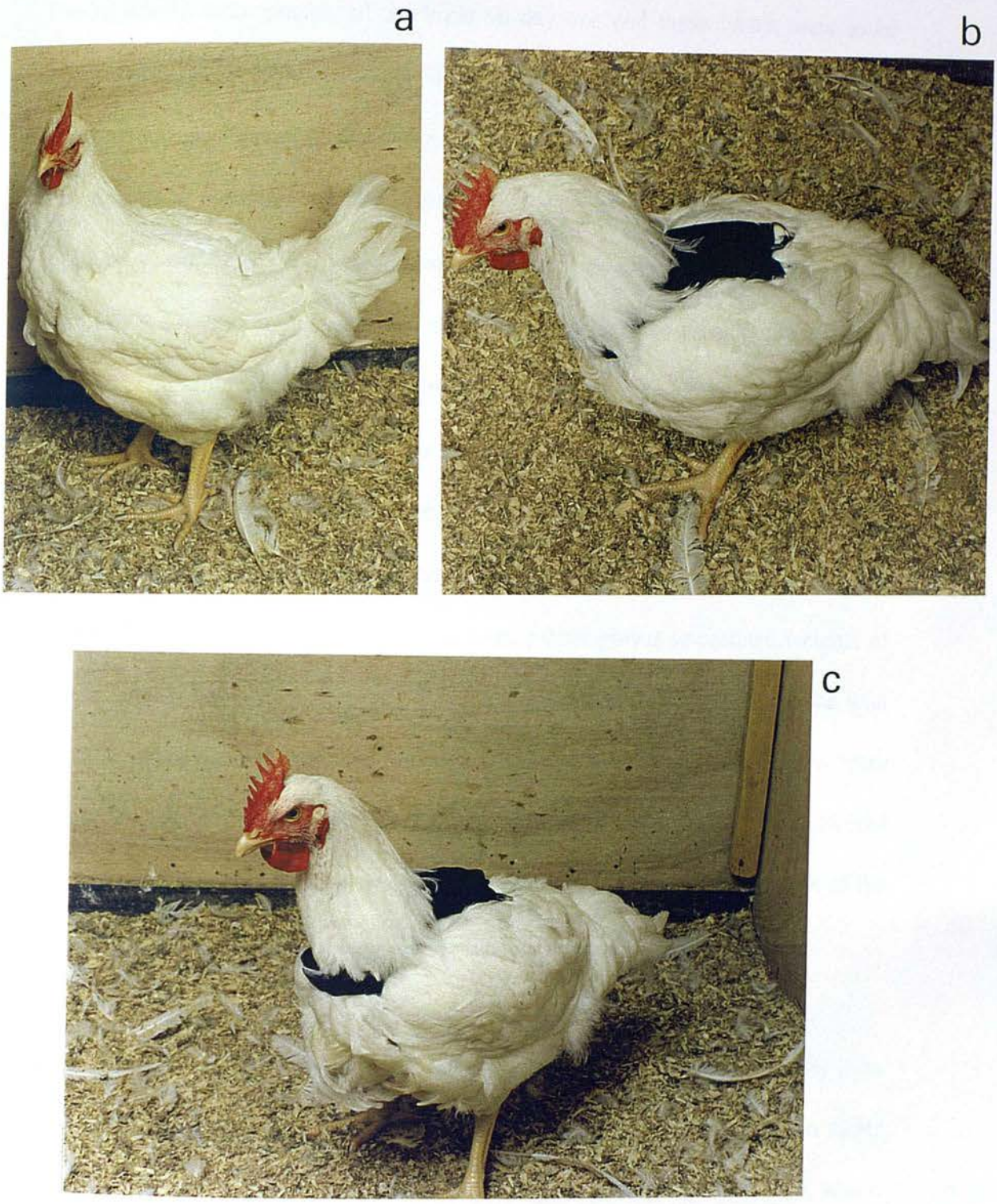


Figure 7.2 - Chickens with rucksacks. In (a) the camouflage of the white rucksack is illustrated, the loop of the backstrap and one D-ring can be seen. In (b) and (c) a black rucksack, dyed for photography, is seen in the middle of the back. The front view (c) shows the front straps, originally designed to be under the wing, in front of the bird whilst the front to back straps are not visible under the feathers.

The birds were allocated to groups destined to have loaded or unloaded rucksacks. The rucksacks were put on all the birds on day one and those which were to be loaded were loaded the next day. This schedule allowed familiarisation to the rucksack and minimised the discomfort of the birds. When the rucksack was first put on, an adverse reaction was observed and the bird was agitated, often exhibiting a backwards running behaviour. However, after 18 hours the birds appeared completely adjusted to the presence of the rucksack as they were walking around normally. The introduction of weight into the rucksack was much less disturbing than the initial introduction of the rucksack and the birds resumed normal walking around within an hour of being re-introduced into the pen. The weights consisted of one penny pieces, each of these weighing approximately 3.5g. They were collected in groups of 14, 7 and 3 into the fingers of latex rubber gloves to produce weights of approximately 50, 25 and 10 grams. Each individual bird was weighed and the load of 10% of the bird's mass was calculated. The rucksack and the weights were weighed together to produce the mass required to within 5 grams. Each rucksack was marked inside and the weights were distributed evenly in the internal pockets of the rucksack, space being padded out with medical tissues.

After one week it became apparent that the D-rings which lay at the shoulder joint were rubbing and starting to cause pressure sores on the less feathered region under the wing. The arrangement of the straps was changed to bring the two straps, which had been one under each wing, to be fastened across the front of the birds. The strap which went from the front two D-rings and looped through the back strap was tightened and this was sufficient to keep the rucksack in place whilst relieving the

pressure on the under-wing area. The D-rings were now lying over the feathered part of the bird which protected the bird from further injury. At 0, 6, 13 and 20 days after the commencement of loading, groups of 12 birds were sacrificed (6 loaded and 6 unloaded). The joint surfaces investigated were the DTT, which showed strong susceptibity to disease in the longitudinal study, and the PTM, the opposing surface in the joint which seems to be somehow protected from disease. Cartilage samples were taken as in the longitudinal study and digested with papain, as in chapter 5. Samples were also taken for histology and processed as in chapter 2.

Behavioural studies were carried out; the birds in sample pens were scan observed every minute during a fifteen minute period. The behaviour was scan observed (Martin and Bateson, 1993); the behaviour of each bird in a pen was noted at a set point in a minute, once a minute for 15 minutes. The behaviour was one of feeding, standing, resting, preen/stand, preen/rest, drinking, walking, peck/wall, peck litter, scratch, dustbathe, feather pull, feather eat, agressive peck or rucksack peck (Copy of observation sheet in the appendix). Each of these behaviours could be categorised as standing or sitting. Observations of 4 pens were made prior to loading, then during the experiment observations were made of 7 unloaded and 8 loaded pens. The percentage of time spent exhibiting standing or sitting behaviour was calculated for each pen. Since the numbers of birds in the pen was decreasing every week the results were calculated as a percentage of total behaviour observed. Some of the observations were carried out by Susan Alexander.

7.3 Results

7.3.1 Behavioural studies

The main purpose of these studies was to monitor the behaviour of the birds and to establish the effects of having both a loaded and unloaded rucksacks. Initially the main concern had been that the birds with loaded rucksacks would exhibit much more sedentary behaviour than the birds with unloaded rucksacks. This would introduce a variation in activity in addition to the variation in load on the joints. The percentage of time standing is shown in Figure 7.3.

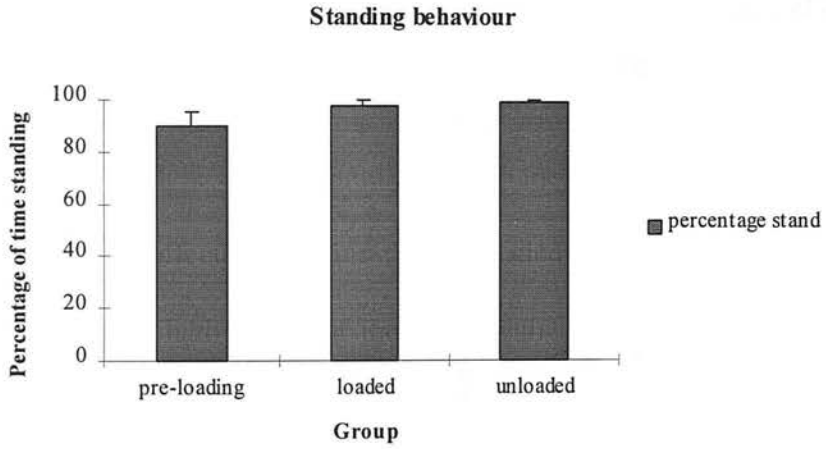


Figure 7.3 - Percentage of time spent standing by the three groups. Error bars are S.E.M.

The rucksacks, either loaded or unloaded, did not have a significant effect on the standing behaviour of the birds. This confirms that the major variable under study in this experiment was, in fact, the increased load on the joint brought about by the additional weight in the rucksack.

7.3.2 Hydration

The hydration of the samples is shown in Figure 7.4. Results for the PTM were not significantly different between the loaded and unloaded groups. This is in sharp contrast to the results from the DTT, where the hydration of the loaded DTT samples was significantly increased ($p<0.05$) over that of the unloaded samples at days 6 and 13 after loading. There was no significant difference at 20 days after loading.

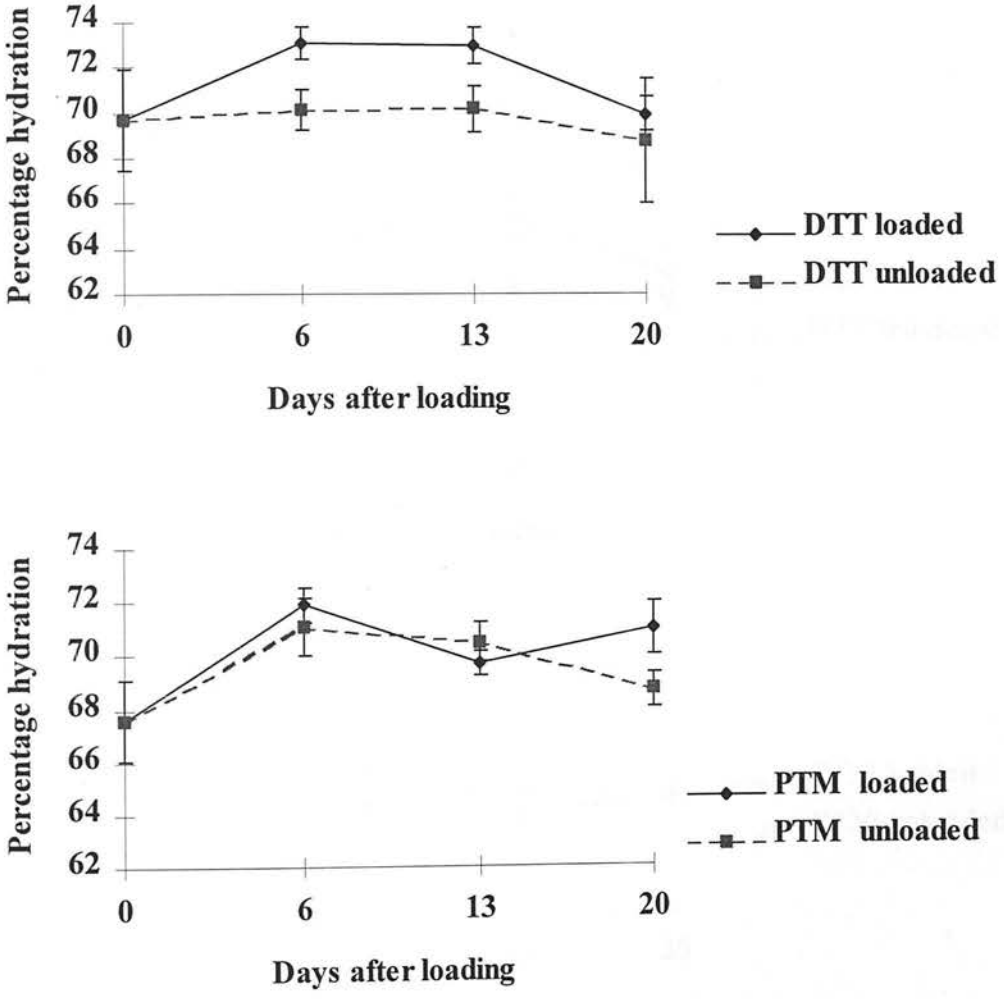


Figure 7.4 - Hydration of the cartilage from the DTT and PTM of the loaded and unloaded birds. Error bars are ±S.E.M.

7.3.3 Sulphated glycosaminoglycan content

The results for the SGAG content (Figure 7.5) closely followed the pattern of the hydration results. The loaded DTT showed an increase in SGAG content over the unloaded DTT (at 13 days, $p<0.02$) There was no difference at 20 days. There were no significant differences in the SGAG content between the loaded and unloaded PTM samples. The SGAG content of the loaded DTT at 13 days after loading was significantly ($p\leq0.01$) higher than that of both the unloaded and loaded PTM at this time.

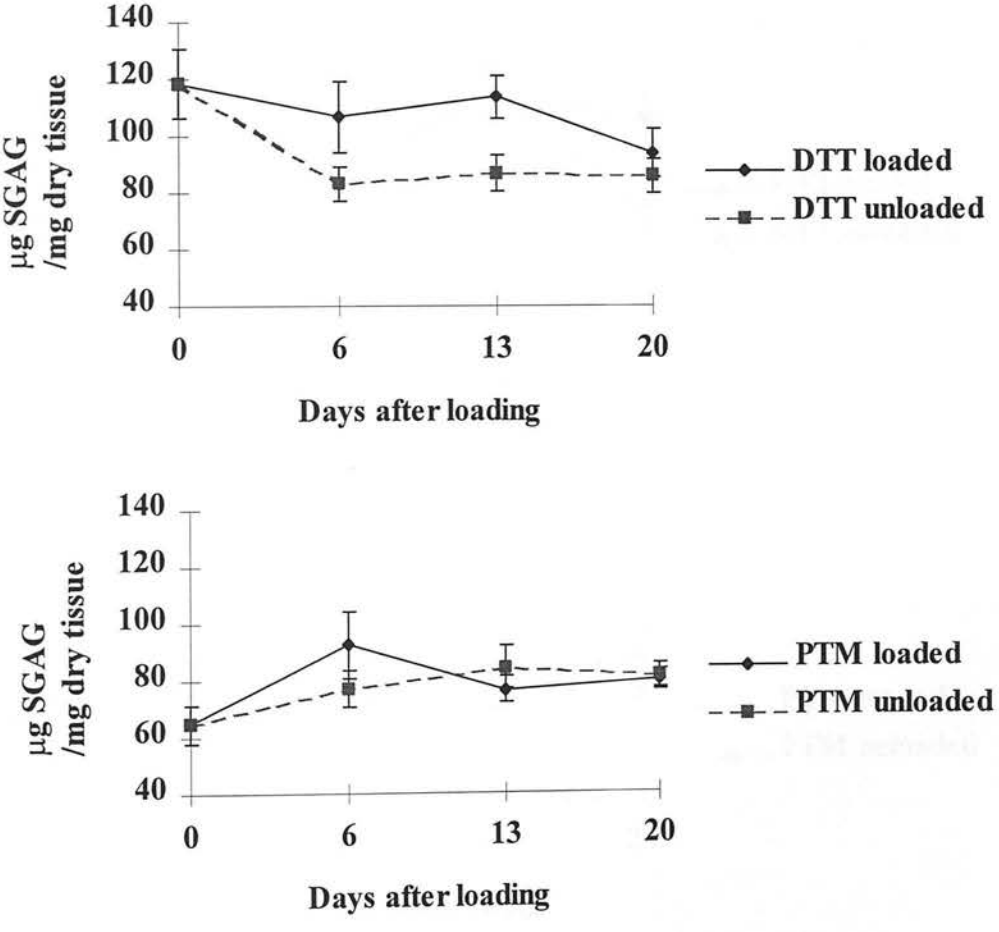


Figure 7.5 - SGAG content of the loaded and unloaded cartilage. Error bars are ±S.E.M.

7.3.4 DNA Content

There was only one significant difference in DNA content between a loaded and unloaded sample. This was the DTT at day 20, where the loaded cartilage had less DNA than the unloaded cartilage (p=0.01). In general, the PTM appeared to have a slightly lower DNA content than the DTT although this was not significant at all time points.

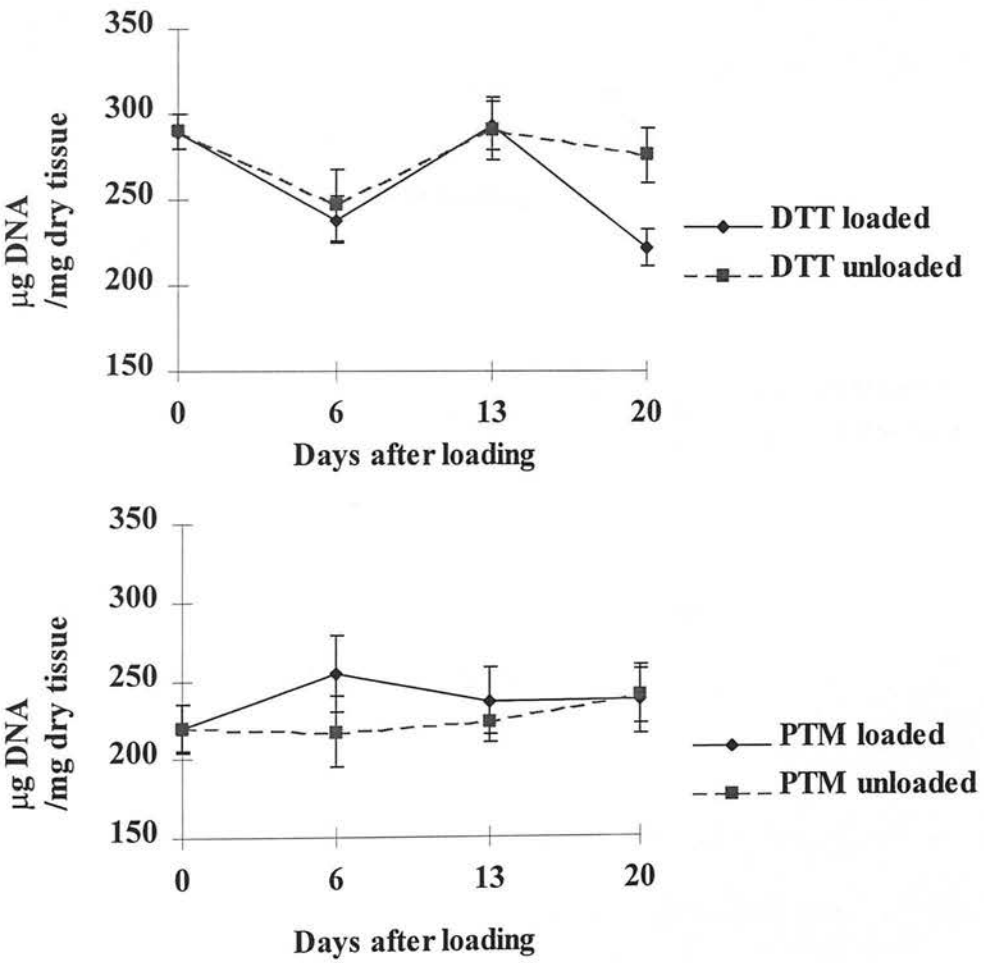


Figure 7.6 - DNA content of the loaded and unloaded cartilage. Error bars are ±S.E.M.

7.3.5 Uronic acid content

There were no significant differences in the uronic acid content between the loaded and unloaded cartilage samples in either the DTT or the PTM (Figure 7.7).

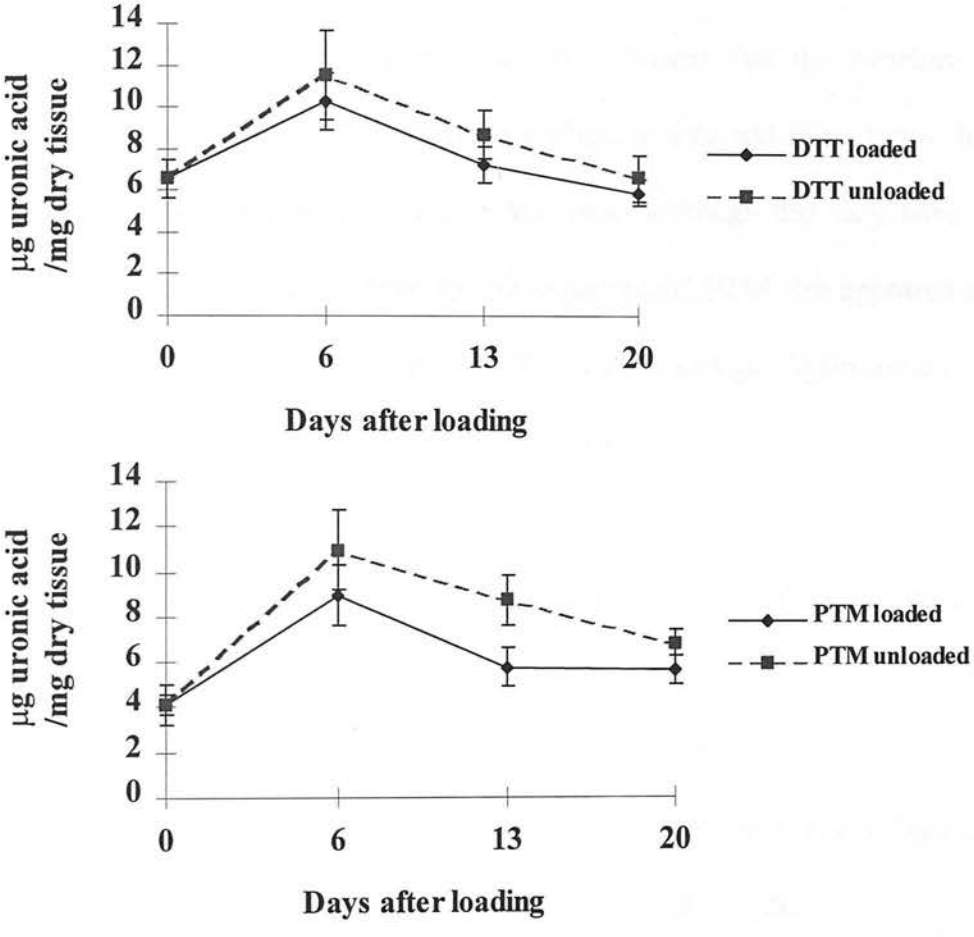


Figure 7.7 - Uronic acid content of the loaded and unloaded cartilage samples. Error bars are ± S.E.M.

7.4 Histology and specialist staining

7.4.1 Histology

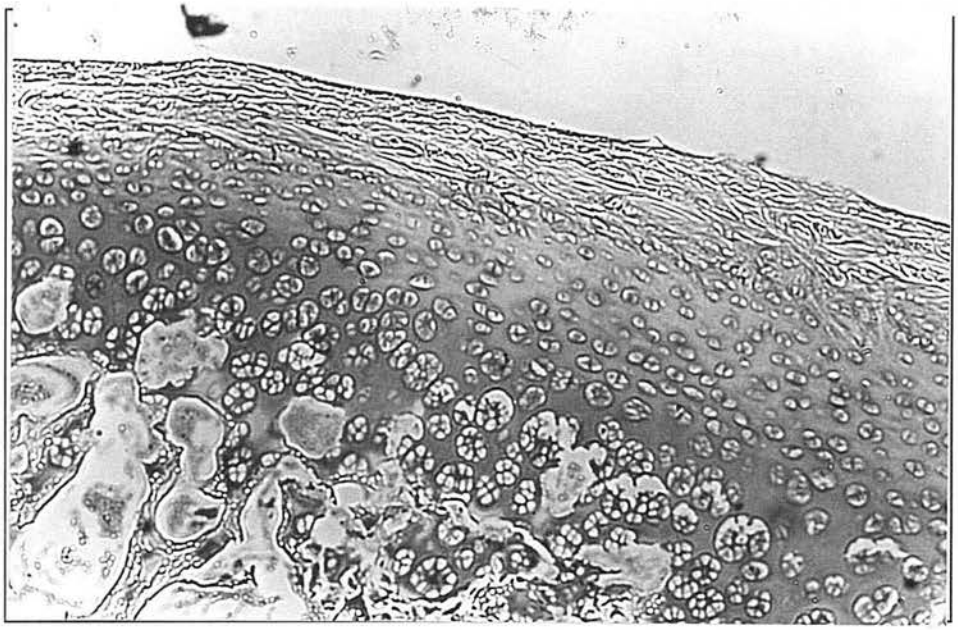
Histological sections were made from all DTT and PTM samples and were stained with haematoxylin and eosin.

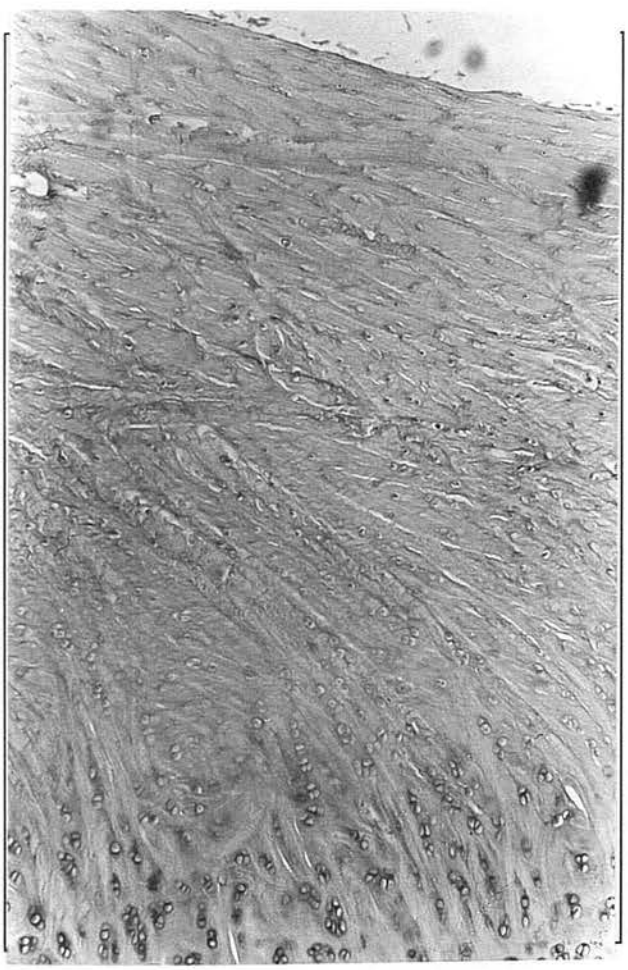
The sections from the pre-experimental DTT showed that the structure of the cartilage was normal, with characteristic surface, middle and deep zones. In some sections there was disruption in the middle zones although this may have been a cutting artefact. The cartilage from the pre-experimental PTM also appeared normal, although it was much thicker than the DTT articular cartilage. Again some mid zone areas appeared to be disrupted (Figure 7.8a and b).

After one week of loading the appearance of the cartilage from the DTT was different in comparison to that of the unloaded cartilage at the same time point (Figure 7.9). There was a certain amount of surface irregularities which, in some sections, gave the surface zone an undulated appearance. There were also instances of cartilage thinning which were not observed in the samples from the unloaded birds.

After two weeks of loading the cartilage of the DTT samples showed various signs of disruption, which included some surface undulation and areas of mid zone acellularity. In addition, some samples showed foci of cartilage thinning and in most samples, the deep zone chondrocytes were beginning to form clusters with basophillic halos (Figure 7.10).

(a)





(b)

Figure 7.8 - Cartilage (a) DTT and (b) PTM from birds before the experiment commenced. Haematoxylin and eosin x70.

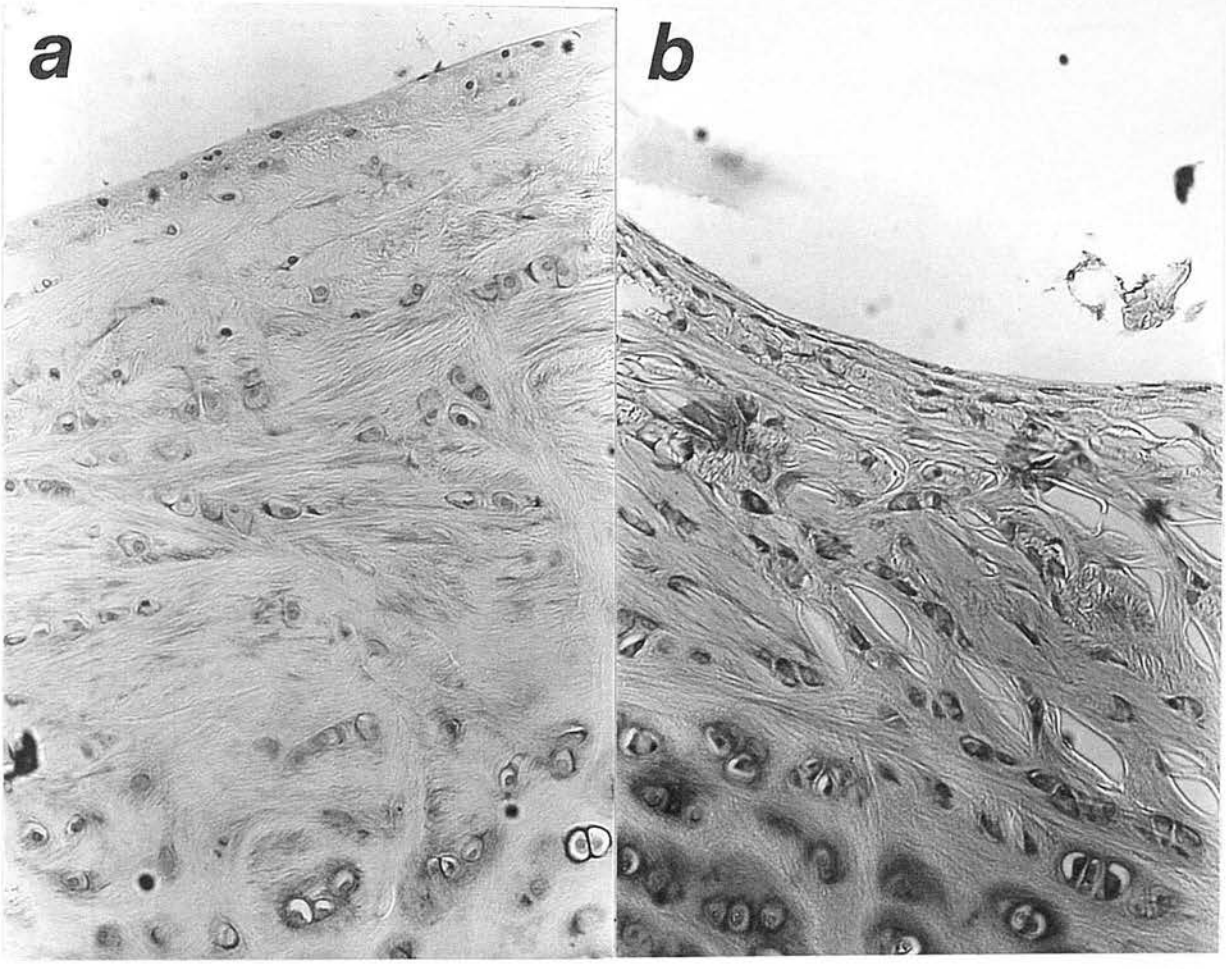
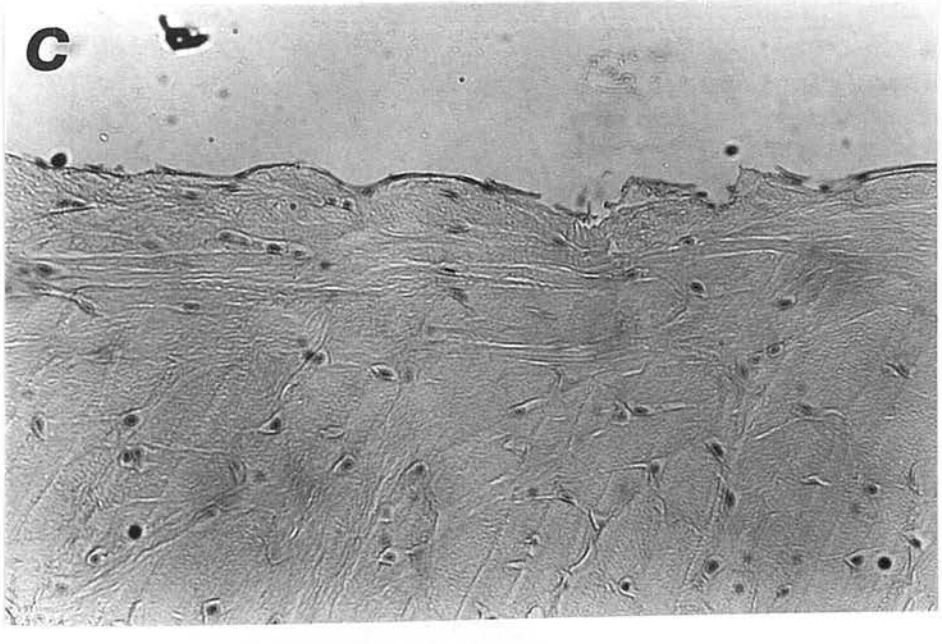


Figure 7.9 - Comparison of loaded and unloaded DTT samples after one week of loading. (a) Unloaded cartilage after one week. (b) Loaded cartilage after one week, displaying considerable thinning. (c) An example of surface undulation of the cartilage. Haematoxylin and eosin x70.



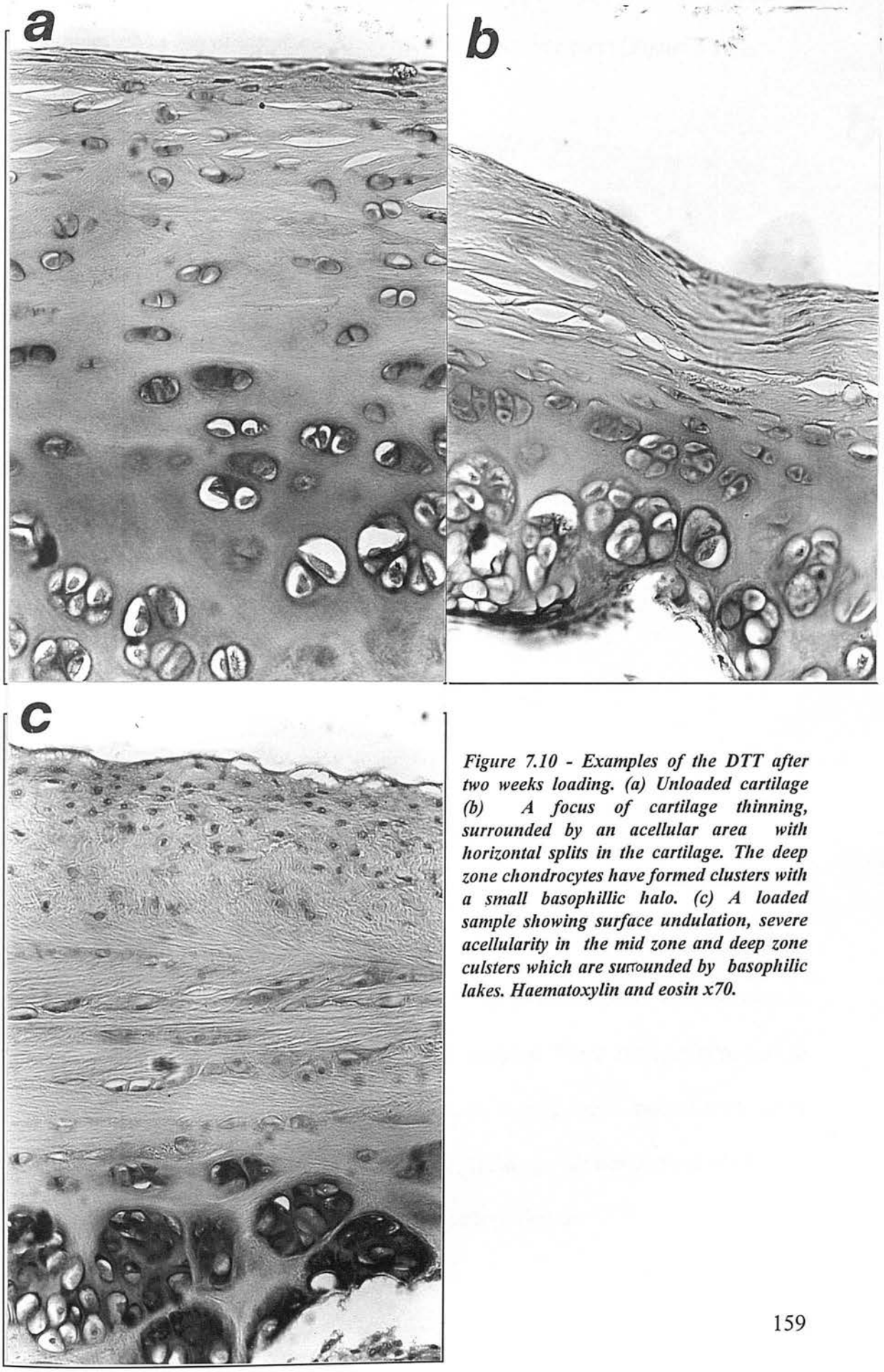


Figure 7.10 - Examples of the DTT after two weeks loading. (a) Unloaded cartilage (b) A focus of cartilage thinning, surrounded by an acellular area with horizontal splits in the cartilage. The deep zone chondrocytes have formed clusters with a small basophillic halo. (c) A loaded sample showing surface undulation, severe acellularity in the mid zone and deep zone culsters which are surrounded by basophilic lakes. Haematoxylin and eosin x70.

After 3 weeks of loading the cartilage of the DTT appeared less cellular in comparison to that of the unloaded samples from this time point (Figure 7.11).

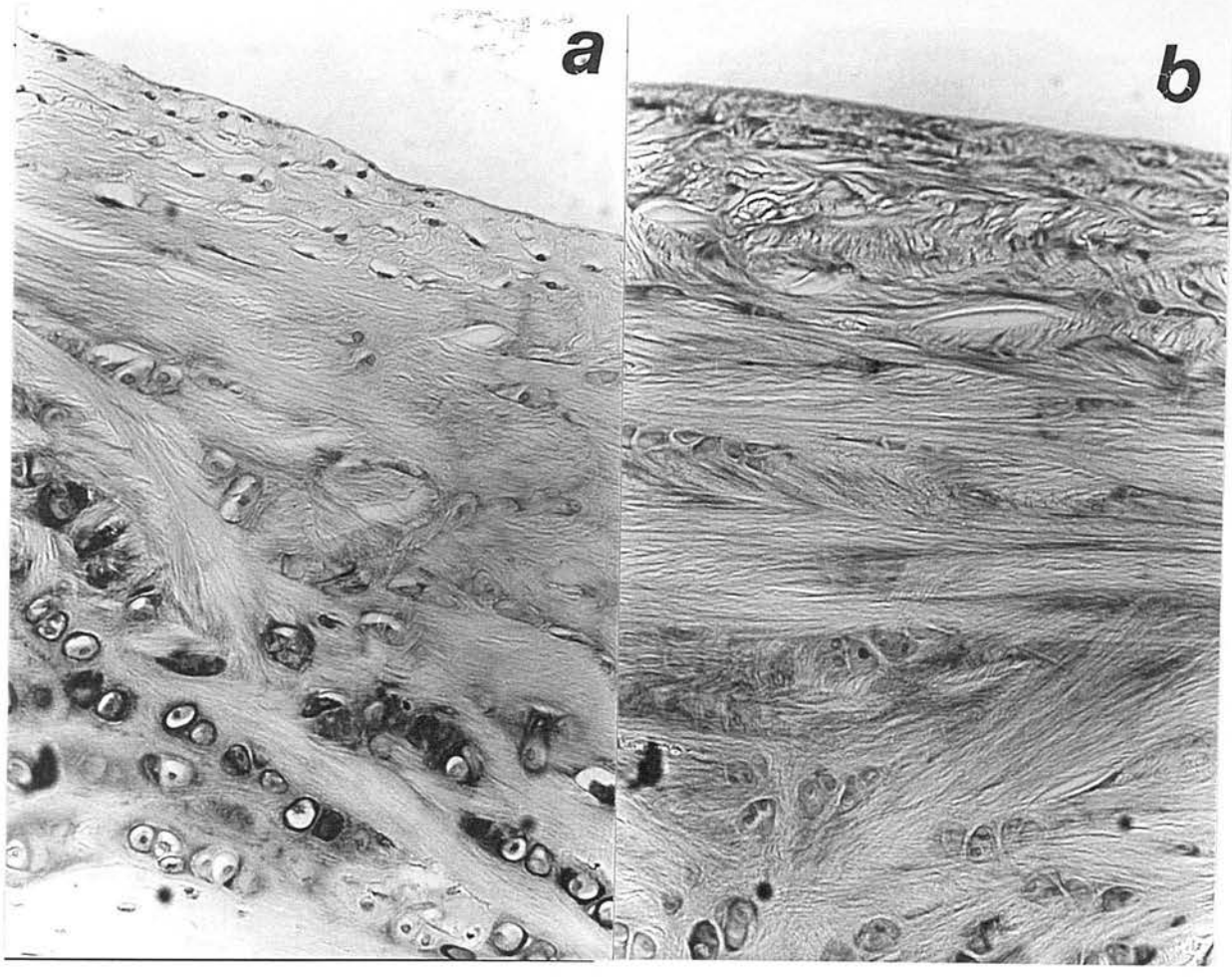


Figure 7.11 - Samples from the three week time point. (a) Unloaded DTT (b) Loaded DTT Note the reduced cellularity and disruption in the surface layer of the loaded sample.

The morphology of the PTM samples appeared less affected by load than that of the DTT. After one week some slight surface and mid zone disruption was observed in loaded samples compared with the unloaded samples. These changes appeared to progress slowly with time and by three weeks of loading some areas of acellularity were observed in the mid zone of the cartilage. However, the morphological changes were not as severe as those seen in the DTT cartilage (Figure 7.12).

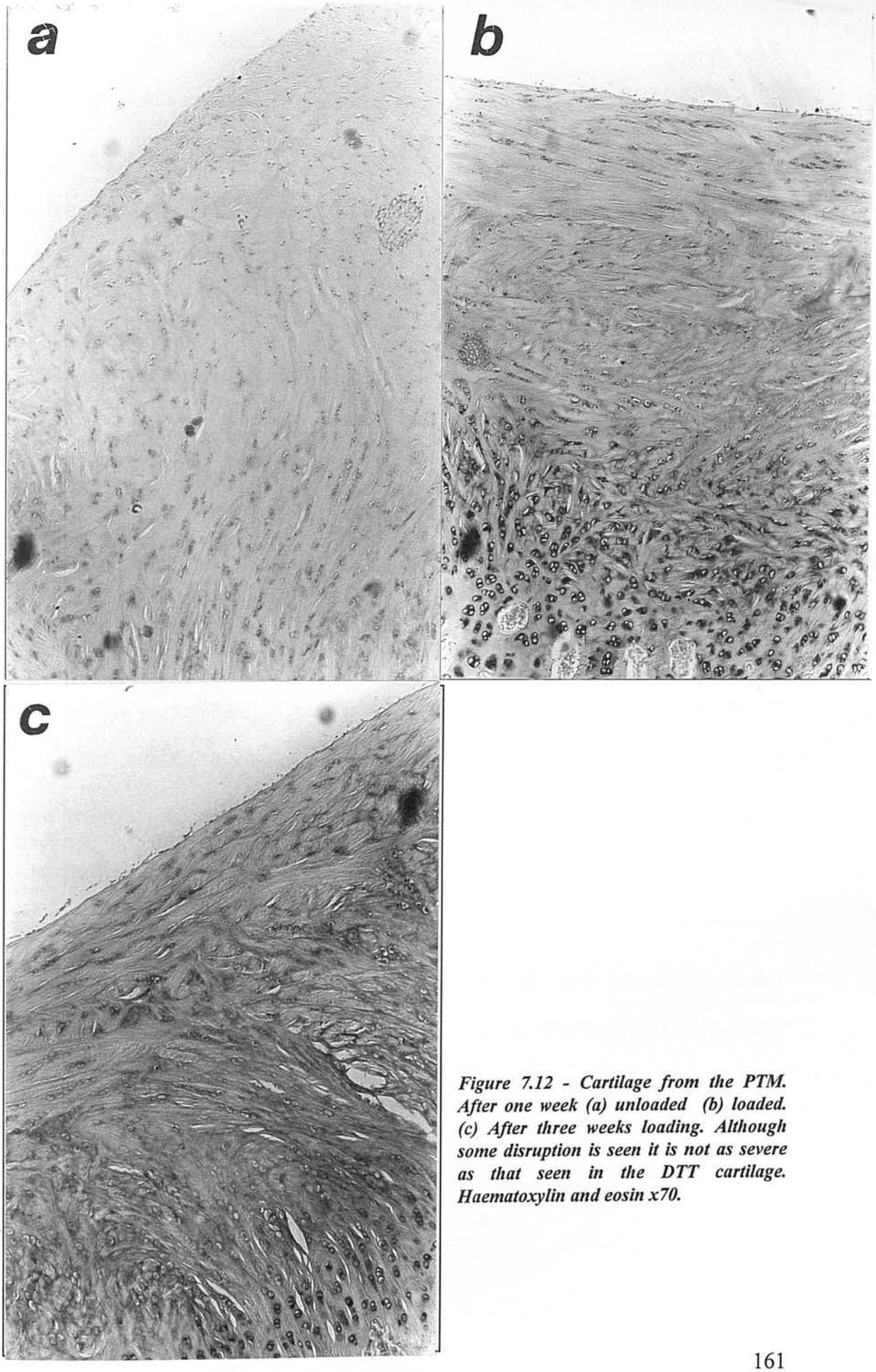


Figure 7.12 - Cartilage from the PTM. After one week (a) unloaded (b) loaded. (c) After three weeks loading. Although some disruption is seen it is not as severe as that seen in the DTT cartilage. Haematoxylin and eosin x70.

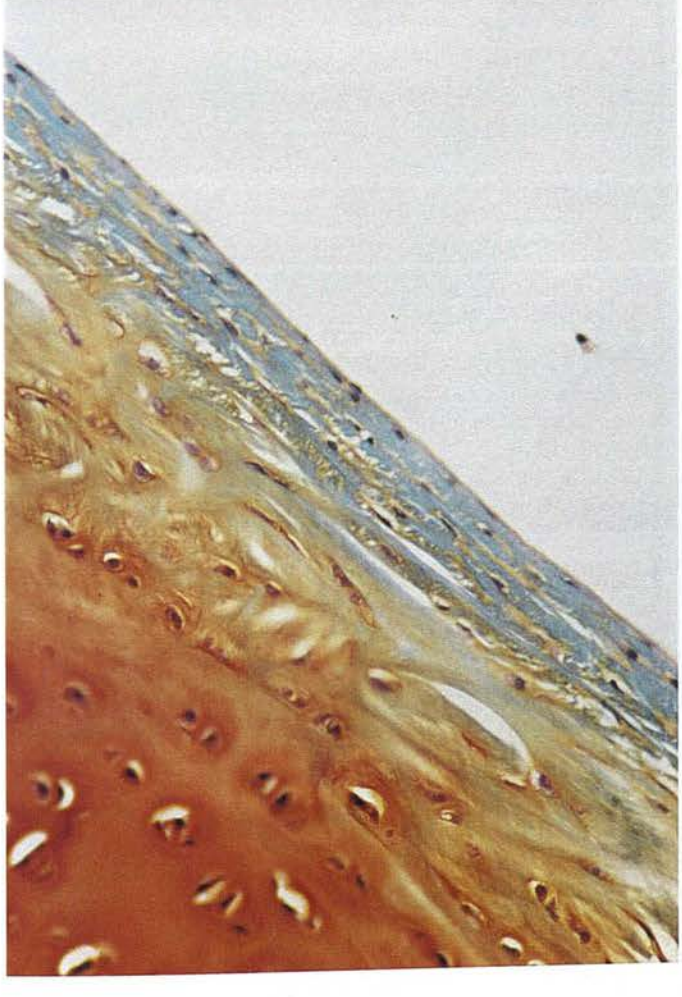
7.4.2 Specialist staining

Since there was a dramatic increase in SGAG content, which appeared to be initiated at an early stage, the pre-experiment and 6 day loaded sections were stained with Saffranin O/Fast Green in order to localise the changes in proteglycan content.

The pre-experiment sections from the DTT show a distinct lack of Saffranin O staining in the surface zone. In the middle zone, the matrix was predominantly unstained and appeared as a green sea which contained islands of chondrocytes. The area immediately surrounding the chondrocytes stained a strong red colour. The deep zone stained strongly for Safranin O.

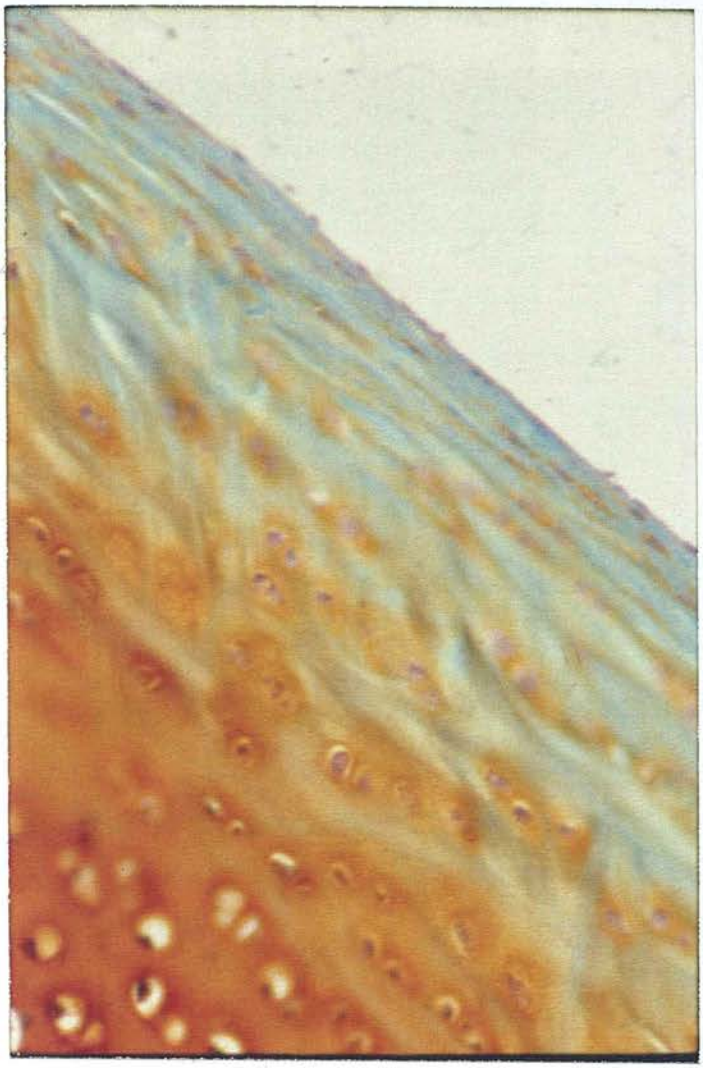
After one week of loading, areas of some DTT sections still appeared similar to the pre-experiment sections. However, there was a change in the staining pattern associated with the disruption of the middle and surface zone. The red staining appeared to spread more completely through the middle zone where previously there was the appearance of a green sea. Where this zone was physically disrupted pink areas could easily be seen. In one area a loss of proteoglycan staining was observed adjacent to an area of relatively abundant staining. This was reminiscent of that observed in the *ad libitum* fed broiler (see section 5.3.2). The Safranin O staining of the unloaded DTT cartilage remained similar to that of the pre-experiment cartilage (Figure 7.13 and Figure 7.14).

(a)



(b)

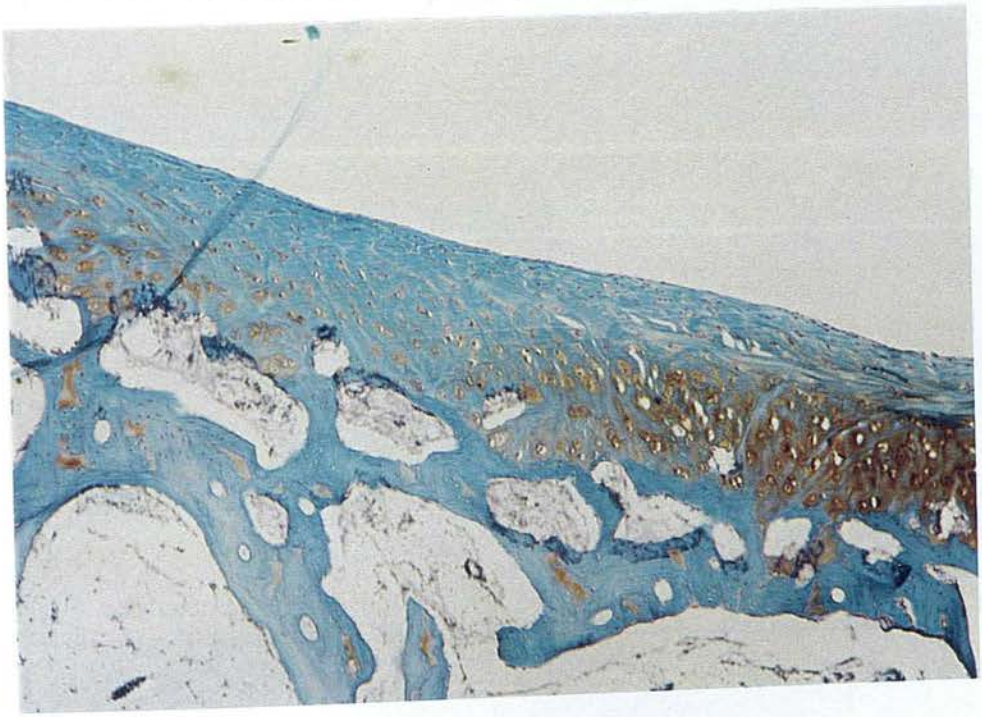
Figure 7.13 - Safranin O stained section from the DTT (a) Pre-experiment, showing the green surface zone, strongly red stained deep zone and middle zone with red chondrocyte islands in a green sea. (b) After one weeks loading the middle zone has gone and some of the surface zone chondrocytes are surrounded by red stain. x70.

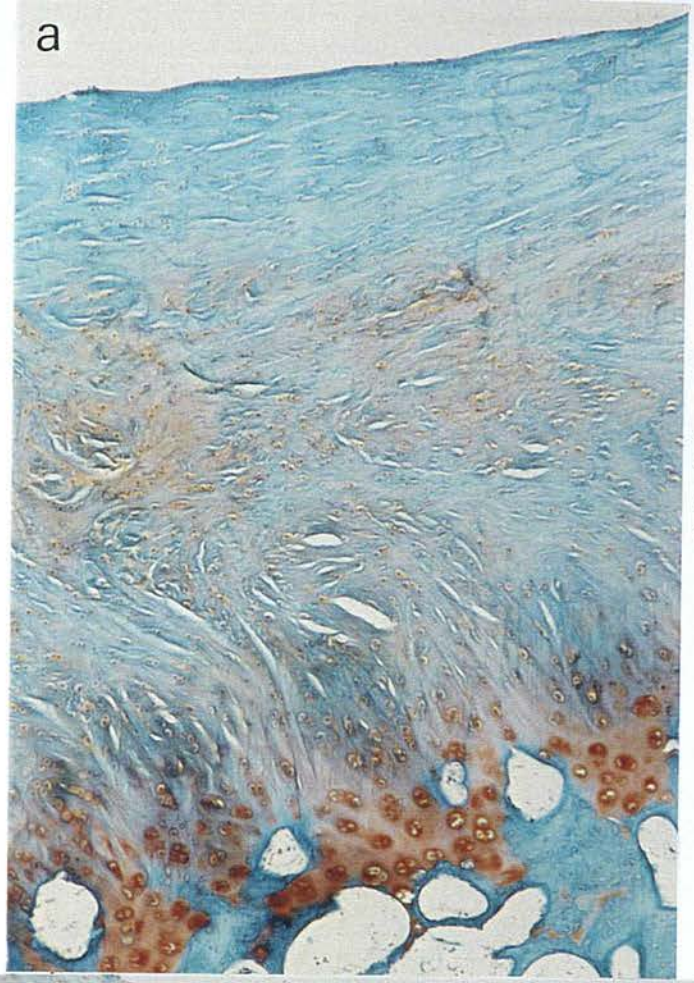


(a)

Figure 7.14 - (a) The unloaded DTT showing a middle zone where there are still areas of green surrounding red staining chondrocytes. x70. (b) An area of cartilage where there is focal loss of Safranin O staining. x20.

(b)





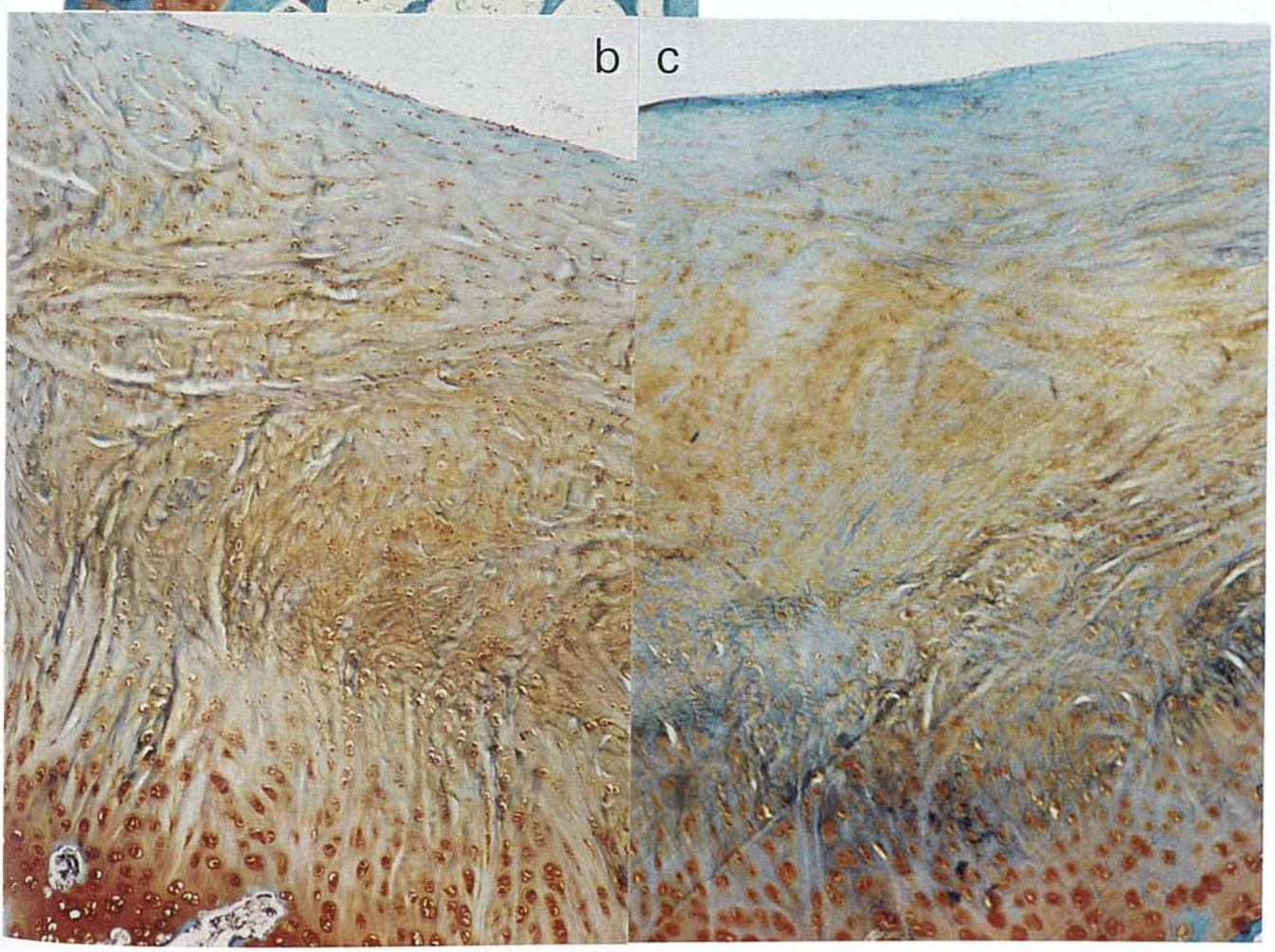


Figure 7.15 - PTM sections stained with Saffranin O. (a) Pre-experiment, (b) after one week of loading and (c) after one week, unloaded.

The pre-experiment sections from the PTM had a distinctly different distribution of Safranin O staining. The surface zone did not stain for Safranin O, but the middle zone had a band of red staining which, unlike the DTT, was spread quite well into the matrix. Below this red band there was a variable blue/green band which did not stain for Safranin O and then the deep zone which stained strongly red.

After one week of loading the Safranin O staining pattern of the PTM sections appeared similar to that of the pre-experiment samples, although there was possibly some more widespread staining through the tissue. The unloaded samples showed similar Safranin O staining to the pre-experiment sections (Figure 7.15).

7.5 Proteoglycan synthesis

Parallel cartilage samples from the birds in this study were taken by N. Venkatesan and the proteoglycan synthesis and degradation of these samples were estimated by investigating the incorporation and release of ^{35}S-Sulphate. The results presented in Figure 7.16 show that proteoglycan synthesis in the DTT was significantly elevated ($p<0.003$) over control cartilage, 6 days after loading, but then decreased to control levels at 13 and 20 days. Degradation of newly synthesised proteoglycans also showed a significant increase with loading ($p<0.002$) at 6 days.

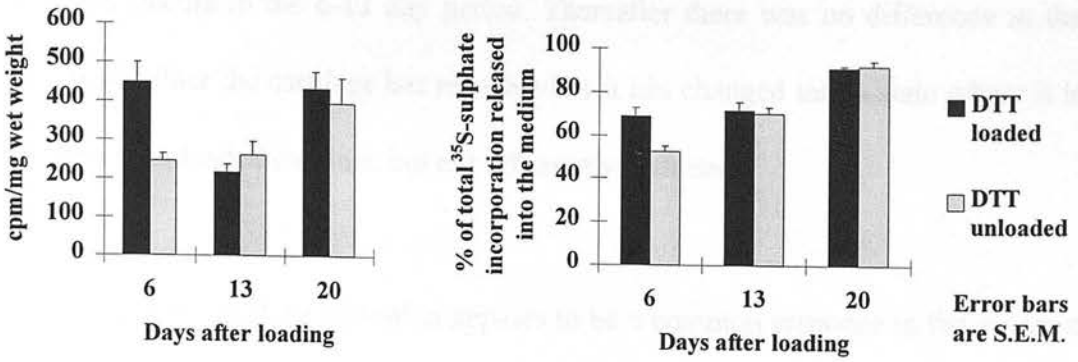


Figure 7.16 - The synthesis and degradation of proteoglycans. (courtesy of N. Venkatesan)

7.6 Discussion

In the original design of the loading experiment a major concern was the way in which the *in vivo* loading might affect the behaviour of the birds. In particular, it was not known whether increased loading would bring about an increase in sitting behaviour thereby introducing unquantifiable effects on loading. It was decided to attempt to assess any major changes in behaviour by using a simple scan observation approach. The results of the behavioural study showed that there was no major difference in the standing behaviour of the pre-loading, loaded and unloaded birds. Since there was no significant difference in activity, the major variable being investigated was therefore the additional load placed upon the cartilage.

The additonal load on the DTT increased the hydration. This appears somewhat paradoxical since load excludes water from cartilage. This increase in hydration is therefore likely to be indicative of a metabolic response occuring in the cartilage, although the birds were loaded with only 10% of their body weight. The increased

hydration occurs in the 6-13 day period. Thereafter there was no difference in the hydration; either the cartilage has recovered or it has changed into a state where it is different to unloaded cartilage but not differently hydrated.

The increase in cartilage hydration appears to be a common response to the increase in load. Arsever *et al.* (1986) found that cartilage water content was increased in the early stages of increased loading produced by selective myectomy in rabbits. An increase in hydration was detected seen in the load alterations in canine OA models (Dedrick *et al.,* 1993).

The increase in hydration with load was not observed in the cartilage from the PTM. In the longitudinal study, the DTT, but not the PTM, was susceptible to disease. The difference in response to load of the PTM and DTT again indicates an inherent susceptibility to change upon loading possibly leading to DJD in the DTT articular cartilage.

There is a remarkable correlation between the hydration results and the SGAG results. Again there was a difference between the DTT loaded and unloaded samples at days 6 and 13 ($p<0.02$ at day 13) but not at day 20. There was no increase in SGAG in the loaded samples from the PTM. These results show that a response has been made to the load, which involves synthesis of sulphated proteoglycans preferentially in the loaded DTT over the unloaded DTT and appears to have subsided by day 20. Such a response was not observed in the PTM. The SGAG

content in the loaded DTT at day 13 was higher than that of the loaded and unloaded PTM at this time.

An increase in proteoglycan synthesis has been reported in the early stages of OA (Mankin *et al.*, 1970, 1971) and in the loaded cartilage of the rabbit myectomy model (Arsever *et al.*, 1986). Increased proteoglycan production has also been observed in the cartilage of contralateral legs in joint immobilisation studies (Behrens *et al.*, 1989) and in cases where load has been increased experimentally through exercise (Kirviranta *et al.*, 1988, Vasan, 1983). Proteoglycan synthesis is increased following *in vitro* loading of chondrocytes within the physiological range (Hall *et al.*, 1991). An increase in proteglycan synthesis and degradation has been produced in the early stages of the load in the present study.

The higher SGAG content of the DTT loaded cartilage appears to be a response to load. The reporting of increased synthesis of proteoglycan is associated with increased load and development of OA, however the uronic acid results show that this is more than a generalised proteoglycan response. The uronic acid content shows no difference between the loaded and unloaded cartilage. Generally used as an indicator of total proteoglycan content, the stability of the uronic acid content shows that a specialised response has been initiated by loading which involves the preferential production of SGAGs, possibly keratan sulphate which is not detected by the uronic acid assay. This hypothesis is supported by evidence that increased loading can lead to an accumulation of keratan sulphate rich proteoglycan (Saamanen *et al.*, 1987).

The only significant difference in DNA content between the loaded and unloaded groups was observed at day 20 in the loaded DTT. Although the hydration and SGAG content have decreased to the same levels as the unloaded joint, the drop in DNA content suggests that a change had occured in the cartilage. A characteristic of the DTT in the longitudinal study was that the DNA content of the cartilage continued to decrease even when the other samples had reached a steady level. This decrease in DNA content could reflect the susceptibility of this cartilage to disease. No similar fall in DNA content was observed in the PTM although this is the apposing joint surface to the DTT, again emphasising the difference between the DTT and PTM in terms of susceptibility to cartilage damage.

The decrease in DNA content of the loaded DTT after 20 days loading was reflected in the apparent acellularity of the histological sections at this time. The other histological results suggest that the cartilage of the DTT was much more severely affected by the loading than that of the PTM. Alterations in the histology of the DTT cartilage, brought about by load, included surface disruption, areas of acelluarity and formation of deep zone clusters. These are similar to the histological signs of DJD which were observed in the *ad libitum* fed broilers in the longitudinal study. The PTM does not show these signs. However, the PTM show some mid and surface zone disruption. This is similar to the results for the PTM from the longitudinal study.

The results of the Saffranin O staining showed that proteolgycan metabolism was altered in the sections from the loaded DTT. The pattern of staining in the middle

zone changed, the proteoglycan was spread much more through the matrix, which could be due to many factors including physical disruption of the cartilage allowing better diffusion away from the cell or possibly synthesis of a more diffusible proteoglycan. The appearance of areas which lacked proteoglycan staining in the loaded DTT again indicates to a discoordinate chondrocyte response (Adams *et al,.* 1996).

Additional load does not always produce DJD. For example DJD was not produced in dogs running with added weight (Buckwalter, 1995). However, load is often associated with development of DJD as can be seen by the association of obesity with progression from unilateral to bilateral OA in humans (Spector *et al.*, 1994). In this avian system, the ability to mimic some of the features of DJD produced in obese birds by artificial loading strongly suggests that load, and not a systemic factor, is mediating DJD progression. The ability to withstand load appears to vary between joint surfaces since PTM cartilage responded to the load in a different manner to the DTT.

7.7 Conclusions

This experiment has shown that artificial loading of feed restricted broiler strain fowl brings about biochemical and morphological changes in the DTT cartilage which are associated with DJD. These changes were not seen in the PTM cartilage. This again emphasises the susceptibility of the broiler strain DTT cartilage to DJD, whilst the PTM was in some way protected.

8. Final Discussion

8.1 Aims

The aim of this chapter is to review the results obtained in these studies in the context of the overall field of degenerative joint disease. The roles of obesity and loading in the progression of DJD are also discussed.

8.2 Susceptibility to disease

8.2.1 Strain susceptibility

These present studies have confirmed in a systematic, controlled study that broiler strain fowl are indeed more susceptible to DJD than laying strain and J-line fowl. This has also been demonstrated by others in the antitrochanter (Hocking *et al.*, 1994) but the work described here shows this to be true for other joint surfaces. The *ad libitum* fed broiler birds were more susceptible to DJD than the feed restricted birds which implicates a role for body mass in mediating susceptibility to disease.

8.2.2 Joint surface susceptibility

The joint surface which was most susceptible to disease in this study was the DTT. In the longitudinal study the DTT showed the first and most severe signs of DJD. The opposing joint, the PTM, was considerably less susceptible to DJD. In the loading study the DTT showed a biochemical response to load, which was not detected in the PTM. Such clearly defined difference in susceptibility is not observed in the

human situation. However the patella groove of the femur, the upper surface in the knee joint, is the first of the two apposing surfaces to suffer from OA, the tibial condyles from the lower surface in the joint are the next most frequently affected area, followed by the medial and lateral femoral condyle (Gardner, 1992).

The mid-zone disruption which was observed in the longitudinal study in the PTM was also seen in the loading study in the PTM. The disruption which was observed in the DTT in the loading study, the cartilage thinning and formation of deep zone chondrocyte clusters, was also observed in the DTT in the longitudinal study. These results suggest that something intrinsic in the articular cartilage, which is particular to the joint surface, mediates the response of the cartilage to the increased load produced either by the large mass in the longitudinal study or that provided artificially in the loading study.

The AT showed some degeneration in the longitudinal study. At the mid time points all 3 groups appeared to be showing some mid-zone degeneration, but this only progressed to fibrillation and cluster formation in the *ad libitum* fed broilers. This again implicates a role for load and body mass in hastening the progression of the disease. Morphologically the size of the AT does not increase in proportion to the increases in bodyweight, which have resulted from the selection of broiler parent strains (Hocking *et al.* 1994). The antitrochanter of the *ad libitum* fed broilers was therefore more loaded than that of the feed restricted birds. The hock joint of the *ad*

libitum fed broilers was also not increased in size in comparison to that of the feed restricted group so a relative increase in loading was also observed in this joint.

8.3 Obesity, hormones and load.

In human DJD many factors are thought to point towards a metabolic /systemic component in the progression of the disease. These include: the female predominance, menopausal onset of generalised OA, the unequal effect of obesity on knee and hip OA, the relationship with excess endogenous oestrogen, and the inverse relationship with osteoporosis. Hypertension, hypercholesteremia and blood glucose are associated with both unilateral and bilateral knee OA, independent of obesity and support the concept that OA has an important systemic and metabolic component in its aetiology (Hart *et al.*, 1995).

Another endocrinological factor in the progression of OA is growth hormone. Patients with growth hormone deficiency have significantly less OA than the normal population (Bagge *et al.*, 1993). Individuals with growth hormone deficiency have a lower bone mineral density in comparison with normals (Rosen *et al.*, 1993a), this may have implications for the articular cartilage of these individuals. The matter is complicated further by the fact that patients with growth hormone deficiency have an increase in body mass index and are obese compared with normals (Rosen *et al.*, 1993b) factors which might otherwise be considered to increase the susceptibility to

OA. Patients with OA have also been shown to have higher levels of basal growth hormone compared to controls (Dequequer *et al.* 1982).

The possible role that these factors may play in the progression of DJD in the *ad libitum* fed broilers cannot be ignored but due to the experimental design some of these factors may be discounted. For example all the birds in the longitudinal study were male, which excludes the effects of female sex hormones and related variations in development. In female fowl the effects of female sex hormones are even more severe than those observed in humans since the laying of eggs produces marked effects on bone structure and mineral density. Another factor which is markedly different between the fowl and the humans is the nature of the increased body mass. In humans obesity is produced by an increase in body fat and it is thought that the metabolism of hormones in the adipose tissue could play a role in the progression of DJD. However in the fowl the broiler strain birds have been bred to produce enormous breast muscles for meat purposes. This reduces the possibility of adipose tissue derived hormones playing a significant role in the progression of avian DJD.

Undoubtedly the *ad libitum* fed broilers will have elevated blood glucose over the feed restricted birds, the extent of the difference should really be checked in further studies by taking blood samples from the birds at sacrifice. One effect of increased blood glucose levels may be manifested in an in increase in the formation of sugar dependent crosslinks such as pentosidine (Monnier 1990). The *ad libitum* fed broilers and the feed restricted birds may have different amounts of growth hormone,

although they are genetically identical, which is another factor which should be confirmed at sacrifice. A higher level of growth hormone in all the broiler strain birds may account in part for the increased susceptibility of the broilers over the J-line to DJD. However since the *ad libitum* fed broilers develop much earlier and more severe DJD than the feed restricted birds body mass must also play an important role.

8.4 Sulphation patterns

The distribution of sulphated proteoglycans appears to be the factor which is differently controlled in both the diseased articular surfaces in the longitudinal study and in the DTT during the loading study. At day 180 an increase in uronic acid content is observed in both the J-line and the *ad libitum* fed broilers. The absolute amounts are higher in the *ad libitum* fed broilers and results from the SGAG assays on this tissue suggest that there is a disproportionately higher proportion of sulphated GAG in the *ad libitum* fed group in comparison to the J-lines. There is also a sulphate related response in the loading study. The results from the DTT show that there is an increase in SGAG and hydration as an early response to loading. The rate of proteoglycan synthesis and degradation 6 days after loading is significantly higher in the loaded DTT in comparison to the unloaded DTT, as measured using ^{35}S-sulphate incorporation. However the results of the uronic acid assays do not show a concomitant increase in the DTT along with that of the SGAG. Again this indicates a specific proteoglycan response involving sulphation. Whether the response that has been detected in these studies is the same in the longitudinal study and in the loading

study is a subject for further research. The control of the sulphation of proteoglycans is vitally important to the function of the cartilage and a malfunction in response to load or another DJD trigger could lead to the increase in hydration seen in the early stages of DJD.

8.5 Nature of the predisposition

The exact nature of the predisposition of the broiler strain fowl to DJD has not been clearly elucidated by these studies. The cartilage of the broiler strain fowl does not contain an inherent cartilage weakness which causes catastrophic failure. This has been shown because the feed restricted fowl do not develop DJD to any where near the same level as the *ad libitum* fed fowl.

There may however be a genetic weakness in the cartilage which prevents the cartilage withstanding the high levels of load it is subjected to by the *ad libitum* fed birds. Alternatively the cartilage may not be intrinsically significantly different to that of the J-line birds, instead it may just not be able to withstand the extreme load placed on it by the extreme body mass of the *ad libitum* fed birds.

The PTM appears in some way to be protected from degeneration, unlike the opposing surface of the joint the DTT. The shape of the joint may well be important

here in mediating the local forces which exist within the cartilage. Alternatively there may be some intrinsic metabolic difference between the articular cartilage of the PTM and that of the DTT. If this is the case, discovering the factor is vitally important since it differentiates between cartilage survival and degradation in these fowl.

The fast growth of *ad libitum* fed broilers may compromise the cartilage indirectly. Valgus and varus deformities predispose to DJD and the strain of rapid growth on bones producing abnormalities in the joint alignment could alter the loading pattern sufficiently to have a significant effect on the articular cartilage. Measuring the torsion angles of the bones from the birds would clarify if this plays a role in development of DJD.

DJD compromises evolutionary fitness in the birds in that it is thought to cause infertility (Hocking, 1992). It has been proposed that the prevalence of OA in humans has developed since it does not affect reproductive fitness (Dieppe, 1995). The broiler strain birds have been strongly selected for the commercial market. If a population of broiler strain birds were left to develop over several generations, then natural selection against DJD would occur, thereby producing healthier birds which retain much of their commercial value.

8.6 Further work

There are various approaches which could be taken in order to address the questions raised in this study.

If the response which is seen in the DTT of the artificially loaded broiler strain birds is specifically related to the way in which the broiler strain cartilage responds to load then repeating the loading experiment in J-line birds would not produce the same results. A more precise understanding of the response of the broiler strain DTT to load would be invaluable. Obviously an alteration in proteoglycan metabolism has occurred, so investigating the types of proteoglycan in loaded and unloaded joints, for example by gel electrophoresis, would be useful. Testing for apoptosis in the loaded DTT would also elucidate if an adaptive or truly degenerative response is occurring. The difference between the response of the DTT and PTM to the load should be investigated. Since two different samples from the same individual would be obtained this is an ideal situation for the application of differential display techniques.

Some questions remain unanswered about the nature of articular cartilage during development. The distribution of collagen types at different stages of development and the distribution within the tissue are important questions which have not been properly addressed here. Cyanogen bromide mapping of samples could be used to investigate collagen types in various stages of development and preparation of samples by cryosection could show how the collagen type varies through the depth of

the tissue. Immunocytochemistry would also elucidate the distribution of collagen types in avian articular cartilage.

8.7 Conclusions

These studies have confirmed that broiler strain fowl are more susceptible to DJD than laying strain fowl. This susceptibility, which may or may not be genetically determined, appears to be related to body mass. Artificial load can produce morphological and biochemical changes in the articular cartilage of the DTT which are similar to those seen in natural DJD. The PTM is somehow protected from disease and does not exhibit the biochemical or morphological changes with age or artificial load which are seen in the DTT.

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10. Appendix

10.1 Biochemical techniques

Reagents were from Sigma Chemical Co. Ltd, Poole, Dorset, unless otherwise stated.

10.1.1 Proteinase K digestion

The dehydrated cartilage samples were suspended in 0.2 ml 1% SDS and boiled for ten minutes. After cooling to room temperature, 0.7ml of 0.01M TRIS buffer (pH 8.0) was added. 0.1ml proteinase K (Boerhinger Manheim) solution (5mg/ml in 0.01M TRIS buffer (pH 8.0) with 1mM calcium chloride), was then added and incubated at 60°C for 18 hours. After this time any cartilage which was undigested was macerated with a blunt seeker and then another 0.1ml of proteinase K solution was added, followed by incubation for a further 18 hours at 60°C. The samples were then aliquoted and stored at -20°C until required.

10.1.2 Papain digestion

The dehydrated cartilage samples were digested in one ml of 0.1M phosphate buffer pH 7.0, 10mM cysteine, 2mM EDTA with 125µg/ml papain. The samples were incubated for 18 hours at 60°C, after this time the undigested cartilage samples were macerated and then 100µl of buffer, which contained 1.25mg/ml papain. was added to each sample and incubated for a further 18 hours at 60°C. The samples were then aliquoted and stored at -20°C until required.

10.1.3 Sulphated GAG assay

DMB 8mg 1,9-dimethylene blue was mixed with 2.5ml ethanol. 1.0 g sodium formate. 1.0ml formic acid and made up to 500 ml and stored in a brown bottle.

Chondrotin 4 sulphate stock solution of 2mg/ml in phosphate buffer, as in papain digest.

The stock solution was diluted to give 100 μg/ml and standards of 5, 10, 25, 50, 75 and 100μg/ml were prepared. A flat bottomed microtitre plate was used. 20μl quadruplicates of blanks, standards and samples were prepared in the plate. 180μl of DMB dye was added to each well, either using a multichannel pippette or the automatic dipsenser facility on the Dynatech MR7000 plate reader. The absorbance was measured at 490nm immediately.

10.1.4 Uronic acid assay

Assay used in chapters 2 and 3.

Borate/sulphuric acid reagent 2.39 g sodium tetraborate decahydrate in 250ml of concentrated sulphuric acid. Left to dissolve overnight.

Water saturated with Benzoic acid

Carbazole reagent 125mg of carbazole in 100ml of ethanol.

Glucuronolactone standards 1mg/ml dissolved in water saturated with benzoic acid.

Standards made up with benzoic acid saturated water to give, 1.6, 8, 32, 64 and 96μg/ml.

750μl of borate/sulphuric acid reagent was added to a clip eppendorf and placed on ice. 125μl of standard or test solution was carefully layered onto the surface of the acid mixture and allowed to diffuse on ice for ten minutes. The tubes were mixed carefully, keeping them cool, once mixed briefly vortex mix. The tubes were heated at 100°C for ten minutes then cooled in an ice bath. 25μl of cooled carbazole was added to the mixture which was vortex mixed and returned to 100°C for 15 minutes. The tubes were then cooled on ice and the absorbance measured at 525nm.

10.1.5 Uronic acid assay using microtitre plate

Method used for chapter 5 and chapter 7.

2.39g of sodium teraborate decahydrate was dissolved in 250ml of concentrated sulphuric acid and left overnight to dissolve. 48mg of carbazole was dissolved into 100ml of the borate solution. 150µl of the acid/borate solution was dispensed into a microtitre plate using a positive displacement repeating pipette, the plate was then stored frozen at -80°C. Standards were prepared as in 1.4, except 0.1M phosphate buffer pH 7.0 was used instead of benzoinc acid saturated water. 30µl of either samples or standards was layered on to each well. This was mixed on a shaker for 15 minutes and then incubated at 80°C for 30-40 minutes. The plate was then allowed to cool for 5 minutes and the absorbance measured at 490nm. The plate was disposed of by tipping into a sink of running water.

10.1.6 DNA assay

TRIS- HCl buffer 0.01M TRIS buffer (pH 8.0)
Citrate/chloride buffer 0.015M sodium citrate, 0.154M sodium chloride.
Hoescht 33258 0.15mM

DNA standards were prepared to a concentration range of 4-160 µg/ml. The samples and standards were incubated at 60°C for 15 minutes and then cooled slowly. 60µl of sample or standard was then mixed in a vial with 75µl TRIS-HCl, 2ml citrate buffer and 1ml dye solution, incubated in the dark for 15 minutes. The fluorescence of these samples was measured at 340nm following excitation at 340nm.

10.1.7 Hydroxyproline assay

Buffer 6.0 5.5g citric acid, 57.0g sodium acetate, 37.5g trisodium citrate, dissolved in water, 385 ml methoxyethanol was then added and diluted to 1 litre with water.
Chloramine T solution 0.1g chloramine, 1 ml water, 4ml buffer pH 6.0.
Ehrlichs reagent 1.0g p-dimethylaminobenzaldehyde, 7ml methyl cellosolve, 1.0ml concentrated hydrochloric acid.

50µl of sample was mixed with 1.2ml of 6M hydrochloric acid. This was placed in a microcentrifuge tube which contained an O-ring and was held shut with a clip. The samples were then incubated at 108°C overnight, then 500µl of this hydrolysate was placed in a shallow glass pot, dried under vacuum. 1ml of water was then added to the sample.

Duplicates of 0, 2.5, 5, 10, 15, 20 and 25 nmoles in 0.5ml distilled water were prepared. Duplicates of the rehydrated samples were prepared. 0.5 ml methoxyethanol was dispensed with a repeating pipette into each tube. 0.2ml of chloramine T solution was added to each tube. The tubes were shaken and left at room temperature for 5 minutes. 0.3ml Ehrlich's reagent was added to each tube, shaken and heated at 110°C in an oven for 5 minutes. The tubes were cooled and the measured read at 553nm.

10.1.8 ELISA method

20mM carbonate buffer - Solutions of $NaCO_3$ 1.06g in 500ml H_2O and 0.84g $NaHCO_3$ in 500ml H_2O were prepared. Solutions were mixed to a pH of 9.6.
PBS for 1 litre 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4.
PBS-Tween 0.05%(v/v) Tween in PBS.
PBS-Tween-BSA As above with BSA added to 1% (w/v).

Substrate - Prepared fresh on day of use. O-phenyldiamine was dissolved in methanol (10mg/ml) and diluted 1/100 with 0.03% (v/v) hydrogen peroxide in distilled water.

A flat bottomed, Nunc Maxisorp plate (Life technologies) was incubated with 100µl antigen solution at 4°C overnight. (22.5 µl of stock antigen (125µg/ml) in 10mM acetic acid diluted with 4.5ml carbonate buffer). This gave an antigen concentration of 0.1µg/well. The plate was washed five times with PBS-Tween. The wells were filled with 150µl PBS-Tween-BSA and the plate was incubated for 2 hours at 4°C. The plate was washed five times in PBS-Tween. 100µl of the antibody preparation at serial dilutions in PBS-Tween was added to the plates, and incubated in the dark at room temperature for two hours. The plate was washed five times with PBS-Tween. The anti-rabbit conjugated with horseradish peroxidase (S.A.P.U.) was added, 100µl at 1 in 500 dilution in PBS-Tween-BSA and the plate was incubated for two hours at room temperature in the dark. The plate was washed five times in PBS-Tween-BSA. 200µl of O-phenyldiamine was added to the plate and incubated for 1 hour in the dark. The reaction was stopped with 50µl of 4M sulphuric acid and abosrbance measured at 490nm in plate reader (Dynatech).

10.1.9 Affinity purification

A sample containing the antigen preparation was run on a well-less macro SDS-PAGE gel. The electrophoresed gel was Western blotted on to nitrocellulose and the blot was stained with Ponceau S (0.2% in 3%TCA). The band required was excised with a scalpel, this was the affinity strip. The strip was washed in TBS until the colour could not be seen. The affinity strip was incubated in blocking buffer (3%BSA in TBS) for 90 minutes, followed by a wash in TBS. The affinity strip was placed, antigen side up, on parafilm in a sealed container. The immune serum was placed on the strip, as much as could be held by surface tension. The box was sealed and shaken gently for two hours at room temperature. The excess serum was removed and the strip was washed three times for five minutes in TBS to remove any

unbound antibodies. The specific antibodies were eluted by placing elution buffer (0.2M glycine/HCl, pH2.8) onto the strip (as much as can be held by surface tension) for 20 minutes. The elution buffer was removed and neutralised immediately with an equal volume of 0.1M TRIS/HCl pH8.5. The affinity purified antibodies were stored at 4°C after the addition of sodium azide (to 0.1%). The affinity strips were washed in TBS and stored at 4°C in TBS, 0.1% sodium azide fo re-use.

10.2 Histology techiniques

10.2.1 Decalcification

Samples were fixed in 10% buffered neutral formalin for 7 days prior to decalcification in Goodin Stewarts Fluid (formaldehyde, formic acid and distilled water at a ratio of 1:1:8). Samples were washed in tap water overnight, returned to buffered neutral formalin and processed through ascending alcohol concentrations and CNP 30 to paraffin wax.

10.2.2 Haematoxylin and eosin staining

After deparaffiniation and hydration the sections were stained in haemalum for 5 minutes (solution A [0.03% sodium iodates, 8% aluminium potassium sulphate, 8% chloral hydrate in distilled water] heated gently and mixed with soluiton B [0.25% haematoxylin in 9% ethanol] at a ratio of 1.66:1. Glacial acetic acid [0.096%] added after 48 hours). After rinsing in water for 5 minutes the sections were stained in 2% aqueous eosin Y for 2 minutes then the slides were dehydrated, cleared and mounted in DPX.

10.2.3 Toludine blue staining

Sections were stained for 30 minutes at room temperature in 1% Toluidine blue in 50% isopropanol. The sections were then washed with isopropanol for 30 seconds, CNP 30 for 30 seconds then clear and mounted with DPX.

10.2.4 Safranin O/ Fast Green staining

Dewaxed sections were transferred to 74 OP for 3 minutes followed by 64 OP for 3 minutes. Sections were stained in Weingharts haemotoxylin for 6 to 8 minutes. Washed in water and then stained in 0.2% aqueous Fast Green FCF for 3 minutes. Following a wash in 1% acetic acid, the sections were stained in 0.1 Safranin O for 4-6 minutes. Then sections were washed in water, transfered to 64 OP for 3 minutes, 74 OP for 3 minutes then in CNP 30 for five minutes, then the slides were mounted.

10.2.5 Immunostaining

The immunostaining was performed using the Sigma vectastain ABC kit. The sections were pre-treated for 30 minutes with 0.6% hydrogen peroxide in methanol. Then incubated for 30 minutes at 37°C with bovine testicular hyaluronidase (2mg/ml in 0.1M sodium acetate pH5.5 containing 0.85% sodium chloride). The anti type IX antibody and the control serum were used at 1/50 dilution.

10.3 Husbandry techniques

10.3.1 Feed restriction

The feed restriction schedule shown below is that recommended for broiler birds by their suppliers Ross Breeders, Newbirdge, Midlothian. The males should be fed on breeder ration.

Age in days	Feed g/bird/day	Age in days	Feed g/bird/day
0-11	ad lib to 24	44-49	51
12-13	25	50-56	55
14-15	26	57-63	60
16-17	27	64-70	66
18-19	28	71-77	72
20-21	29	78-84	76
22-23	32	85-91	78
24-26	35	92-98	79
27-29	38	99-105	79
30-32	40	106-119	80
33-35	42	120-126	83
36-38	44	127-140	86
39-43	48	141-161	90

Age in weeks	Feed g/bird/day
20	95-100
21	100-105
22	105-110
23	110-115
24-30	110-115
30-64	110-115

10.3.2 Low density diet

Per 1000kg this contained; 578kg barley, 287kg wheat, 50kg soya, 34kg wheatfeed, 10kg limestone, 23kg Dicalcium phosphate, 3kg salt, 10kg pellet binder, 2.5kg vitamin mix 4 and 2.5kg vitamin mix 5. Made into 3mm pellets.

10.3.3 Copper deficient diet to produce lathyrism in chicks

	g/kg	for 15kg mix
sucrose	192.6	2.89kg
skimmed milk	600	9kg
$NaCO_3$	12.0	180g
$CaHPO_4.2H_2O$	4.4	66g
$CaCo_3$	8.8	132g
methionine	5.0	75g
arginine	10.0	150g
glycine	15.0	225g
cellulose	49.7	746g
vegetable oil	100.0	1.5kg
mineral mix	as below	
vitamins mix 6	2.5	30g

Mineral mix sufficient for 15kg.

$ZnSO_4.7H_2O$	3.301g
NaI	7mg
$FeSO_4.7H_20$	5.973g
$MnCO_3$	3.138g
Na_2SeO_3	4.9 mg

10.4 Behavioural studies

Total No In Pen					Date				Pen No				Bird Type		
Behaviour\Time		2	3	4	5	6	7	8	9	10	11	12	13	14	15
Feeding															
Standing															
Resting															
Preen/Stand															
Preen/Rest															
Drinking															
Walking															
Peck/Wall															
Peck/Litter															
Scratch															
Dustbathe															
Feather Pull															
Feather Eat															
Aggresive/ Peck															
Rucksack/Peck															

10.5 Gross pathology from the lonigitudinal study

	Day	DTT	PTM	PH	AT
***ad libiutm* fed broilers**	79	1/10 cartilage thinning			
	113	1/10 cartilage thinning			
	180	1/10cartilage thinning 1/10 pannus formation 6/10 cartilage loss approx. 1cm by 0.5cm on medial condyle			
	279	6/12 peripheral cartilage thinning	2/12 visible cartilage loss	4/12 cartilage thinning	6/12 cartilage degeneration

	Day	DTT	PTM	PH	AT
feed restricted broilers	113		1/10 cartilage thinning		
	180				
	279	4/10 cartilage thinning		1/10 peripheral defect and pannus	1/10 mild degeneration
	376	4/10 cartilage thinning			2/10 cartilage degeneration

	Day	DTT	PTM	PH	AT
J-line	376			10/10 cartilage thinning	

10.6 Histopathology

	Day	DTT	PTM	PH	AT
***ad libiutm* fed broilers**	113	4/5 cartilage thinning and focal poor cellularity	1/5 focal defects in superficial and middle zones	1/5 focal developing DJD 1/5 small foci of cartilage thinning	1/5 slight cartilage degeneration
	180	7/10 hisotpathological signs of DJD. Varying from loss of surface zone cellularity to complete focal loss of articular cartilage	6/10 chondrocyte degeneration in the middle or upper zones	3/10 varialbe cellularity of surface zone	1/10 chondrocyte cluster formation 6/10 mid zone chondrocyte degeneration 4/10 surface zone variability
	279	No histological signs of DJD	2/10 focal loss of surface cellularity	1/10 loss of celluarity	2/5 chondrocyte cluster formation 1/5 basophillic matrix surrounding mid zone chondrocytes

	Day	DTT	PTM	PH	AT
J-line	113	1/5 variable cellularity in surface zone	1/5 loss of surface layer		
	180		2/10 chondrocyte degeneration in middle or deep zones		1/10 mid zone chondrocyte degeneration
	279	1/10 focal loss of cellularity and cluster formation	1/10 focal loss of surface cellularity 1/10 basophillic pools around mid zone chondrocytes		
	376	2/10 focal loss of surface or middle zone cellularity	2/10 focal loss of cellularity in surface or mid zone		

Histopathology (Contd.)

	Day	DTT	PTM	PH	AT
feed restricted broilers	113		1/5 focal defects in superficial and mid zones 1/5 focal degeneration in deep zone		
	180	1/10 some cartilage thinning 1/10 focal matrix degeneration and chondrocyte clusters	3/10 chondrocyte degeneration in middle or deep zones	2/10 variable cellularity of surface zone	5/8 mid zone chondrocyte degeneration
	279	1/10 focal loss of surface zone cellularity	1/10 basophillic pools around mid-zone chondrocytes		
	376	1/10 reduced surface zone cellularity	2/10 focal loss of celluarity in surface or mid zone	1/10 loss of cellularity	1/10 reduced surface zone cellularity

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AN AVIAN MODEL FOR OSTEOARTHROSIS ? EARLY RESULTS SHOW SIMILARITY TO DOG MODELS

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Broiler fowl are more susceptible than laying strain fowl to degenerative joint disease resulting in osteoarthrosis. This is a preliminary study of the biochemical constituents of articular cartilage from the two strains.

Two groups of six mature adult birds were studied. Six joints were taken from each bird: right and left distal tibiotarsi, proximal tarsometatarsi (PTM) (both load bearing) and proximal humeri (PH) (non-load bearing). Each joint was divided vertically into two. One half was fixed for histological study. From the remaining half, full-depth articular cartilage was removed and placed in a pre-weighed sealed container. After determination of wet and dry weight (hence hydration) the tissue was boiled with SDS and digested with proteinase K. Sulphated GAG and uronic acid assays were then carried out.

In both groups the loaded joints were hydrated to a similar extent, but significantly ($p<0.05$) less hydrated than the unloaded joint. The broiler joints were significantly more hydrated than the corresponding layer joints. The broiler unloaded joint had significantly higher amounts of uronic acid and sulphated GAG per dry weight than the loaded joints and the layer PH had significantly higher amounts of uronic acid and sulphated GAG than the layer PTM. The uronic acid and sulphated GAG concentrations in the broiler joints were significantly higher than those in the corresponding layer joints. The uronic acid differences imply that there is more chondroitin sulphate per dry weight in the broiler than in the layer joints.

Of the 72 joints examined in this study only two layer and two broiler birds showed any gross articular cartilage lesions. The biochemical differences observed in this study are comparable with and similar to pre-lesion studies in dog models of osteoarthrosis. (McDevitt C.A. and Muir H. (1976) J.B.J.S. 58B:94-101)

Osteoarthrosis is a major welfare problem in man and poultry. Broiler fowl may represent an inexpensive and non-invasive animal model of osteoarthrosis. Further studies may give insights into human osteoarthrosis and suggest possibilties for genetically altering the susceptibilty to osteoarthrosis in birds.

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Cartilage changes associated with mycoplasma-induced arthritis in broiler fowl

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Cartilage consists of chondrocytes embedded in a large volume of extra-cellular matrix which they produce and maintain. Collagen II fibrils are networked together with type IX (and some type I in avian cartilage) to provide a framework which contains proteoglycan molecules. Hydration is controlled in the tissue by the compressive force of the collagen network and the osmotic drawing power of the proteoglycans. This study involved measuring three biochemical parameters of cartilage condition in broiler fowl following experimental infection with *Mycoplasma gallisepticum* or *Mycoplasma synoviae*. The parameters were: hydration, sulphated glycosaminoglycan (SGAG) content and uronic acid content.

The experimental group were five-week-old broiler strain fowl from stock which were free of mycoplasmal infection. Four mycoplasma strains were chosen, two *M. gallisepticum* (S6, B31/85) and two *M. synoviae* (B94/91, B31/88). 0.1ml of culture or control media was injected into the left tibiotarsal joint of the birds at 5 days of age. The birds were scored weekly for swelling and lameness. All the mycoplasma injected birds developed lameness (either lame or very lame). Birds with valgus deformities were excluded as an abberrant gait would affect loading on the joint. After sacrifice by intravenous administration of dilute barbiturate, the articular cartilage was removed from the distal tibiotarsus and wet and dry mass of tissue determined. The tissue was then digested using SDS and proteinase K and assays for SGAG and uronic acid were carried out.

The results showed no differences dependent upon the strain of mycoplasma that had been injected.The media only group showed no signs of lameness with no difference in hydration between the injected and non-injected joints. The joints injected with mycoplasma showed a general, but variable, increase in hydration and uronic acid content (per dry weight) compared to joints injected with control media. With the SGAG results however an opposite trend was observed. When the non-injected (contralateral) joints were examined, however, there were significant changes which correlated to the degree of lameness. The non-injected joints of the very lame birds were significantly ($p<0.05$) more hydrated than the non-injected joints of the lame birds and these in turn were significantly more hydrated than the control joints. A corresponding pattern was seen in the uronic acid data. In contrast an opposite trend was seen in the SGAG results, decreases in SGAG content per mg dry mass accompanying an increase in lameness.

In conclusion, the results showed that infection with either *M. gallisepticum* or *M. synoviae* produced variable results in the injected joints. The non-injected (contralateral) joints developed significant, probably load-dependent, changes corresponding to the degree of lameness. These changes were similar to those seen in surgically induced models of osteoarthrosis.

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Avian Degenerative Joint Disease; an *in vivo* loading study

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Controversy has long surrounded the roles of obesity and genotype in degenerative joint disease (DJD). Abnormal loading can induce degenerative changes in cartilage but the exact cause and nature of the degeneration is not known. This study involves *ad libitum* fed broiler strain fowl which are both heavy and susceptible to DJD, food-restricted broiler strain fowl which do not have such a large body mass and J-line fowl which are both lighter and less susceptible to DJD. The *ad libitum*-fed birds and the food-restricted birds are genotypically identical so variation in cartilage composition is the result of differences in growth rate and *in vivo* loading.

The distal tibiotarsus is a load-bearing joint surface which frequently is affected by DJD in broiler fowl. Cartilage samples are collected, some taken for histology, the others, once hydration has been determined, are digested with papain or proteinase K. The samples are assayed for uronic acid (carbazole), sulphated glycosaminoglycan (dimethylene blue), DNA (Hoechst 33258) and mature pyridinium crosslinks (HPLC).

The *ad libitum*-fed birds developed DJD. By day 113 foci of cartilage thinning and areas of poor cellularity were observed histologically and by day 181 there were cricumscribed areas of cartilage loss and pannus formation; in histological sections fissures and chondroma formation were seen. The gross morphological changes are accompanied with an increase in hydration and uronic acid content; samples from the diseased (day 181) *ad libitum*-fed birds also have a higher sulphated glycosaminoglycan concentration than those from the J-line or food-restricted birds collected at the same time. During the early progression of DJD in the *ad libitum*-fed broilers DNA concentration fell, indicating a reduction in celluarity. Samples from the non-diseased J-line birds and from the diseased *ad libitum*-fed birds have similar amounts of mature pyridinium crosslinks, suggesting that these do not play a role in early DJD.

Because the *ad libitum* fed birds develop DJD first, growth rate and *in vivo* loading are implicated in the development of DJD. These models are proving useful in the study of DJD and should allow more of the factors involved in its development and progression to be identified.

Biochemical variation in articular surfaces liable to develop avian degenerative joint disease.

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Degenerative joint disease in fowl is a major animal welfare problem, particularly in birds selected for meat production (broiler strain). In studies of four articular surfaces in broiler fowl, distal tibiotarsus (DTT), proximal tarsometatarsus (PTM), proximal humerus (PH) and antitrochanter (AT) we have found that the DTT is the joint surface that presents the earliest and most severe signs of degenerative joint disease. On a gross scale these include cartilage thinning and loss. Histologically characteristic chondrocyte clusters and fibrillation can be seen in the cartilage along with areas of acellularity. In order to elucidate the biochemical factors which may determine the particular susceptibility of articular cartilage to disease a study of the joints of day old chicks was carried out.

Cartilage samples, after hydration had been determined, were digested with proteinase K and assayed for uronic acid (carbazole) and DNA (Hoecsht 33258) content. In addition, rates of synthesis and degradation of sulphated glycosaminoglycans were investigated using radioactive pulse-chase studies with ^{35}S-sulphate and dimethylene blue binding. Cartilage from broiler strain fowl was compared to that from J-line fowl, whose suceptiblity to degenerative joint disease is much less.

Within both groups (broiler and J-line), the cartilages from the DTT and PTM were significantly less hydrated than that of the PH ($p<0.001$), and the cartilage from the broiler DTT was significantly more hydrated than the corresponding J-line strain cartilage. Similarly, the cartilage from the broiler strain DTT had a significantly higher uronic acid content than the cartilage from the J-line DTT. In the broiler strain, all four articular surfaces examined were similar in their uronic acid content, wheras in J-line those in both DTT and PTM were relatively low. DNA concentrations showed a similar trend. Furthermore, when all four articular surfaces were examined, in both broiler and J-line strains, rates of synthesis and degradation of sulphated glycosaminoglycans were highest in DTT articular cartilage. Finally, broiler strain showed a degradation rate higher than J-line.

The differences seen between the DTT and the other joints, and between the broiler and J-line DTT suggest that even as early as day old the broiler DTT is biochemically predisposed to disease. The higher rates of turnover in the broiler DTT cartilage may *per se* render it susceptible to metabolic disfunction. By continuing these analyses through development we should be able to elucidate the biochemical processes involved in the progression of degenerative joint disease.

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ARTIFICIAL LOADING OF FOWL INDUCES BIOCHEMICAL CHANGES IN ARTICULAR CARTILAGE.

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Broiler strain fowl, selected for the large mass required in meat production, are known to be susceptible to degenerative joint disease (DJD), which ultimately leads to lameness. Cartilage degeneration in the distal tibiotarsus (DTT) of the hock joint occurs preferentially in heavy, *ad libitum* fed birds, in comparison to lighter, feed restricted birds. Load on the cartilage may therefore play an important role in the progression of DJD.

Sixteen week old feed-restricted broiler strain fowl were loaded with ten per cent of their body weight using rucksacks. The control group had rucksacks but not additional weight. Cartilage samples from the DTT were taken before loading and at 6, 13 and 20 days after loading commenced. Samples were analysed for hydration, uronic acid, DNA and sulphated glycosaminoglycan (SGAG). Synthesis and degradation of proteoglycans were investigated in parallel samples using ^{35}S-sulphate.

After 6 and 13 days loading there was an increase in hydration ($p<0.05$) and SGAG ($p<0.02$ at 13 days) in the loaded cartilage. The hydration and SGAG content of the loaded cartilage then decreased at day 20 to be similar to those of the unloaded joints. DNA and uronic acid content appeared to be unaffected by loading except at day 20 where the DNA content of the loaded cartilage had fallen significantly compared to controls. These changes seen in the composition of the cartilage were also observed in the turnover studies, where proteoglycan synthesis in the DTT was significantly elevated ($p<0.003$) over control cartilage, 6 days after loading but then fell to control levels at 13 and 20 days. Degradation of newly synthesised proteoglycans also showed a significant increase ($p<0.002$) with loading at six days.

Our results suggest that this may be an appropriate model for *in vivo* loading of articular cartilage in bipeds. Our previous (1) and current studies show that the biochemical and morphological changes seen in avian DJD are similar to those seen in mammalian DJD. The increased loading *in vivo* has produced an increase in proteoglycan content and turnover in the cartilage, in agreement with the effect of cyclic loading *in vitro* and increased *in vivo* load due to lameness (2).

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Figure numbers x correspond to 2.x in Chapter 2. Legends also as in Chapter 2.

DEGENERATIVE JOINT DISEASE IN POULTRY- DIFFERENCES IN COMPOSITION AND MORPHOLOGY OF ARTICULAR CARTILAGE ARE ASSOCIATED WITH STRAIN SPECIFIC SUSCEPTIBILITY

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Running title: Avian articular cartilage - composition and morphology

SUMMARY

The morphology and basic biochemical composition of articular cartilage from two strains of fowl were examined. Broiler strain fowl are considered susceptible to degenerative joint disease (DJD); histological examination of one year old broiler breeders showed, in some samples, articular cartilage thinning, fibrillation and chondrocyte cluster formation, features considered typical of DJD. Examination of similar samples from laying strain fowl showed only minor age-related changes such as some slight cartilage thinning and very mild fibrillation. The articular cartilage from the broiler strain birds was significantly more hydrated, with a higher uronic acid content than that of the laying strain birds. In addition unloaded articular surfaces such as the proximal humerus had significantly higher amounts of uronic acid than the loaded cartilage surfaces of the proximal tarsometatarsus and the distal tibiotarsus; this suggested that the joint loading may have a role in any biochemical differences found between joints and between strains of fowl. These findings concur with other reports in mammals that showed increased hydration and uronic acid in association with early DJD and in models of osteoarthritis (OA). Thus, despite some differences between avian and mammalian articular cartilage, studies on avian DJD may give insights on mammalian disease.

INTRODUCTION

Degeneration of avian articular cartilage causes pain and lameness, which is a major welfare issue in commercial chicken flocks (Riddel 1992). Degradation of the articular cartilage exposes the subchondral bone and impairs the ability of the cartilage to provide a smooth articulating surface for the joint (Clyne 1987). Degenerative joint disease (DJD) causes lameness in broiler strain fowl, the pathology including areas of cartilage thinning, surface fibrillation and necrosis (Duff and Hocking 1986). This is similar to that seen in human DJD (osteoarthrosis) (Gardner *et al.* 1987). In contrast DJD is rare in laying strain fowl, reported cases appearing to be limited to extreme old age (Yamasaki & Itikuru 1983).

The biochemical components of the articular cartilage mediate the function of the tissue. The cells, chondrocytes, are enmeshed in a network of collagens (types II, VI, IX and XI; Eyre *et al.* 1992). Unlike that in mammals, avian articular cartilage also contains significant amounts of type I collagen (Eyre *et al.* 1978). In addition the network also contains proteoglycans. The glycosaminoglycans attached to the proteoglycans have a negative charge that produces an osmotic swelling pressure drawing water into the cartilage. The tensile force of the collagen network constrains the proteoglycans to about 20% of their volume in free solution (Keutner *et al.* 1988). The balance of hydration in the cartilage is vital in providing its resilience to deformation. The largest and most abundant of the proteoglycans is aggrecan, which consists of a protein core, attached to which are many chondroitin and keratan sulphate chains. (Heinegard 1992). Several aggrecan molecules interact with hyaluronic acid to form high molecular weight (10^8) aggregates. Smaller non-aggregating proteoglycans are also present, containing dermatan sulphate or keratan sulphate glycosaminoglycan chains.

This is the first study which compares the morphology of articular cartilage from broiler and laying strain fowl, although the morphology of the articular cartilage in fowl has previously been documented (Gentle and Thorp 1994). In this study we examine the morphological and biochemical differences between articular cartilage from mature adult broiler strain fowl (susceptible to DJD), and laying strain fowl (non-susceptible), features of cartilage diversity which may be related to genotypic susceptibility to DJD.

MATERIALS AND METHODS

Two groups of six fowl were selected at random from female flocks of one-year-old adult broiler breeder (commercial type) and laying strain fowl. The flocks were both considered normal and there was no evidence of debilitating disease or noteworthy lameness. All fowl used in this study were killed by an overdose of barbiturate. Six articular surfaces were analysed from each bird; right and left distal tibiotarsi (DTT), right and left proximal tarsometatarsi (PTM) all of which were load bearing and the right and left proximal humeri (PH) which were non-load bearing. Joints were dissected out and divided in half sagitally. One half was preserved for histology in buffered neutral formalin, while the articular cartilage was removed from the other

half and placed in a pre-weighed sealed container. The wet and dry mass of the tissue was determined and hence hydration was calculated. The tissue was solubilised using the proteinase K/SDS method of Lipman (1989) adapted to include two rounds of digestion. An aliquot of the digest was taken for uronic acid determination using a standard carbazole assay (Chaplin 1986). As all glycosaminoglycans except keratan sulphate contain uronic acid, determination of uronic acid gives a general indication of overall proteoglycan content. After 7 days, the formalin fixed specimens were decalcified in Gooding Stewarts Fluid (formaldehyde, formic acid and distilled water at a ratio of 1:1:8). Samples were washed in tap water overnight, returned to buffered neutral formalin and processed through ascending alcohol concentration and CNP 30 to paraffin wax. The resulting sections were stained with haemotoxylin and eosin. The hydration and uronic acid assay results were analysed using unpaired Student t-tests in Microsoft Excel.

RESULTS

Hydration

As shown in Figure 1, within both groups (broiler and laying strain) the cartilage from the loaded joints (DTT and PTM) were similarly hydrated, but significantly less ($p<0.05$) than the unloaded joints (PH). The cartilage from both broiler strain DTT and PH joints was significantly more hydrated than the corresponding layer strain samples, and a similar trend ($p=0.09$) was seen in the PTM joint.

Uronic acid content

Differences in uronic acid content (Fig. 2) corresponded to the hydration pattern. The unloaded cartilage (PH) of the broiler strain fowl had significantly higher amounts of uronic acid than the loaded cartilage (PTM, DTT), while the layer strain PH had significantly higher concentrations of uronic acid than the layer strain PTM. For all joints, the uronic acid content of cartilage from the broiler strain joints was significantly higher than in the corresponding layer strain samples.

Histology

Tissue sections of DTT contained small areas of synovial tissue. In laying strain fowl the synovium was 1-4 cells thick and the synoviocytes were flattened in appearance (Fig. 3). In contrast, the synovium of the broilers frequently showed mild hyperplasia and hypertrophy (Fig. 4), indicative of mild synovitis, as seen in four of the twelve DTT examined.

In all samples of articular cartilage, three zones could be identified. The surface zone contained flattened chondrocytes and fibres, oriented parallel to the surface. The middle zone was identified by its rounded chondrocytes and the presence of fibres oriented at various angles, including perpendicular to the articular surface, but not parallel to it. The chondrocytes of the deep zone were larger than those of the middle

zone and distinct fibres could not be detected visually in the matrix which had the appearance of epiphyseal hyaline cartilage (Fig. 5).

The articular cartilage of the DTT of laying strain fowl varied in apparent thickness both across the surface of the joint and between individuals. In six DTT the articular cartilage appeared thin, approximately 7-8 chondrocytes deep (Fig. 5). The articular cartilage of the remaining six DTT was thicker, approximately 12-15 cells deep. Where present, cartilage thinning did not appear to be specific to any one zone. In one layer strain the articular cartilage the DTT showed reduced cellularity and a pale eosinophilia of the surface zone (Fig. 6).

The articular cartilage from the DTT of the broiler strain fowl differed from that of the laying strain fowl. Only 2 out of 12 broiler strain DTT had articular cartilage considered normal, although both of these joints showed evidence of synovitis. A further 4 DTT showed apparent thinning of the articular cartilage, two of which also had mild synovial hypertrophy and hyperplasia. The remaining 6 DTT showed a loss of cellularity within the surface zone, which was also frequently eosinophilic. Within the articular cartilage, beneath the area with a loss of surface zone cellularity, small clusters of chondrocytes were occasionally seen in some sections (Fig. 7).

The articular cartilage from the PTM was normal (Fig. 8) in 5 of the 12 layer strain samples, but in none of the 12 broiler strain samples. Within the remaining 7 laying strain PTM specimens, two showed focal thinning of the articular cartilage and three showed areas of poor cellularity within the surface zone, with one specimen showing both features. Pale eosinophilia was not seen in the surface zone within any of the laying strain tarsometatarsi. One PTM showed some fibrillation of the surface zone and the adjacent matrix contained some small clumps of chondrocytes. In contrast, the articular cartilage from all but 2 of the 12 broiler strain PTM showed focal loss of cellularity within the surface zone, with frequently associated with pale eosinophilia. Pyknotic nuclei were also frequently noted within surface zone chondrocytes (Fig. 9). In addition, minor fibrillation of the articular surface was noted in the PTM from 3 broiler strain fowl.

The articular cartilage from the 12 PH examined from the layer strain fowl appeared normal, while 9 of the 12 broiler strain joints showed evidence of degeneration. These changes varied from focal cartilage thinning with no other changes (one example) to, focal loss of cellularity of the surface zone, pyknotic nuclei within remaining chondrocytes and pale eosinophilia of the surface zone. Moreover 1 PH also showed focal total loss of articular cartilage with exposure of the underlying subchondral bone. (Fig. 10) The adjacent cartilage contained large clusters of chondrocytes (Fig. 11).

DISCUSSION

The present study shows that there are distinct differences in the biochemical composition of the articular cartilage from the two strains. In addition, the morphology of the articular cartilage from the broiler strain fowl varied markedly from that previously reported for laying strain fowl (Gentle and Thorp 1994). The articular cartilage of the broiler strain fowl showed histological signs associated with DJD; pronounced areas of cartilage thinning, loss of surface zone cellularity, pale eosinophilia and pyknotic nuclei (Gardner *et al.* 1987). Some severe cartilage changes were noted in some of the broiler strain fowl; these included the presence of clumps of chondrocytes deep to the surface zone and one sample showed a total loss of all the articular cartilage with exposure of the underlying bone. No severe cartilage changes were observed in the laying strain fowl and the frequency and severity of minor cartilage changes were far less in the layer strain fowl. This confirms that broiler strain fowl are more susceptible to DJD than laying strain fowl.

Despite the biochemical differences in articular cartilage between avian species and mammals, the broiler strain cartilage in this study shows morphological signs of degeneration which are similar to those seen in human DJD. These include loss of cartilage, chondrocyte multiplication or migration to form clusters and loss of metachromatic material from nearby matrix and fibrillation (Gardner *et al.* 1987). These features are also seen in mammalian models of osteoarthritis (Burton-Wurster *et al.* 1993). This suggests that a common mechanism for articular cartilage degeneration might cross the phylum boundaries.

The biochemical differences between articular cartilage from broiler strain and laying strain fowl also reflect those seen in mammalian DJD. Increased hydration and uronic acid content, as shown for the broiler strain fowl, have previously been reported in dog models of DJD in areas where degeneration is expected to occur (McDevitt and Muir 1976). Increased hydration is a common feature of developing DJD (Muir 1988) and an increase in proteoglycan synthesis is also associated with early stages of cartilage degeneration in dog models (Muir 1977) and in other animals including Rhesus macaque monkeys (Brandt 1993).

The cartilage thinning and occasional fibrillation seen in some of the histological samples from the laying strain birds may not be degenerative changes which would lead to clinical pathology but merely a result of ageing on the articular cartilage similar to that reported in mammals by Meachim (1969). The low incidence of DJD in layer strain fowl (Yamasaki & Itikuru 1983) also suggests that this is the case. Further support for this hypothesis is given by the elevated uronic acid concentrations in the articular cartilage of the broiler strain fowl in comparison to that of the layers. An increase in chondroitin to keratan sulphate ratio is seen in DJD (Muir 1988). This is the reverse of the normal changes seen in ageing (Burton-Wurster *et al.* 1993).

In conclusion, these results have shown that despite the previously reported differences between avian and mammal cartilage (Eyre *et al.* 1978), the morphological and biochemical characteristics of early stage degenerative changes seen in articular

cartilage from broiler strain fowl closely resemble those seen in mammalian joint disease and joint disease models. Further studies with these fowl should help elucidate the progression of DJD in fowl and may provide a non-intrusive model for investigation of the general disease processes which lead to articular cartilage degeneration.

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