THE EFFECT OF EXERCISE ON THE COLLAGEN FIBRIL MORPHOLOGY OF THE DIGITAL FLEXOR TENDONS OF THE THOROUGHBRED

**A thesis presented in partial fulfilment of the
requirement for the
Degree of Doctor of Philosophy
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Abstract

A series of studies was conducted to test the hypothesis that galloping exercise changes the morphology of collagen fibrils in the superficial digital flexor tendon of the equine forelimb. Tendons were examined from three groups of horses, namely wild horses, randomly sourced thoroughbred horses submitted for necropsy, and thoroughbreds from an experimental exercise trial. All tendons examined were from horses with no history of superficial digital flexor tendonitis, and all were macroscopically normal.

Three studies involved the measurement of collagen fibril bundle 'crimp' morphology. The crimp, a planar waveform followed by the fibrils, is believed to determine in part the mechanical behaviour of tendon. The first study involved measurement of crimp angle and period length in central and peripheral regions of tendons from wild ponies ranging in age from two to over ten years, which had obviously never undergone training. Horses of ten years of age or older showed a mean crimp angle in the central region which was lower than that in the periphery. It was concluded that the reduction in central region crimp angle with age in untrained horses is a normal fatigue effect. The second study involved measurement of crimp parameters in central and peripheral regions of tendons from horses of two years and over which had been submitted for necropsy to Massey University. These horses were separated into 'exercised' and 'non-exercised' groups based on whether their most recent function had involved competitive galloping exercise. Five of the eight exercised horses under ten years of age had a lower mean crimp angle and length in the central region than in the peripheral region. No un-exercised horses in this age group showed lower values for either parameter in the central region as opposed to the periphery. It was therefore concluded that rapid high-strain cycling of the tendon occurring during galloping exercise modifies normal age-related changes in the crimp morphology of the superficial digital flexor tendon core. For the third study, crimp angle and length were measured in tendons from five thoroughbreds (39±1 months of age) which had undergone a specific 18 month exercise regime involving galloping on a high-

speed equine treadmill, and from six age- and sex-matched thoroughbreds which had undergone walking exercise only during that period. Central region crimp angle and length were significantly lower in the tendons of exercised horses compared to the controls. Four of the five exercised horses showed a significantly lower crimp angle in the central region than in the periphery, and three of these horses also showed a lower central crimp length. The peripheral angle was significantly greater in the exercised horses than in the controls. This was evidence that a specific and defined regime of high-strain cycling of the superficial digital flexor tendon modified age-related crimp morphology changes in the central region.

Two studies were undertaken to investigate the effect of the above defined exercise regime on collagen fibril diameter distributions in the superficial digital flexor tendons, and in the deep digital flexor tendons and suspensory ligaments from the same horses. Central region fibrils in superficial digital flexor tendons from exercised horses had a significantly lower mass-average diameter than those from the control horses, whereas peripheral region fibrils from the two groups did not differ significantly. In the second study collagen fibril mass-average diameters in both regions of the deep digital flexor tendon and the suspensory ligament were not found to differ significantly in exercised horses compared to controls. It was concluded that the exercise regime had caused breakdown of large diameter collagen fibrils in the core of the superficial digital flexor tendon. The fact that such a change did not occur in the suspensory ligament was suggested to be due to a difference in distribution of load between the suspensory ligament and superficial digital flexor tendon during galloping, or due to differences in distribution of such changes along the lengths of the two structures.

The final study involved an age-related analysis of crimp parameters, collagen fibril diameters and biochemical factors in thoroughbreds from foetuses to 3 years of age. It was suggested on the basis of the limited results, that collagen fibrils in the central region of the superficial digital flexor tendon become mature between one and two years of age.

It is proposed that the observed reductions in crimp morphology and collagen fibril mass-average diameter in the core of the superficial digital flexor tendon in response to galloping exercise represent microtrauma, as they would lead to weakness of the structure and predispose it to overloading and further damage. The mechanisms involved are suggested to involve slippage and/or separation of subfibrils, and a mechanism for the development of tendon core lesions is hypothesized. The use of the present information for the prevention of tendonitis is discussed.

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I would like to dedicate this thesis to my mother,

Jacky Patterson-Kane,

with my love and gratitude.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

Introduction

Injury to the superficial digital flexor tendon (SDFT), or tendonitis, of the thoroughbred racehorse is very common, affecting at least 30% of racehorses in National Hunt training in the United Kingdom (Goodship 1993). Recovery is generally prolonged, and the risk of recurrence is high. The financial impact is considerable, in terms of training and racing days lost, reduced performance, long recovery and high treatment costs, reduced value of lame horses, and failure of horses to return to competition. There are also questions concerning animal welfare, as SDFT injury is rare in non-performance horses, and treatments such as tendon firing are of ethical concern.

Despite the importance of tendonitis, understanding of the causes and pathogenesis of lesions is limited. As a result no currently used treatment method is based on scientific rationale, and it has not been possible to institute successful preventative measures to prevent tendonitis, in contrast to other conditions.

Determination of the aetiology and pathogenesis of SDFT tendonitis could allow prevention of injury. Prevention would probably be more successful than any treatment method, as injured tendons appear to be unable to regain normal structure. The higher incidence of SDFT injuries in galloping racehorses than in either standardbred trotters or non-performance horses suggests that events occurring during galloping are responsible for lesion development. The limited information available on the response of other collagenous structures to exercise indicates that the type and volume of exercise may be important. Knowledge of the response of the SDFT to galloping exercise would therefore increase the understanding of the pathogenesis of tendonitis and could provide the basis for the development of preventative training programmes.

1.1 The importance of superficial digital flexor tendonitis to the thoroughbred industry.

The wastage of large numbers of racehorses is of major concern to the multimillion dollar racing industry. Reproductive inefficiency and failure of horses to be put forward for training due to lack of inherent ability contribute significantly to this wastage (Jeffcott *et al.* 1982), but there are also large

losses among horses which do undergo training and/or racing. A British survey by Jeffcott *et al.* (1982) showed 45% of horses in training did not start to race due to musculo-skeletal injury; 53% of horses which did race experienced a period of lameness during the season, and 20% of those animals did not race again due to that lameness. A subsequent study by Rossdale *et al.* (1985) calculated that 67.6% of training or racing days lost were as a result of lameness. Robinson and Gordon (1987) calculated that 25% of racehorses in one racing season at a track in Minnesota were lost permanently due to musculo-skeletal injury, and that a further 22% of horses experienced injury that temporarily interfered with training and/or racing. None of the above surveys determined the effect of injury on performance itself.

If the reasons for losses due to lameness are analyzed, SDFT tendonitis or "bowed" tendon is of significance. Of the training days lost due to diagnosed cases of lameness, 9% (Rossdale *et al.* 1985) and 5.7% (Jeffcott *et al.* 1982) have been estimated to be due to tendonitis. Of track-recorded breakdowns recorded by Robinson and Gordon (1987) in a season, most of which occurred during racing, 35% were due to bowed tendon, 9% were due to suspensory ligament (SL) rupture and 5% were due to a combination of bowed tendon and SL rupture.

Rooney and Genovese (1981) found that 7% of horses in a 9 month race meeting experienced a bowed tendon, and a further 6% experienced a recurrence of a previous tendonitis. Of the horses which had previously bowed a tendon, 48% rebowed. The incidence of SDFT tendonitis in this study did not show age-dependence, but was related to the number of starts. 25% of injuries occurred before the first start, and further 25% occurred within the first three to four starts.

SDFT tendonitis therefore comprises a significant fraction of musculo-skeletal injuries. The significance of tendonitis may be even greater than these figures indicate, since recurrence on resumption of athletic activity is common (Rooney and Genovese 1981), long recovery periods are required (McCullagh *et al.* 1979), and the effect on performance of horses in which injury does not recur is unknown but probably significant.

1.2 Development of the thoroughbred racing industry

The thoroughbred and all other living breeds of horse belong to the genus *Equus*, which evolved over a period of approximately sixty million years (Simpson 1951). Details of this evolution are given in Appendix A (page 183). The thoroughbreds which developed in Britain are acknowledged as the sole source of horses considered to be 'thoroughbred' in the many countries where horse races are held.

Horse racing in Britain was a natural development at a time when these animals played an important role in society. The first formalised races were held on a local basis. The patronage of Newmarket by King Charles II helped to make racing a national sport, with a central organisation. The resultant connection between racing and the upper classes, the strategic location of Newmarket between London and the major horse-breeding area of Yorkshire, and the advent of railway transport fortuitously combined to provide commercial incentive to breed faster horses (Vamplew 1976). Railway transport also allowed the racing of younger horses, yielding earlier returns. Increases in prize money attracted greater numbers of better horses and encouraged ownership.

A period of intensive selection from the mid-17th century to the first quarter of the 19th century resulted in the thoroughbred (Willett 1975). Much of this progress was made during the agricultural revolution of the 18th century, when scientific principles began to be applied to the feeding and breeding of animals. The average height of the racehorse increased by approximately six inches in an approximately 200 year period (Clabby 1976). There were increases in overall size, strength, length of stride and speed. The original small light breeds had been modified by numerous importations of other breeds, including the Arab. The increase in the size and speed of the horse, culminating in *Equus*, exacted a penalty in terms of injuries to the distal limbs (Simpson 1951). There is evidence of injury to the limbs of fossil horses, in the form of periosteal exostoses (Camp and Smith 1942). The further increase in size and speed in the thoroughbred means it is possible the capacities of the supporting tendons and ligaments are severely taxed. In fact, thoroughbreds are as fast

as is mechanically possible for animals of their size and bodyweight. In certain thoroughbred horses it is likely that the mechanical limits are easily exceeded.

1.3 The collagen fibril

Collagen fibrils are the fundamental units of tensile strength in tendons and other connective tissues. They are submicroscopic cylindrical structures with diameters that range between 20 and several hundred nanometres in tendon. Their lengths are unknown, but have been estimated to be at least several millimetres in mature tendon (Craig *et al.* 1989). The diameters, orientation and packing density are important determinants of the strength and elasticity of a tendon. Interactions with components of the matrix which surrounds them are also thought to be important in this respect, by maintaining interfibrillar cohesion (Cribb and Scott 1995).

1.3.1 The collagen molecule.

Collagens are a family of proteins with regions displaying unique similarity in amino acid sequences. Collagen is the only constituent of connective tissue with appreciable tensile strength, accounting for its high concentration (60-85% of dry weight) in tendon (Elliott 1965). There are numerous genetic types of collagen, type I being predominant in adult tendon.

The collagen molecule is rodlike, measuring 300 nm by 1.4 nm (Parry 1988). It consists of three left-handed helical polypeptides, or α-chains (Grant and Prockop 1972). Short non-helical regions termed telopeptides comprise the ends of each α-chain. The helical regions are composed of sequences of polar and non-polar tripeptides with the arrangement glycine-X-Y (Figure 1.1). X and Y are most commonly the imino acids proline or hydroxyproline respectively, which are thought to be important in stabilizing the α-chain helix. Positioning of the glycine residues in the centre of the collagen monomer allows the formation of a super triple helix by the three α-chains.

Collagen molecules are released from the cell in a procollagen form, with cleavage of the large propeptide sequences being catalyzed extracellularly by

the enzymes procollagen N-proteinase and procollagen C-proteinase (Jiminez *et al.* 1971). Degradation is less well understood, but it is thought that acidic cathepsin enzymes and/or neutral serine proteinases depolymerise fibrillar collagen (Bailey and Light 1989, Miles *et al.* 1994). Extracellular tissue collagenases or intracellular lysosomal cathepsins then attack specific sites on the helical portion of the molecule (Kucharz 1992). The turnover of collagen is the net result of the biosynthetic and degradative processes, and in adult rat tendon has been demonstrated to be slow relative to other tissues, being in the order of 3-4 months(Gerber *et al.* 1960).

1.3.2 Collagen covalent cross-linkages

Covalent cross-linkages form within and between collagen molecules. They are largely responsible for the tensile strength of the collagen fibril due to their resistance to shear forces, and hence slippage, between the molecules (Bailey *et al.* 1974). This is supported by the considerable weakening of fibrils which occurs due to interference with cross-link formation by lathyrogens (Bailey *et al.* 1970) or genetic factors (Eyre 1984). Cross-links also increase the resistance of collagen to chemical or enzymatic breakdown.

With the exception of the carboxytelopeptide of $\alpha_2(I)$ (Parry and Craig 1984), the amino- and carboxy-terminal telopeptides for the α-chains in collagen types I, II and III each contain a lysine residue. This lysine residue may be hydroxylated by the enzyme lysyl hydroxylase. Cross-link formation involves conversion of the specific lysine or hydroxylysine residues to the aldehydes allysine or hydroxyallysine respectively (Bailey *et al.* 1970). The reaction is catalyzed by the copper metalloenzyme lysyl oxidase (Siegel *et al.* 1970), which has a high activity towards collagen aggregated in fibrils and low activity to soluble forms (Siegel 1974). This implies that the formation of cross-links begins after the onset of fibril formation *in vivo*. Subsequent reactions in cross-link formation appear to occur spontaneously, possibly due to 'active sites' in the amino acid sequences (Eyre 1984).

Intramolecular crosslinks arise from the aldol condensation of two allysine residues (Bailey 1968) and have been identified only between N-terminal

telopeptides (Eyre 1984). They are not thought to increase the stability of the fibril, as they are located within one molecule (Bailey and Light 1989).

Intermolecular cross-links form by condensation of allysine or hydroxyallysine in the nonhelical region of one molecule with a hydroxylysine residue in the helical region of an end-overlapped adjacent molecule (Figure 1.1) (Bailey *et al.* 1974). The result is a 'Schiff's base' or aldimine compound (Figure 1.2). Condensation of allysine with hydroxylysine results in the crosslink compound dehydrohydroxylysinonorleucine (dehydro-HLNL), the reduced form of which is hydroxylysinonorleucine (HLNL). The reaction between hydroxyallysine and hydroxylysine results in the compound dehydroxydihydroxylysinonorleucine. The latter can undergo a spontaneous and irreversible Amadori rearrangement to form the more common single-bonded system, an oxo-imine called hydroxylysino-5-oxo-norleucine (HLONL) (Bailey and Light 1989). The reduced form of this compound is dihydroxylysinonorleucine (DHLNL).

The extent of hydroxylation of lysine residues in the nonhelical regions by lysyl hydroxylase therefore determines whether the aldimine or the oxo-imine compound will predominate (Bailey *et al.* 1974, Bailey and Light 1989). The hydroxyallysine pathway predominates in tendon (Barnes et al. 1974), and lysyl hydroxylase activity has been shown to increase in response to increased loading in this tissue (Gerriets *et al.* 1993). Some tissues may also show different levels of hydroxylation during development (Bailey and Light 1989).

Some HLNL and DHLNL compounds are derived from glycosylated hydroxylysine residues, the proportions of which vary in different tissues, and the functional importance of which is not known (Eyre 1984).

Cross-links formed by the allysine and hydroxyallysine pathways rapidly increase in concentration during initial synthesis and laying down of collagen, then gradually disappear as the tissue matures (Robins *et al.* 1973, Eyre 1987). There is a concomitant increase in the thermal stability, acid-insolubility, and collagenase-resistance of the collagen (Bailey 1968, Bailey *et al.* 1974, Bailey and Light 1989, Bigi *et al.* 1992). It was initially hypothesized that the crosslinks underwent a natural reduction to form more stable compounds, but such forms have not been detected by direct analysis (Bailey and Peach 1971, Robins *et al.* 1973, Bailey *et al.* 1974). There is now strong evidence that the

difunctional reducible crosslinks formed via the hydroxyallysine pathway are converted to fluorescent cyclic 3-hydroxypyridinium compounds (Eyre 1984, 1987), which are nonreducible and have greater resistance to enzymatic digestion. Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are two analogues. It has been proposed that two aldimine or oxo-imine compounds condense, with the elimination of one residue (Eyre 1984, 1987). HP is thought to be formed by condensation of DHLNL with a hydroxylysine residue, as it contains three hydroxylysine residues. LP is a minor analogue containing two hydroxylysine residues and one lysine (Eyre 1984). It is not known if the compounds involve residues from two or three separate molecules, but the latter would support known data indicating increased stability of collagen with age. This would indicate that end-to-end stabilisation occurs by the formation of reducible crosslinks between overlapped molecules. Further increases in strength would then occur once the fibril has ceased to grow, by formation of tri- and/or tetravalent crosslinks between the longitudinal units.

Concentrations and relative proportions of both reducible (immature) and nonreducible (mature) crosslinks vary between different tissues (Bailey *et al*. 1970, Sims and Bailey 1992), but the significance of this is poorly understood. It is possible that they are related to mechanical loading experienced by the tissue (Eyre 1984).

1.3.3 The axial and lateral molecular arrangement.

The collagen molecules are aligned in parallel in the long axis of the fibril, and staggered so that a little less than ¼ of the length of each molecule overlaps with the adjacent one (Figure 1.1)(Hodge and Petruska 1963). This 'quarter-stagger' model is supported by the optical synthesis of electron micrographs of segment-long-spacing (SLS) bundles of collagen molecules (Hodge and Schmitt 1960) and amino acid sequence analysis (Hulmes *et al*. 1973, Doyle *et al*. 1974).

Three model types have been suggested to explain the lateral molecular arrangement. Smith (1968) suggested a hollow cylinder of five molecules, termed a 'microfibril', to extend the quarter-stagger arrangement in the
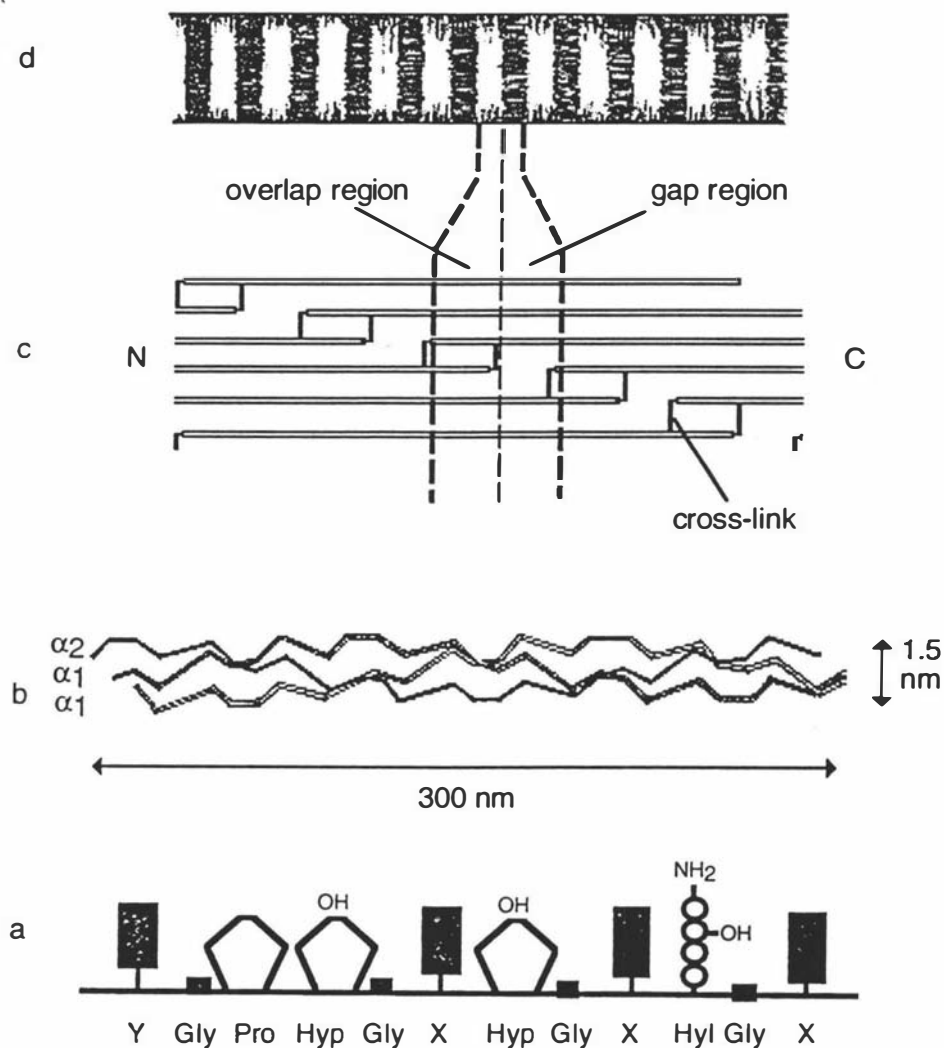
Figure 1.1 Structure and two-dimensional packing of the collagen molecule. a - typical amino acid sequence of an α-chain. Gly = glycine, Pro = proline, Hyp = hydroxyproline, Hyl = hydroxylysine. b - triple helix of the collagen molecule. c - two-dimensional packing of collagen molecules ("quarter stagger "model). N = amino terminal, C = carboxy terminal d - the negatively-stained collagen fibril.

hydroxyallysine

```
—CH—
  |
 (CH2)2
  |
 CH-OH
  |
 CHO          in vivo
  +          ———————→
 NH2
  |
 CH2
  |
 CH-OH
  |
 (CH2)2
  |
_CH_
```

hydroxylysine

in vivo Amadori rearrangement
⇄

```
—CH—              —CH—
  |                 |
 (CH2)2            (CH2)2
  |                 |
 CH-OH             C =O
  |                 |
 CH                CH2
  ‖                 |
  N                NH
  |                 |
 CH2               CH2
  |                 |
 CH-OH             CH-OH
  |                 |
 (CH2)2            (CH2)2
  |                 |
—CH—              —CH—
```

dehydrodihydroxy-lysinonorleucine   *hydroxylysino-5-oxo-norleucine*

KBH4

```
—CH—
  |
 (CH2)2
  |
 CH-OH
  |
 CH2
  |
 NH
  |
 CH2
  |
 CH-OH
  |
 (CH2)2
  |
—CH—
```

dihydroxylysino-norleucine (DHLNL)
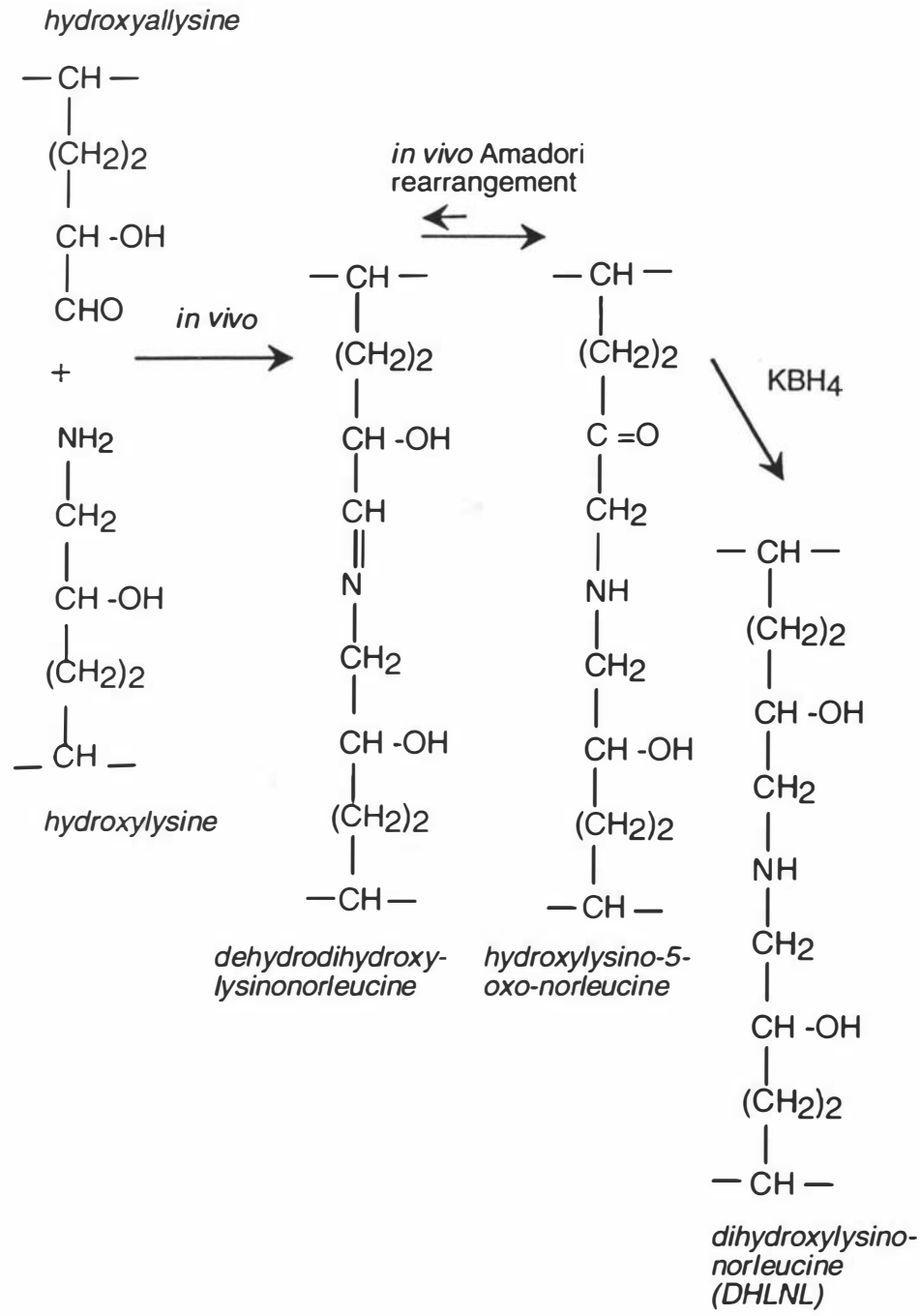
Figure 1.2 The formation, rearrangement and reduction chemistry of the oxo-imine reducible cross-link of collagen (adapted from Bailey and Light (1989)).

transverse direction. Hulmes and Miller (1979) hypothesized quasihexagonal crystalline packing of tilted individual molecules, based on improved X-ray diffraction data. Other researchers have suggested an amalgam of the microfibril and molecular crystal models in the form of compressed (distorted) microfibrils lying on a quasihexagonal lattice (Bailey et al. 1980, Trus and Piez 1980)

1.3.4 The subfibril

It is possible that a structural unit exists which is intermediate in size between the microfibril and the fibril. This is based on observations of embryonic tissue (Jackson 1956), mechanically or chemically disrupted fibrils from adult tissue (Bouteille and Pease 1971, Torp *et al.* 1975a, Lillie *et al.* 1977, Barenberg *et al.* 1978, Parry and Craig 1981, Scott 1990) and freeze-fractured preparations (Rayns 1974, Ruggeri *et al.* 1979, Itoh *et al.* 1982, Leonardi *et al.* 1983). Diameters of observed units ranged between 10 and 20 nm, but have often been dismissed as artefacts of sample preparation procedures (Stolinski and Breathnach 1977). It is possible, however, that subfibrils are not observed in normal electron microscopy preparations due to formation of methylene bridges between them by the glutaraldehyde fixative (Rayns 1974, Itoh *et al.* 1982). Scott (1990) observed 12-15 nm diameter structures in rat tail tendon disaggregated under conditions which were close to physiological. It was suggested that for steric reasons, it is difficult for collagen molecules to form crosslinks to the periphery of units 10 nm in diameter. A corollary of the latter theory is that cross-links do not exist between the 10 nm subfibrils (Scott 1995).

1.3.5 Fibril diameter growth and control mechanisms

The distributions of fibril diameters are characteristic for certain tissues and structures, in order to provide the appropriate mechanical properties (Parry *et al.* 1978a, Flint *et al.* 1984). The mechanisms involved in regulating the diameters have not been defined, but are undoubtedly complicated, given the

number of post-translational modifications of collagen molecules between intracellular synthesis and their addition to the extracellular fibril in monomer or aggregate form.

It has been suggested that further increases in fibril diameter occur by fusion of collagen fibrils as the connective tissue ages, on the basis of the appearance of certain fibrils in electron micrographs (Torp *et al.* 1975a, Scott *et al.* 1981, Scott and Parry 1992), and the fact that increases in diameters of fibrils in mature tendon are not correlated with increases in collagen content (Scott *et al.* 1981, Scott and Hughes 1986). Scott and Parry (1992) suggested that tissues undergoing rapid high-strain cycles have larger fibrils because more forceful contacts are made between them, resulting in fusion. Scott (1990) also hypothesized that subfibrils could separate and reaggregate due to factors such as altered tensile forces, resulting in rapid alterations of fibril diameter with minimal tissue destruction and cellular input.

Numerous factors have been suggested to control collagen fibril diameter. A relationship between mean fibril diameter, glycosaminoglycan (GAG) concentration and relative proportions of the different GAGs has been noted in studies of developing (Flint *et al.* 1984, Parry *et al.* 1982) and surgically manipulated tendon (Gillard *et al.* 1979a). This does not necessarily imply that GAGs control fibril diameter. Certain proteoglycans (proteodermochondans) which associate regularly with collagen fibrils at crosslinking sites may also regulate fibril growth by inhibiting further crosslinking (Scott and Orford 1981). The structural glycoprotein, fibronectin, appears to have a strong association with collagen and to stabilise fibrils either by direct interaction, or indirectly via interactions with proteoglycans (Anderson and Jackson 1972, Kleinman *et al.* 1981). Cetta *et al.* (1982) measured an increase in fibril diameter in developing rabbit Achilles tendon which was preceded by a reduction in glycoprotein concentration.

The extent of glycosylation of hydroxylysine residues in collagen molecules has been suggested to control collagen fibril diameter (Cetta *et al.* 1992), possibly because the large side groups of hydroxylysyl glycosides interfere with fibril growth (Morgan *et al.* 1970). The ratio of type I to type III collagen molecules may also be important (Henkel and Glanville 1982), as diameters of collagen

fibrils formed *in vitro* are inversely proportional to the concentration of type III collagen used (Lapière *et al.* 1977). Other *in vitro* studies have shown that fibril diameter is dependent on whether the immediate precursor has an attached amino- or carboxypropeptide, indicating that the order of removal of the propeptide sequences may be a control mechanism *in vivo* (Miyahara *et al.* 1984).

In tendon, approximately 50% of the fibrils point "up" and 50% point "down" (Torp *et al.* 1975a, Parry *et al.* 1978b, Parry and Craig 1984). Fusion cannot occur between anti-parallel fibrils, as 'hybrids' have not been observed (Scott and Parry 1992). Regulation of polarity could result in different regions in a tendon containing different proportions of "up-" and "down-pointing" fibrils and therefore different potentials for growth by fusion.

1.3.6 Fibril diameter distributions

Collagen fibril diameter distribution appears to be related to the type and magnitude of mechanical stress and strain experienced. Alterations in distributions have been seen in immobilised tendon (Enwemeka 1989), regenerating tendon exposed to varying mechanical stimuli (Postacchini and De Martino 1980, Matthew and Moore 1991), surgically manipulated tendon (Gillard *et al.* 1979a), exercised tendon (Michna 1984, Enwemeka *et al.* 1992) and developing tendon (Jackson 1956).

Fibrils in young and repair tissue are typically of small and constant diameter, resulting in a unimodal diameter-frequency distribution (Figure 1.3)(Parry *et al.* 1978a). The mean fibril diameter increases as the tissue matures. Adult tendons show a wider distribution of diameters, which may be bimodal or unimodal depending on their function (Figure 1.3).

Large diameter fibrils were calculated to have a higher potential density of collagen cross-links, implying that they have a greater tensile strength (Parry *et al.* 1980). This was supported by the positive correlation of mass-average diameters with tendon and ligament ultimate strengths in age-related studies (Parry and Craig 1988, Oakes 1989). Small diameter fibrils by virtue of their higher surface area per volume, were proposed to have stronger interactions
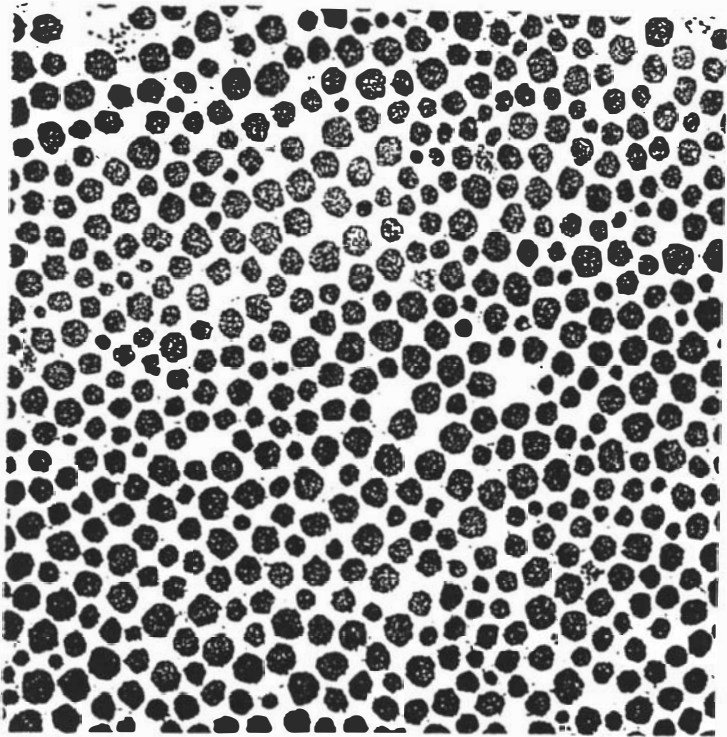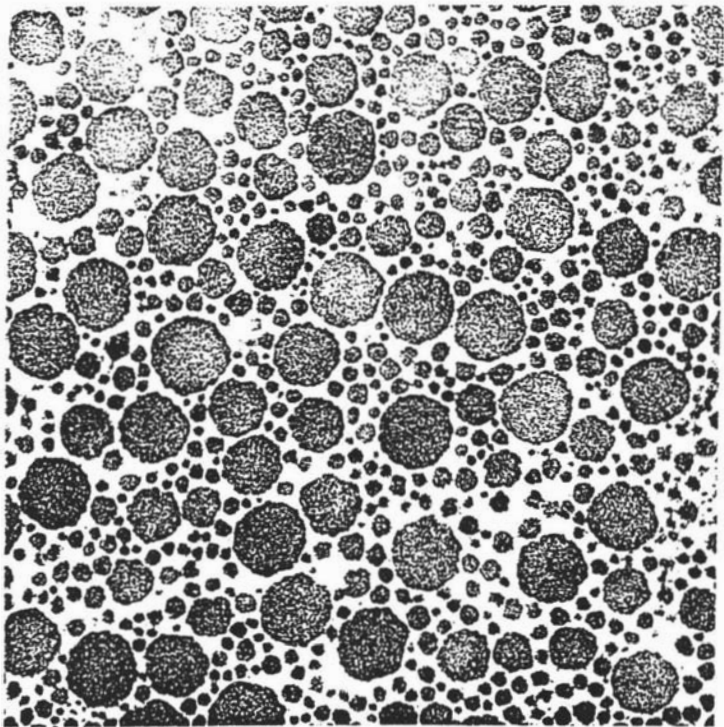
Figure 1.3 Transverse sections of collagen fibrils in foetal (a) and adult (b) equine superficial digital flexor tendon. The foetal tendon contains small fibrils of relatively uniform diameter. The adult tendon contains both large and small diameter fibrils (both 45 100x).

with surrounding matrix components, reducing interfibrillar slippage ('creep'). The proportions of large and small diameter fibrils should therefore determine the strength and elasticity of the tendon. A bimodal distribution containing both small and large diameter fibrils also results in a greater density of collagen per unit area, as small diameter fibrils pack into the spaces between large fibrils. This collagen density is also positively correlated with ultimate tensile strength of the tendon (Parry 1988). There appears to be an increase in the number of small fibrils in a number of senescent tendons. This may be a fatigue effect due to breakdown of large diameter fibrils, as supported by their commonly irregular appearance in such tendons (Parry and Craig 1977), or the small fibrils have been newly synthesized but have not grown in diameter (Parry and Craig 1984).

1.4 The structure and mechanical behaviour of tendon

Tendons are white bands of dense fibrous connective tissue. They are structural elements which, in their simplest form, function to transmit muscular forces to the skeleton. The arrangement allows the muscle to be some distance away from the joint or joints which it affects, and allows transmission of forces around corners. Tendons have a hierarchical structure, the development and maintenance of which is essential for normal function.

1.4.1 Structural organisation.

Tendon has a specific arrangement of longitudinal elements on several levels, from molecular to microscopic (Figure 1.4). The largest units are termed 'fascicles', which range from 100-300 μm in diameter and are roughly hexagonal in cross-section (Kastelic *et al.* 1978). Fasciculation of the tendon has been suggested to increase strength and flexibility of the structure as a whole, and individual fascicles do appear to move independently in the equine SDFT (Goodship *et al.* 1994). Fasciculation is also important for nutrition of tendon cells, as blood vessels can pass between the units (Edwards 1946, Chaplin and Greenlee 1975).
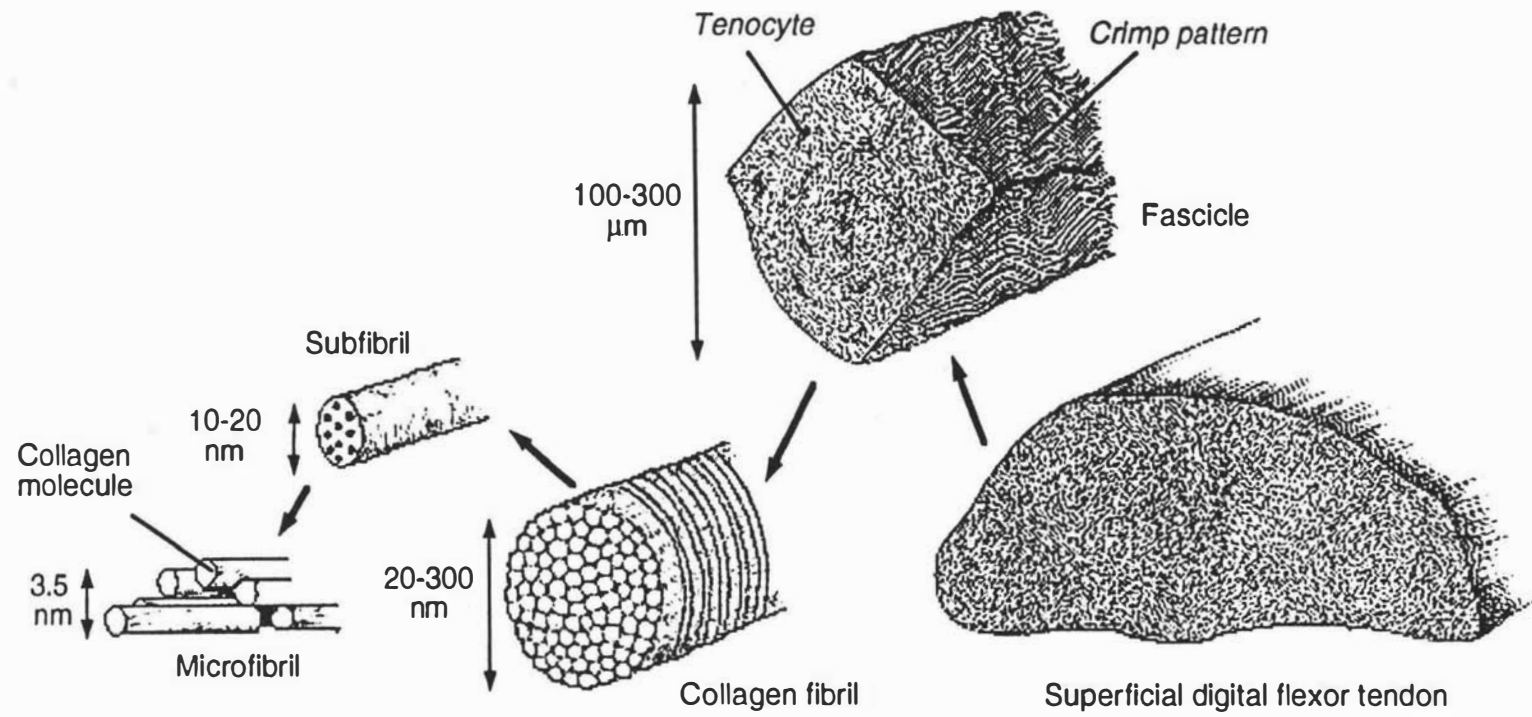
Figure 1.4 The hierarchical structure of tendon.

The fascicles are comprised of collagen fibrils surrounded by an extrafibrillar matrix. This fibril-matrix composition allows the structure of the fascicle to withstand crack propagation, and means that damage to individual fibrils will not necessarily cause mechanical failure of the whole unit.

The matrix has considerable order despite its amorphous appearance, largely due to macromolecules termed proteoglycans. The latter include 'small' proteodermochondans, which associate in a regular and orthogonal fashion with collagen fibrils in tendon and other connective tissues. They are thought to form duplexes which bridge interfibrillar spaces, implying roles in fibril spacing and mechanical behaviour (Cribb and Scott 1995, Scott 1995).

Other constituents of the extrafibrillar matrix include water, simple ions, glycoproteins and elastic fibres. In adult tendon the latter consist of an amorphous core of elastin, surrounded by glycoprotein microfibrils 10-12 nm in diameter (Figure 1.5)(Bailey and Light 1989). In the adult tendon the matrix contains elongated cells with minimal cytoplasm termed tenocytes (Williams *et al.* 1980), which are responsible for the synthesis and maintenance of both collagenous and noncollagenous components of the extracellular matrix. Their viability and level of function is therefore of paramount importance.

1.4.2 Associated structures

At the myotendinous junction there is no direct continuity between collagen fibrils and muscle fibres. The collagen fibrils insert into deep recesses of the muscle cells produced by folding of the sarcolemma, which maximise the surface area for interaction (Kannus *et al.* 1992). Numerous tendon sense organs are located in this region (Stillwell 1957).

Where a tendon changes direction or experiences friction from surrounding structures it is surrounded by a synovial sheath (Elliott 1965). The sheath is composed of outer parietal and inner visceral membranous layers which are continuous with one another along the mesotendon (Ottaway and Worden 1940). Annular ligaments may pass over the tendon at certain points to help maintain it in position as the joint angle changes between full flexion and
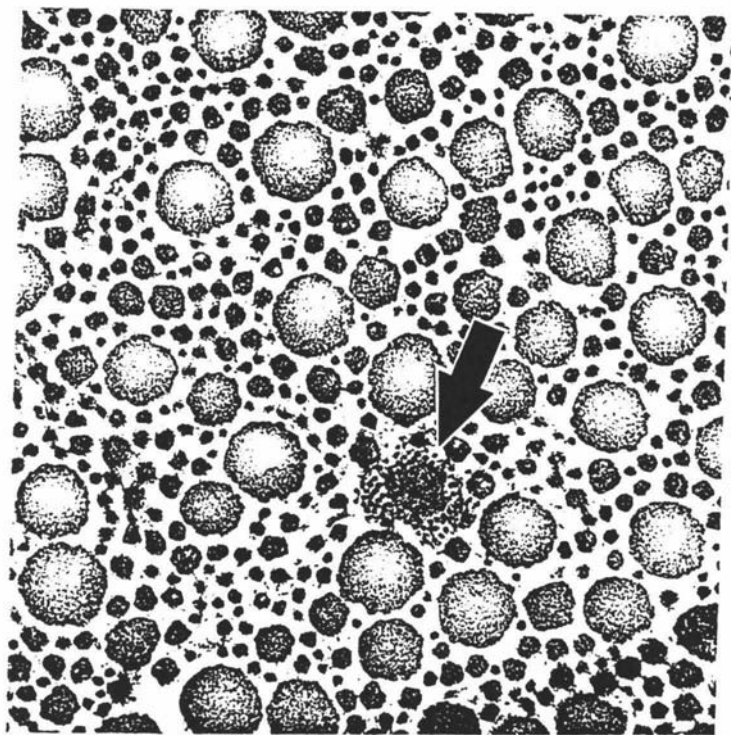
Figure 1.5 Transverse section of a superficial digital flexor tendon from a four year old horse (45 100x), showing an elastic fibre (arrowed).

extension (McIlwraith 1987). Other regions of the tendon are surrounded by a loose, vascular connective tissue layer termed the paratendon. The paratendon is highly elastic and is an essential element in the gliding of the tendon during deformation (Selway 1975). Beneath the synovial sheath or paratendon is a layer of loose connective tissue termed the epitendon, which is closely adherent to the tendon surface. It extends into the tendon substance between fascicles as the endotendon, which is continuous with the perimysium and periosteum at either end of the structure (Edwards 1946). The endotendon carries blood vessels, lymphatic vessels, and nerves. Both myelinated and nonmyelinated nerves are present, to conduct pain and proprioceptive impulses(Fackelman 1973).

At the osseous insertion there is a progression through four distinct histological zones, from normal tendon tissue to fibrocartilage and thence to mineralised fibrocartilage and lamellar bone. Collagen fibrils aggregate to form regularly spaced Sharpey's fibres in the mineralised fibrocartilage which intermingle with collagen fibrils of the bone (Cooper and Misol 1970). The gradation in structure and associated gradation in elastic modulus is thought to allow dissipation of tensile forces, preventing failure at this site (Cooper and Misol 1970).

1.4.3 Blood supply

Within the tendon, long arteriolar vessels and associated venules and lymphatic vessels course longitudinally between fascicles, connected into a plexus by a regular series of transverse vessels (Edwards 1946). In developing tendon there is a rich capillary network to supply the active population of fibroblasts (Peacock 1959). As the amount of intercellular substance increases the relative vascularity reduces. The tenocytes in adult tendon do still require nutrients and oxygen, despite the slow turnover of collagen.

Peacock (1959) identified three sources of blood supply, in isotopic studies of canine digital flexor and extensor tendons. Blood vessels in the perimysium of the associated muscle were able to supply vessels in the proximal third of

the tendon, and those in the periosteum of the bony insertion were able to supply the distal fourth. The paratendon or mesotendon appeared to be a vital supply to the intervening region, as indicated by degenerative changes in artificially sheathed tendons (Peacock 1959).

1.4.4 The crimp morphology

The wavy or 'crimped' appearance of tendon collagen has been known for many years (Elliott 1965, Diamant *et al.* 1972) and can be observed with the naked eye. It is only recently that this morphology has been quantified using polarising optics and objectively related to mechanical behaviour .

Polarised light microscopy

Intact tendon shows typical dark and light periodic banding when examined under crossed polaroids (Figure 1.6), the pattern of which changes in a systematic fashion as the crossed polaroids are rotated (page 67). Diamant *et al.* (1972) examined intact rat tail tendon, and established by mathematical calculation that the observed pattern of orientation variation was due to a planar zig-zag wave. The correlation of the mathematical model with actual morphology was demonstrated using polarised light microscopy (Diamant *et al.* 1972) and scanning electron microscopy of teased tendons (Gathercole *et al.* 1974, Gathercole and Keller 1975, Nicholls *et al.* 1983), transmission electron microscopy (Dlugosz *et al.* 1978, Kastelic *et al.* 1978), and low to medium angle X-ray diffraction measurements (Gathercole and Keller 1975, Kastelic *et al.* 1978). The parallel arrays of collagen fibrils in tendon follow the path of the waveform. The crimp bending sites are very sharp, occurring within one to two 67 nm band periods (Dlugosz *et al.* 1978). Previous studies had suggested a helical arrangement, but experiments involving axial rotation of specimens under polarised light (Shah *et al.* 1979) and in the scanning electron microscope (Gathercole *et al.* 1974) also supported planar organisation. The planar wave has also been demonstrated in other mammalian tendons, in both fresh and preserved tissue (Dale *et al.* 1972, Gathercole and Keller 1975).
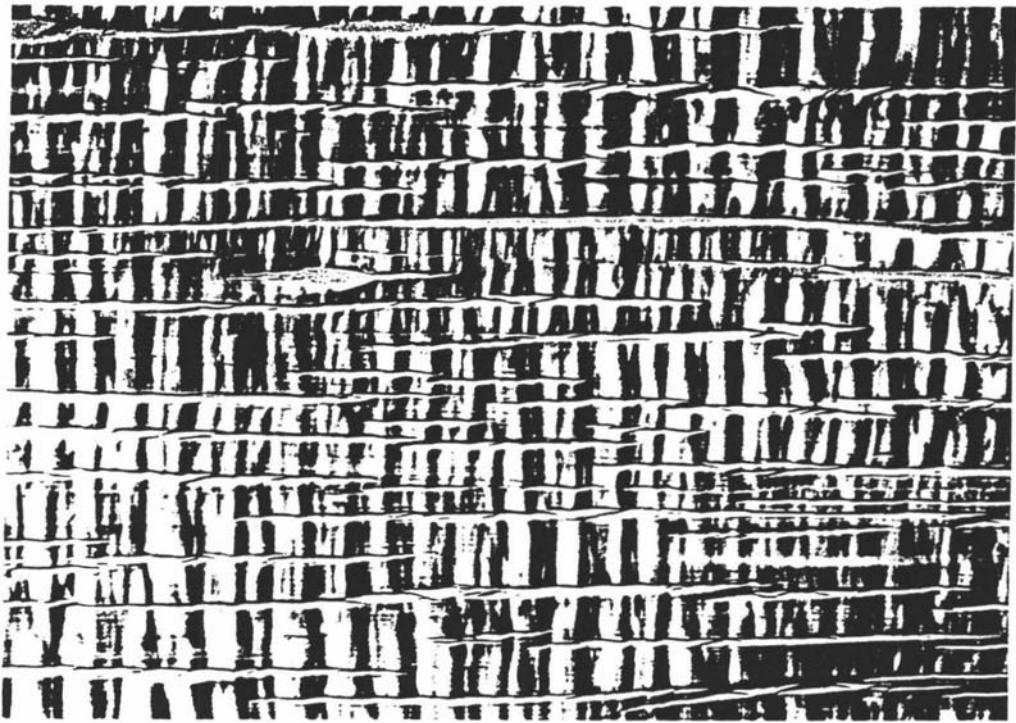
Figure 1.6 The periodic banding pattern of collagen fascicles in the superficial digital flexor tendon of a six year old horse (30 µm thick frozen section, 100x).

The crimp waveform has a specific angle and period length, which may be quantified based on the changing banding pattern on rotation of the polarised light (Diamant *et al.* 1972). These parameters have been demonstrated to change with age in various tendons (Diamant *et al.* 1972, Wilmink *et al.* 1992). Crimp angles typically vary between 5 and 25°, and crimp lengths between 1 and 100 μm (Parry 1988).

Development

The exact mechanism by which crimp develops is unknown, but it is unlikely to be due to spontaneous behaviour of the collagen alone. It is not a feature of isolated collagen fibrils, as obtained by either matrix extraction or collagen reconstitution (Kastelic *et al.* 1978). Examination of the bending sites of crimped fibrils under the electron microscope has not revealed specific locations, differences in appearance, or fibril ends at those points (Gathercole *et al.* 1974, Gathercole and Keller 1975, Dlugosz *et al.* 1978, Kastelic *et al.* 1978).

One model, based on the mechanical behaviour of synthetic fibre-filled composites, suggests that buckling of fibrils occurs due to the changes in relative volumes of collagen fibrils and the intervening matrix during development (Dale and Baer 1974). The loss of large quantities of the space-filling hyaluronic acid (HA) could be particularly important. Shah *et al.* (1982) noted the coincidence of the appearance of crimp and the bending of fibroblasts in embryonic chick tendons, but it could not be determined whether they influenced one another or were responding to a separate event.

Fascicular arrangement

There is evidence for a cylindrically symmetrical arrangement of planar crimped fibrils within the fascicles, with crimping planes at right angles to the outer surface (Kastelic *et al.* 1978). The crimp angle was suggested to decrease towards the fascicle centre on the basis of small angle X-ray diffraction measurements, which would have implications for its mechanical behaviour

(Kastelic *et al.* 1978). This has not been measured directly. Conversely, Nicholls *et al.* (1983) suggested that crimped fibrils are all in the same plane, parallel to the longitudinal axis of the tendon.

Crimp patterns of adjacent fascicles in rat tail tendon have been observed to be in register, despite the absence of direct communications between them (Kastelic *et al.* 1978). This results in considerable lateral cohesion within the tendon, making fascicles difficult to separate (Kastelic *et al.* 1978). Transverse mechanical interaction between fascicles (Kastelic *et al.* 1978) and cellular communication (Oakes 1989) have been suggested to cause this co-crimping.

1.4.5 Mechanical behaviour

Information regarding mechanical behaviour of tendon has largely been derived from mechanical testing *in vitro*. When a tendon is isolated and loaded to failure it shows a characteristic stress-strain curve, stress being the internal force experienced per unit of cross-sectional area, and strain the elongation relative to the original length (Butler *et al.* 1978). Three regions occur, namely the 'toe', 'linear', and 'yield and failure' regions (Figure 1.7)(Torp *et al.* 1975b). Direct measurement of forces and strains *in vivo*, using force transducers and strain gauges, is necessary to determine the region in which a particular tendon functions.

The toe region

Initially the structure is highly compliant, so large increases in strain result only in a small increase in stress. This region corresponds to opening out of the crimp, as evidenced by the loss of the banding pattern under polarised light as the tendon is stretched (Rigby *et al.* 1959, Diamant *et al.* 1972, Shah *et al.* 1979). There is no stretching of the fibrils themselves while this occurs, in adult tendon (Diamant *et al.* 1972). The crimp angle is reduced to zero at the end of the toe region, and the strain at this point is termed the 'toe limit strain' (Diamant *et al.* 1972, Shah *et al.* 1979), determined to be 2-5% in a variety of collagenous tissues. A fibril bundle with a larger crimp angle undergoes a

greater elongation before it straightens, hence crimp angle is correlated with the toe limit strain (Wilmink *et al.* 1992).

Various models have been developed to describe the opening out of the crimp (Diamant *et al.* 1972, Dale and Baer 1974, Kastelic *et al.* 1980). The model of Diamant *et al.* (1972) assumed the properties of a single fibril to represent the properties of the fascicle. The sequential straightening and loading model proposed by Kastelic *et al.* (1980) was a structural one based on a distribution of angles within the fascicle, which fitted well with experimental data.

The crimp morphology allows fibril bundles to undergo additional strain in comparison with initially straight structures of the same elasticity. It has been suggested to act as a shock absorbance mechanism, protecting muscle fibres against sudden increases in tensile loading (Rigby *et al.* 1959, Dale and Baer 1974, Shah *et al.* 1979). Certain tendons possibly operate within the toe region *in vivo* (Elliott 1965), but a tendon which acts as an elastic energy store must operate in the linear region.

The linear region

Once the crimp has been straightened the fibrils themselves begin to be stretched and the tendon behaves as an elastic material. This means that each increment in increased strain results in an equivalent increment in increased stress, and that these changes are recoverable (Torp *et al.* 1975b). The slope of the linear plot is a measure of stiffness.

If the tendon is cyclically loaded and unloaded in this region, some energy is lost as heat due to internal friction. This heat loss is represented by the area of the observed hysteresis loop (Figure 1.8). Rapidly repeated cyclical loading appears to stabilise the mechanical behaviour of the tendon, which becomes more ₁nearly elastic and slightly stiffer (Evans and Barbenel 1975). This reversible effect is referred to as 'preconditioning,' and has been correlated with a sharpening of the X-ray diffraction pattern (Rigby 1964). This was proposed to represent improved orientation of elements within the collagen fibrils (Rigby 1964). If the tendon is subject to a constant load in this region, the deformation will increase with time - a phenomenon termed 'creep'. If
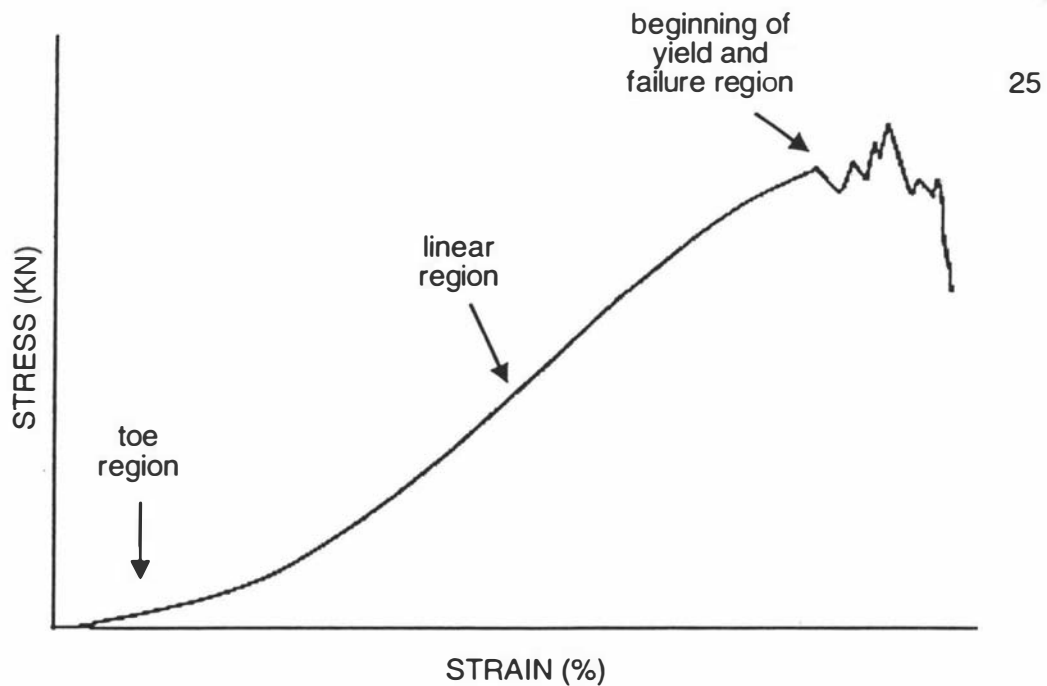
Figure 1.7 Example of a typical stress-strain curve for *in vitro* loading of tendon (adapted from Goodship *et al.* (1994)).
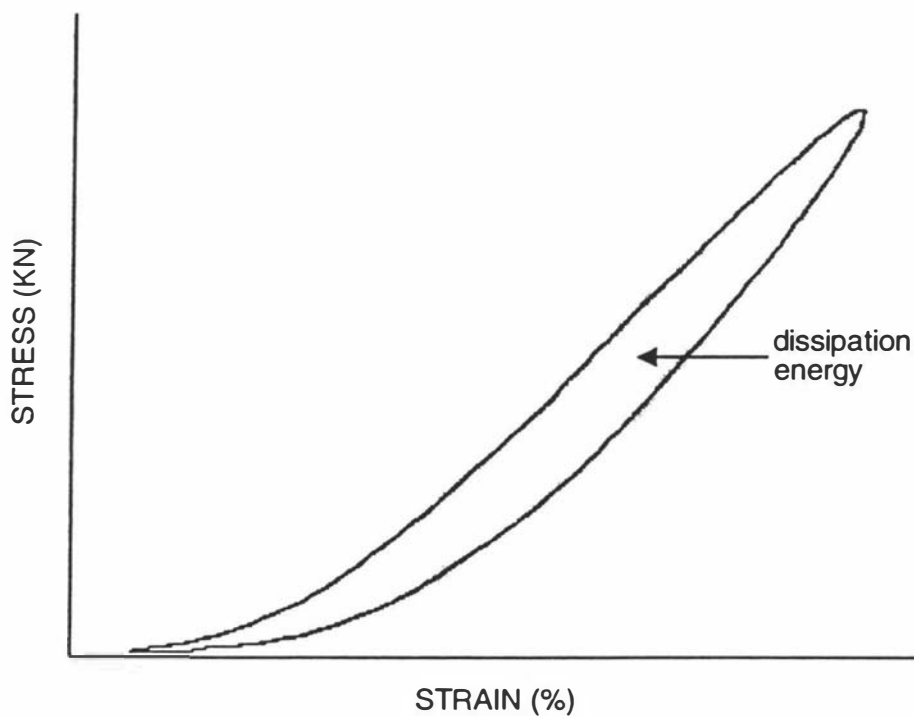
Figure 1.8 Loading cycle of a tendon showing the hysteresis loop. The area of this loop is equal to the energy lost in the cycle (adapted from Goodship *et al.* (1994)).

subjected to a constant deformation, the stress will decrease, an effect termed 'stress relaxation'.

Fibrils under strain in rat tail tendon have been observed to become thinner (Cribb and Scott 1995). It is not certain which parts of the fibril structure deform. Cribb and Scott (1995) proposed a reversible straightening of helically arranged subfibrils, which would correlate with the reduction in fibril diameter. Schwartz *et al.* (1969) suggested that it is the polar regions, based on studies of stretched reconstituted collagen. Mosler *et al.* (1985) studied the synchrotron radiation diffraction spectra of tendons under tension, and concluded that there is an increase in the D period due to sliding of molecules relative to one another, combined with stretching of the molecules themselves. The intermolecular slippage was proposed to stretch covalent cross-links between the telopeptides.

The yield and failure region

With further increases in strain, a yield point is reached. Small stress reductions can sometimes be seen in the loading curve prior to the end of the linear region, representing failure of a few individual fascicles (Butler *et al.* 1978). The ultimate *in vitro* tensile strengths of tendons generally range between 50-100 MPa (Evans and Barbenel 1975). Following this point the tendon is again highly compliant, but is undergoing plastic deformation which is not recoverable. Failure occurs due to breaking of interfibrillar crosslinks, followed by intrafibrillar crosslinks at higher stress and strain levels (Parry and Craig 1988). Torp *et al.* (1975b) cyclically deformed rat tail tendon into the yield region and noted the appearance of a new small-period waveform on relaxation, superimposed on the original one. This phenomenon was termed "recrimping". This was suggested to be due to slippage of elements within the collagen fibrils. Torp *et al.* (1975b) observed voids propagating throughout fibrils which were stretched into the yield region. At failure, the fibrils disaggregated into subfibrils approximately 15 nm in diameter. The primary failure mechanism is proposed to be by this fibrillar dissociation. Parry *et al.* (1980) observed preferential breakdown of the peripheries of large fibrils in rat

tail tendon stretched to rupture. It was proposed that this was due to large diameter fibrils experiencing higher stress levels than small fibrils, at the same level of strain. Complete structural failure occurs rapidly with increasing stress and strain, and the tendon ruptures.

1.5 The equine superficial digital flexor tendon

1.5.1 Anatomy

The small superficial flexor muscle arises from the medial epicondyle of the humerus,with a second tendon of origin arising from the disto-medial radius (Figure 1.9). The latter is a reinforcing ligament known as the accessory ligament of the superficial digital flexor tendon, which is present only in the forelimb. The musculotendinous junction lies just proximal to the carpus, with the accessory ligament joining the main tendon near this point (Getty 1975, Shively 1983). The deep digital flexor tendon (DDFT) originates as three heads; the humeral, ulnar and radial. These merge into the main tendon just above the carpus (Nickel *et al.* 1986). The SDFT and DDFT are closely associated along their lengths, with the deep fascia of the forelimb helping to bind them to each other and to adjacent structures (Rooney *et al.* 1978). They pass together through the carpal canal, in which they are surrounded and protected by the carpal synovial flexor tendon sheath. In the metacarpal region they are surrounded by a paratendon. The two flexor tendons are separated by a groove from the underlying suspensory ligament (SL), and are easily palpable. The comma-shaped SDFT is closely applied to the palmar aspect of the DDFT, which is round in cross-section. The cross-sectional area of the SDFT is always at its smallest in this mid-metcarpal region (Webbon 1973). The accessory ligament of the DDFT, which originates from the palmar carpal ligament, joins the tendon in the middle third of the metacarpus (Getty 1975). Just proximal to the fetlock the SDFT widens and forms a ring through which the DDFT passes. They travel together through the sesamoidean canal, protected by the digital synovial sheath, which extends from the distal third of the metacarpus to the middle of the second phalanx (P2). The palmar aspect
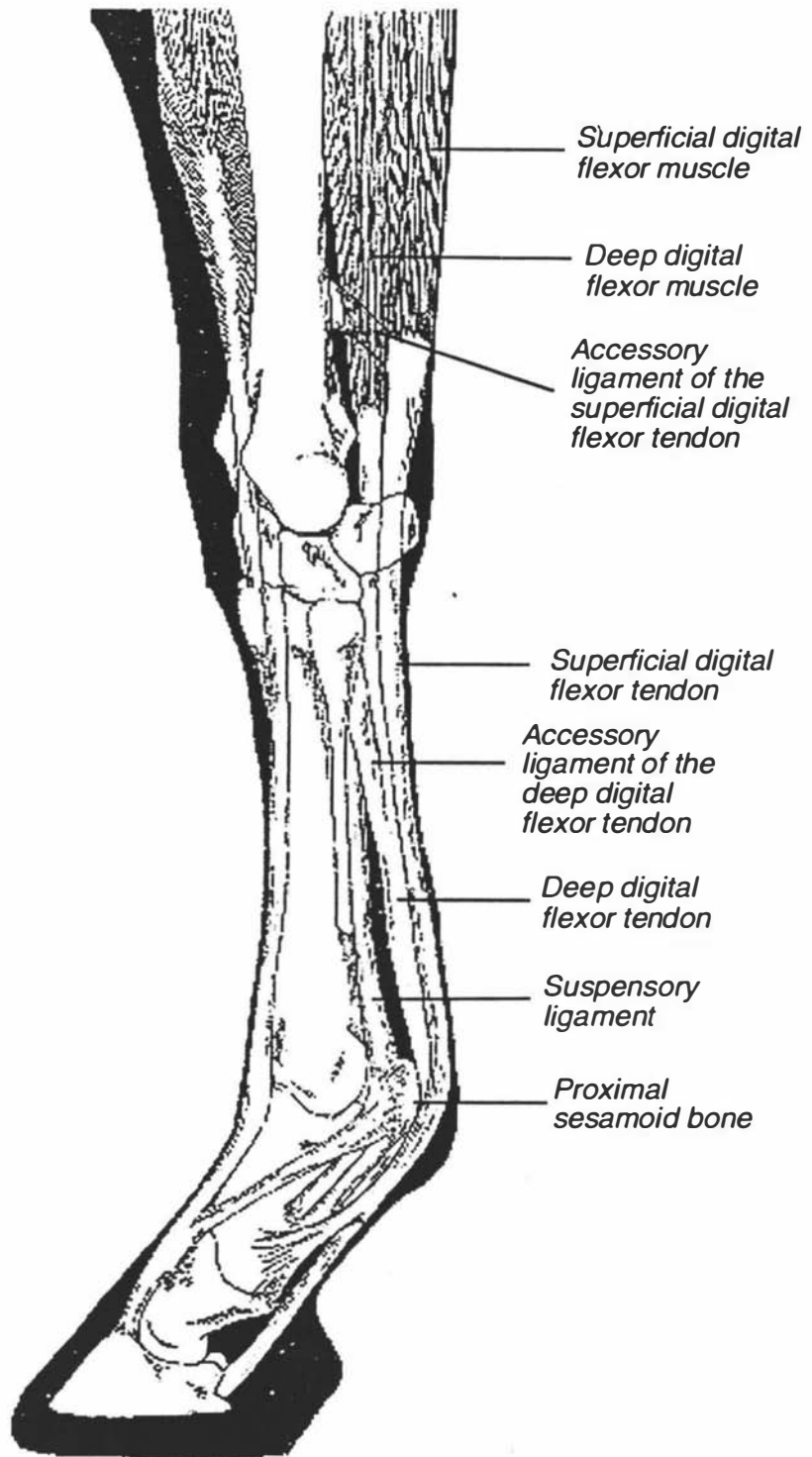
Figure 1.9 Flexor structures of the equine distal forelimb

of this sheath is incomplete, as the ring formed by the SDFT attaches to the overlying fibrous annular ligament of the fetlock (Webbon 1973). At the level of the first phalanx (P1) the SDFT divides into two tendons of insertion which attach to the distal aspect of P1 and proximal aspect of P2 respectively. The DDFT passes between them to its major insertion site on the semilunar crest of the third phalanx.The SL is a modified interosseous muscle which contains small but variable amounts of muscle fibres in the adult horse. It originates from the palmar carpal ligament and the proximo-palmar aspect of the third metacarpal bone, passing distally between the second and fourth metacarpals. The ligament bifurcates above the fetlock with the two branches attaching to the abaxial surfaces of the proximal sesamoid bones. The sesamoid bones act as a pulley between the SL and a triangle of ligaments which insert on the palmar aspects of the phalanges; the straight, oblique, cruciate and short sesamoidean ligaments. A narrow band runs forward from each of the sesamoidean insertions of the SL, to join the common digital extensor tendon on the proximo-dorsal aspect of P1.

Vascular supply and innervation

Proximally, the SDFT receives its blood supply from perimysial vessels, branches of the median artery carried within the median canal, and the mesotendon of the carpal tendon sheath (Stromberg 1971, Kraus-Hansen *et al.* 1992). The latter lies between the SDFT and the DDFT (Ottaway and Worden 1940). Distally it is supplied by vessels carried in the mesotendon of the digital synovial sheath (Webbon 1973), with the periosteum at the insertion site being an unimportant source (Stromberg 1971).

The paratendon which surrounds the middle third of the SDFT is supplied by numerous branches of the median palmar artery, and vessels from it have been observed entering the tendon substance (Stromberg 1971,1973, Webbon 1973). Kraus-Hansen *et al.* (1992) observed a branch of the median or median palmar artery entering the palmar aspect of the tendon via the accessory ligament, in a similar manner to a 'nutrient artery' of bone. Their preliminary studies did not, however, indicate that the paratendon is an important blood

supply to the region, and it was concluded that the region is likely to be relatively deficient. The SDFT is innervated by the ulnar nerve (Getty 1975).

1.5.2 Structural morphology and biochemistry

The cross-sectional shapes and sizes of fascicles in the SDFT are variable (Goodship *et al*. 1994). Tenocytes are arranged in longitudinal rows within the fascicles (Stromberg 1971) and appear to be of three morphological types (Goodship *et al*. 1994). These may be of functional significance, or merely indicate different stages of maturity (Goodship *et al*. 1994). Patches of acellularity have been noted in the core of the mid-metacarpal region of the SDFT in horses of 2 years or older, but the significance of these is unknown (Goodship 1993). No data are available on collagen turnover rate by the tenocytes, due to the large amounts of radioactive label which would be required.

There are only limited data available on both crimp morphology and collagen fibril diameter distributions. Crimp angles in thoroughbred SDFTs vary between 6-24° and crimp lengths between 7-26 μm, depending on the age of the horse and the location in the tendon cross-section (Wilmink *et al*. 1992).

Collagen fibrils have a bimodal diameter-frequency distribution, with peaks at 35-40 nm and 165-215 nm in the adult horse (Parry *et al*. 1978b). Parry et al. (1978b) measured fibril diameters in a limited number of horses, which showed an increase in mass-average diameter between foetal and mature tissue, and a reduction with senescence (Table 1.1).

In the SDFT of horses of three years of age or over, only trace amounts of reducible collagen crosslinks have been detected (Birch 1993). HP is the predominant crosslink, with LP and histidinohydroxylysinonorleucine occurring at very low levels (Birch 1993).

1.5.3 Mechanical properties

The SDFT serves three functions; it assists in supporting weight as a component of a highly specialised collagenous system, it flexes the foot during

the non-weight bearing phase of the gait, and it acts as an elastic energy store to improve the efficiency of locomotion.

Table 1.1 Mass-average diameters (nm) for collagen fibrils in equine superficial digital flexor tendons and suspensory ligaments*

Age (years)	SDFT	SL
-0.2	120.0	146.5
1	176.3	196.7
3	142.5	-
5	187.0	191.0
19	139.0	151.5

* from Parry *et al.* (1980)

Tendon functions

The limbs of the horse form supporting columns, the collapse of which the passive collagenous stay apparatus acts to prevent. Vertical forces are greater in the forelimbs because they bear approximately 60% of the bodyweight of the horse, being closer to the centre of mass. Proximally the SDFT and DDFT assist in supporting the carpus. Distally the SL together with the proximal sesamoid bones and associated sesamoidean ligaments forms the suspensory apparatus of the fetlock, which is reinforced by the flexor tendons and their accessory ligaments. During weight-bearing, vertical forces on the limb act to deform the digit, extending the fetlock and loading the suspensory apparatus. Stress and strain increase in the three flexor structures until they exert a flexing moment which overcomes the extending moment (Jansen *et al.* 1993). The degree of fetlock extension increases with the speed of the gait (Schryver *et al.* 1978), increasing stress and strain in the SDFT, DDFT and SL (Stephens *et al.* 1989, Wilson 1991, Jansen *et al.* 1993). Addition of a rider and saddle has been shown to further increase the SDFT strain (Stephens *et al.* 1989).

The muscles associated with the digital flexor tendons have almost been replaced by the passive apparatus, although they are active just before the limb bears weight, in order to prepare the tendons for loading (Jones 1989). The fibres of these highly pennate muscles are too short to enable them to fully stretch the tendons (Pollock and Shadwick 1994). It has been proposed, however, that the humeral head of the deep flexor muscle may be able to store some energy and exert more precise control over its tendon and accessory ligament, on the basis of its structure and histochemistry (Hermanson and Cobb 1992). Force-time curves obtained from *in vitro* preparations, in which the muscles are not active, show close agreement with *in vivo* measurements (Kingsbury *et al.* 1978). The accessory ligaments form ligament-tendon-bone functional units, thus preventing overstretching of the muscles (Shively 1983). In the resting horse they allow the animal to stand for long periods with minimal muscular exertion (Shively 1983).

Horses have one of the most extreme examples of energy storage by tendons. Internal kinetic energy is stored as energy of deformation in the SDFT, DDFT and SL from the instant at which the hoof begins weight-bearing. From the middle of the support phase until the hoof leaves the ground this elastic energy is released as kinetic energy again, allowing the digit to flex and impart forward impetus. This minimises muscular effort and consequently reduces consumption of metabolic energy. To maximise the energy storage, the tendons must undergo high strains. Therefore, the predicted safety margins for tendons which function as springs are low (Alexander 1988).

In vitro testing

The ultimate strengths and stiffnesses of equine SDFTs have been shown to have a normal distribution with a wide range (Wilson 1991). They do not appear to correlate with age, exercise history or bodyweight. It is possible that certain horses have very high strengths or stiffnesses, but at this time there is no means of identifying such individuals (Goodship *et al.* 1994).

Wilson and Goodship (1991a) stretched 73 forelimb SDFTs to failure and calculated the linear region to extend from 3.6% to 10.6% strain. Those strain

levels corresponded to loads of 2 kN and 8.4 kN respectively. Jansen and Savelberg (1994) loaded 12 forelimb specimens and calculated a failure strain for the SDFT of 10.9% (116 MPa).

In vivo measurements

It has been demonstrated using liquid metal strain gauges or implantable force transducers, that maximal strains and loads differ between the SDFT, DDFT and SL at slower gaits (Lochner *et al.* 1980, Wilson 1991, Platt *et al.* 1991). Platt *et al.* (1991) demonstrated that the combined support of all three structures coincided with the maximal vertical loading on the forelimb at both a walk and a trot, but the SDFT and SL were loaded earlier in the stride than the DDFT. There is only limited information available regarding distribution of loads between the three structures at a gallop.

The SDFT is thought to approach its elastic limit during galloping, however it is not known how closely (McCullagh *et al.* 1979). Stephens *et al.* (1989) measured strains in two galloping thoroughbreds of 11.5% and 16.6% respectively, using Hall-effect strain gauges. This implies that the SDFT has a low safety margin, if the elastic limit is approximately 10%, as suggested by *in vitro* studies (see above). The low safety margin is in keeping with its function as an energy store.

1.6 Equine superficial digital flexor tendonitis

In racing thoroughbreds, the most frequently injured tendon is the forelimb SDFT. The hindlimb SL is most commonly injured in standardbred trotters, and in the forelimbs of the latter, SL desmitis is at least as common as SDFT tendonitis (Nilsson 1970). SDFT lesions are usually located in the midmetacarpal region, involving the tendon core (Webbon 1977).

1.6.1 Clinical tendonitis

The clinical appearance of SDFT tendonitis is variable, but the onset is usually

sudden (Stromberg 1971). Many two year old thoroughbreds show tendon swelling following exercise which has not been thought to result in detectable scarring (McCullagh *et al.* 1979, McIlwraith 1987). At the other end of the scale, there may be complete rupture of both forelimb tendons.

Acutely, the region shows the typical inflammatory signs of heat, swelling and pain. Heat may be palpated or demonstrated thermographically (Stromberg 1971). The typical palmar swelling in the mid-metacarpal region (Figure 1.10) has lead to the colloquial term 'bowed tendon'. Swelling is a more reliable indicator of lesion severity than lameness, as even a minor swelling indicates significant disruption of tendon structure. In chronic stages of injury the tendon may remain thickened due to fibrosis within the tendon and surrounding paratendon. In severe cases the horse may be lame, and the fetlock may drop if there has been significant rupture (McIlwraith 1987). In many milder cases the lameness may be slight, or not observable (Dow *et al.* 1991).

The severity and extent of the lesion often does not correlate well with clinical signs, but is related to the likelihood of returning to work and to the level of post-injury performance (Genovese *et al.* 1986, 1990, Marr *et al.* 1993). Also, as the tendon heals, it may feel normal on palpation when large defects are still present (Genovese *et al.* 1986). Ultrasound evaluation and monitoring of lesions is therefore desirable. An acute lesion will typically appear as a well-defined anechoic or hypoechoic region in the tendon core, surrounded by peripheral tissue of normal echogenicity (Figure 1.11)(Genovese *et al.* 1985). Other diagnostic aids studied, with varying success, have included force plate analysis (Dow *et al.* 1991), infrared and microwave thermography (Stromberg 1971, 1973, Marr 1992) and magnetic resonance imaging (Crass *et al.* 1992).

1.6.2 Pathology

Tendonitis is characterised by fibril slippage and rupture, the extent of which is variable and has not been correlated with clinical signs.
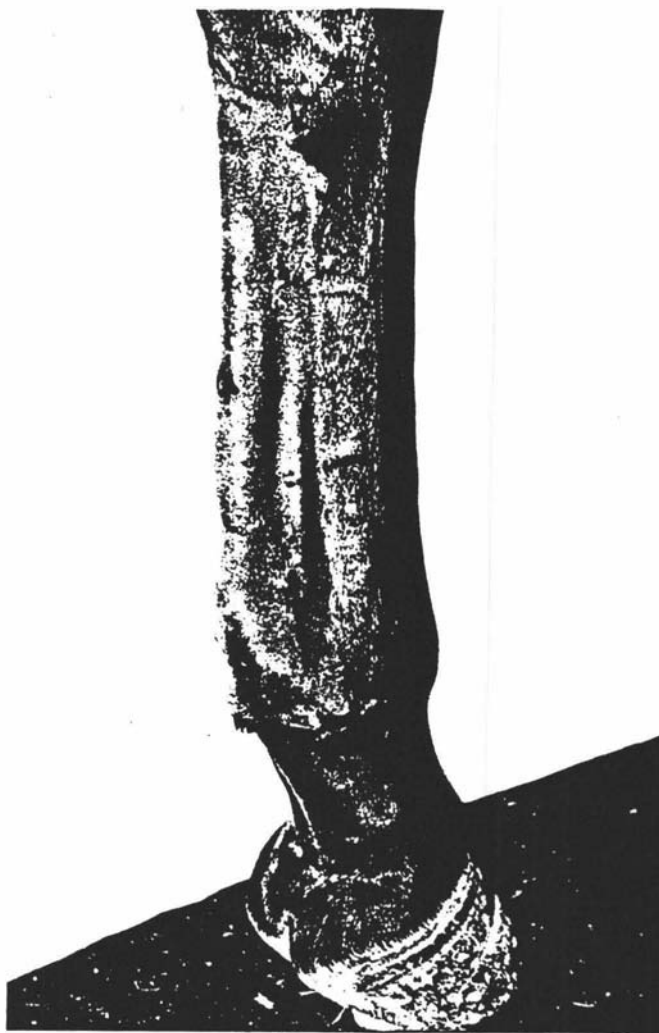
Figure 1.10 An example of superficial digital flexor tendonitis ("bowed tendon").

Core lesion
(hypoechoic)

Superficial digital flexor tendon

Deep digital flexor tendon

Suspensory ligament
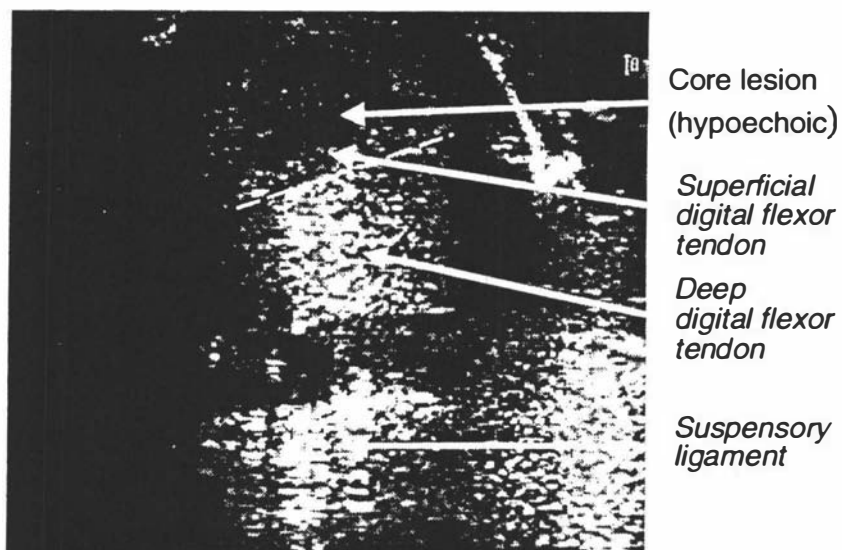
Figure 1.11 An ultrasound image of a typical superficial digital flexor tendon core lesion.

Inflammatory phase

Acute inflammatory processes are similar to those which occur in other tissues. The slippage and rupture of collagen fibril bundles causes capillary haemorrhage, and in more extensive ruptures a haematoma will form. The haemorrhage and inflammatory exudate separates and weakens the remaining intact fibril bundles and may result in a noticeable increase in tendon cross-sectional area (McIwraith 1987). Fibrin and inflammatory cells accumulate, with release of hydrolytic enzymes further contributing to the breakdown of collagen and matrix components. The fibrin filaments will align in the longitudinal axis of the tendon under the influence of slight tensile force (Buck 1953).

Repair phase

As the inflammatory exudate begins to reduce, the formation of granulation tissue begins (Stromberg 1971). Blood vessels appear in the new tissue from as early as 5 days post-injury, and increase in number over the next 10 days (Buck 1953). New cells synthesize the new extracellular matrix. Peritendonous structures are generally thought to provide ingrowing blood vessels and cells (Stromberg 1971, Fackelman 1973), a corollary being the frequent formation of adhesions (Selway 1975). The repair cells resemble 'myofibroblasts', which are intermediate in appearance between smooth muscle cells and fibroblasts (Williams *et al.* 1980). This suggests that repair cells are derived from smooth muscle cells of the blood vessels growing into the lesion (Williams *et al.* 1980, Silver *et al.* 1983). It is possible that endotendon cells are also stimulated to function as collagen-synthesizing tenoblasts, but their relative importance is uncertain (Manske *et al.* 1984). Existing tenocytes in surrounding normal tissue appear to be well-differentiated and incapable of reverting to the more primitive form (Williams *et al.* 1980). The dense surrounding matrix and apparently sparse blood supply may be partly responsible for preventing growth and division of the tenocytes (Buck 1953).

The longitudinally oriented fibrin strands appear to form a scaffold which directs the orientation of the reparative cells in the early stages. *In situ* fibronectin, or

that secreted by the cells, may be responsible for their adherence to this scaffold (McCullagh et al. 1979).

The reparative cells have a well-developed rough endoplasmic reticulum and Golgi apparatus typical of cells involved in active protein synthesis and secretion (Silver et al. 1983). As they replace the inflammatory cells, the synthesis and deposition of type III collagen commences, in the form of small and randomly orientated fibrils (Silver *et al.* 1983, Watkins *et al.* 1985). Type III collagen is not usually synthesized by tenocytes in large amounts. Maximal rates of collagen synthesis and cellularity occur at approximately 3 weeks post-injury. Type III collagen is not detectable in normal tendon by biochemical analysis, but reaches 20-30% of total collagen in the repair tissue (Silver *et al.* 1983). High concentrations of GAGs are also produced, containing a high proportion of hyaluronate (Flint 1972, Reid and Flint 1974). The large concentration of hyaluronate may serve several purposes. It is believed to provide a favourable and protective environment for cellular migration and proliferation (Ivaska 1981). It may also help to disperse fibrils and allow rapid breakdown by relatively low concentrations of collagenolytic enzymes (Flint 1972, Reid and Flint 1974). Finally, it may prevent the collagen from aggregating into large fibrils before mechanical forces are able to align them correctly (Ivaska 1981). The latter author suggests that a 'connective tissue activating peptide' is released by cells at the site of the injury, triggering hyaluronate synthesis by the reparative cells.

Remodelling phase

The first matrix synthesized is a provisional one, to rapidly restore continuity of structure (Ivaska 1981). The slower processes of remodelling and maturation improve the mechanical properties of this matrix, beginning after approximately one month. Remodelling differs from that in other tissues in that in addition to cellular proliferation, collagen synthesis and correct alignment of the new collagen are required. The latter two processes are the most important in replacement of the highly evolved tendon structure.

As the tissue matures, the GAG concentration reduces. The proportion of

hyaluronate decreases and that of the sulphated GAGs increases. A high hyaluronidase activity in wounds has been shown to occur concurrently (Bertolami *et al.* 1978). The proportion of type I collagen increases, as does the concentration of nonreducible (mature) crosslinks and the diameters of the collagen fibrils themselves. The collagen fibrils and tenoblasts align along the longitudinal axis of the tendon, with the latter beginning to assume the normal spindle shape and long cytoplasmic processes typical of the adult tenocytes. The realignment of the collagen fibrils in itself appears to be responsible for some of the increase in stiffness and strength of the tissue which occurs during this phase (Frank *et al.* 1992). A general reduction in vascularity of the lesion occurs at the same time as the collagen reorganisation, with blood vessels also becoming longitudinally orientated within the endotendon septa (Stromberg 1971).

The slow rate of maturation of the scar tissue, and its failure to return to a completely normal structure are of concern. The persistence of unimodal small diameter collagen fibrils with abnormal crimp morphology has been noted 14 months after collagenase-induced injury (Williams *et al.* 1985). This has also been observed in chronic Achilles tendon lesions in humans (Oakes 1996). The large fibrils seen in normal tendon do not appear to be replaced. Levels of type III collagen and reducible crosslinks are also found to be abnormally high, indicating a failure to mature completely (Silver *et al.* 1983, Williams *et al.* 1984). Inadequate mechanical stimulation may be an important factor in the failure to re-establish normal collagen fibril morphology. Increasing numbers of large diameter fibrils have been noted in partially transected tendons of other species, but not in those which were completely transected (Postacchini and De Martino 1980, Matthew and Moore 1991). The scar tissue has similarities to the fibrous plaques seen in the palm of humans suffering from Dupuytren's disease (Williams *et al.* 1984), which have been suggested to persist due to compression from surrounding tension-transmitting tissue (Flint 1981).

The abnormality of structure of the scar tissue and the high frequency of recurrence of SDFT injury imply that it is often weaker and less elastic than normal tendon. Also, the formation of this scar tissue between tendon fascicles will prevent their normal independent movement, further compromising function

(Goodship *et al.* 1994). It is common for subsequent tearing to occur above or below the inelastic scar tissue (Selway 1975, Genovese *et al.* 1986).

1.6.3 Aetiology and pathogenesis

The exact causes of SDFT tendonitis have not been defined, although there is general agreement that overloading and overstretching of the tendon during galloping is a major factor (Stromberg 1971, Selway 1975, McCullagh *et al.* 1979, McIlwraith 1987). This is supported by the high incidence of the condition in horses involved in competitive galloping activity, and the fact that recurrence can be avoided if a racehorse is retired from such activity (Stromberg 1971, Webbon 1973, 1977, McIlwraith 1987).

There has been debate as to whether tendon lesions result from a single episode of non-physiological strain, or whether there is an accumulation of subclinical damage which weakens the tendon structure and predisposes it to rupture. The latter theory originated from the study of Achilles tendonitis in humans (Arner *et al.* 1958), and is supported in the equine SDFT by findings of tendon core discolouration in horses with no record of clinical injury (Webbon 1973). Biochemical analysis of the discoloured regions has shown significant increases in type III collagen and changes in GAGs (Birch 1993). Even in apparently normal SDFTs which are loaded to failure *in vitro*, the fascicles in the centre will often rupture before those in the periphery (Goodship *et al.* 1994). Wilmink *et al.* (1992) interpreted alterations in crimp morphology in the core region of normal SDFTs from older exercised thoroughbreds as evidence of 'microtrauma'. Microtrauma is damage to the ultrastructure or microscopic structure which accumulates over a period of time due to intermittent or continued pathogenic stimuli. The end result may be a discolouration and eventually a clinical lesion in the tendon core.

The mechanical environment

The stresses and strains experienced by the SDFT during galloping have not yet been measured, but are thought to approach the elastic limits of the

structure (McCullagh *et al.* 1979, Wilson and Goodship 1991). This is likely to be due to the low safety margin involved in elastic energy storage (Alexander 1988). The fact that the cross-sectional area of the SDFT is smallest in the mid-metacarpal region has been suggested to result in higher stress levels there (Stephens *et al.* 1989), but has not been definitively proven that this is the case *in vivo*. Non-loadbearing structural components appear to be responsible for differences in the cross-sectional area, meaning that the loadbearing area may be constant along the length of the tendon (Riemersma and Schamhardt 1985).

Other factors have been suggested to further increase the tendon strain, predisposing the structure to injury, although little or no supportive scientific evidence is available. Fatigue of the muscle towards the end of a race in unfit animals has been hypothesized to allow further hyperextension of the fetlock, thus increasing the loads on the tendons and their accessory ligaments (Shively 1983). Dow *et al.* (1992) determined by ground reaction force analysis that some horses lose symmetry of action following exhaustive exercise, and that these horses appeared to be more prone to injury. This loss of symmetry could overload the SDFT in one forelimb. A fatigued horse may also load the limb more suddenly or before the hoof is in the correct position (McCullagh *et al.* 1979). Other individual factors which may increase SDFT strain include poor foot balance and shoeing (Moyer and Anderson 1975, Henninger 1992) and certain conformations such as long and low pasterns (Fackelman 1983). Racetrack surface conditions (Cheney *et al.* 1973, Evans and Barbenel 1975, McCullagh *et al.* 1979), tight fitting bandages or boots and training techniques have also been proposed as predisposing factors.

Ischaemia

It has been suggested that the blood supply to the mid-metacarpal region of the SDFT is deficient, particularly under the metabolic stress of training and racing (Stromberg 1971). In terms of the tensile strength of the tendon, the blood supply is not directly important. Isolated tendon is as strong as that with normal vascular connections (Peacock 1959). A deficient vascularity would

indirectly weaken the structure, by failing to supply metabolic requirements of the tenocytes in the exercising horse. Several studies involving microangiography and/or thermography have suggested lower vascularity in the middle and distal part of the tendon (Stromberg 1971, Kraus-Hansen *et al.* 1992, Marr 1992), although microangiography does not measure functional blood flow (Stromberg 1971, Selway 1975). Fackelman (1973) proposed that repetitive over-stretching of the tendon damages blood vessels in that region. Measured blood flow in the SDFT appears to be similar to that in resting muscle (Goodship et al. 1994), but does not appear to increase during exercise (Stromberg 1971). Training may increase demands on the cells which are not met by the blood supply (Stromberg 1971, Fackelman 1973).

The nutritional requirements of tenocytes in vivo, and the effects of hypoxia upon them are not known. Equine SDFT tenocytes are capable of aerobic metabolism, indicating that hypoxia could interfere with their normal function (Birch *et al.* 1994). Organelles in cells from human achilles tendon lesions have been suggested to show hypoxic changes (Goodship *et al.* 1994). It has also been suggested that production of superoxides during load-induced ischaemia could result in reperfusion injury to tenocytes on relaxation of the tendon (Wilson 1991, Birch 1993).

Tendon core hyperthermia

Wilson and Goodship (1994) developed a mathematical model which predicted that the temperature of the SDFT core would rise to 45-50°C if a horse were galloped for 10 minutes, the cause being heat loss during elastic recoil. A temperature of 45°C was measured in the SDFT core of a horse galloped for 7 minutes, using implanted thermistors (Figure 1.12). Tenocytes cultured from equine SDFT explants proved to be more resistant to such temperatures than equine skin fibroblasts or a commercial line (Birch *et al.* 1992). There was no significant reduction in the viability of the SDFT cells for 45 minutes at 45°C, and it was hypothesized that they might produce 'heat shock proteins' for protection (Birch *et al.* 1992). Effects on the metabolism of the cells were not measured. Miles *et al.* (1994) suggested that a combined effect on
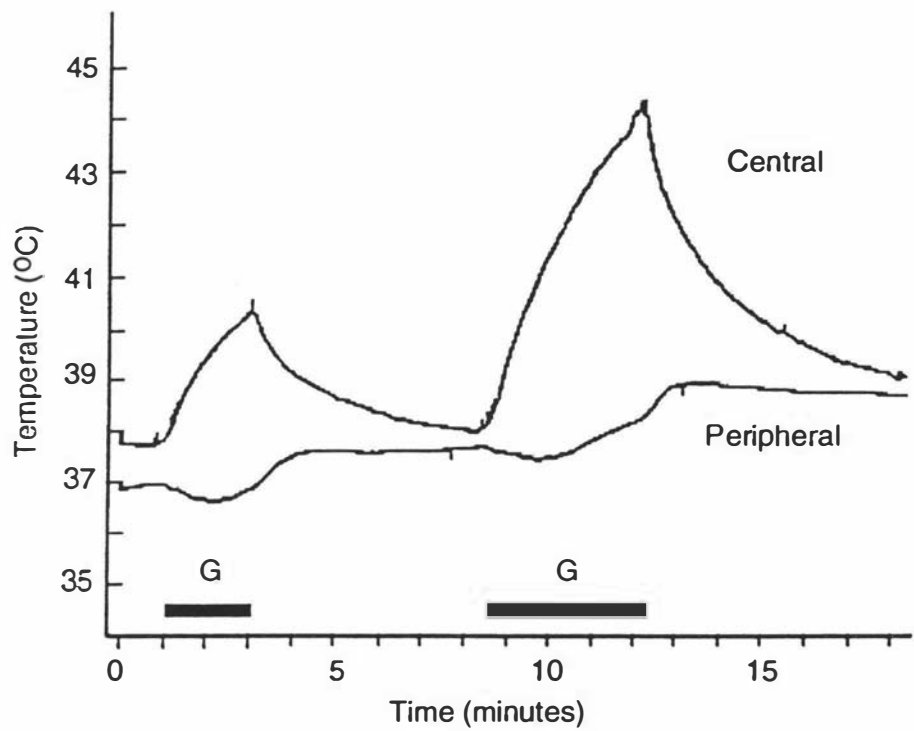
Figure 1.12 **Typical plot from a superficial digital flexor tendon temperature-recording session. Ambient temperature was 2 degrees celsius. G - time spent galloping. (FromWilson and Goodship (1994)).**

hyperthermia and mechanical shearing on tenocytes *in vivo* might increase the release of proteolytic enzymes. This could result in depolymerisation and denaturation of collagen molecules, as seen in subclinical SDFT core lesions using differential scanning calorimetric techniques (Miles *et al.* 1994). A preliminary study showed the activity of cathepsin B, a collagen fibre depolymerisation enzyme, to be increased in the same lesions (Miles *et al.* 1994).

1.6.4 Treatments

The clinical approach to equine SDFT tendonitis at this time relies entirely on treatment of lesions. Successful treatment should re-establish functional microanatomy and minimise the formation of scar tissue, to restore normal strength and movement of the tendon. The poor understanding of the causes and mechanisms of lesion development and repair have meant that current methods do not have a scientific basis and are not directed towards specific ultrastructural events in the tissue. Scientific assessment of methods has not been possible due to difficulties in achieving controlled assessment of similar lesions, long-term follow-up of horses, and objective assessment of post-injury performance. As a result, no treatment procedure has been objectively demonstrated to achieve more favourable results than rest alone.

At this time it can be said that tendonitis in both horses and humans will usually impair or prevent subsequent athletic performance. Recurrence rates of 18-50% have been estimated (Marr *et al.* 1993).

Conservative treatments

In the acute stages the aim is to limit haemorrhage and inflammatory exudation, in order to reduce pain and reduce the area of the eventual scar (McCullagh *et al.* 1979). Measures used include bandage support (Crawford 1988, Wyn-Jones 1988, Henninger 1992), cold hydrotherapy or ice-packs (Farry *et al.* 1980, Henninger 1992), and anti-inflammatory drugs. Non-steroidal anti-inflammatory drugs can suppress tenoblast activity and collagen

synthesis rates (Goodship 1993), and the use of corticosteroids is now discouraged due to detrimental effects on collagen and GAG synthesis (Henninger 1992). Polysulphated GAGs have been injected intralesionally (Smith 1992), as has beta-aminopropionitrile (Genovese 1992). The latter is hypothesized to have angiogenic properties and to delay collagen crosslinking, providing more time for the parallel alignment of fibrils (Genovese 1992). Other methods of treatment studied have included ultrasonic therapy (Morcos and Aswad 1978), electric currents (Norrie 1975) and ionising radiation (Franks 1979). Rest appears to be the most important factor, with severe injuries requiring up to 9 months (McCullagh *et al*. 1979). If training is not discontinued, substantial lesions will usually progress to large ruptures (Genovese *et al*. 1986). More recently, physiotherapy and careful graded exercise during the recovery period has been recommended, to assist alignment and maturation of the repair tissue (Goodship *et al*. 1994).

Firing and other counterirritations

Firing has been in use since at least 1000 B.C., and has caused considerable debate in the racing industry and veterinary profession regarding the ethics of its use. The scar tissue created in the skin and subcutaneous tissue overlying the tendon by superficial firing or application of topical irritants is suggested to act as a mechanical support, but there is no evidence that this occurs (Silver *et al*. 1983). An extensive study by Silver *et al*. (1983) did not indicate that the technique assisted or even altered healing of underlying tendon tissue. It is probable that the enforced rest is more effective than the treatment itself (Asheim and Knudsen 1967, Silver *et al*. 1983).

Introduction of sclerosing agents or firing irons into the tendon substance was believed to induce a 'beneficial hyperaemia' which would encourage the development of the repair tissue (Robinson 1968). This appears to cause further damage by creating bridges of scar tissue between fascicles (Silver *et al*. 1983).

Surgical treatments

Surgical treatment should be considered only in severe cases. Most techniques aim to accelerate one or more phases of the healing process. Many apparent successes, however, may actually result from careful attention to rest following the procedure.

Tendon splitting was first used by Forssell of Stockholm University in the 1940s and later modified (Asheim 1964, Asheim and Knudsen 1967). The aim was to encourage development of new vasculature and connective tissue in chronic lesions by providing channels of ingress for proliferating elements from the paratendon. The splitting may be of some benefit to release fluid from an acute lesion (Goodship 1993), and favourable results have been reported in such cases (Henninger *et al.* 1990, Henninger 1992). It has, however, been demonstrated to cause unacceptable damage to normal tendons (Stromberg *et al.* 1974) and worsening of chronic lesions (Silver *et al.* 1983).

Various grafts or implants have been attempted, with the aim of providing a biocompatible scaffold on which tenoblasts and newly synthesized collagen will align, as demonstrated in vitro (Goodship *et al.* 1980). These have included; the largely unsuccessful autologous lateral digital extensor tendon transplants (Fackelman 1973, Selway 1975, Grant *et al.* 1978) and filamentous carbon fibre implants (Littlewood 1979, McIlwraith 1987). Despite promising histological findings with the latter in tendon lacerations (Valdez *et al.* 1980), clinical results in strain-induced lesions have been disappointing (Goodship *et al.* 1980, Fackelman 1983). More compliant nylon or polyester materials are now preferred, as they have biomechanical characteristics more similar to tendon collagen (Silver *et al.* 1983, Goodship *et al.* 1994). Work is also being directed towards development of composite prostheses (Silver *et al.* 1983, Wyn-Jones 1988).

Desmotomy of the accessory ligament of the SDFT aims to elongate the bone-tendon-bone unit, thereby increasing the elastic limit and preventing recurrence due to overloading of the relatively inelastic scar tissue (Bramlage 1986). Some load is transferred to the muscle and proximal tendon, which do not usually participate in weight-bearing and elastic energy storage. It is uncertain

as to whether the technique influences the healing of lesions already present. An *in vitro* study measured an increase in SDFT strain due to this procedure (Shoemaker et al. 1991). This may not be the case *in vivo*, due to involvement of the superficial digital flexor muscle (Hogan and Bramlage 1995).

Reduction of peritendinous adhesions

Adhesions are a corollary of the extrinsic healing mechanisms, but may interfere with normal tendon movement. Methods described to break down or prevent adhesion formation have included local peritenectomy, drugs, and use of various biologic or synthetic implants (Selway 1975). The effects and benefits of such measures are questionable.

1.7 The current study

The general aim of this study was to determine if galloping exercise affects the morphology of collagen fibrils in the equine SDFT, in terms of both crimp angle and length, and fibril diameter. Current knowledge of the pathogenesis of SDFT tendonitis is very limited, although it is known that animals involved in competitive galloping activity have a significantly higher incidence of the condition than other horses, and that lesions are usually localised in the tendon core. As collagen fibrils are the units of tensile strength in tendon, any significant change in their morphology in response to galloping, particularly in the core of the tendon, would be of importance in terms of understanding the effect of such exercise on SDFT structure and possibly its role in lesion development.

Six hypotheses were tested in relation to the above aim:

1) That in wild horses, which had not undergone imposed exercise, age-related reductions in crimp angle and length, and in collagen fibril mass-average diameter (MAD) were greater in the central region than in the peripheral region of the SDFT (Chapter 3).

2) That imposed but variable exercise regimes involving galloping modify the normal age-related reduction of crimp angle in the SDFT core (Chapter 4).

3) That a controlled 18 month training regime involving galloping exercise would cause crimp angle and length in the central region of the SDFT to reduce below their values for the peripheral region (Chapter 5).

4) That collagen fibrils in both central and peripheral regions of the SDFT would undergo hypertrophy in response to the above 18 month training programme, resulting in an increase in MAD (Chapter 6).

5) That in the same 18 month training programme, the collagen fibril MAD for the SL of exercised horses would be significantly reduced. An increase in MAD was predicted for the DDFT (Chapter 7).

6) That maturity of collagen fibril structure and biochemistry in both the SDFT and the DDFT is reached at around two years of age (Chapter 8).

The following experiments were conducted to test the above hypotheses:

1) Crimp angle and period length were measured in frozen longitudinal sections of central and peripheral regions of the SDFT, from 23 wild horses ranging in age from two to over 10 years. Collagen fibril diameters were measured from micrographs of resin-embedded, ultra-thin transverse sections and the MAD for the resultant distribution calculated. This was done in order to establish normal age-related changes in crimp parameters and fibril MAD, without the influence of imposed galloping exercise (Chapter 3).

2) Crimp angle and period length was measured in frozen longitudinal sections of central and peripheral regions of the SDFT from 18 horses of two years or older which were presented for necropsy to the

Department of Veterinary Pathology and Public Health at Massey University. These horses were separated into 'exercised' (n=9) and 'non-exercised' (n=9) groups, depending on whether their most recent function involved competitive galloping activity (Chapter 4).

3) Six 20-22 month old thoroughbreds underwent a specific 18 month training regime involving galloping, on a high-speed equine treadmill at Bristol University. Six age- and sex-matched horses served as controls, undergoing walking exercise only. Crimp angle and length was measured from central and peripheral regions of longitudinal wax-embedded sections of the SDFT (Chapter 5).

4) Collagen fibril diameters were measured from micrographs of ultrathin-sections from samples of central and peripheral regions of the SDFT, from the same experimental horses as Chapter 5. The MAD was calculated for each of the central and peripheral regions of the SDFT for individual horses (Chapter 6).

5) Collagen fibril diameters were measured, and the mass-average diameter calculated for central and peripheral regions of the SL and DDFT, from the same horses as in Chapters 5 and 6 (Chapter 7).

6) Crimp angle and length and collagen fibril diameter was measured in the central region of the SDFT and the DDFT from 36 thoroughbreds ranging in age from foetuses to three years. Reducible and nonreducible collagen covalent crosslink concentrations were measured for the SDFT and DDFT in 15 of those 36 horses (Chapter 8).

The overall objective of this study was to relate any observed changes in collagen fibril morphology to imposed galloping exercise, and to interpret them with respect to their possible effect on tendon mechanical properties and the high incidence of SDFT core lesions in thoroughbred racehorses.

Chapter 2

Materials and methods

2.1 Horses

Three sources of equine tendons were used; culled wild horses (Chapter 3), thoroughbreds submitted for necropsy (Chapters 4 and 8), and experimental horses (Chapters 5, 6 and 7).

2.1.1 Kaimanawa horses

This population ranges over an area of approximately 24 000 hectares in the plateau region of the central North Island of New Zealand. The first record of wild "horses" in this region dates back to 1876 with most initially being British stock which had escaped or been liberated. In 1941 the army released thoroughbreds into the area during an outbreak of strangles. In terms of genetic markers, these horses are very similar to thoroughbreds (Cothran, personal communication).

Culls were carried out on 11 May 1994 and 9 June 1994 by the New Zealand Department of Conservation, due to unacceptable levels of destruction of rare native plants and straying of horses onto the highway. Younger horses were prepared for sale, with older mares and stallions being immediately sent to the Taumarunui export freezing works.

These horses were sampled because animals with a wide age range and no history of training were required (Chapter 3). Tendons from a total of 23 animals were analyzed, the samples being taken randomly. They ranged in age from 2 to 15 years, as determined from dentition by two veterinary surgeons in consultation. Details of these horses are given in Appendix B (page 189).

2.1.2 Thoroughbreds submitted for necropsy

Eighteen horses of 2 years of age or over which were submitted to the Department of Veterinary Pathology and Public Health at Massey University for necropsy were used as the source of tendons in Chapter 4. Ages were determined from the brands and from owner information. In terms of exercise

history, only the recent function of the horse was taken into account because of the unreliable nature of owner information in this respect. None of the horses sampled had a known history of SDFT tendonitis or tendon core discolouration. Details of the sampled horses are given in Chapter 4, (pp 100-101).

Thirty-six horses from foetuses to 3 years of age were sampled and used in the research described in Chapter 8. These animals were submitted to both Massey University and the Animal Health Laboratory at Ruakura between August 1994 and August 1995. Six of the foals (4-5 months old) were experimental animals from a study on dietary copper levels, these being from a group raised on a copper-sufficient diet (30 ppm). Age was determined from owner information and/or brands, and recent function of the horse noted for horses two years old or more. In total 36 animals were sampled, some also being used in the work described in Chapter 4. Details are given in Appendix B (page 189). None of the foals or horses sampled had a history of clinical tendonitis, tendon core discolouration or congenital tendon deformity.

2.1.3 Experimental horses

Tissue for Chapters 5, 6 and 7 was obtained from twelve experimental thoroughbred horses at Bristol University, United Kingdom. Six horses had undergone an 18 month specific programme of galloping exercise on a high-speed equine treadmill, and six had served as controls which underwent walking exercise only. One horse was withdrawn from the exercised group part-way through the programme due to poor adaptation to treadmill exercise. Details of the exercise regime are given in the relevant chapters. All of the horses were 19±1 months at the beginning of the study.

2.2 Collection of samples

Tendon samples were excised using one-edged razor blades (Gem Scientific, American Safety Razor Co., Virginia, USA). If immediate collection was not possible from some of the horses submitted for necropsy, samples were taken

within 48 hours.

2.2.1 Kaimanawa horses

A section one cm in length was excised from the mid-metacarpal point of the right forelimb SDFT, for analysis of collagen fibril diameter distributions (Figure 2.1). The section was bisected sagitally, and placed in phosphate-buffered glutaraldehyde (see Appendix C, page 191) in a labelled plastic vial. Another section 2-3 cm in length was taken immediately proximal to that, for analysis of collagen crimp patterns. The latter was double-wrapped in cling-film (Hefty Wrap, Cartigny Pty Ltd, Australia), labelled, then wrapped in aluminium foil (Detpak NZ Ltd, Auckland) and stored at -70°C.

2.2.2 Thoroughbreds submitted for necropsy

A 2-3 cm section was excised from the left forelimb SDFT for crimp analysis, from the same longitudinal point as above (Chapter 4).

For work in Chapter 8, four sections were removed from each of the left forelimb SDFT and DDFT, at corresponding levels (Figure 2.2). Samples for collagen fibril diameter analysis were placed in phosphate-buffered glutaraldehyde. Those for collagen crimp analysis and biochemistry were wrapped in cling-film and aluminium foil and stored at -70°C.

2.2.3 Experimental horses

A 1 cm section for collagen fibril diameter analysis was taken from a point just distal to the midpoint of the left forelimb SDFT. Corresponding sections were taken from the underlying DDFT and SL (Chapter 7, page 139). The three sections were placed in separate labelled vials containing Karnovsky's fixative in sodium cacodylate buffer (see Appendix C, page 193), and stored overnight at 4°C. A 2 cm section was taken from the SDFT from a point 2.5 cm proximally, for collagen crimp pattern analysis (see Chapter 4, page 97). The latter was placed in 10% formol buffered saline (see Appendix C, page 195).
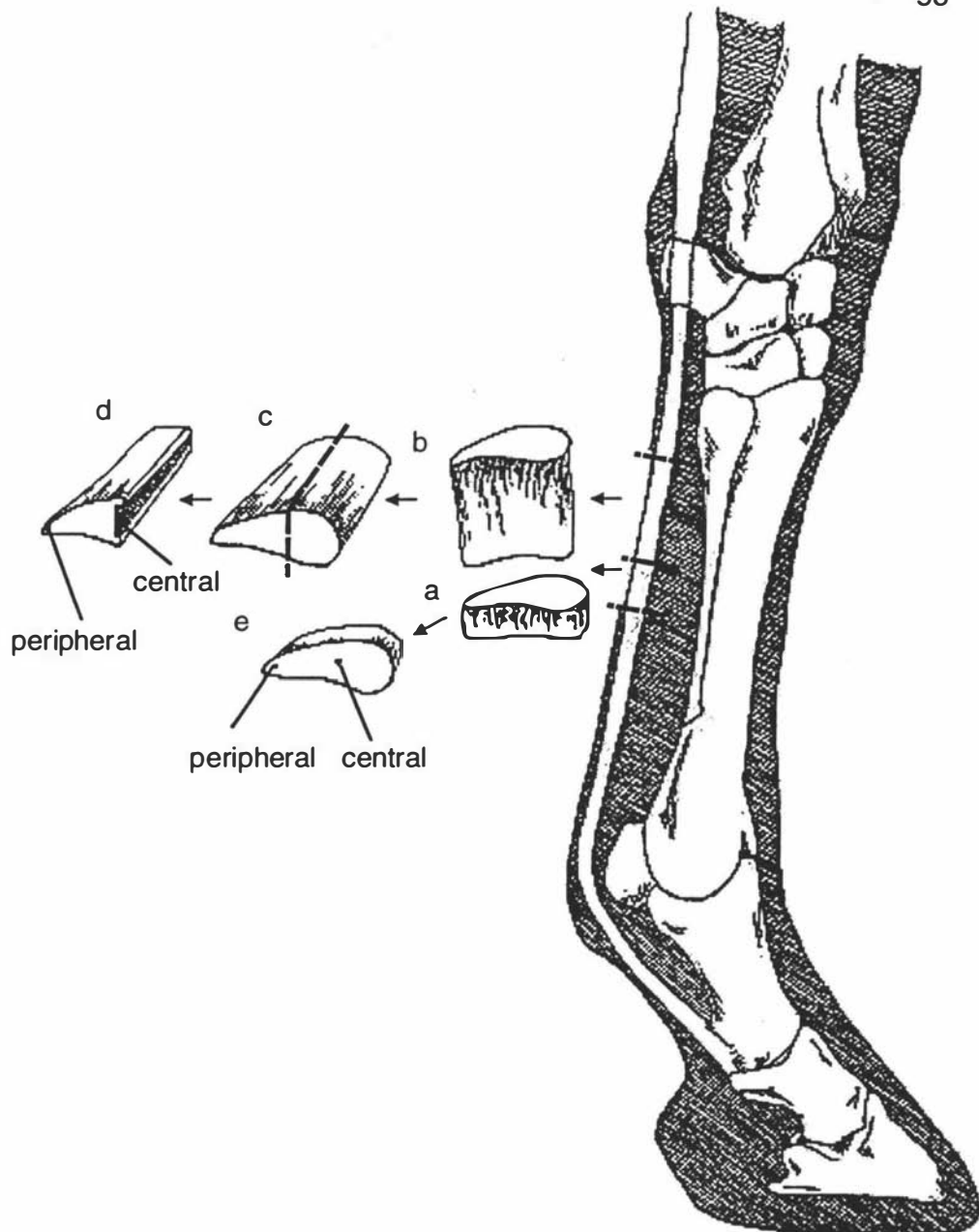
Figure 2.1 Details of the sampling procedure from the superficial digital flexor tendon of the Kaimanawa horses. a - a 1 cm segment was excised from the midpoint of the metacarpal region of the tendon for fibril diameter measurements. b - a segment 2-3 cm in length was taken immediately proximal to that for crimp pattern measurements. c - the latter segment was bisected sagitally. d - shows regions sampled for crimp measurements. e - shows regions sampled for fibril diameter measurements.
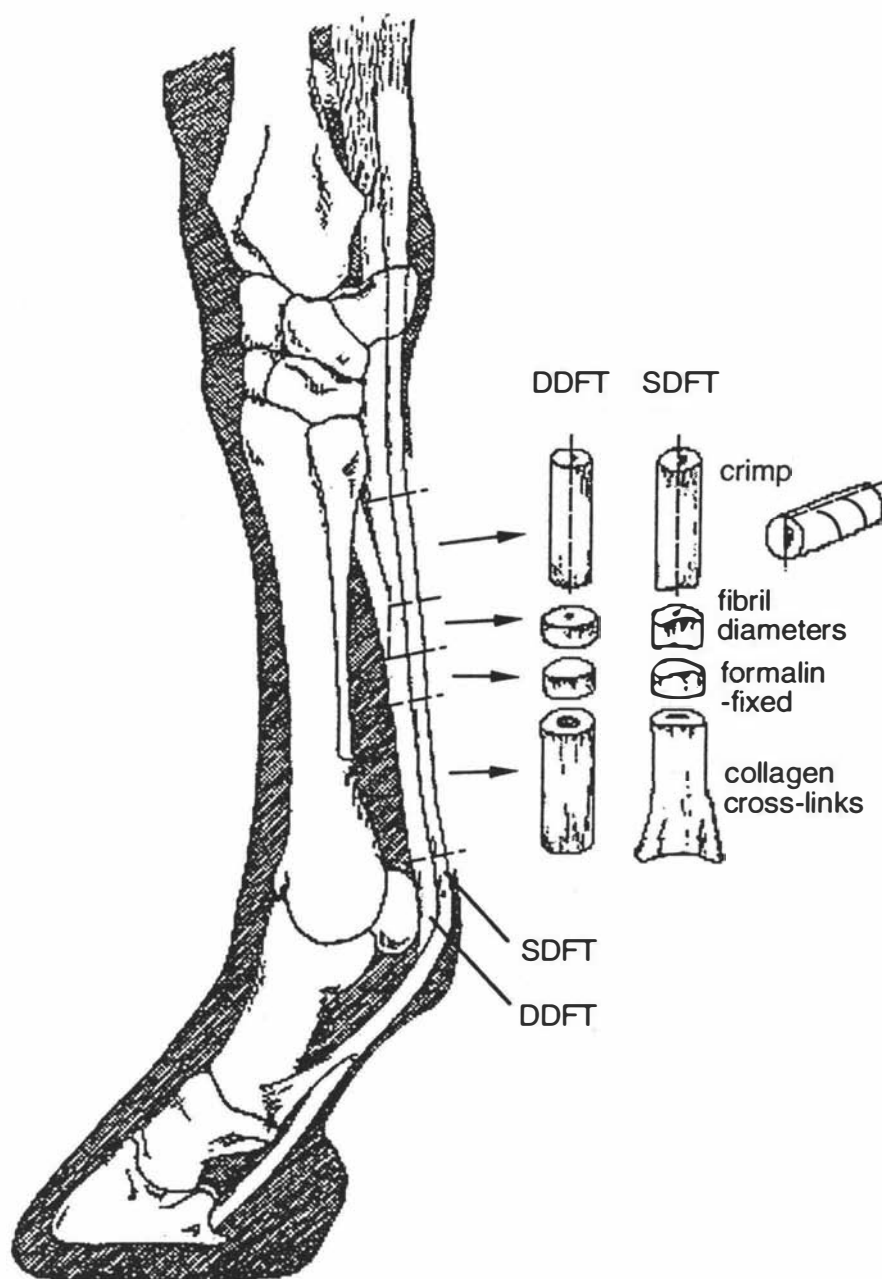
Figure 2.2 Details of the sampling procedure for thorough-breds submitted for necropsy which were 3 years of age or younger. Formalin-fixed sections were not used in this study. Shaded regions in tendon segments represent sampled areas.

2.3 Processing of samples for electron microscopy

2.3.1 Kaimanawa horses (Chapter 3)

The morning following sampling, $1mm^2$ x 2 mm longitudinal slivers were dissected from central and peripheral regions of the sample cross-section using a number 24 scalpel blade (Paragon, Sheffield, England) (Figure 2.1). The overnight fixation of larger blocks made this easier to do without crushing the tissue with the blade. The slivers were placed in fresh phosphate-buffered glutaraldehyde overnight again, in 4 ml screw-cap glass vials (Alltech, Deerfield, Illinois, USA). The optimal time for fixation of tendon tissue is unknown, although glutaraldehyde probably penetrates and fixes the structure more slowly than that of less dense tissues. Labels on the vials were written in pencil, as subsequent procedures often remove ink.

The embedding schedule is detailed in Appendix C (page 192). The sample vials were placed in a rotating stage in a fumehood. This facilitates penetration of dehydrants and infiltration of embedding materials (Weakley 1981), and prevents significant polymerisation of the resin at room temperature. They were only removed from the stage for osmium fixation at 4°C. Samples were too small to move between vials, so solutions were withdrawn and immediately replaced using glass pipettes, taking care not to damage the specimens with the tip. Approximately one ml was added to each vial at each step.

Details for preparation of resin are given in Appendix C (page 191). Following six hours immersion in the pure resin (Procure 812, Probing and Structure, Thuringowa, Central Queensland, Australia) at room temperature, tendon strips were removed from the vials using toothpicks and placed on blotting paper to remove any excess. They were flat embedded in fresh resin in labelled silicon rubber moulds, with one sliver placed at the end of each block in longitudinal orientation. The blocks were cured in an oven pre-heated to 60°C, for 72 hours. Blocks were generally not sectioned until 1-2 days after removal from the oven, as this is suggested to improve the ease of cutting (Hayat 1970).

2.3.2 Thoroughbreds submitted for necropsy (Chapter 8).

Sections from samples processed using the above method were difficult to cut, and tended to fragment. This appeared to be due to insufficient time for infiltration of the resin. Collagenous tissue such as tendon is extremely dense and therefore the embedding process must be extended in comparison with other tissues, in order to allow full penetration of dehydrants, transitional solvents and resin into the structure. Extra steps were added to the ethanol series and a more gradual transition was made from propylene oxide to pure Taab 812 resin (Appendix C, page 190). Several changes of fresh resin were used, the first being at 5 pm the day the propylene oxide had been evaporated by removing the lids of the vials. It was changed again at 8 am and at 5 pm the next day. On the third day the resin was changed at 8 am, and that afternoon the tendon samples were removed from the resin, blocked out and heat-polymerised as detailed above. Because this was a four day process, slivers were usually left in fixative until the beginning of the week. It is known that samples can be left for days in glutaraldehyde without adverse effects on fine structure (Hayat 1970).

2.3.3 Experimental horses

These samples were processed in the Department of Veterinary Anatomy at Bristol University, England. Sodium cacodylate buffer was used in preference to phosphate buffer (Appendix C), osmium fixation was extended, and the ethanol series and transition to propylene oxide were more gradual in comparison to the above method. During infiltration and embedding, the samples were covered with silica gel and placed in a refrigerator overnight. If they are left in a rotating mixer, slight curing of the resin can occur before the next change. The samples were brought up to room temperature before opening in the morning.

2.4 Cutting and staining of ultrathin sections for electron microscopy

Each block was mounted in a chuck in an ultramicrotome (Reichert-Jung "Ultracut E", Austria), and the end trimmed into a pyramid shape using a double-edged razor blade (Figure 2.3). The block face was then smoothed by cutting thick sections (1μm) using a glass knife. The latter were prepared on the day of use using a knife-making machine (LKB Bromma 7800 Knifemaker, Sweden). The resulting thick sections may be collected in a water trough made on the knife using adhesive strapping tape. They may be transferred to a glass slide using a glass rod, stained with methylene blue and examined under the light microscope to ensure correct transverse orientation. This was usually not necessary if the sliver was oriented correctly within the block. It is commonly done with other tissues in order to select certain areas for ultra-thin cutting. The smoothed block face was trimmed to a smaller sized pyramid shape for cutting of ultrathin sections (Figure 2.3). The smaller the section size, the lower the compression forces and the better the quality of the resulting sections (Hayat 1970). This is especially true for tendon, for which it is preferable to have as small a block face as possible. Silver-gold sections (90-130 nm) were cut using a 3 mm diamond knife (DDK, Delaware Knifes Inc., Wilmington, Delaware, USA). For adult equine tendon, a cutting speed of 3-5 mm/second was usually required. This is a fast cutting speed; 1-2 mm/second is usually recommended (Hayat 1970).

The sections were floated out onto clean, freshly distilled water in the trough of the knife (Figure 2.4). References generally refer to sections forming a long unbroken ribbon from the knife edge as indicating correct cutting procedure (Hayat 1970). This is often not possible when cutting tendon, and it is sufficient to pick up a collection of individual sections. An eyelash mounted on the end of a toothpick may be used to shift the sections closer to one another by gently paddling the water, and to a position which allows them to be picked up together on the centre of a grid. Often sections are crumpled due to compression caused by cutting forces. They may be flattened by dipping a grid into chloroform and holding it just above the sections so that the vapour falls onto them. The sections were picked up from the surface of the water
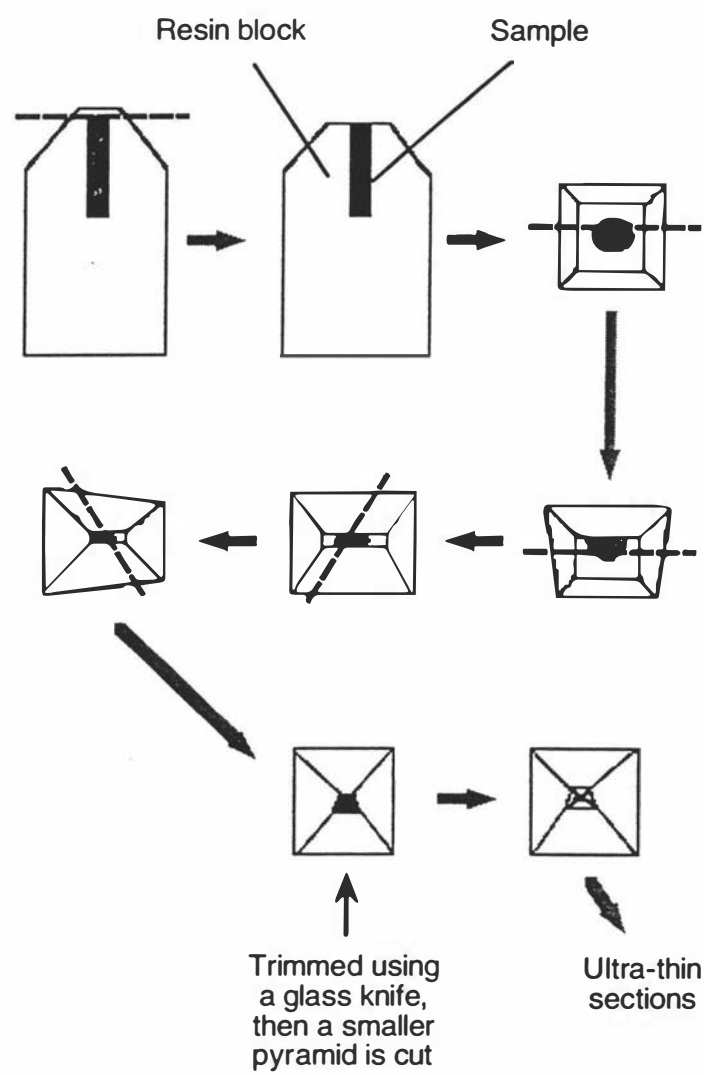
Figure 2.3 Trimming resin blocks for cutting of ultrathin sections.

using 400 mesh regular copper grids (SPI, West Chester, Pennsylvania, USA) (Chapters 6,7) or 200 mesh formvar-supported copper grids (Agar Scientific Ltd., Stansted, England) (Chapters 3, 8). Grids were handled using Dumont curved-pointed fine forceps (size 7, A. Dumont & Fils, Switzerland) and stored in a labelled plastic grid box (Slide-A-Grid, SPI Supplies, West Chester, PA).

2.4.1 Staining

Staining involves the deposition of heavy metal atoms onto the tissue, which can scatter or stop electrons, increasing the contrast of the section. Sections were stained a day or more after mounting on the grids, in saturated uranyl acetate in 50% ethanol, following by a modified lead citrate stain (Sato, 1967). Details for preparing stains are given in Appendix C (pp 194-195). Uranyl acetate increases the contrast of tissue proteins such as collagen when used prior to staining with lead (Griffin 1990).

A pilot project was conducted to determine the optimal staining time. Sections cut from the same block were immersed for 5, 10, 15, 20, 25 and 30 minutes in uranyl acetate and then for the same time in lead. Staining for 15 minutes in each produced optimal contrast between fibrils and extrafibrillar matrix, without formation of a black granular precipitate as seen under the electron microscope, which indicates overstaining (Griffin 1990).

Drops of uranyl acetate stain were placed on laboratory film (Parafilm "M", American National Can™, Greenwich, CT, USA) in a clean covered petri dish, and grids submerged for 15 minutes. Each grid was placed in a separate drop of staining solution. They were then removed, quickly dipped in a wash of 50% ethanol followed by a wash of double-distilled water and then submerged in drops of lead stain for 15 minutes. Following lead staining the grids were dipped in three fresh washes of double-distilled water. Great care must be taken when using lead citrate, as carbon dioxide contamination results in the formation of lead carbonate, inactivating the stain. The stain should be crystal clear, but there is commonly a very thin film of sediment on the surface of the solution which can be avoided with the tip of the pipette when removing stain from the bottle. Grids should not be stained in the first or last drops from the

pipette as these may also have been contaminated.

2.5 Electron microscopy

The sections were viewed using a Philips E.M. 201c high resolution electron microscope. One representative section was examined for each tendon or tendon region. The fibril size analysis programme required uniform and dark staining of fibrils, a certain level of contrast between fibrils and extrafibrillar matrix, and reasonable separation of fibrils from one another. Areas containing elastic fibres, tenocytes and certain stain artifacts were avoided as the programme could not distinguish these from collagen fibrils. Areas of each section of which micrographs were taken were selected randomly within these constraints (Figure 2.4). Four to fifteen micrographs were taken per section, which was sufficient to allow analysis of at least 1000 collagen fibril diameters. Most were examined and photographed at a magnification of 48600 times. Deep digital flexor tendons contained relatively large fibrils, so they were examined at a magnification of 31800 times to avoid taking very large numbers of micrographs. All micrographs were recorded on 35 mm fine grain positive film (Agfa Copex PET-10 positive) and developed in a high contrast developer (Kodak D19B).

A carbon grating replica of 2160 lines/mm was used to calibrate each set of micrographs (Probing and Structure, Thuringowa Central, Queensland, Australia). A pre-pumped airlock allowed the magnification to remain unchanged while the calibration grid was inserted and photographed. This minimised inaccuracy due to fluctuations in the high tension voltage or currents in the electromagnetic lenses (Craig 1984). All magnifications were within 4% of 48600 times.

2.6 Computerised image analysis of electron micrographs.

The micrographs were analyzed using a VIPS-based computer programme (Visual Image Processing System). Micrograph images were captured using a Sony colour video camera (model DXC-3000P, Sony, Japan) and an imaging

card (Imaging Technology PCVISION*plus*, Imaging Technology Inc., USA) connected to a 486 PC computer (Advantage, Palmerston North, New Zealand). They were calibrated using a captured image of a printed black circle measuring exactly 50 mm in diameter. The programme smoothed 'noise' in the micrographs to reduce differences in contrast within stained fibrils. It then calculated an average fibril intensity level, and an average background intensity level. This normalised the contrast within the image so that the fibrils were uniformly dark against a uniformly light background and were detected more reliably. Detected fibril edges were outlined, and any 'holes' in the image considered to be part of the background. Each hole was linked to the nearest background pixel. Where there was significant concavity on the boundary of an object and on the opposite side of the object, this indicated two or more apparently touching fibrils. The holes on each side were joined, separating the fibrils, which were usually not actually touching in the micrograph image, but were situated too closely for their neighbouring edges to be detected by the programme. A guard frame was then calculated to prevent biasing the results towards smaller fibrils, using the largest "height" of a fibril and the largest width of a fibril. Fibrils which were at least partly in the box but not touching the lower or right edges of it were counted and their diameters and areas (corrected for ellipticity) measured. Structures less than 20 nm in diameter were not measured, nor were those with ellipticities greater than 2, to avoid inclusion of non-collagenous components. The total area of each image was also recorded and from this the percentage of each image area occupied by collagen fibrils calculated, the collagen fibril index (CFI) (Enwemeka *et al.* 1992). A more detailed outline of the programme may be found in Appendix D (page 196).

An assumption was made that fibril diameter distributions arose from sampling of a population of fibrils of various diameters, rather than a homogeneous population of tapered structures. This is supported by the fact that fibril ends are rarely seen in longitudinal sections of tendon (Parry and Craig 1984). It was also assumed that 1000 fibril diameter measurements would be representative of the central or peripheral tendon region. Assuming there was no significant local variation within these regions, that number of
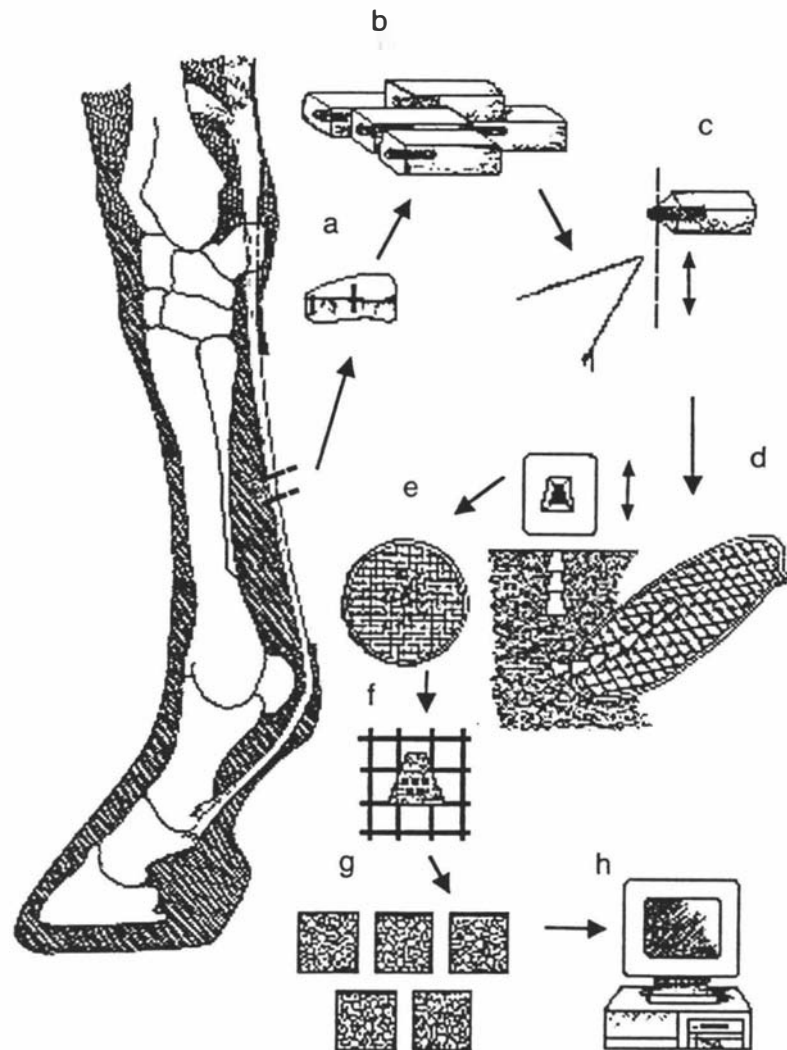
Figure 2.4 Cutting and image analysis of ultra-thin sections. a - a 1 cm segment was excised. b - slivers from central and/or peripheral regions were processed and embedded in resin blocks. c - ultra-thin sections were cut on a diamond knife. d - sections were collected in the water trough and picked up on a copper grid. e - sections on a grid. f - areas of one section were selected randomly. g - micrographs were taken of those areas. h - computerised image analysis of micrographs.

measurements should be sufficient, according to the calculations of Parry and Craig (1977) (Table 2.1). A diameter-frequency distribution was calculated from the data and entered into a computer programme developed by D. A. D. Parry. This programme uses the calibration factor to convert the diameter measurements to nanometres and then calculates the percentage of total measured fibril area occupied by each diameter group. The mass-average diameter (MAD) of a collagen fibril population is derived from the diameter

Table 2.1 Required number of collagen fibril measurements.

Mass average to mean diameter ratio	Minimum number of measurements
1.0	100
1.05 - 1.2	300
> 1.3	1000

versus percentage of fibril area distribution using the formula:

$$\sum n_i \times d_i^3 / \sum n_i \times d_i^2$$

where d is a certain diameter, and n is the number of fibrils of that diameter. Significant lateral shrinkage of collagen fibrils and the volume of matrix between them occurs during processing for electron microscopy. This means that measured diameters will be significantly smaller than those in native fibrils, as indicated by comparative measurements made using low-temperature procedures for electron microscopy (Craig et al. 1986) or X-ray diffraction methods (Eikenberry *et al.* 1982). This is thought to be mainly due to the loss of water during processing. Some extraction of protein may also occur, for example due to osmium tetroxide fixation (Itoh *et al.* 1982). Additional small amounts of axial and lateral shrinkage will take place once the sections are mounted on grids. The fact that diameters are not absolute does not prevent comparison of samples prepared using the same method. Direct comparison of numerical values from samples prepared using different methods is questionable. Statistical analyses of the data are outlined in the relevant chapters.

2.7 Preparation of frozen sections for crimp pattern analysis.

Previous studies of crimp patterns in equine SDFTs have involved conventional histological sections (Williams *et al.* 1985) and isolated fibre bundles (Wilmink *et al.* 1992). For this study conventional wax-embedded sections were not used, as wax in comparison with simple freezing does not provide a dense connective tissue such as tendon with equivalent support. It is easier to cut frozen sections, and the sections obtained are flatter (Disbrey and Rack 1970), and possibly less distorted than tissue subjected to procedures for isolating the highly adherent tendon fascicles.

2.7.1 Sectioning

Each sample was removed from the -70°C freezer and cut into pieces in a cryostat unit from which the cutting apparatus had been removed (-25°C). A pre-cooled single-edged razor blade was used. The section was bisected sagittally, with the medial half being re-wrapped in cling-film and returned to the freezer for possible future study. The lateral half was cut into three blocks, which were divided again into lateral and medial halves. Medial or 'central' blocks were trimmed to remove several mm of tissue from the periphery (Figure 2.5). Lateral or 'peripheral' blocks were used in Chapter 3 only. The central and peripheral samples were re-wrapped in aluminium foil and transferred to a cryotome set to a temperature of -30°C.

Each block was attached to a slice of cork, and the cork to a chuck in the cutting apparatus, using O.C.T* embedding compound (Tissue-Tek, Miles Inc. Diagnostics Division, Elkhart, IN, USA). 30 μm thick sections were cut, with a perspex anti-roll plate preventing them from curling up on the knife. 30-40 sections were thaw-mounted on labelled glass slides for each tendon or tendon region.

* 10.24% w/w polyvinyl alcohol, 4.26% w/w polyethylene glycol, 85.50% nonreactive ingredients.
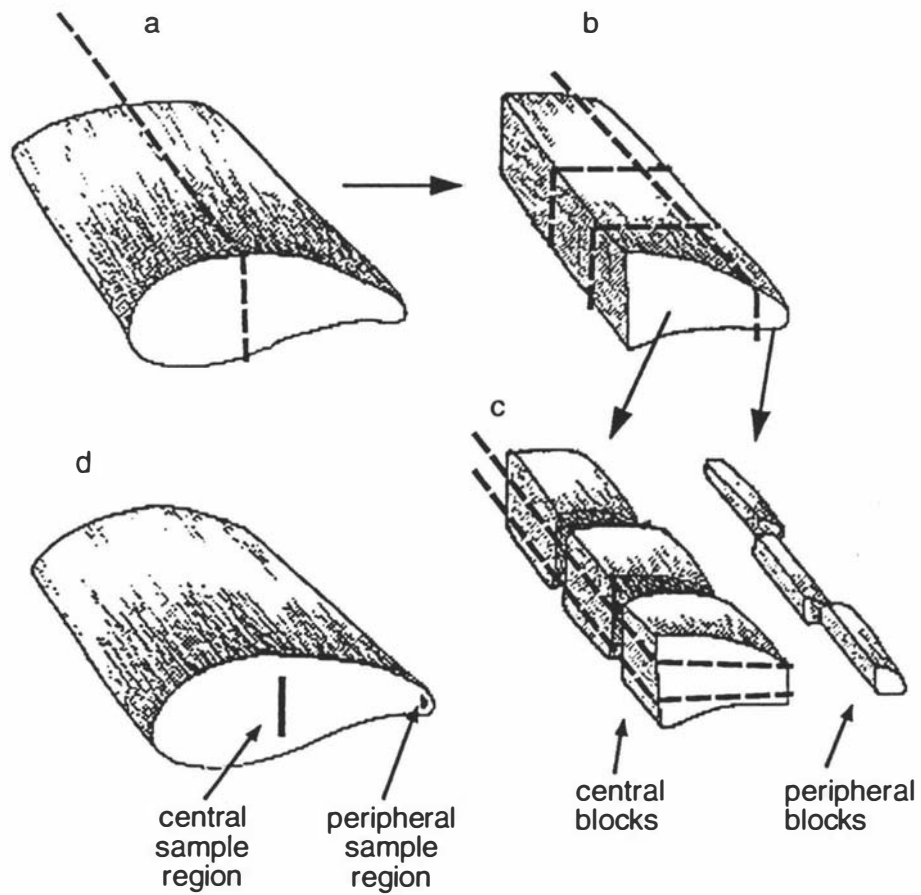
Figure 2.5 Preparation of blocks for sectioning for crimp pattern analysis. a - the segment was bisected sagittally. b - the lateral half of the tendon segment was divided longitudinally, and the resultant two halves then divided transversely into three blocks. c - peripheral regions of the central blocks were removed. d - shows the regions sampled from core and periphery of the original tendon segment.

2.7.2 Processing

The mounted sections were air-dried at room temperature for 30 minutes to one hour. The slides were then dipped for one minute each in; 70% ethanol, two changes of absolute ethanol, and two changes of xylene. DPX mountant was then applied and the slides coverslipped. Sections were examined several days later when the mountant had hardened completely. This processing was carried out for two reasons: to enable the sections to be stored at room temperature and to improve the clarity of the image under polarized light. Unfixed sections must be stored at sub-zero temperatures, resulting in repeated freezing and rethawing if they cannot be examined immediately. Sections which had not been processed were highly light reflective, making the image confusing and analysis of crimp parameters difficult. A far clearer image was therefore obtained from the processed, translucent sections. Alcohol dehydration is known to change crimp parameters only by a small consistent amount (Nicholls *et al*. 1983, 1984) with little observable change in the actual shape of the waveform. Regardless of that fact sections from central and peripheral regions, and tendons from different horses, were treated in the same way and comparisons could therefore be considered valid.

2.8 Polarized light microscopy

A Nikon Labophot2-Pol polarising light microscope (Nikon, Japan) was used to visualise the sections. Crimp angle was measured by placing the slides on the vernier stage and rotating this stage around the axis of the microscope. An intact area of a fibre bundle showing regular in-phase crimp was chosen randomly and the stage rotated until optimal contrast between transmitting (light) and extinguishing (dark) regions was obtained, with no splitting of bands. Dark regions denote fibres running parallel to the plane of one of the polarising filters. Rotation of the stage through twice the crimp angle from this point changed the banding pattern to a complementary one as the tendon waveform is symmetric (Figure 2.6). Bands which previously appeared dark now
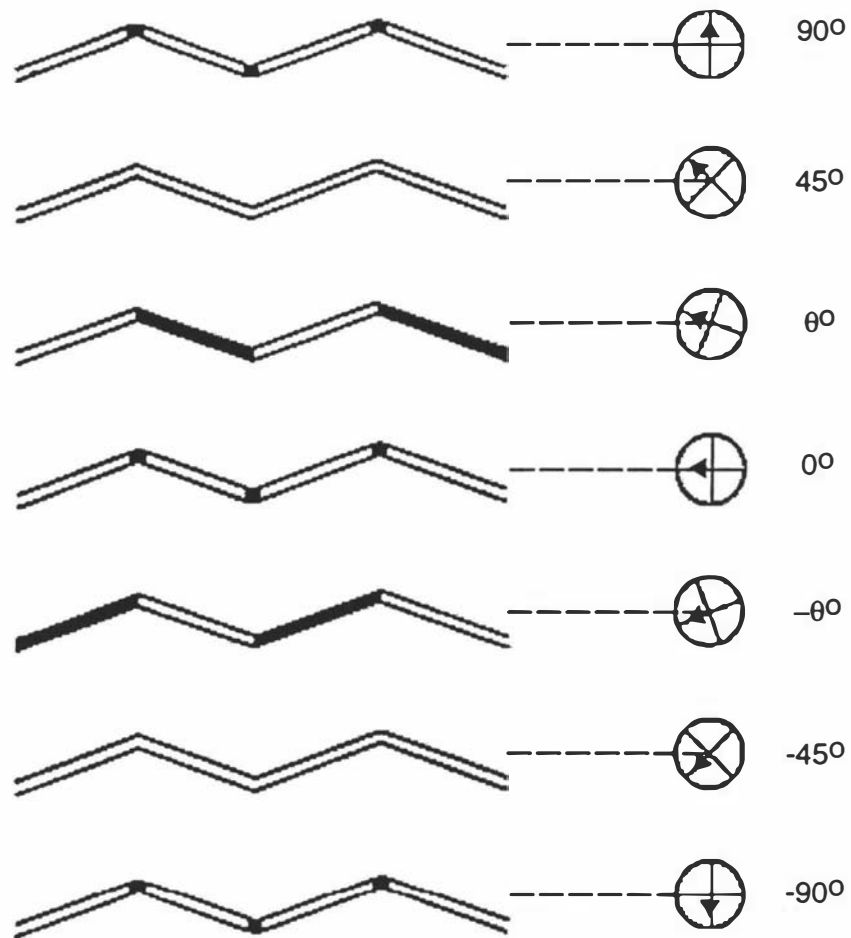
Figure 2.6 Changes in the banding pattern of a tendon fascicle with rotation around an axis at right angles to the surface plane. θ = crimp angle. (Adapted from Shah *et al*. (1979)).

appeared to be light and vice versa (Diamant *et al.* 1972, Dale *et al.* 1972). This measurement was carried out at 50 different sites at a magnification of 100x. The sites were located in 30 to 40 different sections, taken from all of the blocks to avoid measuring from only one small area of the tendon.

Crimp length was measured using an eyepiece graticule in the microscope which was calibrated using a 1mm stage micrometer. Five consecutive crimp periods were measured at the same 50 sites, at 400x magnification. Details of statistical analyses may be found in the relevant chapters.

2.9 Biochemistry

Sections of tendon for biochemical analysis were placed in two resealable plastic bags. These were packed in dry ice in a chilly bin (2 kg of ice per kg of tissue) and transported by air to Bristol University, England. They were then transferred to a -70°C freezer. All samples were still frozen on arrival.

2.9.1 Lyophilisation

Each tendon sample was partially thawed, and peripheral layers removed using a scalpel blade, to ensure that paratendonous tissue was not included in the analysis. The tissue was then sliced into 0.5 cm x 0.5 cm cubes. Approximately 500 mg was weighed out on a digital balance for each sample and transferred to a labelled screw-top plastic vial. All samples were then re-frozen to -70°C and freeze-dried under vacuum (HETOSICC, Heto Laboratory Equipment, Denmark).

2.9.2 Collagen cross-links

Superficial and deep digital flexor tendons from 15 of the horses were selected for analysis of these compounds. The method of Sims and Bailey (1992) was used to prepare and measure crosslinks, which allows detection of reducible and non-reducible crosslink amino acids on the same system. Lyophilised tissue samples were cut into small pieces using scissors, and approximately

15 mg weighed out on a digital balance. 1 ml of 0.025 M phosphate-buffered saline was added to each.

Reduction

Immature crosslinks must be stabilised by reduction before the acid hydrolysis step, or they will be broken down. 100 µl of 20 mg/ml potassium borohydride in N'N'-dimethylformamide (Sigma, Poole, UK) was added. The tissue samples were incubated in this agent for one hour at room temperature. The reduction reaction was stopped at that point by lowering the pH of the solution to 3, using 4 drops of glacial acetic acid. The tissue was spun down and the supernatant pipetted off. 2 ml of de-ionised water was then added, the sample spun down again, and the water pipetted off. The water wash was repeated, and the tissue was then re-frozen to -70°C and freeze-dried overnight.

Acid hydrolysis

The freeze-dried samples were transferred to 15 ml screw-top hydrolysis tubes (Corning Laboratory Science Co.) and 3 ml of 6 M hydrochloric acid added. Hydrolysis proceeded for 24 hours at 110°C in a heat block. The resultant hydrolysates were freeze-dried under vacuum to remove excess acid, and the residue dissolved in 1 ml of de-ionised water. 10 µl was removed from each solution for a hydroxyproline assay and added to 2.5 ml of de-ionised water.

Preparation of a pre-separation column

This column removes non-cross-link amino acids from the sample. An "organic" phase consisting of butan-1-ol, acetic acid and de-ionised water in a 4:1:1 ratio was prepared. A CF1-cellulose column was then prepared for each of the 30 samples. The top of a bulb of a 3 ml disposable Pasteur pipette (Sterilin, Hounslow, UK) was removed, and a small plug of glass wool inserted and pushed to the lower end. Each pipette was labelled for an individual sample, and suspended in a test tube rack over a plastic vial.

30 g of CF1 cellulose (Whatman, Maidstone, UK) was shaken in 500 ml of water, allowed to settle, ,and then the supernatant decanted. This was repeated a further four times, in order to wash the cellulose. The cellulose was then washed three times in organic phase, and finally suspended in 500 ml of this phase to give a concentration of approximately 5% (w/v). Each pipette was filled to the bottom of the bulb with this cellulose slurry. The resultant columns were settled by washing 8 ml of the organic phase through each one.

CF1 column separation

To each hydrolysed sample in 1 ml of water, 1 ml of glacial acetic acid and 4 ml of butanol were added to give a total volume of 6 ml of organic phase. These solutions were applied to separate labelled CF1-cellulose columns. The tubes were washed twice with 2 ml of organic phase, which were also applied to the columns. Cross-linked amino acids were adsorbed to the cellulose, while noncross-linked amino acids were washed through into the effluent using a further 16 ml of organic phase in 4 ml aliquots. Cross-linked amino acids were eluted from the column by washing with 8 ml of de-ionised water, and collected in 20 ml glass vials. The eluent was allowed to settle overnight, and the lower aqueous phase containing the cross-linked amino acids was pipetted off and transferred to screw-capped glass tubes. The latter were freeze-dried, and the resulting residues dissolved in 200 µl of LKB loading buffer (0.2 M sodium citrate, pH 2.2). Each sample solution was then filtered through a 0.2 µm plastic filter (Gelman Sciences, Northampton, UK). Recovery rates of cross-link residues using this method have been determined to lie between 90-95% (Sims and Bailey 1992). The reduced crosslink compounds are very stable to acid hydrolysis and no corrections for losses are necessary (Robins 1982).

Ion-exchange column separation

This allows separation of the individual collagen crosslinks. 50 µl of each sample solution was applied to an LKB 4400 amino acid analyzer (Pharmacia,

Loughborough, UK), which was configured for the separation of collagen cross-links. Ninhydrin was used to detect these compounds, which were identified by comparison with an authenticated standard cross-link preparation. They were quantified by comparison with the amino acid leucine, and expressed as nanomoles of cross-link compound per mg of collagen. Leucine equivalent factors used in the calculation of cross-link concentrations are given in Table 2.2.

Collagen content

The method used is an adaptation of those of Bergman and Loxley (1963) and Bannister and Burns (1970). Each of the 10 μl aliquots removed from the dissolved hydrolysates were dissolved in 2.5 ml of de-ionised water to yield an approximate concentration of 5 μg of hydroxyproline per ml. This volume of water was calculated from dry weights of tissue used for the cross-link assay, assuming a collagen content of 75% (Birch 1993), with 14% of the collagen residues being hydroxyproline. Hydroxyproline assays were carried out using an automated continuous flow colorimeter (Chemlab Instruments Ltd). The reaction involves the oxidation by chloramine T (in the oxidant solution), followed by condensation with dimethylaminobenzaldehyde (in the colour reagent solution). The resulting red-coloured product is measured at 550 nm. Details for preparation of diluent, oxidant and colour reagent solutions are given in Appendix C (page 196). Hydroxyproline concentrations in the samples were calculated by comparison with a standard curve (1-10 μg hydroxyproline/ml). Standards were prepared by dilution of a 1 mg/ml stock solution with de-ionised water (see Appendix C, page 196). The amount of collagen added to the CF1 cellulose column in mg was calculated from the hydroxyproline figure, assuming a hydroxyproline content of 14%.

Table 2.2 Leucine equivalence factors used in the calculation of collagen cross-link concentrations[*].

Cross-link	Leucine equivalence factor
Hydroxylysinonorleucine (HLNL)	1.8
Dihydroxylysinonorleucine (DHLNL)	1.8
Hydroxylysylpyridinoline (HYL-PYR)	1.7

[*] from Sims and Bailey (1992).

Chapter 3

An age-related analysis of collagen fibril diameter distributions and collagen crimp patterns in superficial digital flexor tendons from a sample of wild ponies[1]

3.1 Abstract

The pathogenesis of superficial digital flexor tendon (SDFT) injury in the racing thoroughbred is poorly understood. A previous study demonstrated significant reductions in crimp angles and lengths with age, particularly in the central region, so that in the older horses both parameters were lower in the tendon core than in the periphery. It was not determined at that time if this was an age-related fatigue effect or a result of microdamage induced by high intensity training. This study was therefore undertaken to measure crimp angles and period lengths as well as collagen fibril mass-average diameters in the SDFTs of wild horses to ascertain any age-related changes in the central regions in the absence of imposed exercise. The crimp angle for the central region was found to decrease with age, so that in horses of 10 years or older it was lower than that for the tendon periphery. The crimp angle for the latter region did not alter significantly with age. Crimp period lengths and collagen fibril mass-average diameters did not show significant changes in either region. These results suggest that reduction of the crimp angle in the core of the SDFT occurs normally with age, as tendons of older animals would have undergone a higher number of loading cycles. The additional decrease in peripheral angle and the reductions in crimp length in both regions measured previously in older thoroughbreds may represent a more severe grade of change, which could be regarded as pathological. Athletic training increases the frequency and magnitude of high loading cycles experienced by the tendon, and may accelerate and worsen the normal load-related ageing process in the SDFTs of young performance horses, particularly in the central regions where lesions usually occur.

3.2 Introduction

Injury to the superficial digital flexor tendon (SDFT) of thoroughbred racehorses results in significant losses to the racing industry every year. It has been estimated that 30% of horses in training in Europe suffer from this condition at some stage (Goodship 1993). Prolonged periods out of work and a high likelihood of recurrence on resumption of athletic activity (Rooney and Genovese 1981) further increase its importance. The situation also raises concern for the welfare of racehorses, as such lesions are far less common in animals not used for competition (Webbon 1977).

SDFT lesions occur most frequently in the mid-metacarpal region of the tendon. Often only the centre of the tendon is involved, with the periphery of the cross-section apparently unaffected (Webbon 1977). Most cases probably have degenerative alterations in the tendon structure before the onset of clinical signs. In a post-mortem survey of random thoroughbred SDFTs (Webbon 1977), 48% of tendons had reddish discolouration in the tendon core, but not all horses with this abnormality had been recorded as having had clinical episodes of tendonitis.

A more recent theory is that alterations in structure occur even before the macroscopic changes. These alterations are termed "microtrauma" and are hypothesized to be caused, either directly or indirectly, by certain patterns of cyclical mechanical loading which approach or exceed the physiological limits of the tendon (Becker *et al.* 1994, Platt and Wilson 1994). The microdamage is cumulative, eventually resulting in significant degenerative change, and in some animals, clinical tendonitis.

If evidence for microtrauma in the equine SDFT could be demonstrated in macroscopically normal structures, and some means found for detecting it, programmes could be developed to prevent further progression of damage. Prevention of tendonitis is likely to be more successful than treatment of the clinical condition, as by that stage tendon structure has been severely disrupted.

Tendon has a structure consisting of longitudinally arranged elements at several levels (Kastelic *et al.* 1978). The largest units are termed fascicles,

which are in the order of 50-300 μm in diameter. The fascicles are composed of bundles of collagen fibrils, embedded in matrix. This extrafibrillar matrix is comprised of: proteoglycans, glycoproteins, small amounts of elastin, simple ions and water. It contains small numbers of tenocytes and elastic fibres.

Collagen fibrils are cylindrical structures composed of covalently cross-linked collagen molecules in a specific arrangement. They are the basic units of tensile strength of the tendon, and in the equine SDFT range from 20 to several hundred nm in diameter. In the adult SDFT they may be classified as "large" or "small" diameter, resulting from the observed bimodal diameter-frequency distribution with peaks at 25-35 nm and 165-215 nm (Parry *et al.* 1978b). The morphology of a fibril population may be characterised by the mass-average diameter (MAD), which is effectively the mean of the diameter-area distribution. This parameter takes into account the fact the usually small numbers of large diameter fibrils can occupy a large proportion of the cross-sectional area of the tendon. As the larger diameter fibrils are stronger (Parry *et al.* 1978a), the mass-average diameter has a good correlation with overall tendon strength (Parry and Craig 1988, Oakes 1989).

Collagen fibril diameter has been shown to change with age (Parry *et al.* 1978b) and with exercise (Patterson-Kane *et al.* 1996a). A trend of increase in MADs of SDFTs with increasing maturity, and a reduction with senescence was observed in horses of undetermined breed and exercise history (Parry *et al.* 1978b). The MADs of core regions of SDFTs from thoroughbred horses were shown to decrease significantly in response to a specific 18 month training programme (Patterson-Kane *et al.* 1996a), while those of the peripheral regions were unaffected. In 3 of the 5 exercised horses the MAD of the central region was significantly reduced below that of the periphery. These changes were interpreted as microdamage to the tendon core, involving exercise-induced breakdown of the larger diameter fibrils.

Tendon collagen fascicles show an alternate dark and light banding under polarised light, termed the 'crimp'. This is due to bundles of collagen fibrils following a planar zig-zag wave rather than a straight path along the tendon unit (Diamant *et al.* 1972). The formation of crimp appears to require fibril bundles which are subjected to high levels of tensile strain, as it is not a

feature of isolated fibrils, or fibril bundles in tissues such as cartilage or bone (Gathercole and Keller 1975). The banding pattern of intact tendon at the level of the light microscope has been definitively correlated with the underlying morphology of the collagen fibrils using scanning electron microscopy (Gathercole *et al.* 1974) and low angle x-ray diffraction techniques (Gathercole and Keller 1975).

The periodic extinction effects seen in the banding pattern by rotating the plane of polarised light can be related to the crimp angle and crimp period length (Diamant *et al.* 1972). This is a convenient way of quantifying crimp structure and can be applied to both intact tendon units or histological sections. Mean crimp angles and lengths have been shown to change with age in rat tail tendon (Diamant *et al.* 1972) and the equine SDFT (Wilmink *et al.* 1992), and with exercise in the rat SDFT (Wood *et al.* 1988).

Crimp angle and period length were less in SDFTs of old thoroughbred horses (mean age 11 years) compared with those in younger horses (mean age 3 years), and these reductions were greatest in the central regions, so that the parameters were significantly lower than those in the periphery of the same tendons in the older horses (Wilmink *et al.* 1992). Because only a subjective exercise history was available in the study, it could not be ascertained as to whether this was a result of an accumulation of central region exercise-induced microdamage in the older horses, or an age-related fatigue effect.

This study was undertaken to test the hypothesis that, in wild horses which had not undergone imposed exercise, age-related reductions in crimp angle and length, and in collagen fibril MADs would be greater in the central region than in the peripheral region of the SDFT. Peripheral regions were not predicted to alter as significantly with age.

3.3 Materials and methods

Samples were taken from two culls of "Kaimanawa horses" which were carried out by the New Zealand Department of Conservation. This population ranges over an area of approximately 24 000 hectares in the plateau region of the central North Island of New Zealand. The horses are under 14.2 hands in

height and therefore ponies by definition, but in terms of genetic markers they are closer to the British thoroughbred than any domestic breed (Cothran, personal communication). The culls in 1994 were carried out due to unacceptable levels of destruction of native plants, and horses straying onto the highway.

23 adult animals were sampled for this study (see Table 3.1), which were aged on the basis of dentition by two veterinary surgeons, in consultation. Three fetuses were also sampled, which were determined to be at approximately 210 days of gestation on the basis of crown-rump measurements.

Table 3.1 Age of horses used in this study

Age group (years)	Mean age (years)	Number of horses sampled
<5	3	10
5 to <10	6	9
10 +	12	4

3.3.1 Tissue preparation

Electron microscopy

A one centimetre length was excised from the mid-metacarpal point of the right SDFT, cut in half sagittally, and placed in phosphate-buffered glutaraldehyde. The next day 2mm x 1mm^2 strips were dissected from each sample, from both the centre of the cross-section and a peripheral location approximately one mm from the lateral surface. The small size of the fetal tendons did not allow sampling from different regions. The strips were fixed overnight and then embedded conventionally using Taab 812 resin. Silver-gold ultrathin sections were cut from one block for each tendon region and mounted on 200 mesh formvar-supported copper grids. The sections were stained in saturated uranyl acetate in 50% ethanol followed by modified lead citrate.

Polarised light microscopy

A 2-3 cm length was taken directly proximal to the above sample and double-wrapped in cling-film. Cling-film wrapped samples were stored at -20°C. Frozen tendon segments were cut in half sagittally, and the lateral half cut into "central" and "peripheral" blocks using a single-edged razor blade. 30 μm thick longitudinal sections were cut in the latero-medial plane in a cryotome set to -30°C. Sections from the central blocks were taken from the most central surface. Sections from the peripheral blocks were taken from within one mm of the outside surface. The sections were thaw-mounted on glass slides, 30-40 being taken from each tendon region. After drying for one hour they were dehydrated in 70% ethanol and thence in two washes of absolute ethanol, for one minute in each. They were then cleared in xylene and mounted in DPX.

3.3.2 Measurement of collagen fibril diameters

Sections from each tendon region were visualised using a Philips E.M. 201c high-resolution electron microscope. Details of the image analysis method used to determine the collagen fibril diameter distribution have been described previously (Patterson-Kane *et al.* 1996a). For the measured collagen fibril population of each central or peripheral region the MAD was calculated, from the diameter-area distribution.

3.3.3 Measurement of crimp morphology parameters

Sections were analyzed using a Nikon Labophot2-Pol polarising light microscope. Crimp angle was measured by rotating the sections between crossed polarising filters on the vernier stage of the microscope, which results in a changing pattern of alternating dark and light bands. If the stage is rotated through twice the crimp angle the pattern changes from one series of dark and light bands to a complementary series. Crimp angle was measured at a magnification of 100x at 50 different sites from the 30 to 40 sections, to the nearest 0.1°.

The length of five consecutive crimp segments was measured using a calibrated eyepiece graticule in the microscope, at a magnification of 400x. This measurement was also done at 50 sites.

3.3.4 Statistical analysis

Adult horses were divided into three age groups (see Table 3.1): "young" horses less than 5 years (n=10); "middle-aged" horses between 5 and 10 years (n=9); and "aged" horses of 10 years of age or older (n=4).

Distributions for crimp angles and lengths were determined to be normal using a normal probability plot (SYSTAT for Windows, Systat Inc., Illinois, USA). True crimp lengths for each distribution were calculated using the formula $l = d/\cos\theta$, d being the mean crimp length in projection and θ being the mean crimp angle (see Figure 3.1). Crimp angles and lengths and collagen fibril MADs were compared between groups using t-tests for independent samples and between central and peripheral regions within groups using paired-sample t-tests.

3.4 Results

No areas of discolouration were seen in central regions of tendons sampled which would have indicated degenerative lesions (Webbon 1977).

Previous examinations of crimp in equine SDFTs have used paraffin wax-embedded histological sections (Williams *et al.* 1985) and isolated fibril bundles (Wilmink *et al.* 1992). The images obtained using frozen sections were more distinct. The processing fixed the sections meaning they could be stored at room temperature, and caused them to become translucent, which resulted in a clearer image for analysis under the microscope. Alcohol dehydration is known to only change crimp parameters by a small consistent amount with little observable change in the actual shape of the waveform (Nicholls *et al.* 1983, 1984). Regardless of that fact, sections from central and peripheral regions from the different age groups were treated in the same way, so comparisons

between them were considered valid.

3.4.1 Collagen fibril diameters

The collagen fibril MAD of 60.9±3.1 nm for the foetuses was significantly less than that of 127.8±6.6 nm for the central region of the young group. The increase in MAD between young and middle-aged groups was not significant (p=0.065), nor was the decrease between middle-aged and old groups (Table 3.2). There were no significant differences between central and peripheral regions for any group.

3.4.2 Crimp morphology parameters

Mean crimp angles and period lengths for the fetuses and the three adult age groups are summarised in Table 3.3.

Crimp angle

Crimp angles for samples were normally distributed with ranges of $14\text{-}18^{\circ}$. The mean crimp angle of $10.5 \pm 0.7^{\circ}$ for the central regions of the old horses differed significantly from that of $14.3 \pm 0.6^{\circ}$ for the young group (p<0.01) and that of $13.1 \pm 0.5^{\circ}$ for the middle-aged group (p<0.05). Young and middle-aged groups did not differ significantly from one another (p=0.07). The mean crimp angles for peripheral regions did not alter significantly with age.

The mean crimp angle of $14.3 \pm 0.6^{\circ}$ for the central region was significantly greater than that of $13.2 \pm 0.5^{\circ}$ for the peripheral region in the young group (p<0.01). For the old horses, the mean crimp angle of $10.5 \pm 0.7^{\circ}$ for the central region was significantly lower than that of $12.7 \pm 0.4^{\circ}$ (p<0.05) in the peripheral region. The two regions did not differ significantly in the middle-aged group.

The mean crimp angle for the foetal group was $26.7 \pm 1.3^{\circ}$, which was significantly greater than that for any adult group.

Crimp length

Crimp lengths for samples were normally distributed with ranges of 26-28 µm. Crimp length did not alter significantly with age in either tendon region. The mean crimp length of 23.0 ± 1.2 µm for the central region of the young group was significantly greater than that of 21.2 ± 1.3 µm for the peripheral region (p<0.05).

The mean crimp length for fetal tendon was 37.8 ± 4.5 µm, which significantly exceeded values for all adult groups.

Table 3.2 Collagen fibril mass-average diameters and standard errors (in brackets) for the central and peripheral regions

Age group	Central	Peripheral
Young	127.8 (6.6)	115.8 (7.9)
Middle-aged	143.5 (4.1)	140.3 (11.1)
Old	133.9 (12.9)	130.4 (6.4)

Table 3.3:   Summary of group means and standard errors for crimp angle (°) and length measurements (µm)

	CRIMP ANGLE		CRIMP LENGTH	
	Central	Peripheral	Central	Peripheral
Foetal‡	26.7 (1.3)†		37.8 (4.5)†	
Young	14.6 (0.6)*†	13.2 (0.5)	23.0 (1.2)*	21.2 (1.3)
Middle-aged	13.1 (0.5)†	12.0 (0.8)	19.8 (1.2)	18.0 (1.6)
Old	10.5 (0.7)*	12.7 (0.4)	17.9 (2.4)	20.3 (2.4)

‡ Foetal tendon samples were not differentiated according to region.
* Differs significantly from corresponding peripheral value.
† Differs significantly from the old group value for that region.

3.5 Discussion

3.5.1 Collagen fibril diameters

The MADs had large standard errors for each of the groups and regions due to individual variability, meaning differences between age groups were not significant. In addition, no significant differences were seen between MADs for central and peripheral regions in any age group, so the collagen fibril diameter distributions did not show evidence of age-related central region microtrauma. The increase in MAD between foetal and adult SDFTs has been observed previously (Parry *et al.* 1978b).

3.5.2 Crimp morphology parameters

The *in vitro* stress-strain curve for tendon is non-linear, showing an initially concave portion termed the "toe" region. In this region the tendon is highly compliant, undergoing relatively large extensions for relatively small increases in stress. A steep linear region follows in which the tendon functions elastically and which is demarcated quite clearly from the toe in mature tendon. The end of the linear region signifies the first large-scale failure of fascicular units, and from this point, which marks the beginning of the yield region, the curve dips downwards with complete failure occurring very rapidly (Butler *et al.* 1978). The toe region corresponds to straightening of the waveform, which is completed by the beginning of the linear region. This has been observed directly in loosened tendon (Rigby *et al.* 1959, Abrahams 1967) and in intact rat tail tendon, using polarising optics (Diamant *et al.* 1972). In the latter study crimp angle was observed to decrease on stretching. At the end of the toe region the crimp angle had been reduced to zero. The collagen fibrils themselves are not stretched in the toe region in mature animals. It is from the beginning of the linear region that the straightened fibrils undergo elastic elongation (Torp *et al.* 1975b) and when stretched into the yield region they are damaged, dissociating into subfibrils and eventually rupturing (Torp et al. 1975a). The strain at the point of crimp elimination is termed the "toe limit

strain" (TLS), which has been found to lie between 2 and 5% for a variety of collagenous tissues (Elliott 1965). The larger the crimp angle, the greater the value of the TLS, because the waveform must be stretched further before it straightens. This means that at the same level of tendon strain a fibril bundle with a smaller crimp angle experiences a higher level of stress and will fail before a bundle with a larger crimp angle (see Figure 3.2). The crimp allows the tendon to achieve higher strains than would be possible if the fibrils were straight at the onset of loading and is said to act as a safety mechanism to protect the tendon against sudden forces. The importance of the crimp period length in terms of mechanical behaviour has not been defined, but this parameter has been suggested to be merely a consequence of the crimp angle (Wilmink et al. 1992).

Age-related changes

The crimp angles for central regions decreased from young to old horses, and from middle-aged to old horses. The difference between young and middle-aged groups was not significant, although the higher significance of difference between young and old (p<0.01) versus middle-aged and old (p<0.05) does indicate a trend of reduction in crimp angle with age as observed in other species (Diamant *et al.* 1972). The peripheral crimp angles did not change between young and old groups, which differed from the reduction seen in the study of thoroughbred tendons, but the difference in central region crimp angles was similar in magnitude (Wilmink *et al.* 1992).

The crimp lengths did not alter with age in either tendon region. Crimp length has been observed to increase with age in rat tail tendon (Diamant *et al.* 1992), however this appeared to be related to tail length and may be a specific feature for that structure. Significant reductions in crimp length with age were seen in central and peripheral regions of thoroughbred SDFTs (Wilmink *et al.* 1992). This, together with the reductions in peripheral crimp angle in those horses, may indicate that changes in the tendons of the older thoroughbreds were more severe than those seen in this study which used tendons from horses which were not used for athletic performance.
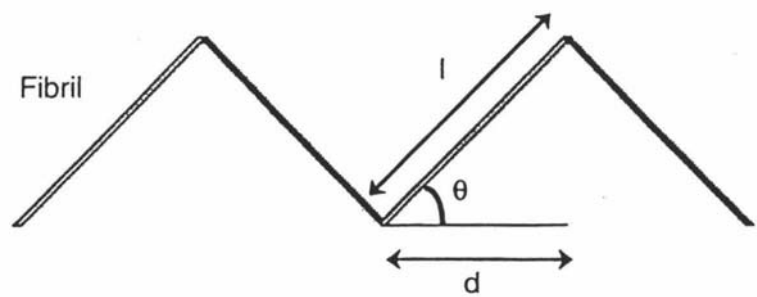
Figure 3.1 Details of crimp angle and length measurements
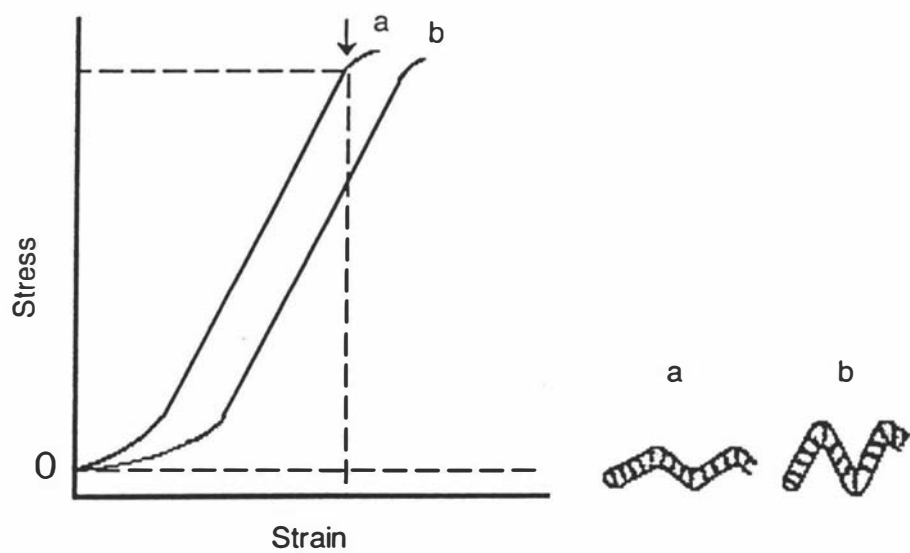
Figure 3.2 Comparison of stress-strain curves for fibrils of differing crimp angle. Arrow denotes the beginning of the failure region for the fibril with the smaller angle (a). At this level of strain the fibril with the greater angle (b) is still in the linear region.

Measurements of crimp parameters for SDFT samples from three fetuses from the same population of ponies gave a mean angle of 26.7° and a mean length of 34.6 μm for the central region of the tendon. It is likely that the crimp angle and length decrease from birth until maturity, at which point the values plateau. The exception to this is the crimp angle of the central region of the tendon, which continues to decrease.

The mean crimp angles for central regions of young horses were significantly greater than those for the corresponding peripheral regions, a difference which had not been seen previously in young thoroughbreds (Wilmink *et al.* 1992). This may have been due to the low number of horses used in that study (n=4), or exercise-induced reduction in the central regions of the tendons from two of the young horses, whose training history was not documented. The lower angle in the periphery in this study implies a differing mechanical environment between the two tendon regions. If stress and strain were experienced uniformly across the tendon, the peripheral fibrils with their lower crimp angles would be expected to provide stress-protection for the central fibrils, as the latter would undergo less elongation before straightening (Kastelic *et al.* 1980). If this were occurring, a higher incidence of peripheral lesions than central lesions would be expected in young horses, but lesions are typically found in central regions in all age groups (Webbon 1977). The implication is that the central region experiences greater stresses and strains. The same regional difference was not noted in the two older groups due to specific age-related reduction in crimp angle the centre of the tendons, which would have increased stresses and strains on fibrils in this region still further above those in the periphery.

The reduction in crimp angle of SDFT cores in wild horses, as observed in thoroughbreds, appears to be a normal age-related change. The additional reduction in peripheral angles in the performance horses may indicate that imposed exercise causes damage, affecting even the peripheral layers which on the basis of this study may experience a lower level of stress and strain than the central regions. Results from this study and the previous study of thoroughbred SDFTs are summarised in Figure 3.3.
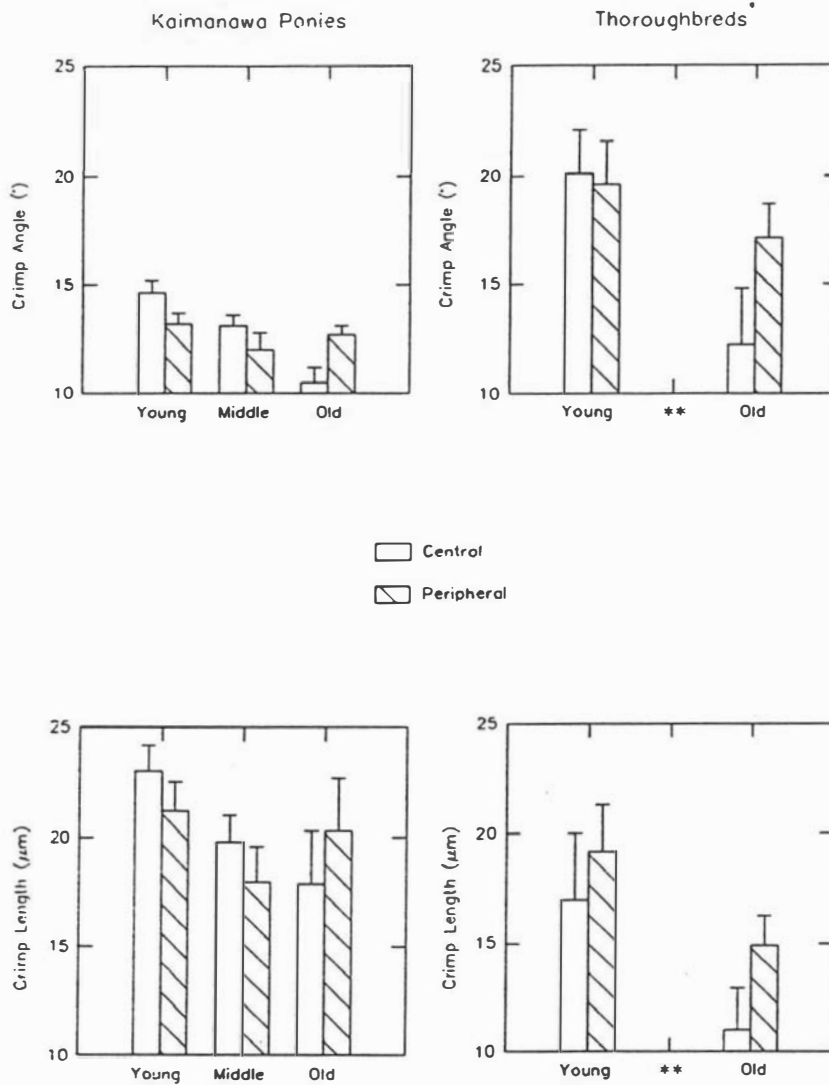
Figure 3.3 A summary and comparison of crimp morphology data for superficial digital flexor tendons from wild horses and from thoroughbreds. * data from Wilmink et al. (1992) ** middle-aged horses were not sampled in this study.

Mechanisms

The mechanisms involved in age or exercise-related changes in crimp morphology have not been determined. The changes may be caused directly by mechanical factors, or may be secondary to alterations in cellular metabolism.

The half-life of collagen in tendon is long in comparison with other connective tissues (Gerber *et al.* 1960), and measurements of collagen glycosylation in equine SDFTs have indicated that less new collagen is laid down as the horse ages (Birch 1993). It therefore seems unlikely that collagen fibrils are broken down en masse and replaced by those with different crimp parameters. Newly synthesized single fibrils would tend to be laid down along the already present waveform, so it would seem more likely that there is reformation of the collagen already present.

It has been hypothesized, using synthetic fibre-filled composites, that the crimp is the result of in-phase buckling of collagen fibrils, due to the effect of strain cycling on the fibril-matrix interface (Dale and Baer 1974). When the synthetic composites were strained above the toe region, a reduction in crimp angle was observed. Even higher strains resulted in a new waveform with a reduced crimp length, which was superimposed on the original one. The new crimp angle was not measured. This effect was also observed in rat tail tendon which was cyclically deformed *in vitro* into the yield region of the stress-strain curve (Dale and Baer 1974, Torp *et al.* 1975b). In the synthetic materials the "recrimping" effect was demonstrated to be due to slippage between fibres and the surrounding matrix. On relaxation, the fibres rebuckled within the channels of matrix material, which was suggested to occur in order to accommodate the resultant increase in strain between the two components. The effect in rat tail tendon was hypothesized to be due to interfibrillar slippage due to failure of the "medium" which transferred stress between them.

Recent work has demonstrated the importance of proteodermochondan sulphates in the mechanical behaviour of tendon (Cribb and Scott 1995). These structures are orthogonally arranged along collagen fibrils and are known to connect them. In rat tail tendon stretched nearly to rupture the

arrangement of these proteoglycans did not appear to be altered. It is possible that in the equine SDFT, which has a low safety margin during galloping (McCullagh et al. 1979, Wilson and Goodship 1991), that disruption of these interfibrillar structures could occur, allowing slippage of the fibril and recrimping within a channel of other matrix components which are not specifically bound. The proteodermochondan linkages could rapidly reform in a new conformation (Cribb and Scott 1995).

Recrimping may also involve disruption of subfibrillar organisation. Rat tail tendon which had undergone this change showed an extended toe region (Dale and Baer 1974, Torp et al. 1975b) and the fibril bundles from the SDFTs of older thoroughbreds showed higher TLSs than those from younger horses, particularly those from the central region (Wilmink et al. 1992). This apparently paradoxical increase in TLS associated with a reduction in mean crimp angle in the latter study could be explained by the reduced mechanical integrity of some fibrils (Torp et al. 1975b, Wilmink et al. 1992). Uneven loading of fibrils which have been 'stretched' by different amounts due to subfibrillar slippage has been suggested (Torp et al. 1975b), which might assist the recrimping process. Slight subfibrillar slippage could also prevent fibrils from fully relaxing to their original crimp angle.

The reductions in crimp angle seen in central regions of SDFTs in this study may occur due to irreversible straightening of the waveform by repetitive loading over the lifespan of the horse, which approaches but does not enter the yield region. More severe changes involving reductions in crimp angle and length as seen in the SDFTs of exercised thoroughbreds may be due to overloading of the fibril-matrix composite, which results in rebuckling of some fibrils into a new waveform, and a consequent shifting of the normal distributions of both crimp parameters. Some degree of disruption of the proteoglycans connecting the fibrils and of the subfibrillar organisation may be involved at both levels. The morphology observed in the tendons of the older exercised thoroughbreds would thus represent a more severe grade of change in comparison to that in older wild horses, and could be regarded as pathological. Scar tissue induced by collagenase injury to equine SDFTs showed increased crimp angle and decreased crimp length, characteristics similar to

that seen in immature tissue in other species (Williams *et al*. 1985). The changes seen in aged wild and thoroughbred horses do not therefore represent scarring, or "new" collagen. One of the older horses in the thoroughbred study did show signs of core degeneration. Crimp morphology seen in aged thoroughbred tendon would seem to indicate degenerative change, or "microtrauma" in its initial stages.

Greater temperature increases have been measured in the core of the equine SDFT during galloping than in the periphery (Wilson and Goodship 1994), which in one horse reached 45°C. This may be another possible mechanism for causing changes in crimp morphology, acting in conjunction with mechanical factors. Thermoelastic fibre buckling is a phenomenon in synthetic fibre-filled composites (Dale and Baer 1974), which occurs during cooling.

High temperatures, free radicals, and low pH levels could assist in subfibrillar disruption. It has been suggested that high temperatures in conjunction with lowered pH could result in the breakage of bonds between subfibrils (Scott 1990).

In summary, based on the results of this age-related study of crimp morphology in SDFTs from wild horses and those of a previous study involving thoroughbreds, it is suggested that reduction of crimp angle in the tendon cores is a normal age-related occurrence, a fatigue mechanism occurring with a large number of loading cycles over the lifespan of the horse. This may involve slight subfibrillar slippage and reorganisation of proteodermochondans, occurring over a period of 10 years. These changes are unlikely to be of clinical importance due to the long time-span involved. In shorter, more extreme mechanical situations such as those experienced by the tendons of exercised thoroughbreds, more severe disruption of fibril-matrix bonds and/or subfibrillar structure may occur, resulting in recrimping of some fibril bundles, particularly in the central tendon regions. This is suggested to represent accumulation of exercise-induced microdamage. The reductions in crimp angle and length may be potentiated by increases in tendon core temperature and changes in other factors such as pH and free radical concentration which may occur during galloping. The fibril mass-average diameters did not reduce significantly, implying that the morphology of the waveform followed by the

fibrils is more sensitive to loading-induced change than the transverse structure of these units.

Now that the effect of age in the absence of imposed exercise has been measured, the effect of high intensity training for racing on the SDFTs of younger thoroughbreds must be elucidated to determine if this accelerates and worsens normal age-related processes, resulting in microdamage and predisposing the tendons to the development of clinical injury.

Chapter 4

Exercise modifies the age-related change in crimp pattern in the core region of the equine superficial digital flexor tendon[2]

4.1 Abstract

One of the current concepts with regard to equine superficial digital flexor tendonitis is that cumulative subclinical microscopic damage weakens the structure, predisposing the tendon to partial or complete rupture. This 'microtrauma' is likely to affect the waveform or 'crimp' of the collagen fibrils, which are the units of tensile strength. Collagen fibril crimp morphology characteristics were determined in superficial digital flexor tendons of 18 horses presented for necropsy. Horses were separated into "exercised" (n=9) and "non-exercised" groups (n=9), based on recent function. Five of the eight exercised horses below the age of 10 years showed significantly lower crimp angle in the central region of the tendon in comparison with the periphery. Three of those five animals also showed significantly lower crimp period length in the centre. No unexercised horses in this age group showed such regional differences.

A lower crimp angle in the core region in comparison with the periphery is abnormal in animals younger than 10 years, on the basis of previous data obtained from wild horses. It is hypothesized that imposed exercise regimes involving galloping modify the normal age-related reduction of crimp angle in the tendon core, probably as a result of the increased number of rapid high-strain cycles experienced by the collagen fibrils.

4.2 Introduction

Injury of the superficial digital flexor tendon (SDFT) of the forelimb in the performance thoroughbred occurs frequently. It has been estimated that 30% of horses in training for the National Hunt in the United Kingdom suffer from SDFT tendonitis at some stage in their career (Goodship 1993). Despite the importance of this condition, knowledge regarding the pathogenetic mechanisms is limited.

It is generally agreed that overloading of the SDFT during galloping is responsible for tendon injury, however this is unlikely to result in catastrophic rupture of a healthy tendon (Fackelman 1973). Lesions usually involve the core of the midmetacarpal region of the tendon, with the peripheral region being apparently unaffected (Webbon 1977). They are suggested to develop by accumulation of subclinical traumatic change or 'microtrauma', which occurs until the structure is weakened sufficiently to allow failure under the appropriate conditions (Fackelman 1973). This is supported by the findings of lesions on post-mortem in horses with no clinical history of tendon injury (Webbon 1977), and the common occurrence of partial rupture of the central region when apparently normal thoroughbred SDFTs are loaded to failure *in vitro* (Goodship 1993).

Several studies have measured alterations in structure (Wilmink *et al.* 1992, Patterson-Kane *et al.* 1996a) and biochemistry (Birch 1993) in the central regions of grossly normal tendons from exercised thoroughbreds which may explain this local structural weakening. Knowledge of the mechanisms involved in these early alterations would therefore increase the understanding of the pathogenesis of SDFT tendonitis in racehorses.

Tendons are composed of longitudinal units termed fascicles which range between 50 and 300 µm in diameter (Kastelic *et al.* 1978). The fascicles are multicomposite, consisting of submicroscopic collagen fibrils embedded in a matrix composed of proteoglycans, glycoproteins, elastic fibres, ions and water. Collagen fibrils are cylindrical structures probably several millimetres in length (Craig *et al.* 1989), which are comprised of type I collagen molecules in a specific axial and lateral arrangement. The fibrils range in diameter from 20

nm to between 200-300 nm in the adult equine SDFT (Parry et al. 1978b, Patterson-Kane *et al.* 1996a), and are the basic units of tensile strength in the tendon.

The collagen fibrils follow a planar zig-zag waveform along the tendon, which is visible under polarised light at the level of the fascicles. It appears as dark and light bands, the pattern of which changes in systematic fashion as the plane of polarised light is rotated (Diamant *et al.* 1972). The waveform may be quantified conveniently by the parameters of crimp angle and crimp period length using the method of Diamant *et al.* (1972).

In a previous study of wild horses, which had obviously not undergone training, there was a reduction in crimp angle in the central region with age, such that it was less than that in the tendon periphery in horses of 10 years and over (Patterson-Kane *et al.* 1996b). A study of SDFTs from thoroughbreds demonstrated a reduction in crimp angle and also in crimp period length in both regions with age, such that both parameters were lower in the central region than in the periphery in horses of 10 years or over (Wilmink *et al.* 1992). Reduction of either parameter in the tendon core below that in the periphery in a horse under the age of 10 years is therefore abnormal.

This study was undertaken to test the hypothesis that training of thoroughbreds which involves galloping modifies the known age-related reduction in crimp angle in the central region of the SDFT.

4.3 Materials and Methods

4.3.1 Horses

SDFT samples were obtained from thoroughbreds of 2 years of age or older which were submitted for necropsy at Massey University between September 1994 and August 1995. None of the 18 horses sampled had a known history of digital tendon injury. Age was determined from brands, and any known exercise history for the horse was noted. Horses were allocated to "exercised" and "non-exercised" groups on the basis of recent function. A horse was classified as exercised only if it had been used for competitive athletic activity

involving galloping, meaning that hacks were not included in this group. A horse used solely for dressage was also not included. The exercised group (Table 4.1) contained 9 horses between the ages of 2 and 15 years (mean age 6 years). A horse was classified as non-exercised if it had not yet begun training or had no record of recent competitive galloping. The latter included a hack, a dressage horse, a broodmare and three horses which at the time of death were not being used for any specific function. The non-exercised group (Table 4.2) contained 9 horses between the ages of 2 and 14 years (mean age 7 years) .

4.3.2 Tissue sampling and processing

A 3 cm long section was taken from each left forelimb SDFT, from a point 0.5 cm proximal to the midpoint of the metacarpal region (Figure 4.1). Each sample was double-wrapped in cling-film, then wrapped in aluminium foil and stored at -70°C. Frozen tendon segments were bisected sagitally, and the lateral half cut into "central" and "peripheral" blocks 1 cm long, using a single-edged razor blade. Sagittal sections 30μm thick were cut from these blocks in a cryotome set to -30°C. Those from the "central" block were taken from the most central surface, and those from the "peripheral" block were taken from within one mm of the outside (lateral) surface (Figure 4.1). For both the central and peripheral regions, 30-40 sections were thaw-mounted on labelled glass slides. After drying for one hour they were dehydrated for one minute each in 70% ethanol and two washes of absolute ethanol. They were then cleared in xylene and mounted in DPX.

4.3.3 Crimp morphometry

Sections were visualised using a Nikon Labophot2-Pol polarising light microscope. Crimp angle was measured by rotating the sections between crossed polarising filters on the vernier stage of the microscope, which results in a changing pattern of alternating dark and light bands. If the stage is rotated through twice the crimp angle the pattern changes from one series of dark and
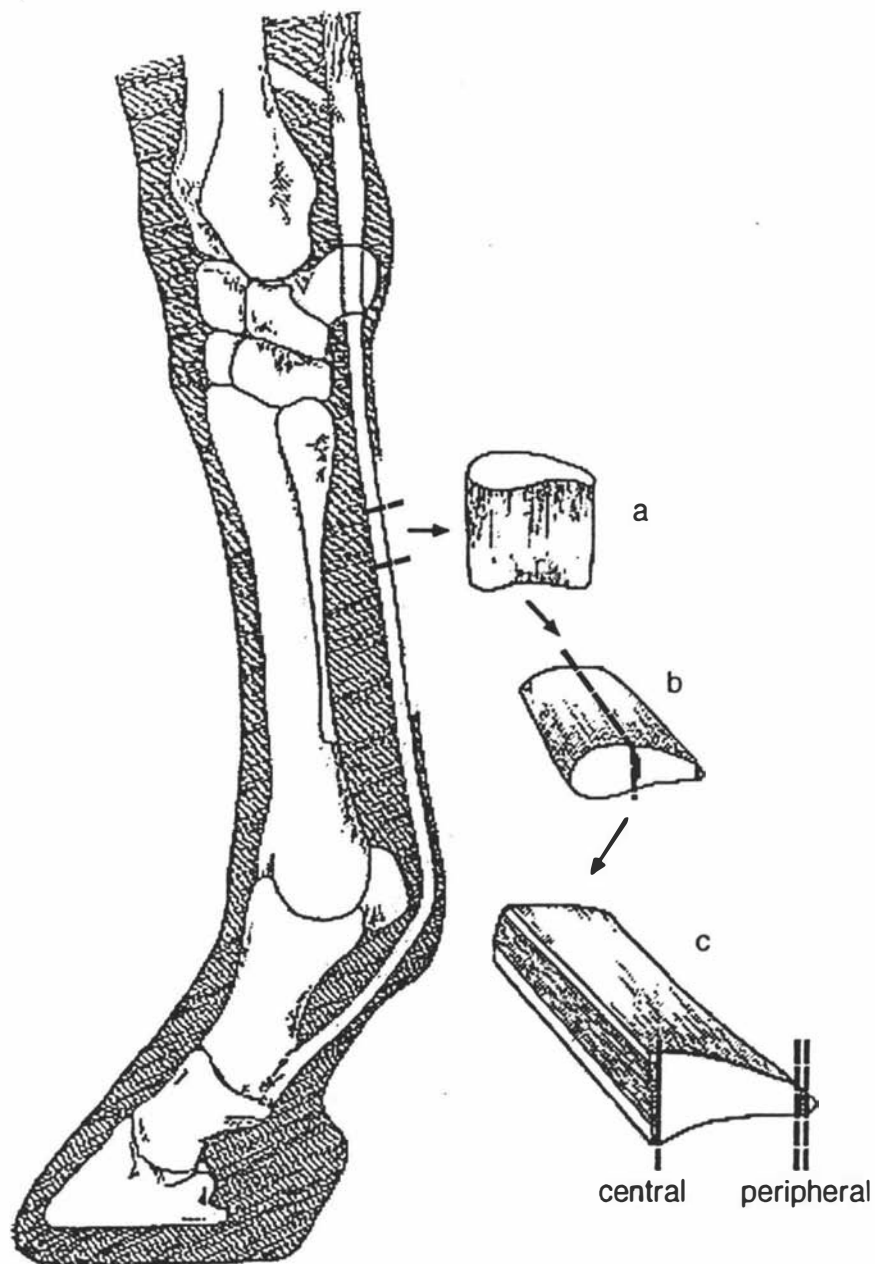
Figure 4.1 Details of the sampling procedure from the superficial digital flexor tendon. a - a 3 cm section was removed from a point just proximal to the midpoint of the metacarpus. b - the section was bisected sagittally. c - the lateral half was divided into blocks and central and peripheral sections cut.

light bands to a complementary series. The angle was measured at 50 different randomly selected sites from the 30-40 sections. The projected lengths of ten consecutive crimp periods were measured at each of the same 50 sites, using a calibrated eyepiece graticule in the microscope. The mean true crimp length for each distribution was calculated using the formula $l = d/\cos\theta$ (Figure 4.2). Mean crimp angles and lengths were compared between central and peripheral regions for each horse using paired-sample t-tests.

4.4 Results

Parameters were compared between regions for individual horses rather than between exercised and non-exercised groups, due to the large age ranges within each group and differences in exercise histories in the trained horses.

4.4.1 Exercised group (Table 4.1)

In the exercised group six of the nine horses sampled had a central region crimp angle which was significantly lower than that for the peripheral region. Only one of these horses was over the age of 10 years. Three of the six horses also had significantly lower crimp period lengths in the central region, these being horses c, e and g. Horses a, d and i showed central region angle changes only. Of the remaining exercised horses, 3 showed no difference in angle between the regions, with two of these showing a significantly higher crimp period length in the centre (f, h).

4.4.2 Un-exercised group (Table 4.2)

In the un-exercised group, three of the nine horses had a central region crimp angle which was significantly greater than that of the periphery (horses 3,5,6). One of these also had a significantly greater crimp period length in the central region (6). Four horses did not show significant differences in crimp angle between the regions (horses 1,2,4,7) but two of these did have a significantly greater crimp period length in the core region. The central region angles and
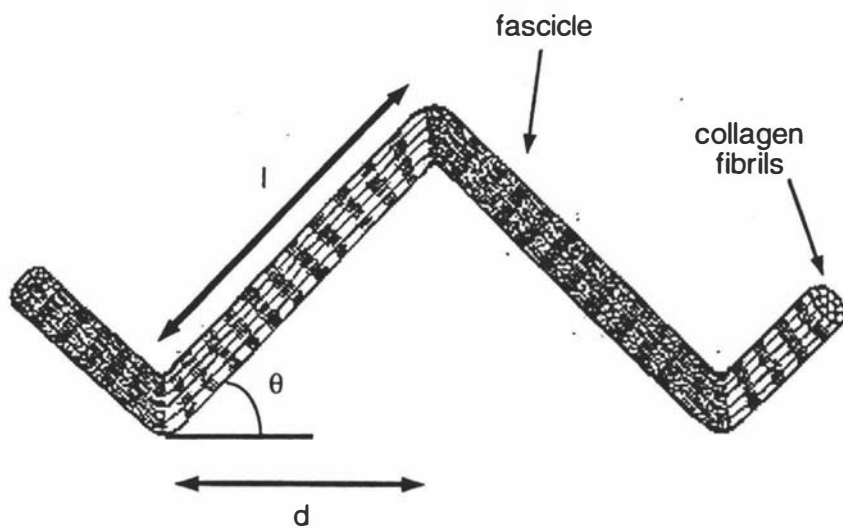
Figure 4.2 Definition of crimp angle and length measurements.
θ - crimp angle, d - measured crimp length, l - true crimp length.
l = d/cosθ

lengths were significantly lower than those for the periphery in the two remaining horses (horses 8,9), which were 10 and 14 years of age respectively.

Table 4.1 Crimp angles (degrees) and crimp lengths (µm) for central and peripheral regions of SDFTs from "exercised" horses (standard errors in brackets)

	Age	Function	Central angle	Peripheral angle	Central length	Peripheral length
a	2	In training	11.2(0.4)	13.0(0.3)*	17.6(0.5)	18.5(0.3)
b	3	"Race-fit"	13.1(0.3)	12.7(0.2)	16.5(0.4)	15.9(0.3)
c	4	In training	9.1(0.2)	10.5(0.3)*	20.1(0.4)	21.9(0.5)†
d	4	Minimal training	12.2(0.3)	13.0(0.3)*	21.3(0.6)	22.2(0.6)
e	5	Racing	13.4(0.3)	15.4(0.2)*	23.1(0.5)	25.7(0.5)†
f	6	Eventer	11.0(0.3)	11.0(0.3)	22.2(0.6)†	20.0(0.5)
g	7	Eventer	9.1(0.2)	11.8(0.3)*	22.8(0.5)	25.3(0.7)†
h	9	Polo "pony"	10.2(0.2)	9.8 (0.2)	22.7(0.4)†	21.0(0.4)
i	15	Eventer	11.1(0.3)	13.4(0.3)*	21.6(0.7)	21.9(0.7)

* crimp angle significantly greater than that for the corresponding region ($p<0.05$).
† crimp length significantly greater than that for the corresponding region ($p<0.05$).

4.5 Discussion

Tendon shows a typical non-linear stress-strain curve when stretched to failure *in vitro*. The "toe" region is a region of high compliance before the linear elastic region of the curve. It corresponds to straightening of the crimp waveform, so that the crimp angle is equal to zero and the fibrils themselves begin to be stretched at the beginning of the linear region. This means that if

a tendon is subjected to increasing strain, a bundle of fibrils with a lower crimp angle will straighten, be loaded and fail at lower strain levels than a bundle with a higher crimp angle (Figure 4.3). The crimp morphology therefore is important with respect to the mechanical behaviour of the tendon.

Table 4.2 Crimp angles (degrees) and crimp lengths (µm) for central and peripheral regions of SDFTs from "un-exercised" horses (standard errors in brackets)

	Age	Function	Central angle	Peripheral angle	Central length	Peripheral length
1	2	untrained	12.7(0.3)	12.6(0.3)	29.8(0.6)	30.9(0.6)
2	2	untrained	13.7(0.3)	13.2(0.3)	28.6(0.5)[†]	26.3(0.5)
3	3	untrained	14.3(0.4)[*]	12.3(0.3)	22.7(0.5)	23.3(0.5)
4	5	hack	13.2(0.3)	13.7(0.3)	30.4(0.4)	29.6(0.7)
5	7	dressage	8.3(0.2)[*]	7.1(0.2)	16.4(0.4)	16.8(0.4)
6	8	not in training	10.2(0.3)[*]	8.6(0.2)	20.8(0.6)[†]	13.1(0.4)
7	8.5	not in training	7.4(0.2)	7.4(0.2)	11.7(0.4)[†]	9.1(0.3)
8	10	not in training	10.5(0.3)	13.1(0.3)[*]	16.0(0.5)	19.6(0.7)[†]
9	14	brood-mare	9.0(0.2)	13.4(0.3)[*]	11.1(0.3)	23.1(0.7)[†]

[*] crimp angle significantly greater than that for the corresponding region ($p<0.05$).
[†] crimp length significantly greater than that for the corresponding region ($p<0.05$).

The previous observation of a reduction in central region crimp angle below that of the periphery in untrained horses of 10 years of age or over was interpreted as a fatigue effect resulting from a high number of loading cycles on the collagen fibrils over that time period, which eventually disrupted subfibrillar structure (Patterson-Kane *et al.* 1996b). In both wild horses (Patterson-Kane *et al.* 1996b) and thoroughbreds (Wilmink *et al.* 1992) younger than 10 years of age, the crimp angle for the central region was equal to or
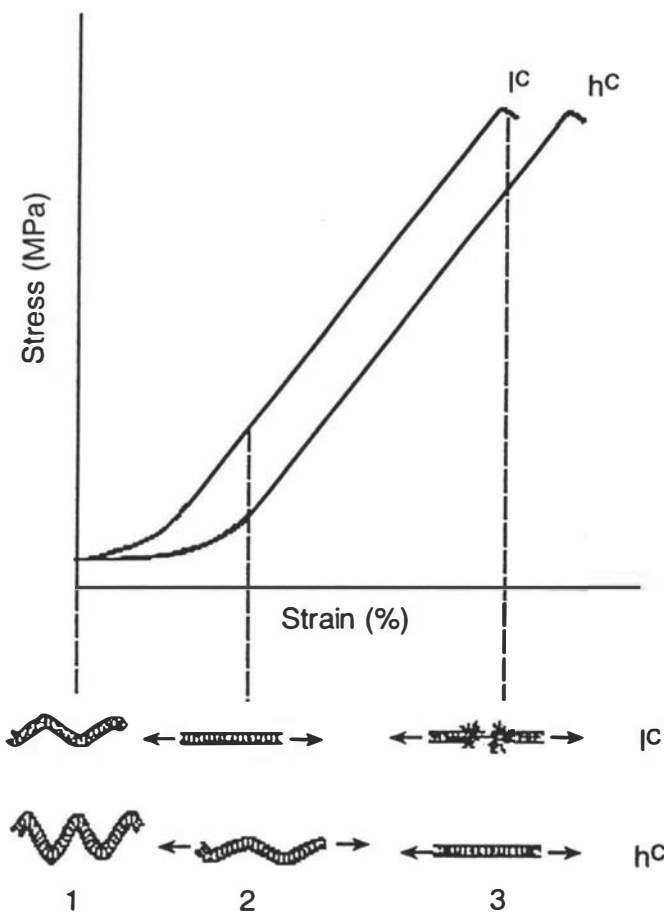
Figure 4.3 Typical stress-strain curve for tendon, showing the effect of variation in crimp angle. l^c = low crimp angle, h^c = high crimp angle. 1 - fibrils at zero strain. 2 - l^c has reached the linear region, whilst the crimp of h^c has not yet straightened. 3 - l^c has reached its elastic limit and failed, h^c is still in the linear region.

exceeded that of the periphery. In the un-exercised group in this study, 7 of the 9 horses were younger than 10 years, and central angles either exceeded or equalled peripheral angles in all of these animals. On the basis of the above findings, a reduction in central crimp angle below that of the periphery in this age group would be considered abnormal.

In the exercised group in this study, eight of the nine horses were under 10 years of age. Five of those eight horses had a peripheral region crimp angle which exceeded that of the central region. The higher number of rapid high-strain cycles experienced by horses in training appears to modify the fatigue mechanism in the central regions of SDFTs in some animals. Two of the five horses showing lower crimp angles in the centre than the periphery also had lower crimp period lengths in those regions. Previous observations that this had occurred in SDFTs from aged exercised thoroughbreds (Wilmink *et al.* 1992), but not in aged wild horses (Patterson-Kane *et al.* 1996b), led to the hypothesis that changes in both parameters in the tendon core represent a more severe grade of disruption of collagen fibril substructure (Patterson-Kane *et al.* 1996b). Some young thoroughbreds in this study therefore may have showed exacerbation of normal age-related changes in crimp morphology.

Three of the nine trained horses in this study did not show abnormality in the central region crimp morphology, and two of the five horses with reduced central crimp angle did not show changes in crimp length. It is possible that tendons of some animals are more resistant to exercise-induced changes, but that cannot be determined from this study as there was variation in ages and in training regimes, the exact details of which could not be obtained from the anecdotal information of owners.

Differences in the volume and intensity of exercise which individual horses experienced may also be responsible for the variation in crimp morphology changes. The relationship between reductions in crimp parameters in the SDFT core and different training histories requires investigation. Controlled studies of the effects of specific exercise regimes on collagen crimp patterns in SDFTs from horses of the same age would increase understanding of this relationship and possible identification of animals resistant to such changes in tendon structure. Resistance could be due to individual variation in distribution

of load onto the SDFT at a gallop, as a result of differences in the geometrical conformation of the limbs and in gait patterns. There may also be variability in tendon matrix composition and morphology.

The problem of obtaining detailed exercise histories from clinical cases is also important when considering the results from the horses over 10 years of age. The only two "un-exercised" horses which showed lower crimp angles in the central regions were 10 and 14 years of age respectively, and central crimp lengths in the two horses were also reduced below those of the periphery. The 15 year old in the exercised group showed a lower central angle only. The reduction in central crimp angle would be expected in horses of this age, but the reductions in length would not (Patterson-Kane *et al*. 1996b). Although the recent histories of unexercised horses did not involve competitive exercise they may have been used as performance horses previously. This is particularly likely in horses in this age group. It therefore is highly possible that exercise-related damage had occurred at an earlier age, and had lifetime exercise histories been recorded, it may have been more appropriate to assign the two aged un-exercised horses to the other group. Another implication of the above finding is that exercise-induced changes to collagen fibrils do not "heal" when the horse ceases competitive galloping activity. This may have been because sufficient time had not elapsed for this to occur, or because tenocytes in the tendon core are not capable of replacing significant quantities of collagen.

The SDFT has a low safety margin as, in addition to forming part of the passive collagenous supporting apparatus of the forelimb, it serves as an elastic energy store. The elastic energy stored in the tendon is reconverted to kinetic energy in the second half of the weight-bearing phase, and high strain levels must be present in the tendon to allow significant energy storage levels. It has therefore been suggested that the safety margins could be exceeded easily during galloping (McCullagh *et al*. 1979, Wlison and Goodship 1991). On the basis of the relationship of crimp angle to the mechanical behaviour of the tendon, it can be stated that a lower central crimp angle, which is abnormal in a young horse, will predispose the fibrils in that region of the SDFT to overstretching and further damage. This is particularly likely in a tendon with such a low safety margin. The microtrauma in the form of reduced crimp angle

could accumulate with further rapid high-strain cycles and eventually result in clinical tendonitis. This is not likely to be of significance in wild horses of 10 years of age or older, the tendons of which are unlikely to experience a further high number of rapid high-strain cycles, if they occur at all. Microtrauma is however of concern in young thoroughbreds in the initial stages of training or racing. One two year old horse in training (horse a) showed a reduction in central crimp angle normally seen only in horses at least eight years older. Such a horse would be likely to subsequently experience a large number of rapid high-strain cycles due to galloping activity which could result in microtrauma accumulation, culminating in the development of clinical tendonitis sometime during its racing career.

In conclusion, a high proportion of horses less than 10 years old with a known history of high-speed competitive activity showed lower crimp angles in the central region of the SDFT than in the periphery, which would predispose those central regions to further damage. This central region crimp angle reduction might be considered as evidence of microtrauma. It appears to be a modification of a normal age-related change, as no un-exercised horse under the age of 10 years showed this effect in either this or previous studies. The microtraumatic insult may be of greater severity in some horses, as indicated by additional significantly lower crimp period length in the central region in comparison with the periphery. Further definition of the relationship between specific exercise regimes and central crimp morphology changes may assist in understanding the pathogenesis of SDFT tendonitis, and in the development of training programmes to prevent predisposing microtraumatic change in the tendon core.

Chapter 5

Exercise-related alterations in crimp morphology in the central regions of superficial digital flexor tendons from young thoroughbreds: a controlled study[3]

5.1 Abstract

Injury to the core of the midmetacarpal region of the superficial digital flexor tendon in thoroughbred racehorses is a very frequent but poorly understood condition. It has been suggested that subclinical changes induced by galloping exercise weaken this region of the tendon, predisposing it to rupture. The crimp is a planar waveform followed by the longitudinally arranged collagen fibrils in tendon. Fibril bundles with a smaller crimp angle will fail at a lower level of strain than those with a larger crimp angle. This study tested the hypothesis that a specific 18 month exercise programme would result in significant reduction of collagen fibril crimp angle and period length in the core region of the superficial digital flexor tendon of young thoroughbreds (21±1 months), compared to the normal change in these parameters with age. Central region crimp angle and length were significantly lower in exercised horses than in control horses (p<0.05). The crimp angle was significantly lower in this central region than in the peripheral region of the tendon in four of the five exercised horses, as was the crimp length in three of the four horses. The crimp angle in the peripheral region was significantly greater in exercised horses than in the controls (p<0.05), which may indicate functional adaptation due to differing mechanical environment between the two tendon regions. The results of this study supported previous evidence that galloping exercise modifies normal age-related changes in crimp morphology in the core of the superficial digital flexor tendon. Such changes are indicative of microtrauma and would be detrimental to tendon strength.

5.2 Introduction

Injury to the superficial digital flexor tendon (SDFT) of the thoroughbred racehorse is a very common and often serious condition, which has been estimated to affect at least 30% of horses in training for the National Hunt in Europe (Goodship 1993). Recovery periods are often lengthy, and recurrence of injury on resumption of athletic activity is common (Rooney and Genovese 1981).

Lesions are generally localised to the core of the mid-metacarpal region of the forelimb tendon (Webbon 1977), often appearing on ultrasound examination as an anechoic or hypoechoic core surrounded by tissue of normal appearance (Genovese *et al.* 1985).

Knowledge of the pathogenesis of this condition is limited, despite its clinical importance. The mechanical factors influencing the tendon during galloping are thought to play a major role (McCullagh *et al.* 1979), due to its functions in preventing overextension of the fetlock (Goodship 1993) and in the storage of elastic energy (Dimery *et al.* 1986). Stephens *et al.* (1989) measured strains of 10-16% in SDFTs of galloping thoroughbreds using Hall-effect strain gauges. An *in vitro* study has indicated an elastic strain limit of approximately 10% (Wilson and Goodship 1991). This implies that to fulfil the energy storing role, the SDFT must have a very low safety margin during the weight-bearing phase of the gallop.

Several recent studies have suggested that galloping exercise causes microtrauma to the core region of the tendon (Wilmink *et al.* 1992, Birch 1993, Patterson-Kane *et al.* 1996a, 1996c). Accumulation of microtrauma is hypothesized to weaken the tendon, eventually resulting in partial or complete rupture, accompanied by clinical signs. Relationships between the intensity and duration of galloping exercise and the occurrence of tendon core microtrauma have not yet been determined, nor have the mechanisms by which it occurs.

The fundamental units of tensile strength in the tendon are the collagen fibrils. These are submicroscopic cylindrical structures composed of mainly type I collagen molecules in a specific axial and lateral arrangement. The fibrils

follow a planar zig-zag waveform along the longitudinal axis of the tendon, which is termed the 'crimp' (Diamant *et al.* 1972). This is a feature of fibril bundles rather than isolated fibrils, and may be conveniently quantified by the crimp angle and crimp period length as measured at the level of the fascicle using polarising optics (Diamant *et al.* 1972). The crimp morphology is related to the mechanical behaviour of the tendon; collagen fibril bundles with a smaller crimp angle will experience higher levels of stress at a given level of strain and will fail before those with a larger crimp angle (Wilmink *et al.* 1992). Crimp angles in the tendons of some young thoroughbreds with variable histories of galloping exercise have been shown to be lower in the centre of the SDFT than in the periphery (Patterson-Kane *et al.* 1996c). The crimp period lengths in some of these horses were also lower in the tendon core. Reduction of crimp angle in the centre of the tendon below that of the periphery in horses known not to have undergone any such training was only seen in horses aged 10 years or more, and no such reductions in central region crimp length were seen in untrained horses in any age group (Wilmink *et al.* 1992, Patterson-Kane *et al.* 1996b, 1996c).

On the basis of the above findings, it has been hypothesized that galloping exercise modifies the age-related reduction in central crimp angle in the SDFTs of young horses due to fatigue of the collagen fibril structure induced by rapid high-strain cycles, and that additional changes in crimp period length in that region indicate more severe disruption (Patterson-Kane *et al.* 1996c). A reduction in crimp angle in the tendon core may be interpreted as evidence of microtrauma, which predisposes the collagen fibrils in that region to overloading and further structural damage.

The relationship between exercise duration and intensity and changes in crimp morphology could not be determined in a previous study due to the wide age ranges available for analysis and the unavoidably variable and incomplete nature of exercise histories obtained from the clinical cases submitted for necropsy (Patterson-Kane *et al.* 1996c). For the same reasons, any variation in individual susceptibility to such changes could not be determined. The aim of this study was to determine the effects of a specific training regime, involving galloping, on crimp angle and crimp period length in central and peripheral

regions of the SDFT, using age-matched and unexercised controls. It was hypothesized that at the end of the 18 month period, crimp angle and length in the central region would have reduced below the values for the peripheral region, in the tendons of the exercised horses. Crimp angles and lengths in peripheral regions were predicted not to be affected by the training regime.

5.3 Materials and methods

5.3.1 Horses and training programme

Twelve thoroughbred fillies were divided randomly into two groups, one of which underwent a specific 18 month controlled exercise programme on a high-speed equine treadmill (Sato, Sweden). This involved galloping exercise three days per week; Monday 4 km @ 12 m/s, 3% slope, Wednesday two times 1.5 km @ 12 m/s and 14 m/s, 4% slope, 5 minutes recovery, Friday three times 1 km @ 12 m/s, 13.5 m/s, 15 m/s, 3% slope, 5 minutes recovery. This was combined with ten minutes trotting on a mechanical horse walker three times weekly and walking exercise on the horse walker (40 minutes) for six days per week. The control group undertook walking exercise only, on six days per week, for 40 minutes per day.

The horses were 39±1 months of age at the time of tissue sampling. One horse had been withdrawn from the trained group before the end of the trial, due to poor adaptation to the treadmill exercise.

5.3.2 Tissue sampling and processing

A 2 cm longitudinal section was cut from the left forelimb SDFT of each horse, from a point 2 cm proximal to the midpoint of the metacarpus (Figure 5.1). Each sample was fixed in 10% neutral buffered formal saline. They were dehydrated in a Shandon Southern 2L Processor Mark II (Life Sciences (U.K.) Ltd), then vacuum embedded in three changes of wax (Paramat Extra Wax 'Gurr'), and blocked out in fresh wax.

Four 5 μm thick longitudinal sections were cut in the latero-medial plane from

the centre of each sample (Figure 5.1) and mounted on glass slides. They were stained with haematoxylin and eosin (HE) and mounted in DPX.

5.3.3 Crimp morphometry

Sections were visualised using a Leitz Wetzlar polarising light microscope. Crimp angle was measured at 50 sites for each tendon region using the method of Diamant *et al.* (1972). The projected lengths of 10 consecutive crimp segments were measured using a calibrated eyepiece graticule in the microscope. This measurement was also carried out at 50 sites. The true mean crimp length for each tendon region was calculated from the mean projected crimp length and the mean crimp angle (Figure 5.2).

5.3.4 Statistical analysis

Mean crimp angles and lengths were compared between groups for each region using t-tests for independent samples. Mean and individual angles and lengths were compared between central and peripheral regions within groups and within individual horses respectively, using paired-sample t-tests.

5.4 Results

5.4.1 Comparison of exercised and control groups

For the central region, the mean crimp angle of $4.7\pm0.1^\circ$ for the exercised group was significantly lower than that of $5.3\pm0.1^\circ$ for the control group ($p<0.05$) (Table 5.1). The mean crimp length of 24.5 ± 2.7 µm for the exercised group was significantly lower than that of 33.2 ± 1.0 µm for the control group ($p<0.05$)(Table 5.2).

For the peripheral region, the mean crimp angle of $5.6\pm0.4^\circ$ in the exercised group was significantly higher than that of $4.6\pm0.1^\circ$ in the control group ($p<0.05$). The mean crimp lengths did not differ significantly, and this was not
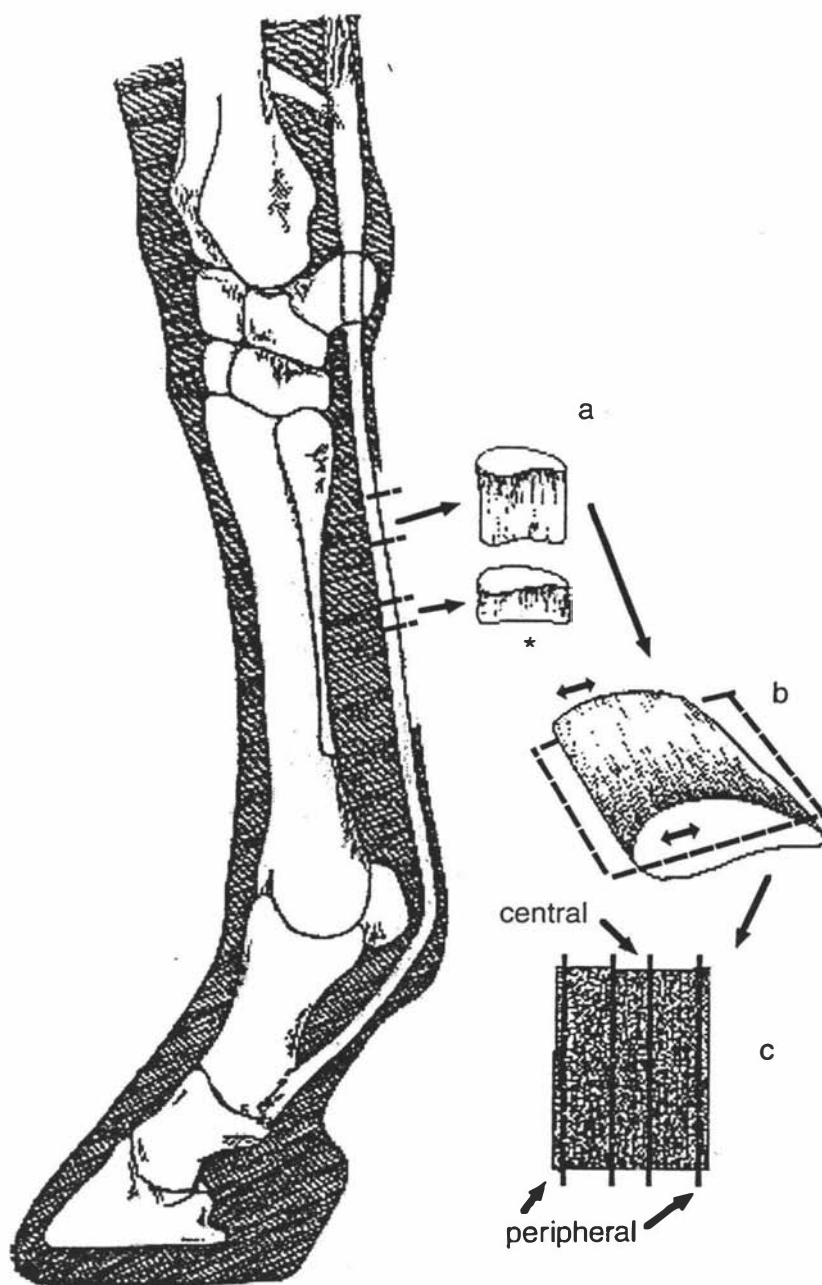
Figure 5.1 Details of the sampling and sectioning procedure.
* - sample for electron microscopy (Patterson-Kane *et al.* 1996a).
a - a 2 cm section was cut from above the midpoint of the metacarpus.
b - sections cut in latero-medial orientation. c - a section, from which
central and peripheral measurements were taken.
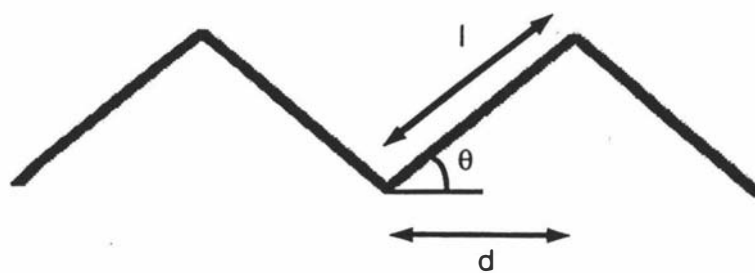
Figure 5.2 Definition of crimp angle and length measurements.
θ - crimp angle, d - projected crimp length, l - true crimp length.
l = d/cos θ.

affected by the apparently abnormal value of 17.6 µm for one of the control horses in this region (horse 2).

5.4.2 Comparison of central and peripheral regions within groups

In the exercised horses, the mean crimp angles of 4.7±0.1° and 5.6±0.4° for central and peripheral regions respectively, did not differ significantly (p=0.065) (Table 5.1). This was probably due to high individual variation in the latter region. The group mean lengths also did not differ significantly between the two regions (Table 5.2). Analysis of regional data for individual horses within this group showed the central crimp angle to be significantly lower than the peripheral crimp angle in four of the five horses (p<0.001). Three of these four horses also has significantly lower crimp lengths in the central region (p<0.05). In the remaining horse (number 5), both the central angle and length were significantly greater than their values in the periphery (p<0.05, p<0.05). In the control horses, the mean angle of 5.3±0.1° for the central region was significantly higher than that of 4.6±0.1° for the peripheral region. Data pertaining to individual horses showed crimp angle and length in the central region to either exceed or equal that in the peripheral region.

5.5 Discussion

The crimp angles measured in this study were considerably lower than those measured in horses of a similar age in previous studies which used either isolated fascicles (Wilmink *et al*. 1992), frozen sections (Patterson-Kane *et al*. 1996b, 1996c) or wax-embedded and HE stained sections (Williams *et al*. 1985). Alcohol dehydration is expected to change crimp parameters by a small consistent amount (Nicholls *et al*. 1983, 1984), but the results in this study were even lower than those from samples processed in a similar fashion (Williams *et al*. 1985). It is possible that the vacuum used during the embedding of these samples straightened the crimp slightly. Regardless of that fact, the samples were processed at the same time, so the results are comparable between groups and horses.

Table 5.1 **Crimp angles** (degrees) **for central** and **peripheral regions of the SDFT from exercised and non-exercised horses (standard errors in brackets).**

	Horse	Central Angle	Peripheral Angle
Controls	2	5.7 (0.1)	4.4 (0.1)
	4	5.3 (0.1)	5.0 (0.1)
	6	5.0 (0.1)	4.5 (0.1)
	8	5.5 (0.1)	4.5 (0.1)
	10	4.9 (0.1)	4.6 (0.1)
	12	5.2 (0.1)	4.4 (0.1)
	Mean	**5.3 (0.1)***	**4.6 (0.1)**
Exercised	1	4.5 (0.1)	5.4 (0.1)
	3	5.0 (0.1)	6.8 (0.2)
	5	5.0 (0.1)	4.6 (0.1)
	7	4.4 (0.1)	5.7 (0.1)
	11	4.7 (0.1)	5.7 (0.1)
	Mean	**4.7 (0.1)†**	**5.6 (0.4)†**

* differs significantly from the peripheral value for the group
† differs significantly from the control value for that region.

When mechanically tested in vitro, tendon shows a typical non-linear stress-strain curve (Butler *et al.* 1978), the initial compliant region of which is termed the "toe". This region corresponds to opening out of the crimp, so when the "toe limit strain" is reached the straightened fibrils are stretched and the tendon begins to behave in a linear elastic fashion. A collagen fibril bundle with a low crimp angle will reach its elastic limit at the end of the linear region at a lower level of strain than a bundle with a larger crimp angle (Wilmink *et al.* 1992). In untrained horses, a crimp angle which is significantly lower in the tendon core than in the periphery has been observed only in animals 10 years old or more (Patterson-Kane *et al.* 1996b). Lower crimp angles in the tendon core have also been observed in younger horses, but only in those animals with a recent history of galloping exercise (Patterson-Kane *et al.* 1996c). It has been suggested that this represents an acceleration of collagen fibril fatigue, induced

by the rapid high-strain cycles during galloping (Patterson-Kane *et al.* 1996c).

Table 5.2 Crimp period lengths (μm) for central and peripheral regions of the SDFT from exercised and non-exercised horses (standard errors in brackets)

	Horse	Central length	Peripheral length
Controls	2	33.3 (0.7)	17.6 (0.5)
	4	36.0 (0.5)	37.0 (0.7)
	6	36.1 (0.5)	37.8 (0.9)
	8	30.9 (0.7)	31.8 (0.7)
	10	31.0 (0.6)	29.7 (0.8)
	12	31.7 (0.4)	23.4 (0.8)
	Mean	**33.2 (1.0)**	**29.6 (3.2)**
Exercised	1	20.3 (0.5)	32.1 (0.7)
	3	20.5 (0.5)	26.6 (0.6)
	5	31.6 (0.6)	25.0 (0.9)
	7	25.6 (0.6)	28.3 (0.9)
	11	29.5 (0.5)	30.1 (0.6)
	Mean	**24.5 (2.7)†**	**28.4 (1.3)**

† differs significantly from the control value for that region

The relationship between crimp angle and mechanical properties implies that such changes predispose the tendon cores to overloading, and therefore provide evidence of likely microtrauma.

The present study allowed controlled assessment of the effect of a clearly defined exercise regime on crimp parameters in horses of the same age. Central crimp angle and length in untrained horses equalled or exceeded that in the periphery. This is in agreement with previous measurements from untrained horses under 10 years of age (Wilmink *et al.* 1992, Patterson-Kane *et al.* 1996b, 1996c). The mean central crimp angle in exercised horses was significantly lower than that for control horses. In four of the exercised horses, the central angle was significantly lower than that in the peripheral region of the

same tendon. These data support previous findings which indicated that exercise modified age-related changes in central crimp morphology. A previous study from the same exercise trial showed significant reductions in collagen fibril mass-average diameters in the same regions, which also indicates structural weakening and was interpreted as further evidence of microtrauma (Patterson-Kane *et al.* 1996a).

The mean crimp length for the central region in the exercised horses was also significantly lower than that for control horses; in three exercised horses, the central crimp length was significantly lower than that for the peripheral region in the same tendon. This additional reduction in central region crimp length has been observed previously in both young (Patterson-Kane *et al.* 1996c) and aged (Wilmink *et al.* 1992, Patterson-Kane *et al.* 1996c) thoroughbreds, and was suggested to represent more severe disruption of fibril structure (Patterson-Kane *et al.* 1996c).

Patterson-Kane *et al.* (1996c) have previously suggested that certain horses may be less susceptible to alterations in central crimp morphology, but were unable to provide evidence for this due to the limited nature of the exercise histories in that study. Horse number 5 in the exercised group in this study had a significantly greater crimp angle and length in the central region than in the periphery ($p<0.05$, $p<0.05$). Horse number 11 showed a typically lower crimp angle in the centre ($p<0.001$), but the crimp length did not differ significantly between the regions. All horses in the exercised group underwent exactly the same training regime. These results therefore imply that tendon structure in some horses is less susceptible to disruption of the crimp morphology. This could be due to different distributions in loading between SDFT, DDFT and SL between individuals (Jansen *et al.* 1993) for reasons of conformation and/or gait. It could also imply differences in matrix composition or rates of collagen turnover.

Three horses in the exercised group (1,7 and 11) showed a reduction in central region collagen fibril mass-average diameter (Patterson-Kane *et al.* 1996a) and crimp angle below their values in the periphery. Horse 5 showed a reduction in the mass-average diameter but not the crimp angle in the central region, and horse 3 showed a reduction in crimp angle but not in mass-average diameter.

The mechanisms involved in crimp morphology changes and alterations in diameters of the collagen fibrils themselves may be independent and influenced by different factors. Alternatively, they may be related, with an apparent anomaly occurring as a result of the 2.5 cm separation between samples for fibril diameter distribution analysis and crimp morphometry analysis (see Figure 5.1).

The significant increase in crimp angle in the peripheral region of exercised tendons above that of the same region in the controls was an unexpected finding. Wood *et al*. (1988) measured an increase in crimp angle in hindlimb superficial digital flexor tendons of treadmill-exercised rats, but it was not clear if this was a genuine exercise-induced effect or an artefact due to the method of selection of animals for exercise and control groups. The results of the present study suggest that it is a response to exercise, as animals were randomly divided between the two groups. Peripheral fibril bundles with a higher crimp angle would be subjected to lower stresses than central fibril bundles if strain is experienced uniformly across the tendon cross-section. It is therefore possible that peripheral angle is an adaptive change which would decrease the susceptibility of that region to overloading. This is of great interest, as functional adaptation to galloping exercise has not previously been demonstrated in the equine SDFT.

In conclusion, the results of this study support the hypothesis that rapid high-strain cycling experienced by the SDFT of young thoroughbreds subjected to a uniform galloping exercise regime causes reduction of crimp angle in the tendon core, as well as reduction of crimp period length in some horses. These changes are disadvantageous to the mechanical properties of this region and thus may be classified as evidence of microtrauma, which will predispose the tendon to further overloading and damage.

Definition of training regimes which do not adversely affect central region crimp morphology, and identification of individuals which are less susceptible to these effects, would assist in prevention of SDFT tendonitis.

Chapter 6

Comparison of collagen fibril populations in the superficial digital flexor tendons of exercised and non-exercised thoroughbreds[4]

6.1 Abstract

The superficial digital flexor tendon (SDFT) is the most frequently injured of the digital flexor structures in racing thoroughbreds, with lesions most commonly occurring in the core of the mid-metacarpal region of the forelimb tendon. The periphery of the tendon often appears to be unaffected.

It is not known how the ultrastructure of the SDFT is influenced by defined levels of training. If functional adaptation occurred in response to exercise as seen in muscle and bone, then appropriate training regimens could be developed to minimise injury. Alternatively, evidence of damage in tendons subjected to a specific programme would provide important information on the pathophysiology of tendonitis.

Collagen fibrils are the submicroscopic units of strength in tendon, and this study was undertaken to test the hypothesis that they would hypertrophy in response to a specific defined training programme. Fibril diameters were measured in central and peripheral regions of SDFT samples from five 18 month old horses which had undergone an 18 month training programme, and six age and sex-matched controls.

Central region fibrils from the trained horses had a mass-average diameter (MAD) of 105.3 nm, which was significantly lower ($p < 0.01$) than that of 131.7 nm for the same region in the control horses. This reduction in fibril diameter in the region of tendon which is predisposed to injury was interpreted as evidence of microtrauma, as it implies the region is weakened by the training regimen. Repeated episodes of microtrauma may accumulate and eventually result in degenerative lesions and clinical tendonitis.

The training programme used was neither extreme nor lengthy, in comparison with a National Hunt racing career. It is possible that training of a different type or duration may not have this effect. If a means of detecting microdamage could be developed, and training programmes found which would induce appropriate functional adaptation, then significant advances could be made in the prevention of clinical tendonitis.

6.2 Introduction

Superficial digital flexor tendon (SDFT) injury is a major cause of wastage in thoroughbred racehorses (Rooney and Genovese 1981; Rossdale *et al*. 1985; Robinson and Gordon 1988). A typical lesion involves the central area of the cross-section of the SDFT in the mid-metacarpal region of the forelimb, with the more peripheral regions being apparently unaffected (Webbon 1977).

The SDFT is loaded early in the support phase of the stride, and this loading increases in magnitude with the speed of the gait (Stephens *et al*. 1989; Platt *et al*. 1994). Overloading and consequent overstretching of the SDFT during galloping is suggested to be a major aetiological factor in the development of lesions (McIlwraith 1987). This is because in addition to their supportive functions, the SDFT and other collagenous flexor structures improve the efficiency of locomotion by storing elastic energy when stretched (Alexander 1988). The elastic recoil in the latter half of the stance phase helps to propel the horse forward and reduces expenditure of metabolic energy by muscula-ture. In order to store useful amounts of elastic energy, tendons must undergo high strains. This means that energy-storing tendons have low safety margins which may be exceeded easily by the repetitive loading in the galloping equine athlete, with consequent damage to tendon structure (McCullagh *et al*. 1979; Wilson and Goodship 1991). The high frequency of lesions in the SDFTs of performance thoroughbreds supports this hypothesis (Webbon 1973, 1977; McIlwraith 1987).

The exact pathogenesis of clinical SDF tendonitis has not been defined. There may be prior degenerative change resulting from cumulative 'microtrauma' due to repetitive episodes of cyclic overloading (Stromberg 1971; Fackelman 1973; Kraus-Hansen *et al* 1992; Becker *et al* 1994; Wilson and Goodship 1994). Clinically observable tendonitis would then result from failure of the degener-ated, and therefore weakened, structure under the appropriate mechanical circumstances. This is a more likely scenario than the catastrophic overload of a previously healthy tendon. The concept of pre-existing degenerative change is supported by the findings of SDFT lesions in clinically sound horses, which appear as a reddish discolouration in the tendon core (Webbon 1977).

It has been recognised for many years that bone is responsive to alterations in mechanical loading, with Wolff's "law of bone remodelling" being described in 1884 (Wolff 1986). There is much evidence that strain histories regulate gene expression in both bone and muscle, so that appropriate training programmes can result in physiological adaptation (see Pearson 1990, Maffulli and King 1992 for reviews). If such functional adaptation is possible in equine tendon there is potential for the development of specific training programmes in order to minimise the incidence of injury. Experiments involving translocation of rabbit digital flexor tendons (Gillard *et al.* 1979a) illustrated the fact that tendons are capable of very rapid alterations in structure, with observable reorganisation of the extracellular matrix occurring within 14 days of the change in mechanical environment. In vitro studies have shown cultured embryonic chick fibroblasts to be responsive to charged synthetic polymers (Gillard *et al.* 1979b) and cultured equine SDFT fibroblasts to be responsive to short periods of heating (Birch 1993). The potential for cellular response to alterations in environment due to exercise therefore exists, resulting in altered synthesis and\or degradation of extracellular matrix components, which will in turn affect the mechanical characteristics of the tendon.

General information on functional adaptation of tendon to exercise in terms of size and/or strength is very limited, and observed responses to training have been variable (Inglemark 1948; Barfred 1971; Tipton *et al.* 1975; Woo *et al.* 1980; Wood *et al.* 1988; Wilson 1991). Previous studies on the effect of exercise on equine tendon have not involved analysis of ultrastructural features (Bramlage *et al.* 1989; Gillis *et al.* 1993).

Mammalian tendons have a complex hierarchical structure (Kastelic *et al.* 1978). They are composed of longitudinally arranged units termed fascicles. The fascicles are composed of longitudinally oriented, submicroscopic collagen fibrils and the extrafibrillar matrix between them. This matrix is comprised of proteoglycans, glycoproteins, small amounts of elastin, simple ions and water. In normal mature tendon the extrafibrillar matrix is of small volume, surrounding tenocytes, elastic fibres and the collagen fibrils. Collagen fibrils in tendon are cylindrical structures of at least several millimetres in length (Parry and Craig 1984), consisting of covalently cross-linked type I collagen molecules. In the

equine SDFT collagen fibrils may be classified as "large" or "small" diameter, resulting in a bimodal diameter-frequency distribution with peaks at 35-40 nm and 165-215 nm in the adult horse (Parry *et al.* 1978b). The collagen fibrils are regarded as the units of tensile strength of the tendon but the extrafibrillar matrix, in particular the proteoglycans, does appear to exert some influence on the mechanical behaviour of the tendon by maintaining inter-fibrillar cohesion (Cribb and Scott 1994).

The effects of training on collagen fibril morphology have been variable in previous investigations involving other species (Oakes *et al.* 1981; Michna 1984; Wilson 1991; Enwemeka *et al.* 1992). No investigation of the response of these structures in equine tendon has been carried out. This study was undertaken to test the hypothesis that collagen fibrils in the equine SDFT would undergo hypertrophy in response to a specific defined 18 month training programme.

6.3 Materials and methods

6.3.1 Horses

Twelve thoroughbred fillies were divided randomly into two groups. Six underwent a specific training programme, however one was withdrawn from the study due to poor adaptation to treadmill exercise. The remaining six horses served as the control group. The horses were 39±1 months at the time of tissue sampling. The mean final body-weights were 504±32 kg for the control group and 452±21 kg for the exercised group.

6.3.2 Training programme

The trained group underwent an 18 month controlled exercise programme on a high-speed equine treadmill (Sato, Sweden). In a typical week the regimen was as follows: Monday 4 km @ 12 m/s, 3% slope; Wednesday two times 1.5 km @ 12 m/s and 14 m/s, 4% slope, 5 minutes recovery; Friday three times 1 km @ 12 m/s, 13.5 m/s, 15 m/s, 3% slope, 5 minutes recovery. This was

combined with ten minutes trotting on a mechanical horse walker three times weekly and walking exercise on the horse walker (40 minutes) for six days per week. The control group undertook walking exercise only, on six days per week, for 40 minutes per day.

6.3.3 Tissue processing and electron microscopy

A section one cm in length was excised from each tendon from the longitudinal midpoint of the metacarpus. Strips 2mm x 1mm^2 were dissected from both the centre of the cross-section and from a peripheral location approximately one mm from the surface on the lateral aspect of the tendon (see Figure 6.1). These central and peripheral strips were fixed conventionally for electron microscopy and embedded in Taab 812 resin. Silver-gold sections (90-130 nm) were cut from one block for each tendon region using a diamond knife.
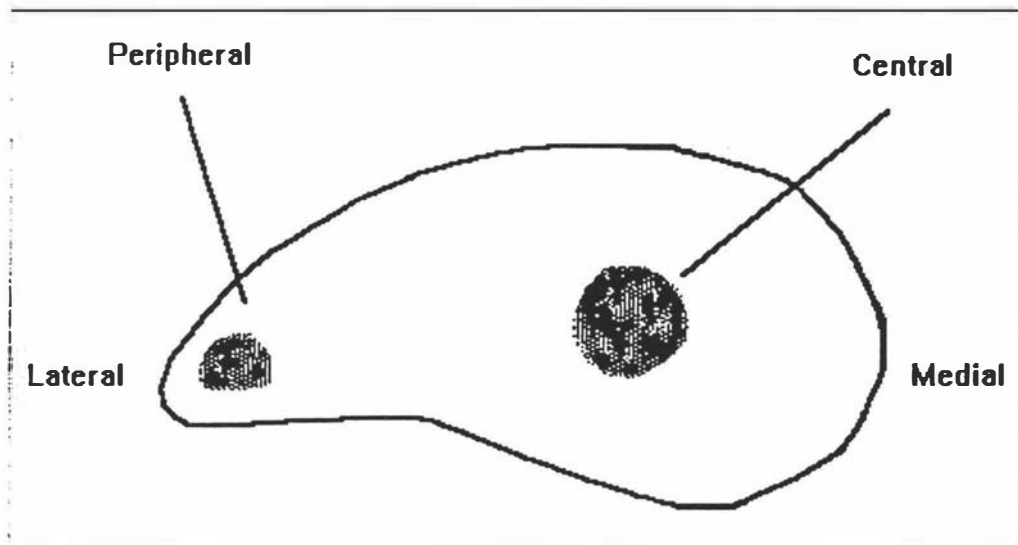
Figure 6.1 Sampling positions from the tendon cross-section

They were mounted on unsupported 400 mesh copper grids and stained in uranyl acetate followed by modified lead citrate (Sato, 1967).

The sections were viewed using a Philips E.M. 201c high resolution electron

microscope. One representative section was examined for the central region and one for the peripheral region of each tendon. The analysis programme required uniform and dark staining of fibrils, a certain level of contrast between fibrils and extrafibrillar matrix, and reasonable separation of fibrils from one another. Areas containing elastic fibres, tenocytes and certain stain artifacts were avoided as the programme could not distinguish these from collagen fibrils. Areas of each section of which micrographs were taken for analysis were selected randomly within these constraints. Three to seven micrographs were taken per section, which was sufficient to allow analysis of at least 1000 collagen fibril diameters for both regions.

A carbon grating replica of 2160 lines/mm was used to calibrate each set of micrographs. All magnifications were within 4% of 48000 times.

6.3.4 Collagen fibril morphometry

The micrographs were analyzed using a VIPS-based computer programme (Visual Image Processing System), which measured the collagen fibril diameters and areas. The total area of each image was also recorded and from this the percentage of each image area occupied by collagen fibrils calculated, the collagen fibril index (CFI) (Enwemeka *et al.* 1992). A diameter-frequency distribution was calculated from this data and entered into a computer programme developed by D. A. D. Parry. This programme uses the calibration factor to convert the diameter measurements to nanometres and then calculates the percentage of total measured fibril area occupied by each diameter group. The MAD of a collagen fibril population is derived from the diameter versus percentage of fibril area distribution.

6.3.5 Statistical analysis

It has been determined that 1000 fibril diameter measurements are sufficient to obtain a histogram which is truly representative of the diameter distribution (Parry and Craig 1977).

Mass-average diameters and CFIs were compared between groups using the

t-test for independent samples, and between central and peripheral regions within groups using the paired sample t-test.

Diameter-area distributions were compared between central and peripheral regions for individual horses from the exercised group, using the one-tailed two-sample Kolmogorov-Smirnov test (Siegel and Castellan 1988). This nonparametric test was used because the distributions were shown to be bimodal, using the method of McLachlan and Basford (1988). The one-tailed test was used on the basis of differences between MADs for the two regions in individual horses.

The maximal diameter for each distribution was recorded, and these were compared between groups and regions using the independent and paired sample t-tests respectively.

6.4 Results

The mean final bodyweight for the exercised group was significantly lower than that for the control group ($p < 0.05$).

The MAD of 105.3 ± 4.3 nm for the central region of the exercised group was significantly lower than that of 131.7 ± 4.9 nm for the same region in the controls (Table 6.1). The maximal diameters were significantly lower for the central regions of the control group ($p < 0.05$), but the corresponding CFIs did not differ significantly, the mean value being 71.7% for the control group, and 66.2% for the exercised group.

MADs for three horses from the exercised group (numbers 1, 7 and 11) were significantly higher in the peripheral region in comparison with the central region ($p < 0.05$) and higher but not significantly so in another horse (number 5). The remaining horse (number 3) had a MAD for the central region which was significantly higher than that for the peripheral region ($p < 0.05$).

6.5 Discussion

The lower final bodyweights in the trained animals are a typical finding in such studies (Tipton *et al.* 1975; Woo *et al.* 1980).

Parry *et al.* (1978a) hypothesised that large diameter fibrils have greater tensile strength due to the higher density of intrafibrillar covalent cross-linkages between collagen molecules. These cross-links prevent slippage between molecules, and possibly subfibrillar units, when the collagen is subject to tension. Larger fibrils have a lower surface area to mass ratio and therefore a lower proportion of peripheral molecules. These peripheral molecules are

Table 6.1 Mass-average diameters and standard errors (in nm) for collagen fibril populations from central and peripheral regions of superficial digital flexor tendons from control and exercised horses

	Horse	Central	Peripheral
Controls	2	119.1	74.8
	4	136.8	107.7
	6	126.8	150.8
	8	127.8	140.8
	10	153.5	104.3
	12	126.0	128.1
	Mean	**131.7 (4.9)**	**117.8 (11.3)**
Exercised	1	110.5	140.0
	3	117.2	86.0
	5	106.3	123.9
	7	100.8	133.2
	11	91.7	152.3
	Mean	**105.3 (4.3)**	**127.1 (11.3)**

unable to form their full complement of intermolecular cross-links. The tensile strength of a large fibril should therefore be greater than that of an equivalent mass of small fibrils. This is accounted for by the mass-average diameter, which is effectively the mean of the diameter-area distribution and allows for the fact that a small number of large diameter fibrils can occupy a large

proportion of the mass of collagen in a tendon cross-section. As these large fibrils have a greater tensile strength per unit area, this parameter is more relevant than a simple mean diameter when considering the fibril diameter distribution in relation to mechanical strength. The smaller fibrils with their greater surface area are more important in terms of fibril-ground substance interaction. The viscous resistance provided by this interaction acts to prevent slippage between the fibrils themselves, endowing the tendon with elasticity. The equine SDFT may experience stresses in the region of the theoretical failure range of 5 to 10 kg/mm^2 during galloping (McCullagh *et al.* 1979), but also requires elasticity to serve its function as an elastic energy store. The SDFT therefore requires an appropriate mixture of small and large fibrils providing an ultrastructure with both strength and elasticity. A fibril population of this type also allows a higher CFI as smaller fibrils can pack into spaces between larger ones.

Both the mass-average diameter and percentage of collagen (CFI) have been positively correlated with the in vitro ultimate strength of tendon (Parry and Craig 1988, Oakes 1989).

An appropriate increase in the magnitude and duration of tensile loads on the SDFT should result in fibrillar hypertrophy if functional adaptation can occur in this tendon. An increase in the mass-average diameter indicates strengthening of the tendon structure. This cannot occur to such an extent as to exclude small diameter fibrils because the reduction in CFI and interfibrillar slippage that this would entail would negate the tendency to an increase in tensile strength and also reduce elastic energy storage. An increase in CFI due to increased diameters of large fibrils and packing of small fibrils between them would also be a desirable response to training.

In the core regions of the SDFTs of the trained horses, where lesions are known to occur with high frequency, there was a significant reduction in mass-average diameter. In three of the five exercised horses the MAD for this region was reduced significantly below that for the peripheral region. The changes in the mean central region MAD occurred due to a reduction in diameters of the largest fibrils, and an increase in the number of small fibrils (Figure 6.2). The increase in the number of small fibrils would in theory confer greater elasticity
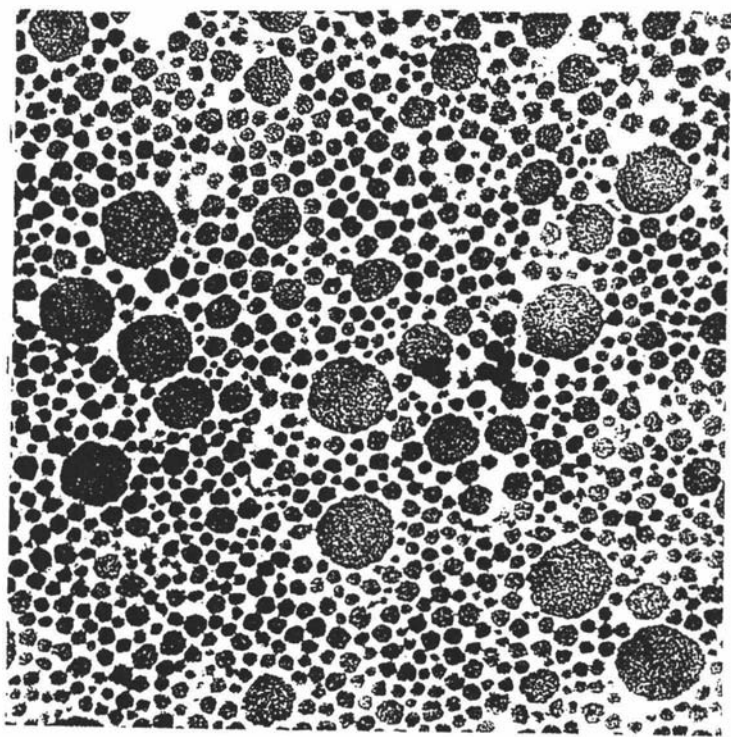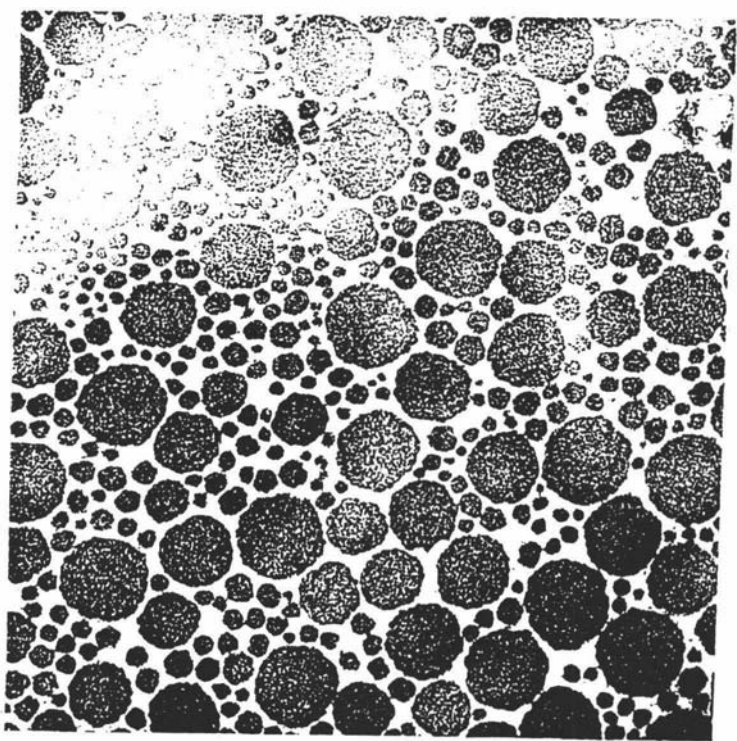
Figure 6.2 Transverse sections from the central region of an exercised SDFT (a) and the peripheral region of the same tendon (b), 45 100x. The central region contains fewer larger fibrils.

and therefore greater potential to store elastic energy. More importantly, however, it would reduce tendon tensile strength in the core region, making it more prone to injury when repeatedly exposed to high tensile forces during galloping.

Similar changes in fibril morphology have been seen in exercise studies involving other species. Oakes *et al.* (1981) demonstrated reductions in mass-average diameters in the anterior cruciate ligaments of exercised rats. The amount of collagen (CFI) did not alter but there was significant remodelling resulting in populations of smaller but more numerous fibrils. Michna (1984) demonstrated significant increases in mean diameters and CFIs in flexor tendons of mice after only one week of training. By ten weeks diameters had reduced below those of controls, with the CFIs remaining the same. In all three studies the largest fibrils had 'disappeared'.

The detrimental effect of exercise on the core fibril populations may have implications in terms of the concept of microtrauma. Differences in structure between core and peripheral regions of SDFTs have been noted previously in older horses with no clinical signs of tendonitis which could be interpreted as microdamage, or the response to it. Wilmink *et al.* (1992) found significantly lower collagen fibre bundle crimp angles, crimp lengths and toe limit strains in core regions of tendon from older horses. Unfortunately the exercise histories of two of the four younger animals in this study were unknown, so it could not be determined if these changes were an exercise-related or an ageing effect, or both. A longer lifespan implies exposure to a potentially higher incidence of trauma-inducing events.

One trained horse (number 3) showed an MAD for the central region which significantly exceeded that of the corresponding peripheral region (p<0.05), in contrast to the MADs for the other exercised horses. This may indicate individual variation in response. The tendon may not have experienced the same forces, or the structure may have been more resistant to 'damage'. The individuality of response requires further study as it may have implications in terms of identifying horses with a predisposition to developing tendonitis.

Wilson and Goodship (1994) proposed that increases in temperature in the core regions of the SDFT during galloping were important in tendonitis

aetiology. Temperatures during five minute gallops were found to rise to approximately 43 degrees celsius with a mean peak differential between peripheral and core regions of approximately five degrees celsius. The exact mechanism of possible hyperthermic damage has not been elucidated. Fibroblasts cultured from the tendon core region have been shown to be highly resistant to such temperatures, but effects on their metabolism could not be ruled out (Birch *et al*. 1992; Birch 1993). It is also possible that mechanical forces differ across the tendon cross-section.

The mechanism by which fibril diameters were reduced in the tendon core requires investigation, and could be important in relation to the pathogenesis of tendonitis if this condition is the ultimate result of repetitive or intermittent microtrauma. Parry and Craig (1988) suggested that at a given level of strain the larger fibrils experience greater stress due to their greater cross-sectional area; tail tendons stretched to rupture showed that the larger fibrils had broken down into smaller ones by fragmentation of their peripheral layers (Parry *et al*. 1980). Scott (1990) induced longitudinal splitting of rat tail tendon collagen fibrils into 12 to 15 nm subunits or "protofibrils" using a pH typical of inflammatory fluid. Michna (1984) claimed to have observed such splitting in the fibrils of trained mice midway through the 10 week programme. This would imply that either there are covalent bonds between subunits which break more easily than the bonds within them, or as Scott (1995) suggests, there are no covalent bonds at all between the proto-fibrils.

Degradative enzymes may also play a role in the breakdown of large diameter fibrils. Birch (1993) suggested that increased mechanical loading could result in increased cellular production of cathepsins, and that enzymatic activity could be stimulated by the high temperatures experienced in the core of the SDFT during galloping.

The new small diameter fibrils could be the subunits of degenerating large fibrils or have been newly synthesized by stimulated tenocytes. It is possible that both mechanisms are occurring. Formation from already existent subunits would lessen the requirements for large increases in collagen synthetic rate (Scott and Parry 1992).

Another important consideration is the age of the horses used in this study.

At the onset of training the rats used by Oakes *et al.* (1981) were less than 3 months of age, the age at which rat tendon reaches maturity in structure (Diamant *et al.* 1972). Michna used mice of only 6 weeks of age. Age-related fibril diameter data for SDFTs from horses of unknown breed and age history does exist (Parry *et al.* 1978b), however it is difficult to assess tendon maturity using this information. Collagen cross-link analysis would be a more definitive index. Fibrils of immature tendons contain greater proportions of reducible collagen cross-links which are more chemically, thermally and mechanically labile than the non-reducible cross-links which predominate at physiological maturity (Bailey 1968; Bailey and Shimokomaki 1971; Bailey *et al.* 1974). Such fibrils could conceivably be more prone to longitudinal breakdown at a given level of force (Davison 1989). Fibril cross-sectional areas and CFIs increased in the calcaneal tendons of mature (6 month old) rats which were trained for 10 weeks (Enwemeka *et al.* 1992). Food-restricted rats which were exercised showed reductions in fibril areas. It was suggested that these tendons were immature in structure, in this case as a result of nutritional deprivation, and that in such tendons collagen turnover becomes so rapid the fibrils are unable to mature, exacerbating the effect. This is supported by the findings of Curwin *et al.* (1988). It is possible that the core fibrils in the horses were attempting to respond to training with this effect being masked by fibril splitting and/or over-accelerated collagen turnover due to immaturity, with hyperthermia and mechanical stress as possible aetiological factors. It is not known if these fibrils did at some time exhibit hypertrophy which was later superseded by fibril breakdown with further loading cycles.

The collagen fibrils in the peripheral regions of trained tendons may have remained similar to those of control tendons as they were not subjected to the same thermal and/or mechanical conditions as the fibrils in the core regions. The forces experienced by these regions do not appear to have been sufficient to stimulate fibrillar hypertrophy.

In conclusion, the large diameter collagen fibrils in the core regions of exercised equine SDFTs underwent breakdown, as evidenced by reductions in the mass-average diameters and maximal diameters. The fibrils in the peripheral regions were not altered by the training. The reduction of the MAD

in response to exercise in the core regions may be interpreted as microdamage, repeated episodes of which may result in development of degenerative core lesions and eventually clinical tendonitis. The implication is that by the time even minor SDFT core lesions are detected clinically, considerable weakening of the tendon ultrastructure will have already occurred. It would seem that the core region of equine SDFT is quite susceptible to such microdamage, as the training programme used in this study was not of extreme intensity and a period of 18 months in training is short in comparison with many racing careers. It is possible that training of shorter duration and/or intensity could produce collagen fibrillar hypertrophy in the SDFT central regions, which might still also be appropriate for developing race fitness in other respects. If a clinical means of detecting SDFT core microdamage could be developed, and training programmes devised which can induce resistance to injury, tendons could be prevented from progression to degenerative change and returned to their normal structure.

Chapter 7

The effects of training on collagen fibril populations in the suspensory ligament and deep digital flexor tendon of young thoroughbreds[5]

7.1 Abstract

Suspensory ligament desmitis is a common problem in the thoroughbred racehorse, although it occurs at a far lower frequency than superficial digital flexor tendonitis. Lesions involving the deep digital flexor tendon are comparatively rare. Collagen fibril mass-average diameters in the core regions of superficial flexor tendons have previously been found to decrease significantly in 39±1 month old thoroughbreds which had undergone a specific 18 month training programme. This reduction in diameter was interpreted as structural weakening or microdamage, as collagen fibrils are the basic units of tensile strength in tendon. The present study involved the measurement of collagen fibril diameter distributions in the suspensory ligament and deep digital flexor tendon from the same horses. It was hypothesized that the mass-average diameter of fibrils in the suspensory ligament would also reduce in magnitude as a result of the exercise regime, as it has been shown to be loaded in a similar fashion to the superficial flexor tendon, at least at the slower gaits. Fibrils in the deep digital flexor tendon were predicted to undergo hypertrophy due to relatively lower loading, which would allow an adaptive response. The mass-average diameter did not significantly alter with exercise in either structure. It is possible that microtrauma did occur in the SL in response to training, but at a different site to that sampled. Another possibility is that loading of the suspensory ligament does not increase to the same extent as that of the superficial digital flexor tendon between the trot and the gallop.

7.2 Introduction

Suspensory ligament (SL) desmitis in the forelimb is a common problem in both thoroughbreds and standardbred trotters, although it occurs with less frequency than superficial digital flexor tendonitis in the former class of horse (Gibson *et al.* 1995). A clinical survey of cases of forelimb digital tendon and ligament injury in New Zealand thoroughbreds showed 19% to involve the SL, and 72% the superficial digital flexor tendon (SDFT) (Gibson *et al.* 1995). Deep digital flexor tendon (DDFT) injury is rare, with only 1% of clinical lesions or post-mortem abnormalities in thoroughbreds found to involve this structure (Webbon 1977; Gibson *et al.* 1995).

It is very important to know why the SDFT and SL sustain injury in racehorses, and why the former is most predisposed to the development of lesions. The distribution of loading between the three tendons must be important, since SL desmitis and SDFT tendonitis occur with approximately equal frequency in the forelimbs of standardbreds (Nilsson 1970; Fackelman 1973). Fackelman (1973) suggested that the first half of the weight-bearing phase is prolonged during an extended trot, and both the SL and SDFT provide their main support in this phase of the stride (Platt *et al.* 1991). Using implantable force transducers, Wilson (1991) found the increase in loading of the SL between the slow trot (2.0 m/s) and the extended trot (6.0 m/s) to be almost twice that of the SDFT or DDFT, which might be associated with the higher incidence of SL desmitis in trotters. SDFT, DDFT and SL loading at the gallop has not been measured. However, knowledge of the effect of a training programme involving galloping exercise on the collagen fibril populations in all three flexor structures may indicate if there is any correlation between the mechanical environments which they experience, and the response of their ultrastructure. This may help to explain the predisposition of the SDFT and the SL to injury, and possibly the different rates of occurrence in the two structures.

Mammalian tendons have a hierarchical structure (Kastelic *et al.* 1978), consisting of longitudinally arranged units termed fascicles, (100-300 μm in diameter), which are composed of collagen fibrils in a matrix of proteoglycans, glycoproteins, elastic fibres, small ions and water. Collagen fibrils are

submicroscopic units believed to be at least several millimetres in length in the adult tendon (Craig *et al.* 1989), which are packed closely together in its longitudinal axis. They are the fundamental units of tensile strength in the tendon structure, and may be classified as "large" or "small" diameter in equine digital flexor tendons (Parry *et al.* 1978b; Deane 1991; Patterson-Kane *et al.* 1996a).

A diameter-area distribution may be derived from the diameter-frequency distribution for a collagen fibril population. The mass-average diameter (MAD) is effectively the mean of this distribution, and is correlated positively with overall tendon strength (Parry and Craig 1988, Oakes 1989). The MAD for core regions of thoroughbred SDFTs was reduced significantly in response to a specific 18 month training programme (Patterson-Kane *et al.* 1996a). This was interpreted as being due to breakdown of larger diameter fibrils, resulting in weakening of the tendon structure in this region. This would predispose it to the development of further microtraumatic changes and could eventually result in clinical tendonitis.

For this study, fibril diameters were measured from samples of SLs and DDFTs from horses which had undergone a specific 18 month training programme involving galloping exercise. Their age and sex-matched controls experienced walking exercise only. It was hypothesized that MADs for fibril populations in the SL of exercised thoroughbreds would be significantly reduced. Fibrils in the DDFT were predicted to undergo an adaptive increase in size in response to training, as that tendon experiences lower loads than either the SL or SDFT.

7.3 Materials and methods

7.3.1 Horses

Twelve thoroughbred fillies were divided randomly into two groups: one underwent the training programme and the other served as the control group. One horse was withdrawn from the trained group before the end of the programme due to poor adaptation to treadmill exercise.

7.3.2 Training programme

The group in training underwent an 18 month controlled exercise programme on a high-speed equine treadmill (Sato, Sweden). In a typical week the regimen was as follows: Monday 4 km @ 12 m/s, 3% slope; Wednesday two times 1.5 km @ 12 m/s and 14 m/s, 4% slope, 5 minutes recovery; Friday three times 1 km @ 12 m/s, 13.5 m/s, 15 m/s, 3% slope, 5 minutes recovery. This was combined with ten minutes trotting on a mechanical horse walker three times weekly and walking exercise on the horse walker (40 minutes) for six days per week.

The control group undertook only a very low volume of low intensity exercise over the same period. This consisted of walking exercise on six days per week, in the mechanical horse walker, for 40 minutes per day.

7.3.3 Tissue processing and electron microscopy

A section one cm in length was excised from both the SL and the DDFT, from the longitudinal midpoint of the metacarpus. 2mm x 1mm^2 strips were dissected from the centre of the cross-section of each piece, and from a peripheral location approximately one mm from the surface of the lateral aspect (Figure 7.1). The central and peripheral strips were fixed conventionally for electron microscopy and embedded in Taab 812 resin. Silver-gold sections (90-130 nm) were cut from one block for each tendon region using a diamond knife and mounted on unsupported 400 mesh copper grids. They were stained in uranyl acetate followed by modified lead citrate (Sato 1967).

The sections were viewed using a Philips E.M. 201c high resolution electron microscope. Sections on a grid were scanned at low and then high magnification, to ensure the section selected for analysis appeared representative. Within the section chosen, areas containing elastic fibres, tenocytes or certain stain artefacts were avoided, as the image analysis programme (see below) could not distinguish these from collagen fibrils. Collagen fibrils could not be obliquely sectioned, and were required to show uniform and dark staining in comparison with the background. Areas of the section of which micrographs
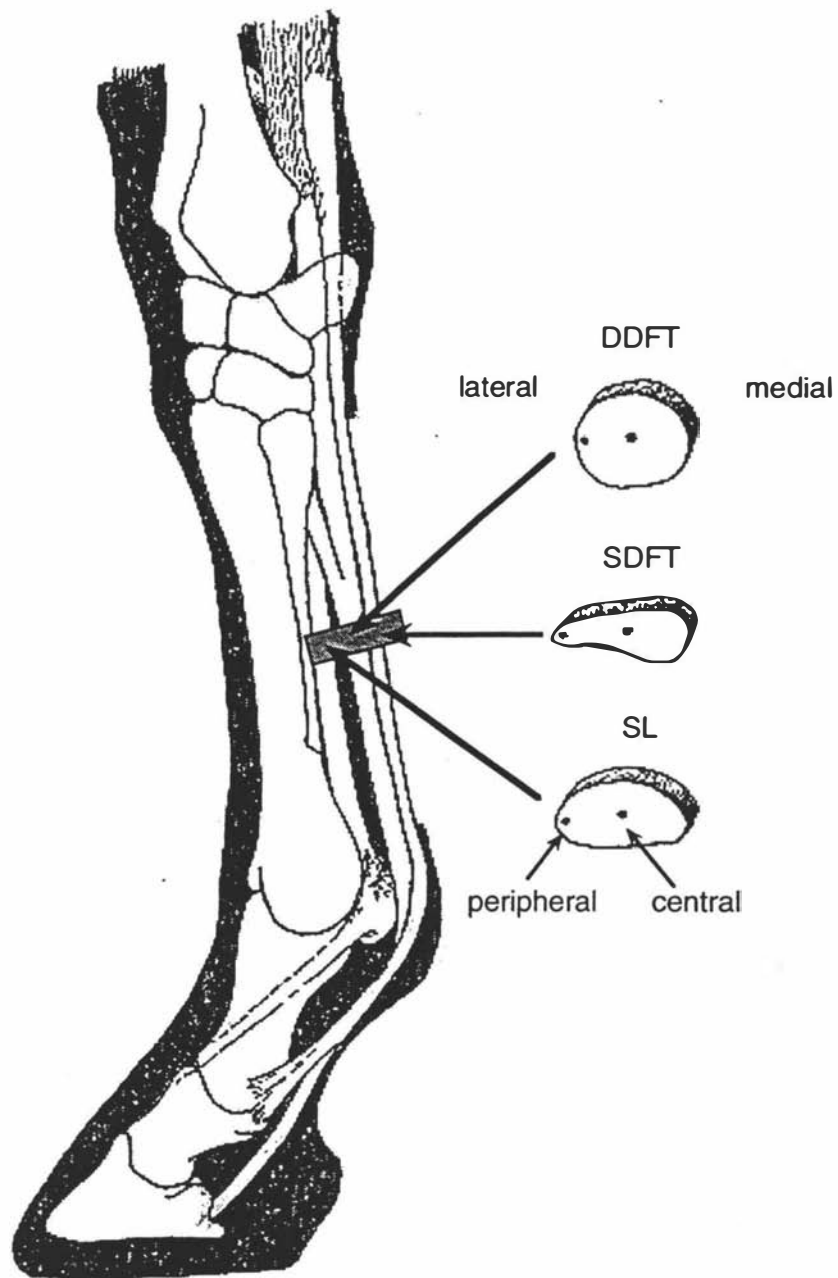
Figure 7.1 Details of the sampling procedure from the deep digital flexor tendon (DDFT), superficial digital flexor tendon (SDFT), and suspensory ligament (SL).

were taken were selected randomly within those constraints. Sufficient micrographs were taken per section to allow analysis of at least 1000 fibril diameters (Parry and Craig 1977), at magnifications of 48600x for the SL and 31800x for the DDFT. A carbon grating replica with 2160 lines per mm was used to calibrate each set of micrographs.

7.3.4 Collagen fibril morphometry

The micrographs were analyzed using a VIPS-based computer programme (Visual Image Processing System) which measured collagen fibril diameters. The total area occupied by each image was also recorded and from this value the percentage of measured area occupied by collagen fibrils was also calculated, termed the collagen fibril index (CFI). Diameter and frequency measurements were analyzed, using the calibration factor to convert the diameter measurements directly to nm. The MAD was then calculated from the diameter-area distribution.

7.3.5 Statistical analysis

MADs and CFIs were compared between groups for both the SL and DDFT, using the t-test for independent samples, and between central and peripheral regions within groups using the paired sample t-test.

7.4 Results

The MAD of 88.0 ± 9.2 nm for the central region of the SL in trained horses did not differ significantly from that of 114.3 ± 10.1 nm for the control horses (p=0.086)(Table 7.1). Similarly, for the peripheral regions, the MAD of 110.1 ± 8.5 for the trained horses did not differ significantly from that of 98.6 ± 11.4 for the control horses (p=0.437). For the DDFTs, MADs of 207.3 ± 15.3 nm and 220.9 ± 26.5 nm for the central and peripheral regions of the trained group respectively, did not differ significantly from those of 202.2 ± 7.3 nm and 200.1 ± 13.8 nm for the same regions in the control group (Table 7.2). There were

no significant differences between central and peripheral regions for either the SL or the DDFT in trained or control groups. Furthermore, CFIs did not differ between groups or regions for either structure (Table 7.3).

Table 7.1: **Mass-average diameters and standard errors (in nm) for collagen fibril populations from central and peripheral regions of suspensory ligaments from control and exercised horses.**

	Horse	Central	Peripheral
Controls	2	116.0	87.7
	4	115.2	124.3
	6	99.1	102.9
	8	131.4	136.3
	10	76.4	66.3
	12	147.5	73.8
	Mean	**114.3 (10.1)**	**98.6 (11.4)**
Exercised	1	80.2	138.3
	3	108.7	105.3
	5	59.1	95.6
	7	85.3	91.9
	11	106.6	119.5
	Mean	**88.0 (9.2)**	**110.1 (8.5)**

7.5 Discussion

During weight-bearing the SL together with the proximal sesamoid bones and associated sesamoidean ligaments, the SDFT and the DDFT, act to prevent hyperextension of the fetlock joint. This suspensory apparatus is loaded maximally in the support phase of the stride (Platt *et al.* 1991) and stores elastic energy (Alexander 1988). The degree of fetlock extension increases with speed (Schryver *et al.* 1978), increasing the load and therefore the elongation experienced by the flexor structures. Increase in strain level with

speed has been measured *in vivo* in both the SL and the SDFT. Jansen *et al.*

Table 7.2: **Mass-average diameters and standard errors (in nm) for collagen fibril populations from central and peripheral regions of deep digital flexor tendons from control and exercised horses.**

	Horse	Central	Peripheral
Controls	2	202.3	235.2
	4	233.2	147.1
	6	206.1	218.0
	8	191.4	226.2
	10	200.4	199.1
	12	179.6	174.8
	Mean	**202.2 (7.3)**	**200.1 (13.8)**
Exercised	1	155.9	256.3
	3	192.3	228.2
	5	214.8	216.2
	7	236.4	255.4
	11	237.3	143.7
	Mean	**207.3 (15.3)**	**220.9 (26.5)**

Table 7.3: **Collagen fibril indices and standard errors (%) for collagen fibril populations from central and peripheral regions of suspensory ligaments and deep digital flexor tendons from control and exercised horses.**

		Central	Peripheral
SL	Controls	59.3 (1.6)	62.4 (3.2)
	Exercised	62.1 (1.7)	61.8 (1.5)
DDFT	Controls	74.0 (1.9)	74.4 (3.5)
	Exercised	74.3 (2.1)	74.2 (1.8)

(1993) calculated the mean strains in the SLs of Dutch warmblood horses as being 3.4% at a walk, 5.6% at a trot and 6.3% at a slow canter, using kinematic data. Stephens *et al.* (1989) measured strains in the SDFTs of two thoroughbreds of 3.1% and 7.6% at a walk and 11.5% and 16.6% at a gallop respectively, using Hall-effect transducers. No such data are available for the DDFT, however correlation of known *in vivo* force measurements (Wilson 1991) with *in vitro* stress-strain curves (Jansen and Savelberg 1994) implies that strains in this tendon also increase with the speed of the gait. High strains are also required for storage of significant quantities of elastic energy, the conversion of which to kinetic energy during the latter half of the weight-bearing phase helps to propel the horse forward, reducing muscular effort and expenditure of metabolic energy (Cavagna *et al.* 1977).

Wilson (1991) measured forces in the SLs, SDFTs and DDFTs of two ponies during walking and three speeds of trotting (2.9 m/s, 4.2 m/s, 6.0 m/s). Stresses (i.e. force per unit area) were calculated using tendon cross-sectional areas measured from photographs of sections taken on post-mortem. At all three gaits, the highest peak stress was found to be experienced in the SL, followed by the SDFT. In both structures this occurred early in the support phase. The SDFT appeared to be loaded on its own for a fraction of a second before the SL in some force-time tracings. Peak stresses in the DDFT were significantly lower, and occurred in the caudal phase of the stride just after the SL and SDFT began to recoil. In one pony the stresses at a trot were calculated as being 27.4, 16.3 and 11.2 MPa in the SL, SDFT and DDFT respectively. So, despite the larger cross-sectional area of the SL (Smith *et al.* 1994), it experienced greater stress than the SDFT.

Large diameter fibrils are thought to have greater tensile strength due to their higher density of intrafibrillar covalent collagen cross-links (Parry *et al.* 1978a). Small fibrils are thought to be important in preventing interfibrillar slippage by virtue of their greater surface area per unit volume, which permits greater interaction with the surrounding matrix. At the same level of increasing strain, a small diameter fibril would be expected to fail before a larger fibril. Larger fibrils would therefore be less likely to fail due to the rapid high-strain cycles experienced during galloping, and so if a tendon were to respond to exercise

by increasing resistance to overstrain, the mass-average diameter would be expected to increase. Functional adaptation was not observed in either the SL or the DDFT due to this specific regime. The MADs in the central regions of the SLs did decrease, but not significantly (p=0.086), due to high individual variability.

A previous study showed the MAD of 105.3 ± 4.3 nm in the central region of SDFTs from trained horses to be significantly lower than that of 131.7 ± 4.9 nm for the same region in the control horses (Patterson-Kane *et al.* 1996a). This was in agreement with the fact that lesions in this tendon tend to occur in the core of the midmetacarpal region in horses which undergo competitive galloping exercise (Webbon 1977). The change in MAD was interpreted as microtrauma which would predispose to overstrain and the development of clinical lesions, because smaller fibrils are weaker (Parry *et al.* 1978a).

SL desmitis does occur in racehorses, albeit at a lower frequency than SDFT tendonitis. Dyson *et al.* (1995) have observed central hypoechoic regions in the body of the SL on ultrasonographic examination of apparently normal horses and ponies of undefined function. In racehorses the body, or middle third, of the forelimb SL is a more common site of lesions than the proximal region of the ligament (Dyson *et al.* 1995). On the basis of the clinical situation, and the known force data (Wilson 1991, Platt *et al.* 1991), the collagen fibrils in the core region of the SLs from the same trained horses would be expected to also have a significantly lower MAD than the same region in the control horses. The lack of significant evidence of microtrauma in this study is therefore surprising. If the change in distribution of load between the SL and the SDFT from a trot to a gallop is sufficient to result in greater stress in the SDFT at the latter gait, it would help to explain the higher incidence of SDFT injury than SL desmitis in gallopers. The apparent rapid loading of the SDFT before the SL could also be a contributory factor. Alternatively, the hyperthermia experienced in the core of the SDFT during galloping as a result of heat loss during the conversion of elastic energy to kinetic energy (Wilson and Goodship 1994) may play a role. It is not known if the temperature in the core of the SL increases significantly during galloping. The mid-metacarpal site from which the two structures were sampled may
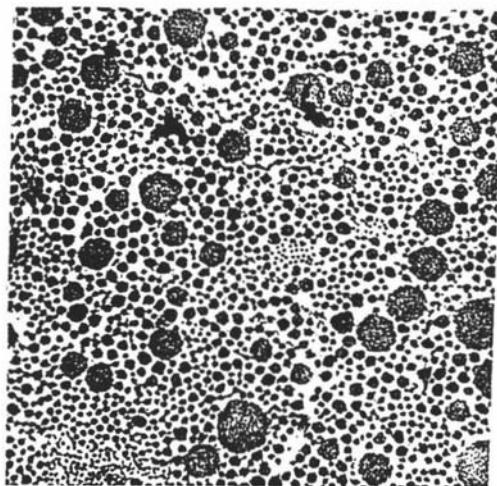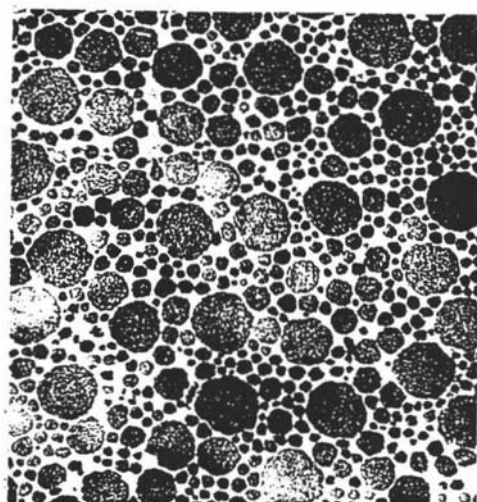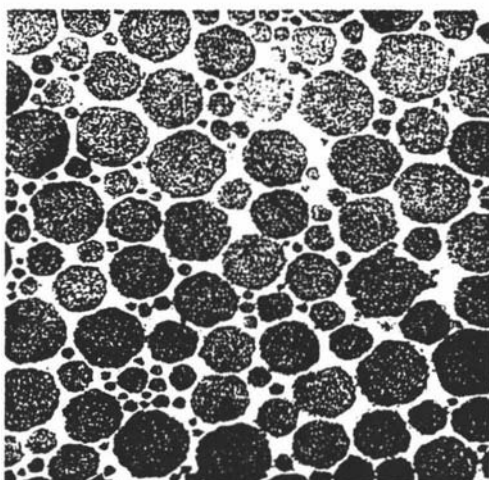
Figure 7.2 Transverse sections of collagen fibrils in the SL (a), SDFT (b) and DDFT (c) of a control horse (number 10), 34 750x

however be of importance (Figure 7.1), if it is taken into account that desmitis in this part of the ligament is often considered to be secondary to splint bone fractures or periostitis (McIlwraith 1987), neither of which occurred in the exercised horses in this study. Suspensory branch and proximal lesions are stated to be more likely to be purely strain-induced (McIlwraith 1987), and if the SL had been sampled in either of those two regions, it is possible a significantly lower MAD may have been observed in the trained horses.

The unaltered MADs for the DDFT imply that the loading was not sufficient to stimulate hypertrophy, in keeping with the current data (Wilson 1991) which shows that this tendon experiences relatively low loads. The fact that the DDFT has larger fibrils (Figure 7.2), and its maximal load occurs when the SL and SDFT have already begun to recoil, indicate a more important role in support than in elastic energy storage. Any increase in training may not therefore have caused this tendon to approach its mechanical limits and necessitate adaptation, because a tendon which was not required to store levels of elastic energy similar to those of the SL and SDFT would not have to undergo such high strains.

In conclusion, the fact that the SLs and DDFTs did not undergo significant changes in the ultrastructure of their tensile units in response to the training regime, whereas the SDFT showed evidence of microtrauma, may indicate thatthe SDFT is preferentially loaded at a gallop. Indeed injury incidence, if other factors such as core hyperthermia are more or less equal, points to a higher loading of the SDFT at a gallop. Direct measurements of the distribution of loading among the three flexor structures at a gallop is now required. Another possibility is that strain-induced microtrauma occurs in both the SL and the SDFT in response to training, but at different sites.

Chapter 8

An age-related study of the morphology and crosslink composition of collagen fibrils in the digital flexor tendons of young thoroughbreds

8.1 Introduction

Despite the importance of injury to the equine superficial digital flexor tendon (SDFT), knowledge of the normal age-related structure and composition in thoroughbreds is limited. Such information is required in order to fully understand the effects of maturation and ageing, which may influence susceptibility to injury.

Galloping exercise is recognised as a major factor in the pathogenesis of tendonitis (McCullagh *et al.* 1979), and research in other species has indicated that immature and mature tendons may respond differently to exercise (Curwin *et al.* 1988, Enwemeka *et al.* 1992). The age at which the SDFT attains a 'mature' structure has not yet been established. It may not have occurred in horses which begin training in order to race as two year olds. Gibson *et al.* (1995) found 27% of cases of SDFT tendonitis in horses in training which had not yet raced. One reason for this high figure may be that the tendon structure in some of the horses was immature, and therefore not as resistant, or as capable of adapting, to an increased number of rapid high-strain cycles.

Increasing age has been correlated with increased mechanical strength of tendon (Rigby *et al.* 1959, Torp *et al.* 1975a, Vogel 1978, Cribb and Scott 1995) and increased physical and chemical stability of the collagen (Hamlin and Kohn 1971, Bailey *et al.* 1974), in various species and structures. The increase in strength is an adaptation to increased mechanical loading, due to greater muscle and ground reaction forces resulting from increased body weight (Curwin *et al.* 1994). Mechanical strength does not appear to correlate simply with the collagen content of a tendon (Danielsen and Andreassen 1988). The types of crosslinks between collagen molecules within fibrils, the structure and packing of these collagen fibrils, and interactions between fibrils and interfibrillar matrix are all important factors. An accurate estimation of the age at which the equine digital flexor tendons reach maturity would need to take into account all of those aspects.

The intermolecular covalent crosslinks within fibrils (see 1.4.2) play a major role in reducing shear forces between adjacent collagen molecules and possibly subfibrillar units. They are therefore considered to be responsible for a large

proportion of the tensile strength of the fibril. The reduction in concentration of reducible crosslinks with age, and the concomitant increase in nonreducible crosslinks is associated with increased insolubility (Bailey *et al.* 1974), thermal stability (Bigi *et al.* 1992), collagenase resistance of the collagen (Jolma and Hruza 1972), and increased mechanical strength of the tendon (Fujii and Tanzer 1974). The age at which reducible crosslinks reach very low levels is therefore a good index of tendon maturity (Curwin *et al.* 1988).

Collagen fibril diameters have been measured as a function of age in tendons from various species (Parry and Craig 1984). There is a general increase in mean fibril diameter throughout development, larger fibrils having greater tensile strength by the definition of Parry *et al.* (1980). The increase in fibril diameter with age in rat tail tendon does not continue beyond 4-5 months, which is considered to be the age at which maturity is reached (Torp *et al.* 1975a, Scott *et al.* 1981). The collagen fibril index has been shown to increase up to the age of maturity in rat tail tendon, remaining unchanged thereafter (Parry and Craig 1984).

Collagen fibril bundles with a larger crimp angle will fail at a higher strain level than those with a smaller crimp angle (Wilmink *et al.* 1992). The age-related morphology of the crimp waveform has also been studied in rat tail tendon. In the latter structure crimp length increases until maturity and then remains unchanged, but there is a continuous but moderate decrease in crimp angle with age (Diamant *et al.* 1972, Torp *et al.* 1975b). The noted change in crimp length appears to be related to tail length, and may be specific for that structure (Diamant *et al.* 1972).

Birch (1993) analyzed crosslinks from SDFTs and deep digital flexor tendons (DDFTs) of thoroughbreds 3 years of age or older, and did not detect significant levels of reducible crosslinks. This indicates that maturity in structure in that respect is reached before the age of three years. Parry *et al.* (1978b) found an increase in collagen fibril mass-average diameter in the equine SDFT up to an age of 5 years, and a decline thereafter. The number of samples in the latter study was limited and the exercise histories of the horses unknown. Patterson-Kane *et al.* (1996b) noted a significant reduction with age in the crimp angle in the core region of SDFTs in horses from two to

over 10 years of age, but no significant changes in crimp period length.

For this study, samples were taken from the SDFTs and DDFTs of thorough-breds ranging in age from foetuses to 3 years, the latter age limit being chosen on the basis of the previous collagen crosslink concentration results of Birch (1993). Reducible and non-reducible crosslink concentrations, collagen mass-average diameters, collagen fibril indices and collagen crimp angles and lengths were measured, these all being parameters thought to be related to mechanical strength of tendon. It was hypothesized that maturity in structure of both the SDFT and the DDFT is reached at around two years of age.

8.2 Materials and methods

8.2.1 Horses and tissue sampling

The SDFTs and DDFTs of thirty-six horses ranging in age from foetuses to three years of age were sampled (see Appendix B, page 189). Four sections were excised from both of the tendons, at corresponding levels (Figure 2.2). The sections, proximal to distal, were frozen for crimp morphology analysis, fixed in glutaraldehyde for electron microscopy of collagen fibrils, fixed in formalin, and frozen for biochemical analysis. The formalin-fixed sections were not used in this study. Fibril diameters and crimp angles and lengths were measured in the SDFT and DDFT of all 36 horses. Collagen crosslink concentrations were calculated for the SDFT and DDFT from 15 of those horses (see Table 8.1). Details for both the processing and analysis of samples are given in the general materials and methods section (page 68).

8.2.2 Data analysis

Collagen crosslink concentrations

The method for calculating collagen cross-link concentrations is outlined in the general materials and methods section (page 71). Calculations were made using a spreadsheet programme (Quattro Pro for Windows Version 5.00,

Borland International Inc., Scotts Valley, CA, USA).

Collagen fibril mass-average diameter

100-1000 fibril measurements were made, depending on the mass-average diameter to mean diameter ratio (see Table 2.1, page 63). The collagen fibril index was obtained from the image analysis programme. The mass-average diameter was obtained from the diameter-frequency distribution using a programme developed by D.A. D. Parry.

Table 8.1   Horses sampled for measurement of collagen crosslink concentrations in the SDFT and DDFT

No.*	Age	No.	Age	No.	Age
1	foetus	17	25 days	28	1 year
3	premature	18	3 months	30	1½ years
13	newborn	23	5 months	31	2 years
14	newborn	26	8 months	34	2 years
16	7 days	27	1 year	36	3 years

* see Appendix B, page 189.

Crimp angle and length

The mean and standard error for the 50 angle measurements for each sample were calculated using a statistics programme (SYSTAT *for Windows Version 5 Edition*, SYSTAT Inc., Evanston, IL, USA). The mean for the 50 projected length measurements was also calculated (d), and converted to the true mean length (l) using the equation $l = d/\cos\theta$, where θ is the mean crimp angle (see Figure 3.1, page 85).

Statistics

The small numbers of horses within each age group did not allow valid statistical analysis of age-related changes. Values for each parameter were plotted against age, and a smoothing line drawn using the distance-weighted least-squares method (SYSTAT *for Windows*).

8.3 Results

8.3.1 Collagen crosslink concentrations

Three forms of covalent collagen crosslink were observed in the tendon samples; hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL), the reduced forms of immature crosslinks, and hydroxylysylpyridinoline (HP), a mature crosslink.

In the SDFT (Figure 8.1), the concentration of DHLNL (immature crosslink) declined rapidly to low levels at birth (approximately 0.25 moles/mole collagen), then continued to decrease at a slower rate, so that by 2 years negligible levels were reached (0.01-0.02 moles/mole collagen). The concentration of HLNL decreased rapidly to zero within a few months of birth. HP (the mature crosslink) increased rapidly from approximately 0.35 moles/mole collagen at birth up to approximately 0.8-0.85 moles/mole collagen between 1 and 1½ years of age, and at a slower rate thereafter, reaching 0.9 moles/mole collagen at 3 years.

In the DDFT (Figure 8.2) the DHLNL concentration followed a similar pattern to that in the SDFT. The HLNL concentration increased to a maximum at approximately 6 months, than gradually reduced to zero by 2 years. HP reached a maximum of 0.75-0.8 moles/mole collagen at approximately 2 years, and then began to decrease. Comparison of HP concentrations for the two tendons showed the line for the DDFT to be consistently lower than that for the SDFT from birth, although there was greater variation in the former tendon (Figure 8.3).
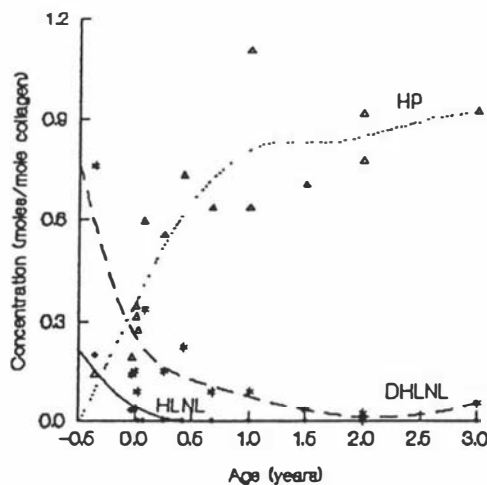
Figure 8.1: Concentrations of reducible (HLNL, DHLNL) and nonreducible (HP) collagen crosslinks in the SDFT versus age for a random sample of thoroughbreds (n=15).
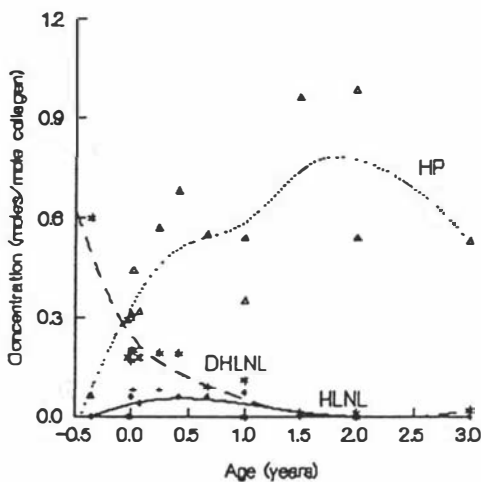
Figure 8.2: Concentrations of reducible and nonreducible collagen crosslinks in the DDFT versus age for a random sample of thoroughbreds (n=15).

8.3.2 Collagen fibril mass-average diameter and collagen fibril index

The MAD for the SDFT increased to a peak of approximately 170 nm between 1 and 1½ years, then began to decline (Figure 8.4). The MAD for the DDFT followed a similar trend, reaching its maximum of approximately 240 nm just before 2 years of age. From birth, the MAD for the DDFT was greater than that for the SDFT.

The CFIs for the SDFT and DDFT peaked at values of approximately 70% and 79% respectively. These maxima occurred at approximately the same age as their respective MAD peaks (Figure 8.5). The CFI for the DDFT exceeded that for the SDFT from 3-4 months of age.
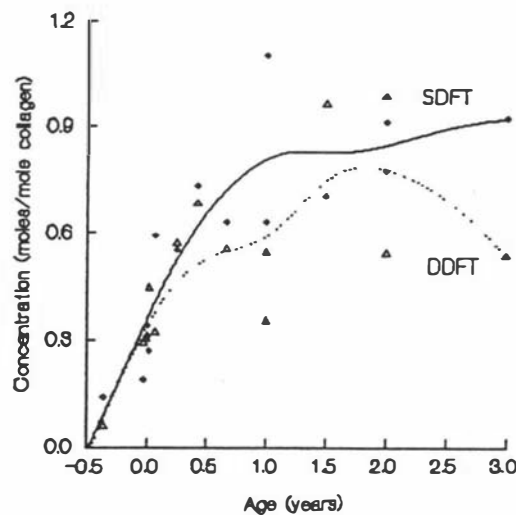
Figure 8.3 Concentration of HP in the SDFT and DDFT versus age for a random sample of thoroughbreds (n=15)
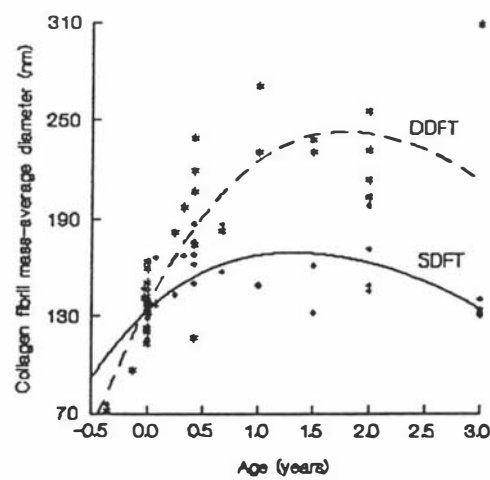
Figure 8.4   Collagen fibril mass-average diameter versus age for the SDFT and DDFT of a random sample of thoroughbreds (n=36).
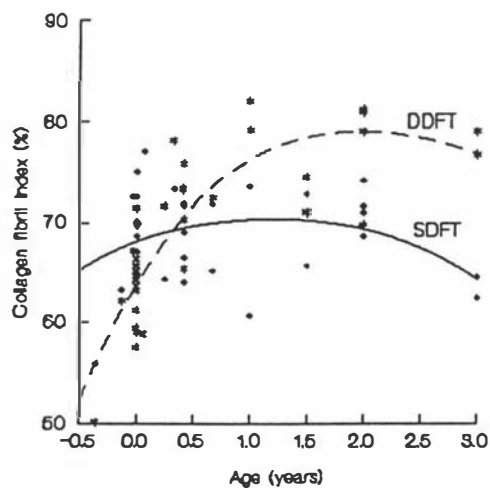
Figure 8.5 Collagen fibril index versus age for the SDFT and DDFT of a random sample of thoroughbreds (n=36).

8.3.3 Crimp angle and length

Crimp angle and length for both the SDFT and the DDFT decreased rapidly until approximately two years. At that time the crimp angle plateaued at approximately 13° for both tendons, and the crimp length plateaued at approximately 22 μm for the SDFT and 36-39 μm for the DDFT (Figures 8.6 and 8.7). The slight upturn in angle and length for the DDFT closer to 3 years appears to be due to an abnormally high value for one horse of that age. Crimp angles for the two tendons were approximately equal over the age range studied, whereas the crimp length for the DDFT was greater than that for the SDFT.

8.4 Discussion

Two factors are likely to be associated with maturation of the digital flexor tendons, the first being the increase in bodyweight, and the second the increase in tendon length. A study of growth in 1992 thoroughbred foals by Hintz *et al*. (1979) showed them to have attained 46, 67 and 80% of their mature weight at 6, 12 and 18 months. By the age of 2 years the horse should have attained close to 100% of mature weight (Frape 1986). Early growth was found to be very rapid, with foals gaining on average 110 kg, 75 kg, 60 kg and 45 kg in the first, second, third and fourth 3 month periods respectively (Hintz *et al*. 1979).

Increases in tendon length with age have not been directly measured. The muscle of the SDFT arises from the humerus, and its accessory ligament from the distal radius. The muscle heads of the DDFT arise from the humerus, ulna and radius. The SDFT inserts on P1 and P2, and the DDFT on P3. Fretz *et al*. (1984) measured long bone growth in nine thoroughbred foals, showing the growth of the distal radius to cease by 60 weeks, and that of the third metacarpal bone and phalanges to cease by 10 weeks. Campbell and Lee (1981) showed growth of the distal end of the humerus to have stopped by 60-70 weeks.

Therefore, by 2 years of age the horse has attained nearly all of its mature
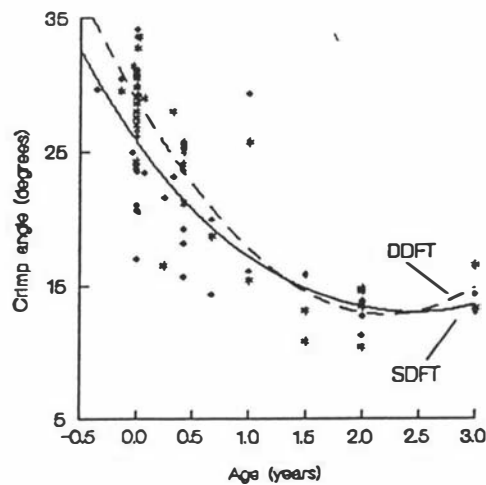
Figure 8.6 Collagen crimp angle versus age for the SDFT and DDFT of a random sample of thoroughbreds (n=36).
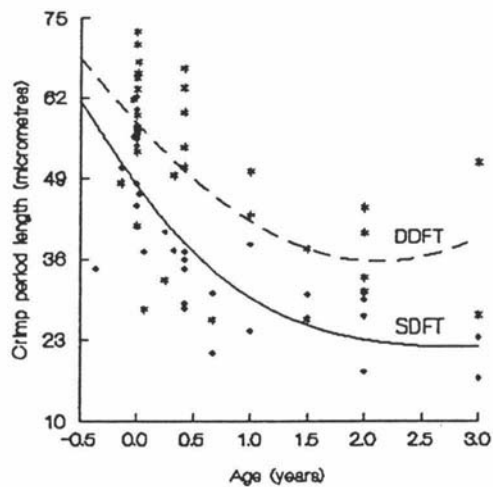
Figure 8.7 Collagen crimp period length versus age for the SDFT and DDFT of a random sample of thoroughbreds (n=36).

weight, and there are no further increases in the lengths of the long bones connected by the SDFT and DDFT and their respective muscles.

The small numbers of horses that could be obtained for this study did not allow statistical analysis of trends, and were very low in certain age groups. Perinatal mortality among foals can be high, as evidenced by the fact that 16/36 samples were from foals 1 week old or younger. Mortality in foals between 1-2 weeks and 1 year appeared to be very low, and horses 1-3 years in age were also difficult to obtain in large numbers. Consequently, the only way to present the data was to plot it, using a smoothing line to indicate any possible trends. As a consequence, some trends seen may actually be artefacts. The decrease in the HP concentration in the DDFT after the age of 2 years is probably a good example of such an artefact, due to the wide variation of points in that region of the plot (Figure 8.2). Any conclusions regarding the age at which maturity of tendon collagen fibrils is attained are therefore very general and approximate only. It may be possible in the future to sample further horses and add to this data, to increase its validity.

The DHLNL (reduced) crosslinks in the SDFT reached their lowest levels between one and two years. The most rapid increase in the nonreducible crosslink (HP) occurred up to the same period, although it continued to increase more gradually thereafter. The age at which these intermediate crosslinks have decreased to negligible levels, being replaced by nonreducible compounds, may be defined as the age at which the collagen fibrils in the tendon have reached maturity (Bailey and Light 1989).

The HLNL concentration trends were of interest. This crosslink is formed via condensation of a hydroxylysine residue with a lysine aldehyde residue. The rapid decrease to zero shortly after birth implies increased lysyl hydroxylase activity, which has previously been shown to occur with increased mechanical loading (Gerriets et al. 1993). In the DDFT the HLNL reduced to zero only after one year. It may be that the increased load experienced by the SDFT in comparison with the DDFT during normal locomotion (Wilson 1991) stimulates a higher lysyl hydroxylase activity in the former tendon.

Investigations of fetal tendon structure in horses and other species have demonstrated large numbers of uniformly small fibrils surrounded by abundant

matrix (Parry and Craig 1984). The distribution of fibril diameters widens by the time of birth in precocious animals such as the horse, which need to be immediately capable of locomotion. The change from a sharp unimodal population to a wide unimodal distribution in the sheep was shown to occur in the fetal SDFT, from mid-gestation (Craig and Parry 1981). Collagen fibril diameters have been observed to increase up to maturity in tendons from various species (Torp *et al.* 1975a, Nakagawa et al. 1994) and to decrease with senescence (Parry *et al.* 1978b, Nakagawa *et al.* 1994). In this study the fibril mass-average diameters increased from before birth and peaked before 2 years of age (Figure 8.8), as did the CFIs. Only two 3 year old horses were sampled, so this may be an artefact, but it is of importance if the MAD does begin to decrease at 2 years of age. This would imply that the collagen fibril population has reached maturity and has begun to weaken at the age at which horses begin to race.

It can therefore be stated that between one and two years, the collagen fibrils in the SDFT and DDFT attain maturity, as indicated by collagen crosslink, collagen fibril MAD and collagen fibril index data.

It is difficult to compare relative concentrations of crosslinks in the two tendons, due to the higher individual variability in the DDFT. The higher MAD of the DDFT in comparison with the SDFT has been discussed previously in terms of their differing mechanical functions (Patterson-Kane *et al.* 1996d). The SDFT is thought to play a more important role as part of the energy storage mechanism, therefore requiring large numbers of small diameter fibrils, to provide higher elasticity and allow higher strains. The fact that the MAD for the DDFT increases above that for the SDFT only after birth further supports the importance of increased mechanical loading on the increase in mass-average diameter of tendon collagen fibrils.

In both the SDFT and DDFT the crimp angle decreased rapidly until approximately 2 years of age, when it appeared to plateau at approximately 13° in both tendons. It is likely that there is a continued, very gradual decrease in the central region of the SDFT (Patterson-Kane *et al.* 1996b). It is possible that the increased loading due to increases in bodyweight gradually straightens the collagen fibrils in both tendons, causing the reduction in crimp angle. The fact

SDFT

DDFT

a

b

c
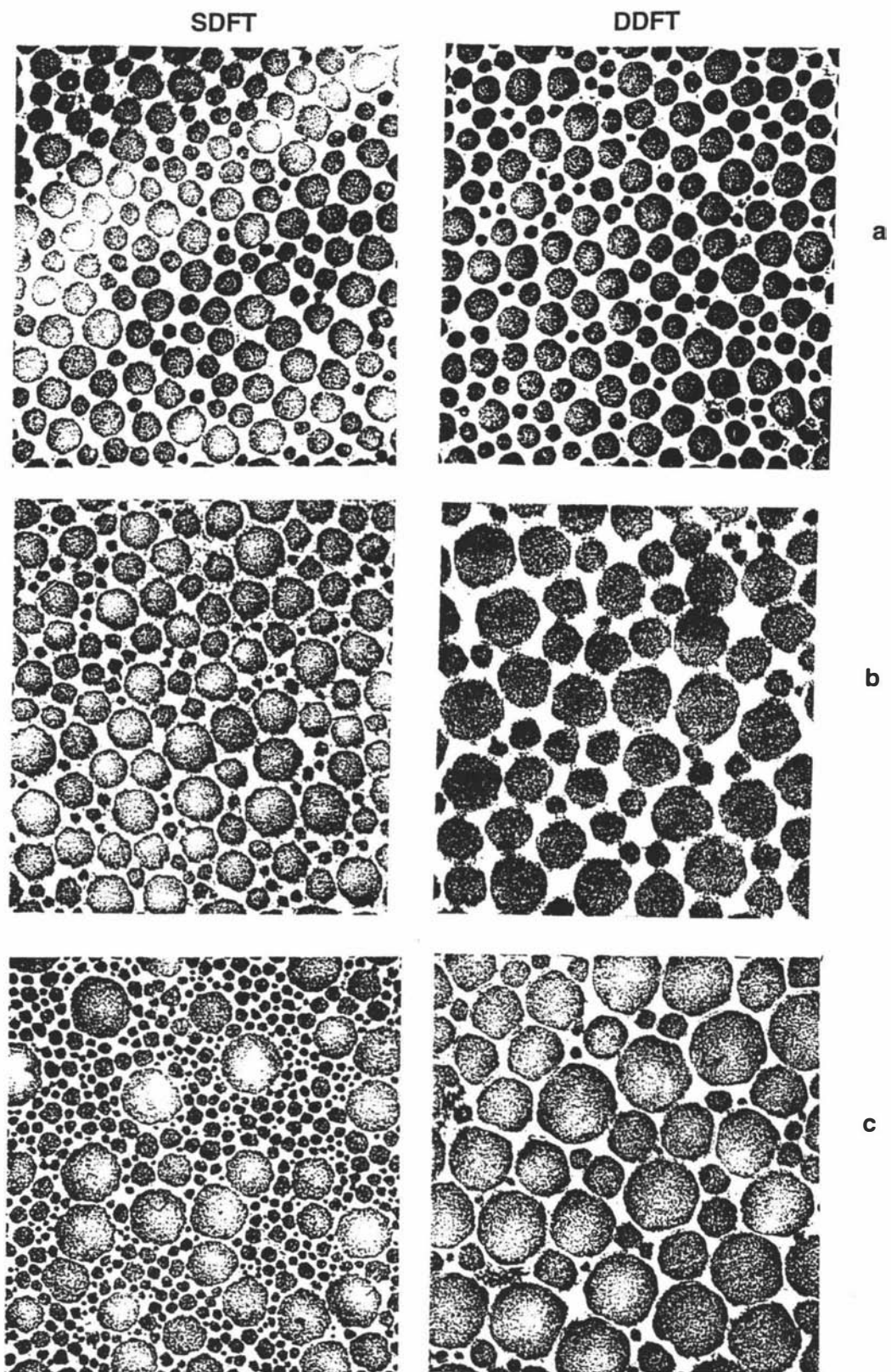
Figure 8.8 Transverse sections of the SDFT and DDFT from a newborn foal (a), a five month old foal (b) and a two year old (c) (37 500x).

that the age at which the crimp angle ceases its rapid reduction coincides with the age at which over 90% of the mature bodyweight is attained (Frape 1986) is supportive of this theory.

The difference in crimp length between the SDFT and DDFT is of interest. The DDFT crimp length is greater at all ages, but the difference increases, so by the age of two years the SDFT length (22 μm) was only 60% of that of the DDFT (36 μm) (Figure 8.7). A fibril which has a smaller crimp length by definition has a greater number of bending sites per unit length, if crimp angle remains constant. So, there is a greater increase in the number of bending sites per unit length in SDFT fibrils with age, up to the age of two years. The amount of new collagen is decreasing over that time as indicated by the rapid reduction in reducible crosslinks, implying that existing fibrils are undergoing changes in crimp morphology. It is possible that a rebuckling mechanism is occurring in both tendons, similar to that suggested by Dale and Baer (1974), possibly due to alterations in fibril-extrafibrillar matrix proportions. Differences in loading known to occur between the SDFT and DDFT (Wilson 1991) may result in the differing crimp lengths.

In conclusion, the collagen fibril populations of the SDFT and DDFT reached maturity in terms of nonreducible crosslink concentration, mass-average diameter, collagen fibril index and crimp morphology between one and two years of age. This is in agreement with previous data on the growth of young thoroughbreds, which indicates that they reach close to 100% of mature weight by the age of 2 years and that all of the long bones of the limb have ceased to grow shortly after one year. It is therefore concluded that immaturity of tendon structure is not a major factor in the development of SDFT lesions in most horses.

Chapter 9

General Discussion

The association between galloping activity and the incidence of SDFT tendonitis is well known, but the mechanisms of pathogenesis are poorly understood. The result has been the use of treatment methods which do not have a scientific basis, and the lack of any means of prevention of lesion development. The aim of this thesis was to determine if galloping exercise can affect the morphology of collagen fibrils in the SDFT of the thoroughbred. Three studies (Chapters 4,5 and 6) demonstrated morphological alterations, which are hypothesized to weaken the tendon core, which is the typical location of clinically evident lesions. The studies therefore provided evidence for microtrauma in the SDFT core, which could accumulate and eventually result in frank core lesions and clinical tendonitis.

In Chapter 3, the normal age-related crimp morphology in both the core and peripheral regions of the SDFT was established, using a sample of horses from a wild (untrained) population. It was demonstrated that crimp angle in the core region reduces with age, such that it is lower than the crimp angle in the periphery in horses 10 years of age or over. In Chapters 4 and 5, using randomly sampled thoroughbreds and those from an experimental exercise trial respectively, it was determined that galloping exercise modifies the normal age-related changes in the tendon core. Central region crimp angle was reduced below that in the periphery in 5 of 7 randomly sourced exercised horses under 10 years of age, and in 4 of 5 exercised experimental horses aged 38-40 months. Central crimp length was reduced below that in the periphery in some of the exercised horses in both groups.

In Chapter 6, it was demonstrated that the collagen fibril mass-average diameter was also reduced in the SDFT core of experimental exercised horses. The mass-average diameters in the SL and DDFT did not reduce significantly in these horses (Chapter 7).

A collagen fibril with a lower crimp angle will fail at a lower level of strain than a fibril with a greater crimp angle, because its waveform straightens at a lower strain. Fibrils of smaller diameter presumably break at a lower level of strain than large diameter fibrils, as the former are believed to have a lower density of covalent collagen crosslinks. Reductions in both collagen fibril crimp angle and in diameter in the SDFT core were therefore evidence of microtrauma.

The following discussion covers firstly the observed changes in central region crimp morphology in exercised horses, and suggests possible mechanisms. The reduction in central region MAD which was also observed in the exercised experimental horses is then addressed. The findings in the SDFT, SL and DDFT are discussed in relation to the predisposition for lesions to occur in the SDFT in racehorses. A mechanism by which the fibril diameters may have reduced is hypothesized, and the significance of the age at which collagen fibrils become mature in structure (Chapter 8) is discussed in relation to it. On the basis of both crimp morphology and collagen fibril diameter changes, a model for the development of SDFT tendonitis is outlined, and its possible relationship to exercise volume and intensity. Potential reasons for the localization of microtrauma to the tendon core are also related to this model. Functional adaptation of the tendon to galloping exercise is then discussed with respect to both crimp morphology and collagen fibril diameters. The increase in crimp angle as seen in the SDFT periphery of experimental exercised horses is put forward as a possible example for functional adaptation, and a mechanism for this change is hypothesized.

Limited evidence from the experimental horses for resistance of certain individuals to exercise-induced collagen fibril microtrauma is presented.

Finally, the possible clinical implications of detecting central region microtrauma are discussed. Future areas for research into mechanisms of development of collagen fibril microtrauma and its prevention, and into the possible functional adaptation of these structures, are suggested.

9.1 Collagen crimp morphology

This work aimed to establish normal age-related changes in crimp angle and/or length in the central and peripheral regions of the SDFT (Chapter 3), and then to determine if galloping exercise modifies such changes in a way which would predispose the tendon core to injury (Chapters 4 and 5).

Wild horses were sampled to determine the normal age-related crimp morphology, as these represent a population which had not been subjected to

training regimes. It would not be possible to obtain significant numbers of untrained thoroughbred horses in the middle-aged or older age groups. This is because it is not economic to keep horses which serve no function. The significant reduction in crimp angle in the centre of the SDFT with age, such that it had reduced below that of the periphery in horses of 10 years or older was interpreted as a normal age-related occurrence.

The reduction of crimp angle and also crimp length in the SDFT core region of random source horses under the age of 10 years which had undergone recent competitive galloping exercise (Chapter 4) indicated that the rapid high-strain cycling does modify the normal age-related changes. It is possible that this is actually an acceleration of the age-related changes, so that noticeable changes in crimp morphology occur earlier.

The reduction in crimp angle and length due to training involving galloping, in horses from a controlled exercise trial (Chapter 5), allowed quantification of these changes in response to a certain volume and intensity of exercise. Similar exercise trials could allow determination of a possible correlation between galloping exercise intensity and/or volume and the magnitude of central region crimp angle and length reductions. It is possible that there is a "safe" level of galloping exercise which does not cause microtrauma in terms of crimp morphology but is still adequate for aerobic and muscular fitness.

This study did not indicate the distribution of crimp angles and lengths within and between individual fascicles within the tendon. The fascicles do appear to function independently in the equine SDFT (Goodship 1993). If all fibrils within a fascicle have approximately the same crimp morphology the overall reduction in crimp angle in the SDFT core may be due to alteration of the crimp angle of collagen fibrils in certain fascicles. If crimp angle is influenced by fascicle radius as suggested by Kastelic *et al.* (1980), decreasing towards the centre of the structure, the size distribution of fascicles may influence the crimp angle distribution in a tendon region. It is unknown if fascicle radius is influenced by age or exercise in the SDFT. Measurement of fascicle diameters in transverse sections of the SDFT from experimental horses could be undertaken.

9.1.1 Mechanisms

It is hypothesized that the reduction in crimp angle occurs with age because collagen fibrils may be stretched and relaxed only a finite number of times, straightening slightly thereafter (Figure 9.1). This straightening could occur by axial slippage of subfibrillar units within fibrils. Scott (1995) has suggested that few or no covalent crosslinks occur between subfibrils, meaning that this slippage could potentially occur relatively easily over a period of approximately 10 years.

It is unlikely that the changes are due to the synthesis of large numbers of new fibrils with a smaller angle. The turnover rate of collagen in tendon is low (Gerber et al. 1960), and a study of collagen glycosylation in the equine SDFT has indicated that older collagen accumulates with age (Birch 1993). Also, newly synthesized fibrils would be likely to follow the waveform and alignment of already established fibrils.

If fascicles could be maintained in *in vitro* culture conditions, it may be possible to induce collagen fibril fatigue by cyclically loading fascicles (rapid high-strain cycles) for certain periods of time and relaxing them in between these short periods of cycling. Collagen fibril crimp angle/length could be periodically measured during periods of relaxation using a polarised light microscope, to determine any permanent deformation.

Collagen fibrils in the SDFT core of a racehorse may experience at least the same number of rapid high-strain cycles over a period of months or years, depending on the training regime, as those in the tendon of a wild horse over a period of 10 years. This could result in the observed reduction in central crimp angle, which is abnormal in that age group. The additional change in crimp period length in some of the younger and older exercised thoroughbreds (Chapters 4 and 5) is hypothesized to indicate more severe disruption of tendon ultrastructure, as it implies a shift in the bending points of the crimp (Figure 9.1). This could occur via a 'rebuckling' mechanism, as observed in synthetic fibre-filled composites (Dale and Baer 1974) and rat tail tendon (Torp et al. 1975a), at stress and strain levels approaching their elastic limits. Rebuckling in the synthetic composites was demonstrated to be due to
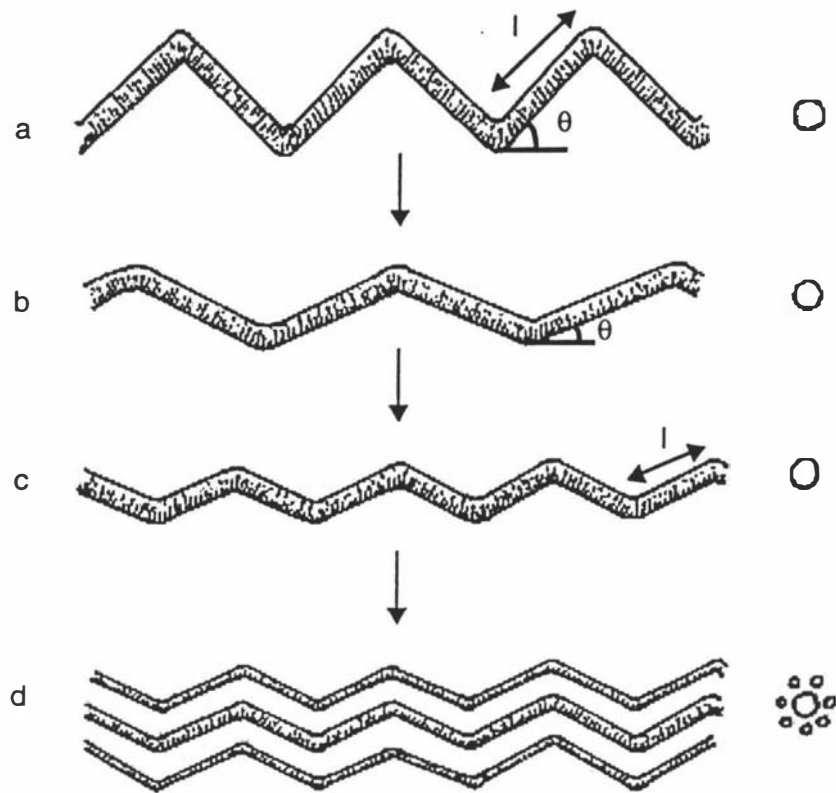
Figure 9.1 A model for the gradation of microtrauma to the equine SDFT core region collagen fibrils. a - normal fibril, θ = crimp angle, l = crimp period length. b - reduction in crimp angle. c - reduction in crimp angle and crimp period length. d - reduction in fibril diameter due to longitudinal splitting.

slippage between fibrils and the surrounding matrix (Dale and Baer 1974). The SDFTs in some galloping horses may experience loads closer to or exceeding the elastic limit, resulting in more severe subfibrillar slippage and disruption of proteodermochondan linkages between the fibrils themselves. The fibril could then recrimp, followed by establishment of new proteodermochondan duplexes. This could be a gradual process, rather than the more dramatic and instantaneous phenomenon observed in the above two studies (Dale and Baer 1974, Torp *et al.* 1975a). The SDFT of wild horses may not experience such extreme high-strain conditions, or experience them only infrequently, explaining why no reductions in crimp period length were seen in those samples.

9.2 Collagen fibril morphology

Measurement of collagen fibril diameters in samples from horses involved in the controlled galloping exercise trial demonstrated a reduction in the MAD for the central region of the SDFT (Chapter 6). A reduction in MAD would weaken the tendon core, as a smaller diameter fibril has been postulated to break at a lower strain than a larger diameter fibril. In contrast, an increase in the MAD would be expected to increase resistance to overstrain.

Although it has been demonstrated that the SL is loaded similarly to the SDFT (Wilson 1991), the collagen fibril MAD for the ligament did not reduce significantly (Chapter 7). It is possible that a greater proportion of the load is distributed to the SDFT at a gallop, or that the site at which microtrauma occurs differs between the two structures (Figure 9.2).

9.2.1 Mechanisms

It is hypothesized that the reduction in collagen fibril MAD is due to rapid high-strain cycles causing separation of subfibrillar units from the largest diameter fibrils, which experience higher stresses than smaller fibrils. Parry *et al.* (1980) observed separation of small units from the peripheries of the largest fibrils in rat tail tendon stretched to failure and hypothesized that at a given level of strain, these large fibrils experience higher stress than those of smaller

accessory carpal bone

deep digital flexor tendon

Suspensory ligament microtrauma

Superficial digital flexor tendon microtrauma

Sampling site for collagen fibril diameter measurements

proximal sesamoid bone
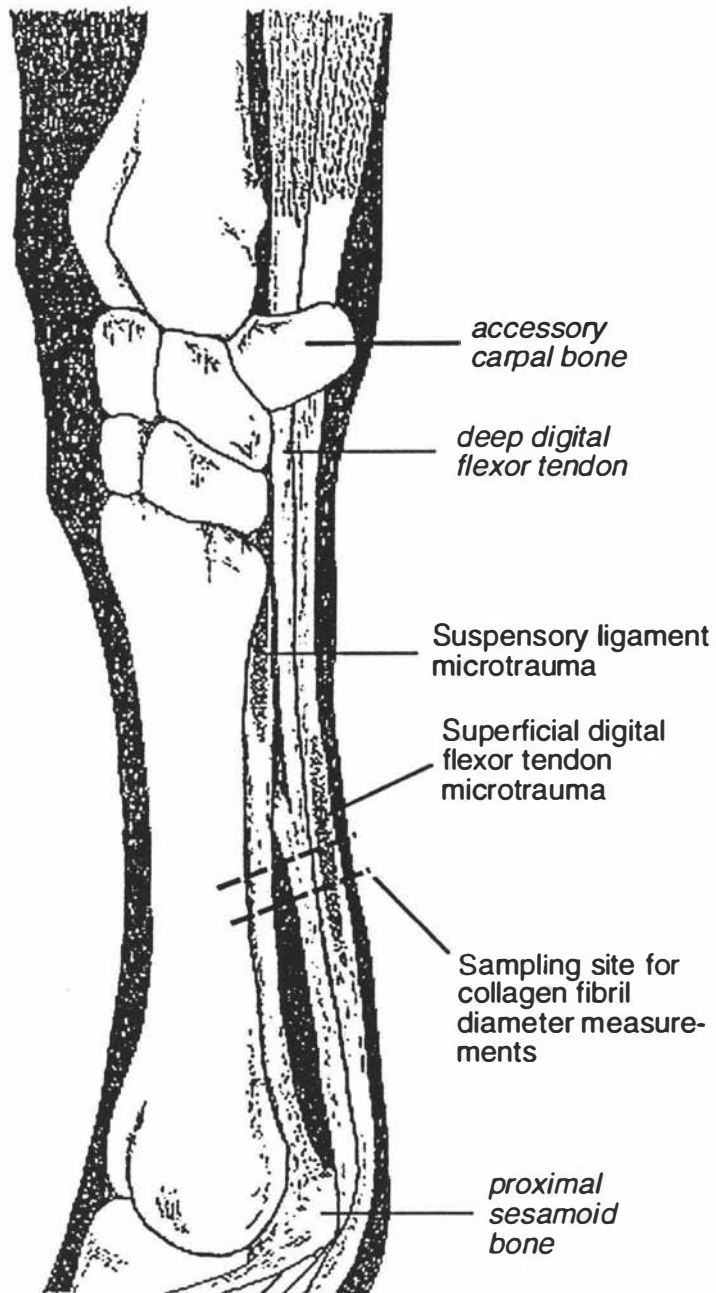
Figure 9.2 Diagram of possible sites of exercise-induced microtrauma in the superficial digital flexor tendon and suspensory ligament of the experimental horses in this study.

diameter. The diameters of the largest fibrils in core regions of exercised tendons were smaller than those from control horses (Chapter 6), therefore a similar splitting process could be occurring in the largest core region fibrils of an equine SDFT which is strained to approach or exceed its elastic limit at a gallop. Michna (1984) observed fibrils which appeared to be splitting longitudinally in the flexor tendons of trained mice. Longitudinal sections were not examined in the present study.

The splitting of small units from large diameter fibrils due to subfibrillar separation would allow the fibril diameter distribution to alter without increased turnover of collagen by tenocytes. It is not known if cellular activity increased in the tendons of the experimental exercised horses in terms of synthesis and/or degradation of collagen. Increased concentrations of reducible collagen crosslinks would indicate increased collagen synthesis. If the latter did not occur, it would support the theory that the 'new' small fibrils arose from already existing structures.

9.3 A model for SDFT tendonitis

This model is summarized in Figure 9.3. It is hypothesized that reductions in crimp angle and length, and in collagen fibril mass-average diameter, occur due to disruption of subfibrillar organisation. It is also hypothesized that these changes lie on a scale, with the least severe involving a reduction in crimp angle only, and the most severe involving reductions in both crimp parameters, and in collagen fibril mass-average diameter (Figure 9.1). The reduction in crimp angle would be considered abnormal only in a horse under 10 years of age.

Changes in crimp morphology would require an axial slippage of subfibrils, whereas changes in collagen fibril diameter would require a complete transverse separation of groups of subfibrils. This implies that more severe disruption is required for the reduction in diameter. The latter statement is supported by the fact that the age-related reduction in central region crimp angle in wild horses was not accompanied by a significant reduction in mass-average diameter. Normal collagen fibril fatigue would involve axial slippage

GALLOPING EXERCISE

↓

Rapid high-strain cycles experienced by
the superficial digital flexor tendon

Increased
tendon
core
temperature

Subfibrillar
slippage

+/-

Interfibrillar slippage
(disruption of proteodermo-
chondan linkages)

Impaired
tenocyte
repair
capacity

Reduction in
crimp angle

Reduction in
crimp length

Altered crimp morphology
in the tendon core

Separation of subfibrillar
units from the periphery of
the largest diameter
collagen fibrils

Relative overloading
of tendon
core fascicles

Partial or complete
rupture

CLINICAL TENDONITIS

**Figure 9.3 A model for the pathogenesis of equine superficial
digital flexor tendonitis.**

of subfibrils only, over a period of 10 years or more. Certain galloping exercise regimes such as that used in the exercise trial caused both axial slippage and transverse separation due to higher strain cycles or greater numbers of such cycles (Chapters 5,6 and 7). It is possible that there could be a progression along this scale, as a reduction in crimp angle in the tendon core would cause increased stress on those fibrils during weight-bearing, which could be sufficient to disrupt subfibrils sufficiently to reduce the diameter of the largest fibrils.

The exact relationship between exercise intensity and volume and the occurrence of the two manifestations of microtrauma is unproven. The reduction in central crimp angle and length seen in randomly selected exercised horses (Chapter 4) and additonal reduction in collagen fibril MAD seen in experimental exercised horses (Chapter 6) indicates that the rapid high-strain cycles experienced by the tendon during galloping are responsible. Further information is now required regarding the speed of galloping, the duration of each exercise period, and the time period over which the exercise regime occurs, in relation to the type and magnitude of microtrauma which develops in the tendon core.

9.3.1 The central localisation of microtrauma

The reduction in the angle and/or MAD of central region fibrils but not peripheral region fibrils suggests a differing mechanical environment between these two regions of the SDFT. In young horses from the wild population, the central angle was significantly higher than the peripheral angle. If strains were experienced evenly across the tendon cross-section this would imply higher stresses are experienced by peripheral fibrils. This is not supported by the occurrence of clinical lesions in the tendon core (Webbon 1977). An alternative explanation is that higher strains are experienced in the core. If higher levels of strain were experienced by fibrils in the core than by fibrils in the periphery of the SDFT of the wild horses, this could explain why there was a significant reduction in crimp angle with age in the core region only. Higher strain levels would be more likely to fatigue collagen fibrils by causing axial subfibril

slippage.

The high temperatures in the tendon core which occur during galloping (Wilson and Goodship 1994) may contribute to fibrillar disruption. Thermoelastic fibre-buckling has been observed in synthetic fibre-filled composites (Dale and Baer 1974) and a similar mechanism could facilitate crimp angle and length reductions. A temperature of 45-50°C is not sufficient to denature fibrillar collagen, but it is hypothesized that these temperatures assist strain-induced axial and transverse slippage between subfibrils. Other factors such as pH may also play a role. Scott (1990) demonstrated disaggregation of subfibrils in rat tail tendon at a pH of 5.9, and suggested that high temperatures could assist this process. The pH level during galloping has not been measured in any region of the SDFT.

The combined effects of different stress and strain levels and other (non-mechanical) factors on collagen fibril transverse structure and crimp morphology could be studied in *in vitro* tests. *In vivo* it is difficult to quantify stresses and strains experienced by collagen fibrils in the two regions of the SDFT. *In vitro*, tendon samples can be subjected to defined mechanical inputs, temperatures and pH. Isolated fascicles, or perhaps tendon sections, could be subjected to various durations and magnitudes of strain combined with high temperature and possibly low pH, and then rapidly fixed *in vitro*. Some samples could be fixed while still under strain, and some after having been allowed to relax. They could then be embedded and sectioned longtudinally and transversely for observation of possible subfibrillar or proteodermochondan disruption. Directly before fixation, crimp parameters could be measured in strained and relaxed samples using a polarized light microscope.

The theory of subfibrillar reorganization in the core of the tendon does not preclude cellular damage (Birch 1993). The high temperatures and other factors such as hyperthermia, hypoxia and high concentrations of oxygen-free radicals (Birch 1993) may inhibit or alter the metabolism of tenocytes in the central region. Increased levels of cathepsins (Miles *et al*. 1994) and inappropriate types of collagen (Birch 1993) have been identifed in subclinical lesions. Compromised cells would be less capable of repairing and/or replacing fatigued or disrupted collagen fibrils, which would assist accumulation of microtrauma.

9.4 Functional adaptation

9.4.1 Crimp angle

If collagen fibrils in tendon develop higher crimp angles in regions where they undergo higher strains, as observed in exercised rat tendons (Wood *et al.* 1988), then the significant increase in crimp angle in the peripheral region of the SDFT of the experimental exercised horses represents functional adaptation. This may occur because mechanical stresses and strains (and other factors) in this region were sufficient to stimulate this adaptation but not extreme enough to cause subfibrillar slippage.

If the crimp angle of a fibril increases, but its crimp period length remains unchanged, this implies that the fibril must shorten. Therefore, in order for the crimp angle to increase *in vivo*, the fibril would need to grow in length. Birk and Trelstad (1984) have suggested that fibril ends lie in narrow cellular cytoplasmic recesses, allowing transduction of tension between fibril and cell. An increase in stress and strain on the fibril could result in a stimulation of collagen synthesis and a growth in fibril length, the fibril retracting axially so the tendon can accommodate the greater length, and the crimp angle increasing as a result (Figure 9.4). The latter retraction requires interfibrillar slippage, and hence the breakage and reformation of proteodermochondan duplexes linking the fibrils. This could be indirectly supported by an increase in reducible crosslinks in that region, or by differences in appearance of the cells in the peripheral region under the electron microscope, in comparison to those from the tendon core. Cells which are more active in protein synthesis have a more extensive rough endoplasmic reticulum and Golgi apparatus. The metabolism of cells in the tendon core has been suggested to be inhibited by the high temperatures, low pH, hypoxia, and oxygen free radicals (Birch 1993). This could include a reduction in collagen synthesis.

9.4.2 Collagen fibril mass-average diameter

It is possible that certain exercise regimes might induce an increase in fibril
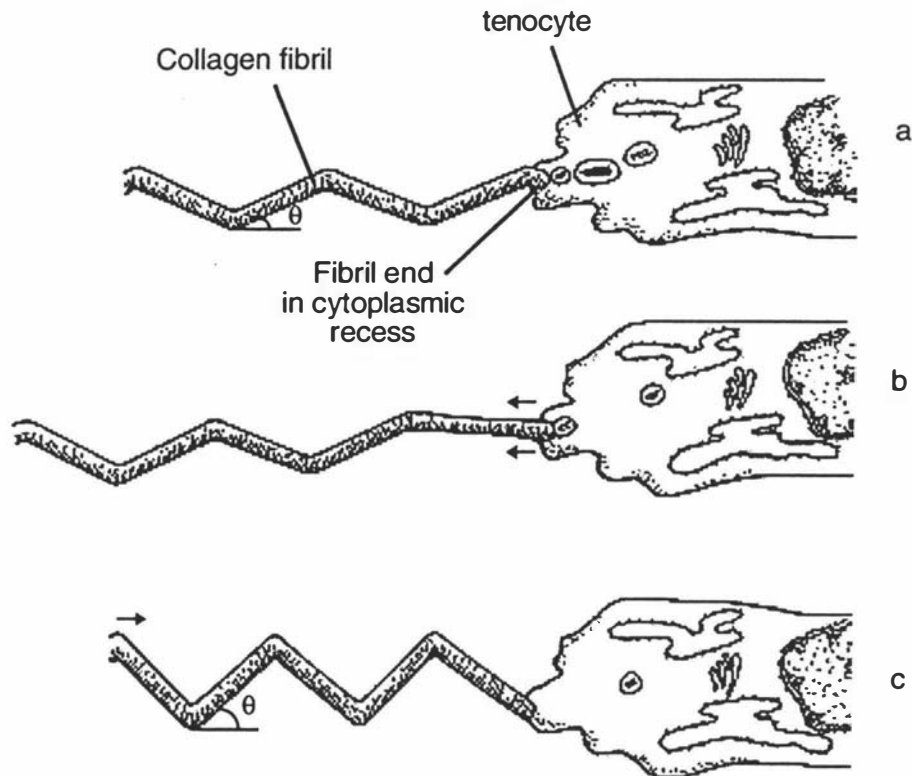
Figure 9.4 A possible mechanism for the increase in crimp angle in the peripheral region of an exercised equine superficial digital flexor tendon. a - a collagen fibril, the end of which lies in a cytoplasmic recess. b - collagen synthesis is stimulated by the increased number of rapid high-strain cycles experienced by the fibril, and new molecules are added to the fibril end. c - the increased fibril length is accommodated by retraction of the fibril, resulting in an increase in crimp angle (θ).

MAD. This would increase the strength of the tendon and hence its resistance to rupture, but would reduce the elasticity which is important in the storage of elastic energy. A correlation of MADs and *in vitro* mechanical strengths of equine SDFTs or isolated fascicles from those tendons would allow an estimation of the increase in MAD which might be possible without significantly compromising energy storage capacity.

Certain exercise regimes of differing intensity and/or volume might induce an increase in collagen fibril MAD. For example, there may have been an increase in MAD in the SDFT core of exercised experimental horses at an earlier stage of the training programme. Michna (1984) noted an increase in fibril diameters in flexor tendons of exercised mice after one week of training, followed by a reduction in diameter. If an increase in MAD occurred in the SDFT core at an early stage of training of the same intensity as that used for the experimental horses in this study (Chapters 5,6 and 7), this would imply that functional adaptation in terms of collagen fibril hypertrophy can occur, but is overcome by the cumulative effect of high strain cycling on subfibrils. The latter would imply that microtrauma was volume-dependent. A regime involving galloping at a slower speed or one with a more gradual increase in intensity and volume might also induce fibrillar hypertrophy and maintain it, by avoiding overloading. This would indicate that microtrauma was intensity-dependent.

9.5 Individual variation

One experimental horse in the exercised group (horse 3) showed a reduction in crimp angle in the central region of the SDFT, but did not show a reduction in MAD. One limitation of analyzing ultrastructure using the electron microscope is that only very small areas may be visualised. It was assumed that the small areas of tendon in which fibril diameters were measured were representative and this appeared to be the case, based on observation of sections from several blocks for each tendon region. Determination of any possible variation in collagen fibril MAD between closely situated areas in the tendon would require an extensive study. It cannot therefore be discounted that the individual variation seen was due to difference in sampling position, or

variation in the extent and location of collagen fibril diameter changes over the tendon cross-section.

It is also possible that some horses are more resistant to collagen fibril disruption. This may be due to lower loading of the SDFT as a result of conformation and/or gait differences, or due to factors within the tendon. Some horses may distribute a greater proportion of weight-bearing load onto the SL, for example. Within the tendon, some horses may have differences in fibril and extrafibrillar composition endowing those components with greater strength, or a more active tendon core tenocyte population, capable of replacing fatigued fibrils more rapidly.

Another horse in the exercised group (horse 5) showed a reduction in mass-average diameter but not in crimp angle. This is explained by the hypothesis that the severity of the microtrauma can differ along the length of the tendon. Samples for crimp morphology and fibril diameter were taken from different levels from the experimental horses. The situations in horses 3 and 5 mentioned above may have been as illustrated in Figure 9.5.

It is difficult to make firm conclusions based on only two horses. Further exercise trials may provide further evidence for individual variation in the response of SDFT ultrastructure.

9.6 The clinical detection of microtrauma

The implication of changes in ultrastructure in the absence of macroscopically observable changes on postmortem is that the core of the SDFT is weakened for some time before clinical signs are observed. The detection of such changes using noninvasive methods would be of use in prevention of further damage which could lead to clinical tendonitis. Biopsies would not be useful in this respect, as the likely damage caused to the tendon (Webbon 1982) would far outweigh the advantage of knowledge of the collagen fibril morphology.

Ultrasonography is the only possibility at this time for noninvasive detection of a reduction in central fibril diameters. Martinoli *et al*. (1993) demonstrated a 'fibrillar echotexture' in calf and sheep calcaneal tendons using a high-

Horse 3   Horse 5
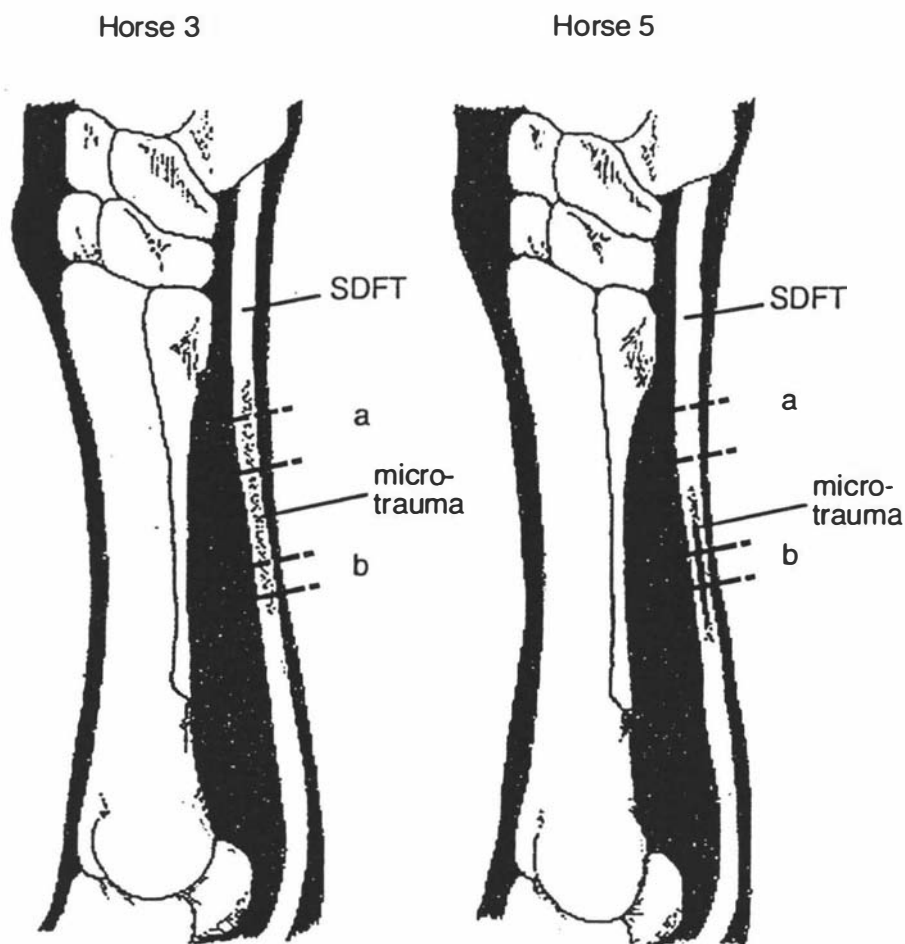
Figure 9.5 Possible individual variation in severity and localization of microtrauma to the superficial digital flexor tendon in two experimental horses in this study. a - sampling site for crimp pattern analysis. b - sampling site for collagen fibril diameter analysis. Dotted areas - disruption of collagen fibrils sufficient to cause changes in crimp morphology. Black areas - disruption of collagen fibrils sufficient to cause a reduction in mass-average diameter.

frequency 'small-parts' probe. The structures seen were 100-300 μm in diameter, and were therefore fascicles. These fascicles could be observed at different depths in the tendon, and could be marked using a fine needle. It is possible that the echogenicity of the fascicles could be related to the collagen fibril diameter distribution within them. It is not likely, however, that any correlation between MAD and echogenicity would be found, because the wavelength of the ultrasound beam is much larger than the diameters of collagen fibrils.

Magnetic resonance imaging also does not appear to have the required resolution at this time. Martolini *et al.* (1993) could not demonstrate images of tendon fascicles using this technique.

If tendon core microtrauma could be detected, it would be useful to know whether changes in crimp morphology and/or collagen fibril diameter may reverse once the horse ceases training. A repetition of the 18 month exercise trial could be undertaken. Three groups would be required: controls, and two exercised groups. One exercised group would be sacrificed at the same time as the controls. The second exercised group would revert to walking exercise for a further 18 months before sacrifice.

9.7 Conclusions

These studies have demonstrated a relationship between galloping exercise and reductions in crimp angle, crimp length, and collagen fibril diameter in the core of the SDFT. Such changes would weaken the tendon core and predispose it to overloading and eventual lesion development, and are therefore defined as microtrauma. The occurrence of precursors to clinical lesions is of importance with respect to understanding the pathogenesis of tendonitis, and indicates that preventative measures may be possible.

The fact that the SDFT is an energy-storing tendon and an important component of the suspensory apparatus of the fetlock require it to undergo high strains, and therefore to have a low safety margin. Strains during galloping are likely to approach the elastic limit of the tendon (Stephens *et al.* 1989, Wilson and Goodship 1991) and are proposed to directly affect collagen

fibrils in the tendon core, causing axial slippage of subfibrils, disruption of proteodermochondans connecting fibrils, and in more extreme situations, transverse separation of subfibrils from the peripheries of large diameter fibrils. Other factors such as tendon core hyperthermia and low pH may assist in fibrillar disruption.

The previously proposed effects of temperature, hypoxia and oxygen free radicals on tenocyte metabolism are of secondary importance in this model, in that this would delay or prevent repair of the above fibrillar damage.

Prevention of SDFT tendonitis could signficantly reduce losses of horses, training and racing days, and racing performance. There are three possible approaches: to develop training methods which allow positive adaptation of tendon ultrastructure to rapid high-strain cycles, to identify individual character-istics associated with a predisposition to SDFT injury, and to identify very early precursors of tendon lesions and alter training accordingly to prevent further development. At present there is very little data on individual responses of tendon structure to exercise, and no non-invasive method for detecting collagen fibril microtrauma. At this time the best approach therefore appears to be to further define the relationships between volume and intensity of rapid high-strain loading and the changes in SDFT central region crimp morphology and collagen fibril mass-average diameter. Both *in vitro* and *in vivo* studies are required to establish these relationships. The eventual aim would be to develop training programmes which cause minimal or no changes, or which stimulate functional adaptation.

9.8 Suggestions for further research

Further investigations of SDFTs from the experimental horses:

- Examination of longitudinal sections under the electron microscope.

- Comparison of the morphology of tenocytes from central and peripheral regions of both groups.

- Measurement of fascicle diameters in central and peripheral regions of

the SDFT for both groups.

In vitro experiments:

- Correlation of the mass-average diameter and *in vitro* strengths of isolated fascicles from equine SDFT.

- *In vitro* fatiguing of collagen fibrils in fascicles in tissue culture.

- *In vitro* subjection of collagen fibrils (in fascicles) to various combinations of high stresses and strains, hyperthermia, low pH and oxygen free radicals.

In vivo experiments

- Measurement of force or strain in the SDFT, SL and DDFT at a gallop.

- Measurement of pH in the SDFT core during galloping.

- Repetition of the exercise trial using three groups of thoroughbreds: controls, horses subjected to galloping exercise regime for 18 months before sacrifice, and horses subjected to galloping exercise for 18 months followed by walking exercise only for 18 months. Measurement of collagen crimp parameters and collagen fibril mass-average diameters in the SDFT, DDFT and SL.

- Repetition of the exercise trial for a shorter period.

Appendices

Appendix A: The evolution of the digit of *Equus*

The reasons for the high frequency of SDFT tendonitis and certain other distal limb injuries lie in the basic anatomical structure of the modern horse. It is therefore important to understand how the horse has evolved in this very specialised way, and the biomechanical implications of that evolution for the lower limb, particularly the forelimb of the thoroughbred racehorse.

The grassland habitat

Eohippus, the 'dawn horse' is the earliest known direct ancestor of the modern horse (Figure A.1). The anatomical changes which occurred from eohippus through to *Equus* took place over a period of approximately 50 million years (Clabby 1976).

During the Eocene epoch, 54 million years ago, tropical and subtropical forests covered most of the land mass of the Northern Hemisphere. Eohippus was a small and successful forest-dwelling herbivore, of which an abundance of specimens have been found in the fossil records in Europe, Asia and North America (Clabby 1976). Downward temperature trends during the Tertiary period reduced the forest area significantly, resulting in the appearance of large areas of grassland by the following Miocene and Pleistocene epochs. The evolution from forest-browser to grazer allowed the exploitation of an increasingly abundant food supply, and was a critical factor in the success and persistence of the family Equidae. The main evolution of *Equus* seems to have occurred in North America, with resultant species migrating to South America, Eurasia and Africa (Simpson 1951, Clabby 1976).

The ancestors of *Equus*

Eohippus was very unlike the modern horse, standing at a height of 2.5-5 hands at the withers (1 hand = 4 inches). The limbs were relatively unspecialised, with four toes on each front foot and three on each hind foot. They were capable of lateral movement as the radius and ulna were separate,

Figure 4. *Molecular structure of collagen in a tendon.* Diagrammatic representation of tendon structure as envisaged by (a), protein chemistry; (b), (c) and (d), x-ray diffraction; (d), electron microscopy; (e), light microscopy; and (f), as seen grossly in a large mammal.
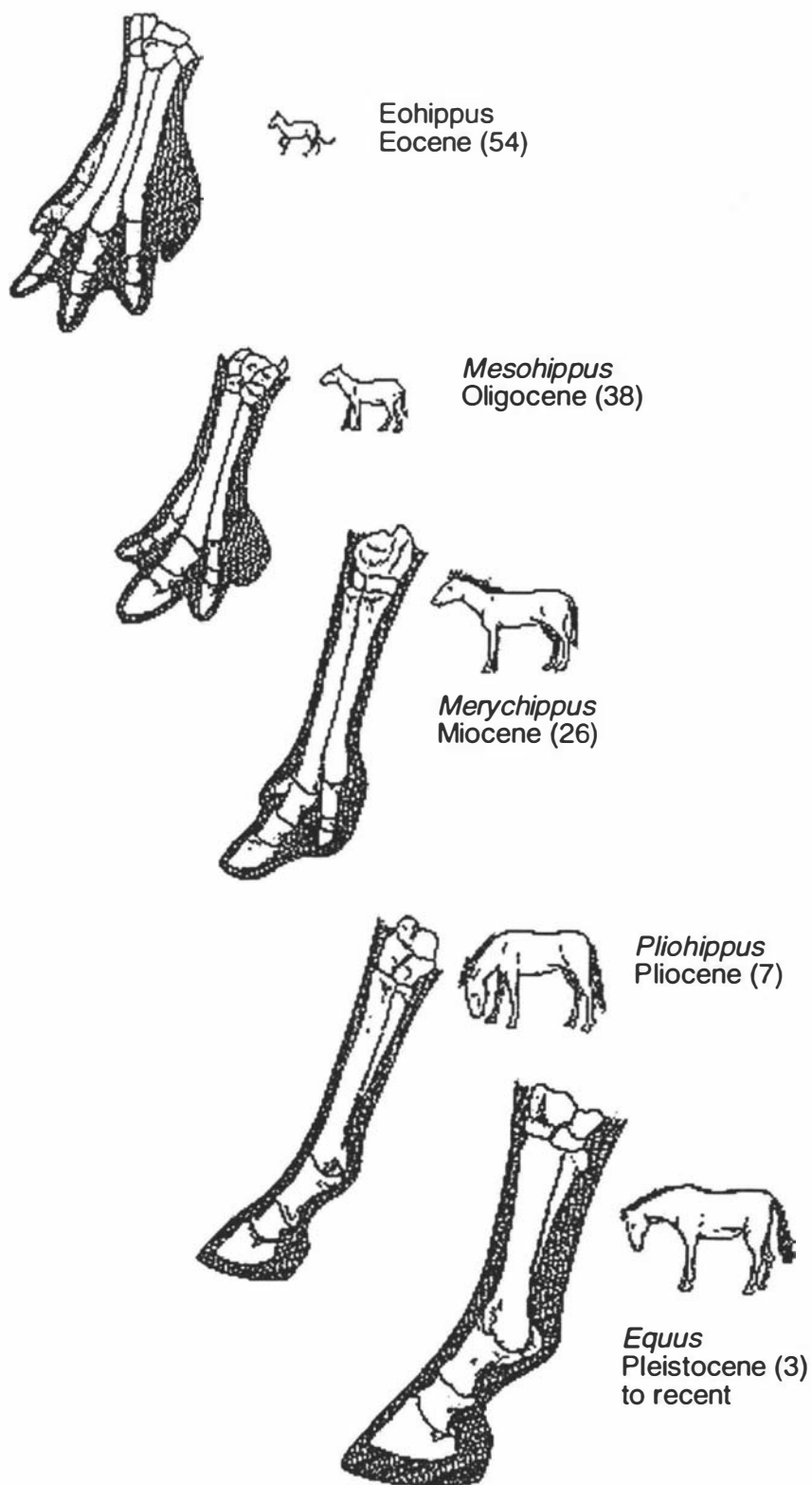
Figure A.1 The evolution of the digit of *Equus*. Figures in brackets refer to millions of years before the present. (Adapted from Simpson (1951) and Clabby (1976)).

as were the tibia and fibula in the hindlimb (Clabby 1976). Although each toe ended in a small hoof, these were non-weight-bearing, with the feet resting on dog-like pads. The hooves were used for thrusting off during running (Clabby 1976). Based on the appearance of its dentition, Eohippus would have been unable to eat grass, and is likely to have subsisted mainly on succulent leaves (Simpson 1951).

The genera of the following Oligocene epoch, *Mesohippus* and *Miohippus*, were 4.5-7 hands in height (Clabby 1976), and the tendency to increase in size continued from this point. The limbs were longer, particularly distally, and more slender. The lateral flexibility of the limbs was reduced, although the radius and ulna remained separate. There were only three toes on each foot, all hoofed and fully functional. The loss of one toe from the forelimb was suggested to provide increased stability for support of the increased bodyweight (Simpson 1951). These animals were still basically forest-dwelling browsers.

The transformation from browsing to grazing dentition occurred entirely with the Miocene epoch in certain evolutionary lines, with modification of other body structures occurring less rapidly. Miocene genera *Parahippus* and *Merychippus* were three toed, but the side toes did not touch the ground in the standing animal, and may only have contacted at fast gaits (Clabby 1976). The central weight-bearing toe was the largest and ended in an *Equus*-like hoof. There may have been a vestige of the foot pad in these species (Simpson 1951). In *Merychippus* adults the ulna and radius had fused, as had the fibula and tibia. Some species of *Merychippus* were as large as many modern-day ponies. *Parahippus* was a transitional form between browsers and grazers. In later species of *Merychippus* the grazing dentition was fully established (Simpson 1951).

In the Pliocene genus *Pliohippus*, the side-toes were finally lost, with the apparently functionless splint bones remaining as vestiges. *Equus* arose from the *Pliohippus* line at the end of the Pliocene, being a one-toed genus of larger animals adapted specifically for running and grazing over large areas of grassland. It spread rapidly to many parts of the world, and was domesticated at approximately 3000 B.C. (Clabby 1976, Simpson 1951). All domestic breeds

belong to the species *Equus caballus.*

General structural changes to the distal limbs

The increase in body size allowed the horse to survive on food of relatively low nutritive value, and to roam over large areas in search of suitable food and water. It also allowed the development of longer limbs in order to increase speed, and allow evasion of predators (Hildebrand 1960). The structural adaptations which occurred in the limbs were therefore influenced by two requirements, namely the capacity to support the higher bodyweight and the ability to run at high speeds with sufficient stamina to escape natural enemies. The consequence was the development of a very specialised and effective means of locomotion.

Speed is the product of stride length and stride rate, a stride being a full cycle of motion of a limb (Hildebrand 1974). A longer limb increases the stride length, but this could not be achieved simply by enlargement of the animal. A larger but unmodified animal would most likely have a slower stride due to overloading of the musculoskeletal system (Clabby 1976). Therefore, the length of the legs relative to the body size increased by elongation of the distal segments, particularly the metacarpals. The effective length was increased still further by adopting a digitigrade or 'finger-walking' stance and by moving the pivot point from the shoulder joint to a point on the scapula by loss of the clavicle, to increase freedom of scapular movement (Hildebrand 1974).

Stride rate was increased by certain modifications of the limb structure which reduced its resistance to acceleration. This was achieved by shifting the centre of mass away from the fastest moving distal part. The heavy musculature shifted proximally and the proximal bones became short and stout. The extensions of muscles to the elongated distal bones became tendonous, which allowed the upper limb muscles to generate maximum displacement with minimal contraction.

Adaptations were also required to reduce the load on locomotor structures, in order to maintain the speed and efficiency of movement. Lateral movement of the limbs became unnecessary and predisposed them to dislocations. The

mass of the limbs was therefore further reduced by reduction of muscles which adducted, abducted or rotated the legs, and the ulna and radius fused in the forelimb (Hildebrand 1974). A fluid fore-and-aft motion of the limbs was adopted, with the leg bones forming bracing, pulley-like joints. Reduction of the toes into one compact unit and fusion of their basal elements into the cannon bone also reduced limb mass while maintaining strength.

It has been estimated that a galloping racehorse can cover approximately 7 metres per stride and complete 2¼ strides per second (Hildebrand 1960). A speed of 63.7 kilometres per hour for a distance of 0.4 km has been recorded (Hildebrand 1974).

Collagenous support structures of the distal limb

The complex system of ligaments and tendons in the distal limb reduces muscular work, and also absorbs a large proportion of the impact force and improve the efficiency of locomotion by elastic energy storage.

The fetlock hyperextends during weight-bearing due to vertical forces on the limb which increase with the speed of the horse. This is resisted by a ligamentous sling consisting of the suspensory ligament and the distal sesamoidean ligaments, assisted by the digital flexor tendons and their associated muscles. Camp and Smith (1942) in their study of attachment scars on fossil bones, showed that the development of this arrangement began in *Mesohippus*, and was associated with the reduction of the digital pads and development of a single-toed foot. The extent of structural change is illustrated by the alteration in the area of the scar of the oblique sesamoidean ligament insertion, which increased by 200-fold from *Mesohippus* to *Equus*, while the palmar area of the first phalanx increased only 30 times (Camp and Smith 1942).

The elastic recoil of the suspensory ligament and digital flexor tendons flicks the foot back after weight-bearing, and helps to propel the body forward (Figure A.2). This is important for endurance as it allows speed to be sustained, by reducing muscular work. Clabby (1976) suggested it may also have developed to compensate for the loss of the supportive and elastic foot pad. The side
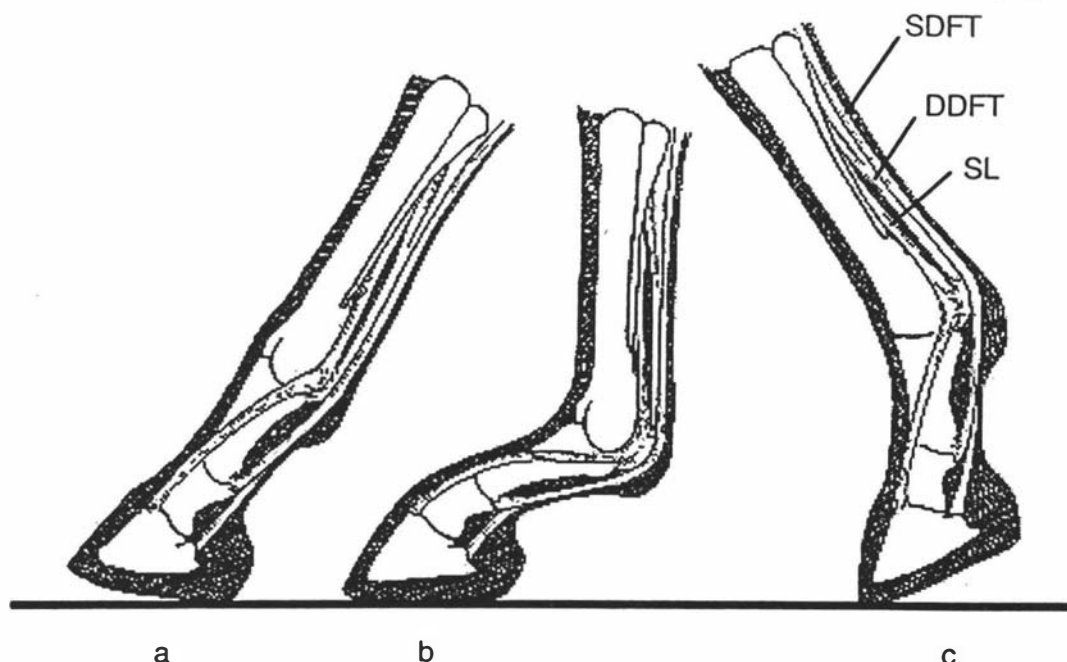
Figure A.2 Suspensory apparatus of the fetlock. a - heel-strike, b - middle of weight-bearing phase, c - toe -off. (Adapted from Hildebrand (1960)).

toes may also have acted as safety buffers in earlier forms, protecting the central weight-bearing toe (Simpson 1951). Development of the accessory ligaments of the SDFT and DDFT appears to have compensated for the reduction of the foot pad (Simpson 1951).

The 'spring' effect in the digit of the horse has become most highly developed in *Equus* due to conversion of the interosseous medius muscle to the suspensory ligament (Camp and Smith 1942). Simpson (1951) stated that "...the springing mechanism did reach its highest development in *Equus* and this is an essential feature in permitting these animals to reach such large size and still to retain great speed". It was one of the most important occurrences in equine evolution.

Appendix B: Horses sampled

(1) Kaimanawa ponies (Chapter 3)

Horse	Age*	Horse	Age	Horse	Age
1	2	*9†*	4	*17†*	7
2	2.5	*10†*	4.5	*18†*	7
3	3	*11†*	5	*19†*	7
4	3	*12†*	5	*20†*	10
5†	3	*13*	6	*21*	11
6	3.5	*14†*	6	*22*	15
7	3.5	*15†*	6	*23*	10+
8	4	*16†*	7		

* ages in years.
† denotes ponies from the second cull (9 June 1994).

(2) Thoroughbreds submitted for necropsy, of 3 years of age or younger (d = days, m = months, yr = year) (Chapter 8).

No.	Age	No.	Age	No.	Age	No.	Age
1	7 m foetus	10	newborn	19	4 m	28	1 yr
2	premature*	11	newborn	20	5 m	29	1½ yrs
3	premature†	12	newborn	21	5 m	30	1½ yrs
4	stillborn	13	newborn	22	5 m	31	2 yrs
5	stillborn	14	newborn	23	5 m	32	2 yrs
6	stillborn	15	3 d	24	5 m	33	2 yrs
7	stillborn	16	7 d	25	8 m	34	2 yrs
8	stillborn	17	25 d	26	8 m	35	3 yrs
9	stillborn	18	3 m	27	1 yr	36	3 yrs

* 6 weeks premature.
† 11 days premature.

Appendix C: Techniques

Electron microscopy

Safety aspects

The solutions and vapours of many chemicals used in processing for electron microscopy are toxic. The arsenic found in sodium cacodylate buffer may be absorbed through the skin, and dust and vapour can cause respiratory irritation (Weakley 1981). Glutaraldehyde is highly toxic and cumulative, and some individuals may become sensitised to it. It should be handled inside a fumehood, with double-gloved hands. Osmium tetroxide is poisonous and highly volatile. The vapour may cause severe conjunctivitis or irritation to the entire respiratory tract (Weakley 1981, Griffin 1990) and the liquid is corrosive. It should also be used in a fumehood, and protective glasses worn. Propylene oxide is a carcinogen and may cause allergic reactions via skin or vapour contact. The vapours of epoxy resins are toxic and prolonged or repeated skin contact may result in chronic dermatitis.

(1) 0.1 M phosphate buffer (pH 7.2)

Make up the following two solutions:

0.2 M di-sodium hydrogen orthophosphate: dissolve 5.68 g of di-sodium hydrogen orthophosphate anhydrous (BDH Laboratory Supplies, Poole, England) in 200 ml of distilled water.

0.2 M sodium di-hydrogen orthophosphate: dissolve 1.56 g of sodium di-hydrogen orthophosphate (BDH Laboratory Supplies, Poole, England) in 50 ml of distilled water.

Mix the two solutions, then make up to 500 ml with distilled water. Check the pH is between 7.2 and 7.4 using narrow range pH paper (BDH Laboratory

Supplies, Poole, England). Store at 4°C for no more than 3 weeks to avoid bacterial overgrowth.

(2) Phosphate-buffered 2.5% glutaraldehyde fixative

Mix 10 ml of 25% glutaraldehyde (Merck, Darmstadt, Germany) with 90 ml of 0.1 M phosphate and store at 4°C. The glutaraldehyde stock solution should be discarded when the pH reaches 3.5 (Griffin 1990).

(3) 1% osmium tetroxide fixative in 0.1 M phosphate buffer

Add 1 g of osmium tetroxide to 100 ml of 0.1 M buffer. Make up at least the day before. The solution remains stable for several weeks at room temperature, if kept away from light.

(4) Preparation of resin

Component	Volume	Volume
Resin (Procure 812)	4.8	9.6
DDSA (hardener)	1.9	3.8
NMA (hardener)	3.3	6.6
DMP-30 (accelerator)	0.2	0.4
	10 ml	20 ml

The resin, hardeners and accelerator were purchased as separate components, and mixed on the day of use. Components were measured into a glass vial, which was sealed with a screw-on cap and mixed thoroughly in a mechanical agitator (Whirlimixer, Fisons Scientific Apparatus, Loughborough, England) for at least 30 minutes before use.

(5) Processing schedule for Chapter 3

Procedure	Time
0.1 M phosphate buffer, pH 7.4	Two 30 minute washes followed by one 1 hour wash.
1% osmium tetroxide	1 hour (4°C)
0.1 M phosphate buffer, pH 7.4	Three 20 minute washes
70% ethanol	overnight
95% ethanol	20 minutes
Absolute ethanol*	One 20 minute wash followed by one 1 hour wash
Propylene oxide*	Two 20 minute washes
Propylene oxide:resin (1:1)	Overnight, with lids off the vials to allow the propylene oxide to evaporate.
Resin (Procure 812)	6 hours

*   99.7-100% v/v ethanol, 1,2-propylene oxide (BDH Laboratory Supplies, Poole, England).

(6) Processing schedule for Chapter 8

Procedure	Time
0.1 M phosphate buffer	Two 30 minute washes followed by one 60 minute wash
0.1 M osmium tetroxide	60 minutes (post-fixative)
0.1 M phosphate buffer	Three 20 minute washes
Dehydration in an alcohol series 25%, 50%, 75%, 95%, 100% (absolute)	20 minutes in each solution, followed by 60 minutes in fresh absolute alcohol
Propylene oxide	Two 20 minute washes
Propylene oxide:resin mixtures	60 minutes in a 2:1 mixture then left overnight in 1:1. Remove lids in the morning and allow the propylene oxide to evaporate

(7) Sodium cacodylate buffers

Prepare a 0.4 M solution of sodium cacodylate by dissolving 21.4 g in 250 ml of distilled water.

To prepare a 0.2 M solution of sodium cacodylate buffer (to make up Karnovsky's fixative), mix 50 ml of the above 0.4 M sodium cacodylate solution with 8 ml of 0.2 M hydrochloric acid (HCl). Add 25 ml of distilled water and adjust to pH 7.2 with 0.2 M HCl, then make up to 100 ml with distilled water. To prepare a 0.1 M solution of sodium cacodylate buffer mix 50 ml of the above 0.2 M solution with approximately 4.3 ml of 0.2 M HCl. Add 25 ml of distilled water and adjust to pH 7.2 with 0.2 HCl, then make up to 100 ml with distilled water.

(8) Karnovsky's fixative (after Karnovsky 1965).

Add 4 g paraformaldehyde to 40 ml of de-ionised water in a flat-bottomed flask. Heat to 60-65°C (n a fume cupboard) or approximately 5 minutes. Add a few drops of 1.0 N NaCl to clear the solution. A slight milkiness may persist, but this will clear as the solution cools.

When cooled, add: 0.2 M Na cacodylate buffer   100 ml

25% glutaraldehyde   20 ml

Adjust pH to 7.2 and make up to 200 ml with distilled water.
Make up the night before tissue sampling, or preferably fresh that morning.

This mixture is thought to provide better fixation than glutaraldehyde alone, because the formaldehyde penetrates tissue more rapidly and may stabilise areas before the glutaraldehyde diffuses to those points and permanently fixes them.

(9) Processing schedule for Chapters 6 and 7

Procedure	Time
0.1 M sodium cacodylate buffer*	Three 20 minute washes followed by four 10 minute washes.
1% osmium tetroxide* in 0.1 M buffer	2½ hours
De-ionised water	Four 10 minute washes
Dehydration in an ethanol series: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 90%, 95%, absolute, absolute	10 minutes in each
Absolute ethanol from a new bottle (to ensure no water is present)	Three 10 minute washes
Absolute ethanol:propylene oxide*	10 minutes each in 2:1, 1:1, 1:2
100% propylene oxide	Two washes, 10 - 15 minutes in total
Propylene oxide/Taab 812 resin*	10 minutes in 1:1 followed by 1 hour in 1:2
100% resin	3-4 resin changes over 2-3 days

* Taab Laboratory Equipment Ltd., Reading, England.

(10) Preparation of uranyl acetate stain

Sufficient uranyl acetate powder is mixed with 50% ethanol in a test tube to produce a saturated solution. This compound is mildly radioactive and care must be taken to avoid skin contact or inhalation. The solution is then centrifuged for 5 minutes, and the supernatant used immediately. The stain must be stored in an amber bottle, away from light. Shake the day before use to resaturate the solution.

(11) Modified lead citrate stain (Sato 1967)

Mix: lead nitrate   1.5 g

           lead acetate   1.5 g

           lead citrate   1.5 g

           distilled water 90 ml

Heat to 40°C and stir for one minute, then add 3.0 g of sodium citrate and stir for a further minute. Add 24 ml of 1.0N decarbonated sodium hydroxide (Pauling Industries Ltd, Auckland, NZ), then 24 ml of distilled water. Stand for 2-3 days before using.

The stain should be crystal clear, with any cloudiness indicating that the stain should be discarded due to carbon dioxide contamination. Very clean glassware should be used and the stain kept in a place where it will not be disturbed. Lead can be absorbed through the skin, and the dust may be inhaled. Gloves should therefore be worn and any spilled stain should not be allowed to dry on the bench (Weakley 1981).

Histology (Chapter 5)

Preparation of neutral buffered formol saline (10%)

Formalin	50 ml
Sodium dihydrogen orthophosphate	1.75 g
Di-sodium hydrogen orthophosphate	3.25 g
Sodium chloride	4.5 g

Make up to 500 ml with distilled water.

Biochemistry (Chapter 8)

Hydroxyproline concentration (Bannister and Burns 1970)

Diluent:: Mix 400 ml propan-2-ol and 200 ml distilled water. May be stored in a clear glass bottle.

Stock buffer (pH 6.0): Dissolve 57.0 g sodium acetate trihydrate, 37.5 g trisodium citrate and 5.5 g citric acid in approximately 500 ml distilled water. Add 400 ml propan-2-ol and mix, then make up to 1 litre with distilled water.

Oxidant: Dissolve 3.5 g of chloramine T (N-chloro-4-toluenesulphonamide sodium salt) in 50 ml of distilled water. Dilute with 250 ml of stock buffer. This must be prepared on the day of use and stored in a dark bottle.

Colour reagent: Dissolve 30 g 4-dimethylaminobenzaldehyde in 45 ml perchloric acid (60% w/v), then add 250 ml propan-2-ol. This may be stored for up to 5 days at 4°C if protected from light. Protective clothing must be used when handling perchloric acid.

Preparation of hydroxyproline (hyp) standards: A 1 mg/ml stock solution was diluted with distilled water as follows:

[Hyp] µg/ml	0	1	2	3	4	5	6	8	10
mls stock	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.40	0.5
mls water	5.0	4.95	4.90	4.85	4.80	4.75	4.70	4.60	4.5

Appendix D: Computerized Image Analysis

Statements in quotation marks are those which appear on the screen.

"Sample identification"

"Capture image"

"Recalibration required?" (recalibrate if necessary).

"Smooth internal noise"

    Average using a 9x9 window to smooth everything.

        Take a range within the 9x9 window - will be high around edges of fibrils.

        Find a good threshold - using the shape of the histogram and threshold range image.

        Use this as a mask.

        Where range is high (around edges) use original image.

        Where range is low use smoothed image.

    Median filter to remove isolated light or dark points around edges.

"Finding fibril intensity level"

    Threshold the filtered image at the mean pixel value.

    Distance transform the image (codes each background pixel with the distance from the nearest fibril).

    Find the maximum distance (maximum background radius), rmax.

        Let box size = rmax * 2 + 5 (enough to remove background + a bit to spare).

        Take the minimum of this using box size + 2.

            This removes the background from the image.

        Take the maximum of this using box size + 2.

            This puts the dark pixels back again so that isolated dark points don't give problems.

    Subtract this fibril image from the filtered image.

        The overall effect is to make the fibrils reasonably uniform in pixel value.

"Finding background intensity level"

A similar approach is taken for the background:

Threshold the image at the mean of the values above the mean.

The mean is most likely dominated by the fibrils, so this ensures we are above them.

Distance transform the image (codes each fibril pixel with the distance from the background).

Find the maximum distance (minimum fibril radius), rmax

Let box size = rmax * 2 + 5 (enough to remove the fibrils + a bit to spare).

Take the maximum of the filtered image using this box size.

This removes the fibrils from the image.

Take the minimum of the filtered image using the box size + 2.

This puts the light pixels back again so isolated points don't give problems.

Divide the fibril image by this background image.

This normalises the contrast within the image so that the fibrils are uniformly against a uniformly light background. This allows the fibrils to be detected more reliably.

"Segmenting fibrils from background"

Two threshold levels are used: 120 and 170. Thresholding at 170 is the right level for detecting the fibrils (the boundaries are in the correct place) but also picks up a lot of noise. Thresholding at 120 detects only the fibrils, but some of the boundaries are broken.

The two thresholded images are combined as follows: objects detected with the 170 level are used, but only if they include objects detected with the 120 level. This throws away the noise.

"Linking in the holes"

Any holes in the image are considered to be part of the background, i.e. a group of fibrils is touching, surrounding some background pixels.

Each hole in the image is linked to the nearest background pixel. This assumes that the fibrils are approximately elliptical in shape, and that they are not overlapping. In these conditions, the cut will be made between touching fibrils (and not through the middle of the fibril).

"Separating blobs"

> Using the same assumptions, any significant concavity on the boundary of an object indicates that there are two (or more) touching fibrils. The nearest point on the opposite side of the concavity should also be in a concavity. A cut is made between these two points. This process is repeated until no further significant concavities remain.

"Getting size"

> A guard frame is calculated to prevent biasing the results towards smaller fibrils. The height of the tallest, and width of the widest fibrils is found. These define the guard frame - fibrils which have at least part of them inside the box but are not touching the edge of the image are measured.

"Processing individual fibrils"

> Objects smaller than 0.25 mm are too small to be fibrils (1mm for SDFTs, which were photographed at a higher magnification). Objects more than twice at long as they are wide are not fibrils.
>
> All other objects within the guard frame are measured:
>
>> The area of the fibril
>>
>> The minimum diameter of the fibril
>>
>> The elipticity of the fibril (maximum diameter/minimum diameter)
>>
>> Corrected area (area/elipticity, assuming the fibril is circular)
>
> When all objects have been measured, the following are also calculated:
>
>> Number of fibrils measured
>>
>> Proportion of area covered by fibrils
>>
>> The area represented by the image

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201

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