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A STUDY OF THE DISTAL HINDLIMB MUSCLES AND NERVES IN NORMAL AND LARYNGEAL HEMIPLEGIC HORSES

A Thesis Presented in Partial Fulfilment of the
Requirements for the
Degree of
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ABSTRACT

Idiopathic laryngeal hemiplegia has long been recognised as a disease of horses which primarily affects the left recurrent laryngeal nerve, resulting in atrophy of the left intrinsic muscles of the larynx and subsequent left-sided laryngeal paralysis. Recent investigations, however, have resulted in classification of the disease as a distal axonopathy, so that in addition to the recurrent laryngeal nerves, other long nerves in the horse may be affected. This present study was undertaken in order to compare the distal hindlimb muscles and nerves in horses clinically free of neuromuscular disease, and in those suffering from idiopathic laryngeal hemiplegia.

A total of nineteen Thoroughbred horses and two ponies were used in this study. Endoscopic examination and the histological appearance of the left dorsal and lateral cricoarytenoid muscles, and the recurrent laryngeal nerve, were used to classify horses into clinical, subclinical and control groups. A number of samples were taken from multiple sites within several of the most distal hindlimb muscles, in both left and right hind legs. Those examined were the deep digital flexor, the cranial tibial, and the long, lateral and short digital extensor muscles. Histological and histochemical staining techniques were used, which allowed extensive morphologic and morphometric assessment of muscles. Morphometric analysis included calculation of the proportion of fibre types; measurement of fibre size; calculation of atrophy and hypertrophy factors; and histographic analysis of fibre diameter distribution.

The nerves examined were those supplying the lower hind limb muscles, and their distal continuations. These were the common and deep peroneal, tibial,

plantar, and plantar digital nerves. Samples taken from these nerves were embedded in resin and transverse sections examined under light microscopy, enabling an assessment of morphological abnormalities and measurement, using computer-assisted image analysis techniques, of fibre density, and the cross-sectional area of a large number of individual axons. Subsequently the mean and total cross-sectional axonal area were calculated and distribution histograms of cross-sectional axonal area were also established. In five horses teased fibre examination was undertaken to further define the nerve changes.

The results of examination of muscle revealed that abnormalities consistent with those of neurogenic disease were commonly present in the hindlimb in control horses and those affected by idiopathic laryngeal hemiplegia. In the latter group these abnormalities were of both greater frequency and severity, and, in the deep digital flexor muscle, were more severe distally.

Abnormalities were also commonly seen in the nerve samples in control, subclinical and clinical horses. These changes, which included regenerating clusters, thinly myelinated fibres, onion bulb formation, demyelination and remyelination, active axonal degeneration and fibre loss, were found to increase in severity from proximal to distal sites in the limb nerves. As was found in the muscle samples, clinical laryngeal hemiplegic horses were more frequently and more severely affected than control horses. The abnormalities were considered to be indicative of a distal axonopathy.

It was concluded that many apparently normal horses, possibly including smaller breeds, have changes in the hindlimb muscles and nerves, which are associated with peripheral nerve disease, and that the disease

process causing idiopathic laryngeal hemiplegia has an effect on distal hindlimb muscles and nerves.

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PART I INTRODUCTION

For over 100 years laryngeal hemiplegia has been known to be a very common and important, incurable disease of horses. Its relationship to recurrent laryngeal nerve damage has long been suspected (Dupuy, 1825; Cadeac, 1897), and was confirmed shortly after these early reports. Since that time many researchers interested in laryngeal hemiplegia have concentrated their investigations on the pathological changes characteristic of the disease in the intrinsic laryngeal muscles and recurrent laryngeal nerves (Cole, 1946; Cook, 1970; Gunn, 1972). Most of these researchers found changes in the distal end of the left recurrent laryngeal nerve and the intrinsic muscles which receive their motor supply through this nerve. In the early 1970's, however, Duncan and his co-workers in Glasgow (1974) found that the nerve and muscle damage in the disease were also present, albeit to a lesser degree, in the right intrinsic laryngeal muscles and the distal end of the right recurrent laryngeal nerve. An important further development in the search for the aetiology of this disease occurred in still more recent times when Cahill and Goulden (1986a,b,c,d,e; 1987) demonstrated that the pathological alterations associated with this disorder were present, not only in the laryngeal muscles and their nerves, but also in other parts of the body. These authors showed that, in animals affected by the disease, pathological changes occurred in distal hind limb muscles and nerves (Cahill and Goulden, 1986a,b,c,d), and possibly also in long central nervous tracts (Cahill and Goulden, 1986e). They concluded that laryngeal hemiplegia in the horse was the result of a process which caused widespread damage to long peripheral, and possibly, central nerves.

A group of conditions with a similar distribution of neurological changes has been known to exist in humans (Cavanagh, 1964b; Spencer and Schaumburg, 1976), and other species (Duncan and Griffiths, 1977) for some time. These diseases were often referred to as distal axonopathies as their major changes occurred in the distal regions of nerves and primarily affected their axons. Cahill and Goulden (1987) classified laryngeal hemiplegia as one of these diseases. The implication of this classification is important in two aspects. Firstly, if correct, it would seemingly dismiss many of the previously conceived hypotheses on the aetiology of equine laryngeal hemiplegia, since most of these incriminated focal damage to the left recurrent laryngeal nerve as a major factor in the creation of the disease. Secondly, if hind limb damage did occur in laryngeal hemiplegic horses, it could be contributing to the limitation of athletic performance known to be associated with the disorder (Baker, 1987). To date the performance limitations of laryngeal hemiplegia has been considered to result from dynamic collapse of the left arytenoid cartilage consequent upon an increased negative intralaryngeal pressure during exercise (Derksen et al, 1986; Shappell et al, 1988). However, since the hind limb muscles of horses provide much of their propulsive power, widespread neuromuscular damage to these could conceivably affect an animal's competitiveness.

The purpose of this study is to establish normal parameters for hindleg muscles and nerves in the horse and to use these to enable identification of any pathological alterations which may result from idiopathic laryngeal hemiplegia.

Idiopathic Laryngeal Hemiplegia - A Summary

A number of clinical conditions associated with the inability of the arytenoid cartilages to abduct efficiently are known to exist. In approximately 10% of cases the cause of unilateral arytenoid cartilage dysfunction can be identified (Goulden and Anderson, 1981). These include irritant perivascular or perineural injection (Marks et al, 1970), trauma to the neck (Gilbert, 1972), guttural pouch mycosis (Cook, 1976), neoplasia (Hillidge, 1986), organophosphate poisoning (Rose et al, 1981), plant poisoning (Neal and Ramsey, 1972; Schebitz, 1964; Williams, 1945), abscess formation (Klein and Deegen, 1988), and anatomical abnormalities (Cook, 1981; Kannegieter et al, 1986). In remaining cases, which constitute the majority of horses with arytenoid cartilage dysfunction, the aetiology is unknown.These animals are considered to have "idiopathic" laryngeal hemiplegia.

The disease process in idiopathic laryngeal hemiplegia affects the left recurrent laryngeal nerve and, as a consequence, the left intrinsic laryngeal muscles. For this reason the disease is always recognized clinically as an inability to efficiently abduct the left arytenoid cartilage. The clinical prevalence of laryngeal hemiplegia has been estimated to vary between 2.6% and 8.3% (Raphal, 1982; Baker, 1983; Hillidge, 1986; Lane et al, 1987), although pathological changes characteristic of the disease occur in up to 70% of thoroughbred horses (Anderson, 1984).

While the aetiology is not known, a number of factors predisposing horses to the disease have been discussed in the literature. The most consistent factor mentioned is the size of the horse, and many authors consider taller horses to be predisposed to the disease (Cook, 1975; Goulden and Anderson, 1981). Male horses are also considered by most

authors to be predisposed to the condition, although this has recently been disputed by Cook (1988).

Other possible predisposing factors which have been discussed include management (Hobday, 1935; Marks et al, 1970); climate and geography (Hutyra et al, 1938; Mason, 1973); and inheritability (Saks, 1927; Smith, 1927; Cook, 1981).
While the cause of idiopathic laryngeal hemiplegia is unknown, a number of postulated aetiologies have been documented in the literature. These include direct damage to the nerve; intoxications by plants or chemicals; bacterial or viral agents, and vitamin deficiencies.

Mechanical damage has been suggested to occur either by stretching of the left recurrent laryngeal nerve (Rooney and Delaney, 1970; Cook, 1976), or compression in the area of the aortic arch, the so-called Haslam's anomaly (Haslam, 1893). In view of recent findings indicating involvement of the right recurrent laryngeal nerve and lack of evidence of focal axonal degeneration at specific sites in the left nerve, such theories appear to be incorrect.

Various intoxications have also been considered as aetiological agents in this disease. Intoxication by plants of the genus Lathyrus and Cicer arientinum, and lead poisoning, have been implicated (Flemming, 1888; Hutyra et al, 1938; Schebitz, 1964). Such toxicities are probably responsible for less than 5% of all cases of laryngeal hemiplegia (Cook, 1970).

Bacterial and viral agents have been strongly associated with laryngeal hemiplegia because of the frequent observation that horses are found to have arytenoid cartilage dysfunction shortly after a severe viral or bacterial infection of the upper respiratory tract (Maguire, 1958; Mahaff ey, 1962; Neal and Ramsey, 1972). While such observations may

be fortuitous, the possibility that viral or bacterial toxins may damage the distal ends of long nerves cannot be dismissed as a possible causative factor of laryngeal hemiplegia.

Vitamin deficiencies have also been suggested as a cause of laryngeal hemiplegia, particularly thiamine (vitamin B_1) deficiency. Although a similarity in the neuropathology of idiopathic laryngeal hemiplegia and thiamine deficiency in other species has been noted by a number of authors (Marks et al, 1970; Cymbaluk et al, 1977; Duncan et al, 1978), there have been few studies which have investigated this relationship (Loew, 1973) in the horse.

While the aetiology of idiopathic laryngeal hemiplegia is obscure, the pathology of the disease has been reasonably well documented (Cole, 1946; Gunn, 1972, 1973; Duncan and Griffiths, 1973; Duncan et al, 1978; Anderson, 1984; Cahill, 1985). The pathological changes involve a progressive loss of large myelinated nerve fibres from the left and right recurrent laryngeal nerves. The presence of signs of axonal degeneration, chronic demyelination and remyelination with onion bulb formation, indicate the pathology results from continuous or repetitive insults to the recurrent laryngeal nerves. Because these changes have been shown to be consistent with primary axonal damage, most severely affecting distal portions of the nerve, the disease has been classified as a distal axonopathy (Cahill 1985).

Distal axonopathies may affect a number of long nerve tracts, including those in both the central and/or peripheral nervous systems, with damage being more severe in those parts of the axon most distant from the nerve cell body. Such a classification explains why the primary effects in horses are manifested as left laryngeal paralysis, as the left recurrent laryngeal nerve is considerably longer than any other

nerve present in the horse.

Anatomy of muscles examined

Anglicized forms of anatomical terms have been used in this study wherever possible. However, for those muscles which are not readily anglicized or well recognized in this form, the latinized nomenclature is used (Getty, 1975; Popesko (1977).

(i) Laryngeal muscles

Dorsal cricoarytenoid muscle

This muscle has its origin from the lamina of the cricoid cartilage. Its fibres converge to a single insertion on the muscular process of the arytenoid cartilage. Its rôle is in abduction of the arytenoid cartilages.

Lateral cricoarytenoid muscle

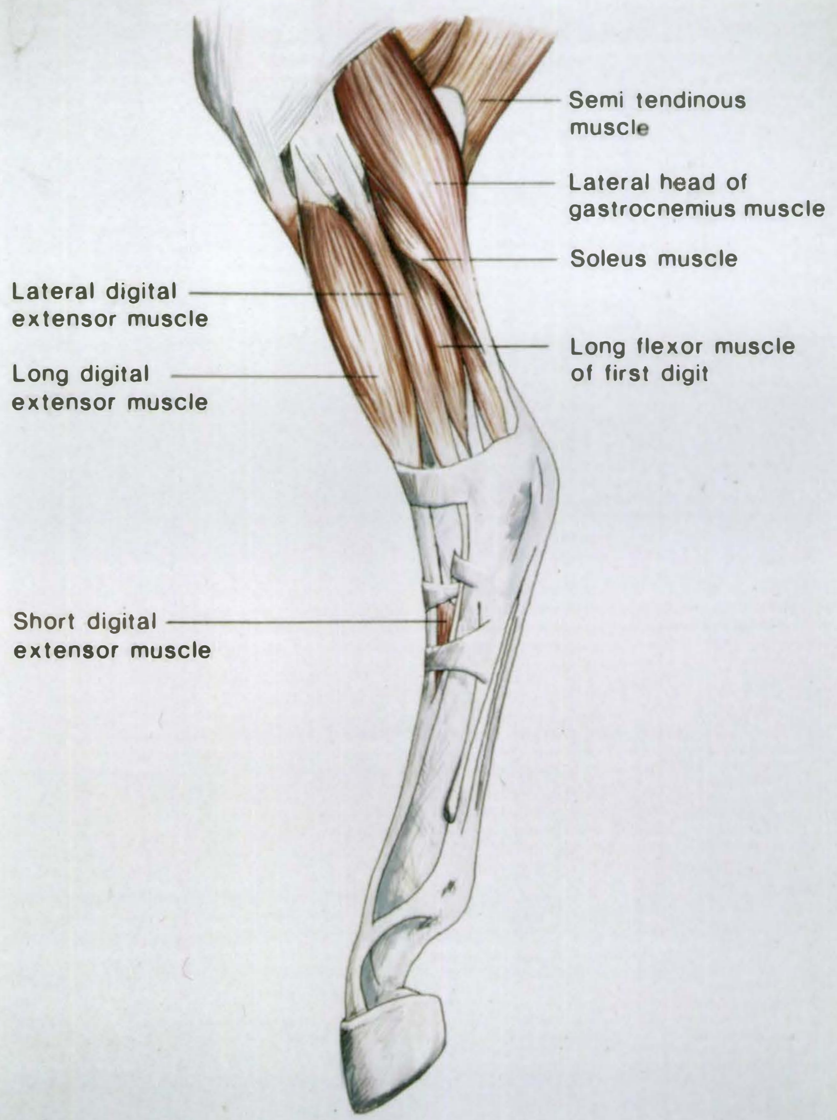
This muscle is situated between the lamina of the thyroid cartilage laterally and the vocal process of the arytenoid cartilage medially. It travels from the arch of the cricoid cartilage to the muscular process of the arytenoid cartilage. Its rôle is in adduction of the arytenoid cartilages.

(ii) Hindlimb muscle

Long digital extensor muscle (fig. 1)

This is a flattened fusiform muscle located on the craniolateral aspect of the hind leg. It has its origin from the peroneus fossa of the femur, in common with the peroneus tertius muscle. The tendon of insertion begins in the middle of the belly of the muscle and is free of muscle tissue at the level of the hock. It continues over the cranial aspect of the metatarsus and is joined by the tendon of the lateral digital extensor a third of the way down the metatarsus. This common tendon of insertion is known as the common digital extensor tendon and inserts on the dorsal aspect of the first and second phalanx and on the extensor process of the third phalanx.

Figure 1. Lateral view of the superficial muscles in the distal hind limb.



Distal muscles of the hind limb. Lateral view.

The main action of the long digital extensor is in extending the digit and flexing the hock.

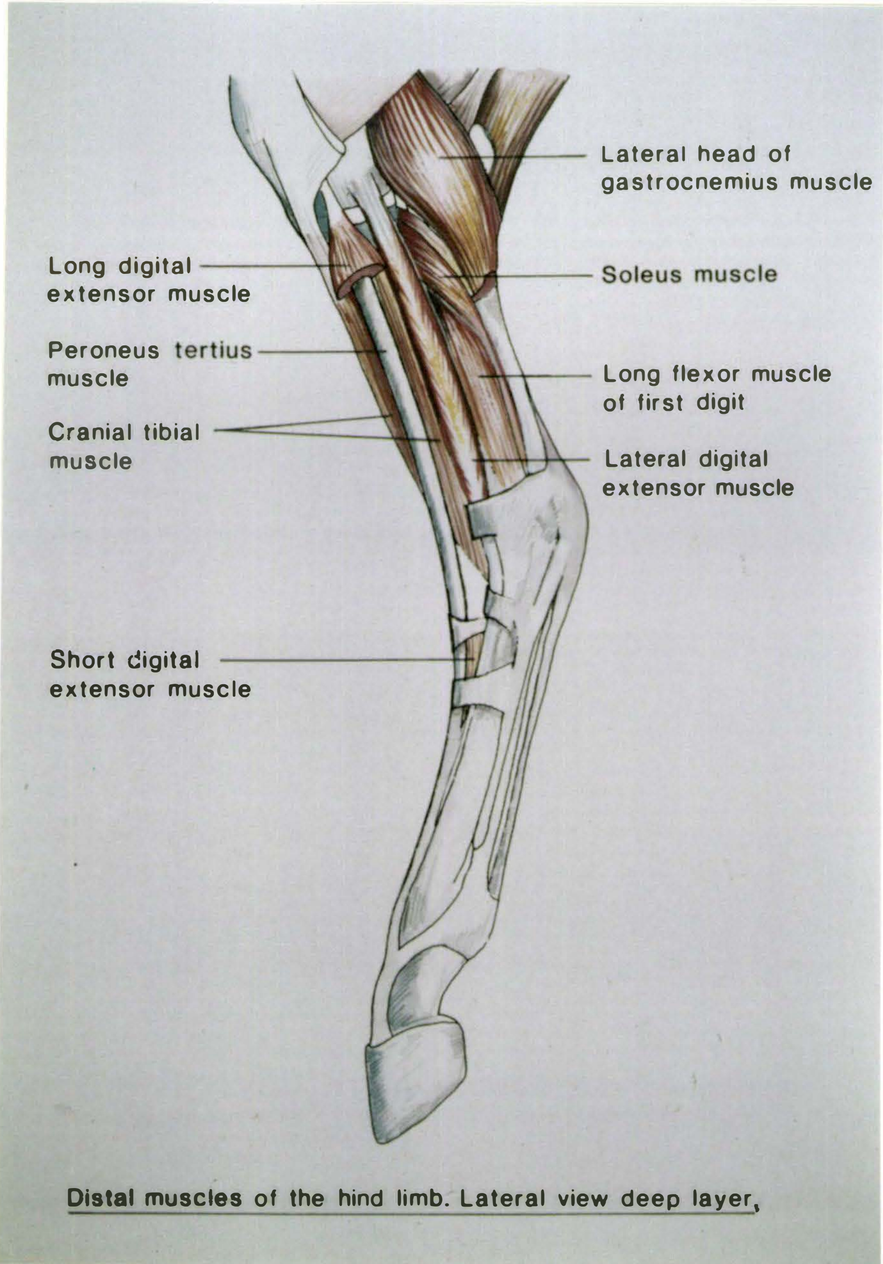
Lateral digital extensor muscle (figs.1,2)
This is a flattened fusiform muscle which lies superficially on the lateral aspect of the hindlimb. It has a wide area of insertion consisting of attachments to the lateral collateral ligament of the stifle, the fibula, the lateral border of the tibia and the interosseus ligament. The tendon of insertion, which is present throughout the muscle, attaches to the long digital extensor one third of the way down the metatarsus. The main action of the muscle is to assist the long digital extensor.

Cranial tibial muscle (fig. 2)
This is a wide, flat muscle which lies directly against the cranio-lateral surface of the tibia, from which many fibres originate. Most of the muscle is deep to the long digital extensor and the fibrous peroneus tertius muscle. Its origin is mainly from the lateral condyle and lateral border of the tibia. Distally it divides into cranial and medial branches. These insert onto the third metatarsal and first tarsal bones respectively. The cranial tibial muscle acts to flex the hock.

Deep digital flexor muscle (figs. 1,2,3)
The deep digital flexor muscle is found on the caudal aspect of the limb, adjacent to the tibia. Although composed of three closely united muscular bellies it has a common tendon of insertion.

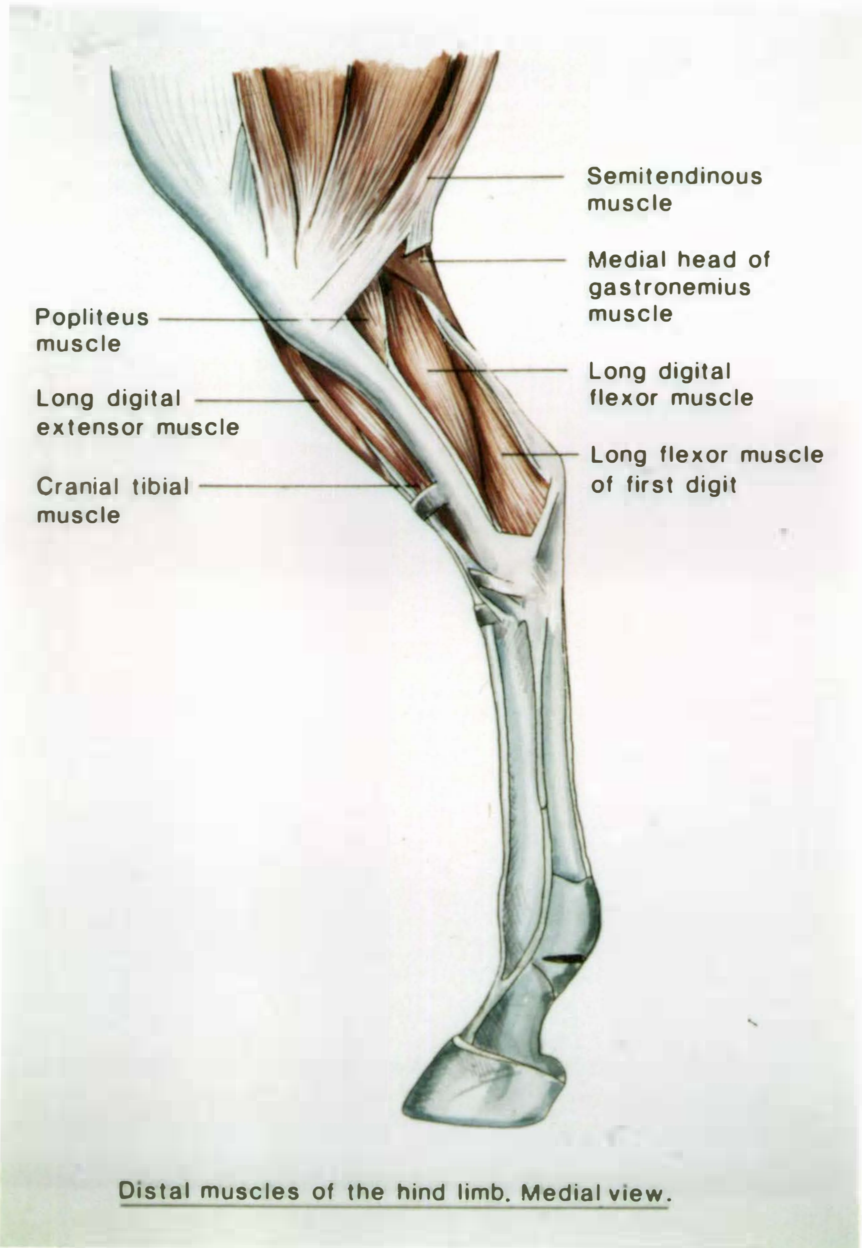
The medial head, or long digital flexor, and the superficial head, or caudal tibial muscle, originate from the lateral condyle of the tibia. The deep head, or long extensor of the first digit, is the largest head and contains extensive tendinous tissue within it. This head arises from the proximal end of

Figure 2. Lateral view of the muscles of the distal hindlimb. The belly of the long digital extensor muscle has been removed.



Distal muscles of the hind limb. Lateral view deep layer.

Figure 3. Medial view of the muscles of the distal hindlimb. Two heads of the deep digital flexor muscle are visible, the long digital flexor muscle and the long flexor muscle of the first digit. The remaining head, the caudal tibial muscle, is obscured by the long digital flexor muscle.



Distal muscles of the hind limb. Medial view.

the tibia and fibula and the interosseus ligament. The common tendon of insertion is found on the caudal aspect of the hock and travels distally to insert on the third phalanx. This muscle acts to flex the digit and extend the hock.

Short digital extensor muscle (figs.1,2)

This is a small muscle found in the angle of the union between the tendons of insertion of the long and lateral digital extensor muscles. It has its origin on the lateral aspect of the peroneus tertius and the middle extensor retinaculum of the hock. It inserts on the tendons of the long and lateral digital extensor muscles. Little is known about the function of this muscle although Getty (1977) states it assists the long digital extensor muscle.

Anatomy of nerves examined

The nerves innervating the muscles involved in this study are the recurrent laryngeal nerves, the terminal branches of the sciatic nerve, the common peroneal and tibial nerves and their tributaries.

(i) Laryngeal nerves

Recurrent laryngeal nerve (fig. 4)

The course of the right and left recurrent laryngeal nerves differs slightly. Both have their origins in the central motor neurones of the mid-brain and initially form part of the vagus nerve as it runs, closely associated with the carotid artery, to the base of the neck on both left and right sides. The right nerve leaves the vagus at the level of the second rib, passes around the costocervical trunk and returns up the neck to the larynx. The left recurrent laryngeal nerve winds around the aortic arch prior to returning to the larynx. This anatomical variation results in the left nerve being longer than the right nerve by up to 31 cm (Cole, 1946). The total length of the left recurrent laryngeal nerve, from the mid-brain to its

Figure 4. Diagram showing the course of the left recurrent laryngeal nerve. Arrows indicate sampling sites.

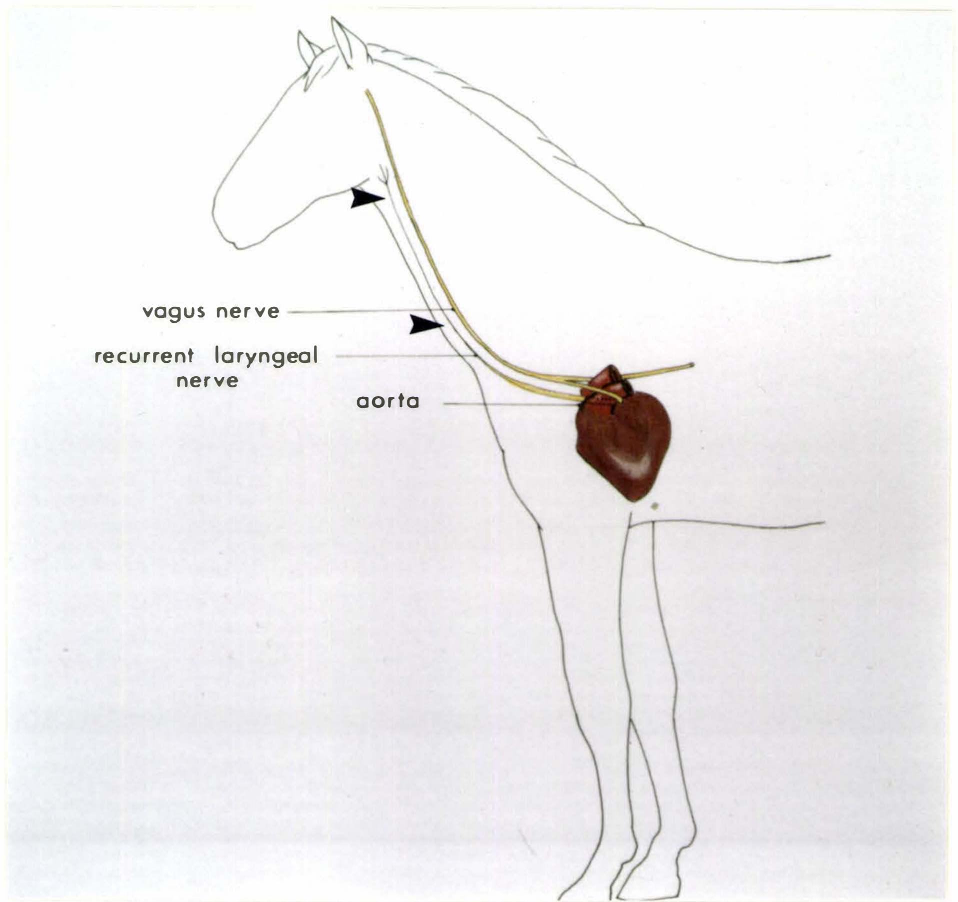
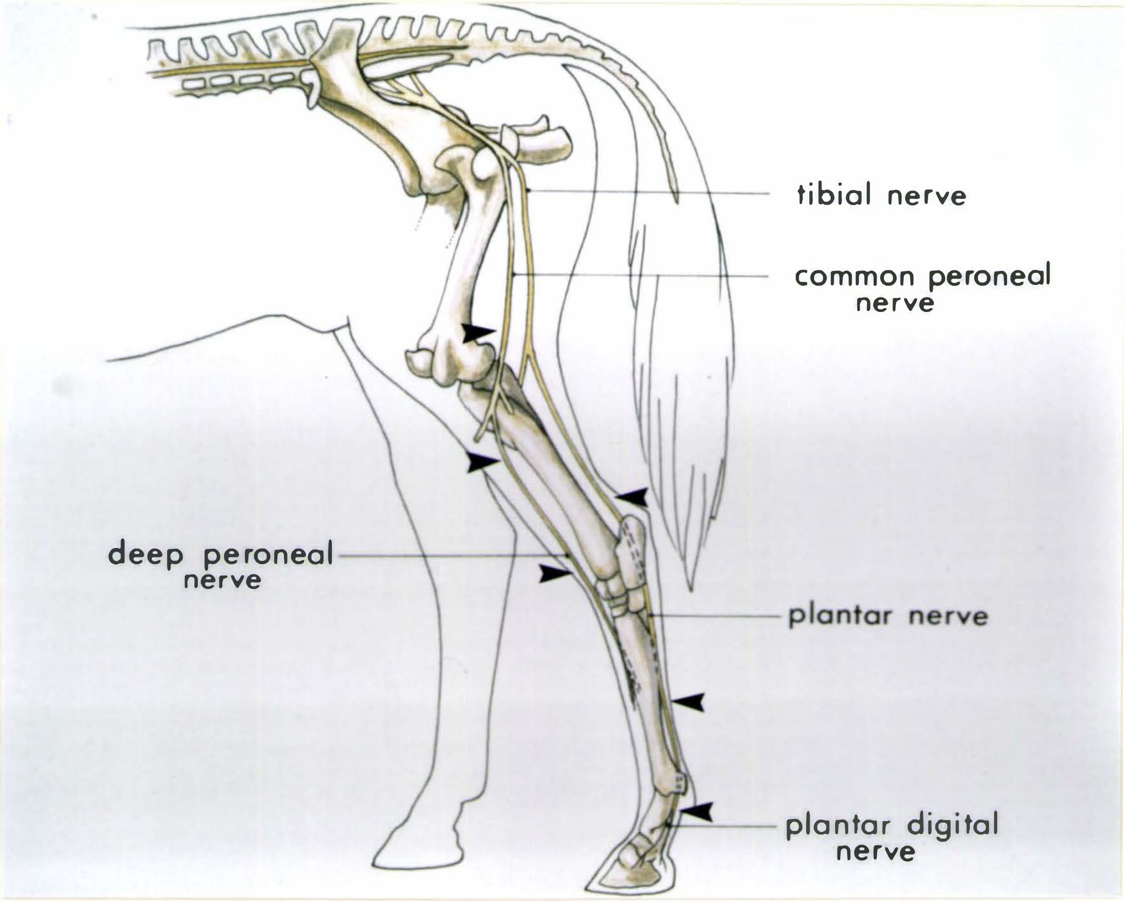

vagus nerve
recurrent laryngeal nerve
aorta

Figure 5. Lateral view of the nerves sampled in the hindlimb. Arrows indicate sampling sites.


tibial nerve
common peroneal nerve
deep peroneal nerve
plantar nerve
plantar digital nerve

innervation of the larynx, can be up to 250 cm (Duncan and Griffiths, 1973).

(ii) Hindlimb nerves

Common peroneal nerve (fig. 5)

This nerve, the smaller terminal branch of the sciatic nerve, arises from its parent shortly after its emergence from the pelvic cavity. It enters the leg between the biceps and lateral head of the gastrocnemius and continues distally down the back of the thigh in company with, and immediately anterior to, the tibial nerve. At the level of the gastrocnemius muscle the two nerves separate. The common peroneal passes over the lateral head of the gastrocnemius to a superficial position at the level of the head of the fibula, over the origin of the lateral digital extensor muscle. Here it divides into superficial and deep peroneal branches referred to as the superficial and deep peroneal nerves.

Small collateral branches of the common peroneal exist and pass from the main nerve to the triceps muscle and skin on the surface of the leg.

Deep peroneal nerve (fig. 5)

This nerve continues distally between the long and lateral digital extensor muscles. It lies deeper than its superficial counterpart and most of its fibres enter the long digital extensor, cranial tibial and <u>peroneus tertius</u> muscles. A small continuation of the nerve runs down the groove between the lateral border of the long digital extensor and anterior tibial muscles to the front of the tarsus where it divides into medial and lateral branches. The medial branch provides nerve fibres to the skin over the dorsal and medial aspect of the limb, while the lateral branch provides nerve fibres to the short digital extensor muscle and skin over the lateral surface of the lower limb.

Tibial nerve (fig. 5)

This nerve, the more posterior and larger terminal branch of the sciatic, passes distally down the back of the thigh between the biceps and semi-membranous muscle and enters the gap between the two heads of the gastrocnemius muscle. It supplies nerve fibres to most of the muscles in the caudal region of the thigh. Just proximal to the tarsus it divides into medial and lateral plantar nerves which pass distally medial and lateral to the flexor tendons and provide the sensory nerve supply to much of the lower limb.

Plantar nerve (fig. 5)

The medial and lateral plantar nerves, which are continuations of the tibial nerve, run adjacent to the deep digital flexor tendon, one medially and the other laterally. They provide much of the sensory nerve supply to the lower limb.

Plantar digital nerve (fig. 5)

The medial and lateral plantar digital nerves are direct continuations of the respective plantar nerves. They pass distally in close association with the corresponding digital artery, and lie along the dorsal border of the superficial digital flexor tendon, proximal to the pastern joint, and along the deep digital flexor tendon distal to the pastern joint. They provide sensation to the plantar areas of the pastern and foot.

PART II - MUSCLES

INTRODUCTION - Skeletal muscle and its reaction to disease

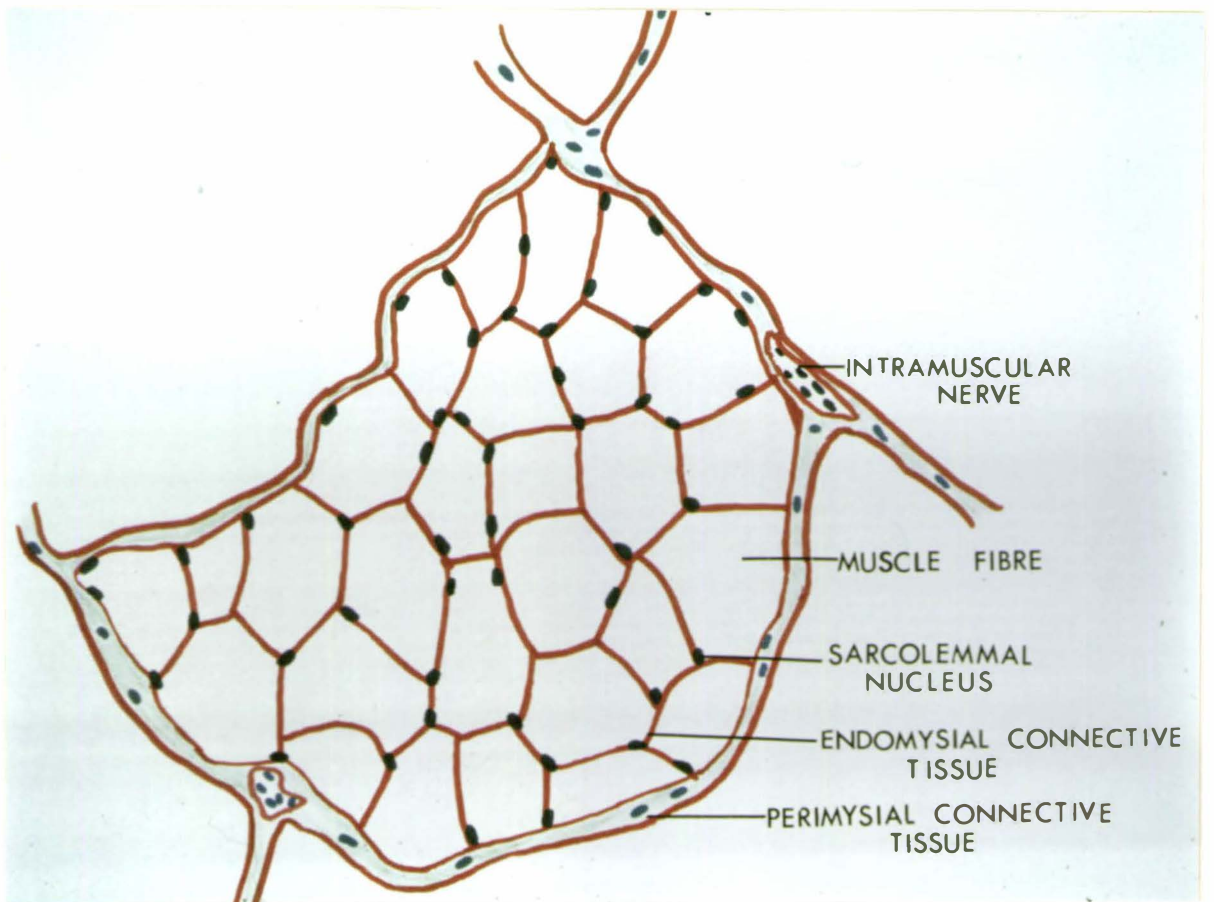
In order to appreciate the effects that the disease of idiopathic laryngeal hemiplegia may have on hindlimb muscles a knowledge of the structure of skeletal muscle, its variation in normal animals, and how it reacts to disease is required. In the following sections of this thesis these are discussed. In addition, the clinical findings and pathology of some myopathies which are known to affect equine skeletal muscle are presented, as well as techniques which can be used to investigate muscle disease in the horse.

(i) Composition of skeletal muscles

Muscle is composed of a large number of individual muscle fibres, often referred to as myofibres. Groups of these myofibres are enclosed in a connective tissue sheath, the endomysium, to form muscle fascicles. Each fascicle is connected to adjacent fascicles by perimysial connective tissue (fig. 6). Groups of fascicles are surrounded by a connective tissue sheath, the epimysium, which forms the outer surface of muscle tissue.

Each myofibre obtains its nerve supply from an axon, which closely adheres to the muscle at the neuromuscular junction. Each axon, by virtue of extensive branching, may innervate up to 150 muscle fibres (Buchthal and Schmalbruch, 1980). Most branching of axons occurs within the muscle itself (Coers and Wolf, 1959), with adjacent muscle fibres often innervated by different axons. The property of each axon will, to a large extent, determine the metabolism of the muscle fibre it innervates (Pette and Vrbová, 1985), resulting in adjacent muscle fibres with different metabolic abilities.

Figure 6. Diagram of a cross-section through a muscle fascicle showing muscle fibres and sarcolemnal nuclei and their supporting connective tissue.



INTRAMUSCULAR NERVE
MUSCLE FIBRE
SARCOLEMMAL NUCLEUS
ENDOMYSIAL CONNECTIVE TISSUE
PERIMYSIAL CONNECTIVE TISSUE

Over recent years classification of muscle fibre types has been based on their metabolic properties. Presently the system proposed by Dubowitz and Brooke (1973) is most commonly used for classification of muscle fibre types into several groups. The main groups proposed are Type I and Type II fibres, with sub-grouping of Type II fibres into Type IIA, IIB and the rarer IIC forms. Some evidence is available which indicates Type I fibres may also be divided into subgroups (Askanas and Engel, 1975; Khan, 1978; Harriman, 1984), but this is not frequently attempted. Differentiation into subgroups is based upon slight differences in enzyme activity within the main fibre groups. With increased sophistication of techniques employed to classify muscle fibres it is likely that additional subgroups will be identified. In fact it has been suggested that, rather than fibres being divided into distinct categories, a continuum of fibre types from one extreme to the other can be identified (Pette and Vrbová, 1985).

Type I fibres are slow twitch and fatigue resistant. They have a high concentration of oxidative enzymes, low concentrations of glycolytic enzymes, and low myosin adenosine triphosphatase (myosin ATPase) activity. Type II fibres are fast contracting, have low levels of oxidative enzymes, higher levels of glycolytic enzymes, and greater myosin ATPase activity than Type I fibres.

Muscle spindles are structures which serve as twitch receptors and regulate muscle length and tone, They are frequently found in muscle tissue, and consist of three to eight specialized fibres which differ histologically and histochemically from other muscle fibres. The distribution of muscle spindles varies between muscles. Some muscles, such as the intrinsic laryngeal muscles of the horse, contain very few, if any, spindles. Identification of pathological alteration in muscle spindles has not been well

defined, and does not generally assist in the diagnosis of neuromuscular disease (Sarnat, 1983).

Variation of fibre type proportions in normal muscle

The proportion of fibre types varies between different muscles and different parts of the same muscle. It also may be influenced by other factors such as age, breed, and athletic fitness.

(i) Variation in fibre type proportions between muscles

In man (Edstrom and Nystrom, 1969; Harriman, 1984), and in the horse (Gunn, 1978; Snow and Guy, 1980; Taylor & Brassard, 1981) fibre type composition of muscles is partly determined by the predominant activity of that muscle. Thus muscles which are slowly contracting and fatigue resistant, such as those used to support posture, generally have a high proportion of Type I fibres, whereas muscles used for locomotion are composed predominantly of Type II fibres. In the Thoroughbred, Snow and Guy (1980) suggested that an increase in proportion of Type I fibres in the muscles of the forelimbs when compared to those of the hindlimbs was related to the function of these limbs, in that the forelimbs are generally used as a braking force while the hind limbs are used predominantly for propulsion. Further support for this concept was provided by Ariano et al (1973) who examined the muscles of five small mammals. These workers found the composition of muscles was consistent across all species and depended upon anatomical location and functional utilization.

In the horse Billeter et al (1987) found the masseter muscle to be composed entirely of Type I fibres, while the subcutaneous muscle contained only Type II fibres, which probably represents an adaption to very specialized functions of these muscles.

A number of studies (Essen et al, 1980; Essen-Gustavsson et al, 1983; Henckel, 1983; Snow, 1983) have concluded that age has no effect on the proportion of Type I fibres, although some changes between subgroups of Type II fibres may occur.

However, more recently Raub et al (1986) found foetal middle gluteal muscle to have a lower percentage of Type I fibres than adults, while Betchel and Kline (1987) found a slight increase in Type I fibre percentage from birth to one year of age. Davies (1972) felt that this change in fibre type proportions was a result of the conversion of one fibre type to another, as he and other workers have shown there is little change in the total number of fibres in the post-natal period (Chiakulas and Pauly, 1965).

(ii) Variation of fibre type proportions within a muscle

Specific regional variation in fibre type proportions within a muscle are well recognized in the horse (Bruce and Turek, 1985; Raub et al, 1985), in man (Nyegaad and Sanchez, 1982; Lexell, 1983 ; Pullen, 1977), and in other species (Gonyea and Ericson, 1977; English and Letbetter, 1982; McConathy et al, 1983).

In the horse a number of muscles have been examined to determine if there is any variation in the proportions of fibre types at different sites. These include the middle gluteal, biceps femoris, triceps brachii, longissimus dorsi, extensor carpi radialis, semimembranous, semitendinous, long digital extensor and soleus muscles (Bruce and Turek, 1985; Raub et al, 1985, 1986; van den Hoven et al, 1985; Andrews and Spurgeon, 1986). The greatest variation has been found in the middle gluteal muscle where changes cranial to caudal, medial to lateral, and superficial to deep have been identified. Differences in the

proportion of Type I fibres from 4% to 70% (Bruce and Turek, 1985; Van den Hoven et al, 1985; Raub et al, 1985), have been recorded from different sites in this muscle. Muscles identified in the horse that show almost no variation in fibre type include the soleus (Raub et al, 1985), laryngeal muscles (Anderson, 1984), masseter and subcutaneous muscles (Billeter et al, 1987), while only slight variation exists in different areas of the longissimus dorsi (Raub et al, 1985; van den Hoven et al, 1985). The most consistent variation has been found to be an increase in the proportion of Type I fibres from superficial to deep areas of many muscles, including the middle gluteal, triceps brachii, biceps femoris and semitendinous muscles of the horse (Bruce and Turek, 1985; van den Hovenet al, 1985; Raub et al, 1986; Kline et al 1987). Such a trend has also been recognized in man (Jennekens et al, 1971b; Lexell et al, 1986) and in rats (Pullen, 1977). These findings contradict earlier reports in man (Johnson et al, 1973; Edgerton et al, 1975) and in the horse (Snow and Guy, 1980), which suggested no significant variation in fibre proportions between superficial and deep portions of the muscle exists. It has been proposed that regional variation in fibre type proportions in a single muscle are present because individual muscles may perform many different functions (Gunn,1978).

(iii) Distribution of fibre types within muscle fascicles

Within a given area of muscle some authors consider that Type I and Type II fibres are distributed randomly (Edstrom and Kugelberg, 1968; Jennekens et al, 1971b, Nyegaard and Sanchez, 1982), but geometrical studies (James, 1971; Lexell et al, 1983) indicate that this is erroneous.

Furthermore, several researchers working in various species of animals and man, have found that Type II fibres tend to predominate at the periphery of muscle

fascicles (Talesara and Goldspink, 1978; Salmons, 1987; James, 1971b; Sjostrom *et al*, 1986; Lexell *et al*, 1984).

(iv) Effect of age on fibre type proportions in muscles

Age apparently affects the proportions of fibre types in immature muscles but has little effect on mature muscles. For example, immature muscles of rats (Kugelbert, 1976) and pigs (Davies, 1972, Suzuki and Cassens, 1980) have an decreased proportion of Type I fibres compared to adult animals. Whether a similar situation occurs in the horse is not as yet clearly documented. In man Anniansson *et al* (1986) found that old age had little influence on fibre proportions, although Jennekens *et al* (1971c) stated that definitive conclusions on the relationship between age and fibre type proportions are difficult to make because of the frequent occurrence of neurogenic changes in older muscle.

(v) Relationship between breed of horse and muscle fibre types

The relationship between breeds of horses and muscle fibre types has been studied by a number of authors (Lindholm and Piehl, 1974; Snow and Guy, 1976, 1980, 1981; Stull and Albert, 1981; Taylor and Brassard, 1981; Andrews and Spurgeon, 1986; Hodgson *et al*, 1986). Their results indicate that horses bred for speed over short distances, such as the Thoroughbred and the Quarterhorse, have a higher proportion of fast-contracting Type II fibres in the muscles of propulsion, in particular the middle gluteal. In contrast, heavy draught breeds and those bred for endurance, have a higher proportion of Type I fibres in these muscles. A similar situation is found in man where sprinting athletes have a higher proportion of Type II fibres compared to endurance runners (Gollnick *et al*, 1972; Saltin *et al*, 1977; Howald, 1982).

(vi) Effect of training on muscle fibre type proportions

The altered activity of muscle can lead to changes in the proportion of fibre types. This has been shown to occur in non-physiological and physiological experiments. In a number of non-physiological experiments in laboratory animals (recently reviewed by Edstrom and Grimby, 1986), it has been shown that conversion of fibre types may occur between Type I and Type II fibres and between sub-groups of the latter. The experimental techniques used to demonstrate this have included cross-innervation studies, direct stimulation of motor nerves and muscles, and treadmill exercise.

Physiological experiments, usually involving altered training methods or observations on human athletes during and after training periods, have supported many of the non-physiological experimental findings. For example, a number of researchers (Lindholm et al, 1983; Nimmo et al, 1982; Hodgson et al, 1986; Edstrom and Grimby, 1986), have clearly shown that an alteration from Type IIb to Type IIa fibres occurs with increasing exercise in both man and the horse.

Physiologically derived evidence for the interconversion of Type I and II fibres is less convincing than that obtained in the non-physiological experiments. Metabolic change in muscle fibres could not be identified with increasing exercise in both man or horses by several researchers (Lindholm et al, 1983; Hodgson et al, 1985, 1986; Edstrom and Grimby, 1986). However, indirect evidence that conversion between these two major groups of fibres occurs with exercise, was produced by Howald (1982) and Larsson and Ansved (1985). These authors observed a dramatic decrease in the percentage of Type I fibres in highly trained athletes who suddenly ceased training. They

considered that this decrease implied that the proportion of Type I fibres increases in the high intensity exercise but rapidly returns to basic levels following reduced activity.

In the horse some confusion as to the effect of training on the proportion of Type I and II muscle fibres exists. Most researchers (Lindholm and Piehl, 1974; Gunn, 1978; Taylor and Brasaard, 1981; Nimmo et al, 1982; Wilson et al, 1987) have been unable to detect changes in the proportions of these fibres with exercise. However, Guy and Snow (1977) found a slight but significant increase in Type I fibres in six limb muscles after exercise, and Henckel (1983) found a similar increase in the middle gluteal muscle of Standardbreds after six months' training. Snow (1983) however reported a decrease in these fibres after eight months' training of Thoroughbreds but he could not determine whether this change was a training effect or a result of variation in biopsy site.

Two explanations as to how interconversion of Type I and Type II fibres may occur as a result of exercise have been advanced in the literature. The first of these is related to the recent finding (Gambke et al, 1985) that a proportion of fibres are constantly undergoing degeneration and being replaced by newly formed fibres from satellite cell proliferation and possibly fibre splitting. This has been shown to occur in response to exercise, but varies between muscles and possibly species (Edgerton, 1970).

The second explanation of fibre type transformation is that, instead of fibres falling into distinct categories, a range of fibre types exists. Each fibre has the genetic coding for expression of either Type I or Type II characteristics, the dominant expression being determined by a combination of physical, neuronal, genetic, positional and

functional demands. An alteration in either of these parameters may result in variation in phenotypic expression of the fibre types involved. In support of this theory is the identification of Type IIC fibres which contain elements of both Type I and Type II fibres and are said to represent a transitional stage between them. Such transitional fibres are frequently found in mature muscle and muscles undergoing changes in workload.

Failure to demonstrate interconversion between fibre types in many physiological experiments may possibly result from a number of factors (Edstrom and Grimby, 1986). These include: variability in biopsy sites and methods; within muscle variation; incorrect muscles being sampled with regard to the training being undertaken; and the training schedules adopted by athletes in experimental studies when compared to the programmes undertaken by high class competitive athletes. Such training programmes have been of decreased intensity, frequency and duration of exercise. Thus subtle alterations in fibre type proportions may not have been detectable because of the experimental variables.

Variation in size of muscle fibres in normal muscle

A small variation in normal muscle fibre size has been demonstrated in many species. In the horse and in man (Brooke and Engel, 1969a; Reniers et al, 1970; Jennekens et al, 1971a), there is general agreement that Type II fibres are larger than Type I, although the reverse may occur in human females (Brooke and Engel, 1969a) and in human postural muscles (Jennekens et al, 1971a). Human Type II muscle fibres also vary more in size than Type I (Brooke and Engel, 1969). In man, the commonest cause of increased muscle fire size is work hypertrophy, which can result in a 30% increase in a few weeks.

The effect of athletic training on muscle fibre size in horses has been studied by several authors but the results are conflicting. Some consider that training increases fibre size (Lindholm and Piehl, 1974; Taylor and Brassard, 1981) while others consider that it either has no effect (Lindholm et al, 1983; Snow, 1983) or actually shrinks them (Henckel, 1983).

Reaction of skeletal muscle to disease

Muscle reacts to a wide variety of influences in a limited number of ways. In man, these changes are frequently investigated by sophisticated techniques capable of detecting subtle alterations. Thus precise diagnoses can be made. In the horse, however, such techniques are not commonly used and, as a consequence, our understanding of many equine muscle disease processes is limited.

A number of morphologic, metabolic and pathologic changes can be recognised in various muscular diseases. The more common of these are discussed below.

(i) Alteration in proportions of fibre types

A change in the proportion of fibre types is frequently used to identify the presence of neuromuscular disease. Such changes have been identified in a number of diseases in man (Telerman-Toppet et al, 1985) and in the horse (Andrews et al, 1986; Roneus and Essen-Gustavsson, 1986). The precise cause of an alteration in fibre type proportions in many diseases is not clearly understood, but a number of aetiological theories have been proposed. Firstly, because of the different innervation of Type I and Type II fibres it is possible that preferential loss of one fibre type may occur. The increased susceptibility of Type II fibres to disease has been demonstrated in a number of conditions, including Duchenne dystrophy (Johnson and Kucukyalcin, 1978), some neuropathic diseases

(Reniers _et al_, 1970) and exertional rhabdomyolysis in the horse (Lindholm _et al_, 1981).

A second mechanism of altered fibre proportions may be due to the response to different functional demands placed upon individual muscles. This may occur as a result of exercise, as previously discussed, or be due to pathological abnormalities in the muscle, resulting in remaining healthy fibres having to perform an altered function. The frequent appearance of Type IIC fibres in such muscles is cited as evidence supporting the possibility of fibre type transformation (Johnson and Kucukyalcin, 1978; Ringqvist, 1973).

Thirdly, selective deficiency of certain fibre specific enzymes has also been suggested as causing abnormal fibre type proportions (Telerman-Toppet _et al_, 1985).

Finally, reinnervation following denervation may also be a cause of altered fibre type proportions (Jennekens _et al_, 1971c). This may be particularly pronounced in muscles which already have a predominance of one fibre type, as reinnervation is likely to result in further fibres of the predominant type (Reniers _et al_, 1970).

Identification of these abnormalities within an affected muscle relies on comparison with muscle samples from similar sites in the same muscles of healthy individuals. The extensive variation in apparently normal muscle, which was previously mentioned, can lead to erroneous results unless these factors are taken into account.

(ii) Alteration in size of fibres

Muscle fibres may increase or decrease in size as a result of pathological as well as physiological processes. An increase in size is recognised as

hypertrophy and a decrease as atrophy. The changes may affect all fibres or those of a particular metabolic type and, since they may be difficult to detect visually under light microscopy, often morphometric examination is required to identify them.

When the nerve supply to a muscle fibre is damaged, there is a decrease in muscle fibre size. In man this proceeds at an even rate to a point where after 120 days the weight of the muscle is reduced to 20-30% of normal (Astrom and Adams, 1981).

Selective Type I or Type II atrophy is frequently found as a result of neuromuscular pathology but is not pathognomonic of any one disease. Type I atrophy is associated with fewer diseases and, in humans, is frequently found in myotonic muscular dystrophy and congenital muscle fibre type disproportion (Sarnat, 1983). Type II atrophy is the more common and occurs in a wide range of conditions such as in disuse atrophy, early denervation atrophy, or following the prolonged administration of steroids. Group atrophy is usually a feature of chronic neurogenic disease but it may also be seen, although infrequently, in some myopathies (Sarnat, 1983).

In addition to the physiological causes of muscle cell hypertrophy, increase in fibre size may occur in primary myopathic diseases, especially muscular dystrophies, and may be seen as a compensatory phenomenon when adjacent fibres have been deinnervated or destroyed (Hofmann, 1980). It may also occur moderately after denervation in some muscles, such as the diaphragm (Harrison, 1981; Niederle and Mayr, 1978).

(iii) Alteration in shape of fibres

Muscle fibres normally have a polygonal cross sectional shape. When this is altered they usually

become rounded or angular although occasionally surface undulations or localised splitting may occur. The common changes in shape usually result from changes in intramuscular pressures or surrounding connective tissue proliferation. The less common surface undulations, or localised splitting are thought to be related to focal necrosis and extrusion of necrotic remnants from the cells (Swashi and Schwartz, 1977).

Rounding of fibres is commonly seen in neonatal muscles and in diseases in which the numbers of fibres per unit area decreases. In neonatal muscle the rounding seemingly results from the process of preparation of the muscle sections (Sarnat, 1983).

(iv) Alteration in numbers of fibres

A decrease in the absolute number of fibres is the best evidence of primary myopathic disease. In contrast, in denervation and disuse atrophy, the first sign of abnormality is a volumetric, rather than numerical, change in the fibres.

(v) Connective tissue proliferation

Increased amounts of intramuscular collagen and fat indicate chronicity of neuromuscular disease. The distribution of the fibrosis may assist in determining if the changes are neurogenic or myogenic in origin (Sarnat, 1983). In most diseases of neurogenic origin, perimysial connective tissue proliferation is more prominant than endomysial. In the terminal stages of neurogenic disease, despite widespread replacement of muscle fibres by fibrous and fatty tissue, a few partial or complete fascicles may usually be found scattered throughout the muscle (Sarnat, 1983). This can also result in the formation of angular rather than rounded fibres as atrophic fibres are compressed by adjacent fibres which distort their shape.

In contrast to diseases of neurogenic origin, in primary myopathic disease there is usually more pronounced endomysial connective tissue proliferation. Collagen often surrounds individual muscle fibres and disrupts fascicular organization as muscle fibres become increasingly isolated. This also has the effect of producing rounded fibres due to an even pressure distribution around them. Also, in contrast to neurogenic disease, connective tissue proliferation occurs shortly after a primary myopathic insult, often as early as 6 weeks. Ultimately there is almost complete replacement of muscle by fat and connective tissue with only a few widely scattered hypertrophic and atrophic fibres remaining (Sarnat, 1983).

(vi) Internal changes to muscle fibres

(a) Internal nuclei

The normal peripheral position of nuclei in mature muscle may become altered in chronic disease states. In such conditions nuclei may migrate to central or eccentral positions in the interior of muscle fibres. In normal neonatal muscle some three per cent of fibres have nuclei which are internally placed. In normal adult muscles the percentage is less, usually around one percent, although an increased number may be found in extraocular muscles, or at myotendinous junctions (Harriman, 1984).

(b) Split and fragmented fibres

Complete longitudinal splitting of muscle fibres is normal in the development of muscle (Harriman, 1984). In normal mature muscles splitting occasionally occurs but is more common in muscular dystrophies, chronic inflammatory myopathies, metabolic myopathies and chronic denervation. They are often found in association with internal sarcolemmal nuclei which may act as a wedge and result in splitting of the muscle fibre. On other occasions they are thought to result from shearing stresses which occur

within a muscle during contraction (Sarnat, 1983). Some fibres may be split in several places. These are referred to as fragmented fibres and may occur as a result of incomplete fusion of regenerating myofibres during repair of muscles.

(c) Myofibrillar whorls

Myofibrillar whorls are a non-specific change in muscle fibres seen in chronic disease states. They result from the loss of normal parallel alignment of the myofibrils along the longitudinal axis and appear as whorl-like formations within the myofibre (Sarnat, 1983). They are usually associated with migrated internal nuclei. It is uncertain if the abnormal position of the nuclei results in faulty coding for orientation of the myofibrils or if both abnormalities are a result of the same adverse influence. Both Type I and Type II fibres can be affected.

(d) Target fibres and central cores

These fibres are best identified using histochemical staining techniques. The target fibre is one which has a ringed or bull's-eye appearance. Fibres with central cores are identified as having central or eccentric longitudinal areas with different histochemically staining properties. Both of these changes appear to result from nervous disorders (Schmitt and Volk, 1975; Sarnat, 1983), and they probably represent similar intracellular lesions. Target fibres are found frequently in biopsies of denervated muscles in man (Engel, 1967) but according to Astrom and Adams (1981) are uncommon in denervation diseases of animals.

(e) Internalization of capillaries

In cross sections of muscles, capillaries are usually seen in the endomysium but occasionally they may be found within a muscle fibre. Such an alteration in capillary position is regarded as a vasodegenerative

change and is observed in chronic muscular diseases of either myopathic or neurogenic origin (Schmitt, 1981). Capillaries occur more frequently in Type I fibres presumably because these fibres have an increased number of capillaries surrounding them (Sarnat, 1983).

(f) Dense or hypercontracted fibres

These fibres appear darkly stained in haematoxylin and eosin and histochemical sections. They are enlarged, rounded fibres that are reported to result from a defective or damaged sarcolemma that is abnormally permeable to calcium. This allows an increased entry of calcium into the sarcoplasm and results in strong contractions of muscle fibres which causes further fibre damage (Cullen and Fulthorpe, 1975). Dense or hypercontracted fibres can be seen in normal muscle as a post-mortem artefact, particularly if there has been a delay in freezing during processing. They are also found in some myopathies, including Vitamin E deficiency (Bradley and Fulthorpe, 1978).

(vii) Degeneration and Necrosis of muscle fibres

Necrosis of muscle fibres is more indicative of a primary myopathic insult than a feature of early neurogenic disease. In man four types of muscle fibre degeneration are recognised (Sarnat, 1983). These are:

1. Focal zones of degeneration within muscle fibres involving some, but not all, myofibrils in the same sarcomere.
2. Segmental necrosis involving some sarcomeres along the fibre.
3. Degeneration involving whole muscle fibres.
4. Fields of necrosis involving adjacent muscle fibres.

Necrosis of a segment of muscle fibre, and subsequent regeneration is more common in myopathies than in neurogenic muscle disorders. Common causes of necrosis include ischaemia, metabolic diseases, autoimmune sensitization, inflammation, toxic insults, and trauma (Sarnat, 1983).

The necrotic fibre loses cellular detail and assumes a finely granular, floccular or hyaline mass, occasionally containing fragments of myofibrils. These changes were first described by Zenker in 1864 (Harriman, 1984), and became known as Zenker's hyaline degeneration. Following muscle necrosis, myophages invade the cell and digest the necrotic material. Phagocytosis of this material often results in the appearance of empty sarcolemnal sacs, which are referred to as "ghost fibres". A small number of inflammatory cells are also usually present.

(viii) Regeneration of muscle fibres

Regenerating muscle fibres have three main histological characteristics (Perl et al, 1968; Adams et al, 1968; Sarnat, 1983). These are:

1. Sarcoplasmic basophilia, due to their high RNA content.
2. True proliferation of sarcolemmal nuclei, which occur in clusters or chains and are closely packed or contiguous. The nuclei are large and vesicular and contain coarse but dispersed chromatin and prominant nucleoli. This resembles the nuclear detail seen in foetal myoblasts. Mitotic figures may occasionally be seen in actively proliferating nuclei during regeneration. This true increase in sarcolemnal nuclei must be distinguished from an apparent increase seen as a result of atrophy of muscle fibres.

3. Reduction in size compared with normal fibres. Histochemically the enzymatic activity is increased in regenerating fibres, which are therefore darker staining, as opposed to the decreased activity and pale staining fibres associated with degenerating changes. These changes are most readily identified with myosin ATPase staining.

Complete regeneration can occur following segmental necrosis, but does not usually take place when large areas are involved. It begins shortly after degenerative processes have been completed, and the regenerative fibres arise from budding of activated satellite cells (Mair and Tome, 1972).

(ix) Denervation and Reinnervation of Muscle Fibres

The nervous supply to muscles plays a major rôle in determining the physiological, metabolic, histochemical and ultrastructural properties of individual fibres. When innervation of a fibre is lost, it will atrophy initially but not degenerate. The earliest change seen in denervation is the presence of scattered fibres undergoing atrophy. This is due mainly to a loss of contractile proteins. As there is no proliferation of endomysial collagen, the atrophic fibres assume an angular, rather than rounded appearance. The loss of cytoplasmic mass results in an apparent increase in sarcolemnal nuclei. Healthy fibres adjacent to those which have become atrophied may undergo compensatory hypertrophy. Occasionally deinnervated fibres may undergo hypertrophy rather than atrophy. This can last for several weeks. These early changes are manifest as a variation in muscle fibre diameter and scattered atrophic angular fibres, although such changes are not specific for neurogenic muscular disease.

If some healthy axons remain, they will undergo collateral sprouting and subsequently reinnervate

atrophied fibres (Wohlfart, 1957; Peyronnard and Charron, 1980). This results in a large number of adjacent fibres being innervated by a single axon, hence they will be of similar histochemical type. This produces the phenomenon of fibre type grouping. Initially these atrophic fibres will appear smaller than normal as the motor neurone innervating large groups of muscle fibres is unable to exert its usual trophic influence on each fibre. Such fibres will eventually increase in size after several weeks or months. Fibre type grouping will be apparent in both Type I and Type II fibres. The predominance of a single fibre type in large groups must be interpreted with caution, as Lexell et al (1983, 1984) demonstrated such findings in apparently normal muscle.

Because of this a number of techniques have been used by different authors in an attempt to quantify, and statistically analyze, the presence of fibre type grouping (James, 1971a, b; Johnson et al, 1973; Lester et al, 1983; Lexell et al 1983, 1984, 1986; Cohen et al, 1987 and Venema, 1988). The large number of methods, all of increasing complexity, indicates the inadequacy of most techniques. The main problems in identifying fibre type grouping arise from the non-random distribution of fibres in normal muscle, and the variation in fibre type proportions, both within and between muscles, which have been previously mentioned.

If continued denervation occurs, the large groups of reinnervated fibres may undergo group atrophy. This is often seen after months, or years of chronic denervating disease. With continued or repetitive axonal destruction there will be an increase in perimysial connective tissue, without a corresponding increase in its endomysial counterpart. This will allow groups of healthy fibres to be surrounded by collagen, yet be adjacent to areas of severe muscular

atrophy, fibre type grouping and group atrophy. This is particularly characteristic of neurogenic muscular disease.

The presence of internal nuclei, split fibres, whorls, and some degenerative changes with foci of mononuclear cells, may also be associated with chronic denervation atrophy.

Some primary diseases of equine skeletal muscle

The pathological alterations found in primary equine muscle disease are poorly documented. Most attention has focussed on clinical findings and response to treatment. A brief description of the clinical signs and pathological changes found in those equine skeletal muscle diseases in which some histological and/or histochemical investigation has been undertaken, are presented here. Other conditions affecting muscle, such as idiopathic laryngeal hemiplegia and stringhalt, which are primary neurogenic diseases with secondary muscular involvement, are discussed later.

Exertional rhabdomyolysis

Exertional rhabdomyolysis is a disease in which breakdown of individual muscle fibres occurs as a result of exercise. It is known by a variety of names, all of which describe various aspects of the disease. These include exertional myoglobinuria, azoturia, tying-up syndrome, cording-up syndrome, equine paralytic myoglobinuria, myositis, cold back and Monday morning disease.

The clinical signs of exertional rhabdomyolysis may vary from mild stiffness and reluctance to perform to complete unwillingness to move, or even recumbancy. The muscles most frequently affected are those of

exertion, such as the gluteal, dorsal back and triceps muscles.

The degree of muscle pathology relates directly to the severity of the skeletal muscle damage. Biopsies reveal muscle degeneration and necrosis, invasion of inflammatory cells, and depletion of glycogen, adenosine triphosphate and creatine phosphate (Van den Hoven, 1987). There is also an accumulation of lactate and glucose (Arighi et al, 1984). Type II fibres, which have a high glycogen content, are most affected (Lindholm et al, 1981).

Malignant hyperthermia

Malignant hyperthermia is an infrequent complication of inhalational anaesthesia in the horse. The syndrome is characterised by changes seen during anaesthesia which include an increase in body temperature, muscle fasciculations, sweating, rigid muscles, hyperventilation, tachycardia and rapid alterations in blood pressure (Manley et al, 1983). Severely affected horses have extensive myositis and myoglobinuria, with significant elevations in muscle enzymes and potassium. Muscle biopsy has revealed mild disparity in fibre size, multifocal areas of eosinophilic granularity, and loss of cross-striation in longitudinal sections (Waldron-Mease et al, 1981).

Nutritional myopathy

Nutritional myopathy refers to a selenium and/or vitamin E responsive disease. It is also known as dystrophic myodegeneration, polymyositis, white muscle disease, muscular dystrophy and maxillary myositis. It is recognized worldwide, but has a comparatively low incidence in horses when compared to other species. The disease is seen mainly in foals (Dodd et al, 1960; Dill and Rebhun, 1985), but mature horses may be affected (Irwin, 1977; Owen et

al, 1977).

The major clinical sign in affected animals is that of muscle dysfunction. The specific muscles affected will determine, to some extent, the clinical signs exhibited. These muscles commonly involved include pectoral, pelvic, cervical, cardiac, diaphragmatic and the masticatory muscles.

Post-mortem findings in this disease include pale discoloration or white streaks in affected muscles with longitudinal calcification and intramuscular oedema. Histologically there may be necrosis and fragmentation with some mineralization of muscle fibres. Muscle necrosis may be present, together with macrophage and mononuclear cell infiltration, and an increase in the number of sarcolemmal nuclei.

Extensive histochemical evaluation of muscles from horses with this disease has recently been undertaken by Roneus and Essen-Gustavsson (1986). Affected foals have higher proportions of Type IIC fibres and a lower proportion of Type I and IIA fibres when compared to healthy foals. Type IIC fibres appear to be more affected in the acute phase. In those foals surviving the disease, normal fibre population is seen about 1-2 months after initial onset of clinical signs. In these recovered foals some fibre type grouping may be observed.

Hypothyroid myopathy

Hypothyroid myopathy is seen in foals as a congenital condition or in adults as an induced hypothyroidism. Foals with congenital hypothyroid myopathy are born weak and have difficulty in walking and suckling. The disease is associated with a variety of musculo-skeletal changes (McLaughlin and Doige, 1981; McLaughlin et al, 1986), including ruptured common digital extensor tendon, forelimb contracture,

mandibular prognathism, and maldevelopment and collapse of carpal and tarsal bones. Histologically widespread degenerative changes have been observed in the common digital extensor muscles. Many fibres are centrally vacuolated, while others have fine granular central areas of calcification. Central nuclei are occasionally observed (McLaughlin *et al*, 1986).

Induced hypothyroid myopathy has also been suggested as a cause of poor performance, muscle pain and exertional rhabdomyolysis (Waldron-Mease, 1979). Muscle biopsy from the semimembranous muscle in such cases shows only minor changes. These consist of mild focal mononuclear cell infiltrate and some areas of increased numbers of sarcolemmal nuclei.

Post-anaesthetic myopathy-neuropathy

A common complication of general anaesthesia in the horse is post-anaesthetic neuro-muscular damage. Affected animals may show an inability or unwillingness to stand, and may have severe myoglobinuria.

The most common lesion associated with this condition is myositis of those muscles subjected to pressure during anaesthesia. In lateral recumbency these include the masseter, triceps and flank muscles, while in dorsal recumbency the gluteals and longissimus dorsi muscles are most affected (Brownlow and Hutchins, 1981). A neuropathy affecting the radial nerve may also be present.

Histopathological muscle lesions can vary within the one horse (Friend, 1981). There may be massive, acute degeneration of muscle fibres which appear swollen, rounded, homogeneous, intensely eosinophilic and show loss of cross-striations. In other cases muscle fibres may appear fragmented, disorganized, vacuolated, focally mineralized and widely separated

by oedema. Often only empty sarcolemnal sheaths remain. Erythrocytes, neutrophils, fibrin strands and mononuclear cells may also be present within the endomysium and perimysium. There may also be some muscles which appear to have undergone ischemic necrosis.

Histochemical examination of affected triceps muscle in the horse shows initial degeneration and necrosis of Type II muscle cells which is similar to the lesion and sequence of early Type II cell degeneration reported in both experimental ischemic myopathy (Lindsay et al, 1980), and in horses with exertional rhabdomyolysis (Lindholm et al, 1974). In severe cases the affected muscles may undergo fibrosis, contraction and atrophy (Brownlow and Hutchins, 1981).

Pasture myopathy

A further acute myopathy seen in horses at pasture, and not associated with exercise, has been described on several occasions (Bowen and Craig, 1942; Pope and Heslop, 1960; Hosie et al, 1986; Whitwell et al, 1988). The clinical and haematological findings resemble those of exertional rhabdomyolysis. The muscles affected are generally those of posture and respiration, rather than movement. Pathological changes are consistent with severe, acute muscle necrosis.

Contracted Tendons

This condition has been included here as muscle fibre pathology has been implicated as a cause of "contracted tendons (Gunn, 1976). This author found evidence of hypoplasia of muscle fibres as well as an increase in fibrous tissue in deep digital flexor muscles in two foals suffering from forelimb

contracture. He suggested the disease was similar to "myopathic" arthrogryposis. However, the aetiology and pathological changes remain speculative.

Myotonia

Myotonia is an uncommon disease of skeletal muscle the horse (Farnbach, 1982; Reed et al, 1988). It is characterized by prolonged after-contracture of muscles with difficulty in relaxation. The hindlimb muscles appear most affected.
Histologic evaluation of muscle reveals variation in fibre diameter and a range well beyond twice normal size. The largest myofibres generally occur in groups, have extremely irregular borders and may be folded within the muscles. On occasions Type I or Type II fibres may not be present (Jamison et al, 1987). Central nuclei are frequently observed, with degenerate and necrotic fibres being common. There is little inflammatory reaction present. Chronically the muscles may become deformed by fibrous pseudohypertrophy. Andrews and Spurgeon (1986) biopsied the middle gluteal and semitendinous muscle in a horse suspected of having myotonia and found hypertrophy of Type I fibres, with atrophy of Type II fibres, as well as significant increases in perimyseal and endomyseal connective tissue.

Histochemical investigation of muscle disease in the horse

A wide variety of techniques are available for the investigation of muscle disease. These include electronmicroscopy, biochemical analysis, electromyography, radionuclide and fluorescent tagging of myofibres, and light microscopic examination of histologically and histochemically stained muscle samples. In the horse not all these

techniques are routinely utilized. In this study histological and histochemical examination of muscle was used to provide information for morphological and morphometric analysis of each sample.

(i) Selection of muscle

Before selecting a muscle for the investigation of a neuromuscular disease, several considerations are required. Firstly, the muscle should be only moderately involved in the disease process. If the muscle is too severely affected then end-stage muscle may be present. Conversely, if only mildly affected, minor or insignificant changes may be observed. In either case the changes may be difficult or impossible to differentiate from alterations caused by non-neurogenic disease processes. Secondly, since fibre type grouping is an important early sign of neuro-muscular disease, muscles with single fibre type predominance should be avoided. Consideration may also have to be given to the size and position of a muscle, particularly when the samples are to be obtained from the conscious animal. In this situation the muscle to be examined should be readily accessible and large enough to provide an adequate specimen for investigation.

(ii) Selection of site within a muscle

The sampling site within each muscle should be made constant as, in some, large differences in fibre type proportions have been shown to exist in different areas. Moreover the myotendinous junction should be avoided as variation in fibre size exists in this area.

(iii) Technique of muscle collection

The two major means of obtaining a muscle sample are by surgical excision or needle biopsy. The techniques for surgical excision of muscle have been well described (McGavin, 1983; Sarnat, 1983;

Korenyi-Both, 1983). Its main advantage is that large amounts of untraumatised muscle tissue can be obtained, while maintaining accurate orientation of muscle fibres.

Needle biopsy techniques have been used extensively in recent years in man (Edwards 1971; Bergstrom, 1975; Edwards, 1971), and in the horse (Snow and Guy, 1976; Hodgson, 1985). The technique of percutaneous needle biopsy has been well described in the latter species (Snow and Guy, 1980; Lindholm and Piehl, 1974). Several biopsy needles are available but all are variations of the Bergstrom needle used in man. The muscle biopsy is usually collected under local anaesthesia via a stab incision through the skin. The main advantages of needle biopsy over surgical excision are that it is less traumatic to the surrounding tissue and causes less patient discomfort. The disadvantages are that the samples obtained are frequently too small and the muscle fibres poorly orientated (Engel, 1967).

A major argument against the use of a needle biopsy is that it represents only a small sample of the entire muscle. A 15-20% difference in fibre proportions, mainly due to "biological inhomogeneity", has been found between repeated needle biopsies at the same site in muscles of different people and a difference of 6-8% between biopsies within the same muscle of a single subject (Halkjaer-Kristensen and Ingemann-Hansen, 1981). Moreover, Sandstedt (1981) studying human muscles and Van den Hoven et al, (1985) investigating equine muscles, have shown that the proportion of fibre types vary between fascicles. For these reasons several authors (Raub et al, 1985; Van den Hoven et al, 1985) have recommended that small biopsy samples should be interpreted with caution and that researchers should precisely define the area from

which samples are collected.

(iv) Processing of muscle samples

Once obtained, muscle samples may be processed in a number of ways. In the past, the most common method used for histological processing was by formalin fixation and paraffin embedding. This method, however, results in a significant degree of artefactual fragmentation and separation of myofibrils (Sarnat, 1983). Additionally, significant fibre shrinkage and distortion of the cross-sectional contour of the muscle fibres may occur.

Fixation of muscle by rapid freezing has superseded formalin fixation for use in studies on muscle disease. This method not only allows histological evaluation but also histochemical, biochemical and electron microscopic assessments to be made. Several methods of freezing are available. To minimise the formation of ice crystals in the muscle as it freezes, it has to be frozen as quickly as possible to -70°C. Liquid nitrogen or Isopentane (2 methylbutasine) alone, or in combination, are used for this purpose. When using liquid nitrogen alone, a coating agent is required to produce a more rapid rate of cooling. Such agents include starch, silicone, talcum or aluminium foil. Without such a coating agent a gas layer forms around the muscle, acting as insulation and slowing the rate of cooling. Isopentane can be used either mixed in a slush with liquid nitrogen, or held in a separate container and cooled by the nitrogen (Moline and Glenner, 1964). Other variations of these techniques have been described (Wu et al, 1985). Once frozen, the muscle can be stored at -70°C. Measures must be taken to prevent freezer burn, for example, wrapping the muscle in aluminium foil or placing it in a vial containing some ice, prior to insertion in the freezer.

After freezing, or storage at -70°C, the muscle sample should be allowed to warm up to the temperature of the cryostat, generally around -20°C to -30°C, prior to cutting. More tissue artifacts occur in stored, frozen samples than in freshly frozen samples, so cutting as soon as possible after freezing is recommended (Sarnat, 1983). After cutting, samples can be stored overnight at -20°C in the cryostat, or returned to -70°C for longer storage.

(v) Histochemical staining of muscle

The enzymes commonly used in muscle to produce a histochemical profile are myosin adenosinetriphosphatase (myosin ATPase) to demonstrate a fibre's contractile properties; an oxidative enzyme to demonstrate aerobic metabolic capacity, for example, succinate dehydrogenase; and a glycolytic enzyme to demonstrate anaerobic metabolism, for example, glycogen phosphorylase (Buchthal and Schmalbruch, 1980).

The most common enzyme used for the histochemical differentiation of fibre types is myosin ATPase. Staining of this enzyme allows differentiation between fibre types based on their speed of contraction (Barany, 1967; Burke et al, 1971; Buchthal and Schmalbruch, 1980; Snow et al, 1981), although some controversy over the relationship exists (Maxwell et al, 1982). In this present study it was the only histochemical technique used. For this reason other histochemical enzymes are not discussed further.

For the in vitro demonstration of myosin ATPase, calcium chloride solution is added to the sample. This reacts with the phosphate released during the conversion of ATP to ADP to form calcium phosphate. At an alkaline pH calcium phosphate is insoluble and

is deposited at the site of enzyme activity. By rinsing in a cobalt solution the calcium is replaced by the cobalt to form cobaltous chloride. The tissue is then placed in ammonium sulphate and a black insoluble precipitate of cobaltous sulphide forms (Sarnat, 1983). Following incubation in an alkaline pH, Type 1 fibres stain lightly and Type II stain darkly. Incubation at a low pH results in reversal of the staining of Type I and II fibres. There are some exceptions to this; for example, neonatal and cardiac muscle, which stain intensely for myosin ATPase at pH 9.4, despite containing mainly Type I fibres and hence being slowly contracting. This is because of the very high level of mitochondria within these muscle fibres (Guth and Sumara, 1972).

(vi) Methods of analysis of muscle

Muscle may be analysed by qualitative or quantitative means.

(a) Qualitative analysis

Qualitative assessment involves a subjective analysis of the muscle noting factors such as variation in fibre size, shape and grouping, the position and frequency of nuclei, and the degree of connective and fatty tissue infiltration. The degree of changes can then be classified into several grades from slight through to severe (Anderson, 1984).

(b) Quantitative analysis

The proportion of different fibre types within a muscle and the size of muscle fibres can be calculated quantitatively to investigate alterations which result from various disease processes.

Several studies have investigated the number of fibres to be counted to provide an accurate assessment of fibre proportions. These indicate that counting 400 to 600 fibres is as accurate as counting

several thousand (Nyegaard and Sanchez, 1982; Bruce and Turek, 1985).

Muscle fibres size can be calculated by measuring either fibre diameter or fibre cross sectional area. For assessment of fibre diameter, the technique of choice is to measure the minimum diameter of the fibre (Brooke and Engel, 1969; Reniers et al, 1970; Jennekens et al, 1971a; Dubowitz and Brooke, 1973; Larsson and Ansved, 1985; Andrews and Spurgeon, 1986). This is defined as the minimum distance, taken through the centre of a muscle fibre, between opposite sides of the fibre (Brooke and Engel, 1969a). This measurement has the advantage over the calculation of fibre area, as it is less influenced by oblique sectioning and is less tedious to perform than area measurements (Jennekens et al, 1971a). Histographic analysis of the distribution of fibre diameters can provide information on the extent of atrophy or hypertrophy present in muscle (Adams et al, 1968; Brooke and Engel, 1969a,b; Andrews and Spurgeon, 1986).

Based on the minimum fibre diameter histograms, atrophy and hypertrophy factors can be calculated using the system of Brooke and Engel (1969). This involves determining a normal range of values for fibre diameter, which is calculated for each muscle as the mean fibre diameter plus and minus 2 standard deviations. The number of fibres falling outside this range is then multiplied by between 1 and 4, the precise value corresponding to the number of standard deviations away from the normal range of fibre diameters. For example, the number of fibres the diameters of which are up to one standard deviation outside the normal range are multiplied by one; the number between 1 and 2 standard deviations are multiplied by two; for between 2 and 3, multiplied by 3; and the number greater than 3 multiplied by 4. These values are then added to give a final atrophy

and hypertrophy factor for each muscle and fibre type. The factors give a numerical indication of the number of excessively small or large fibres, thus providing the degree of atrophy or hypertrophy present.

Some authors have suggested that measurement of cross-sectional fibre area may be a superior technique to other methods of analysis in identifying fibre size changes in neuromuscular disease (Renier et al, 1970). However, it is well recognized as being a very complex procedure (Adams et al, 1968). It can be performed using manual or semi-automatic techniques. Manual techniques include placing graph paper under a muscle fibre and determining the number of squares superimposed on the fibre. Semi-automatic methods rely on the outline of fibres traced onto a digitizer and the area calculated. The number of randomly selected muscle fibres required to be counted for an accurate evaluation of cross-sectional area is around 20 (Halkjaer-Kristensen and Ingemann-Hansen, 1981) to 25 (Sandsted, 1981). The major disadvantage of this technique is that even slightly oblique sectioning of the muscle can result in widely differing results.

MATERIALS AND METHODS

Experimental horses

Nineteen Thoroughbred horses and two ponies ranging in age from six weeks to 27 years were used in this investigation. They were divided into three groups on the basis of clinical, endoscopic and microscopic observations (Table 1).

The first group (clinical) contained eight horses which had an increased inspiratory sound at exercise and endoscopic evidence of an inability to abduct or maintain abduction of the left arytenoid cartilage during quiet respiration. There were obvious

pathological changes in the laryngeal muscles and recurrent laryngeal nerve.

The second group (sub-clinical) consisted of four horses in which no abnormal respiratory noise was detected at exercise and movements of the arytenoid cartilages was similar to the control group. However, pathological changes were seen in the intrinsic laryngeal muscles and recurrent laryngeal nerves.

TABLE 1 Experimental animals

Group Status	Horse	Age (years)	Height (cm)
Clinical	2	5	170
	4	6	165
	7	2	160
	9	5	158
	14	4	157
	15	25	160
	19	3	158
	21	27	155
Subclinical	1	7	155
	6	1-2	150
	16	2	153
	17	5	171
Control	3	2	150
	5	1	146
	8	7	152
	10	1	145
	11	3	152
	12	0*	-
	13	6	155
	18+	7	122
	20+	7	127

* 6 wks old

+ pony

The third group (control) contained nine horses in which there was no detectable abnormal respiratory noise made at exercise. Movements of the left and right arytenoid cartilages appeared symmetrical on endoscopic examination and no, or negligible, changes

were found on light microscopic examination of sections of the intrinsic laryngeal muscles and recurrent laryngeal nerves.

In order to determine if disease processes other than idiopathic laryngeal hemiplegia were present in any case, a full clinical examination was performed. This included observation while walking, trotting and lungeing, as well as measurement of temperature, respiration rate and heart rate. Palpation of the larynx, laryngeal cartilages, and jugular groove was performed. Horses included in this study showed no physical abnormalities of these structures. The height of each horse was also recorded. Endoscopic examination was carried out at rest using a flexible fibreoptic endoscope with a nose twitch applied to the animal for restraint.

Following examination all horses were euthanased by the intravenous administration of pentobarbitone and autopsied.

Collection of muscle samples

The larynx was dissected free and in all horses a one centimetre wide sample was taken from the middle of the left and right dorsal and lateral cricoarytenoid muscles. In all except two clinical animals (horses 14 and 21) one to two cubic centimetre samples were taken from proximal, middle and distal sites from each hindlimb muscle sampled (Fig. 7) except the short digital extensor. In this muscle, because of its small size and flattened shape, a single sample was taken across the middle of the muscle belly. In the two clinical horses mentioned above, only the middle sample was collected from the hindlimb muscles.

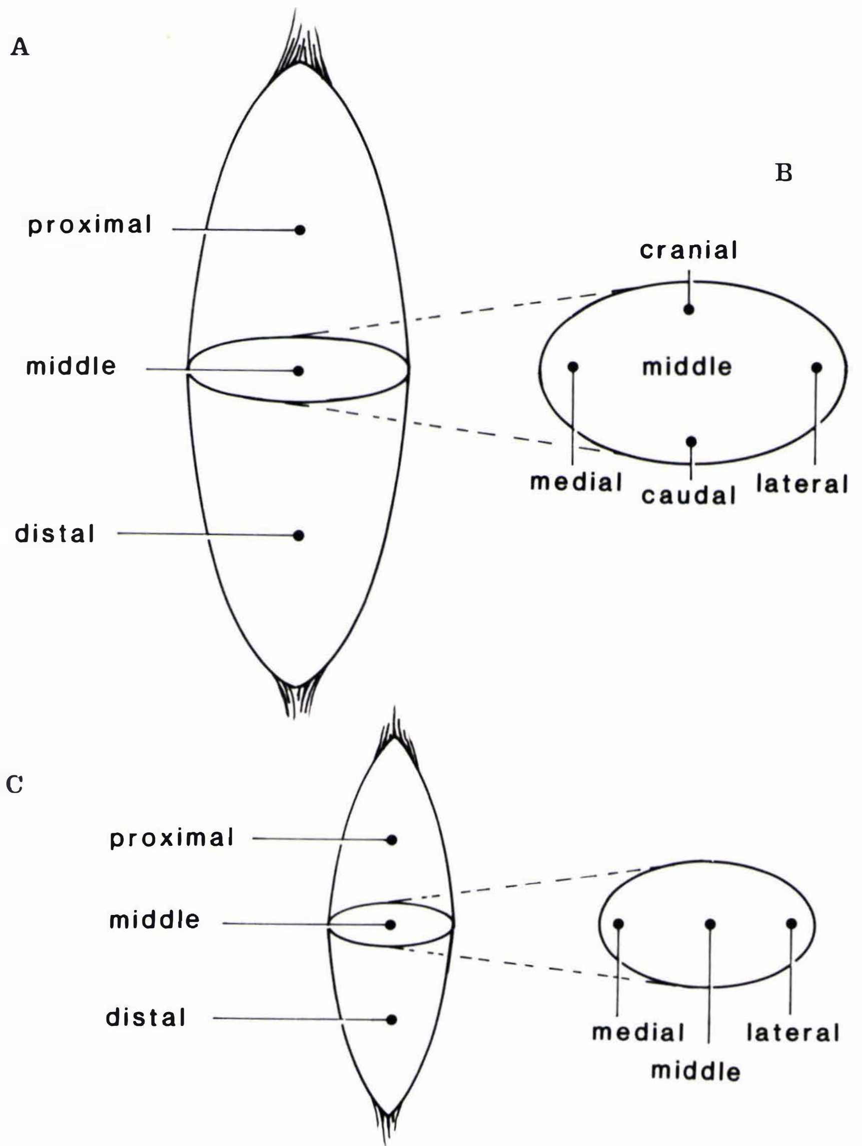
In order to assess the degree of variation which might occur in a cross-section, through the middle of

Figure 7. **A** line drawing illustrating the sites of sampling from the large limb muscles.

A. In the deep digital flexor, long and lateral digital extensors, and cranial tibial muscle samples were collected from proximal, middle and distal sites in all horses.

B. In five control horses additional samples were collected through the middle of the muscle, from cranial, medial, caudal and lateral sites in the deep digital flexor and long and lateral digital extensor muscles.

C. Because the cranial tibial muscle was flatter than the remaining large limb muscles additional samples through the middle of the muscle were collected from only medial and lateral sites in the same five control horses.


A
proximal
middle
distal
B
cranial
middle
medial
caudal
lateral
C
proximal
middle
distal
medial
lateral
middle

the larger hindlimb muscles, samples were collected in 5 control horses from additional sites at this level, as illustrated in Figure 7.

Processing of muscles

Muscle samples were processed as soon as possible after collection. Each sample was examined visually to ensure a cross sectional surface of at least 1 cm^2 was available for sectioning. The muscle was then rapidly frozen in Isopentane[1] cooled by liquid nitrogen to its melting point of -51°C. This freezing took about 20 seconds. The muscle was then mounted either directly onto a chuck or onto a small piece of cork, using a liquid tissue mounting gel[2]. During this procedure the sample was aligned so that the muscle fibres ran transversely to the edge presented for sectioning. They were then individually wrapped in aluminium foil, labelled and stored at -70°C. Frozen sections were cut[3] at 10 µm in a cryostat at-25°C. Two sections of each sample were collected onto glass slides and allowed to air dry. One section was then stained histochemically to detect myosin ATPase activity after pre-incubation at pH 9.4 using the technique of Padykula and Herman (1955) as modified by Davies and Gunn (1972) (Appendix 1). The other section was stained with haematoxylin and eosin. Each sample was then examined under light microscopy. They were rejected if excessive ice artefact was present, if the section was not transverse, inadequately stained, or, with the myosin ATPase sections, if excessive cobalt sulphide precipitate was present. Wherever possible, where these problems were identified, samples were re-cut

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1. Isopentane, Koch-Light Laboratories Ltd., Buckinghamshire, England

2. Tissue-Tek 2, Lab Tek Products, Naperville, USA

3. Lipshaw Cryotome, Lipshaw Manufacturing Co., Michigan, USA

and stained after the problem was corrected.

Collection and analysis of data

(i) Muscle morphology

For morphological assessment of muscle the following features were noted :

Muscle fibre size and shape variation
Density and distribution of nuclei
Amount of connective tissue and fatty tissue
Presence of abnormally stained fibres such as split fibres
Presence of fibre type grouping
Other notable staining characteristics

Changes were then graded according to a system modified from Anderson (1985) as follows:

<u>No pathology (0)</u>

1. Muscle fibre shape and distribution normal.
2. Normal number and distribution of nuclei.

<u>Mild pathology (+)</u>

1. Rounding of, and an apparent increase in the number of sarcolemnal nuclei.
2. Occurrence of a higher than normal number of nuclei within the body of muscle fibres.
3. Minor variation in fibre size

<u>Moderate pathology (++)</u>

1. Slight fibre atrophy and hypertrophy, with associated changes in shape of fibres.
2. An apparent increase in the amount of endomysial and perimysial connective tissue.

<u>Marked pathology (+++)</u>

1. Widespread atrophy and hypertrophy of fibres.
2. Marked endomysial and perimysial fibrosis.

Severe pathology (++++)

1. Widespread fatty and fibrous replac… muscle.
2. Pyknotic nuclear clumps.

Signs indicative of chronic deinnervation and reinnervation, such as fibre type grouping and group atrophy of fibres were also noted.

(ii) Muscle morphometry

For quantitative analysis of muscle the sections were then projected onto tracing paper using a microprojector mounted on the tracing table.

(a) Proportion of Type I fibres

Approximately 200 fibres were randomly selected and differentiated into Type I and Type II fibres, and the mean percentage of Type I fibres calculated. The data was then grouped according to the disease status, age, and site of sampling and statistically analysed using the students' t-test and F-test where applicable. The two ponies in the control group were excluded from all morphometric analysis, which was confined exclusively to Thoroughbreds.

In horses 1 and 2, over 1000 fibres were examined and the percentage of Type I fibres calculated. These results were then compared with those obtained by counting 200 fibres to determine if any variation existed when counting less fibres.

(b) Muscle fibre diameter

The minimum cross-sectional diameter, as defined by Brooke and Engel (1969a), was then measured in 100 Type I and Type II fibres in the middle sample of each muscle from both legs. In addition minimum fibre diameters were measured in the cranial, caudal, medial and lateral muscle samples from 5 control horses.

These measurements were then transferred to a Prime Computer system and means and standard deviations of minimum fibre diameter were calculated for each muscle. Samples were then grouped according to disease status, age, and site of sampling, and analysis of variance performed for both Type I and Type II fibre types, and left and right legs. Where analysis of variance indicated a significant difference existed, a paired students' t-test was used to analyze the results further.

(c) Histograms of fibre diameter distribution

Individual fibre diameter measurements from all horses were then grouped according to the muscle sampled, the fibre type and the disease status of the animal. This enabled a comparison of the distribution of fibre diameters in both Type I and II fibres, between clinical and control horses in each muscle.

Because of the small number of subclinical horses these were not included in the histographic analysis.

(d) Atrophy and hypertrophy factors

Based on the fibre diameter histograms, atrophy and hypertrophy factors were then calculated as previously mentioned (Brooke and Engel,1969b) for Type I and Type II fibres for each muscle in all horses. These were then combined to produce mean atrophy and hypertrophy factors for Type I and II fibres for each muscle for the control, subclinical and clinical groups.

RESULTS

Muscle morphology

The results of morphological grading of muscles are presented in Appendix 4, while the incidence of morphological changes at each muscle site for all groups of horses and the average degree of changes at these sites is presented in Table 2.

Table 2. Incidence of pathology and average morphological grading in samples from the limb muscles in horses in the clinical, subclinical and control groups.

	GROUPS OF HORSES											
	CLINICAL				SUBCLINICAL				CONTROL			
MUSCLE	Samples examined	Samples affected	% samples affected	Average grading	Samples examined	Samples affected	% samples affected	Average grading	Samples examined	Samples affected	% samples affected	Average grading
Deep digital flexor	38	19	50	++/+++	22	10	45	+/++	93	32	34	+
Cranial tibial	39	8	21	0/+	22	2	9	0/++	92	13	14	0/+
Long digital extensor	39	4	10	0/+	24	3	13	0/+	96	4	4	0/+
Lateral digital extensor	38	3	8	0/+	23	0	0	0	95	5	5	0/+
Short digital extensor	15	7	47	++	8	5	63	-	18	6	33	0/+

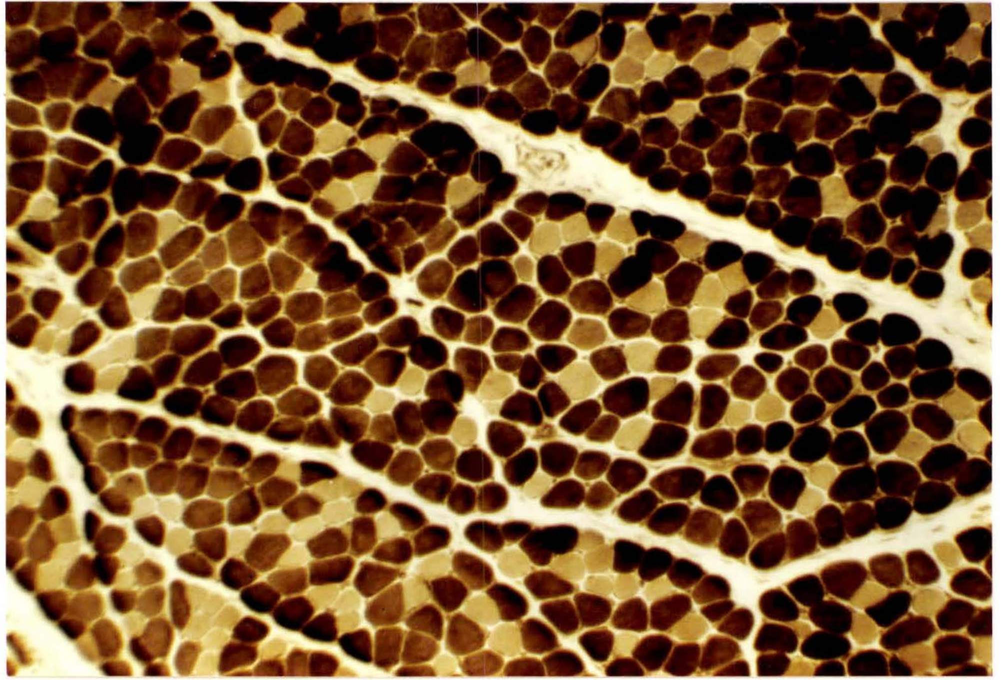
0 = no changes
+ = mild changes
++ = moderate changes
+++ = marked changes
++++ = severe changes

Figure 8. Photomicrograph of a transverse section of the left lateral cricoarytenoid muscle in a six week old foal, showing the typical distribution of Type I and Type II fibres in the control group.

Myosin ATPase staining x 60.

Figure 9. Photomicrograph of a transverse section of the left lateral cricoarytenoid muscle in a horse from the subclinical group showing fibre type grouping of Type I and Type II fibres.

Myosin ATPase staining x 60



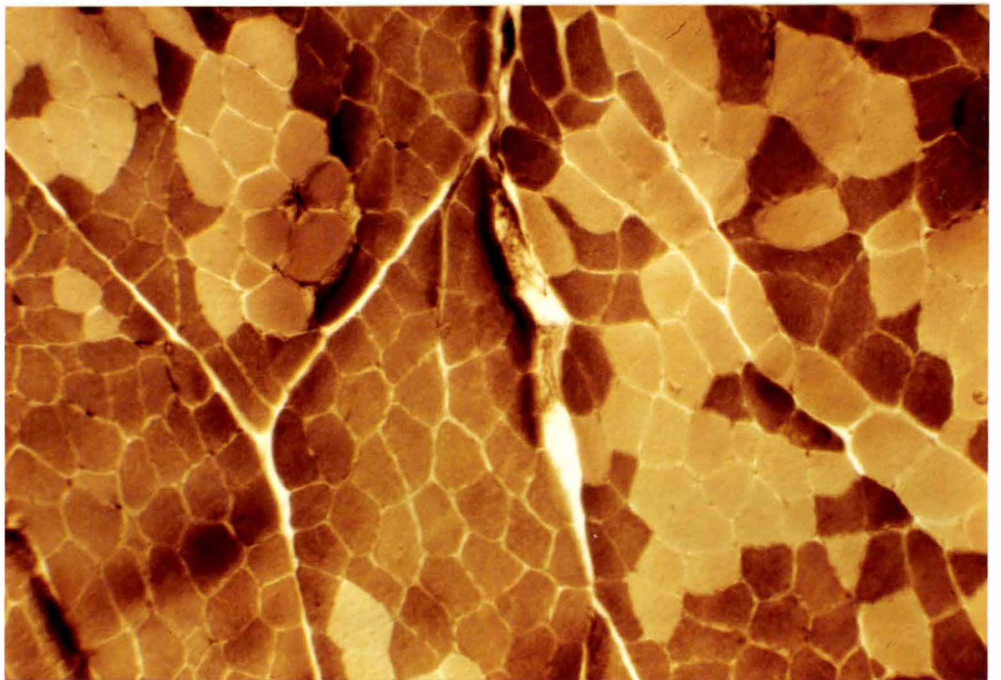


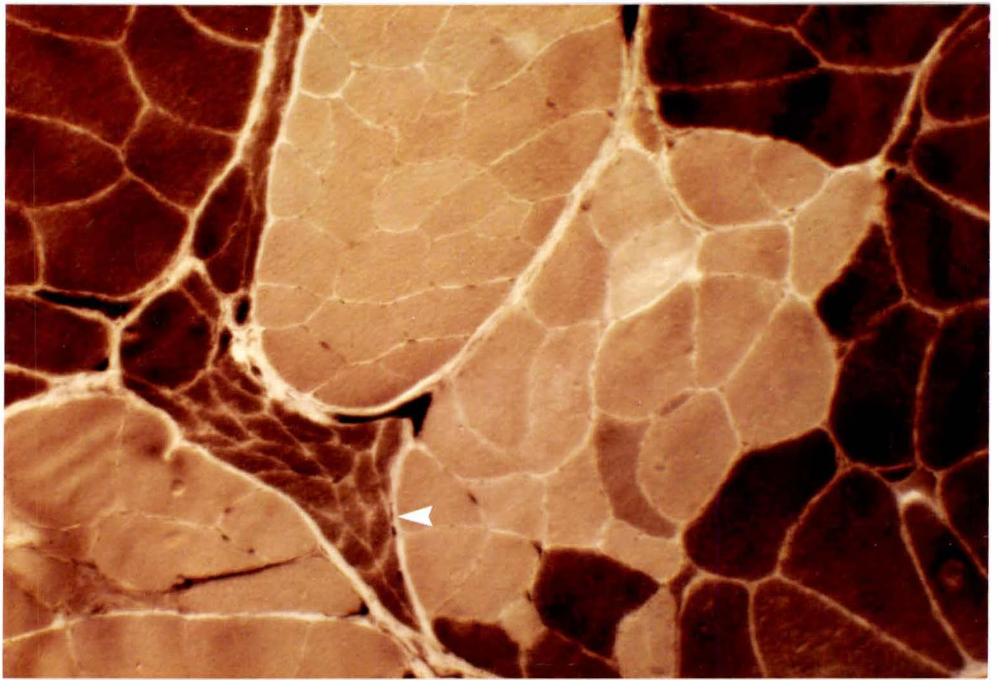
Figure 10. Photomicrographs of a transverse section of the left lateral cricoarytenoid muscle in clinical laryngeal hemiplegic horses, showing:-

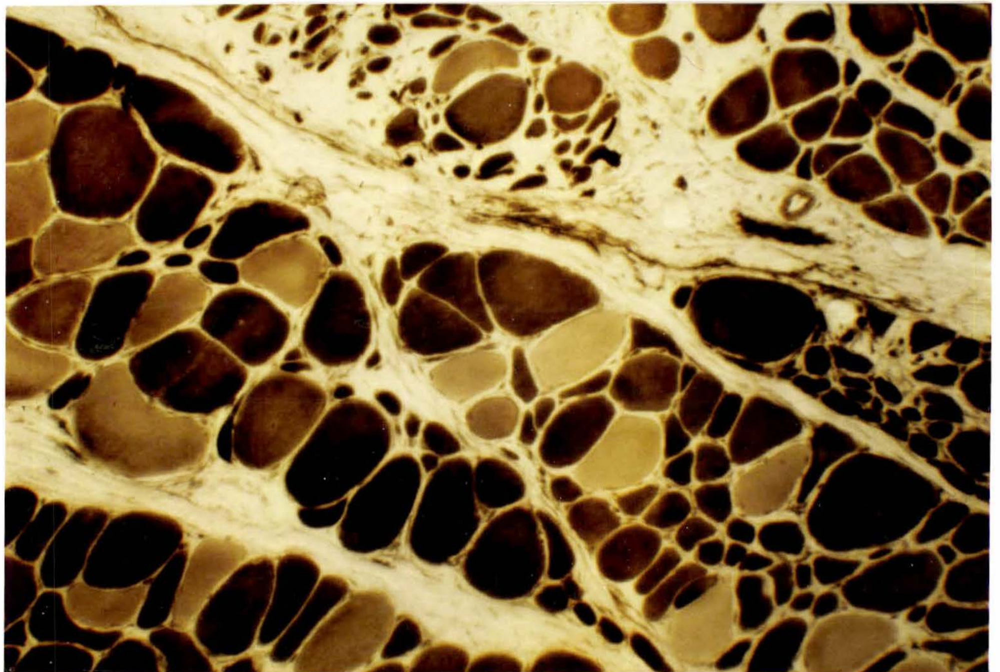
A. Atrophy of groups of fibres (arrow) and fibre type grouping.

B. Gross variation in fibre size and increased connective tissue.

C. Only a few recognisable muscle fibres are visible amongst the degenerate muscle and connective tissue.

Myosin ATPase staining x 60





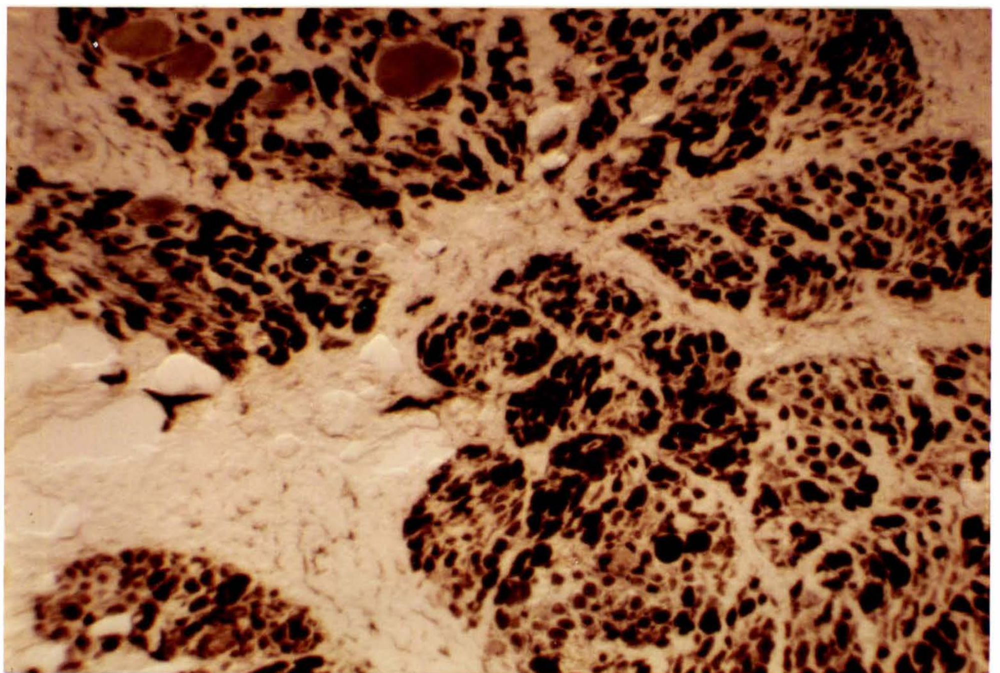


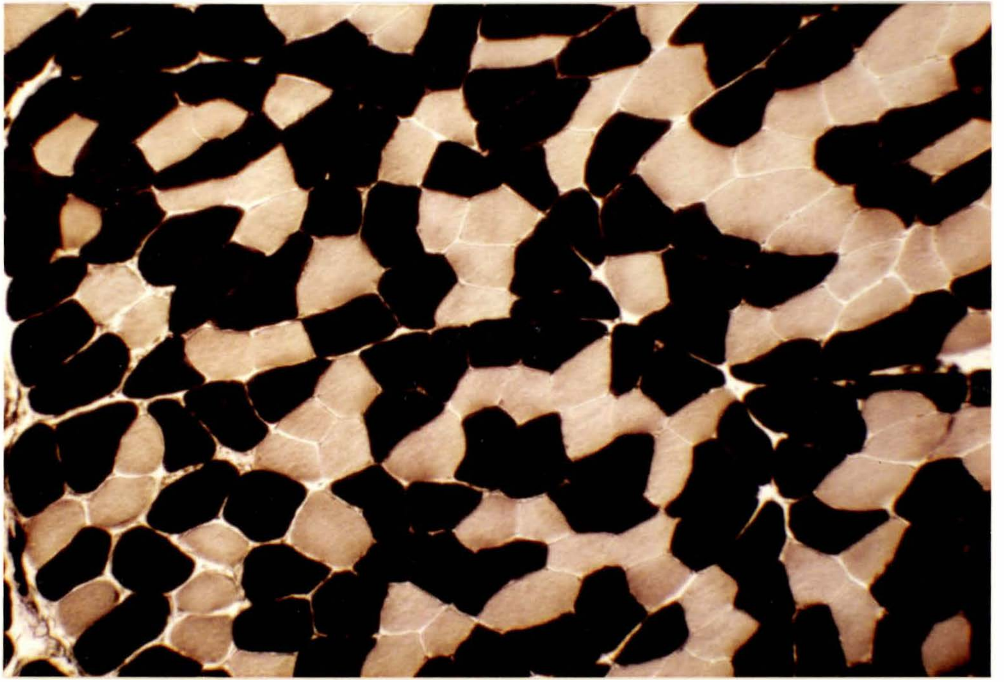
Figure 11. Photomicrographs of transverse sections of the deep digital flexor muscle in control horses. These sections illustrate the variation in the proportions of fibre types which may be found in this muscle.

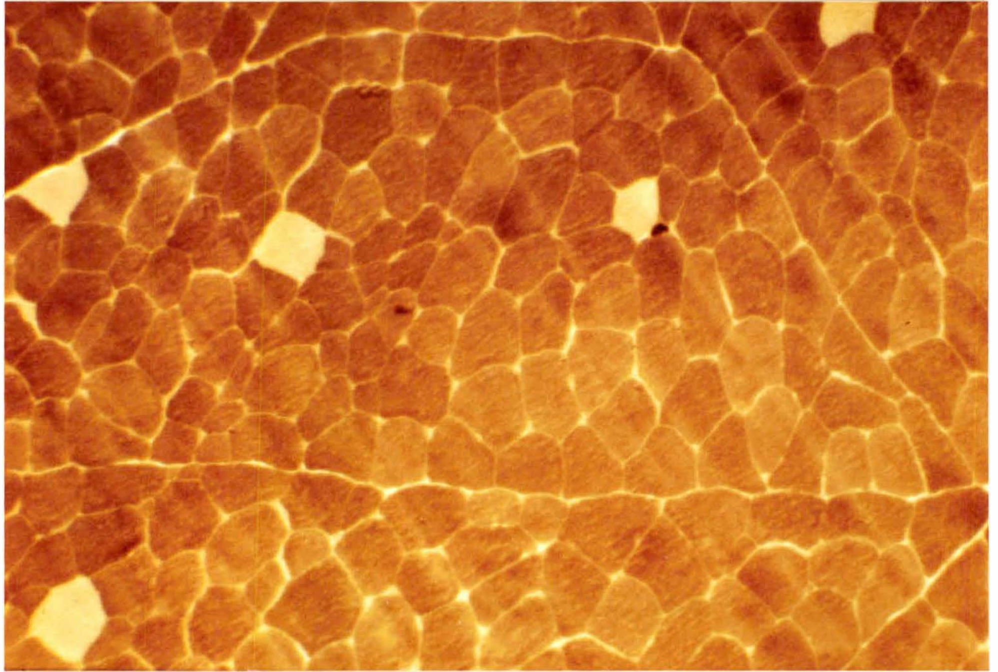
A. Approximately equal proportions of Type I and Type II fibres, with little evidence of morphological abnormalities.

B. Type II fibre predominance. Only five Type I fibres are evident.

C. Type I fibre predominance.

Myosin ATPase staining x 60





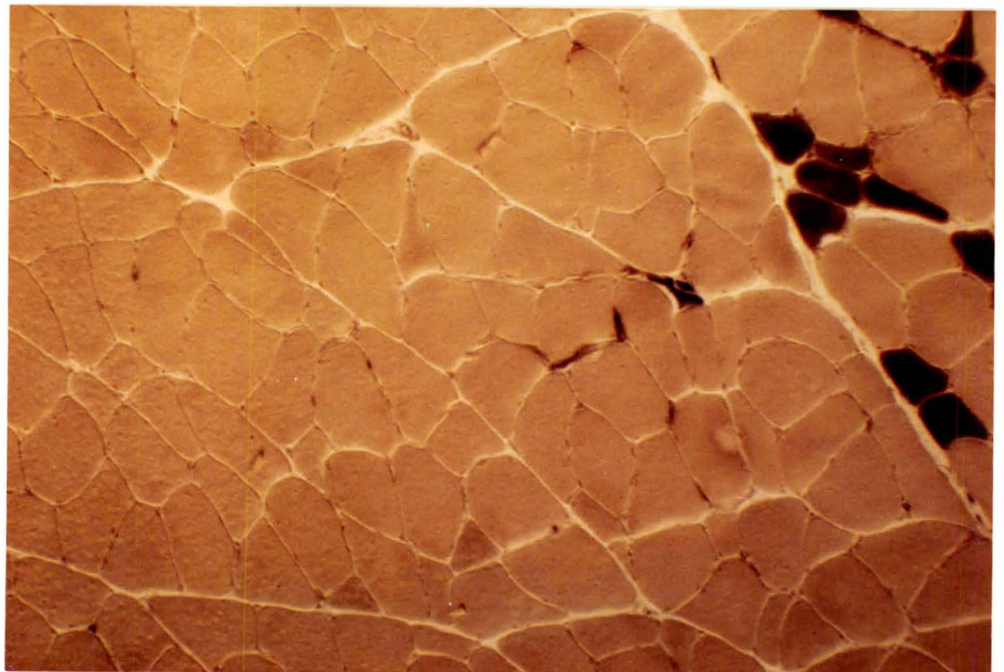


Figure 12. Photomicrographs of transverse sections taken from the deep digital flexor muscle of a control horse showing:-

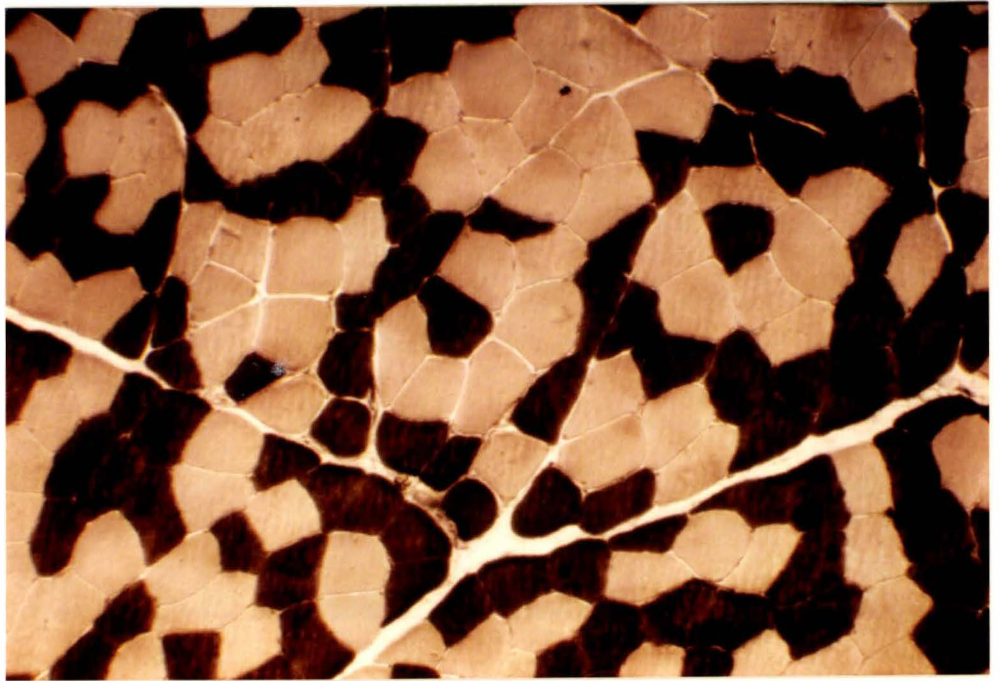
A. Similar size of Type I and Type II fibres.

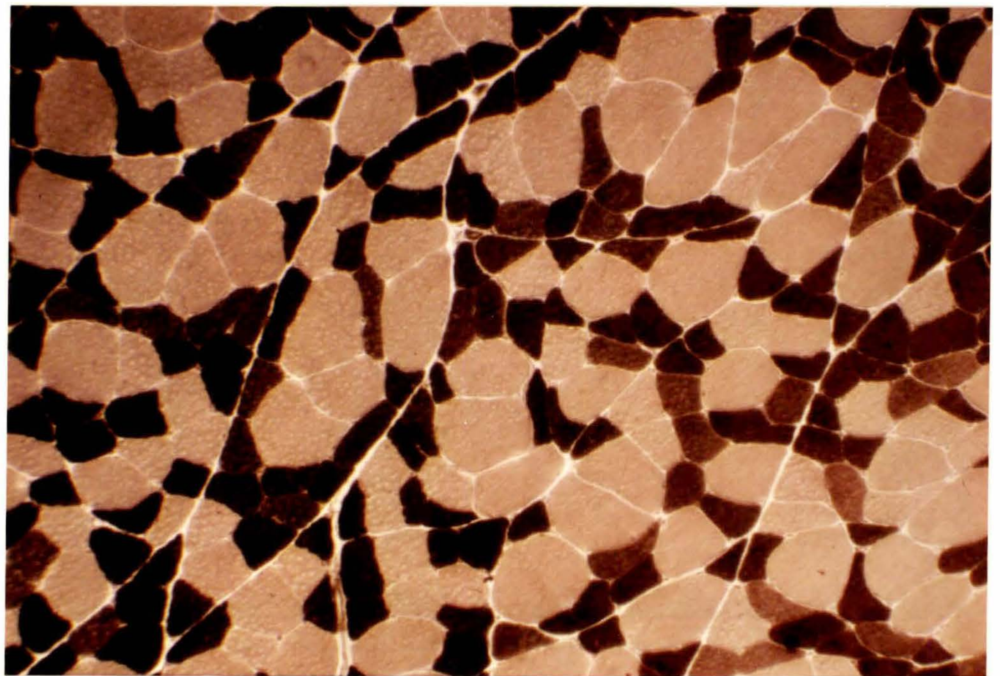
B. Type I fibres consistently larger than Type II fibres.

Myosin ATPase staining x 60

Figure 13. Photomicrograph of a transverse section of the deep digital flexor muscle in a clinical laryngeal hemiplegic horse showing fibre type grouping.

Myosin ATPase staining x 60





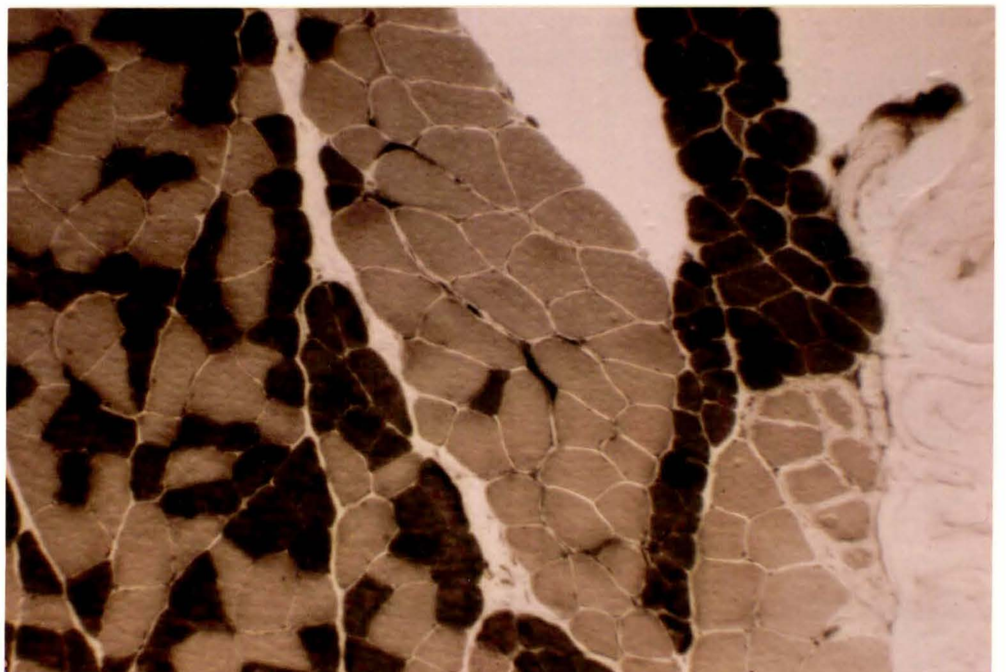
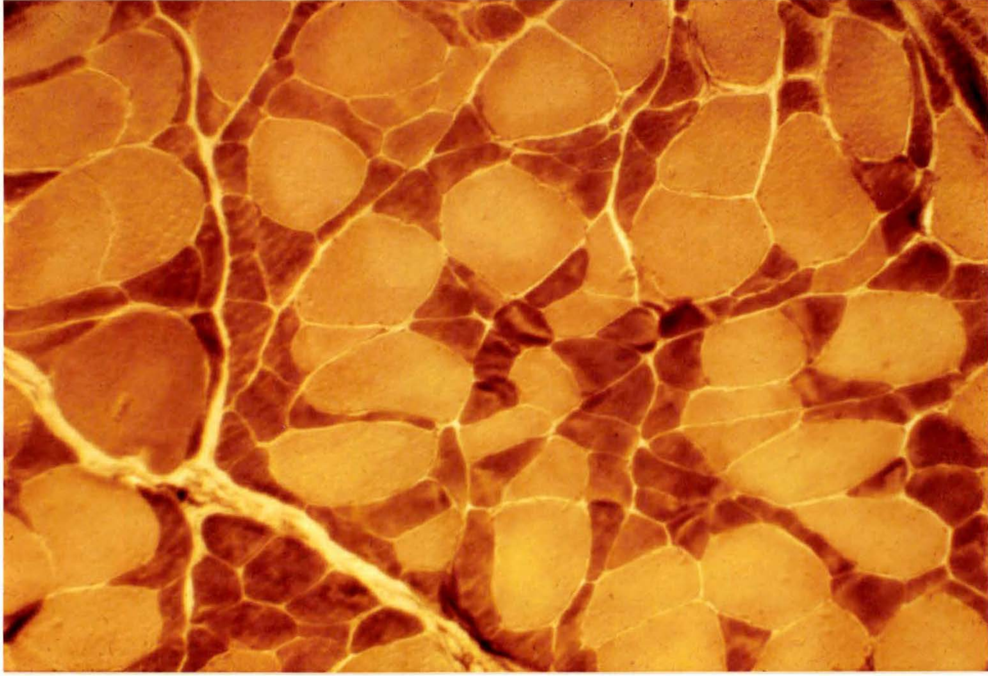


Figure 14. A photomicrograph of a transverse section of the deep digital flexor muscle in a subclinical laryngeal hemiplegic horse showing fibre size variation and Type I fibre hypertrophy.

Myosin ATPase staining x 60

Figure 15. A photomicrograph of a transverse section of the deep digital flexor muscle in a clinical laryngeal hemiplegic horse showing fragmented Type I fibres (arrows), fibre size disproportion and fibre type grouping.

Myosin ATPase staining x 60



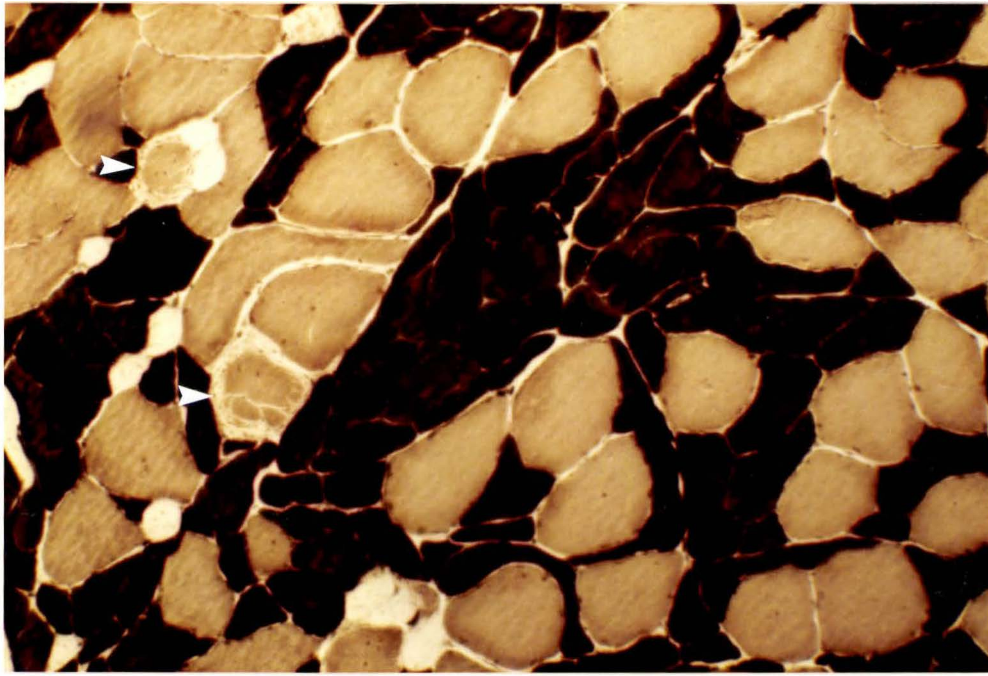


Figure 16. A photomicrograph of a transverse section of the cranial tibial muscle in a control horse, showing normal distribution of Type I and Type II fibres.

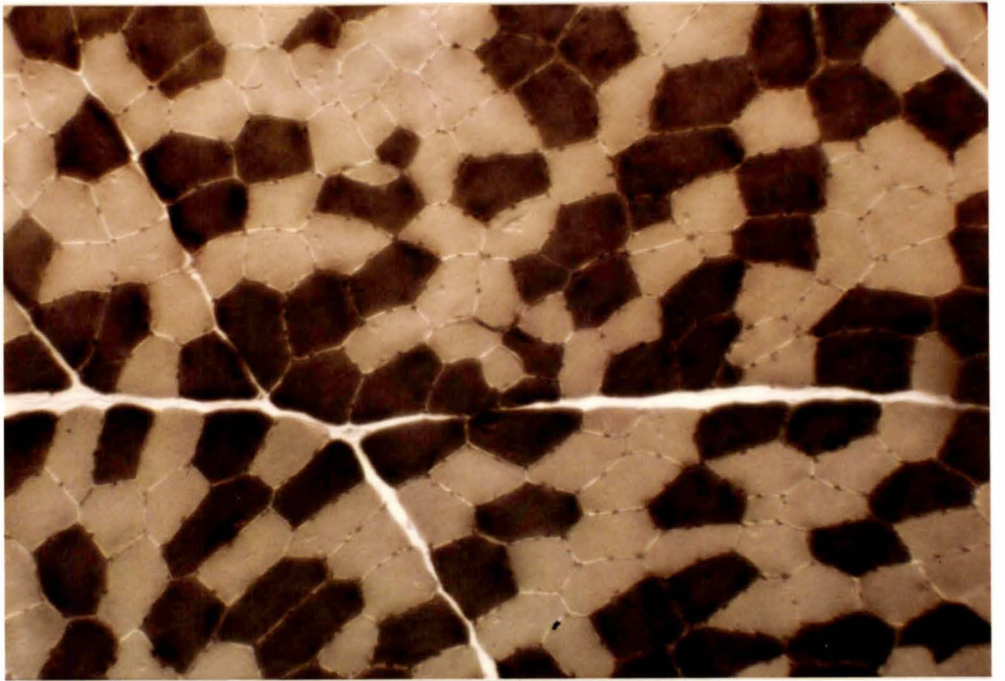
Myosin ATPase staining x 60

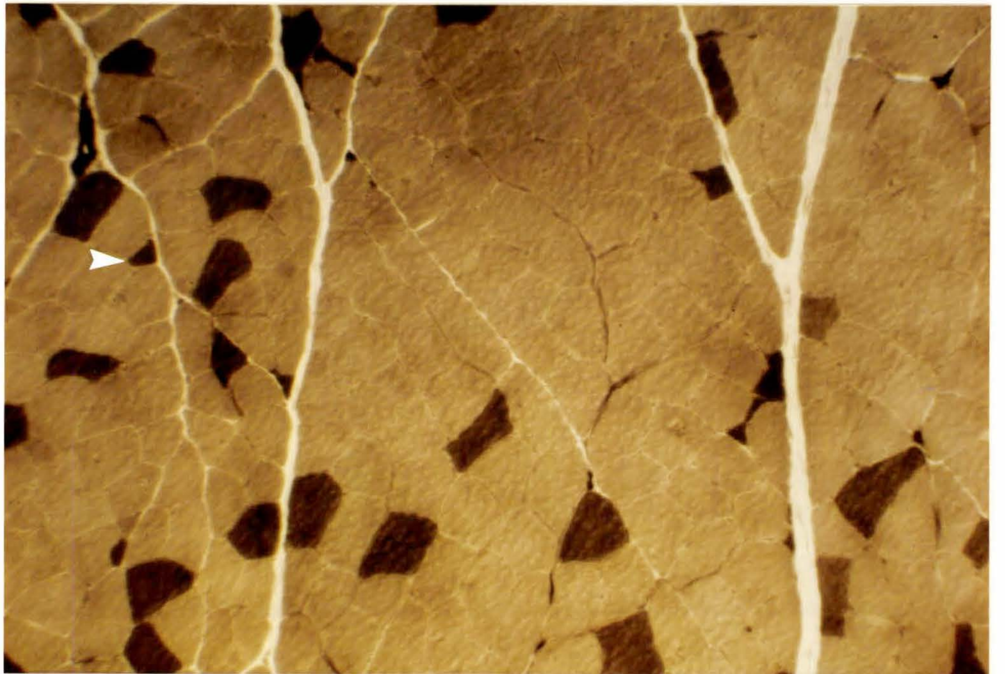
Figure 17. A photomicrograph of a transverse section of the cranial tibial muscle in a subclinical laryngeal hemiplegic horse showing occasional small Type II fibres (arrow) and Type I fibre predominance.

Myosin ATPase staining x 60

Figure 18. A photomicrograph of a transverse section from the cranial tibial muscle in a control horse showing a muscle spindle (arrowed).

Myosin ATPase staining x 150





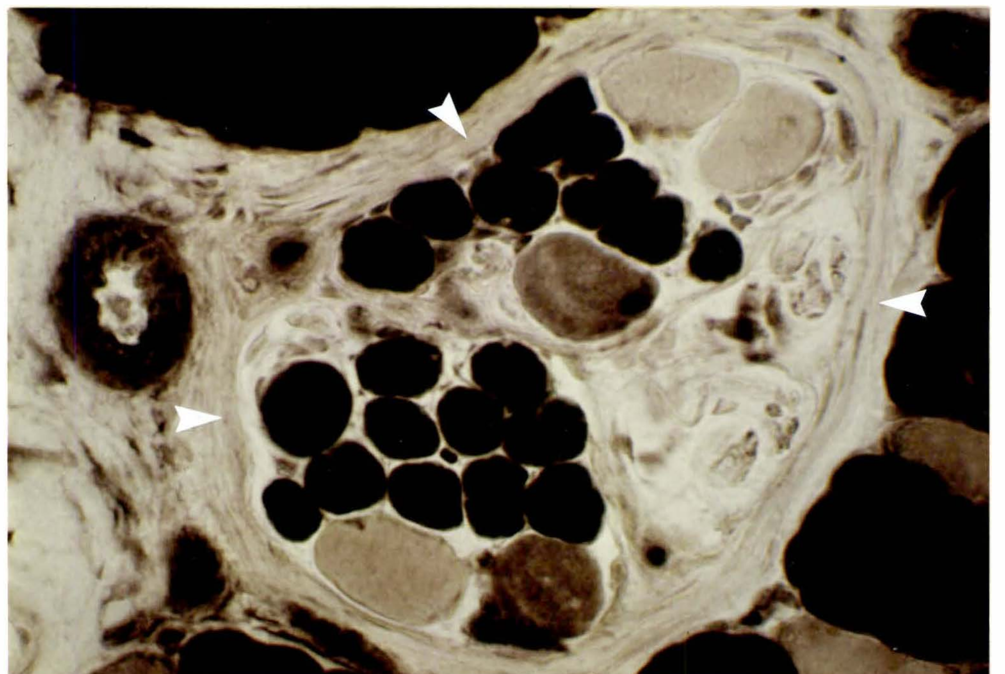


Figure 19. Photomicrograph of a transverse section of the cranial tibial muscle in a clinical laryngeal hemiplegic horse showing fibre type grouping.

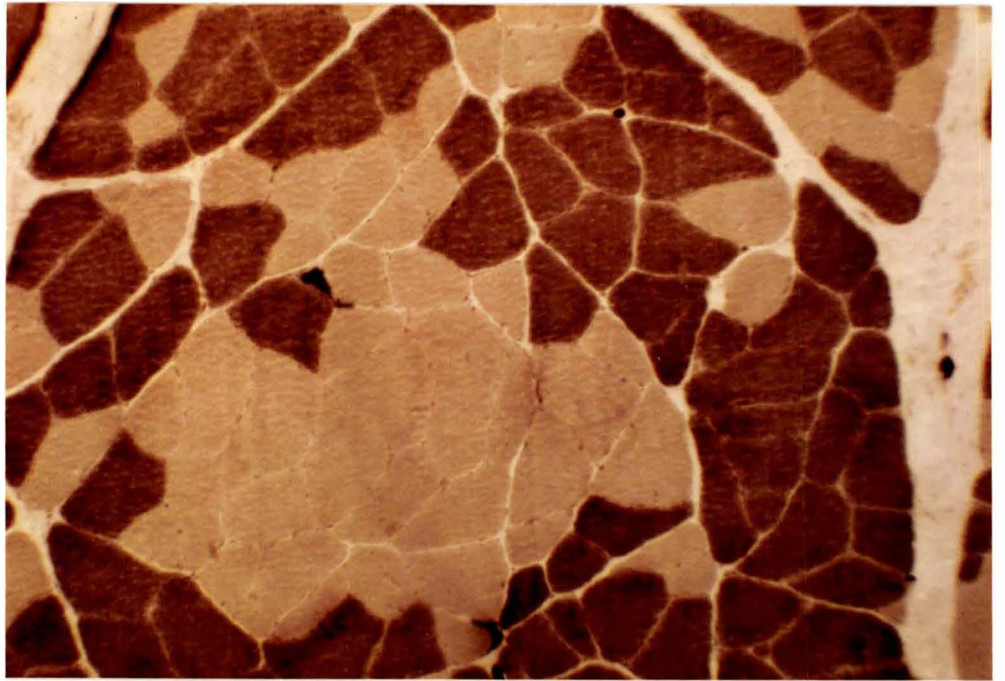
Myosin ATPase staining x 60

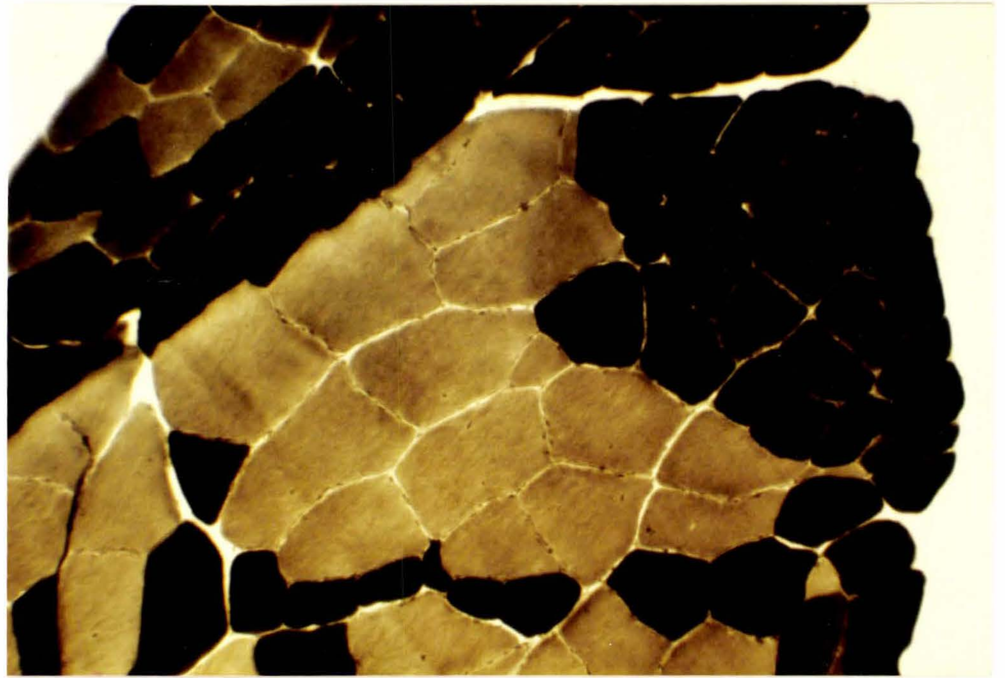
Figure 20. Photomicrograph of a transverse section from the cranial tibial muscle in a control horse showing evidence of fibre type grouping and Type I fibre hypertrophy.

Myosin ATPase staining x 60

Figure 21. Photomicrograph of a transverse section of the cranial tibial muscle in a control horse showing hypertrophy of Type I fibres and Type II fibre predominance.

Myosin ATPase staining x 60





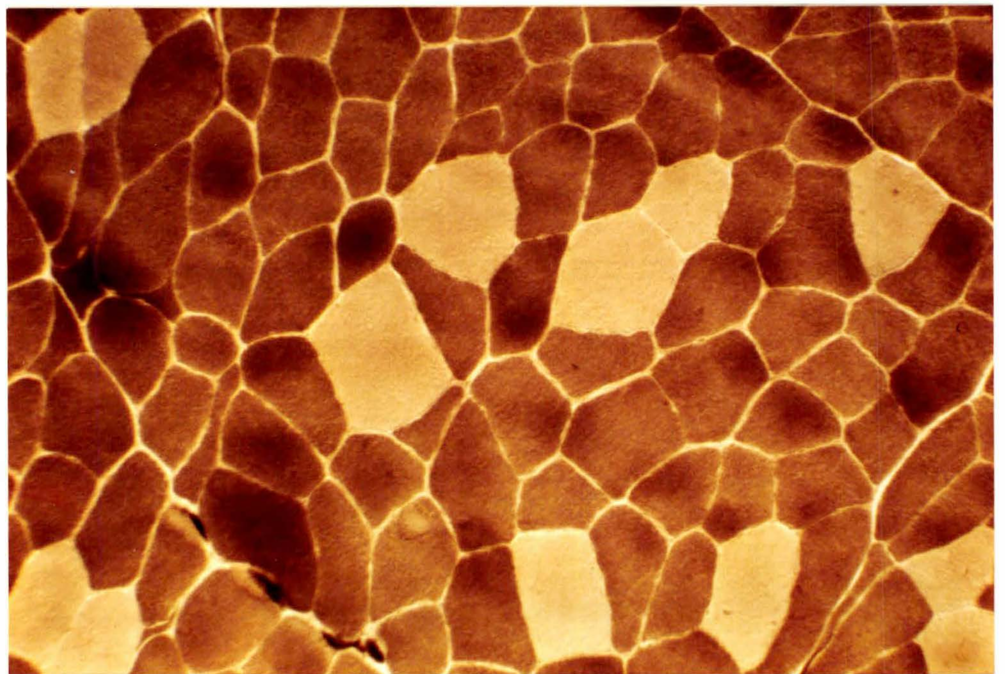
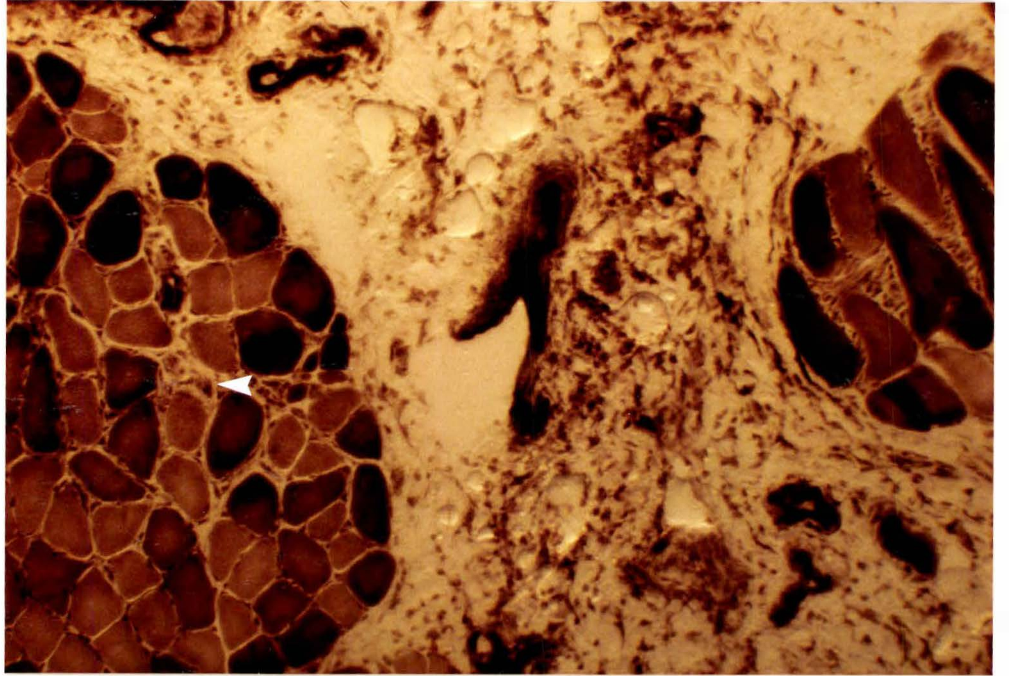


Figure 22. Photomicrograph of a transverse section of the cranial tibial muscle in a subclinical laryngeal hemiplegic horse showing interstitial fibrosis (arrow), fibre size variation and replacement of muscle by connective tissue.

Myosin ATPase staining x 60

Figure 23. Photomicrograph of a transverse section of the cranial tibial muscle in a control horse showing a focal accumulation of inflammatory cells (arrow).

Haematoxylin and eosin staining x 60.



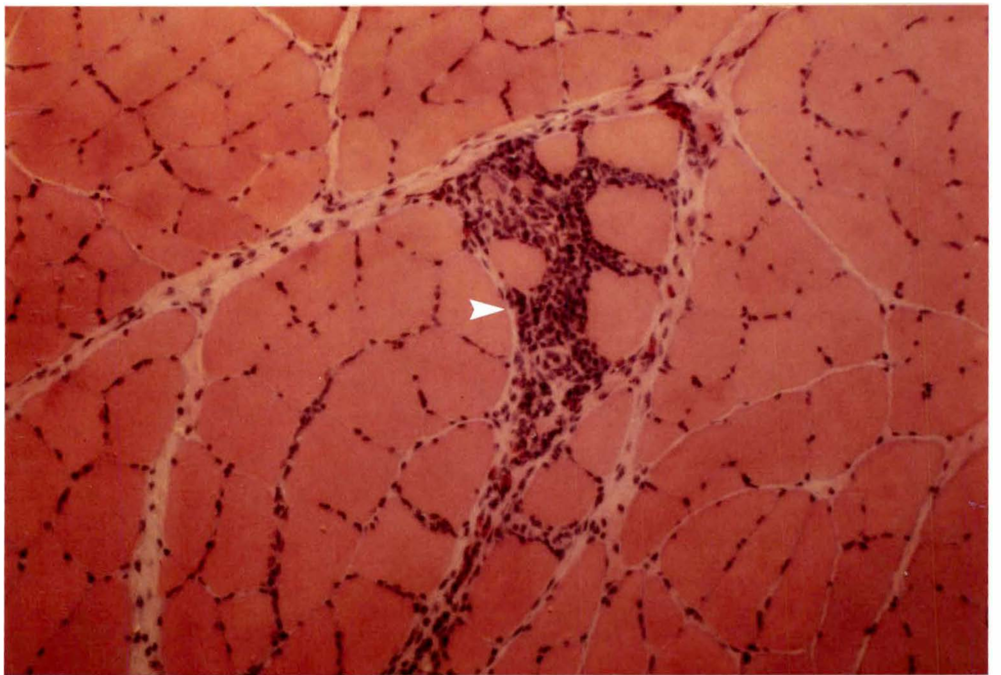
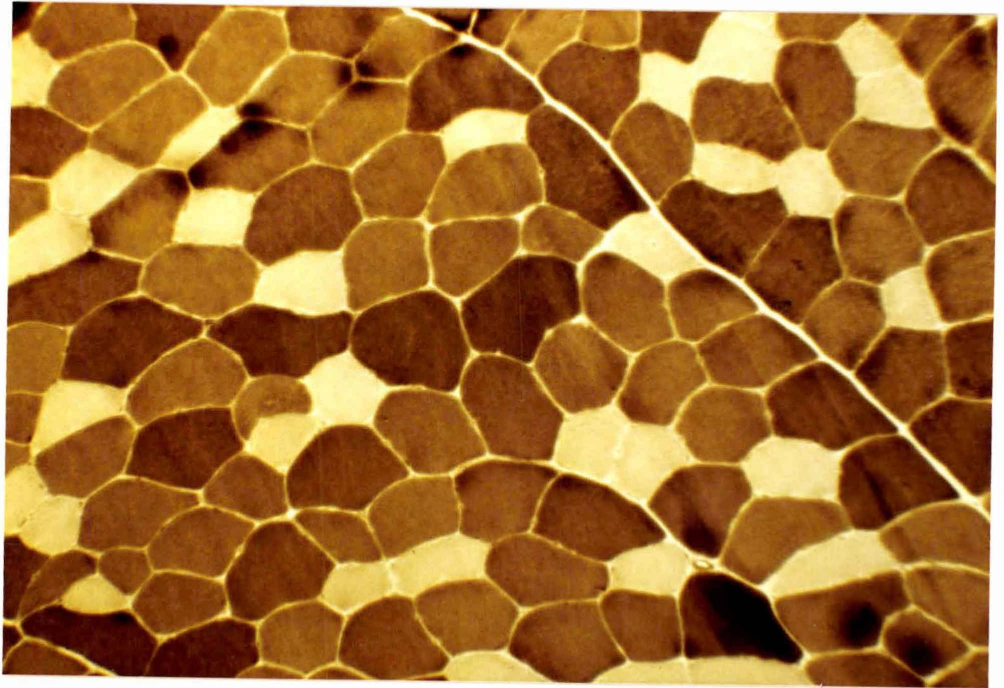


Figure 24. Photomicrograph of a transverse section of the long digital extensor muscle in a control horse showing the normal distribution of Type I and Type II fibres.

Myosin ATPase staining x 60

Figure 25. Photomicrograph of a transverse section of the long digital extensor muscle in the control horse which had fibre type grouping.

Myosin ATPase staining x 60



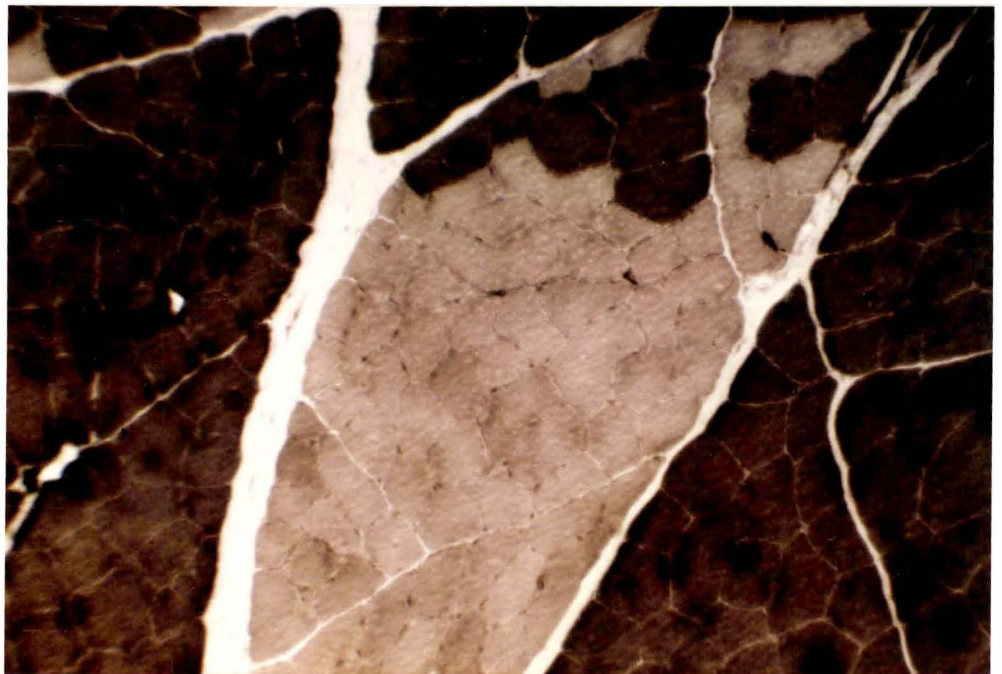


Figure 26. Photomicrographs of transverse sections of the long digital extensor muscle in a six week old control horse showing:-

A. Severe variation in fibre size, rounding of fibres and increased endomysial connective tissue.

Myosin ATPase staining x 60

B. Increased amounts of both endomysial and perimysial connective tissue and rounding of muscle fibres.

Myosin ATPase staining x 60

C. An increased number of central nuclei (arrows), including one associated with a split fibre (large arrow).

Haematoxylin and eosin staining x 150

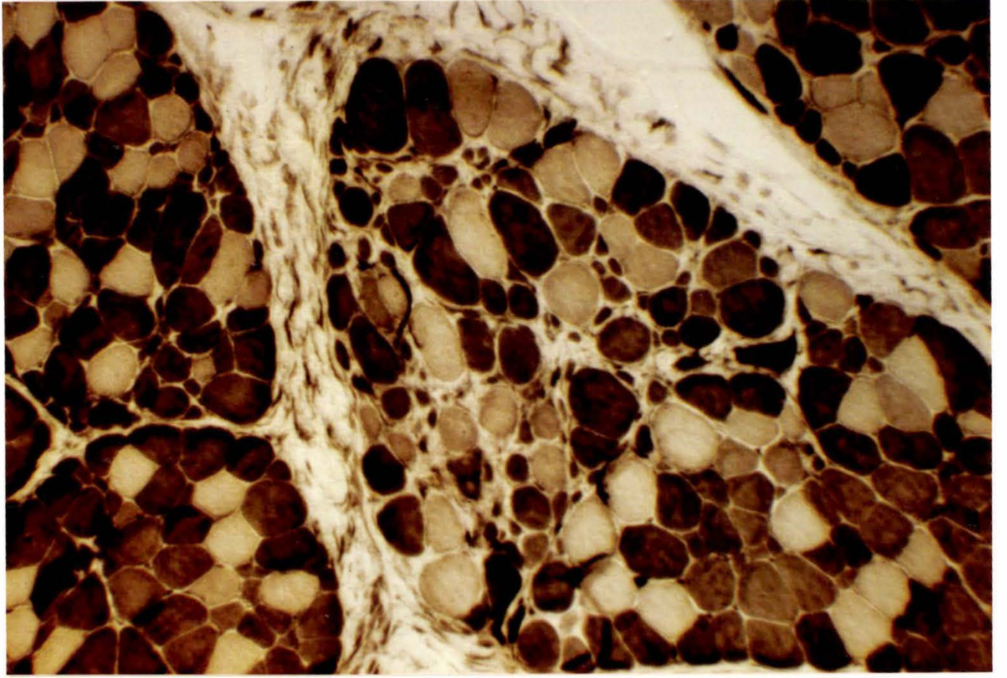
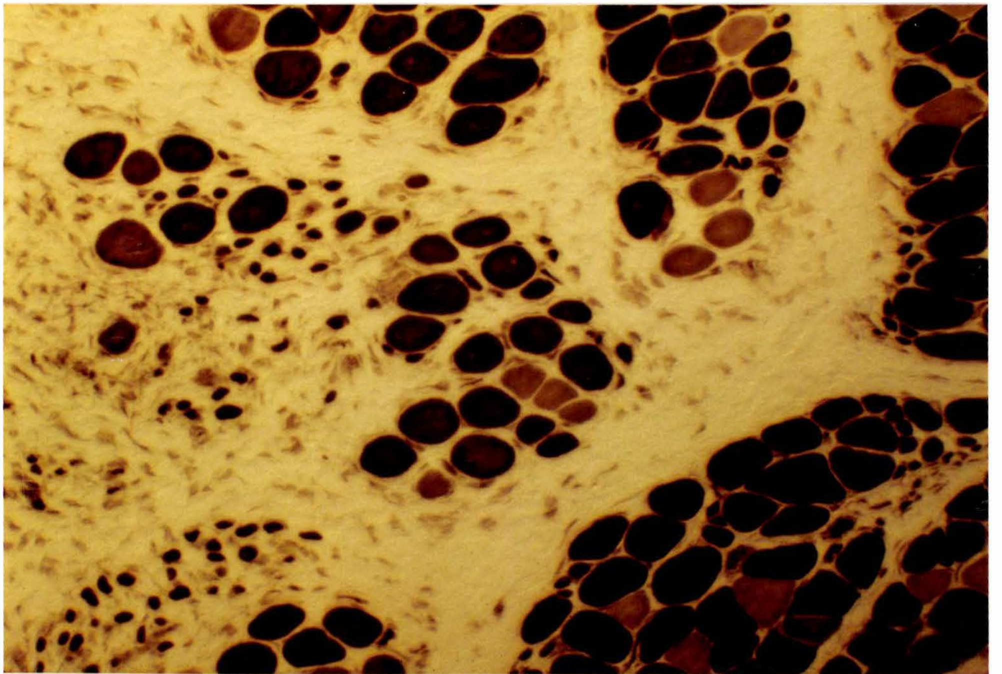
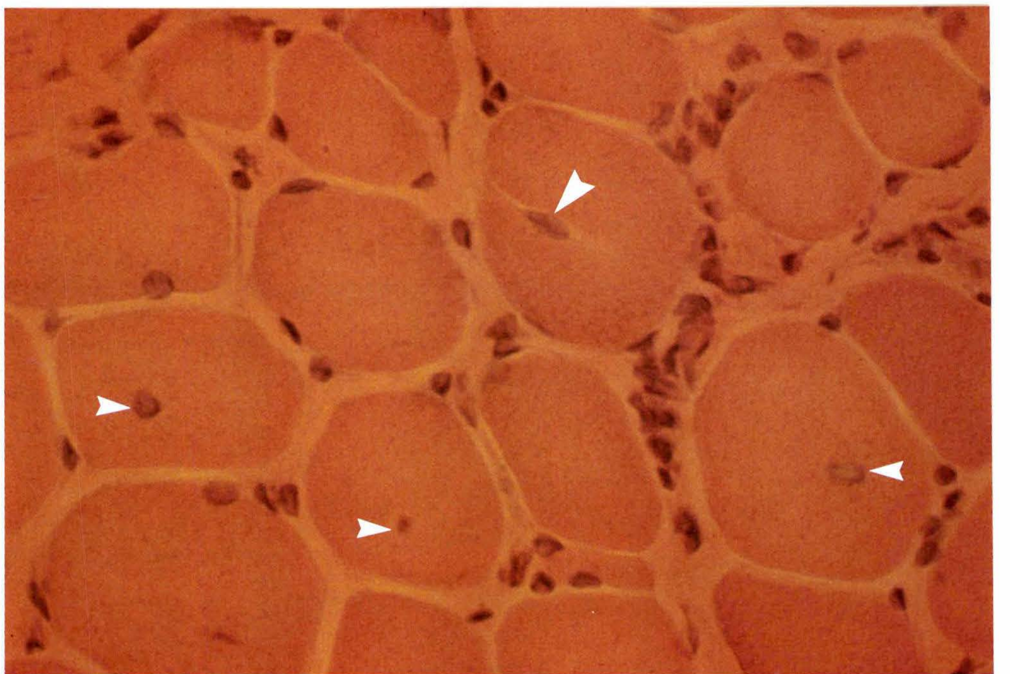
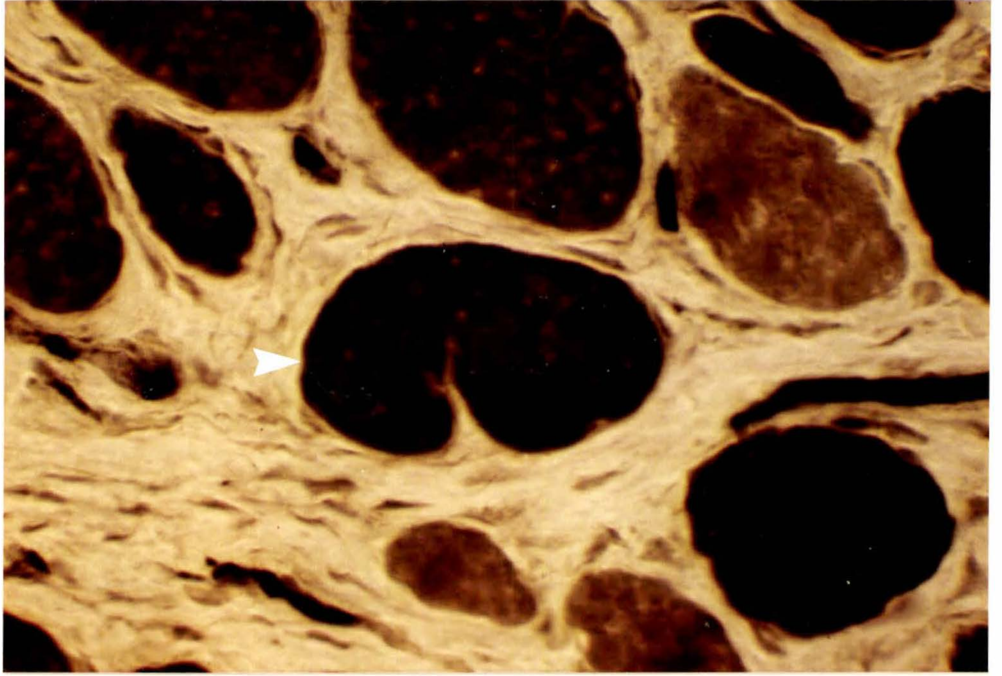




Figure 27. Photomicrographs of transverse sections of the long digital extensor muscle in a six week old control horse showing:

A. A split muscle fibre (arrowed) and increased endomysial connective tissue.

B. A fragmented muscle fibre.

Myosin ATPase staining x 150



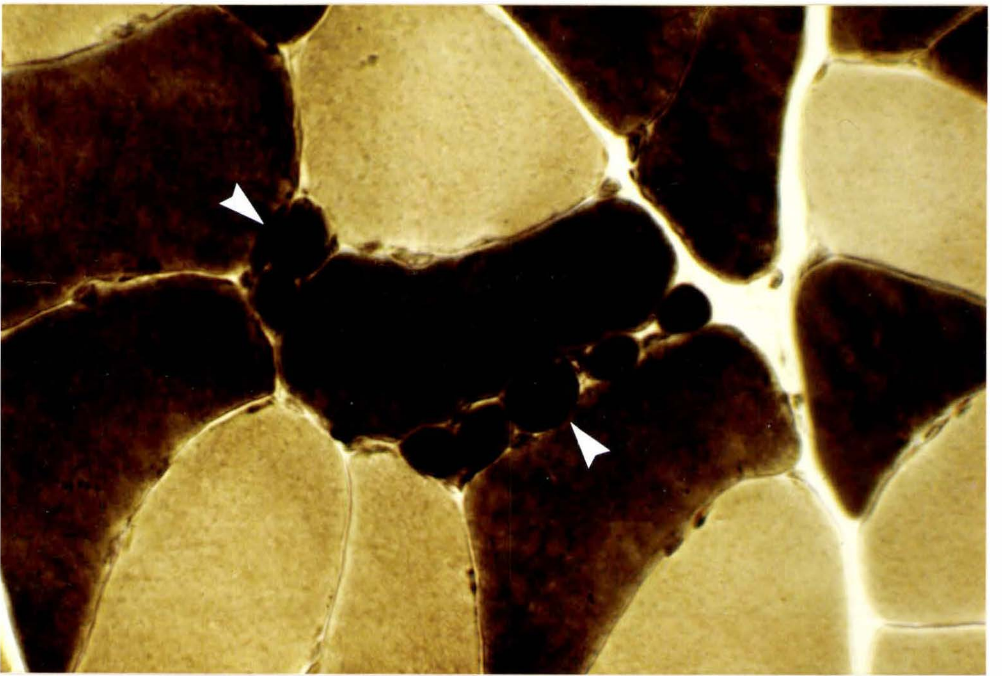
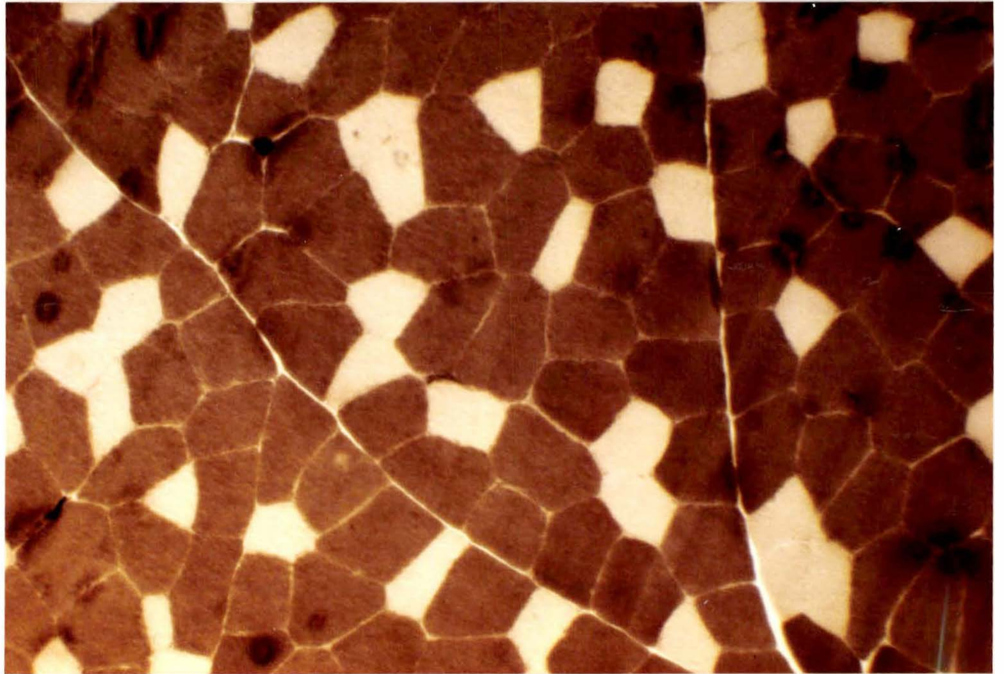


Figure 28. Photomicrograph of a transverse section from the lateral digital extensor muscle in a control horse, which is representative of the appearance of this muscle in most horses.

Myosin ATPase staining x 60

Figure 29. Photomicrograph of a transverse section from the lateral digital extensor muscle in a clinical laryngeal hemiplegic horse. The only recognizable change in this muscle was slight fibre size disproportion.

Myosin ATPase x 60



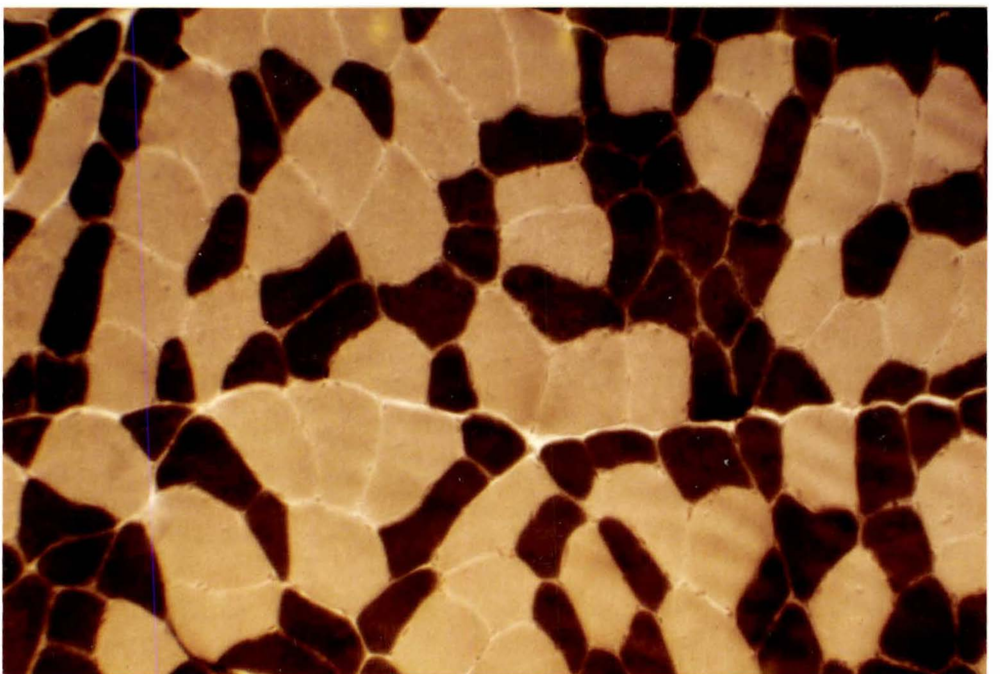
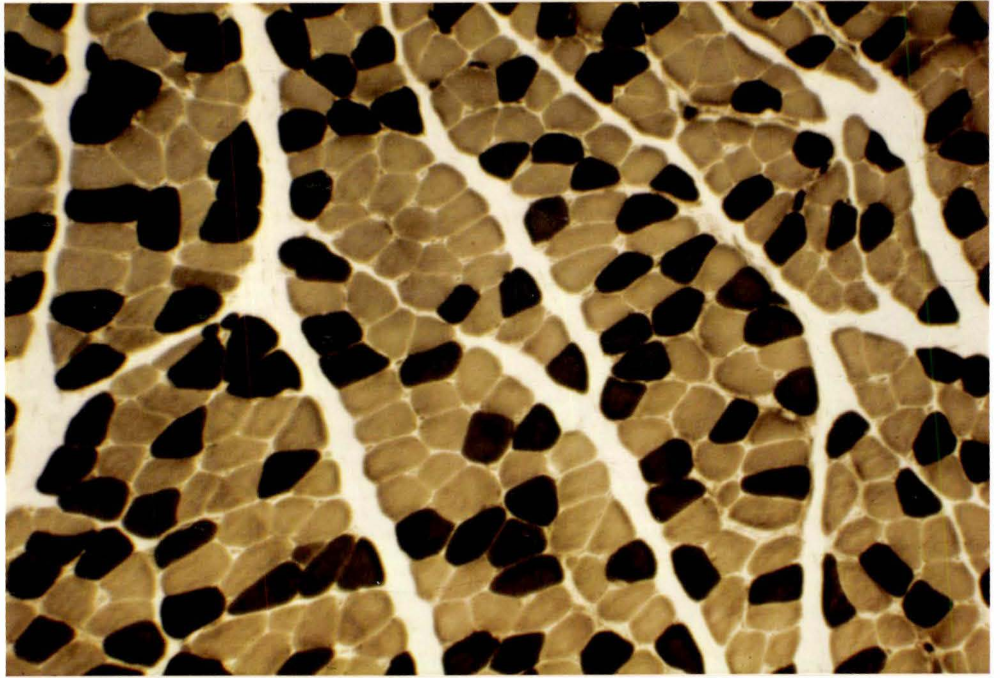


Figure 30. Photomicrographs of a transverse section of the short digital extensor muscle in a control horse showing:-

A. The presence of muscle fascicles containing a smaller number of fibres than other limb muscles examined and few morphological abnormalities.

B. A muscle spindle (arrowed), many of which were frequently observed in this muscle.

Myosin ATPase staining x 60



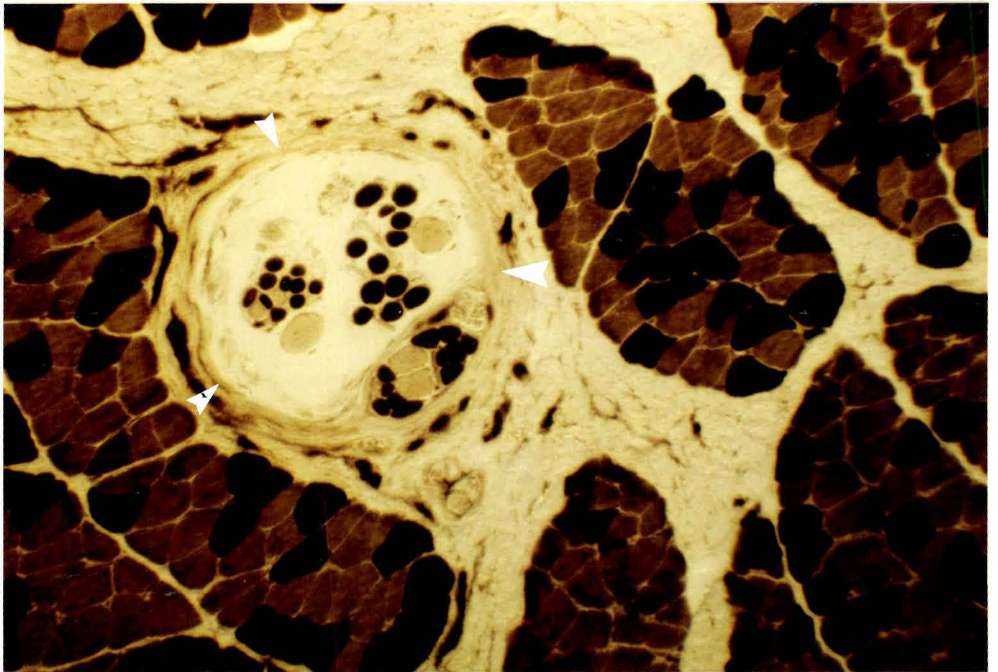
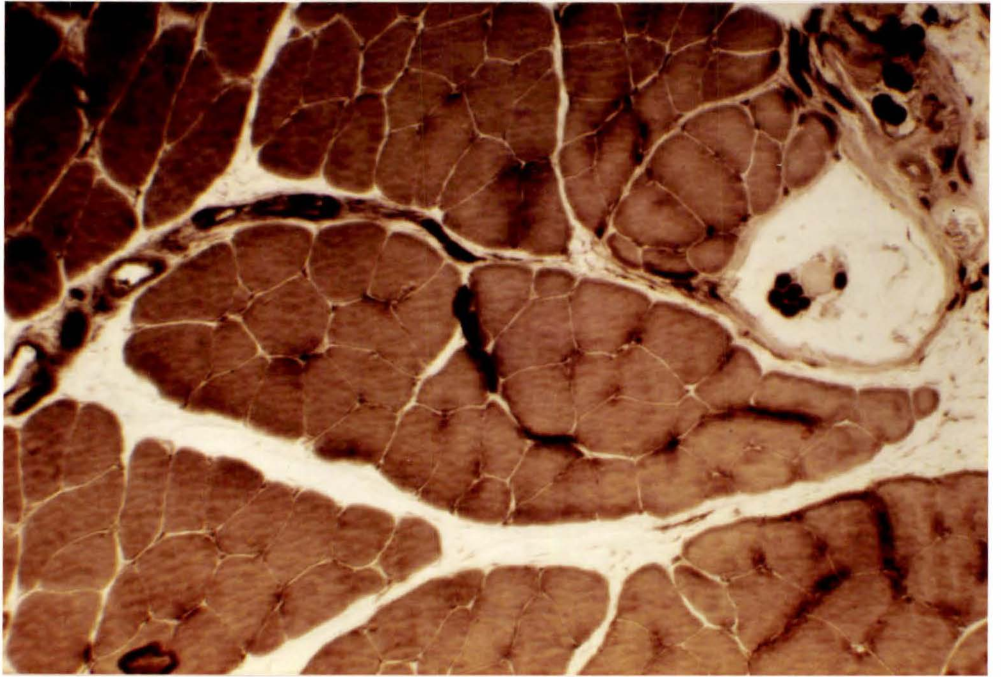


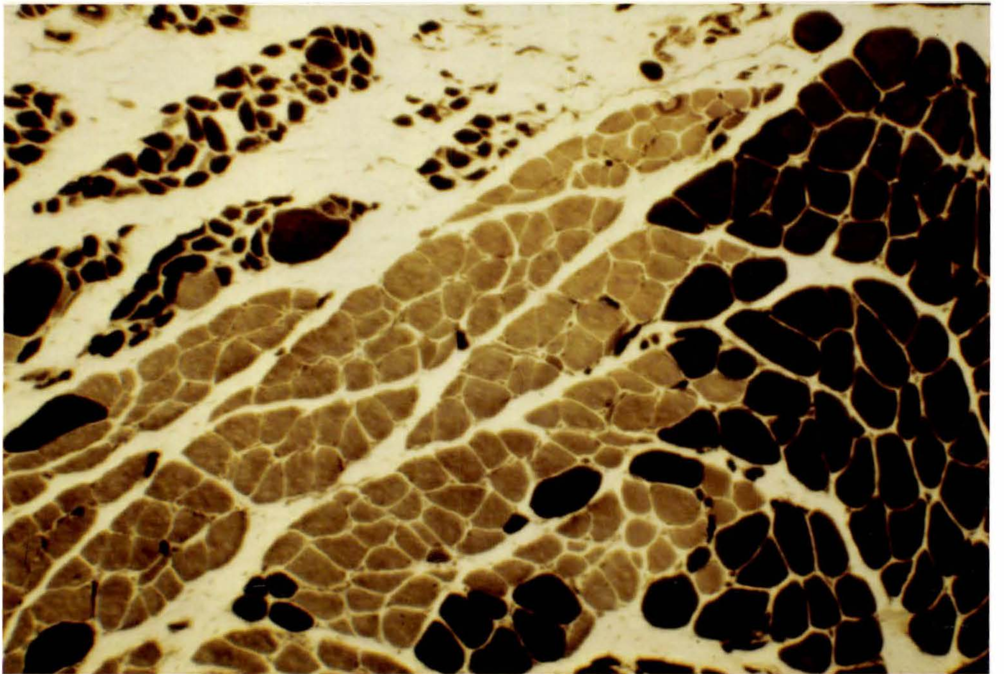
Figure 31. Photomicrograph of a transverse section of the short digital extensor muscle in a control horse containing exclusively Type I fibres. Note the presence of darker staining fibres in the muscle spindle.

Myosin ATPase staining x 60

Figure 32. Photomicrograph of a transverse section of the short digital extensor muscle in a clinical laryngeal hemiplegic horse showing evidence of fibre type grouping, group fibre atrophy, and variation in fibre size.

Myosin ATPase staining x 60





A description of the morphological changes in each muscle is presented below.

Left cricoarytenoid muscle

There was some overlap in the degree of change present in the control and subclinical horses, as division into these groups was based on endoscopic and neurological findings, in addition to muscle pathology. Nevertheless, generally the lateral cricoarytenoid muscle in control horses showed no, or only mild changes (fig. 8), while in the subclinical horses mild to moderate changes were present (Fig. 9).

All clinical laryngeal hemiplegic horses had severe muscle damage and obvious fibre type grouping in this muscle (Fig. 10A & B). In some of these horses only small areas of recognizable muscle tissue remained, the majority of the sample being replaced by fibrous tissue (fig. 10C).

Deep digital flexor muscle

The normal morphologic appearance of the deep digital flexor muscle is shown in figures 11A, B and C, which illustrate samples containing an approximately equal proportion of fibre types, and Type I or Type II fibre predominance. In 31% of samples in control horses mild variation in fibre size was observed (fig. 12). In addition to this, one control animal (horse 13) had severe pathology and fibre type grouping, in both left and right legs, which was indistinguishable from that seen in some clinical laryngeal hemiplegic horses. The two ponies were found to have moderate changes in one sample from both left and right legs in each animal. In control animals, including the ponies, the changes were most frequently observed in samples from the distal portion of the muscle.

Of the five control horses, in which additional samples were examined from the middle of the muscle, for the purpose of determining if any variation in muscle morphology existed between sites, the degree of pathology found was similar to that identified in the original 3 biopsy sites, with the exception of horse 11. In this animal mild to moderate pathology with fibre type grouping existed in both the cranial and medial samples, but no or mild pathology in the remainder.

Changes occurred in subclinical animals in a higher proportion (45%) of samples than in control horses. Some of these changes were similar to that seen in control horses but were more obvious in others, as shown in fig. 14.

Moderate to marked pathology, as well as fibre type grouping and group fibre atrophy, was seen in four of the clinical laryngeal hemiplegic horses (figs. 13, 15), while no or mild changes were seen in the remaining four clinical cases. Overall, signs of muscle damage were identified in 50% (19 of 38) of the samples collected from this muscle in laryngeal hemiplegic horses. These changes consisted of: variation in fibre size, rounding of fibres, split fibres, and an increased frequency of central nuclei. The changes were observed more frequently and were also more severe in the distal portions of the muscle.

<u>Cranial tibial muscle</u>

Horses in all groups showed few signs of pathology in this muscle (fig. 16), although variation in fibre size (figs. 17, 21), and rounding of fibres was observed in some samples. Muscle spindles were often observed but appeared normal (fig. 18). There was evidence of fibre type grouping in two clinical and two control horses and one pony, but these changes were usually confined to small areas of samples in a

single leg (figs. 19, 20).

In addition large groups of a single fibre type were frequently observed resulting in Type I or Type II fibre predominance (fig. 17). In more proximal areas of the muscle, type I fibre predominance was more frequent, while distally Type II fibre predominance was most often seen (fig. 21). Similar changes were observed in all groups of horses.
In two horses, one subclinical and one control, isolated areas of increased interstitial fibrosis and accumulations of inflammatory cells (indicative of a primary myositis) were found to affect this muscle in one leg (fig. 22 and 23).

Long digital extensor muscle

The most consistent feature of this muscle was the lack of morphological alteration in most samples (fig. 24). Where changes were detected these mostly consisted of slight variation in fibre size. Notable exceptions to this in the control group included one animal which had an isolated area of fibre type grouping in one leg (horse 8, fig. 25), and the 6-week-old foal which had evidence of inflammatory muscle disease (fig. 26A, B & C; Fig. 27A & B). One subclinical horse had moderate pathology in one sample from each leg, while one clinical horse had an isolated area of marked pathology in one sample.

There was no evidence of a difference between groups in the severity of pathology in this muscle.

Lateral digital extensor muscle

This muscle was also very consistent in its appearance (fig. 28), and abnormalities were few. When present they consisted of slight variation in fibre size, which was most obvious in one sample from each of two clinical horses (fig. 29). The degree of change in clinical horses was similar to that found in controls.

Short digital extensor muscle

This muscle was composed of unusually small fascicles (fig. 30A) when compared with other muscles examined. It also contained a large number of muscle spindles (fig. 30B). The majority of samples were found to contain a predominance of Type I fibres and on three occasions no Type II fibres could be identified (fig. 31).

Of the control horses, 6 out of 18 samples (33%) were found to have some evidence of pathology, although this was mild in four samples and moderate in the remaining two. One pony had marked changes in one sample. Abnormalities were noted in three subclinical horses, although these changes were only mild.

Almost half the samples from clinical horses were found to have evidence of pathology. In 3 of these moderate to marked pathology, including extensive variations in fibre size, fibre type grouping and group atrophy was evident (fig. 32). Two clinical horses were found to have mild changes, while in the remaining three no abnormalities could be detected.

When comparing groups, clinical and subclinical horses were more frequently affected than controls. In those samples which were affected, the most severe pathology was seen in clinical horses.

Muscle morphometry

(a) Proportion of Type I muscle fibres

The percentage of Type I fibres obtained when counting 200 or 1000 fibres is presented in Appendix 5. The difference in values obtained counting a different number of fibres was not statistically significant.

The mean percentage of Type I fibres for all samples is presented in Appendix 6.

The mean percentage of Type I fibres in the middle sample of all horses in the three groups examined is shown in Table 3, while the variation in the mean percentage of type I fibres in proximal, middle and distal samples in the limb muscles in each group is shown in Table 4. The effect of age on the mean percentage of Type I fibres in the limb muscles of control horses is shown in Table 5.

The variation in fibre proportions across the middle of the hindlimb muscles in the 5 control horses examined in this way is shown in Table 6.

The prominent findings concerning the percentage of Type I fibres in each muscle are summarised below. The two ponies in this study had a significantly higher ($p < 0.01$) mean proportion of Type I fibres in all their muscles than the control thoroughbred horses.

Left lateral cricoarytenoid muscle

The mean proportion of Type I fibres in this muscle was similar in each group of horses. However, there was significantly greater variation ($p < 0.02$) in the range of values within the clinical group (4-47%) when compared to subclinical (20-31%) or control (16-37%) horses. Evaluation of fibre type proportions was difficult in some clinical animals due to the severe atrophy of the muscles, and the presence of large numbers of small darkly staining fibres (fig. 10C). In such cases results were excluded from the statistical analyses.

Left dorsal cricoarytenoid muscle

There was a graded increase in the mean proportion of Type I fibres between normal (25%), subclinical (31%)

TABLE 3: Mean percentage of Type I fibres in the left laryngeal and middle sample of the limb muscles of clinical, subclinical and control horses

Muscle	Clinical % ± S.D.	Subclinical % ± S.D.	Control % ± S.D.
Left lateral cricoarytenoid	30 ± 16	27 ± 5	25 ± 5
Left dorsal cricoarytenoid	49 ± 18	31 ± 18	25 ± 5
Deep digital flexor	35 ± 11	32 ± 10	32 ± 11
Cranial tibial	51 ± 10	51 ± 14	46 ± 22
Long digital extensor	23 ± 6	24 ± 6	25 ± 7
Lateral digital extensor	24 ± 5	25 ± 9	25 ± 5
Short digital extensor	70 ± 18	67 ± 18	73 ± 18

TABLE 4: The mean percentage of Type I fibres in the proximal middle and distal samples of the limb muscles in clinical, subclinical and control horses

Muscle	Area of muscle	Groups of horses		
		Clinical % ± SD	Subclinical % ± SD	Control % ± SD
Deep digital flexor	Proximal	21 ± 14	25 ± 14	26 ± 14
	Middle	35 ± 11	32 ± 10	32 ± 11
	Distal	43 ± 11	40 ± 10	38 ± 5
Cranial tibial	Proximal	66 ± 11	60 ± 8	59 ± 18
	Middle	51 ± 10	51 ± 14	46 ± 22
	Distal	25 ± 10	35 ± 17	23 ± 10
Long digital extensor	Proximal	22 ± 10	22 ± 7	19 ± 4
	Middle	23 ± 6	25 ± 6	25 ± 7
	Distal	21 ± 5	22 ± 4	22 ± 7
Lateral digital extensor	Proximal	28 ± 8	28 ± 8	27 ± 9
	Middle	24 ± 5	24 ± 9	25 ± 5
	Distal	27 ± 8	28 ± 6	27 ± 6

TABLE 5 **The effect of age on the mean percentage of Type I fibres in various areas of the limb muscles in control horses**

Muscle	**Area of Muscle**	**Age Groups**	
		< 2yrs	**> 3yrs**
		% ± SD	% ± SD
Deep digital flexor	Proximal	18 ± 22	34 ± 10
	Middle	29 ± 12	36 ± 9
	Distal	37 ± 6	40 ± 2
Cranial tibial	Proximal	50 ± 20	69 ± 5
	Middle	32 ± 18	65 ± 5
	Distal	18 ± 9	30 ± 9
Long digital extensor	Proximal	18 ± 4	20 ± 3
	Middle	21 ± 7	30 ± 6
	Distal	21 ± 8	25 ± 5
Lateral digital extensor	Proximal	21 ± 5	34 ± 7
	Middle	24 ± 6	27 ± 3
	Distal	27 ± 8	28 ± 5
Short digital extensor	Middle	80 ± 21	64 ± 6

TABLE 6 **Mean percentage of Type I fibres in samples taken from various areas in the middle of the limb muscles in 5 control horses**

Muscle	**Area of Muscle**				
	Medial	**Lateral**	**Middle**	**Cranial**	**Caudal**
	% ± SD	% ± SD	% ± SD	% ± SD	% ± SD
Deep digital flexor	31 ± 9	44 ± 5	32 ± 11	46 ± 11	22 ±12
Cranial tibial	49 ± 13	70 ± 7	46 ± 22	–	–
Long digital extensor	27 ± 4	21 ± 5	25 ± 7	23 ± 5	20 ± 5
Lateral digital extensor	29 ± 8	27 ± 3	25 ± 5	28 ± 7	27 ± 4

and clinical horses (49%). The variation in range of values within both the clinical (27-79%) and subclinical (18-44%) groups was significantly greater ($p < 0.02$) than in normal horses (21-30%).

Deep digital flexor muscle

There was a proximal to distal increase in the proportion of Type I fibres in this muscle in each group of horses (Table 4). In control horses they increased from 26% proximally to 38% distally. When the control horses were divided into two age groups, 2 years and less and those 3 years or greater, a significant increase ($p < 0.01$) from 18 to 37% was found in the younger animals, while the lesser increase in the older horses, from 34 to 40% was not statistically significant. There was also a significant difference ($p < 0.05$) in the proportion of Type I fibres in the proximal sample of young horses (18%), compared with older horses (34%) (Table 5).

At the middle level of this muscle in normal horses there was a significant increase ($p < 0.05$) in Type I fibre proportions from medial (31%) and caudal (22%) to cranial (46%) and lateral (44%) areas of the muscle (Table 6).

This variation in Type I fibre percentage is illustrated in figs. 11A, B & C.

In both the subclinical and clinical groups there was also an increase in Type I fibres from proximal to distal. In the clinical animals this increase was significant from proximal to middle samples ($p < 0.01$) and middle to distal samples ($p < 0.01$).

Comparing control with subclinical and clinical groups of horses, no statistically significant differences in the mean proportion of Type I fibres

was found at similar sites within the muscle. In the distal most sample the variance of the clinical cases was significantly greater than found in normal horses ($p < 0.02$).

Cranial tibial muscle

The main features of this muscle was a marked variation in the percentage of Type I fibres throughout the muscle and, on occasions, within individual samples irrespective of disease status (figs. 16, 17 & 21). In control horses the percentage of Type I fibres ranged from 5 to 78%, and in clinical cases from 6 to 70%. The proportion of fibre types was found to be influenced by the site of sampling and the age of the horse. In all groups there was a significant ($p < 0.05$) decrease in proportion of Type I fibres from proximal to distal, and in 5 control horses, in which additional samples were examined, there was a significant decrease ($p < 0.01$) in Type I fibre proportions from lateral to medial. In addition young control horses (two years old or less) also had a significantly lower ($p < 0.01$) proportion of Type I fibres at all levels than older horses.

There was no significant difference in the proportion of Type I fibres at each sampling site between normal, subclinical or clinical groups of horses

Long digital extensor muscle

This muscle was very consistent in its appearance with no significant difference between any age or group of horses. While proximal middle and distal samples were also similar, there was a significantly ($p < 0.01$) lower percentage of Type I fibres in the caudal and lateral areas of the muscle when compared with the medial area.

Lateral digital extensor muscle

This muscle showed very little variation in fibre type proportions with no significant difference between any sites, age or groups of horses.

Short digital extensor

Most samples contained a majority of Type I fibres and in three of these no Type II fibres were observed (fig. 31). There was considerable variation in the percentage of Type I fibres between horses, although this was not related to the disease status of the animal. Older horses in the control group were, however, found to have a slightly lower percentage of Type I fibres than younger horses.

(b) Muscle fibre diameters

The mean diameter of Type I and Type II fibres for each muscle in all horses is presented in Appendix 7, while the mean fibre diameter for each muscle for the three groups of horses is presented in Tables 7 and 8. There was no difference in fibre diameter between left and right legs of young and old horses, with the exception of the 6 week old foal which had consistently smaller fibres in all muscles. In the majority of cases the diameter of muscle fibres of horses in the subclinical group was less than that found in both normal and clinical cases, although there were only a small number of observations in this group. The foal and subclinical horses were excluded from statistical analysis of fibre diameter and are not discussed further. The main findings in each muscle are presented below.

Left lateral cricoarytenoid muscle

The Type I and II fibres had different mean diameters in clinical and control horses. In clinical animals Type I fibres were larger (50 um vs. 43 um) and Type II fibres smaller (22 um vs. 45 um) than in control animals. There was also a significantly greater

TABLE 7: Mean fibre diameter (µm) and standard deviation of Type I and Type II muscle fibres from laryngeal and limb muscles (middle site) in clinical, subclinical and control horses

Group	Muscle	Mean fibre diameter (µm)	
		Type I	Type II
Clinical	Left lateral cricoarytenoid	50 ± 18	22 ± 18
	Left dorsal cricoarytenoid	47 ± 11	30 ± 4
	Deep digital flexor	50 ± 10	49 ± 4
	Cranial tibial	47 ± 7	52 ± 6
	Long digital extensor	42 ± 7	52 ± 5
	Lateral digital extensor	39 ± 9	55 ± 6
	Short digital extensor	34 ± 5	37 ± 10
Subclinical			
	Left lateral cricoarytenoid	51 ± 5	48 ± 9
	Left dorsal cricoarytenoid	48 ± 1	46 ± 4
	Deep digital flexor	43 ± 7	42 ± 3
	Cranial tibial	44 ± 6	45 ± 3
	Long digital extensor	36 ± 4	45 ± 6
	Lateral digital extensor	31 ± 6	43 ± 3
	Short digital extensor	35 ± 5	35 ± 5
Control	Left lateral cricoarytenoid	43 ± 5	45 ± 3
	Left dorsal cricoarytenoid	47 ± 3	45 ± 4
	Deep digital flexor	49 ± 4	45 ± 3
	Cranial tibial	45 ± 5	47 ± 3
	Long digital extensor	44 ± 4	52 ± 6
	Lateral digital extensor	41 ± 5	51 ± 3
	Short digital extensor	35 ± 5	37 ± 6

TABLE 8: Mean diameter (µm) and standard deviation of Type I and Type 2 fibres from different areas in the middle of the same limb muscles in control horses.

Muscle	**Area of Muscle***	**Mean fibre diameter (um)**	
		Type I	**Type 2**
Deep digital flexor	Middle	49 ± 4	45 ± 3
	Cranial	49 ± 4	38 ± 5
	Caudal	43 ± 8	48 ± 12
	Medial	48 ± 6	46 ± 9
Cranial tibial	Middle	45 ± 5	47 ± 3
	Medial	44 ± 4	47 ± 5
	Lateral	44 ± 5	48 ± 6
Long digital extensor	Middle	44 ± 4	52 ± 6
	Cranial	42 ± 5	51 ± 4
	Caudal	41 ± 3	52 ± 2
	Medial	44 ± 6	51 ± 3
	Lateral	42 ± 4	49 ± 4
Lateral digital	Middle	41 ± 5	51 ± 3
	Cranial	44 ± 2	53 ± 7
	Caudal	36 ± 3	51 ± 3

* Fibre diameter not measured in proximal and distal samples

variation in the size of both these fibre types in clinical horses than in control horses ($p < 0.02$).

Dorsal cricoarytenoid muscle

While the mean diameter of Type I fibres was found to be similar in both control and clinical groups, the range of mean fibre sizes in the clinical group (31 um to 74 um) was significantly different ($p < 0.02$) from those horses in the control group (35 um to 49 um).

Type II fibres were found to be significantly smaller ($p < 0.01$) in the clinical group (mean 30 um) compared with that seen in both control (mean 45 um) and subclinical horses (mean 46 um).

Deep digital flexor muscle

The mean Type I fibre diameter in both control and clinical groups was similar, however the range in mean fibre diameter of horses in the clinical group (33 um to 64 um) was significantly greater ($p < 0.02$) than in control horses (41 um to 55 um). Type II fibres were larger in clinical horses than in control horses, although this was not statistically significant because of abnormally large type II fibres in one control horse (horse 8). When the values for this horse were excluded from the mean for this group, the difference between clinical and normal horses was statistically significant ($p < 0.05$).

Fibre diameter measurements at medial, caudal, lateral and cranial sites in five control horses examined showed some deviation from the values obtained for the middle sample. Type I fibres were smaller in the caudal (superficial) portion of the muscle, while Type II fibres were smaller cranially (deeper).

Cranial tibial muscle

There was no significant difference between groups of horses for fibre diameters of Type I fibres in this muscle. However, clinical cases had significantly greater ($p < 0.05$) Type II fibre diameters than control horses.

Fibre diameters from the medial and lateral aspects of five control horses were not significantly different from the middle sample.

Long digital extensor muscle

In this muscle there was a significantly greater ($p < 0.01$) variation in mean Type I fibre diameters in clinical (30 um to 57 um) compared to control (41 um to 52 um) horses, although the mean fibre size was similar for Type I and II fibres in each group.

Overall Type II fibres were found to be significantly larger ($p < 0.01$) than Type I fibres in all horses.

There was no significant difference in mean fibre diameter at cranial, medial, caudal or lateral sites compared with the middle site for each fibre type, however Type II fibres were again significantly larger ($p < 0.01$) than Type I fibres at all sites.

Lateral digital extensor muscle

There was a greater range of both type I and Type II fibres in clinical horses compared with control horses, although this was significant at a low level ($p < 0.1$). The mean diameter of Type I fibres ranged from 33 um to 46 um in control horses and 17 um to 57 um in clinical horses. Type II fibres ranged from 44 um to 56 um in control horses and 43 um to 65 um in the clinical group. The size of Type II fibres in clinical cases was slightly larger than in control horses, although this was not significant.
Type I fibres were significantly smaller ($p < 0.01$)

than Type II fibres for all groups. No significant difference was found in fibre diameter between different sites.

Short digital extensor

There was no significant difference in mean fibre diameter or variance for any group or fibre type in this muscle.

(c) Muscle fibre diameter distribution

In order to evaluate the differences in fibre size distribution between clinical and control animals histographic analysis of fibre diameter distribution was undertaken by analysis of individual fibre diameters from the middle sample of each muscle in both groups. This involved approximately 1200 measurements of fibre diameters for both Type I and Type II fibres in each muscle in both control and clinical horses.

The results are presented as histograms in figures 33-37.

In all muscles, for both Type I and Type II fibres, a statistically significant difference existed between normal and clinical horses. This was highly significant ($p < 0.0001$) for most muscles and fibre types. The exceptions were Type I fibres in the long digital extensor muscle ($p = 0.017$) and Type II fibres in the short digital extensor muscle ($p = 0.038$).

In each muscle this difference arose from an alteration in the distribution of muscle fibre diameters. In the clinical group there are more smaller and larger diameter fibres, corresponding to an increased number of atrophic and hypertrophic fibres. This is particularly evident for Type I fibres in the deep digital flexor muscle (fig. 33). Such changes result in a wider, flatter fibre diameter distribution curve for those horses in the

clinical group compared to controls.

(d) Atrophy and hypertrophy factors

The normal ranges of fibre diameters for each muscle and fibre type in the control horses used to calculate atrophy and hypertrophy factors are shown in Appendix 8. These values were obtained from samples in those limb muscles in control horses in which little or no pathological changes were observed.

The atrophy and hypertrophy values for each muscle for all horses are presented in Appendix 9. A comparison of the mean atrophy and hypertrophy factors for hindlimb muscles for each group of horses is shown in Table 9. This table shows that, with one exception, horses in the clinical group had either higher atrophy or hypertrophy factors, or both, for Type I and II fibres in the limb muscles than those found in control animals. The one exception to this was Type II fibres in the long digital extensor muscle, in which both atrophy and hypertrophy factors were similar. Those values obtained for subclinical cases were more variable, although there appeared to be severe atrophy of Type I fibres in the deep digital flexor and long digital extensor muscles, and both Type I and Type II fibre atrophy in the lateral digital extensor.

TABLE 9. Mean atrophy and hypertrophy factors for Type I and Type II muscle fibres in the hindlimb muscles of clinical, subclinical and control horses.

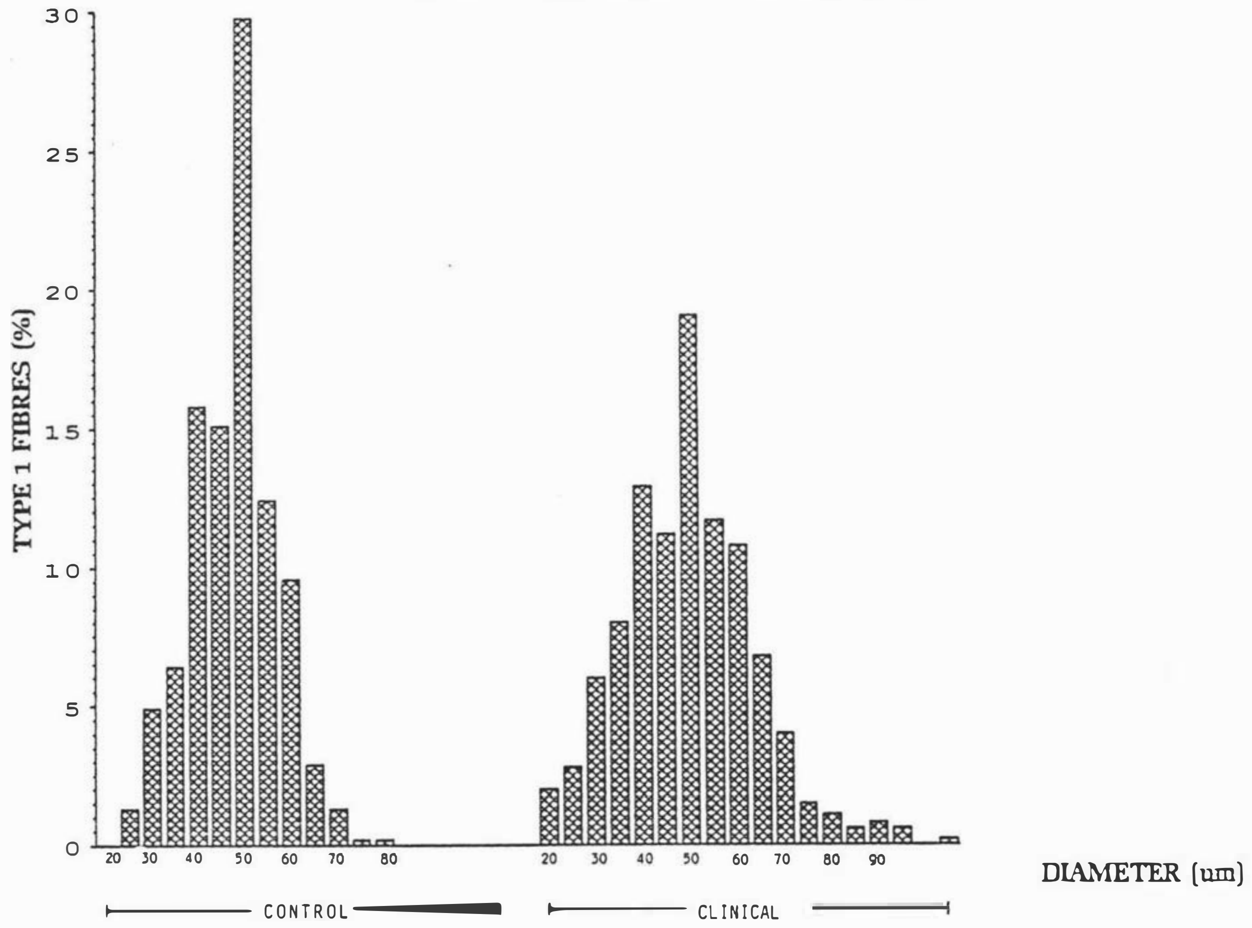
Group	Muscle	Type I fibre		Type II fibre	
		A*	H*	A	H
Clinical					
	Deep digital flexor	18	16	7	19
	Cranial tibial	6	16	2	29
	Long digital extensor	16	7	3	5
	Lateral digital extensor	13	12	8	24
	Short digital extensor	7	9	10	11
Subclinical					
	Deep digital flexor	27	4	10	4
	Cranial tibial	8	5	6	7
	Long digital extensor	24	1	6	2
	Lateral digital extensor	27	1	20	1
	Short digital extensor	3	12	5	2
Control	Deep digital flexor	9	6	9	9
	Cranial tibial	8	6	7	12
	Long digital extensor	6	5	3	4
	Lateral digital extensor	5	5	7	10
	Short digital extensor	6	5	3	4

* A - Atrophy factors
H - Hypertrophy factors

Figure 33. Histograms of muscle fibre diameter distribution in the deep digital flexor muscle in control and clinical laryngeal hemiplegic horses.

A. Type I fibres. The wider, flatter distribution curve seen in clinical horses in all muscles is most readily observed in this histogram.

B. Type II fibres


DEEP DIGITAL FLEXOR MUSCLE – TYPE 1 FIBRES
TYPE 1 FIBRES (%)
30
25
20
15
10
5
0
20 30 40 50 60 70 80
20 30 40 50 60 70 80 90
DIAMETER (um)
CONTROL
CLINICAL

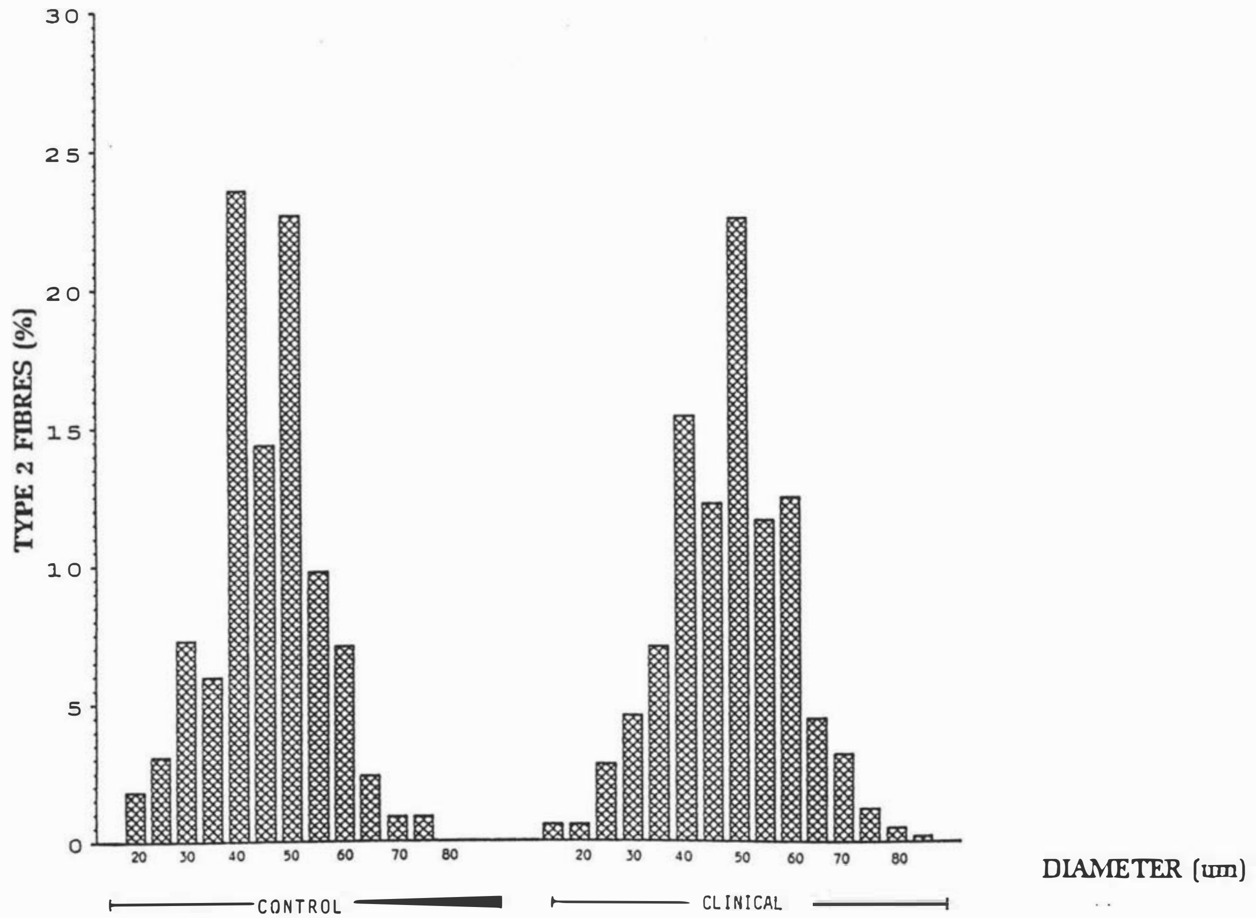
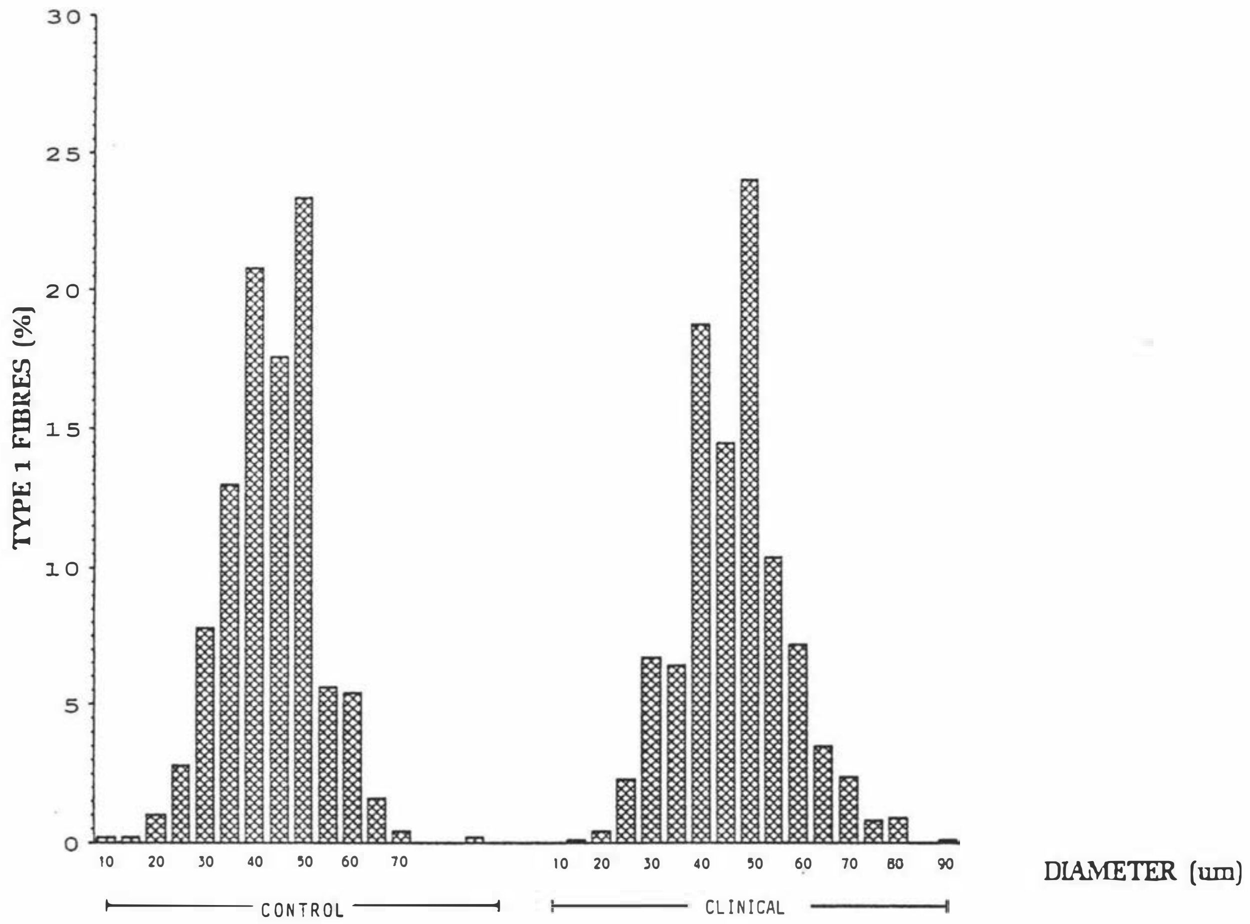

DEEP DIGITAL FLEXOR MUSCLE – TYPE 2 FIBRES
TYPE 2 FIBRES (%)
30
25
20
15
10
5
0
20 30 40 50 60 70 80
20 30 40 50 60 70 80
DIAMETER (um)
CONTROL
CLINICAL

Figure 34. Histograms of muscle fibre diameter distribution in the cranial tibial muscle in control and clinical laryngeal hemiplegic horses.

A. Type I fibres.

B. Type II fibres


CRANIAL TIBIAL MUSCLE - TYPE 1 FIBRES
TYPE 1 FIBRES (%)
30
25
20
15
10
5
0
10 20 30 40 50 60 70
10 20 30 40 50 60 70 80 90
DIAMETER (um)
CONTROL
CLINICAL

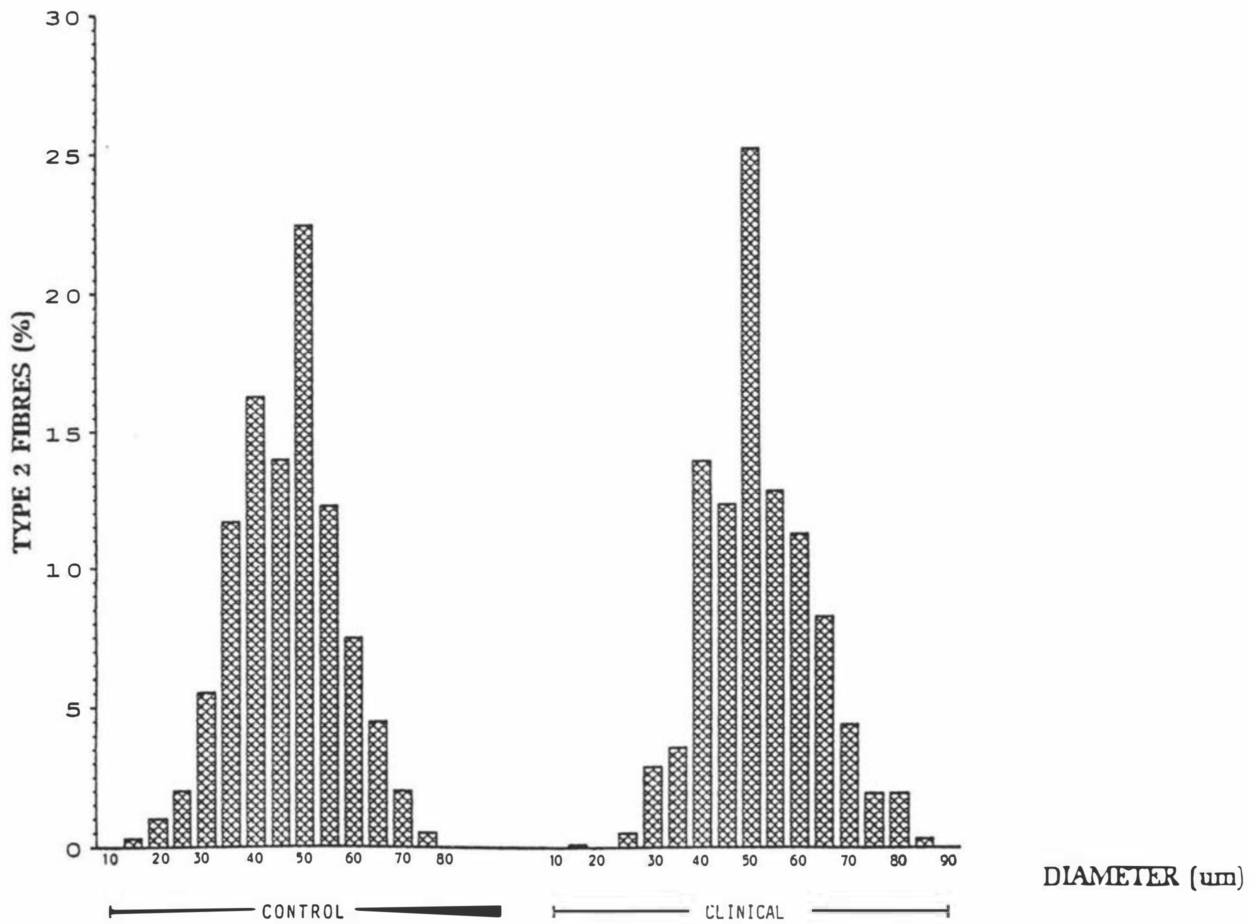
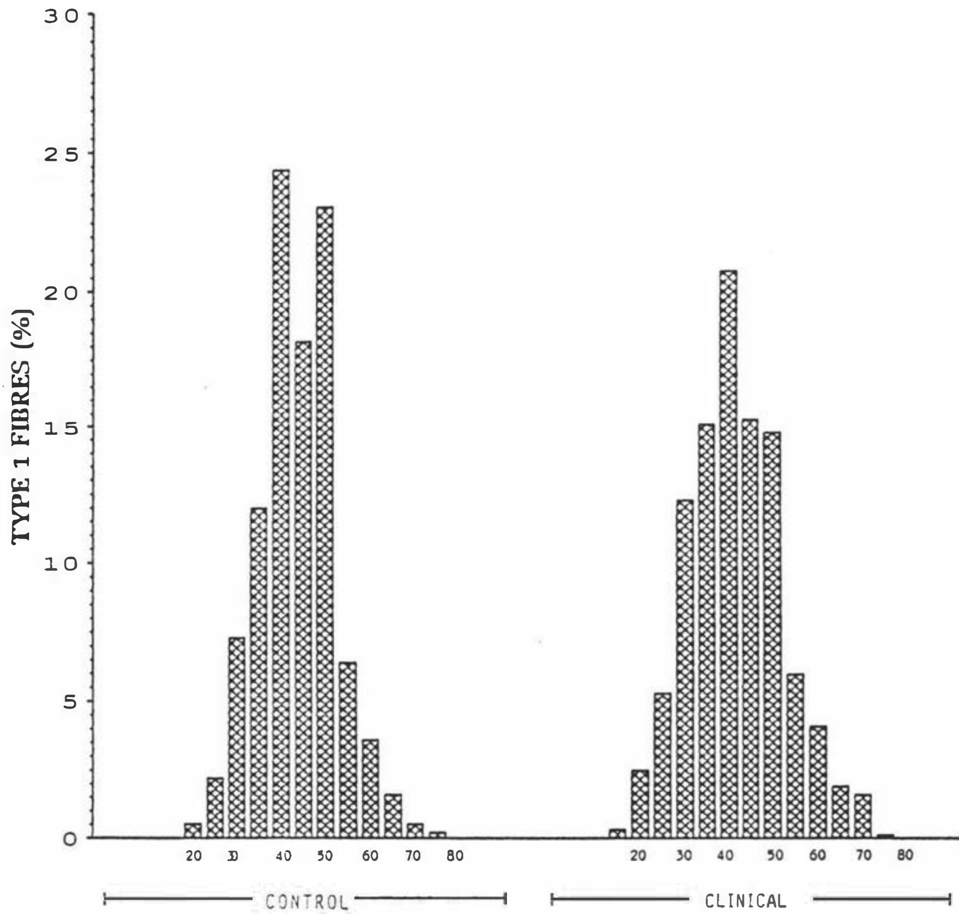

CRANIAL TIBIAL MUSCLE - TYPE 2 FIBRES
TYPE 2 FIBRES (%)
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80
10 20 30 40 50 60 70 80 90
DIAMETER (um)
CONTROL
CLINICAL

Figure 35. Histograms of muscle fibre diameter distribution in the long digital extensor muscle in control and clinical laryngeal hemiplegic horses.

A. Type I fibres.

B. Type II fibres


LONG DIGITAL EXTENSOR MUSCLE - TYPE 1 FIBRES
TYPE 1 FIBRES (%)
30
25
20
15
10
5
0
20 30 40 50 60 70 80
20 30 40 50 60 70 80
DIAMETER (um)
CONTROL
CLINICAL

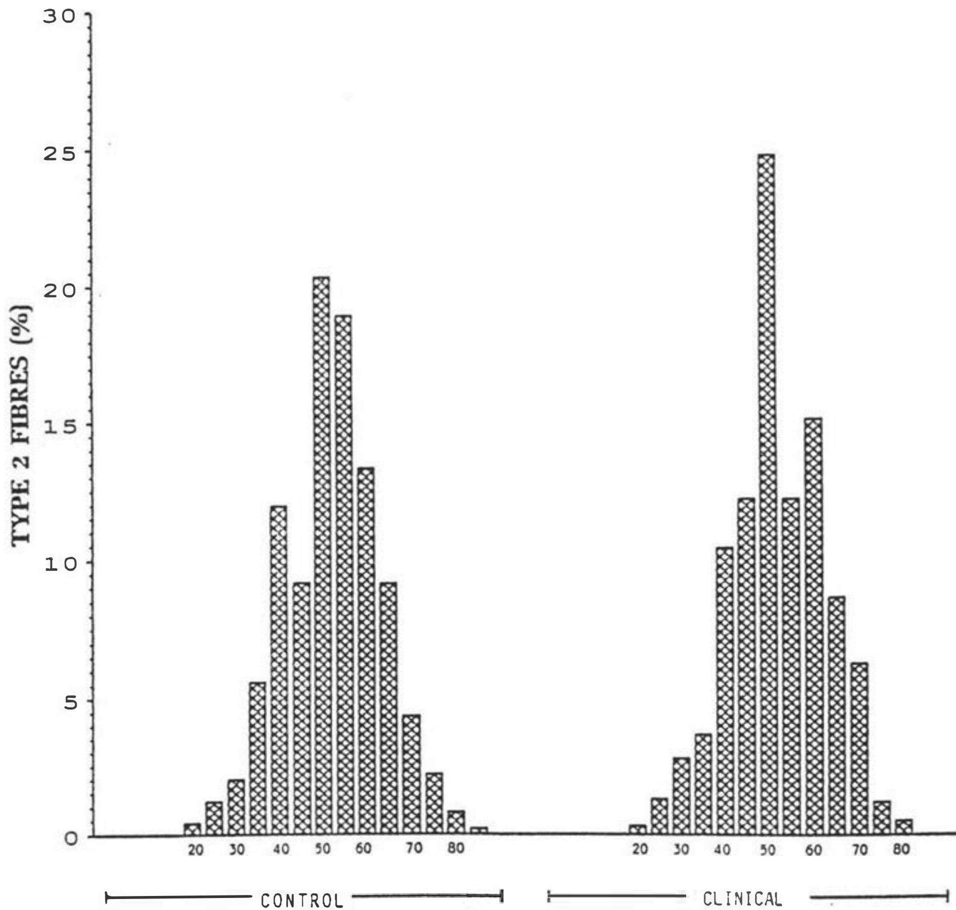
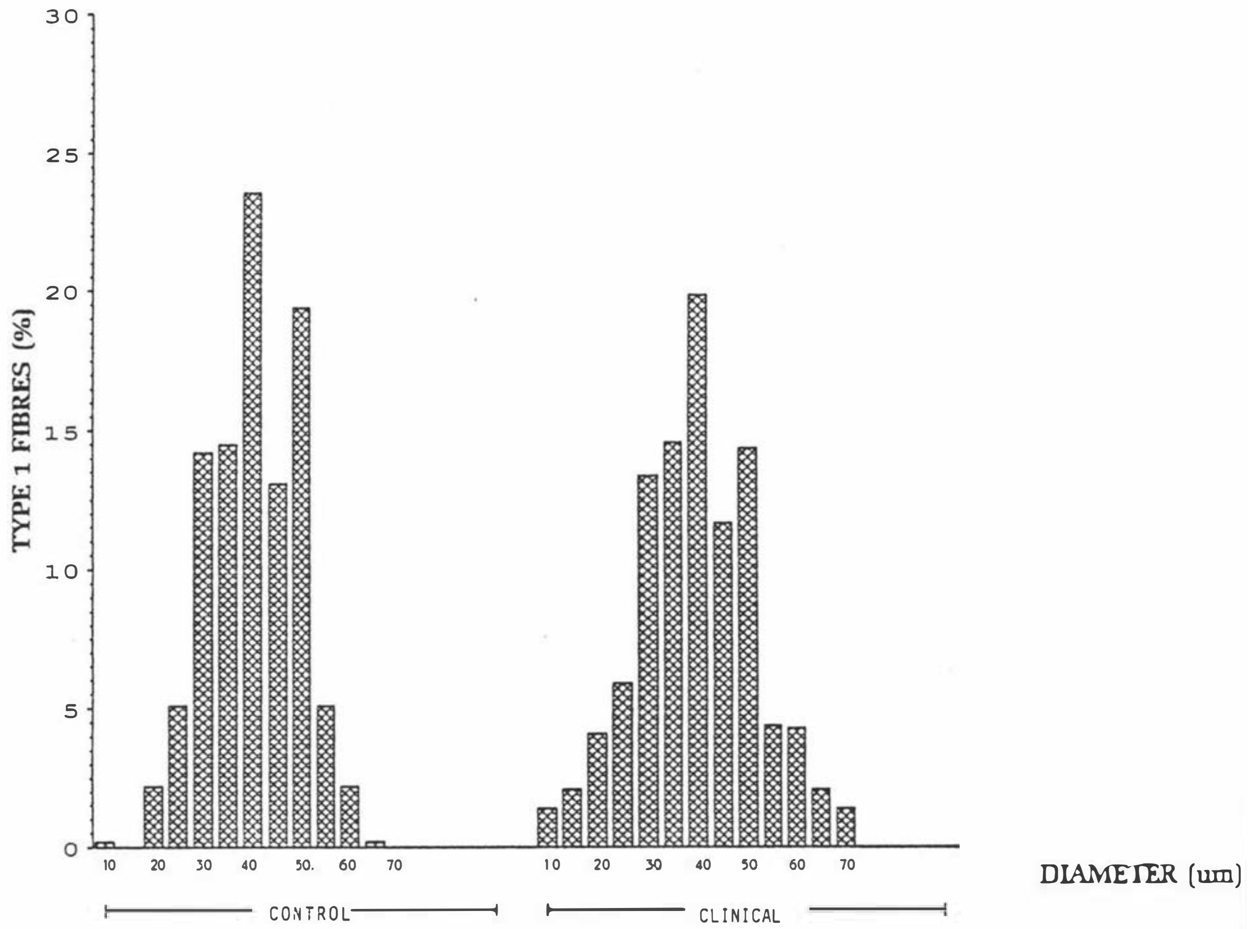

LONG DIGITAL EXTENSOR MUSCLE - TYPE 2 FIBRES
TYPE 2 FIBRES (%)
30
25
20
15
10
5
0
20 30 40 50 60 70 80
20 30 40 50 60 70 80
DIAMETER (um)
CONTROL
CLINICAL

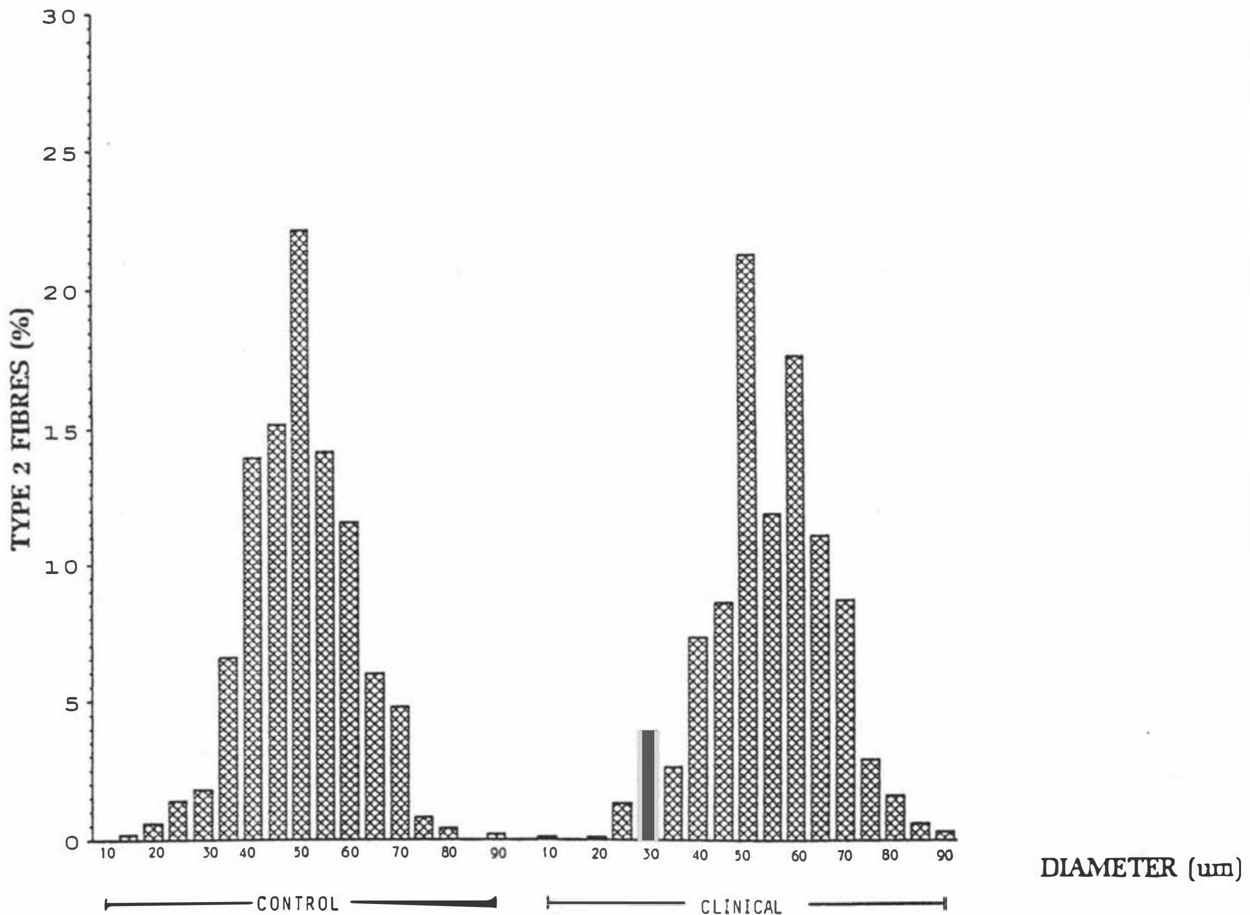
Figure 36. Histograms of muscle fibre diameter distribution in the lateral digital extensor in control and clinical laryngeal hemiplegic horses.

A. Type I fibres. Although little morphological evidence of a difference between clinical and control groups was evident, the histogram for clinical animals has a much wider distribution, indicative of increased atrophy and hypertrophy of fibres in this group.

B. Type II fibres



LATERAL DIGITAL EXTENSOR MUSCLE - TYPE 1 FIBRES
TYPE 1 FIBRES (%)
30
25
20
15
10
5
0
10 20 30 40 50. 60 70
10 20 30 40 50 60 70
DIAMETER (um)
CONTROL
CLINICAL

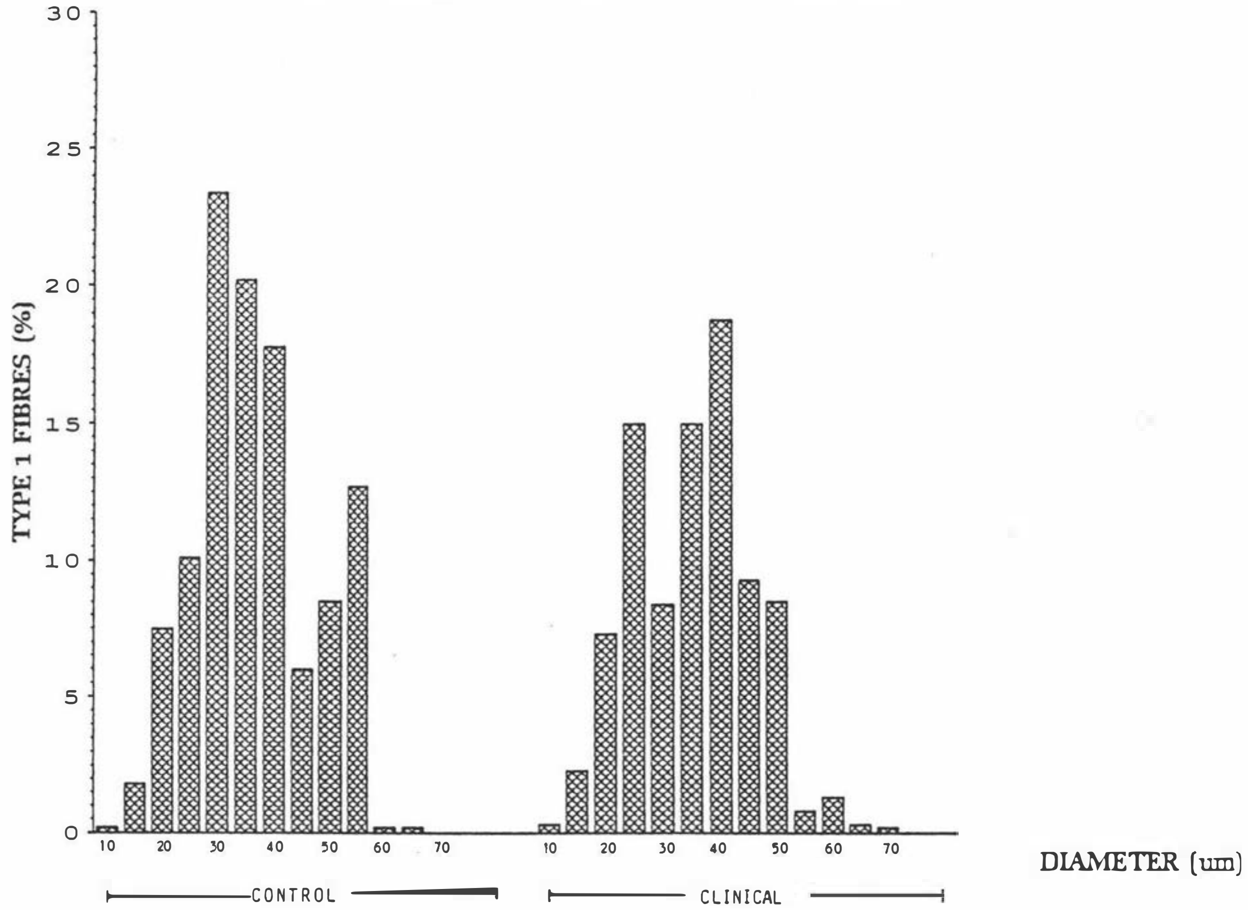


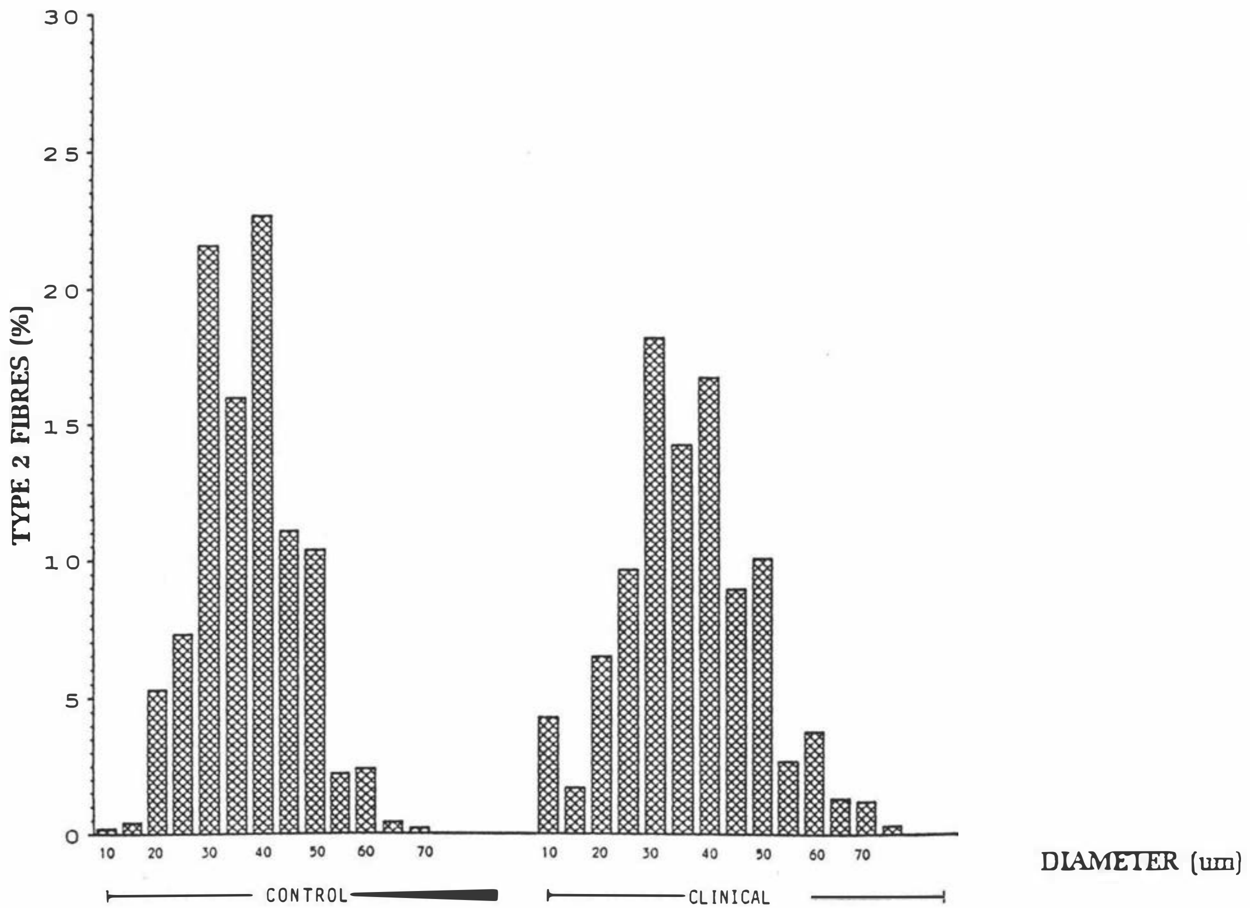
LATERAL DIGITAL EXTENSOR MUSCLE - TYPE 2 FIBRES
TYPE 2 FIBRES (%)
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90
10 20 30 40 50 60 70 80 90
DIAMETER (um)
CONTROL
CLINICAL

Figure 37. Histograms of muscle fibre diameter distribution in the short digital extensor muscle in control and clinical laryngeal hemiplegic horses.

A. Type I fibres.

B. Type II fibres


SHORT DIGITAL EXTENSOR MUSCLE - TYPE 1 FIBRES
TYPE 1 FIBRES (%)
30
25
20
15
10
5
0
10
20
30
40
50
60
70
DIAMETER (um)
CONTROL
CLINICAL


SHORT DIGITAL EXTENSOR MUSCLE - TYPE 2 FIBRES
TYPE 2 FIBRES (%)
30
25
20
15
10
5
0
10
20
30
40
50
60
70
DIAMETER (um)
CONTROL
CLINICAL

DISCUSSION

In comparison to the situation in man there have been few reports in the veterinary literature on the use of histochemical techniques for the evaluation of neuromuscular disease in the horse. Of these some have investigated recognized muscular diseases such as exertional rhabdomyolysis (Van den Hoven et al, 1987), myotonia (Reed et al, 1988), and nutritional myopathy (Roneus et al, 1986). Others have evaluated muscle changes in neurogenic diseases, for example, in idiopathic laryngeal hemiplegia (Anderson, 1984; Cahill, 1985), stringhalt (Cahill et al, 1986), and suprascapular neuropathy (Duncan and Schneider, 1985). As a result of the rapid increase in the use of histochemistry for evaluation of muscle, particularly in equine exercise physiology, these techniques have become more widely used to investigate the evaluation of horses suspected of having neuromuscular disease (Cardinet and Holliday, 1979; Andrews et al, 1986; Van den Hoven et al, 1988).

Muscle morphology

(i) Laryngeal muscles

Several previous investigations on equine intrinsic laryngeal muscles (Gunn, 1972, 1973; Duncan, 1975; Anderson, 1984; Cahill, 1985) have compared the morphology of these muscles in normal and clinical idiopathic laryngeal hemiplegic horses. The reason for re-examining these muscles in the present study was to assist in providing a definitive diagnosis of clinical and subclinical laryngeal hemiplegia, so that these animals could be differentiated from control horses. The results confirmed the histological and histochemical findings of previous researchers and demonstrated that severe changes in these muscles reflects the presence of clinical disease. They also demonstrated that even in control animals some slight changes are present, and that no

definite division in terms of histological changes to these muscles exists between control and subclinically affected horses.

(ii) Hindlimb muscles

Most investigations on equine limb muscles have involved the middle gluteal muscle, because of its function, easy access, large size and, more recently, its well documented histological, histochemical and biochemical composition. There are few reports in the veterinary literature of detailed histological or histochemical investigation of the lower hindlimb muscles in horses (Andrews and Spurgeon, 1986; Cahill, 1985; Cahill et al, 1986). Of these only Andrews and Spurgeon (1986) studied muscles from horses free of clinical disease. Thus at the commencement of this study, little was known about the normal histological anatomy of the lower hindlimb muscles in the horse. For this reason it was important to examine muscle samples from horses free of clinical disease in order to enable interpretation of changes seen in muscles of horses known to have idiopathic laryngeal hemiplegia.

Deep digital flexor muscle

The frequent finding of morphological abnormalities in this muscle in control horses, including ponies, was unexpected, as these animals had been selected because of the absence of clinical evidence of muscular disease. While the changes observed were less severe in the control, and also the subclinical animals, compared to clinical horses, there were a number of similarities in the types of abnormalities seen in each group. These changes, which ranged from slight variation in shape and size of fibres to extensive atrophy and hypertrophy of fibres, and fibre type grouping were indicative of neurogenic muscle disease. The increased severity of these changes in clinical animals, selected because of the presence of a known neurological disease, further

supports this and indicates that a similar process may be affecting both laryngeal and hindlimb muscles. This is reinforced by the type of changes found in this muscle, and although less severe, are similar to those identified in the laryngeal muscles of horses with idiopathic laryngeal hemiplegia.

Almost identical findings to that described in the deep digital flexor muscle in this present study have been reported in other muscles in horses showing clinical evidence of neuro-muscular disease of unknown origin (Van den Hoven et al, 1988). In that report, several horses were found to have changes including atrophy of Type I fibres and hypertrophy of Type II fibres, increased frequency of central nuclei, fibre type grouping, and fibre splitting. These were considered to result from lower motor neurone disease.

The observation that the distal parts of this muscle were more often and more severely affected with changes than the proximal part, could indicate that these changes result from a neurogenic disease which preferentially affects the distal ends of longer peripheral nerves. Alternatively, the regional variation in pathology could be related to the large size of the muscle as it contains several bellies and numerous fibrous divisions of muscle within these bellies. Thus some areas may be subject to variations in nerve and blood supply, or be subject to differing metabolic requirements and stresses, which could result in focal muscle disease. However, the frequent association of fibre type grouping with these changes indicates that a neurogenic cause is more likely.

The extensive amount of fibrous connective tissue in the deep digital flexor when compared to other muscles was evident both grossly and under light microscopy. The precise rôle of this connective

tissue is uncertain, but may be related to the function of this muscle in the stay apparatus of the horse.

Cranial tibial muscle

A feature of the cranial tibial muscle was the frequent occurrence of Type I or Type II fibre predominance. The presence of such large groups of a single fibre type probably represent a variation of normal in this muscle, as similar observations have been reported in other muscles, including the cranial tibial, in other species (James, 1971a; Lexell _et al_, 1983, 1984). Large groups of a single fibre type can on occasions be indicative of fibre type grouping, particularly in a muscle which normally has a predominance of one type (Reniers _et al_, 1970). The lack of morphological evidence of disease associated with these changes suggests that a chronic neurogenic process was probably not the cause of the fibre type predominance in these animals.

While the aetiology of the larger groups of a single fibre type is uncertain, the finding of smaller groups of both Type I and Type II fibres together in the same fascicle in 5 animals was indicative of fibre type grouping. The most likely cause of this is neurogenic disease in these animals.

There appeared to be little difference in the morphological appearance of the cranial tibial muscle between the three groups of horses examined, suggesting that the disease causing idiopathic laryngeal hemiplegia had little detectable effect on the nervous supply of this muscle. This finding is similar to that reported by Cahill (1985) who found only mild changes in this muscle in a single laryngeal hemiplegic horse examined.

Long and lateral digital extensor muscles

The morphology of the long digital extensor muscle has been previously examined by Andrews and Spurgeon (1986), who found no abnormalities in normal horses, and by Cahill (1985) in horses with neurological disease. A feature of this present study was that in both the long and lateral digital extensor muscles there was minimal evidence of morphological abnormalities, irrespective of the disease status of the horse. These findings are, however, in contrast to Cahill (1985) who found evidence of mild to moderate pathology in the long digital extensor in two of four clinical laryngeal hemiplegic horses, and one subclinical horse, while no changes were detected in six control horses.

Short digital extensor

In the short digital extensor muscle features of interest included the absence of Type II fibres in some samples, the high incidence of morphological changes and the frequent occurrence of muscle spindles. Few other muscles in any species have been identified in which a single fibre type exists. Two muscles in the cat are composed of one fibre type: the soleus, which consists exclusively of Type I fibres, and the short digital extensor, which is composed almost exclusively of Type II fibres (Sarnat, 1983). More recently in the horse two muscles have been identified which contain only a single fibre type (Billeter _et al_, 1987). The masseter muscle contains only Type I fibres while the subcutaneous muscle contains only Type II fibres. While not all samples of the short digital extensor muscle in the horse were exclusively Type I fibres, their high proportion in many samples is commensurate with its function as a slow, chronically contracting muscle.

The frequent presence of abnormalities found in the short digital extensor muscle is analogous to the

situation in man. Jennekens et al (1971c) found that the short digital extensor muscle frequently had signs of pathology, and was more severely affected than other muscles during old age. The pathology in this muscle in man is highly suggestive of chronic denervation and reinnervation (Jennekens et al, 1972), and although the signs appeared primarily age related, young adults were frequently affected. Gairns et al (1960) documented fibre loss in the nerve supplying this muscle and suggested a compression neuropathy may be responsible as both young and old people were affected. They suggested that poorly fitting shoes may have been the cause of this compression neuropathy, although others authors disagree with this theory (Jennekens et al, 1972). While poorly fitting shoes would not cause a compression neuropathy in the horse, other forms of compression may be possible. The most likely site of compression of this nerve in the horse would be at the level of the hock joint, where the tendons of the long and lateral digital extensor muscles and their retinacular sheaths pass over the dorsal aspect of this joint. The presence of nerve fibre loss above this site suggests that a compression neuropathy at this point is in most cases not the cause of pathology in the short digital extensor muscle in the horse.

As in man, the findings of fibre size variation, fibre type grouping and group atrophy in this muscle is highly indicative of a neurogenic cause of these changes. Further support for this is the presence of the severest pathology in clinical laryngeal hemiplegic horses.

The frequent presence of muscle spindles in the short digital extensor muscle indicates that one of its functions may be to regulate tension and co-ordinate contraction between the long and lateral digital extensors, on which it has its insertions.

There are a number of conclusions that can be drawn from the morphological examination of the hindlimb muscles in this study.

The first of these is that abnormalities are present in apparently normal horses free of clinical muscular or neurological disease, particularly in the deep digital flexor and short digital extensor, and, to a lesser extent, the cranial tibial muscles. The precise aetiology of these changes in normal horses is unknown. The morphological changes, particularly fibre type grouping, are, however, indicative of a primary neurogenic disease.

The presence of abnormalities in muscles of individuals free of clinical disease has been reported in a number of other species. In man, such changes can result from cachexia, subclinical disease, inactivity, the remote effect of neoplasms (Engel and Askanas, 1976) and old age (Jennekens _et al_, 1971c). However, the method of selection of horses for this study would preclude many of these as a cause of the changes.

In the cricopharyngeus muscle of man and the guinea pig (Rosenfield _et al_, 1982; Bonnington _et al_, 1987, 1988), there is extensive variation in fibre size, and abundant endomysial connective tissue in apparently healthy individuals. A number of explanations for these findings in "normal" muscle were proposed by these authors. The first is that the increased amount of connective tissue acts as a supporting framework and anchorage through which the muscle can tonically contract. As a result the muscle fibres do not travel the entire length of the muscle, but instead possibly insert at various levels into the connective tissue framework. This could explain the large variation of fibre size in this

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muscle, with small fibres representing the tapering ends of otherwise normal fibres just prior to insertion onto the connective tissue. A similar situation may exist in the deep digital flexor of the horse.

A further example of abnormalities being detected in healthy individuals is the internal abdominal oblique muscle of sheep bred intensively, where almost identical changes to those seen in the deep digital flexor muscle in this study have been described (Wilson et al, 1978). Muscle pathology consisting of atrophy and hypertrophy of both Type I and Type II fibres, fibre splitting, increased fibrous tissue, fibre degeneration and regeneration, and fibre type grouping, were described in these sheep. It was suggested these changes occurred in response to chronic stretching or an increased work load, as has been documented in other species (Tabary et al, 1972; Goldspink, 1977). The presence of fibre-type grouping in such cases was interpreted as being indicative of some neurogenic involvement.

A final example of such abnormalities in "normal" muscle is in the horse is where Blythe et al (1983) found changes in the palatal muscles, which were considered as being indicative of myositis, although later Anderson (1984) considered that these changes were a normal characteristic of this muscle.

Muscle morphometry

(a) Proportion of Type I fibres

Measurement of the percentage of Type I fibres can provide valuable information regarding the function of normal muscles, while alterations in fibre proportions can be a sensitive indicator of muscle disease. In order to correctly interpret the effects of diseases on fibre type proportions, a knowledge of the percentage of Type I fibres in normal horses, and how this may vary because of

factors other than disease, is required.

In the laryngeal muscles the percentage of Type I fibres found in control horses was consistent with previous reports (Gunn, 1972; Anderson, 1984). The much higher proportion of Type I fibres found in the left dorsal cricoarytenoid muscle in clinical horses may have resulted from a decrease in the total number of Type II fibres, which have been shown to be more susceptible to damage in a variety of disease processes, including neurogenic atrophy (Reniers et al, 1970; Johnson and Kucukyalcin, 1978; Lindholm et al, 1981; Rouleau et al, 1987).

In the hindlimb muscles the percentage of Type I fibres was found to be dependent on which muscle was sampled, the site of sampling, age and, to a lesser extent, the disease status of the horse. These influences on fibre proportions are discussed below.

(i) Variation between muscles

The variation in the percentage of fibre types between different muscles has been well documented in man as well as animals. As has been previously mentioned, many authors (Ariano et al, 1973; Gunn, 1978; Lexell et al, 1983b) feel this variation is the result of the different anatomical, regional and mechanical functions of each muscle. Therefore, in accordance with this theory, the proximal part of the cranial tibial, the distal part of the digital flexor, and the short digital extensor muscles, which were found to have a higher proportion of Type I fibres, are probably slow, chronically contracting muscles used mainly for the maintenance of posture. Conversely, those muscles which contained a majority of Type II fibres such as the long and lateral digital flexor, are probably used mainly for propulsion. The only other report on fibre type percentages in the distal hindlimb muscle is that of

Andrews and Spurgeon (1986), who found a similar percentage of Type I fibres in the long digital extensor muscle to that identified in this study.

(ii) Variation within muscles

Several studies have investigated the variation in Type I fibre proportions which may occur within a muscle, although there is some disagreement in the literature as to whether any variation does exist. In many earlier studies, in both man and the horse (Johnson et al, 1973; Edgerton et al, 1975; Snow and Guy, 1980) and in one more recent report (Wood et al, 1988), the proportion of Type I fibres was found not to be affected by sampling site. In contrast to these, most later reports again in man (Lexell et al, 1983b) and the horse (Kai, 1984; Bruce and Turek, 1985; Raub et al, 1985; Van den Hoven et al, 1985), have found a consistent increase in the proportion of Type I fibres from superficial to deep areas of muscles.

In this present study a similar finding of an increase in the proportions of Type I fibres from superficial to deep areas of muscles was found in the deep digital flexor, cranial tibial and long digital extensor muscles. In the only other large muscle studied, the lateral digital extensor, no consistent variation in fibre proportions could be identified.

In addition to this, a less well documented form of variation, from proximal to distal sampling sites, was observed in the cranial tibial and deep digital flexor muscles. Other documented reports of this form of variation are in an investigation of the vastus lateralis muscle in man (Lexell et al, 1983), and by Bruce and Turek (1985), who found a cranial to caudal variation in Type I fibres in the middle gluteal muscle in the horse.

(iii) Variation with age

Considerable controversy as to the effect of age on the proportion of Type I fibres exists in the literature. An increasing proportion of Type I fibres with growth has been reported by Kugelburg (1976) in laboratory animals; by Davies (1972) and Suzuki and Cassens (1980) in pigs; and by Bechtel and Kline (1987) and Raub et al (1986) in the horse. In contrast to these findings in the horse, several studies have not been able to detect an effect of age on Type I fibre proportions (Essen et al, 1980; Henckel, 1983; Kline et al, 1987).

The reasons for the lack of obvious changes in Type I fibre proportions with increasing age may be related to the muscle studied. In most reports of age-related changes in the horse, the middle gluteal muscle is examined. As this muscle is primarily involved in propulsion, demand for slow contracting Type I fibres may be less than in lower limb muscles. Consequently age-related changes in fibre type proportions may not be as readily apparent in this muscle. In addition, the extensive variation in fibre proportions which occurs in the middle gluteal muscle could result in small changes which may occur as a result of aging, being overshadowed by sampling variables.

In this present study a higher percentage of Type I fibres in older compared to younger horses was found in all muscles examined, except the short digital extensor. This increase may be related to changes in postural and physical demands as the horse matures, and a greater requirement for tonically contracting, slow Type I fibres. It would appear from this study that most of these changes are completed before a horse reaches two years of age. The exception to this increase in Type I fibre percentage with age was the short digital extensor muscle, in which there was a slight decrease in older horses, although the

reasons for this are unclear. It may be related to the very high percentage of Type I fibres in younger horses, as evidenced in the 12-week-old foal in this study, and the natural increase in activity which occurs shortly after birth in horses, resulting in a requirement for a higher proportion of Type II fibres in the muscle. This change may also be related to the insertion of the short digital extensor onto the tendons of the long and lateral digital extensor muscles, both of which contain a high proportion of Type II fibres.

(iv) Variation between horses

The lack of a significant difference in the percentage of fibre types in each muscle between groups might suggest that calculation of fibre type proportion is of little value in determining the presence of subtle muscular disease in the horse. Despite this, however, examination of fibre type proportions of individual horses within the clinical group, revealed a much greater range of values in some muscles, particularly the deep digital flexor muscle, than in control horses. As a consequence, extremely high or low percentages of Type I fibres, falling outside the range considered normal for that muscle and site, which is generally calculated as the mean plus and minus two standard deviations, may be of use in detecting muscle disease in the absence of other morphological alterations.

The change in fibre proportions in such cases could result from a number of disease processes as similar variations in fibre type proportions, particularly after deinnervation and reinnervation, have been documented in man (Telerman-Toppet et al, 1985) and the horse (Andrews et al, 1986; Roneus and Essen-Gustavsson, 1986; Van den Hoven et al, 1988). In the report of Van den Hoven et al, (1988), Type I fibre predominance was identified in the trapezius, serratus ventralis and brachiocephalicus muscles, in

horses suspected of having neuromuscular disease. The lack of information regarding the normal range of fibre proportions in these muscles makes interpretation of this finding difficult, and reinforces that muscle biopsies are of most value when taken from muscles of known composition. If such techniques are to be used extensively in the horse, all muscles accessible to biopsy should be evaluated in normal animals.

The greater percentage of Type I fibres found in the two ponies examined probably reflects differences in activity of these animals compared to thoroughbreds which have been selected for speed over hundreds of years. This finding is in agreement with the reports of Lindholm and Piehl (1974), Stull and Albert (1981), Taylor and Brassard (1981), and Hodgson _et al_ (1986), who also found that breed had a similar effect on the percentage of Type I fibres.

(b) Muscle fibre diameters - Atrophy and Hypertrophy factors, and Histographic analysis

Measurement of muscle fibre diameter and the histographic analysis of results has proved useful in identifying the presence of neuromuscular diseases in man, often revealing subtle variation in fibre size prior to morphological indications of disease (Brooke and Engel, 1969b; Jennekens _et al_, 1971a). However, in this present study, the mean fibre diameter for a group of horses was often of little value in detecting the presence of disease. In contrast, the mean fibre diameter in individual horses for both Type I or Type II fibres was, on occasions, found to be outside the range calculated as normal. In such cases, the calculation of minimum fibre diameter may be of value in detecting muscle disease.

In only one other report to date have fibre diameters of the lower hindlimb muscles been used to detect neuromuscular disease in the horse (Andrews and Spurgeon, 1986). In that report the minimum

diameters for Type I (54 um) and II (65 um) fibres for the long digital extensor muscle were found to be greater than in this present study (44 and 52 um respectively). This difference may have arisen because of differences in measurement techniques used in each study.

Of greater use than mean fibre diameter is the analysis of the distribution of fibre sizes. This can be quantified by the calculation of atrophy and hypertrophy factors, and by histographic representation of fibre diameter distribution. Atrophy and hypertrophy factors were initially described by Brooke and Engel (1969a,b), while the first reports of its use in the horse appear to be that of Andrews and Spurgeon (1986) and Andrews et al (1986). In this present study atrophy and hypertrophy factors allowed the quantification and detection of fibre atrophy and hypertrophy, which was indicative of abnormalities in these muscles, even though the mean fibre diameter was within normal limits.

There have been few other reports in the veterinary literature of the use of fibre diameter distribution histograms in the evaluation of neuromuscular disease in the horse. Duncan and Griffiths (1973) used a similar technique, although measuring fibre area, in evaluation of the laryngeal muscle in horses suffering from idiopathic laryngeal hemiplegia, while Andrews et al, (1986) used fibre diameter distribution histograms to evaluate four horses with neuromuscular disease. In man attempts have been made to correlate changes in distribution histograms to specific diseases (Brook and Engel, 1969b; Reniers et al, 1970). In this present study the value of fibre diameter histograms was most evident in the long and lateral digital extensor muscles where, although there was little difference in morphological appearance between clinical and control

animals, there was a noticeable difference in the distribution histograms.

Therefore although measurement of fibre diameter, or area, is tedious to undertake, it can provide useful quantitative information regarding the effects of disease on muscle samples, which may be overlooked during morphological examination.

PART III - NERVES

INTRODUCTION

Normal neuroanatomy

For an understanding of the pathological changes in nerves which result in neuromuscular disease, some knowledge of normal neuroanatomy is required. For this reason a brief description of the peripheral nervous system as it relates to this topic is presented.

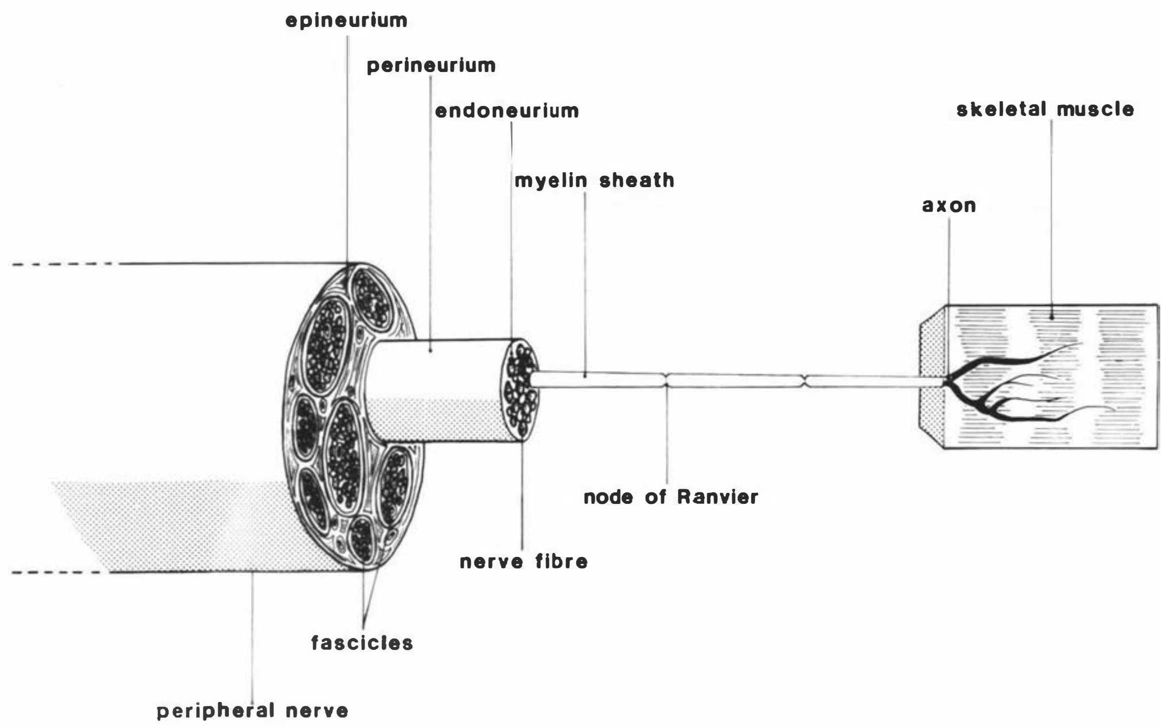
Peripheral nerves supplying skeletal muscle arise from cell bodies within the central nervous system. They communicate directly with muscle by way of an axon. Each axon is surrounded and supported by Schwann cells and, in myelinated axons, each Schwann cell surrounds a single axon (figs. 38A,B). In non-myelinated axons a single Schwann cell is associated with several axons and does not completely enclose each one. In normal nerves the thickness of the myelin sheath is proportional to the product of axonal circumference and internode length (Friede and Bischausen, 1982). Sequential Schwann cells covering myelinated nerve fibers meet at the nodes of Ranvier, which are complex histological structures with high metabolic requirements (Weller and Cervos-Navarro, 1977.) There is fluting of the myelin sheath in the paranodal area which results in bulbous expansion at this site (Williams and Kashef, 1968). At the nodal area there is a marked reduction in axon diameter which is more evident in large diameter fibers (Hess and Young, 1952).

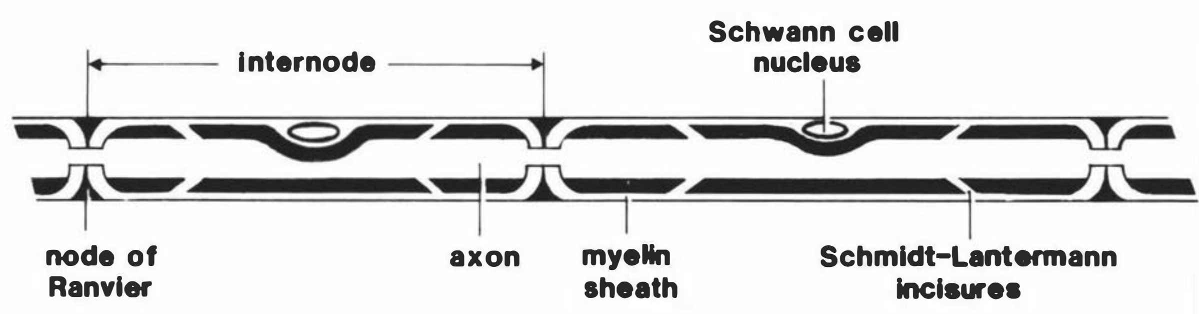
Groups of axons and Schwann cells, often numbering many hundred, are supported by connective tissue, known as the endoneurium, which also contains blood and lymphatic vessels (Fig. 38A). All of these are surrounded by a strong connective tissue sheath, the perineurium. Groups of axons thus formed are known as fascicles, which are bound together by the

Figure 38. Diagrams showing the normal structure of a nerve fibre.

A. A diagram showing a cross-section through a peripheral nerve and through supporting connective tissue showing a single fascicle. A single axon with its attachment to skeletal muscle is also shown.

B. A more magnified view of a single axon showing the internode distance and other structures associated with each myelinated axon.


epineurium
perineurium
endoneurium
myelin sheath
skeletal muscle
axon
node of Ranvier
nerve fibre
fascicles
peripheral nerve


internode
Schwann cell nucleus
node of Ranvier
axon
myelin sheath
Schmidt-Lantermann incisures

epineurium to form a nerve trunk.

Reaction of peripheral nerve to disease

Peripheral nerve, like muscle, reacts to a wide variety of pathological processes in a limited number of ways. These have recently been reviewed by Cahill (1985), and can be divided into three categories, namely Wallerian degeneration, axonal degeneration and segmental demyelination. This classification is dependent upon whether there is primary involvement of the axon, as in the former two, or the myelin sheath, as in the latter. Frequently a combination of these processes will be present at any one time (Webster et al, 1967; Dyck, 1973, 1975).

(i) Wallerian degeneration

Wallerian degeneration describes those changes that occur in the distal portion of a nerve following severe damage, such as transection or direct trauma, to a more proximal part of the nerve. All nerve fibers may be affected. The changes found in this type of nerve damage include an alteration in shape and fragmentation of the axon and paranodal retraction of myelin. The axons and myelin sheath break down and are progressively digested into rows of smaller ovoids and lipid droplets (Blackwood et al, 1971).

(ii) Axonal degeneration

Although axonal degeneration can result from a wide range of factors, including chemical intoxications, vitamin deficiencies and genetic abnormalities, most are thought to result in damage to the nerve because of an effect on the metabolic function. Although the entire axon is thought to be affected, histological changes are most obvious in the distal portions (Bennington, 1978). In contrast to Wallerian degeneration, axonal degeneration is most often indicative of an abnormality of the individual axon,

and as such, selective involvement of certain populations of fibers may occur. Linear rows of myelin ovoids and balls may be seen distally, while at more proximal levels, where the axon is still intact, myelin sheath irregularities, such as focal thickening of the myelin sheath and paranodal demyelination, as well as segmental demyelination and remyelination, may be seen. As many primary axonal diseases are chronic in nature, repeated episodes of demyelination and remyelination result in the appearance of "onion bulbs". Fibre loss may also be evident, ranging from no to mild loss proximally to severe loss and fibre degeneration in more distal parts of the nerve (Bennington, 1978).

Those diseases where the ends of the longest nerve fibers are affected are known as distal axonopathies, and constitute the majority of diseases in which axonal degeneration occurs, although on less frequent occasions degeneration may be unassociated with axonal length (Sabin, 1986).

(iii) Segmental demyelination

Segmental demyelination describes the segmental loss of myelin with preservation of the axon. This may result from a primary myelin abnormality, or be secondary to an axonal abnormality (Dyck et al, 1971). Demyelination is first apparent histologically because of a widening of the node of Ranvier followed by disruption of the myelin sheath and subsequent fragmentation and phagocytosis of the myelin. This progressive loss of myelin may be restricted to the paranodal area or involve the entire internode (Dinn, 1970). Remyelination usually commences within a few days when the length of demyelination is less than 15µm and the existing Schwann cell will reconstitute the myelin sheath. If the length of demyelination is greater than 15µm or an entire internode has been demyelinated, one or several short internodes, each with its own Schwann

cell, will be formed (Bennington, 1978; Cavanagh and Jacobs, 1964).

Investigation of peripheral nerve disease in the horse

A number of techniques can be used to identify peripheral nerve disease. These include light and electron microscopic examination of transverse, longitudinal or teased fibre samples, motor and sensory nerve conduction studies, electromyography, biochemistry, histochemistry, autoradiography, and tissue culture (Dyck, 1975). In this study light microscopic examination of transverse sections and of teased fibre samples of nerve were used to assess the degree of damage present in nerves. Some of the changes frequently identified using these techniques are discussed below.

(i) Common changes in transverse sections of nerves

(a) Onion bulbs

Onion bulbs result from the proliferation of Schwann cells in response to degeneration or demyelination of nerve fibers. Repeated insults to the nerve result in waves of proliferation and subsequent production by the Schwann cell of multiple interleaving lamellar processes which encircle the axon. They are usually, but not always, seen surrounding thinly myelinated fibers (Webster et al, 1967).

Onion bulbs are direct evidence of chronic neurogenic disease due to repeated demyelination and remyelination, either in response to an external insult or as an inherent defect in the Schwann cell (Weller et al, 1983). They are most readily identified in transverse sections of nerve fibers.

(b) Thinly myelinated fibers

Relatively thin myelin sheaths in relation to axon diameter are a useful indicator of remyelination following demyelination or regeneration of an axon (Schroder, 1972). They occur as a result of regenerated nerves which have internodes of smaller length and diameter and a decreased number of lamellae in relation to the axon perimeter (Dyck, 1975; Weller and Das Gupta, 1968). Relatively thinly myelinated fibers may also result from axonal swelling.

(c) Thick myelin sheaths

In regenerating nerves a proportion of axons have inappropriately thick myelin sheaths. These are thought to represent regenerating axons that have failed to reach their end organs and are undergoing axonal atrophy (Schroder, 1972).

(d) Regenerating clusters

Regenerating clusters are found following Wallerian or axonal degeneration. Several small axons sprout from the end of a previously damaged axon and remain closely associated as they progress distally. They appear histologically as discrete groups of three or more myelinated fibers (Madrid et al, 1977). Regenerating fibers often have disproportionately thin myelin sheaths and are surrounded by abundant Schwann cell cytoplasm. They are found in a number of neuropathies, especially if there have been repetitive insults to the nerve (Weller and Cervos-Navarros, 1977).

(e) Axonal degeneration

Evidence of active axonal degeneration, while most readily observed in teased nerve fibers, may occasionally be seen in transverse sections. Evidence includes the presence of myelin ovoids or balls in the Schwann cell cytoplasm, and the more frequent presence of nuclei as a result of an

increased number of Schwann cells, macrophages, fibroblasts and mast cells. Occasionally a myelin sheath may be seen with no central axoplasm (Dyck, 1975).

In transverse sections, accurate interpretation of the abnormalities requires electron-microscopic examination.

(f) Decreased fibre density

If regeneration does not take place after axonal degeneration, fibre loss will be evident. This loss may be obvious under light microscopy in severe cases or be only slight and require detection by morphometric analysis. An assessment of the degree of fibre loss at different sites of a nerve can be used as an indicator of the type and severity of the disease process. Similarly morphometric studies on cross-sections of axons will enable identification of selective damage to fibers of a particular size (Cahill 1985). However, interpretation of this requires prior knowledge of the nerve fibre size and density at the site examined.

(g) Renaut bodies

Renaut bodies are "loosely textured, whorled cell-sparse structures" (Asbury, 1973), which are found in the sub-perineural space in peripheral nerves. They are seen in both normal and pathological states, and are therefore probably relatively non-specific as indicators of neuropathology (Asbury, 1973). Some authors, however, have seen an increased incidence of Renaut bodies in pathogenic conditions (Jefferson et al, 1981). In man an increase in the frequency of these structures with age has been reported (Dyck, 1975). In the horse, Duncan et al (1978) considered Renaut bodies were more frequently observed in the recurrent laryngeal nerve of horses with idiopathic laryngeal hemiplegia, a finding not supported by Cahill (1985).

(ii) Common changes in teased fibres

(a) Myelin ovoids

Linear rows of myelin ovoids and balls are characteristic of Wallerian and axonal degeneration. Initial changes consist of irregular beading of the myelin sheath followed by division of the sheath into larger segments. As degradation continues this progresses to linear rows of myelin ovoids of varying sizes. Finally clumps of myelin lying in rows can be seen (Dyck et al, 1968). These changes may be used to assist in determining whether axonal degeneration or segmental demyelination is present (Cahill, 1985). The presence of small myelin balls is more indicative of segmental demyelination, while larger ovoids are seen more frequently with axonal degeneration. The distribution of degeneration is also slightly different, with damage along the entire myelin sheath seen in axonal degeneration, while more focal damage occurs with segmental demyelination (Madrid and Wisniewski, 1977).

(b) Paranodal demyelination

Widening of the nodal gap appears in early segmental demyelination, both primary and secondary. Myelin loss may be restricted to the paranodal region or spread to involve the entire internode (Dinn, 1970). The distribution of demyelinated regions is random in primary segmental demyelination but clustering on particular fibers will occur in secondary demyelination.

(c) Thick myelin sheaths

As in transverse sections, thick myelin sheaths may also be seen in teased fibre preparations, and are said to represent regenerating axons that are undergoing atrophy as a result of not reaching an end organ (Schroder, 1972).

Excessive variability in myelin thickness within internodes has been associated with peripheral nerve

disease (Dayan _et al_, 1967). Although the myelin thickness appears greater in these regions, it is probably a result of infolding and reduplication of myelin, rather than excessive production (Dyck, 1975).

(d) Thinly myelinated segments and short internodes

In normal nerve fibers the thickness of the myelin sheath and the internode distance, that is the distance between nodes of Ranvier, is relatively constant (Stevens _et al_, 1973). Thinly myelinated segments of nerves with decreased internode distance occurs when a large portion, or all of an internode is demyelinated as a result of disease. Remyelination occurs by the formation of several short internodes, each with its own Schwann cell (Bennington, 1978). Generally the myelin sheath appears proportionately thinner, but in some instances may almost return to normal thickness (Ludwin, 1981).

Axonal degeneration and subsequent regeneration results in uniformly short internodes over the length of the damaged axon (Hiscoe, 1947; Vizoso and Young, 1948). The presence of short internodes in teased nerve fibers is a permanent record of previous demyelination (Morgan-Hughes, 1968; Dinn, 1970).

Segmental demyelination and remyelination also result in decreased internode length, but with greater variability in the length of the intercalated segment (Vizoso and Young, 1948; Schroder, 1975). As a result of this an assessment of randomness of distribution can be used to assist in determining if axonal degeneration or segmental demyelination is present.

Diseases of equine peripheral nerve

A wide range of abnormalities are known to affect peripheral nerves in the horse. Many are the result of trauma or secondary to central nervous system dysfunction. In a small number of diseases, however, abnormalities which primarily affect peripheral nerves, have been documented. In order to further understand peripheral nerve disease in the horse, those diseases which have been examined using modern investigative techniques will be briefly discussed below.

Idiopathic laryngeal hemiplegia

The best studied of the equine peripheral nerve diseases is idiopathic laryngeal hemiplegia. As discussed previously, this disease, which is known to be a distal axonopathy, results in severe neurogenic atrophy of the laryngeal muscles, with the left side more severely affected than the right. It has been recently demonstrated (Cahill, 1985) that some abnormalities indicative of neuromuscular disease are also present in the distal hindlimb muscles and nerves of affected horses. The significance of these findings in terms of clinical disease is uncertain.

Stringhalt

Stringhalt is another disease which can be the result of a peripheral axonopathy (Friend and Jeffcott, 1985; Cahill et al, 1986). A toxin is considered the most likely cause of this disease, and a number of plants, including dandelion and mallow, having been incriminated (Pemberton and Caple, 1980; Cahill et al, 1986). Clinically the disease is characterized by exaggerated hyperflexion of the hindlimbs, but in severe cases the forelimbs may also be involved. Axonal degeneration of large myelinated nerve fibers with a proximal to distal increase in severity have been found in this disease (Cahill et al, 1986). Neurogenic atrophy of distal fore and hind limb muscles has been shown to occur. Many horses recover

spontaneously from the disease within 12 months of the onset of clinical signs.

Toxic neuropathies

Another recognized peripheral nerve disease in the horse is organophosphate toxicity. There have been two reports of this condition in the horse (Rose *et al*, 1981; Duncan and Brook, 1985). In each case the presenting clinical sign has been that of laryngeal dysfunction. When the laryngeal and hindlimb muscles and nerves of such cases have been examined (Duncan and Brook, 1985) abnormalities consistent with severe peripheral nerve disease and associated neurogenic muscular atrophy are evident. Clinical signs in the limbs associated with this condition have not been documented.

A further reference to possible toxins resulting in peripheral neuropathies is made by Robertson-Smith *et al.*, (1985) who examined 12 horses. No specific toxin could be identified, although a diagnosis of peripheral neuropathy was made in four animals. The *semimembranosis*, *semitendinosis*, *pectineus* and *biceps femoris* muscles contained moderate neurogenic atrophy. This consisted of bundles of atrophic fibers, and prominent sarcolemnal nuclei surrounded or interspersed with normal or hypertrophic fibers. More severe neurogenic atrophy was present in the *gastrocnemius* and long and lateral digital extensor muscles.

Suprascapular neuropathy

A further commonly recognized disease of peripheral nerves is suprascapular paralysis, which occurs because of damage to the suprascapular nerve resulting in atrophy of the *supraspinatous* and *infraspinatous* muscles of the shoulder. This results in prominence of the spine of the scapula, and is often referred to as "sweeney". The condition has recently been investigated in detail by Duncan and

Schneider (1985) who considered it to result from pressure on the nerve as it runs over the border of the scapula. Histologically there is good evidence to support the finding that this disease is a compression neuropathy. Treatment by resection of a block of bone directly under the course of the nerve, which supposedly releases pressure on the nerve, was reported to significantly improve recovery rates (Adams et al, 1985).

MATERIALS AND METHODS

The horses used in this section were the same as those described in Part II of this thesis. Division into clinical, subclinical or control groups (Table 1) was based on the same criteria as previously described.

Collection of nerve samples

The left recurrent laryngeal nerves were sampled at two sites (fig. 4)

(i) immediately caudal to the larynx (distal sample);

(ii) at approximately the mid-cervical level (proximal sample).

Nerve samples were collected from six sites in both the left and right hind leg (fig. 5). These sites were:

(i) the common peroneal nerve immediately prior to its bifurcation into deep and superficial branches;

(ii) the deep peroneal nerve approximately 6-8 cm after its bifurcation from the common peroneal;

(iii) the deep peroneal nerve immediately proximal to the hock joint;

(iv) the tibial nerve immediately proximal to

the hock joint;

(v) the medial plantar nerve in the mid-metatarsal region;

(vi) the medial plantar digital nerve distal to the fetlock joint.

Careful dissection of nerves was undertaken to minimize the chance of producing artefactual damage.

Following isolation of the nerve to be sampled, suture material was placed through the proximal end and a 200 gm hooked weight attached to the distal end. Gentle tension was applied to the sample as it was cut free with sharp scissors. The weighted nerve was then immediately suspended in 2% glutaraldehyde[1] (Dyck and Lofgren, 1966). For those samples from which teased fibers were to be taken, the nerve was removed after 10 minutes of fixation and divided into two parts, one section to be used for light microscopic examination and the other for teased fibre preparation.

The techniques used for processing of nerve samples have been adapted from Cahill (1985), Katrak *et al* (1980), and Nukada *et al* (1981). The composition of solutions and embedding resin used during processing are shown in Appendices 2 and 3.

Preparation and examination of transverse sections

Sections of nerve to be used for light microscopic examination was fixed for a total of 90 minutes in 2% glutaraldehyde and then transferred to 0.1M cacodylate buffer. The samples remained in the buffer for between 30 minutes and 12 hours. During this period the nerve was trimmed into smaller sections for further processing and embedding. This involved placing the nerve in a pool of buffer on a sheet of dental wax and examining it under a

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1. Appendix 2

dissecting microscope at a magnification of 40x. Connective tissue was removed and the nerve cut into sections using a fresh portion of a new razor blade for each transection. Three samples of each nerve were prepared. One contained a complete cross-section of the nerve and the other contained several fascicles of nerves only. The length of the samples was always kept greater than the width so that correct orientation could be maintained.

The nerves were then transferred from the buffer to 1% osmium tetroxide for 90 minutes and placed on a tilted revolving platform to facilitate mixing. Following this the nerves were washed in two changes of cacodylate buffer for 30 minutes.

The sections were then stained en bloc by immersing the samples in a freshly prepared 1% phenylenediamine solution for 30 minutes. Following staining, the samples were dehydrated in a series of graded alcohols, by placing each one for 10 minutes in 80% and 95% alcohol and then for two ten-minute periods in 100% alcohol. They were then immersed in propylene oxide[2] for two 10-minute periods. Following this they were immersed in a 50:50 mixture of propylene oxide and polarbed resin overnight. For this last procedure the sample bottles were placed in a fume hood, again on a revolving mixer, with their lids removed to allow evaporation of the propylene oxide. The following morning the nerve samples were placed in fresh sample bottles containing 100% polarbed resin for 60 minutes. The samples were then embedded with individual labels in polarbed resin in flat rubber moulds[3]. For each site of the nerve sampled the trimmed pieces of nerves were embedded

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2. Propylene oxide, BDH Chemicals Ltd., Poole, England

3. Silicone rubber mould, Polaron Equipment Ltd., Watford, England

longitudinally to ensure a transverse section of the nerve was available for cutting. The moulds were then placed in an oven in the fume hood at 60°C for polymerisation of the resin to occur. After 2 hours the orientation of the specimens was checked and corrected when necessary. The specimens were then left at 60°C for 48 hours to allow for hardening of the resin.

Following removal from the mould, "thick" sections of the nerve were cut[4] at 1.5 µm using a glass knife. They were then examined under the light microscope. Pathological abnormalities were graded subjectively, based on individual fibre pathology and loss of myelinated fibers (Cahill, 1985).

Individual fibre pathology was then graded according to the frequency of observing changes consisting of disproportionately thin myelin sheaths, regenerating clusters, "onion bulb" formations and myelinated fibers with myelin debris in their Schwann cell cytoplasm
assessed. These changes were graded on the basis of finding no abnormalities (zero grading): of observing these abnormalities only rarely (+, 1 or 2 in a transverse section of the entire nerve); occasionally (++, 1 or 2 changes per fascicle): frequently (+++, 5-10 per fascicle); and numerously (++++, more than 10 changes per fascicle).

Morphometric analysis was undertaken using computerized image analysis techniques. This involved representative areas of each nerve sample being photographed, and then analysed to determine the number of nerve fibers in each photograph, as well as the area, calculated in pixels, of each individual nerve fibre in the photograph. The

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4. LKB 8806A Ultratome 3, LKB, Bromma 1, Sweden

technique used to determine these values is illustrated in Fig. 39. From this data the density of nerve fibers (no. per square millimetre of nerve), mean nerve fibre area (um^2), and histographic distribution of the range of individual nerve fibre sizes, was determined. Results for each group were then analysed and compared statistically using the students' t-test and chi-squared analysis where appropriate.

Because of the variability in fibre density and size encountered in some limb nerves, particularly the common peroneal nerve and its deep branch, the total cross-sectional axonal area (Quilliam,1956) was determined in all hindlimb samples. This was calculated by multiplying the mean nerve fibre area by the nerve fibre density to obtain a value of total cross-sectional axonal area per square millimetre (um^2/mm^2) at each level of sampling.

Preparation and examination of individual teased fibers

Following the 10 minute fixation in 2% glutaraldehyde the samples were transferred to 0.1M cacodylate buffer for at least 30 minutes. During this period excessive connective tissue was trimmed away in a similar manner as previously discussed. The entire nerve was then placed in 1% osmium tetroxide for 90 minutes and rotated constantly during this time on a revolving platform in a fume hood. A further wash in cacodylate buffer for 30 minutes was then undertaken, with a change of buffer after 15 minutes to ensure elimination of all the osmium residue. The nerves were then placed in 66% glycerol at 60^oC overnight. The next day the samples were placed in 100% glycerol and stored in a refrigerator until required.

Teasing of nerve fibers was carried out on a glass microscope slide using a dissecting microscope at a

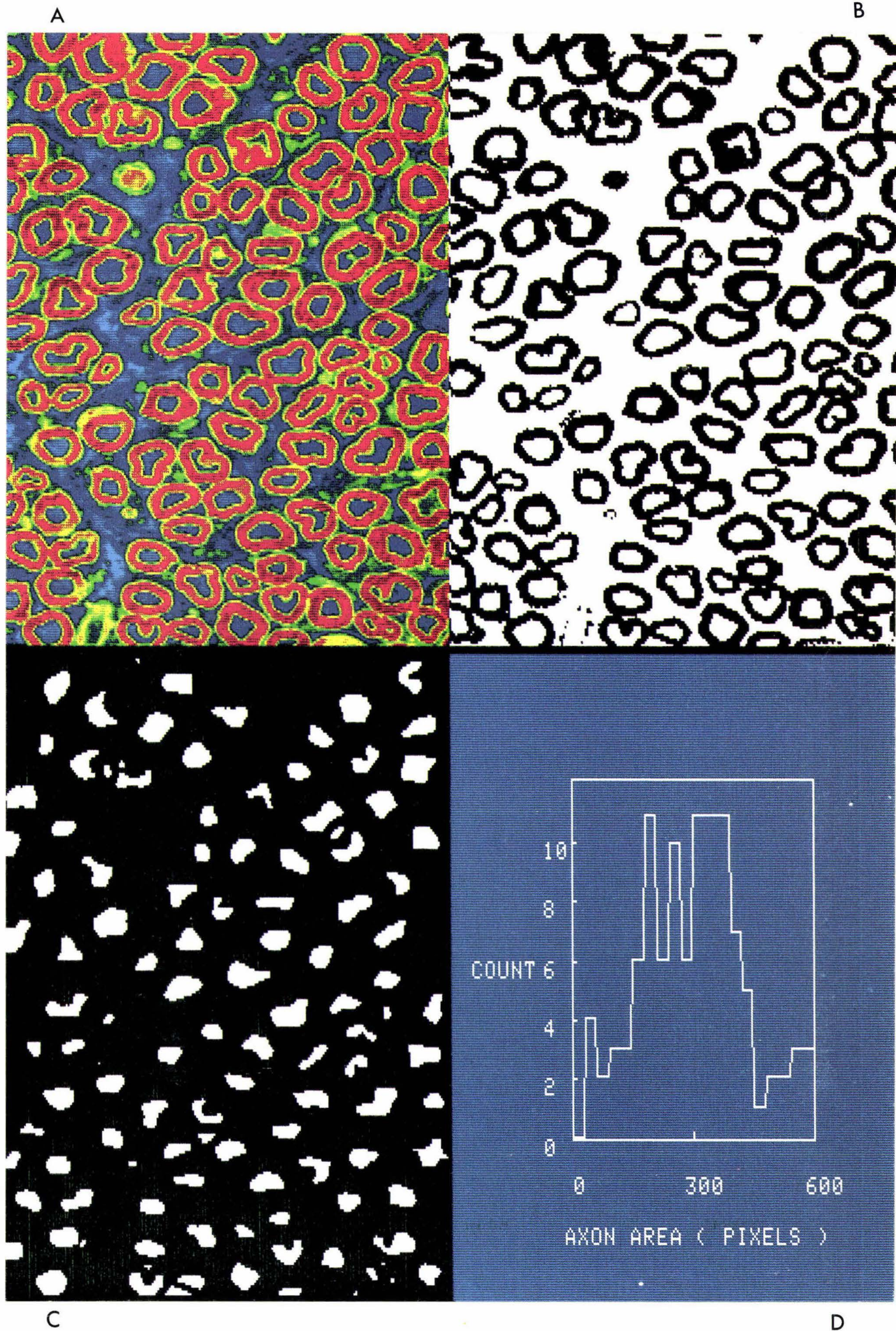
Figure 39. Some of the steps involved in determining fibre density and mean cross-sectional axonal area of nerve samples using image analysis.

A. Computer representation of a photomicrograph of a nerve sample, in this case the recurrent laryngeal nerve in a control horse.

B. The image is converted to black and white to provide a sharp contrast between the myelin sheath, which is black, and each axon.

C. The area outside each nerve fibre is then filled in black as well, so that the only remaining white areas represent individual axons. Each axon is then individually numbered and the area measured (not shown here). Manual checking of each area was then undertaken to ensure structures other than axons, such as blood vessels and enclosed areas between axons, were excluded from the final histogram.

D. The cross-sectional area of each individual axon, as measured in pixels, was converted to square micrometres and transferred to a Prime computer system to enable histographic and statistical analysis.


A
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COUNT 6
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600
AXON AREA (PIXELS)

magnification of 40x (fig. 40). Fine forceps[5] (with sharp points), and a fine pointed needle were used for dissection. The length of nerve was placed in a drop of glycerol and the epineurium removed, thus exposing the nerve fibers and endoneurium. The fascicles were separated into small groups of fibers and repeatedly divided until individual fibers were isolated. Ten fibers with at least five nodes of Ranvier were transferred to a glass slide and laid side by side for grading and measuring. (A total of 100 teased fibers randomly taken from each sample was collected). To dry off excess glycerine, the slides were kept at 60°C for 48 hours and then mounted in glycerine jelly.

Each fibre preparation was then examined under the light microscope and graded according to Dyck (1975) (fig.41), and the mean internode length calculated. Because of the extensive amount of time required to prepare teased fibers from nerves only five horses were examined in this way.

---

5. Number 7 forceps, 4.5 inch, curved, fine pointed. Dumoxel A. Dumont & Sons

Figure 40. Technique for obtaining single teased nerve fibres (after Dyck, 1975).

A. The nerve is placed on a microscope slide in a drop of glycerine and placed under a dissecting microscope.

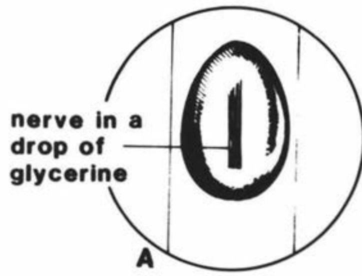
B. Using fine forceps the nerve is divided into individual fascicles.

C. Epineurial collagen and the perineurium is then carefully removed.

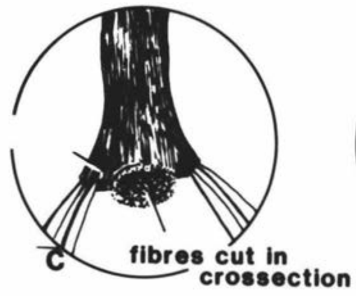
D. The fascicles are then repeatedly divided in half to obtain single fibres.

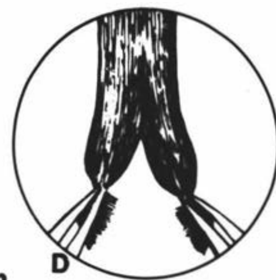
E. Single fibres are then transferred to a clean microscope slide.

F. After groups of 10 fibres are collected on the slide, a coverslip is placed on them. A total of 100 teased fibres is obtained for each nerve sample.


nerve in a
drop of
glycerine
A

separating
fascides
B


C
fibres cut in
crossection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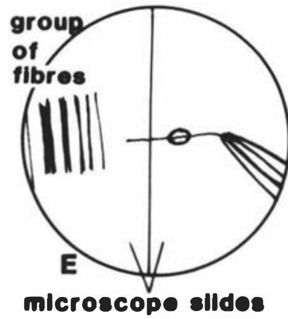

group
of
fibres
E
microscope slides

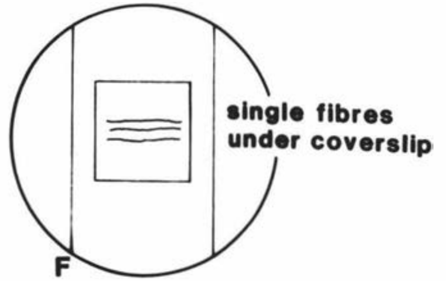

single fibres
under coverslip
F

Figure **41**. System of classification of individual teased fibres (after Dyck, 1975, as modified by Cahill, 1985).

A. A fibre of normal appearance

B. A fibre with irregularity of the myelin sheath, but otherwise normal

C. A fibre with segmental demyelination

D. A fibre with segmental demyelination and remyelination

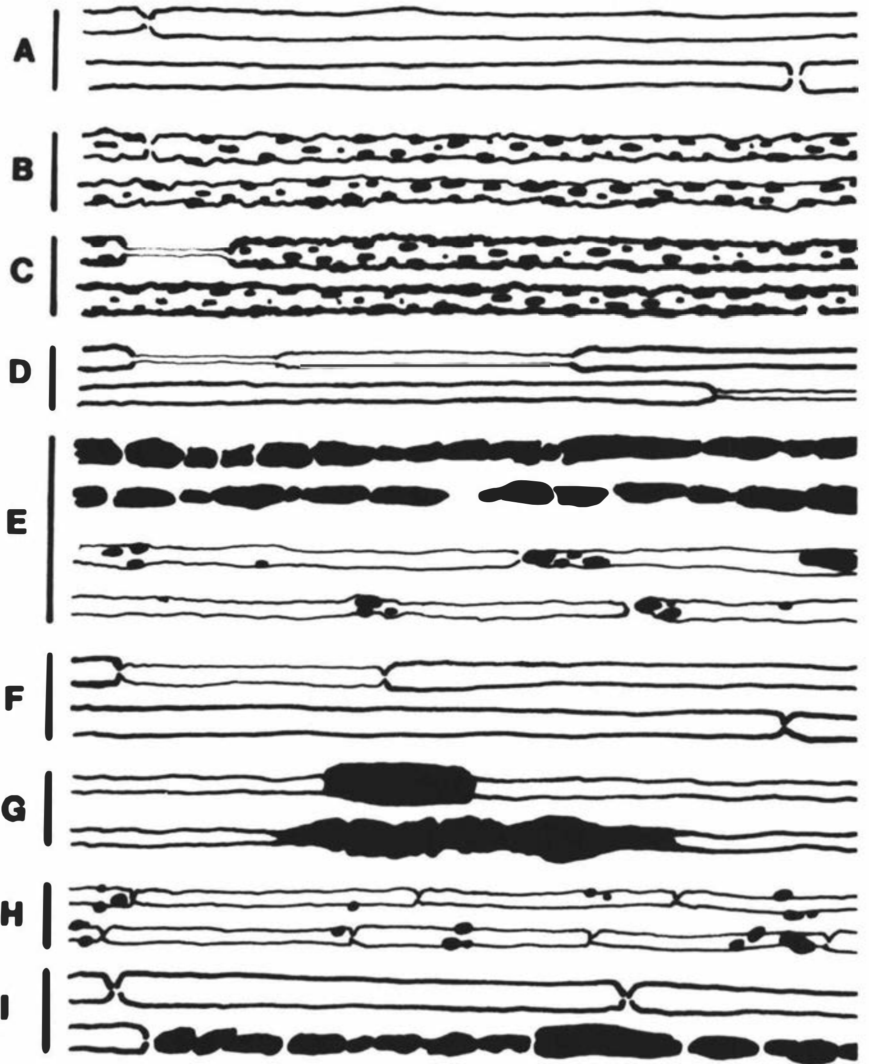
E. A fibre that has undergone degeneration into a linear row of myelin ovoids or balls

F. A fibre with remyelinated internodes which appear as short intercalated segments

G. A fibre with excessive irregularity of the myelin sheath which forms thickenings

H. A fibre with myelin ovoids or balls adjacent to it.

I. A fibre with linear rows of myelin ovoids and balls distally, with or without paranodal demyelination and remyelination on proximal internodes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RESULTS

Individual nerve fibre pathology

The estimated frequency of nerve fibre pathology for each individual horse is presented in Appendix 10. The incidence of individual nerve fibre changes, and the average degree of changes at each sampling site in all horses, is shown in Table 10.

Left recurrent laryngeal nerve

There was little evidence of individual nerve pathology at this site in control horses, although rare changes, consisting of thinly myelinated fibres (fig. 42) or regenerating clusters, were occasionly seen. Abnormalities of nerve fibres were frequently seen in subclinical and clinical horses at both proximal and distal levels of this nerve (fig. 43). However, because of extensive nerve fibre loss at the distal level in the clinical horses, there were less individual nerve fibres present and consequently fibre damage was less evident (fig. 44).

Common peroneal nerve

There was only minimal evidence of individual nerve fibre abnormalities in this nerve (fig. 45A & B). When present, these changes were similar in all groups, and consisted mainly of thinly myelinated fibres (fig. 46B). There were two notable exceptions to this. One, a clinical animal (horse 15) in which occasional to frequent changes, including the presence of onion bulbs, thinly myelinated fibres and regenerating clusters, were present in both legs. The other exception was a pony (horse 18) in which occasional changes were observed in one leg. Unfortunately no sample was available from the contralateral leg in this animal.

Deep peroneal nerve, proximal sample

In the control and subclinical groups, 14 samples from a total of 22 samples examined were considered

Table 10. Incidence and average grading of individual nerve fibre changes at each level of sampling in the nerves in clinical, subclinical and control horses.

GROUPS OF HORSES

NERVE LEVEL	CLINICAL				SUBCLINICAL				CONTROL			
	Samples examined	Samples affected	% samples affected	Average grading	Samples examined	Samples affected	% samples affected	Average grading	Samples examined	Samples affected	% samples affected	Average grading
Common peroneal	13	5	38	0/+	6	4	67	0/+	15	5	33	+
Deep peroneal, proximal	13	11	85	++	7	2	29	0/+	15	6	40	+
Deep peroneal, distal	13	11	85	++	7	5	71	+	15	7	47	0/+
Tibial	13	8	62	+	7	0	0	0	15	8	53	0/+
Plantar	9	8	89	++	2	0	0	+	11	11	100	++
Plantar digital	13	13	100	++/+++	8	6	75	+	16	13	81	++

0 = no individual nerve fibre pathology
+ = rare
++ = occasional
+++ = frequent
++++ = numerous

to have no individual nerve fibre changes. The remaining samples had rare to occasional changes (figs. 47A,B,C).

Individual nerve fibre pathology was both much more frequent and more severe in clinical cases than subclinical and control horses at this level of the deep peroneal nerve. Only two nerve samples from a total of 13 examined in the clinical group were considered free of abnormalities, while the remaining samples had rare, occasional or frequent changes. Regenerating clusters, thinly myelinated fibres and onion bulbs (figs 48A,B,C) were often observed in this group.

Renaut bodies (fig. 48B) were seen more frequently in the two eldest clinical laryngeal hemiplegic horses examined when compared to the younger animals.

Deep peroneal nerve, distal sample

In over half the samples of this nerve from the control horses no abnormalities were observed (fig. 49). The remainder were graded as having rare changes, with the exception of one animal (horse 11) which had rare to occasional changes in one leg.

The frequency of individual fibre pathology was much greater in the subclinical (71% affected) and clinical (85% affected) groups (figs. 50A,B). As was found more proximally in this nerve, the degree of change was greater in clinical animals.

Tibial nerve

The incidence of individual nerve fibre pathology was, at this site, similar in the clinical and control groups. Although many samples contained no evidence of abnormalities (fig. 51),in over half, rare changes were observed. In addition, one clinical animal (horse 15) had occasional changes (fig. 52) in both samples, while one control horse (horse 13) had

frequent changes in one leg. In this latter horse onion bulbs, thinly myelinated fibres and regenerating clusters were frequently seen (fig. 53).

Plantar nerve

Almost all samples of this nerve had some individual nerve fibre changes present. These changes, which included regenerating clusters, degenerating nerve fibres, onion bulb formation, myelin ovoids in the Schwann cell cytoplasm (figs. 54A, 55, 56A,B) and thinly myelinated fibres, were graded as being occasional to frequent. There appeared to be little obvious difference in the degree of individual nerve fibre changes between clinical and control groups of horses. Insufficient samples from the subclinical horses were collected to enable accurate comparison with other groups.

Plantar digital nerve

As seen at the more proximal level of this nerve, most samples contained evidence of individual nerve fibre damage at this level. In control horses only three samples were considered free of pathological changes (figs, 57A,B), while in the remainder the abnormalities were graded from rare to frequent. Regenerating clusters, thinly myelinated fibres and onion bulbs were observed in samples from control horses (fig. 58). In the small number of subclinical horses only rare changes were seen.

The changes identified in samples from the clinical group, which included split myelin sheaths, onion bulbs, thinly myelinated fibres, regenerating clusters, and debris in the myelin sheath (figs. 59A,B,C) were slightly more severe than those found in the remaining horses. In one clinical horse there were few incidences of individual nerve fibre changes in some fascicles because of extensive fibre loss (figs. 60A,B,C), although in other fascicles frequent abnormalities were seen.

Figure 42. Photomicrograph of a transverse section of the proximal left recurrent laryngeal nerve in a control horse. There is minimal individual nerve fibre pathology, although a thinly myelinated fibre is present (arrow).

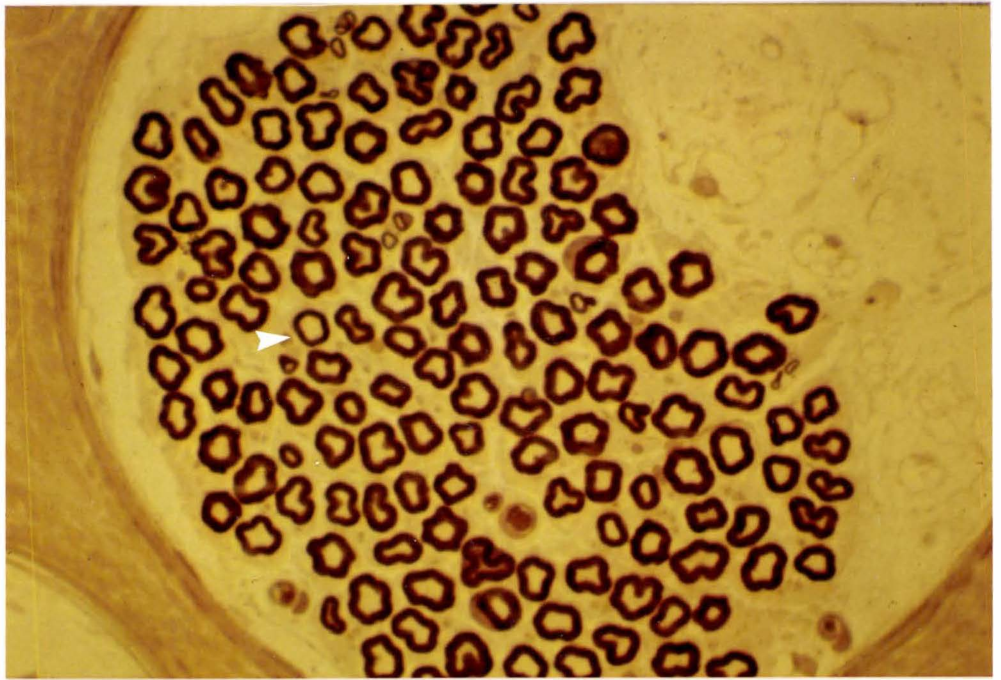
Phenylenediamine staining x 150

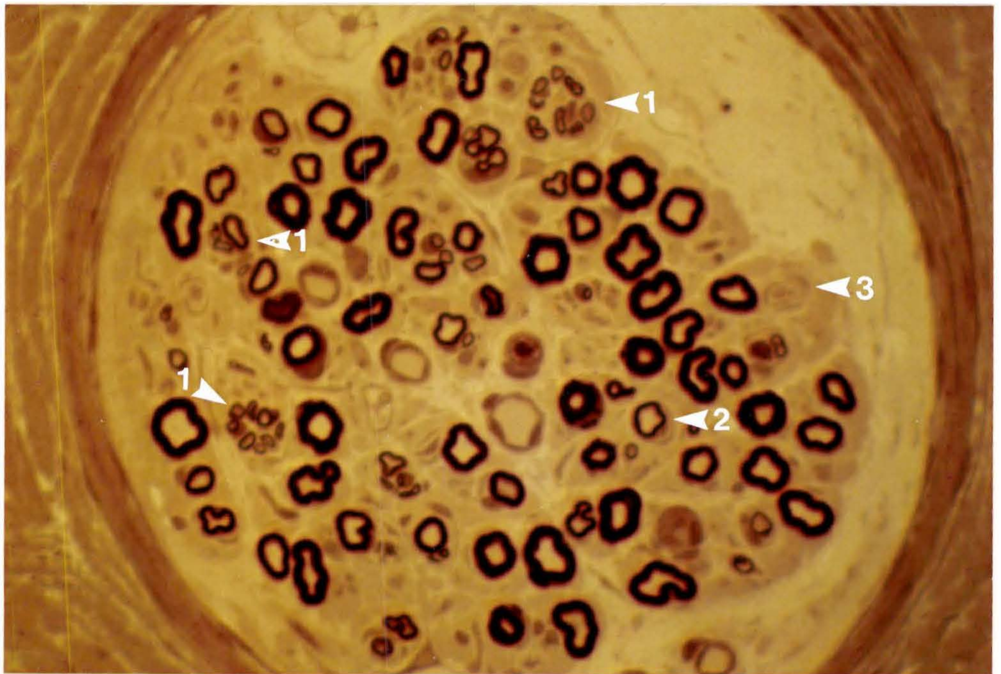
Figure 43. Photomicrograph of a transverse section of the proximal left recurrent laryngeal nerve in a clinical laryngeal hemiplegic horse showing numerous regenerating clusters(1), thinly myelinated fibres surrounded by onion bulbs(2), and an empty Schwann cell surrounded by an onion bulb(3). Note the decreased fibre density.

Phenylenediamine staining x 150

Figure 44. Photomicrograph of a transverse section of the distal left recurrent laryngeal nerve showing severe fibre loss in all fascicles. Renaut bodies are also frequently seen (arrow).

Phenylenediamine staining x 60

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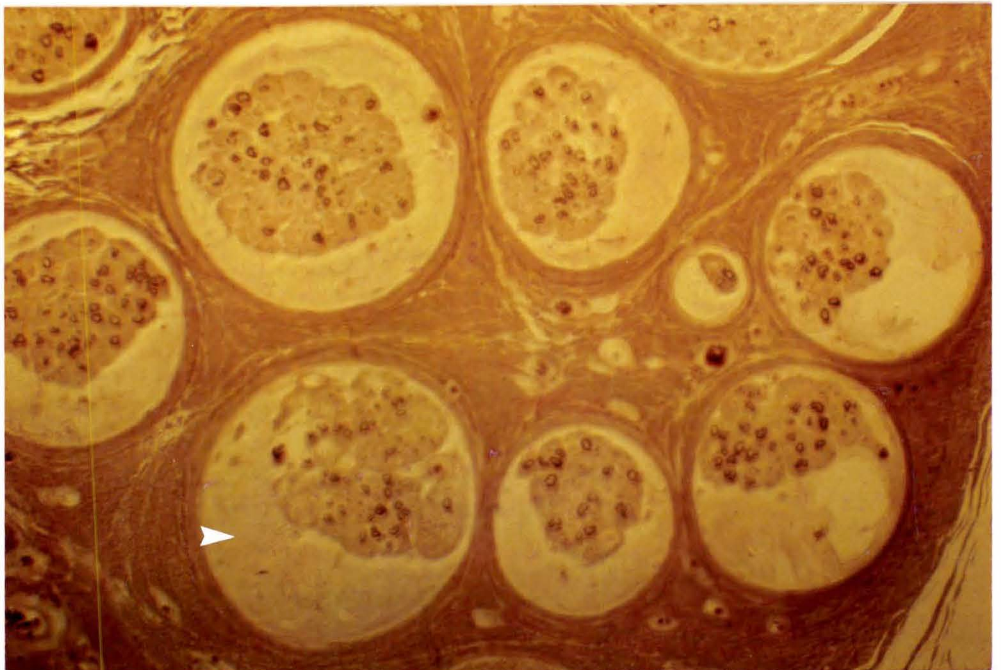


Figure 45. Photomicrographs of transverse sections of the common peroneal nerve in a control horse showing:

A. Few abnormalities of individual nerve fibres, although rare thinly myelinated fibres are evident (arrow).

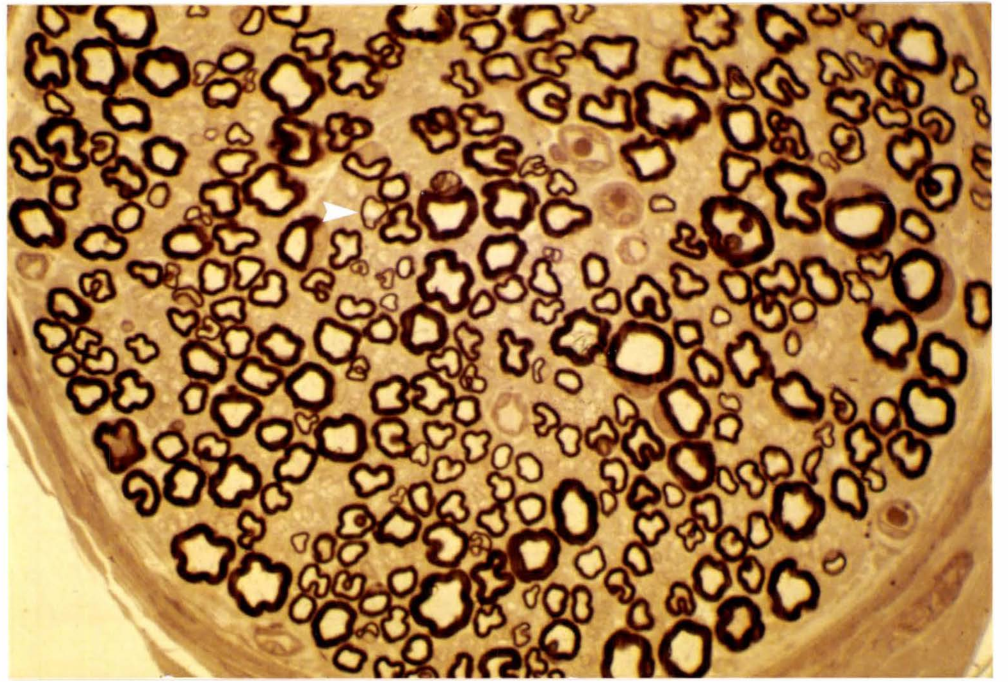
Phenylenediamine staining x 150

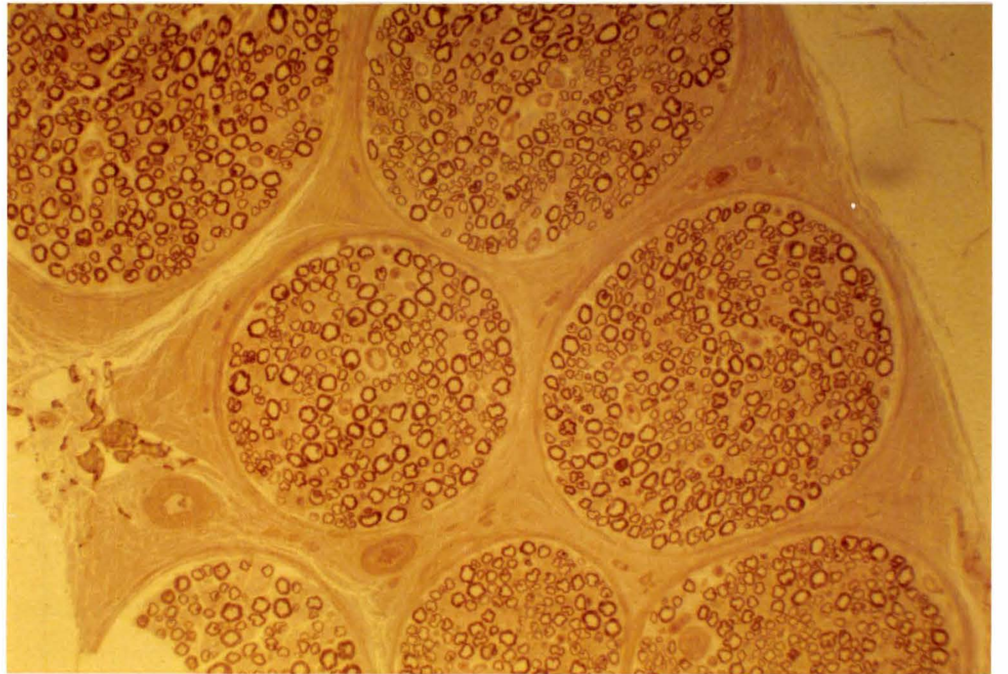
B. Normal fibre density in a number of fascicles, with little evidence of morphological abnormalities.

Phenylenediamine staining x 60

Figure 46. Photomicrograph of a transverse section of the common peroneal nerve in a subclinical laryngeal hemiplegic horse showing a thinly myelinated fibre and onion bulb formation (arrow).

Phenylenediamine staining x 150





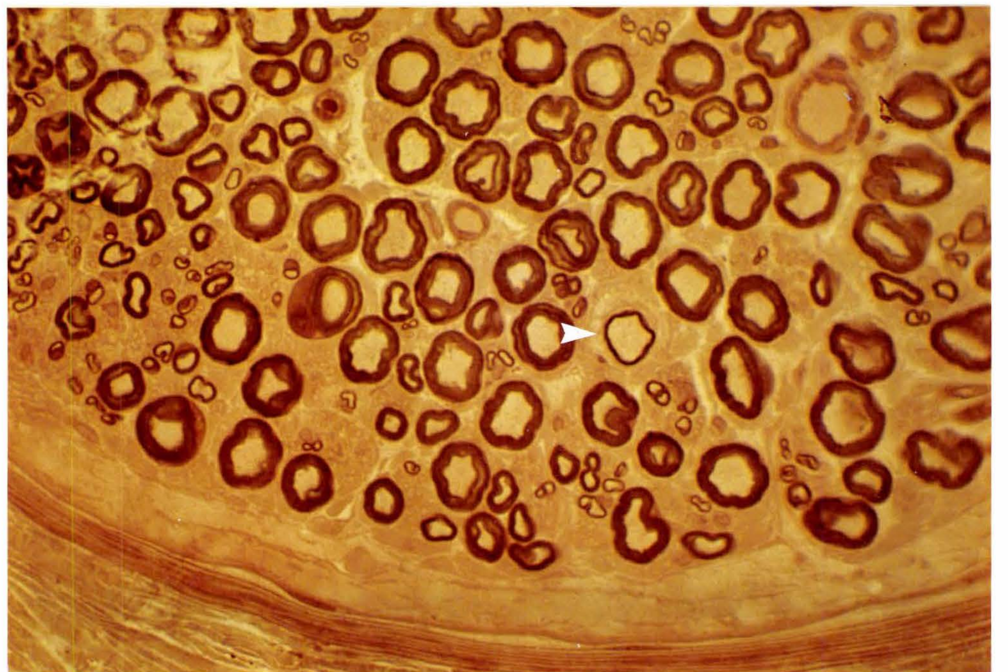


Figure 47. Photomicrographs of transverse sections of the proximal deep peroneal nerve in a control horse, showing the range of fibre populations seen in this nerve.

A. This fascicle was found to have a high fibre density, mainly as a result of an increased proportion of smaller fibres. Thinly myelinated fibres were seen only rarely (arrows).

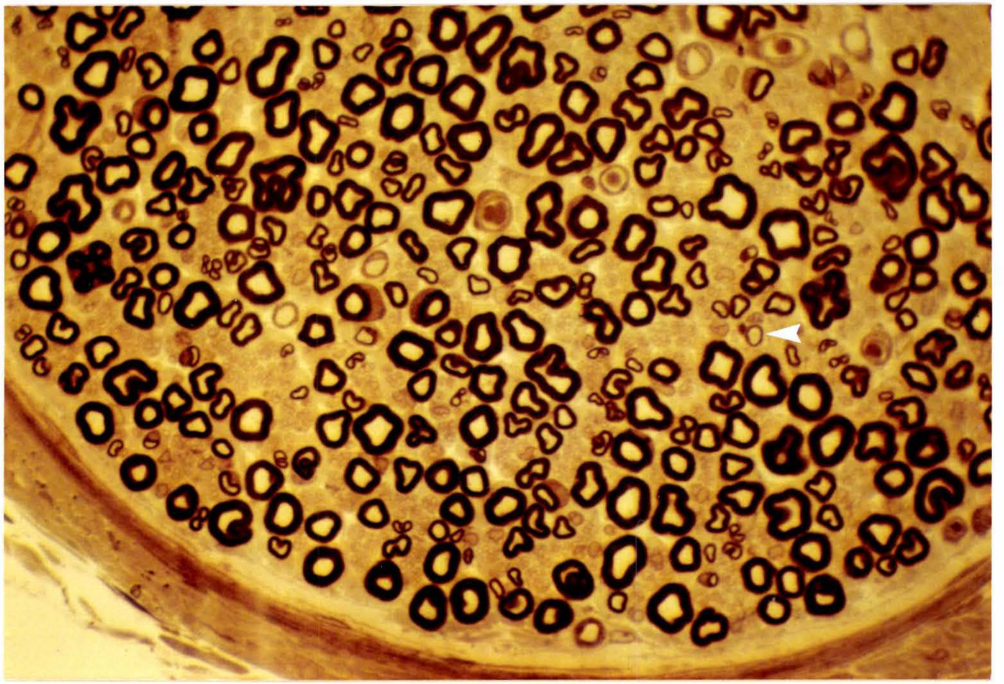
Phenylenediamine staining x 150

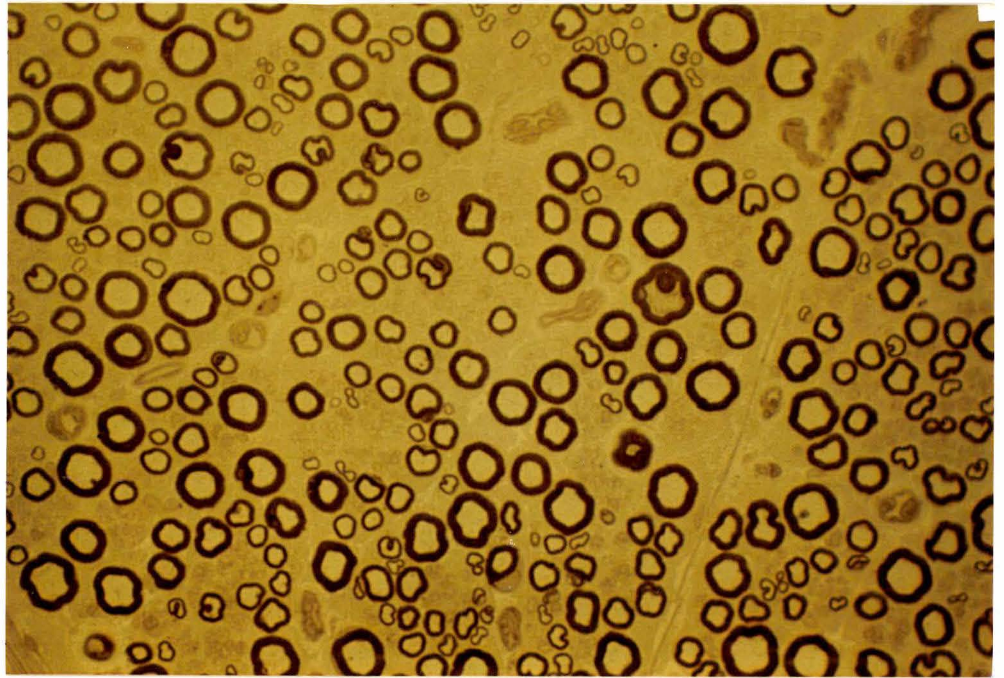
B. The density in this fascicle was less than in A as a result of an increased proportion of larger fibres. A number of fibres appear to have thin myelin sheaths, although in comparison to other fibres in the sample it was difficult to determine if they were inappropriately thin for that axon size.

Phenylenediamine staining x 150

C. Two different fibre populations are present within the same fascicle. Adjacent fascicles further illustrate the range of fibre populations in this nerve.

Phenylenediamine staining x 60





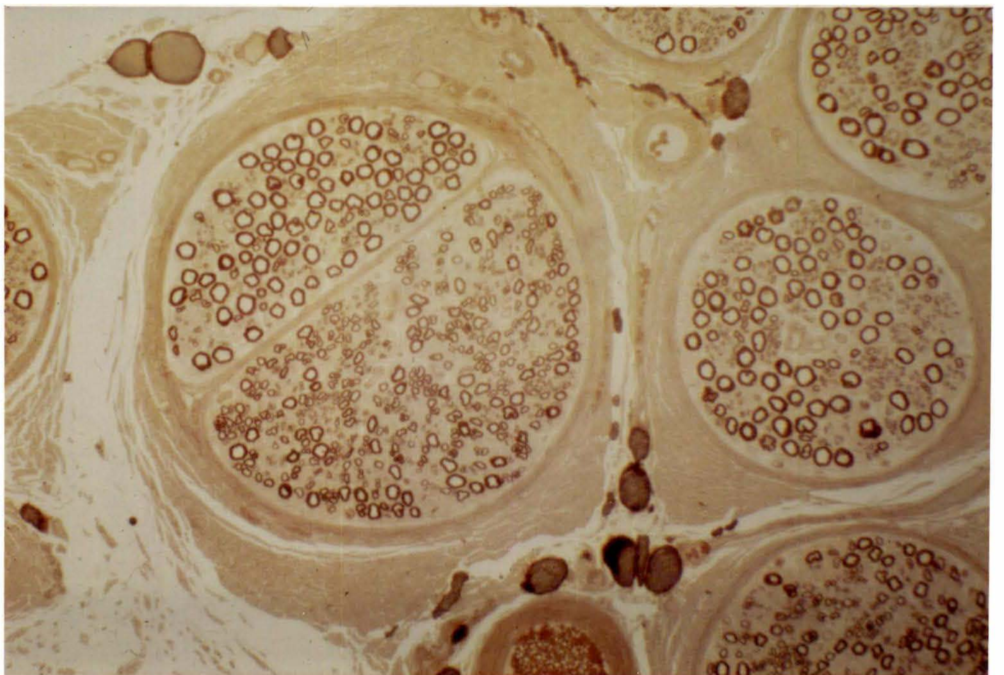


Figure 48. Photomicrographs of transverse sections of the proximal deep peroneal nerve in clinical laryngeal hemiplegic horses showing:

A. The frequent presence of regenerating clusters(1), inappropriately thin myelin sheaths(2), and nerve fibre loss.

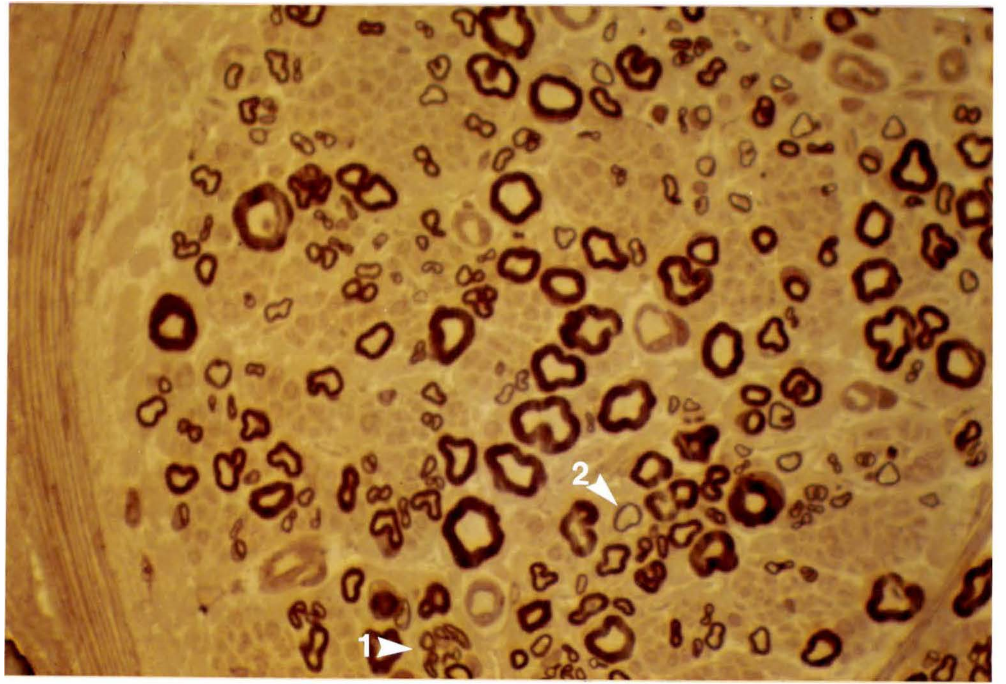
Phenylenediamine staining x 150

B. Renaut bodies (arrow) which were seen more frequently in the two eldest horses.

Phenylenediamine staining x 60

C. A thinly myelinated fibre and onion bulb formation (arrow).

Phenylenediamine staining x 400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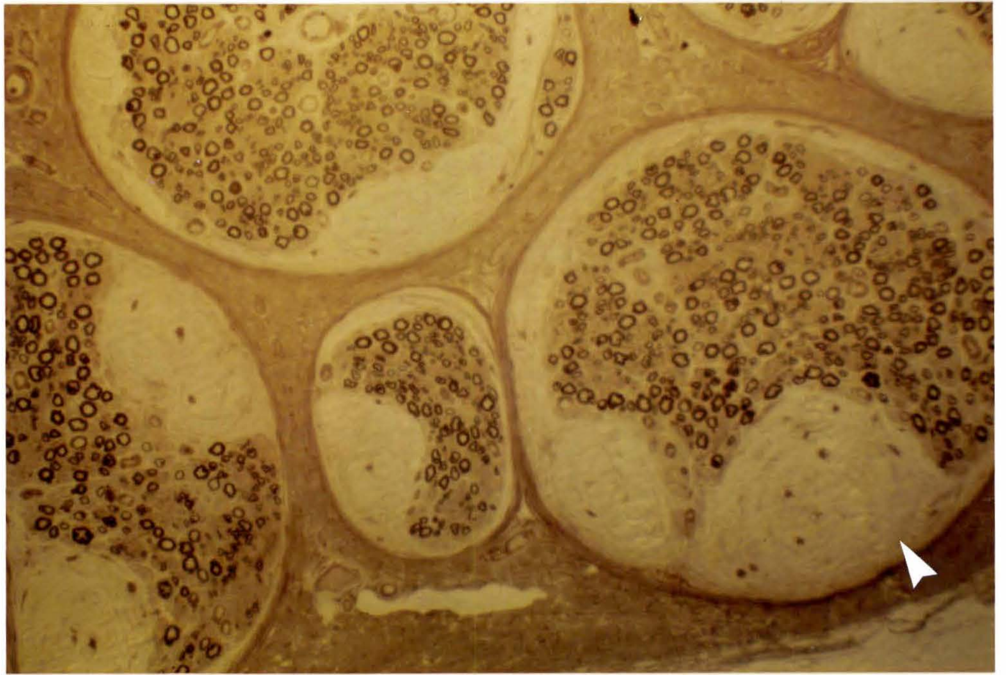


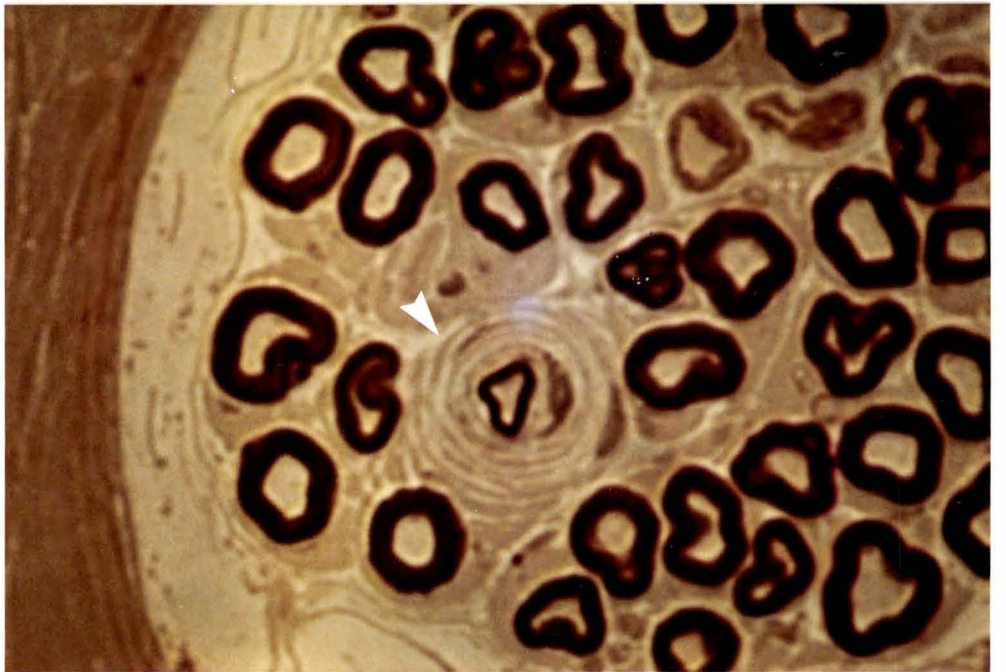


Figure 49. Photomicrograph of a transverse section of the distal deep peroneal nerve in a control horse, in which individual nerve fibre changes were not commonly observed.

Phenylenediamine staining x 150

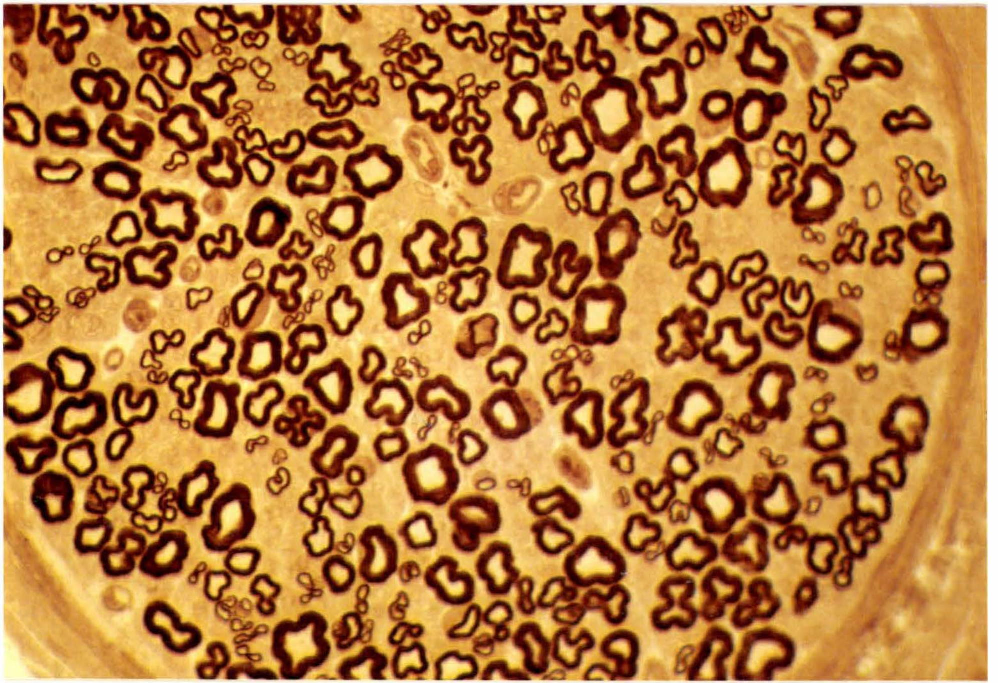
Figure 50. Photomicrogrphs of transverse sections of the distal deep peroneal nerve in a clinical laryngeal hemiplegic horse showing:-

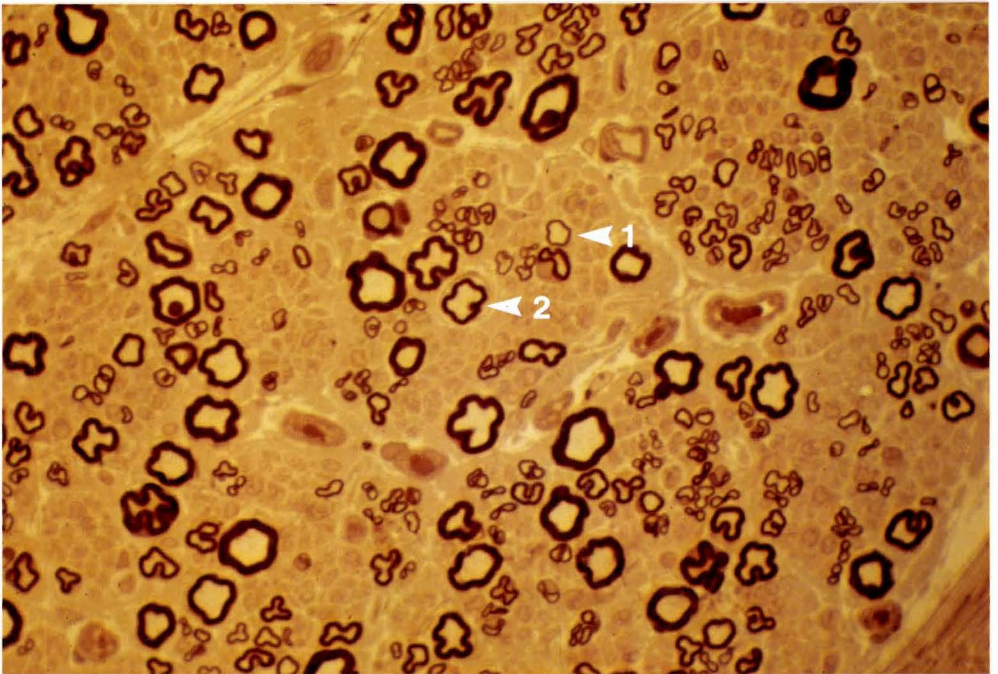
A. Thinly myelinated fibres (1), onion bulb formation around a thinly myelinated fibre(2), and fibre loss.

Phenylenediamine staining x 150

B. Fibre loss is evident in all fascicles

Phenylenediamine staining x 60

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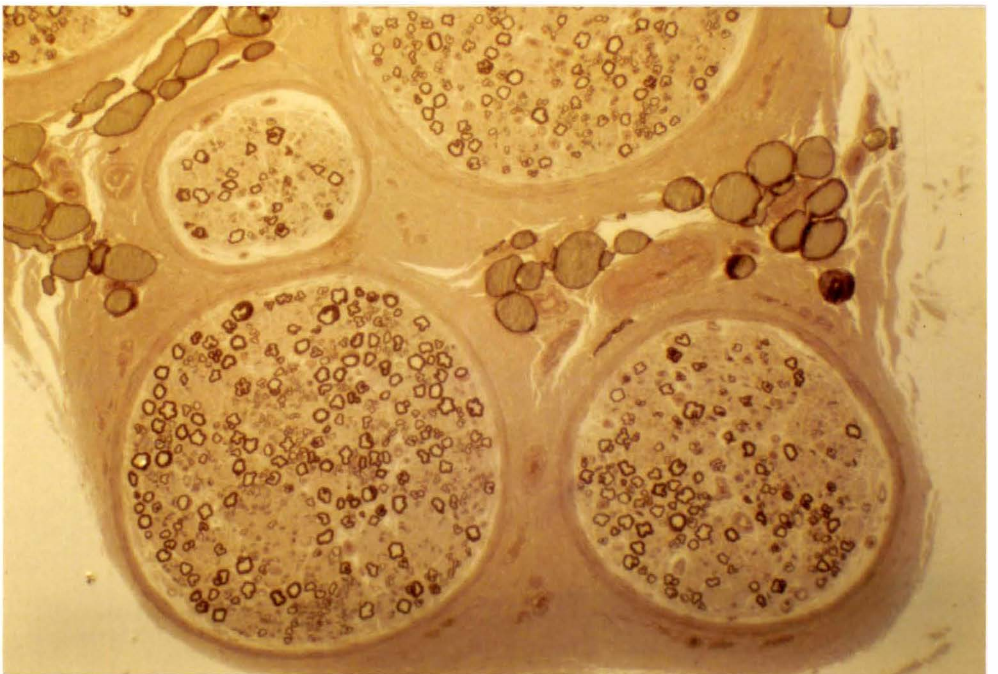


Figure 51. Photomicrograph of a transverse section of the tibial nerve in a control horse showing the normal morphological appearance and density at this level of sampling.

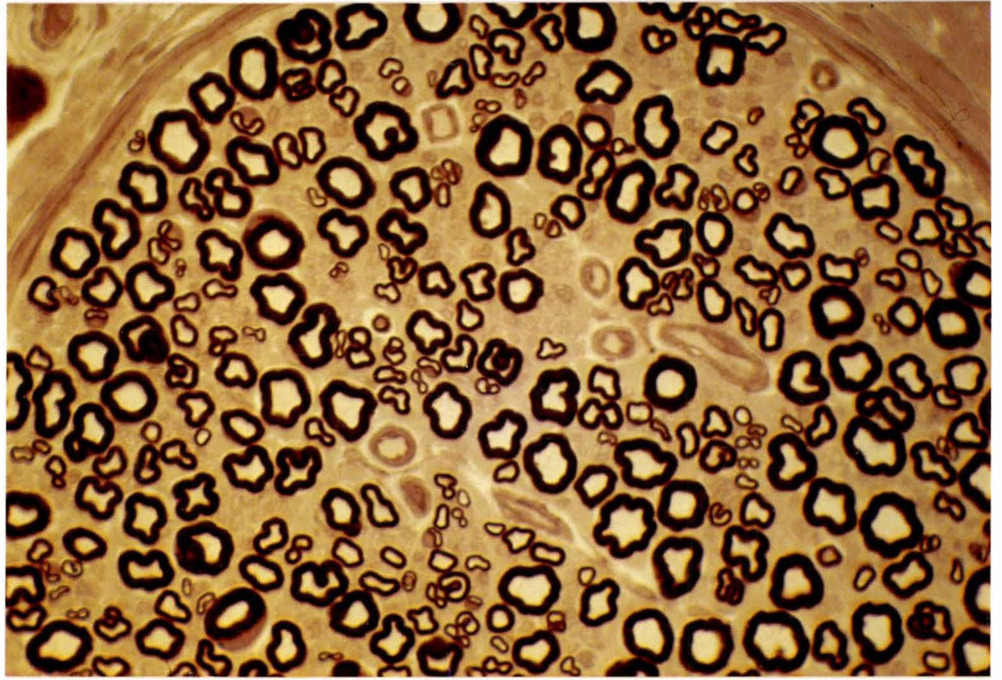
Phenylenediamine staining x 150

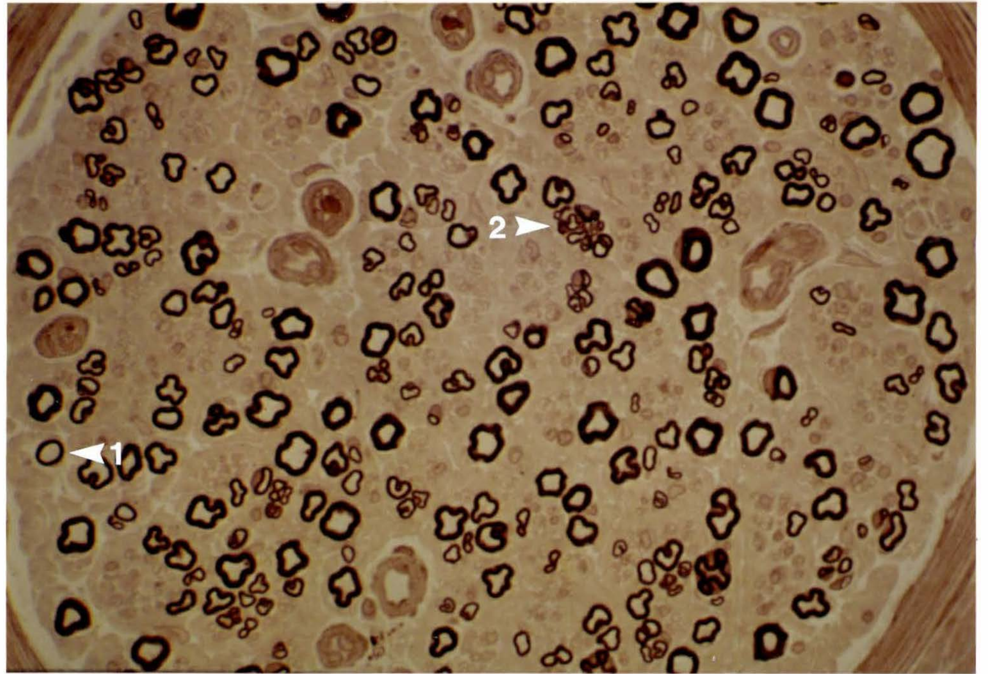
Figure 52. Photomicrograph of the tibial nerve in a clinical laryngeal hemiplegic horse showing occasional individual nerve fibre abnormalities, including the presence of a thinly myelinated fibre(1) and a regenerating cluster(2). Some fibre loss is also evident.

Phenylenediamine staining x 150

Figure 53. Photomicrograph of a transverse section of the tibial nerve in a control horse with frequent abnormalities, including onion bulb formation and thinly myelinated fibres(1), regenerating clusters(2), and fibre loss.

Phenylenediamine staining x 150

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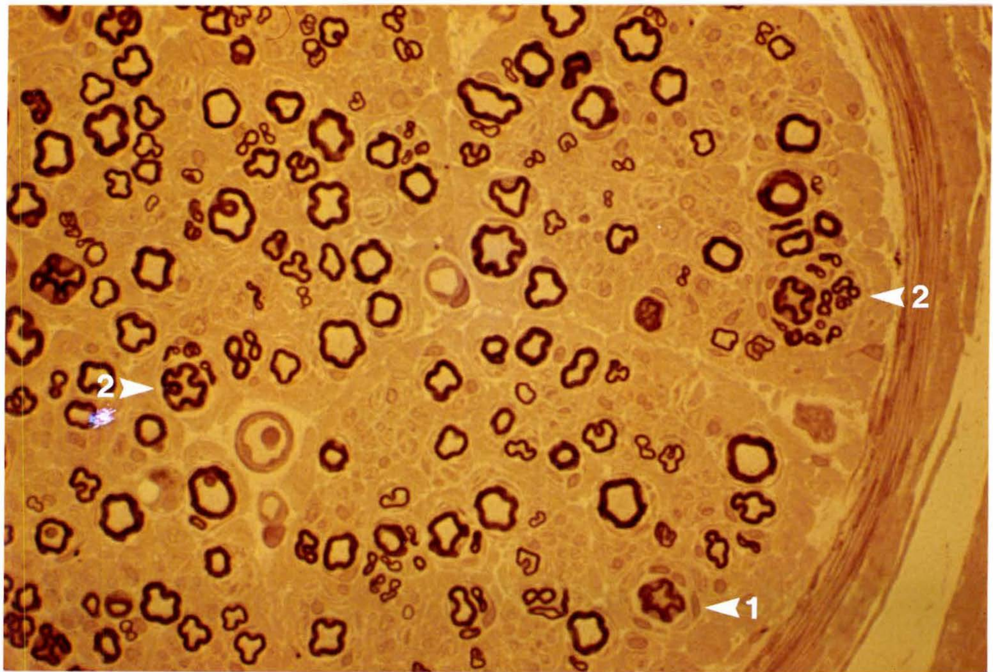
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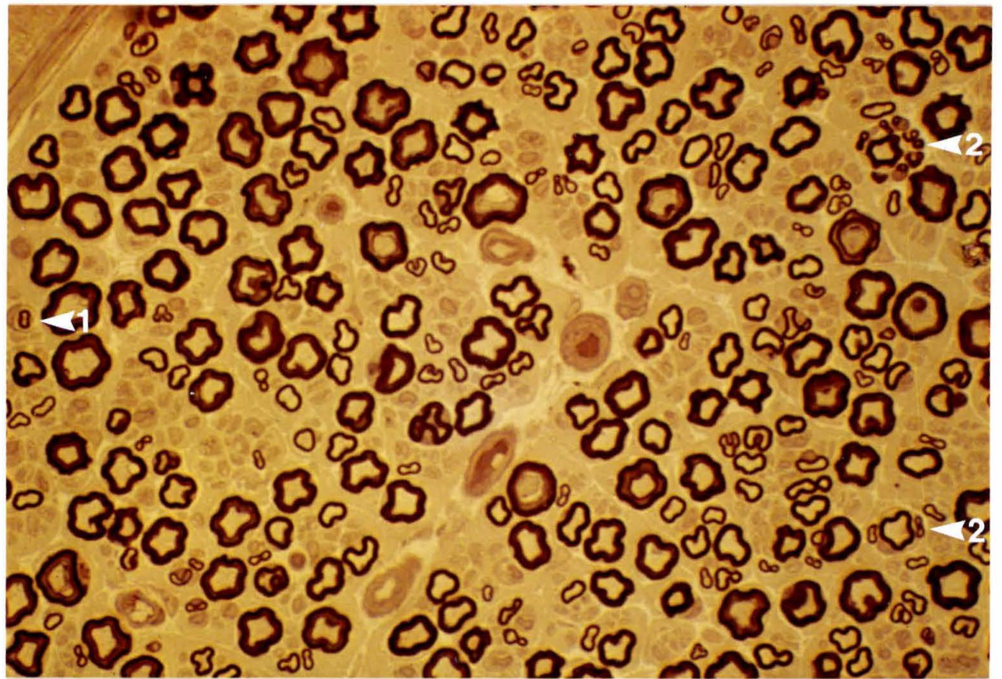
Figure 54. Photomicrographs of transverse sections of the plantar nerve in a control horse showing:-

A. Onion bulb formation(1) and regenerating clusters(2).

Phenylenediamine staining x 150

B. Normal density of nerve fibres at this level of sampling.

Phenylenediamine staining x 60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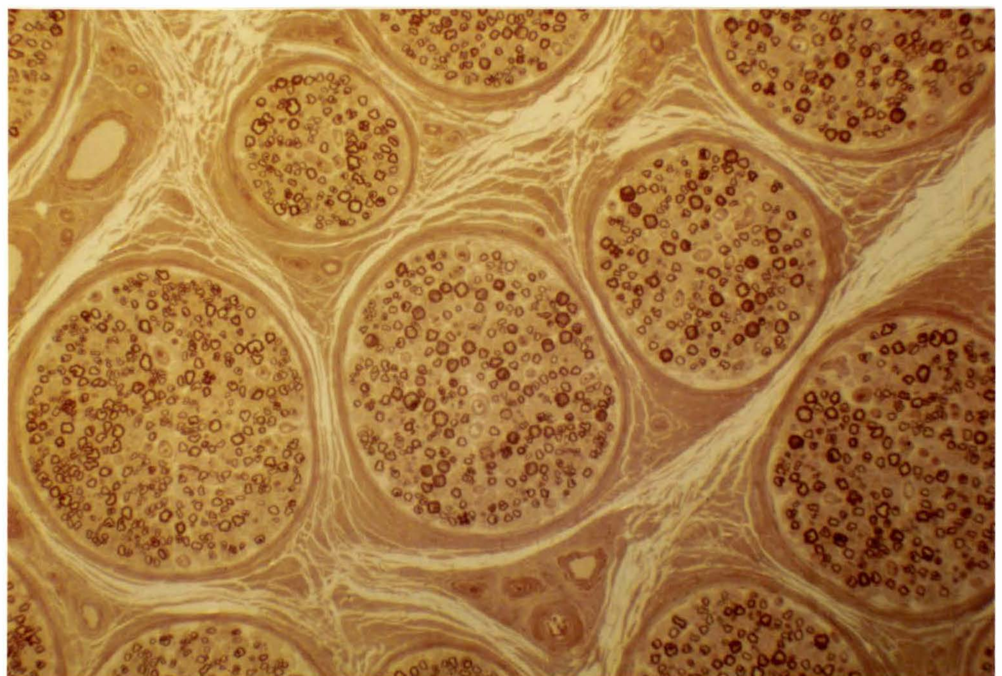


Figure 55. Photomicrograph of a transverse section of the plantar nerve in a control horse showing numerous regenerating clusters (large arrows) and onion bulb formation (small arrows).

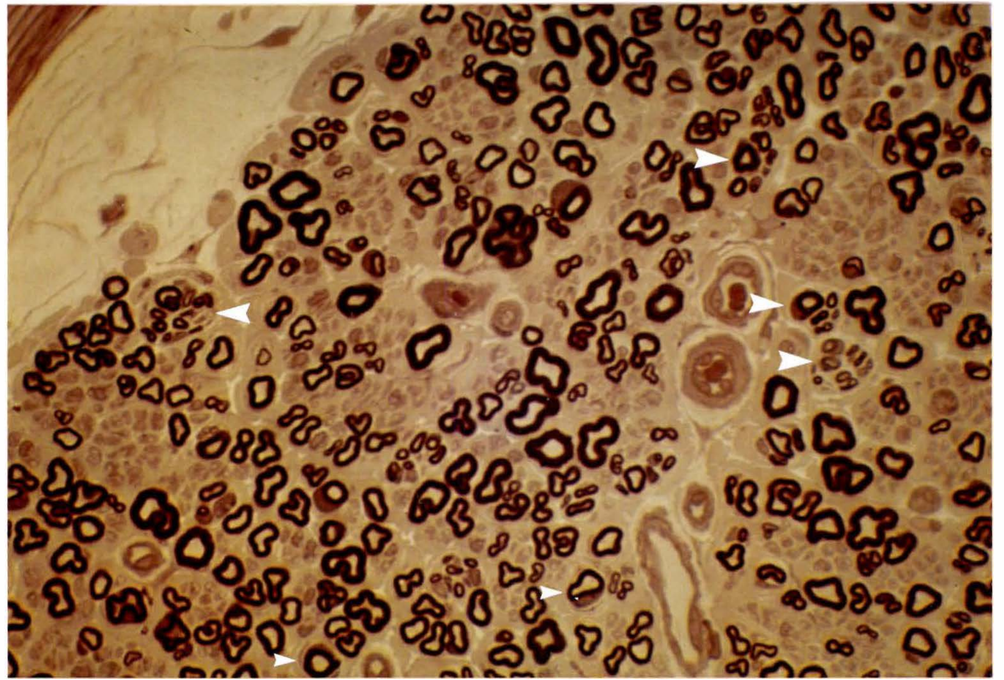
Phenylenediamine staining x 150

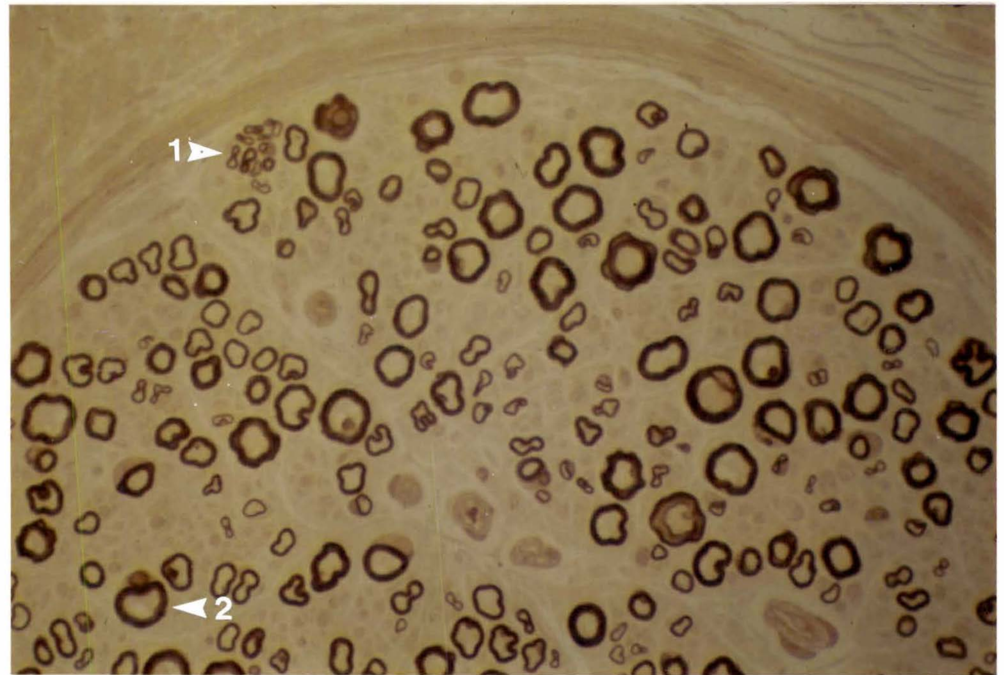
Figure 56. Photomicrographs of transverse sections of the plantar nerve in a clinical laryngeal hemiplegic horse showing:-

A. A regenerating cluster(1), and a myelin ovoid in the Schwann cell cytoplasm(2).

B. A regenerating cluster(1), a degenerating nerve fibre and myelin debris(2), and myelin ovoids in the Schwann cell cytoplasm(3).

Phenylenediamine staining x 150

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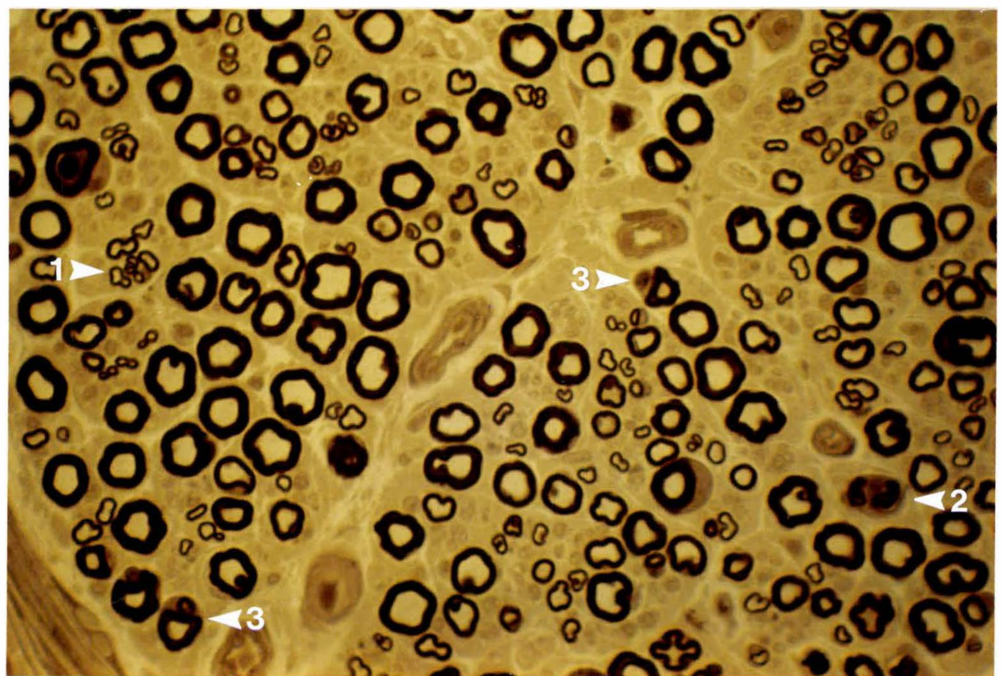
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Figure 57. Photomicrographs of transverse sections of the plantar digital nerves in control horses showing:-

A. The morphological appearance of one of only three samples considered free of individual nerve fibre abnormalities, although slight fibre loss is present.

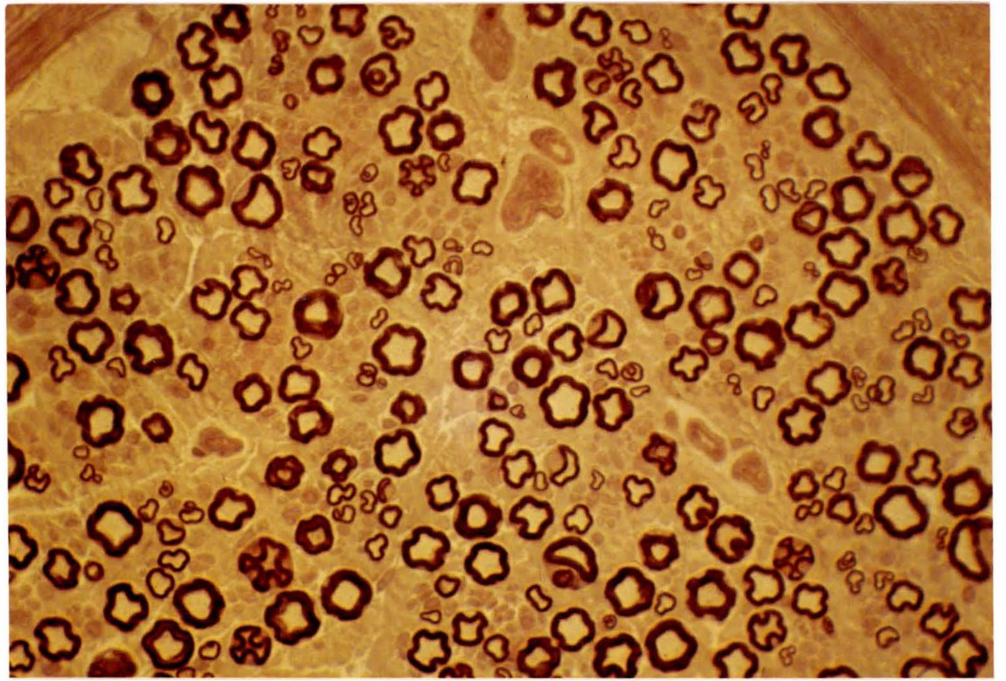
Phenylenediamine staining x 150

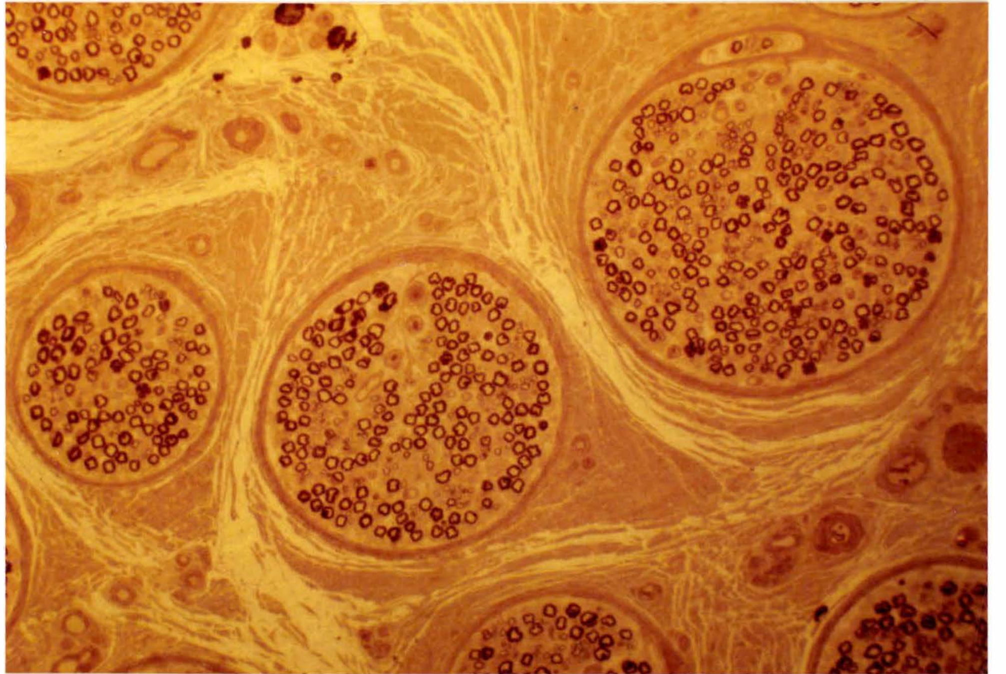
B. Fascicles in which nerve fibre density was considered to be minimal.

Phenylenediamine staining x 60

Figure 58. Photomicrograph of a transverse section of the plantar digital nerve in a control horse showing the frequent presence of regeneratring clusters (arrows) and also fibre loss.

Phenylenediamine staining x 150





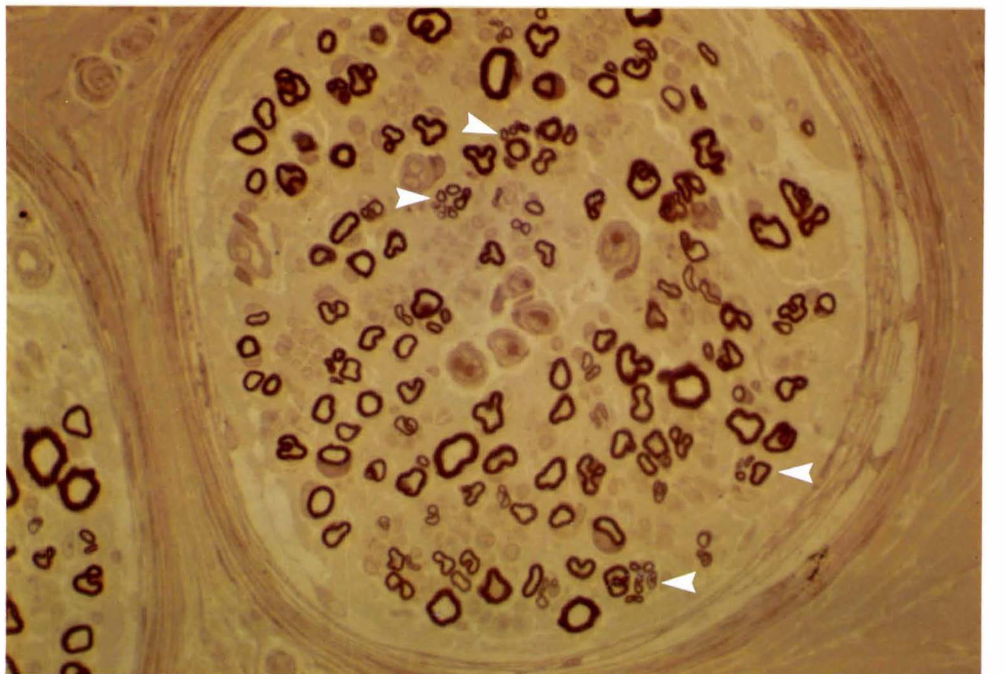


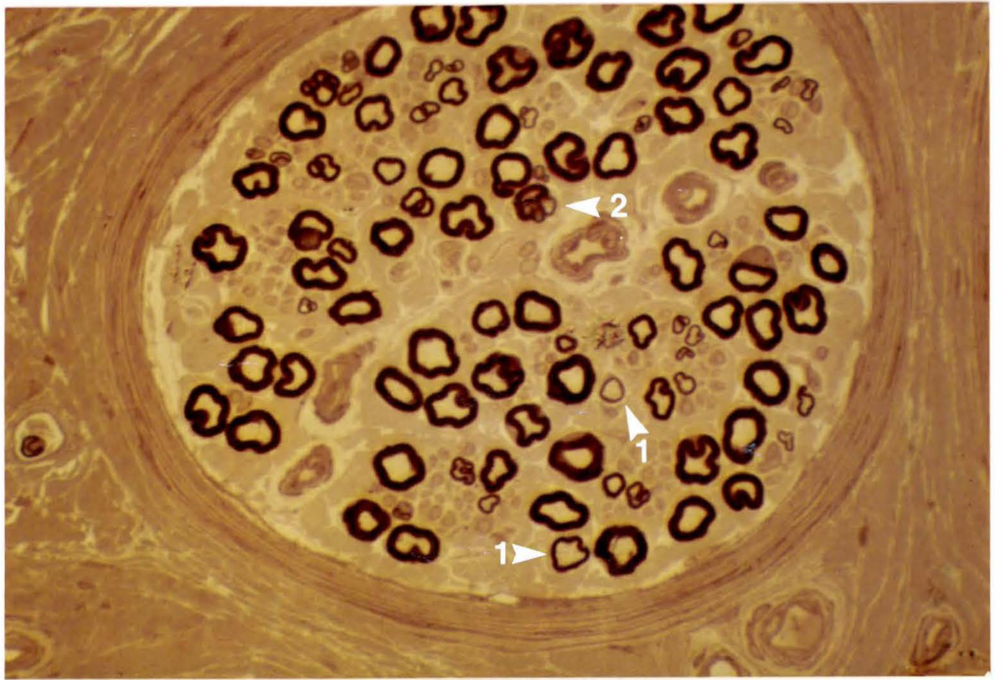
Figure 59. Photomicrographs of transverse sections of the plantar digital nerve in clinical laryngeal hemiplegic horses showing:-

A. Thinly myelinated fibres(1) and a split myelin sheath(2).

B. Fibre loss and thinly myelinated fibre and onion bulb formation(1), a regenerating cluster(2) and debris in the myelin sheath of a nerve fibre(3).

C. A Schwann cell or macrophage containing myelin debris(1), debris in the myelin sheath(2) and numerous regenerating clusters(3).

Phenylenediamine staining x 150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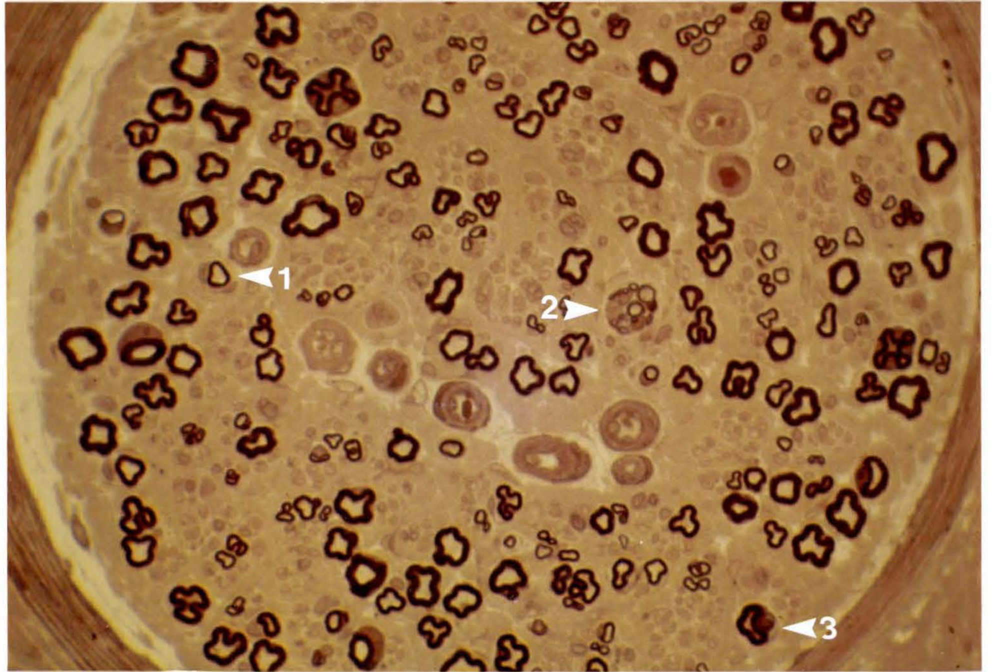
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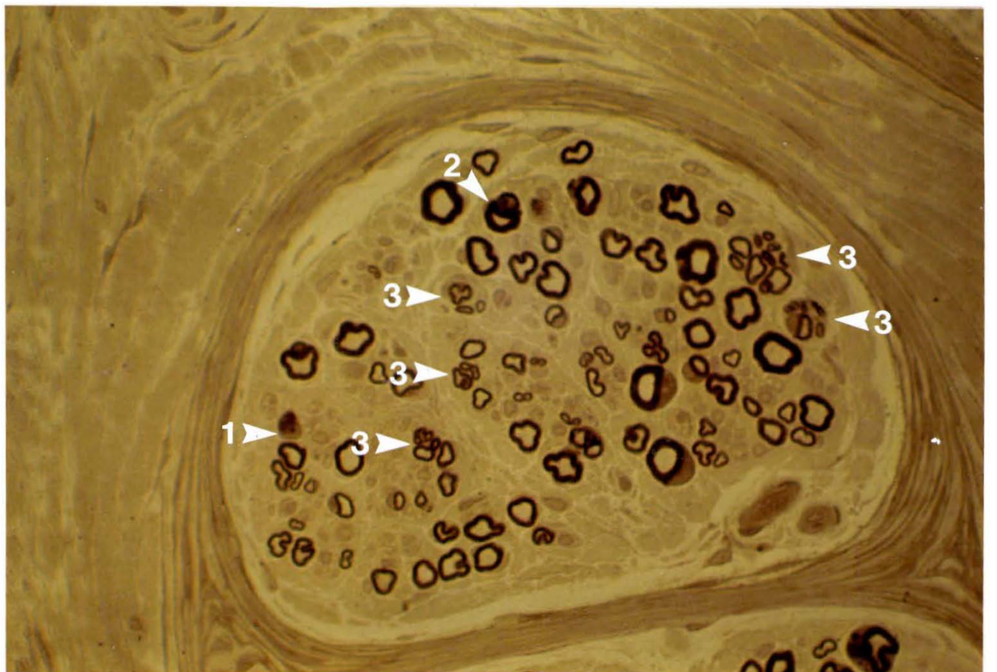
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Figure 60. Photomicrographs of transverse sections of the plantar digital nerve in clinical laryngeal hemiplegic horses showing:-

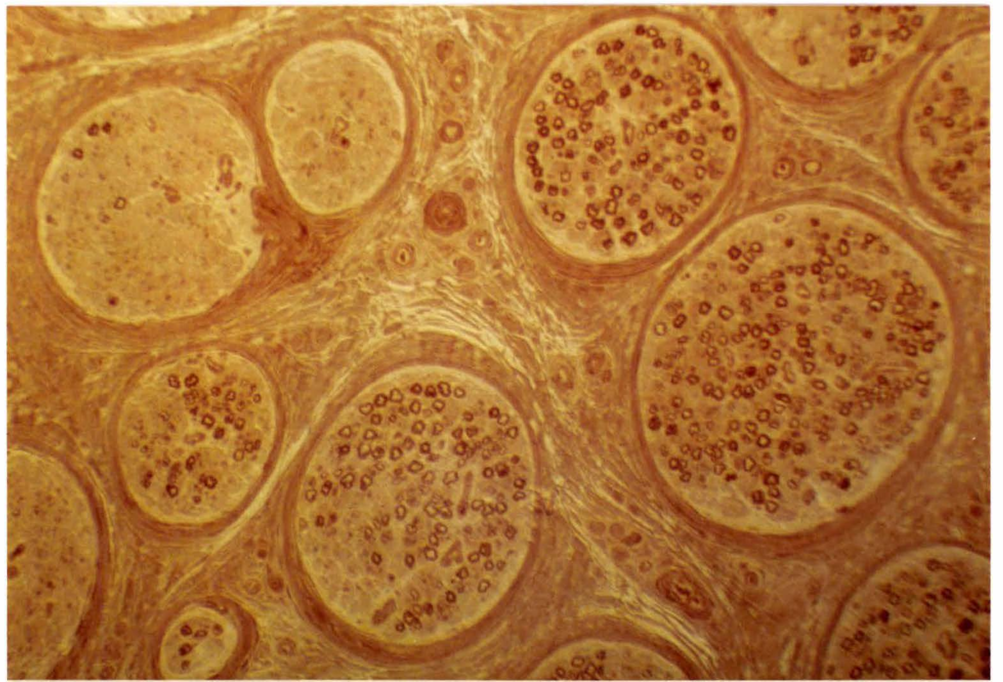
A. Severe fibre loss in some fascicles

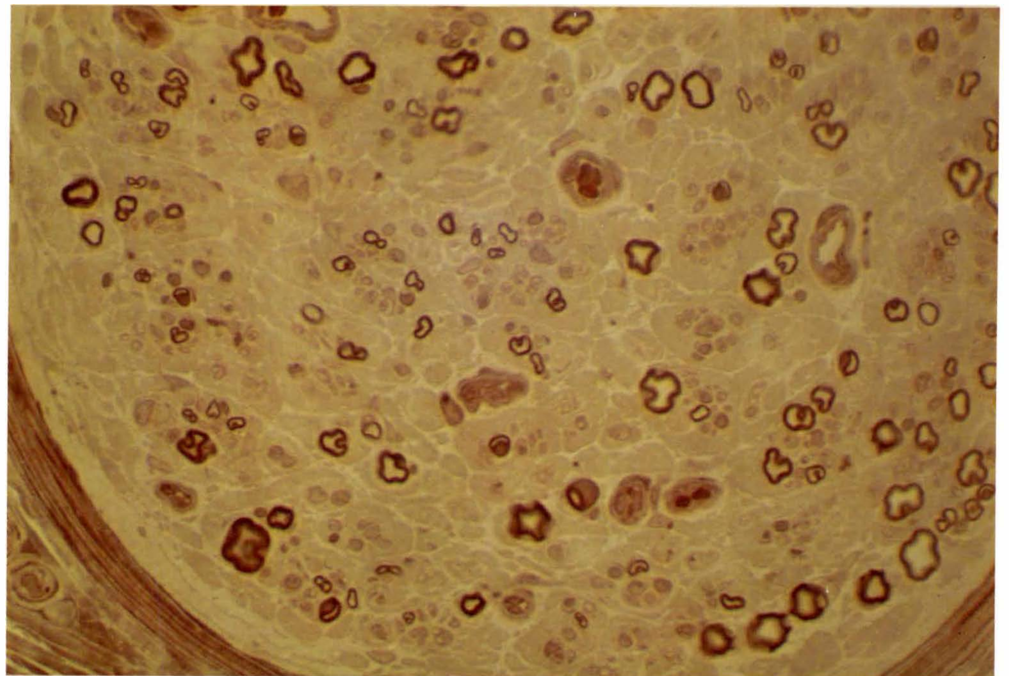
Phenylenediamine staining x 60

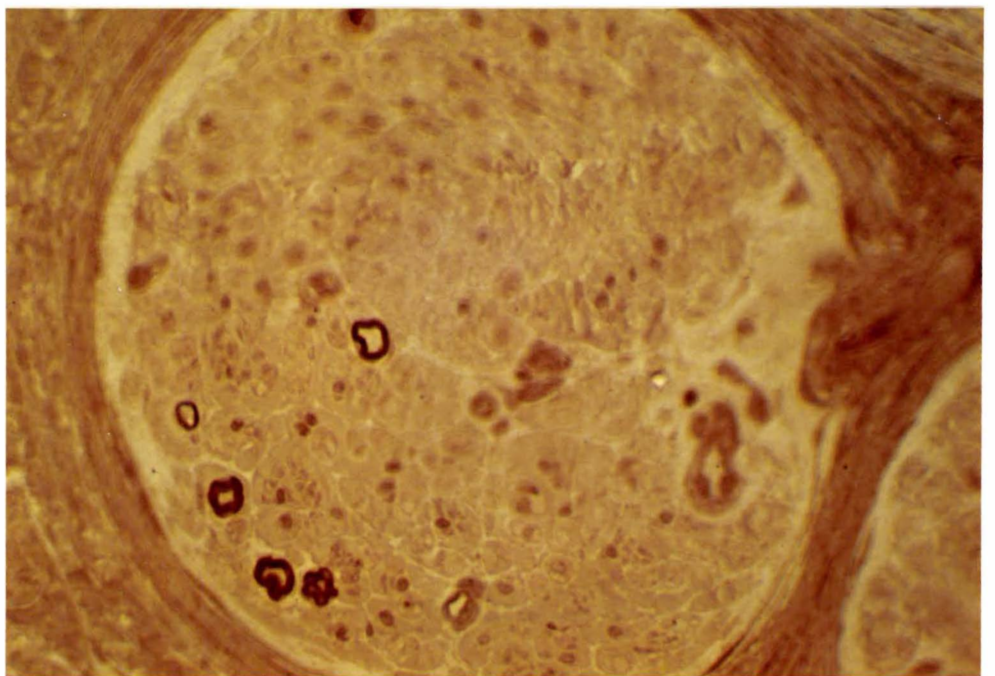
B. Few abnormalities of individual teased fibres are evident because of extensive fibre loss, although some thinly myelinated fibres are present.

C. Almost no myelinated fibres remain in this fascicle.

Phenylenediamine staining x 150







Nerve fibre density

The density of nerve fibres, which was measured as the number of fibres per square millimetre of nerve, at each level of sampling in all horses, is shown in Appendix 11. No morphometric analysis was performed on the nerves of horse 1, 12 or 14.

The mean nerve fibre density at each level of nerve for each group of horses is shown in Table 11.

A description of the findings in both the common peroneal nerve and its deep continuation, and the tibial plantar and plantar digital nerves, is presented below.

TABLE 11 **Mean density (no/mm^2)** of **nerve fibres in the limb nerves in clinical, sub-clinical and control horses**

	Groups of horses		
Nerve	**Clinical**	**Subclinical**	**Control**
Common peroneal	8917	5250	6875
Deep peroneal, proximal	7000	5500	6833
Deep peroneal, distal	7917	7750	7250
Tibial	7416	6583	7250
Plantar	5542	5833	6833
Plantar digital	4792	5542	5958

Common and deep peroneal nerves

The common and deep peroneal nerves were found to contain two distinct populations of nerve fibres (figs. 47A,B). The density of each of these was quite different, as one group was found to have almost twice as many fibres per square millimetre as the other. Although the density of fibres was consistent throughout a single fascicle, adjacent fascicles were often found to have different

populations (fig. 47C). Therefore as the technique of image analysis used in this study measured the fibre density in a single fascicle, there was considerable variation in the mean density found at each site of sampling in the same horse and also between horses. This is well illustrated in horses 9 and 10 in which the fibre density varied greatly between samplings (fig. 61), as a result of measuring different fibre populations.

Because of the differing fibre populations the mean value of fibre density was considered to be an inaccurate measure of fibre loss in these nerves. To overcome this problem, and to provide a more accurate assessment and comparison of fibre density at each site in the nerve, two other techniques of fibre density measurement were used. These were;

i) Fascicles were divided into high and low density groups according to the number of fibres per square millimetre. The high density group consisted of fascicles with approximately 8,000 to 10,000 fibres/mm^2 (Fig. 47A), while in the low density groups contained approximately 5,000 to 7,000 fibres/mm^2 (Fig. 47B). Once placed into these two groups the mean density of high and low values for clinical, subclinical and control horses was determined. These are shown in Table 12.

2) Calculation of total axonal area, the results of which are presented later in this thesis.

Figure 61. Histograms showing the distribution and density of cross-sectional axonal area in the common and deep peroneal nerves in two horses, illustrating the variation found in this fibre.

A. Histograms in a control horse showing an increased number of smaller fibres in the proximal deep peroneal nerve. The total density was 7,626; 9,334; and 5,292 fibres/mm^2 for the common, and proximal and distal deep peroneal nerves respectively.

B. Histograms in a clinical laryngeal hemiplegic horse showing an increased number of small fibres in the common peroneal and distal deep peroneal nerve samples. The total density at each site was 12,959; 5,375; and 11,876 fibres/mm^2 for the common and proximal and distal deep peroneal nerves respectively.

A

FIBRE NUMBER (%)

30
20
10
0

25 50 75

COMMON PERONEAL

25 50 75

DEEP PERONEAL
PROXIMAL

25 50 75 100

DEEP PERONEAL
DISTAL

Axonal area (um^2)

B

FIBRE NUMBER (%)

70
60
50
40
30
20
10
0

25 50 75 100

COMMON PERONEAL

25 50 75 100

DEEP PERONEAL
PROXIMAL

25 50 75 100

DEEP PERONEAL
DISTAL

Axonal area (um^2)

NERVES

TABLE 12 Mean density (no/mm^2) of nerve fibres in the common and deep peroneal nerves in clinical, subclinical and control groups of horses when divided into high and low density groups.

Nerve	Clinical		Subclinical		Control	
	High	Low	High	Low	High	Low
Common peroneal	1179	2675	-	5250	7500	6500
Deep peroneal, proximal	9125	5167	7541	4792	9250	6250
Deep peroneal, distal	8333	5667	8292	5458	8272	6041

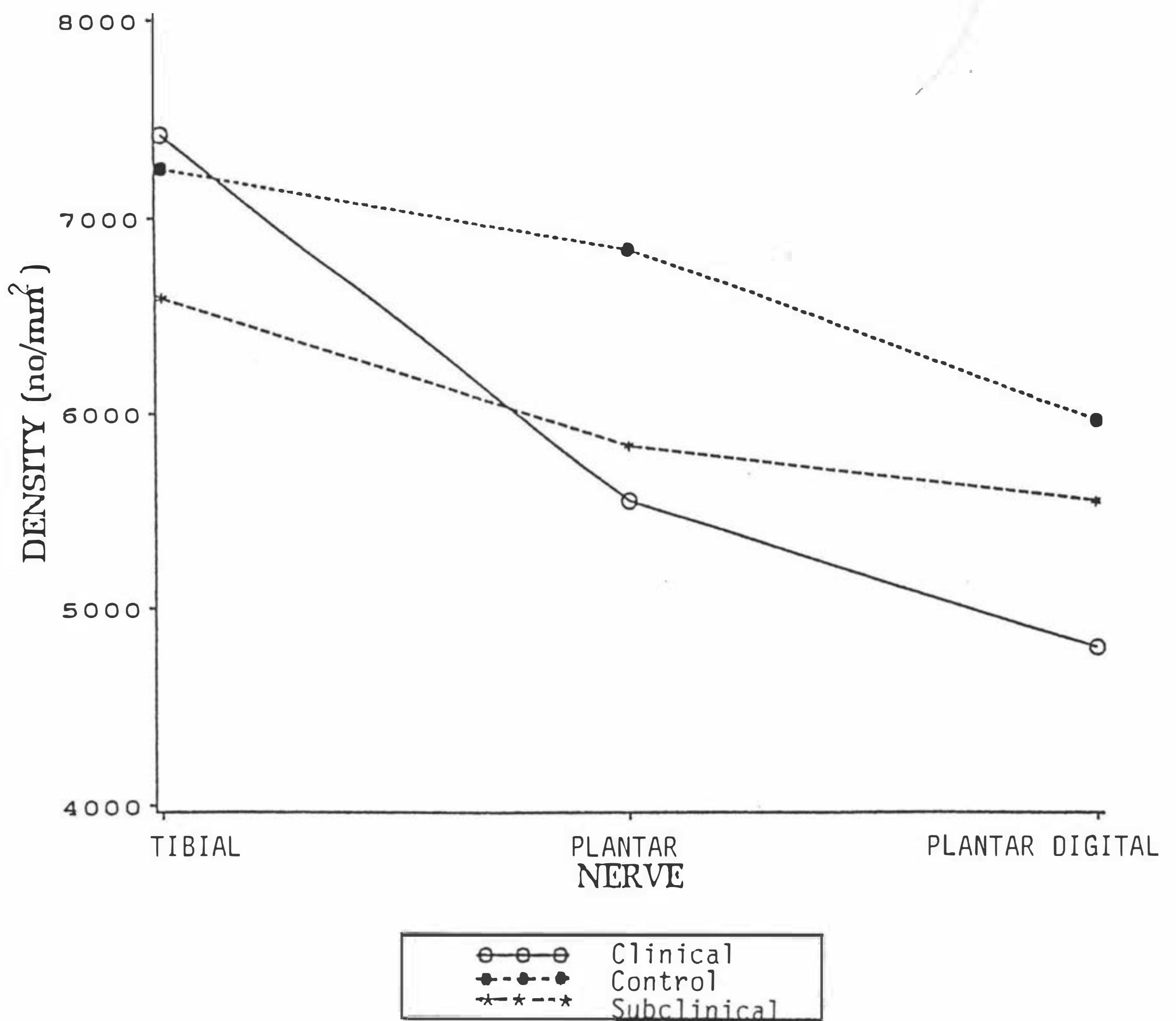
The mean fibre density measurement at each site revealed no consistent difference between groups of horses, with the exception of the common peroneal nerve. In this nerve,the density of fibres in the clinically affected horses was higher than in the other animals.

Loss of nerve density was, however, clearly evident in a number of samples from individual horses. In the common peroneal nerve, one clinical (horse 7) and one control (horse 3) animal had densities of below 3,700 fibres/mm^2 in one sample each. In the case of the clinical horse this was also present in the proximal deep peroneal sample. At the proximal deep peroneal site one subclinical horse was found to have a decreased fibre density of less than 3,500 fibres/mm^2 in one leg.

<u>Tibial, plantar, and plantar digital nerves</u>

In all groups studied there was a proximal to distal decrease in nerve fibre density (Fig. 62). In the control and clinical groups, this difference between the most proximal sample, the tibial nerve,

Figure 62. Graph of nerve fibre density in the tibial, plantar, and plantar digital nerves in clinical, subclinical and control horses. There is a decrease in nerve density from proximal to distal in all groups; however, this decrease is greatest in the clinical group.



DENSITY OF MYELINATED NERVE FIBRES
8000
7000
6000
5000
4000
DENSITY (no/mm²)
TIBIAL
PLANTAR
PLANTAR DIGITAL
NERVE
Clinical
Control
Subclinical

and the most distal sample, the plantar digital nerve was statistically significant($p = 0.01$). In the subclinical group this trend was also evident although statistical evaluation was not undertaken due to the small number of samples available.

In the tibial nerve samples no significant difference in fibre density between groups existed, but at the more distal sampling sites the mean density was significantly lower ($p < 0.05$) in clinical compared to control horses.

The most striking example of fibre loss was seen in the plantar digital nerve in a clinical animal (horse 7), in which the mean density was 2,709 fibres/mm^2 and in some fascicles almost no fibres remained (fig. 60).

Individual cross-sectional axonal area - Mean values and distribution histograms

The result of analysis of mean cross-sectional axonal area for each group of horses at each level of sampling is presented in Table 13 and the results for all individual horses in Appendix 12.

This table shows a trend in all groups towards a decrease from proximal to distal in the mean nerve fibre area in both sets of nerves examined. This decrease from the most proximal to the most distal sample was significant in each group of horses ($p < 0.05$).

The mean fibre area of the common peroneal nerve and its deep continuation was found to be significantly less ($p = 0.05$) in clinical than control horses at all levels, while there was no significant difference between groups in the tibial, plantar, or plantar digital nerves.

TABLE 13 Mean (um^2) and standard deviation of cross-sectional axonal area in the limb nerves of clinical, subclinical and control horses.

	Groups of horses		
Nerve	**Clinical**	**Subclinical***	**Control**
Common peroneal	16 ± 25	27 ± 4	23 ± 22
Deep peroneal, proximal	18 ± 27	28 ± 20	20 ± 19
Deep peroneal, distal	13 ± 18	14 ± 6	17 ± 18
Tibial	17 ± 20	23 ± 2	17 ± 19
Plantar	17 ± 18	20 ± 0	14 ± 13
Plantar digital	12 ± 13	12 ± 4	13 ± 12

* An average of only 2 samples were collected at each level of nerve for subclinical horses.

Values obtained by measurement of cross-sectional axonal areas of all fibres at each level of sampling in clinical and control horses were analyzed to enable comparison of the distribution of fibre sizes between these two groups. A total of 20,162 individual fibres were measured from samples in control and clinical animals. The results are presented as histograms in figures 63 and 64.

The histographic representation of the distribution of fibre sizes in the hind limb nerves studied illustrated the following:-

a) in the control group of horses an increasing percentage of smaller fibres as the nerve progressed distally. This was most obvious in the peroneal nerves (fig 63).

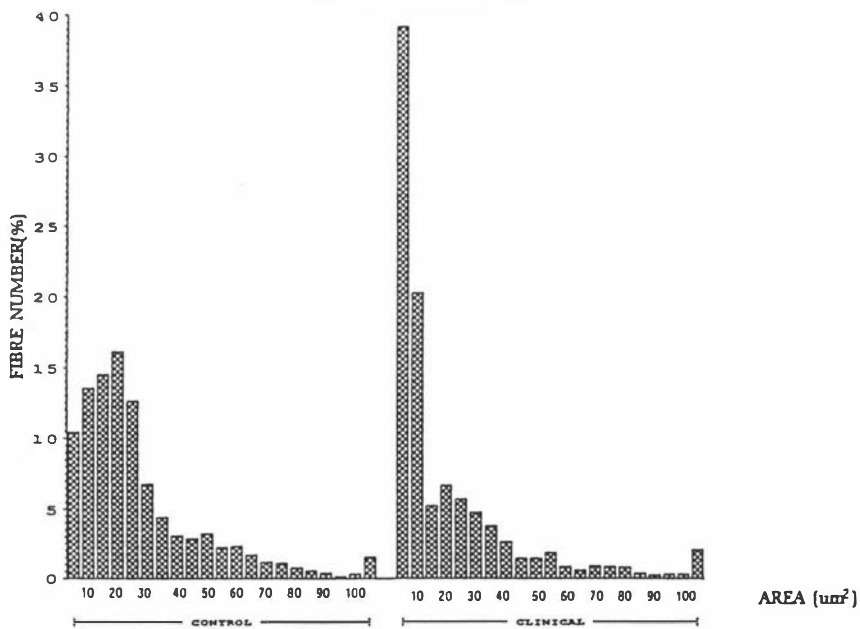
b) in the clinical group, a higher proportion of

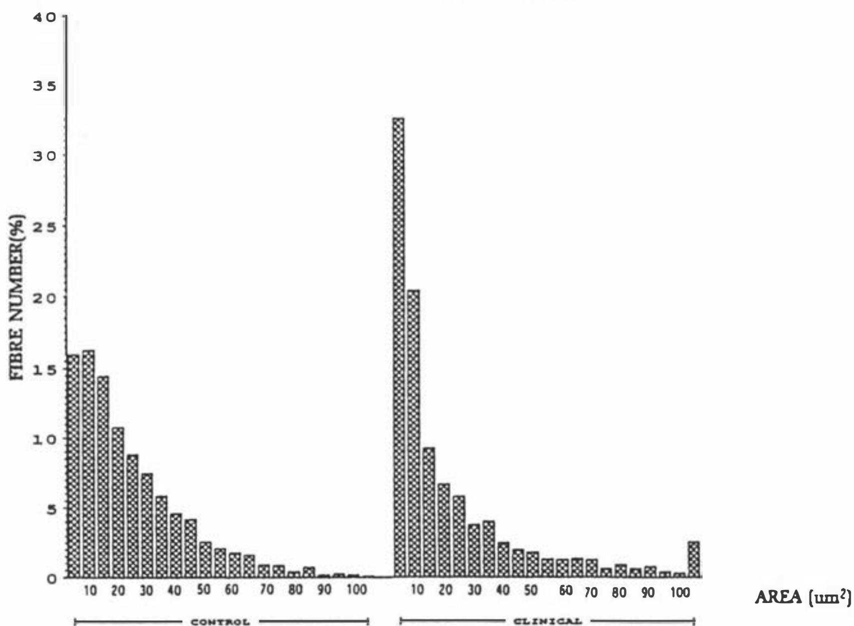
Figure 63. Histograms of cross-sectional axonal area distribution in control and clinical horses in the common and deep peroneal nerves. In both clinical and more obviously in control horses there is an increase in the proportion of small nerve fibres from proximal to distal. At each sampling level there is also an increase in the percentage of small fibres in clinical compared with control horses.

A. Histograms of the common peroneal nerve from control and clinical horses.

B. Histograms of the proximal site of sampling in the deep peroneal nerve from control and clinical horses.

C. Histograms of the distal site of sampling in the deep peroneal nerve from control and clinical horses.


COMMON PERONEAL NERVE
FIBRE NUMBER(%)
40
35
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
10 20 30 40 50 60 70 80 90 100
AREA (um²)
CONTROL
CLINICAL


DEEP PERONEAL NERVE – PROXIMAL
FIBRE NUMBER(%)
40
35
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
10 20 30 40 50 60 70 80 90 100
AREA (um²)
CONTROL
CLINICAL

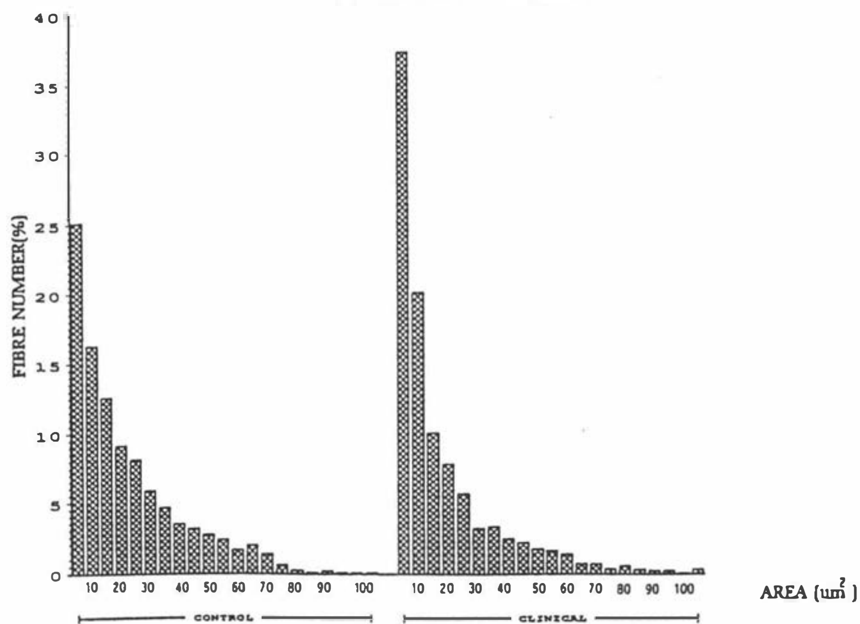
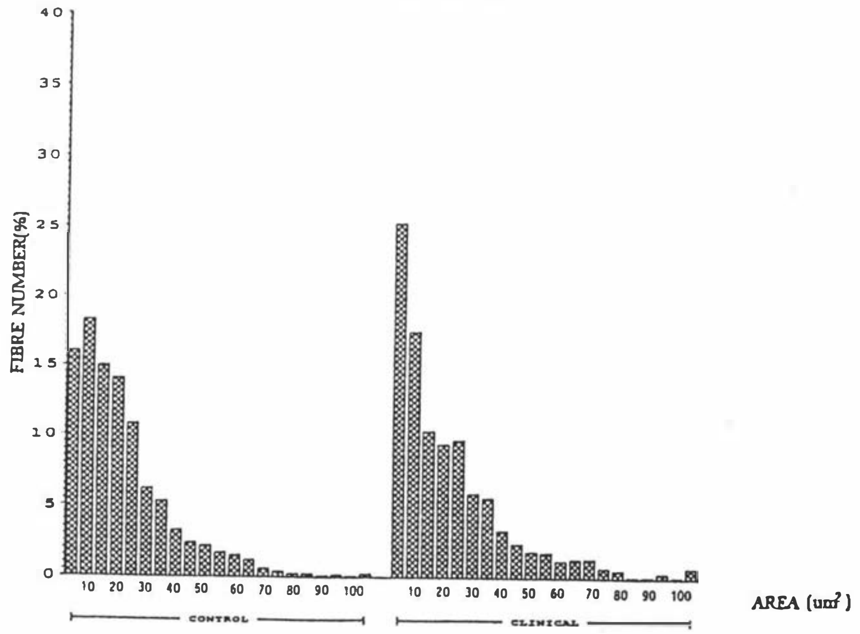

DEEP PERONEAL NERVE – DISTAL
FIBRE NUMBER(%)
40
35
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
10 20 30 40 50 60 70 80 90 100
AREA (um²)
CONTROL
CLINICAL

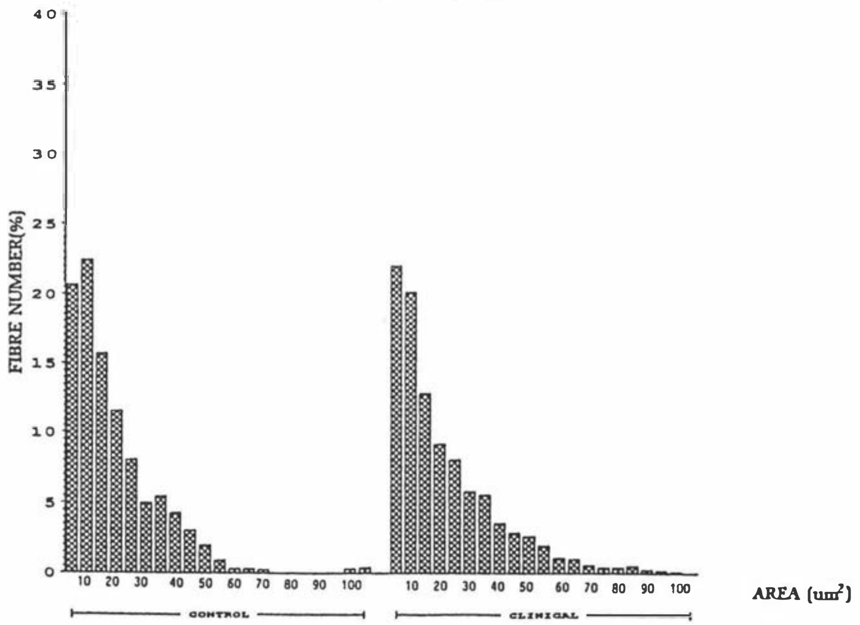
Figure 64. Histograms of cross-sectional axonal area distribution in control and clinical horses for the tibial, plantar, and plantar digital nerves. In both control and clinical groups there is an increase in the proportion of small fibres from proximal to distal sampling sites.

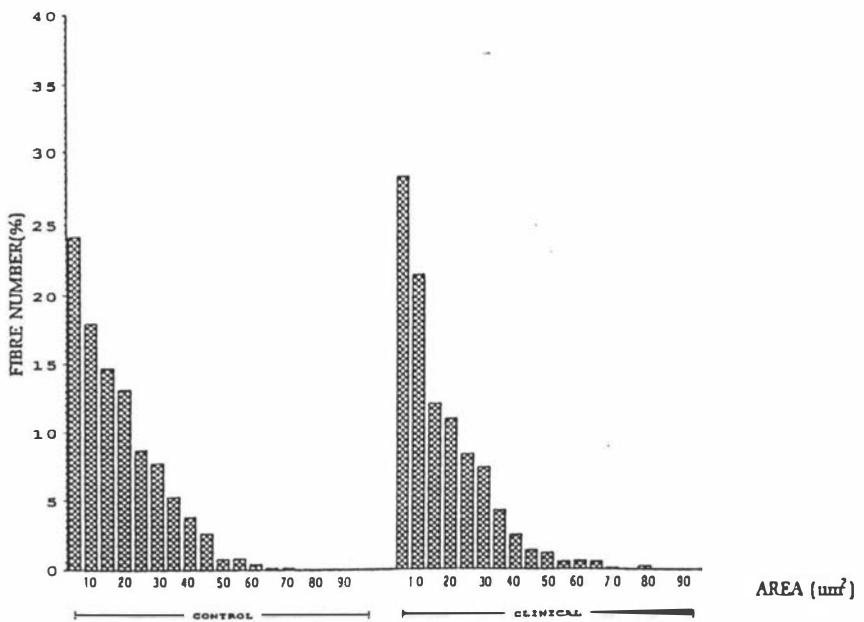
A. Histogram of the tibial nerve which shows an increased proportion of small fibres in clinical compared with control horses.

B. Histogram of the plantar nerve in which there are less obvious differences between the clinical and control groups.

C. Histogram of the plantar digital nerve also shows an increase in the proportion of small fibres in the clinical group.


TIBIAL NERVE
FIBRE NUMBER(%)
40
35
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
10 20 30 40 50 60 70 80 90 100
AREA (um²)
CONTROL
CLINICAL


PLANTAR NERVE
FIBRE NUMBER(%)
40
35
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
10 20 30 40 50 60 70 80 90 100
AREA (um²)
CONTROL
CLINICAL


PLANTAR DIGITAL NERVE
FIBRE NUMBER(%)
40
35
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90
10 20 30 40 50 60 70 80 90
AREA (um²)
CONTROL
CLINICAL

small fibres (and correspondingly lower proportion of larger fibres) when compared to the control group. This difference was particularly obvious and highly significant ($p < 0.0001$) in the peroneal nerves (fig 63) and less obvious, although still significantly different ($p < 0.001$), in the tibial nerve.

Total cross-sectional axonal area

The results of calculating total cross- sectional axonal area at each site in the hindlimb nerves of each group of horses are presented in table 14 and figures 65A and B

TABLE 14 Mean total axonal area ($um^2/mm^2 \times 10^3$) in the limb nerves of clinical, subclinical and control horses.

Nerve	Clinical	Groups of horses Subclinical*	Control
Common peroneal	135	144	167
Deep peroneal, proximal	125	133	134
Deep peroneal, distal	102	100	123
Tibial	129	154	130
Plantar	93	117	94
Plantar digital	60	82	81

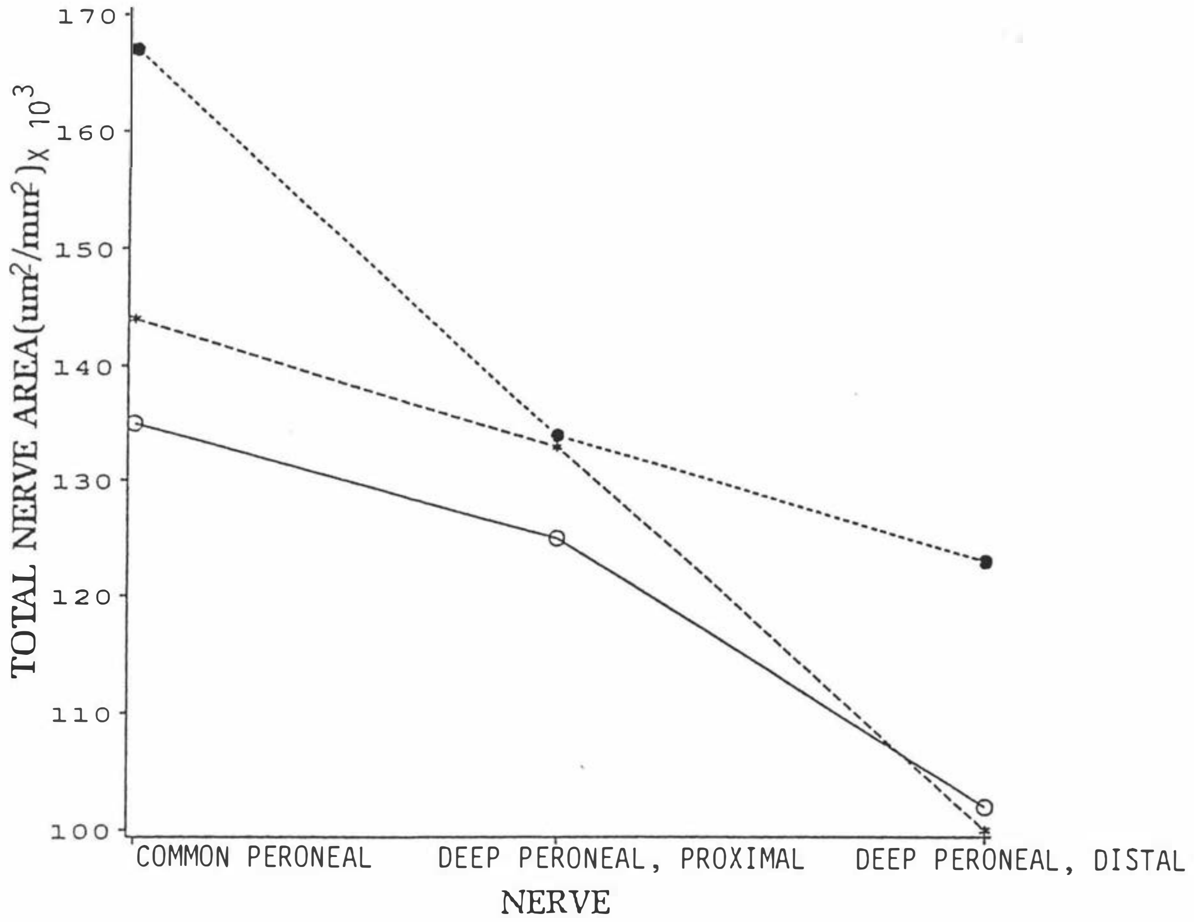
* An average of only two samples were collected at each level of nerve for the subclinical group.

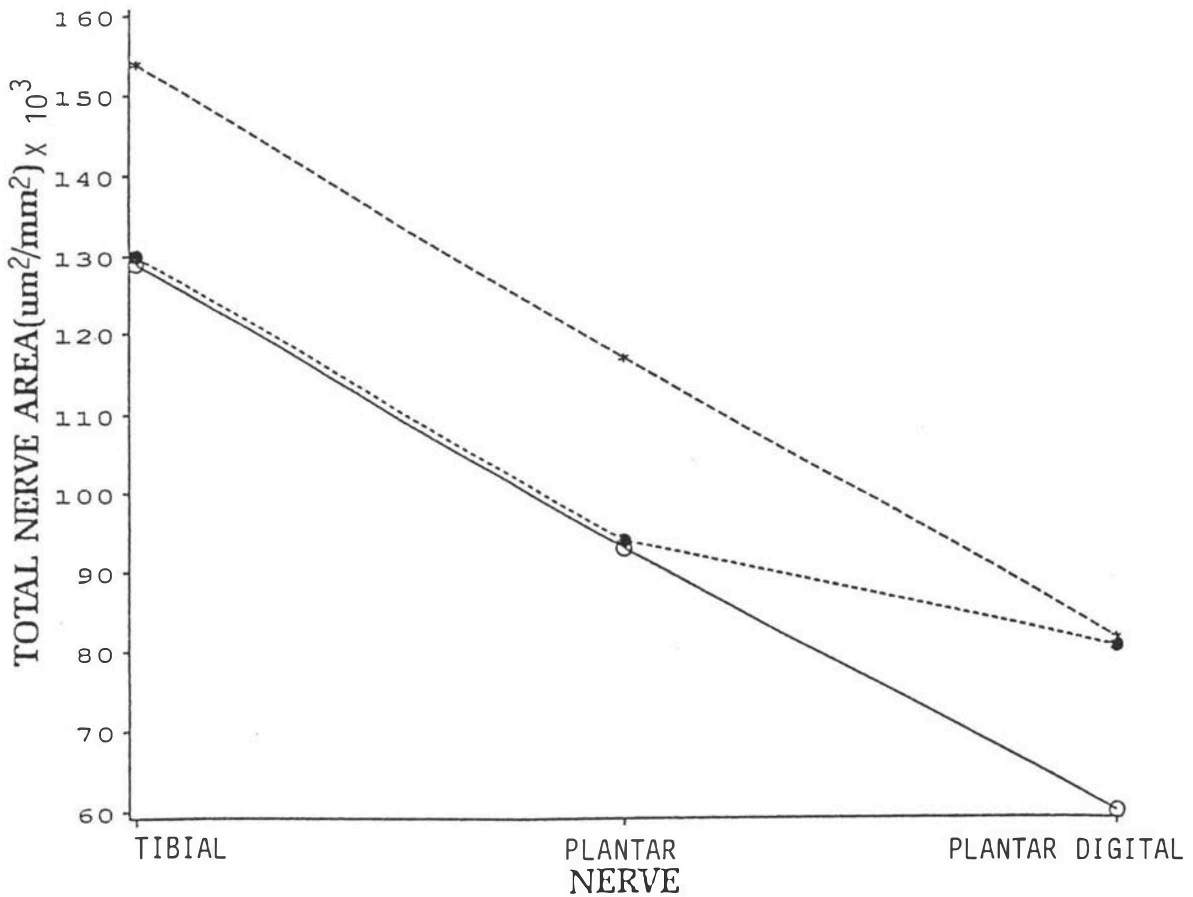
A decrease in mean, total, cross-sectional, axonal area from proximal to distal samples,in all groups of horses, in both the hindlimb nerve trunks examined, was observed.

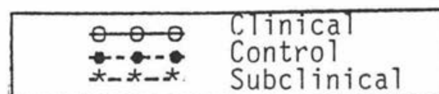
Figure 65. Graphs of total cross-sectional axonal area per square millimetre for all groups of horses at each sampling site.

A. There is a decrease in total area from proximal to distal in all groups of horses. The total axonal area for clinical and subclinical horses was less than found in control horses at all levels.

B. There is also a decrease from proximal to distal in the tibial nerve and its branches. In clinical horses the decrease in area from the plantar to the plantar digital nerve sample is greater than in control horses.


TOTAL NERVE AREA
TOTAL NERVE AREA(um²/mm²) x 10³
170
160
150
140
130
120
110
100
COMMON PERONEAL
DEEP PERONEAL, PROXIMAL
DEEP PERONEAL, DISTAL
NERVE


TOTAL NERVE AREA(um²/mm²) x 10³
160
150
140
130
120
110
100
90
80
70
60
TIBIAL
PLANTAR
PLANTAR DIGITAL
NERVE


Clinical
Control
Subclinical

In the peroneal nerves, the clinical group of horses had lower mean values than the controls at all levels(fig 65A). In the tibial nerve and its distal continuation , the values were similar in control and clinical groups , except at the most distal level. At this site the mean area was less in the clinical group compared to the controls(fig 65B).

Teased fibre examination

(i) Morphology

The results of grading teased fibres from the limb nerves of 5 horses is presented in Table 15.
The teased fibres in the control horse were mostly normal (fig. 66). When changes were seen they usually consisted of demyelination and remyelination (type F change), and occasionally a variation in myelin thickness (type G change). The tibial and plantar nerves were the most severely affected in the control horse. In the subclinical laryngeal hemiplegic horses a wider range of abnormalities were seen. These included demyelination and remyelination, variation in myelin thickness, and degeneration of nerve fibres (fig. 67). Abnormalities in the teased fibres of horse 17 were more frequently noted than in the control horse or horse 16.

The two clinical laryngeal hemiplegic horses had a range of changes similar to that seen in the subclinical horses, although these changes were more frequent. In both clinical horses the plantar digital nerve was most severely affected. Paranodal and segmental demyelination (figs. 68,69), demyelination and remyelination (fig. 70), axonal degeneration (fig. 71), short internodes (fig. 72), variation in myelin thickness (fig. 73), and myelin ovoids adjacent to the fibre (fig. 74), were all frequently identified in the clinically affected horses.

The incidence of abnormal teased fibres in each group of horses is shown in Table 16. Although only a small number of horses were evaluated the incidence of abnormal fibres was higher in the common and deep peroneal and plantar digital nerves in clinical laryngeal hemiplegic compared to control or subclinical horses.

TABLE 15 **Grading (after Dyck, 1975a; Cahill, 1985) of teased myelinated nerve fibres in the limbs of control, subclinical and clinical horses (See fig. 41 for description of grades).**

Group & Nerve	A & B	C	D	E	F	G	H	I
CONTROL								
Horse 13								
Common peroneal	98				2			
Deep peroneal, proximal	96		1			3		
Deep peroneal, distal	97				3			
Tibial	89				10	1		
Plantar	91				6	3		
Plantar digital	97				3			
SUBCLINICAL								
Horse 16								
Common peroneal	99	1						
Deep peroneal, proximal	97				2	1		
Deep peroneal, distal	98			1		1		
Plantar	99					1		
Plantar digital	97	1	1			1		
Horse 17								
Common peroneal	94	1	2	1	1	1		
Deep peroneal, proximal	92	1	2	1	2	2		
Deep peroneal, distal	90	2	3	2	2	1		
Tibial	95	2			1	2		
Plantar	95	1	1	1	2			
Plantar digital	93	2	1		4			
CLINICAL								
Horse 15								
Common peroneal	80	6	2		6	6		
Deep peroneal, proximal	70	8	4		7	10	1	
Deep peroneal, distal	84	3	2		6	5		
Tibial	89	1			3	7		
Plantar	90	1	1		5	3		
Plantar digital	69	10	3		14	4		
Horse 19								
Common peroneal	97	1	1			1		
Deep peroneal, proximal	90	1		1	3	5		
Deep peroneal, distal	94		1		1	4		
Tibial	98	1			1			
Plantar	95	1		1				
Plantar digital	85	5	3	1	5	1		

Figure 66. Photomicrographs of consecutive portions of a normal teased nerve fibre from a control horse which is classified as a Type A fibre. Internodes are arrowed.

x 60

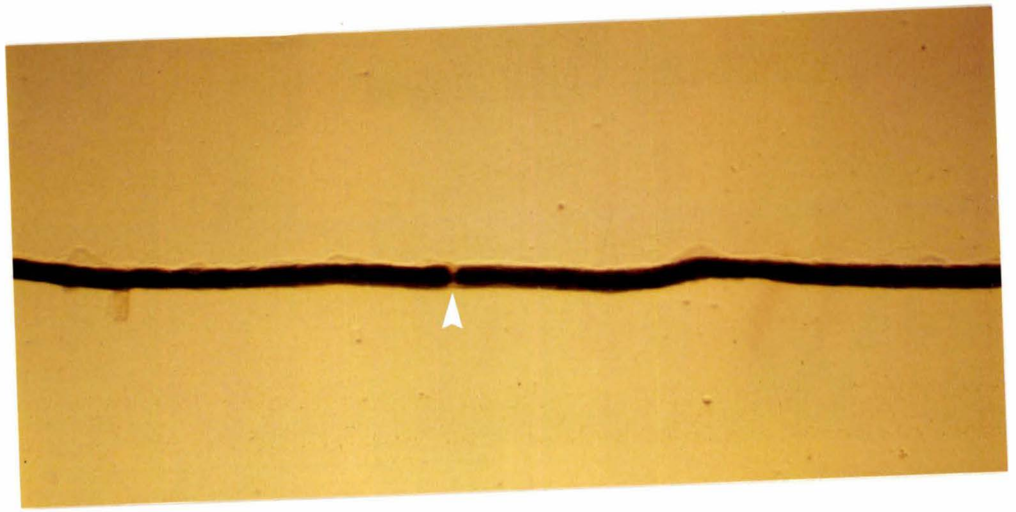
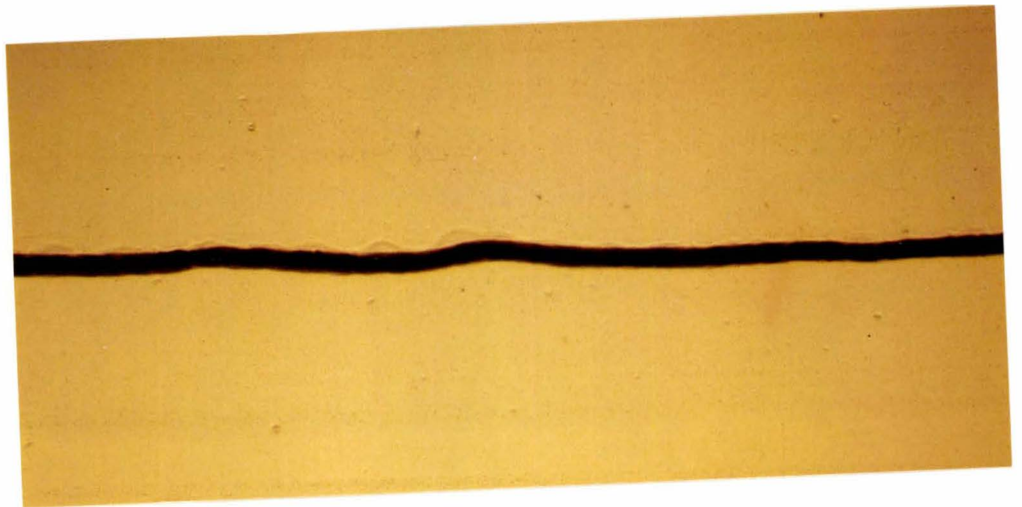
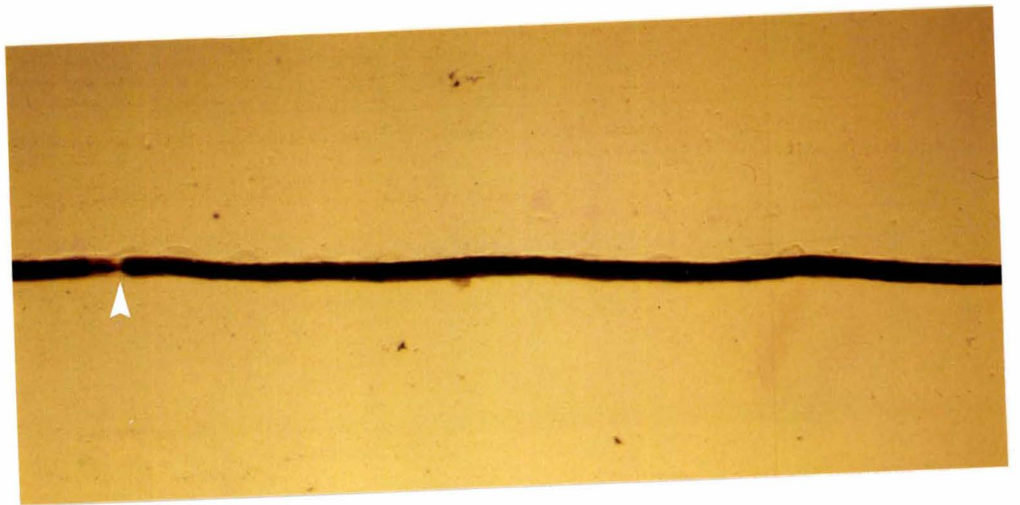
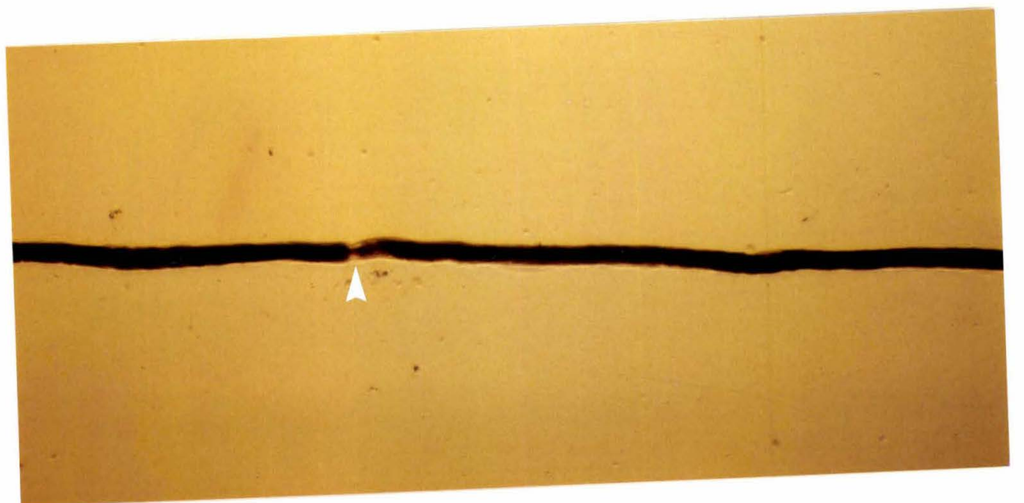
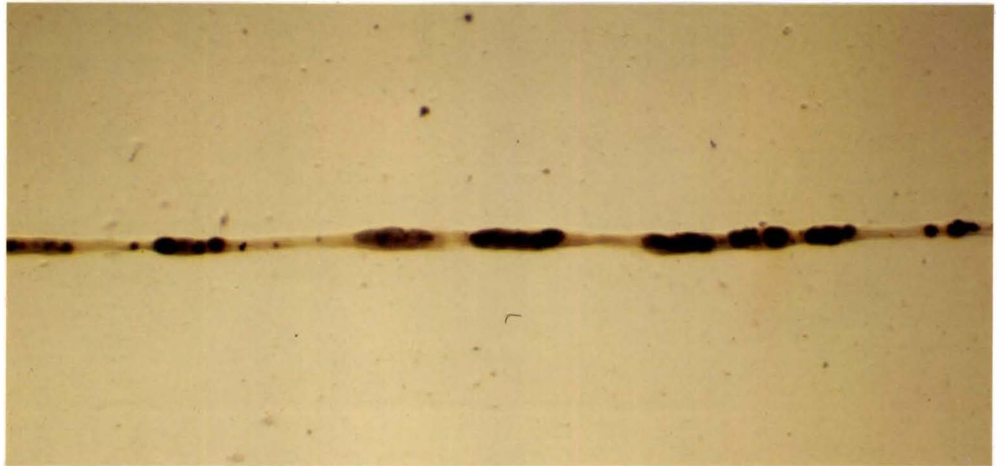
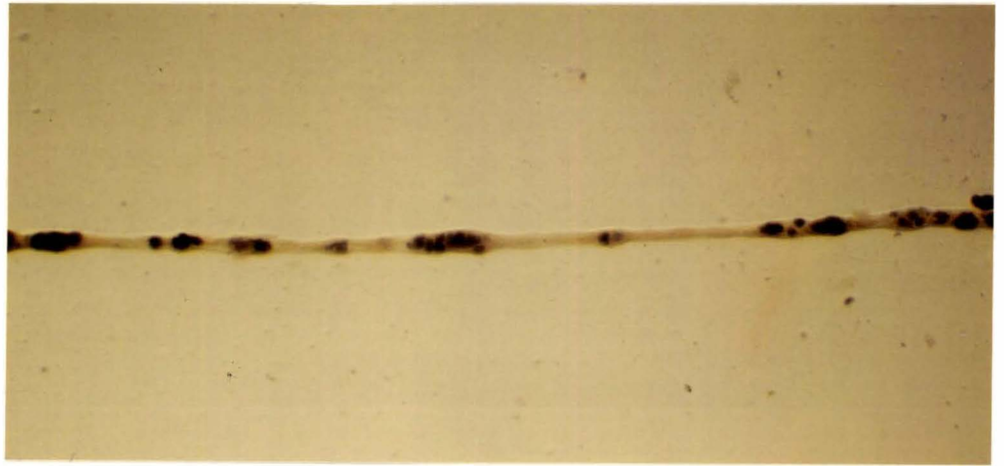





Figure 67. Photomicrographs of consecutive protions of a teased myelinated fibre from the distal deep peroneal nerve in a subclinical laryngeal hemiplegic horse showing axonal degeneration and myelin ovoids and balls, classified as a Type E change.

x 60





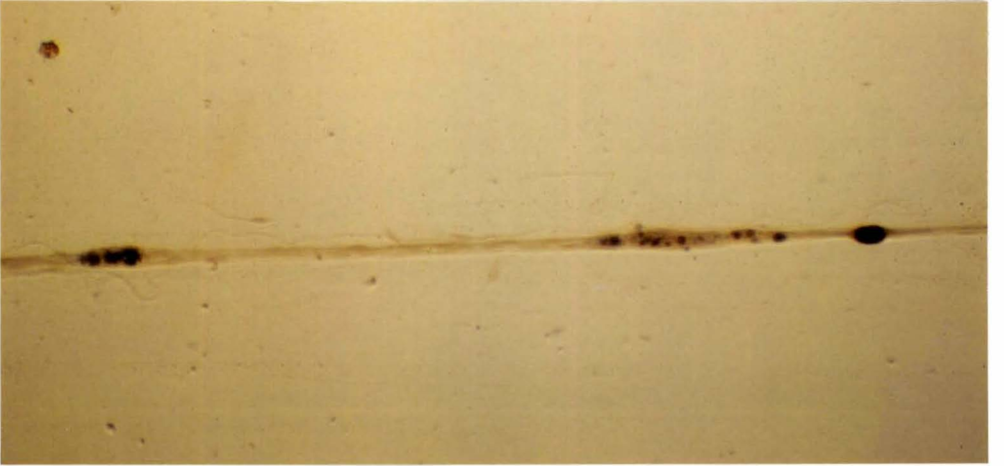


Figure 68. Photomicrograph of a teased myelinated fibre from the plantar digital nerve of a clinical laryngeal hemiplegic horse showing changes strongly suggestive of paranodal demyelination.

x 60

Figure 69. Photomicrograph of a teased fibre from the distal deep peroneal nerve in a clinical laryngeal hemiplegic horse showing segmental demyelination (Type C change).

x 60

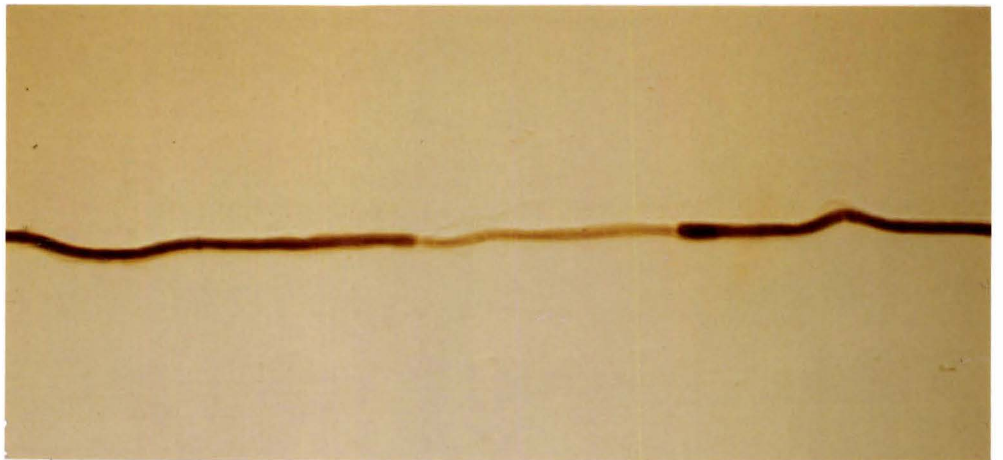
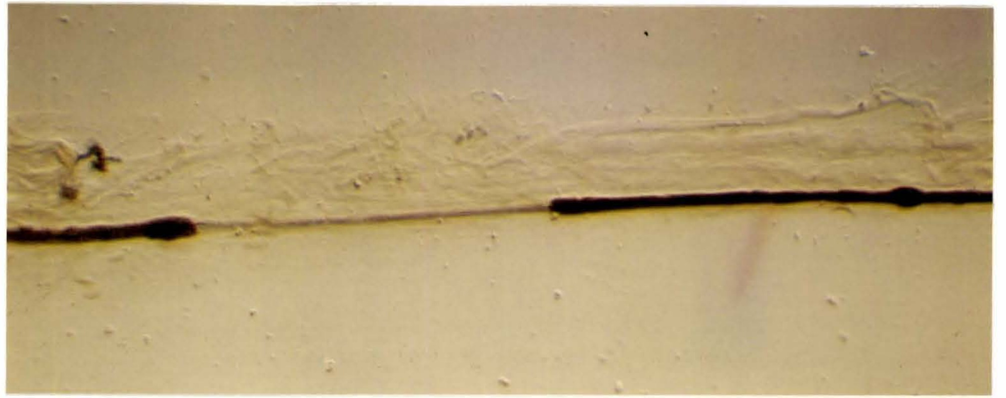
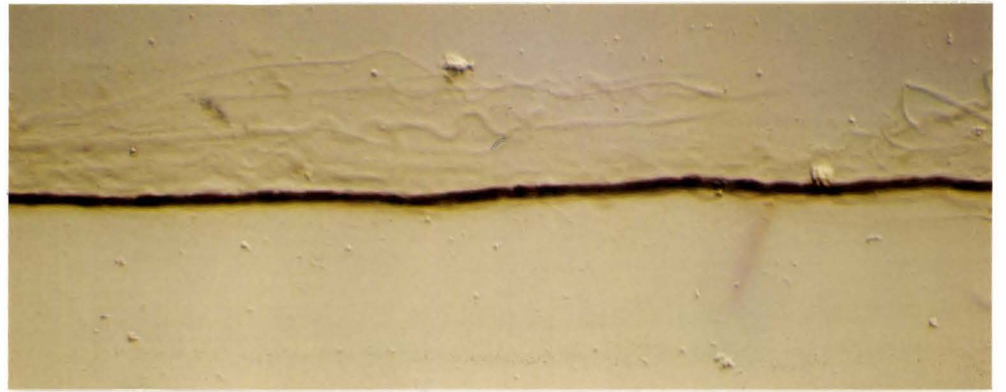


Figure 70. Photomicrographs of consecutive portions of a teased myelinated fibre from the proximal deep peroneal nerve in a clinical laryngeal hemiplegic horse showing segmental demyelination and remyelination (Type D change).

x 60





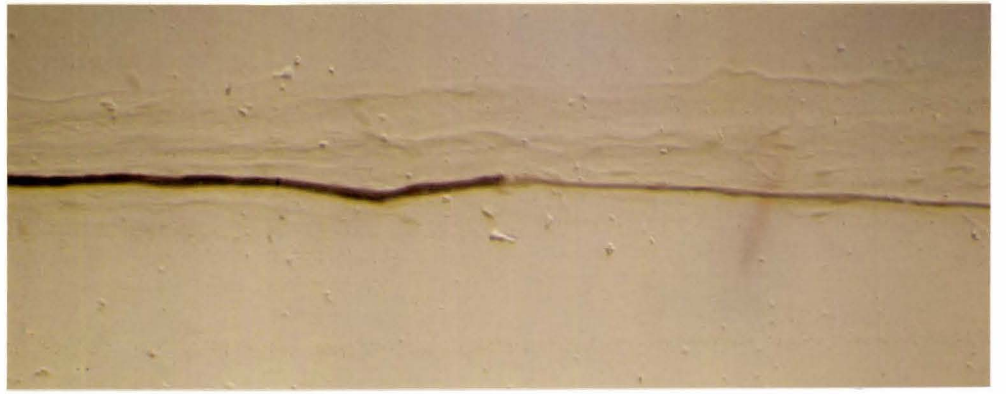
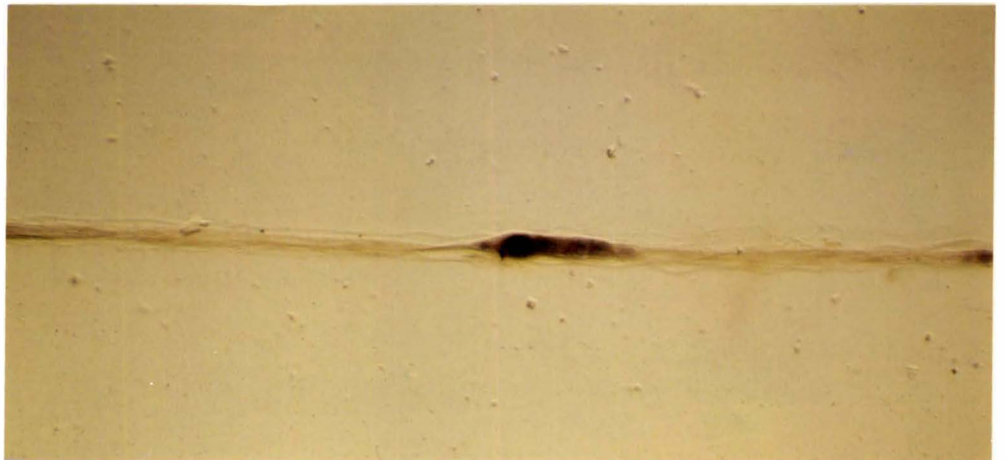
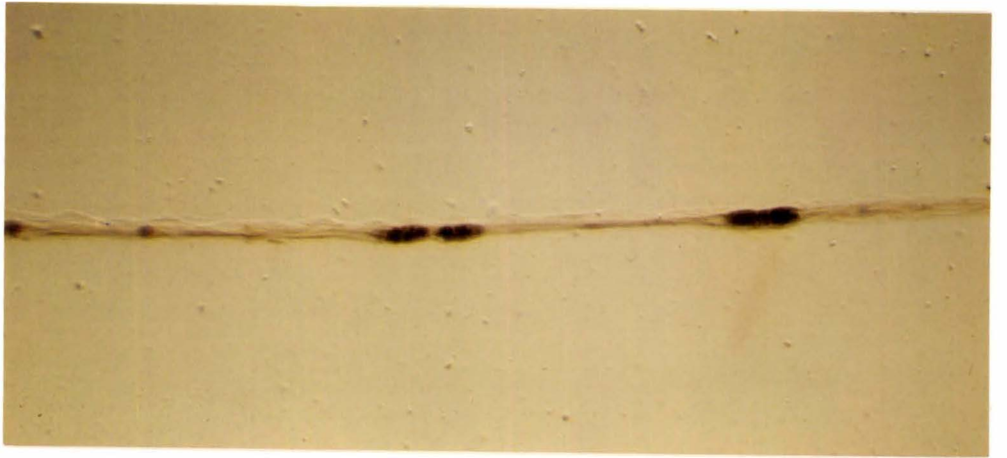


Figure 71. Photomicrographs of consecutive portions of a teased myelinated fibre from the common peroneal nerve in a clinical laryngeal hemiplegic horse showing degeneration of the nerve (Type E change).

x 60





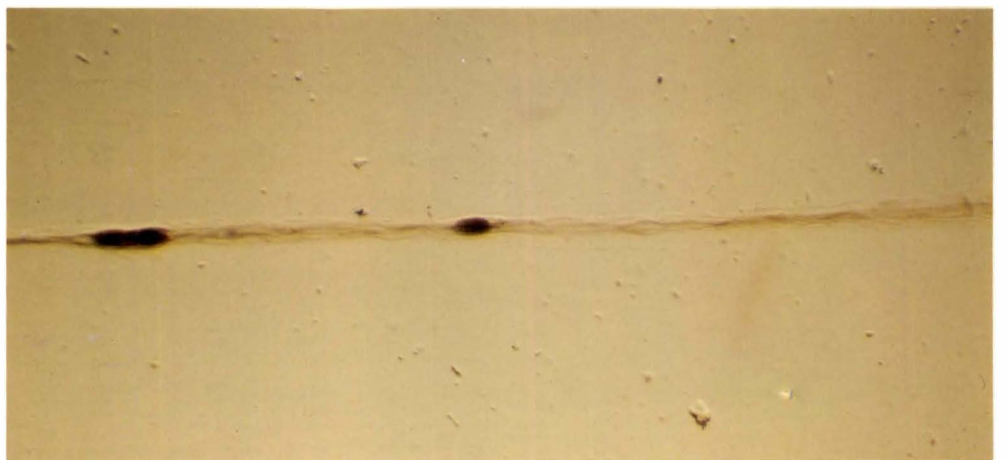


Figure 72. Photomicrographs of consecutive portions of a teased myelinated nerve fibre from the plantar nerve in a clinical laryngeal hemiplegic horse showing remyelination and short internodes (Type F change). Nodes of Ranvier are arrowed.

x 60

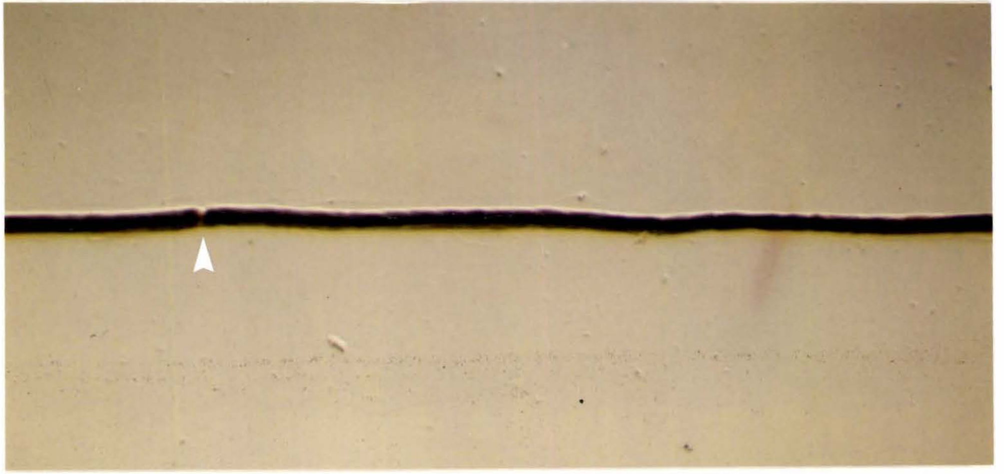
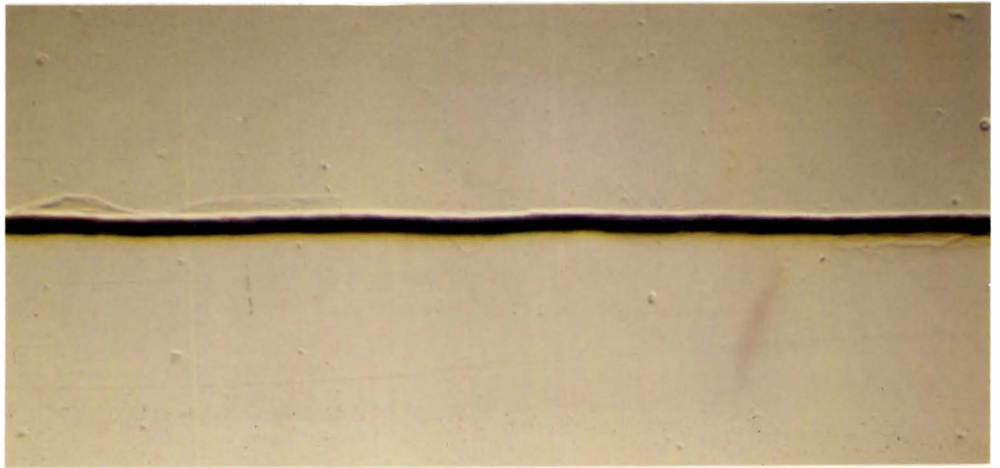
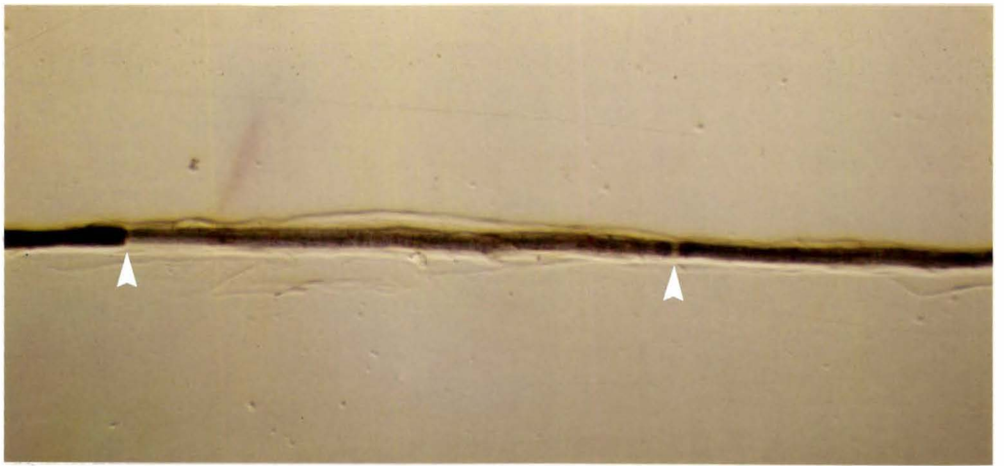
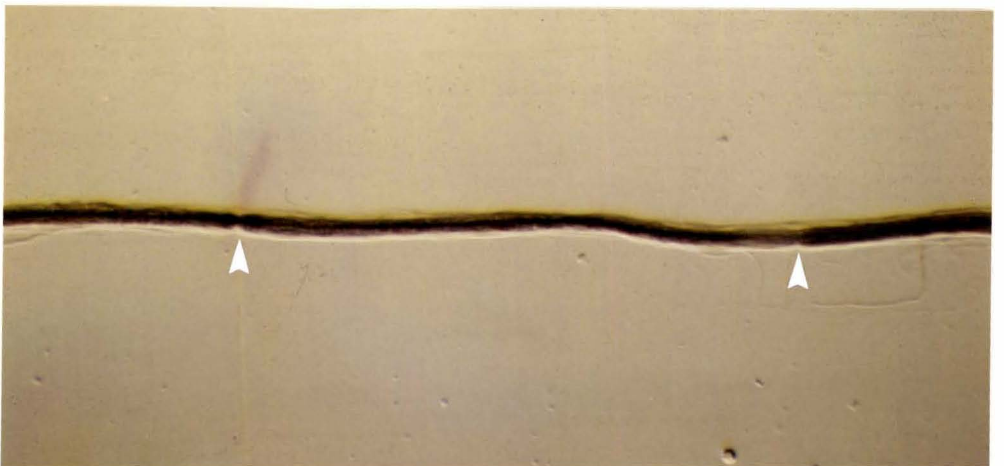





Figure 73. Photomicrographs of teased myelinated nerve fibres from clinical laryngeal hemiplegic horses, showing thickening of the myelin sheath (Type G change).

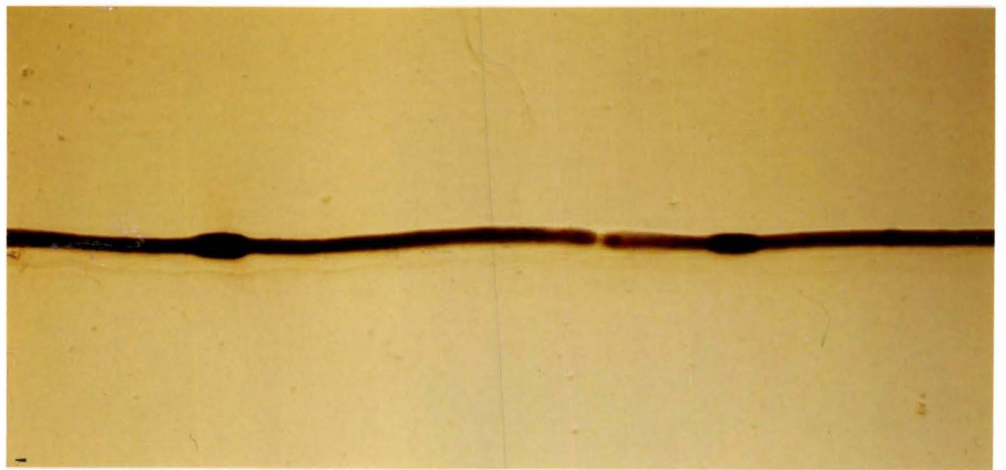
A. Type G changes in the proximal deep peroneal nerve.

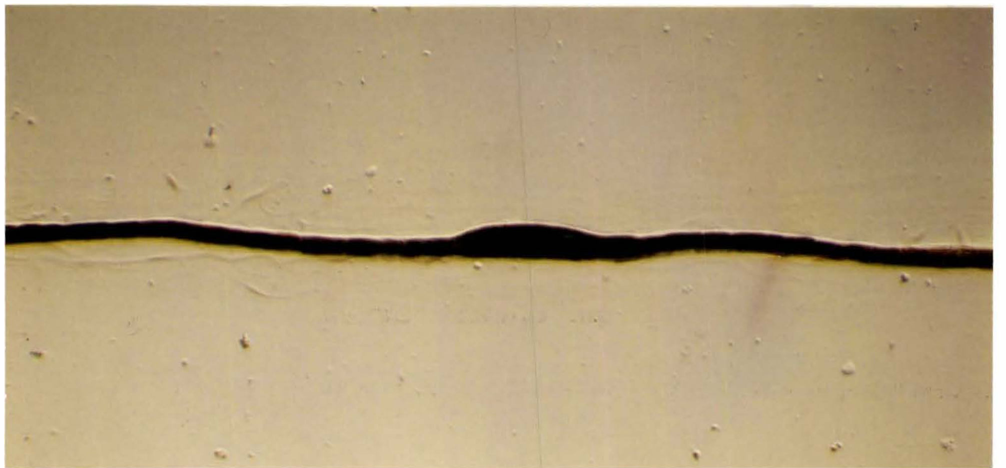
B. Type G changes in the common peroneal nerve.

x 60

Figure 74. Photomicrograph of a teased myelinated fibre from the proximal deep peroneal nerve in a clinical laryngeal hemiplegic horse showing a fibre with myelin ovoids or balls adjacent to it (Type I change).

x 60





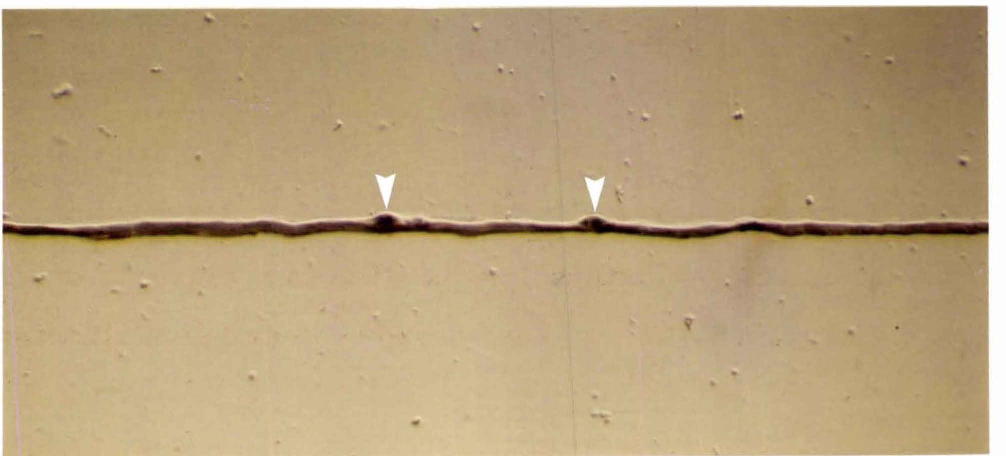


TABLE 16 The percentage of abnormal teased fibres in the limb nerves of clinical, subclinical and control horses.

	Groups of horses		
Nerve level	**Clinical**	**Subclinical**	**Control**
Common peroneal	12	4	2
Deep peroneal, proximal	20	6	4
Deep peroneal, distal	11	6	3
Tibial	7	4	11
Plantar	8	3	9
Plantar digital	23	5	9

(ii) Morphometry

Mean internode distance

The results of analysis of mean internode length at each level in 5 horses is presented in Table 17.

The small number of horses in each group made statistical analysis of teased fibre observations of little value, although the control horse had slightly shorter internode lengths than the others. For each horse there was a trend towards shorter internode lengths in the more distal portions of the nerve. However, the decrease in mean internode length for horse 15, a clinical laryngeal hemiplegic horse, was greater than the remaining horses. This was most evident in the common peroneal nerve and its deep continuation, although the mean internode length of the tibial, plantar, and plantar digital nerves, was also decreased.

TABLE 17 Mean internode length (um) in the limb nerves of a control horse, and subclinical and clinical laryngeal hemiplegic horses.

	Mean internode length				
Group	**Control**	**Subclinical**		**Clinical**	
HORSE NO:	13	16	17	15	19
Common peroneal	1105	1354	1249	1471	1268
Deep peroneal, proximal	976	1115	1316	1262	1251
Deep peroneal, distal	984	1258	1105	1081	1146
Tibial	887	1121	1200	928	1213
Plantar	860	1078	1088	826	931
Plantar digital	841	1154	980	873	1061

DISCUSSION

Individual nerve fibre pathology

The degree of individual nerve fibre changes in laryngeal hemiplegic horses, found at proximal and distal sites of the left recurrent laryngeal nerve in the present study, is similar to that recorded by other authors (Cole, 1946; Duncan, 1975; Duncan et al, 1978; Cahill, 1985; Duncan and Hammang, 1987). The presence of some abnormalities in the distal level of this nerve, in apparently normal horses, described in previous reports was also found in the present study.

There have been few reports in which modern investigative techniques have been used to study peripheral nerves in the horse, other than the recurrent laryngeal. Those reports which are available have generally examined nerve changes in specific conditions such as stringhalt (Friend and Jeffcott, 1985; Cahill et al, 1986), organophosphate toxicity (Duncan and Brook, 1985), compression of the suprascapular nerve (Duncan and Schneider, 1985), limb nerves in horses with idiopathic laryngeal hemiplegia (Cahill, 1985) or in horses with neurological disease of uncertain aetiology (Robertson-Smith et al, 1985). The ultrastructural appearance of palmar digital nerves in the horse has been examined by Henry and Diesem (1980), while more recently Wheeler (1987) reported the findings of teased fibre analysis of the palmar digital nerve of horses apparently free of clinical disease. Prior to the commencement of this study, therefore, little was known about the histological appearance of the distal hindlimb nerves in the horse.

The most noticeable feature of the cross-sectional appearance of the hind limb nerves in this study was

the obvious difference between the peroneal nerves and the tibial nerve and its branches. In the former, a wide variation in density and size of fibres existed between cross-sections and even between fascicles, irrespective of the level of nerve examined, or the disease status of the animal. In contrast, the cross-sectional appearance of the tibial nerve and its branches was much more consistent.
The large variations found in the peroneal nerves were present in all horses and were apparently unassociated with individual nerve fibre changes. For these reasons they considered to reflect the normal variation in fibre size and density for the common and deep peroneal nerves.

A possible explanation for some of the variation in fibre size and density seen in adjacent fascicles of peroneal nerves, is that they are related to the unusually high number of muscle spindles present in the muscles they innervate. A feature of these muscles, the short digital extensor and cranial tibial muscles,is the presence large numbers of these spindles. It is known that muscle spindles are innervated by large nerve fibres, which may account for the range in appearance of fibres in differing fascicles (Fernand and Young, 1951; Lewis and Ridge, 1981).

The presence of individual nerve abnormalities in apparently normal animals is similar to observations reported in other species. In man, up to 10% of fibres may be affected in people free of clinical disease (Arnold and Harriman, 1970; Stevens et al, 1973), while in the horse, one study detected an incidence of 9% of abnormal fibres (Wheeler, 1987). The aetiology of these changes in normal individuals is uncertain. The presence of regenerating clusters, onion bulbs and thinly myelinated fibres in nerves from control horses, indicated a chronic process of

continual demyelination and remyelination, and attempts at regeneration of nerve fibres (Weller and Das Gupta, 1968; Weller and Cervis-Navarro, 1977). These findings, together with the distribution of lesions, namely an increase in the severity of the changes in the most distal portions of the nerve sampled, particularly the plantar digital nerve, are consistent with the effects of a distal axonopathy (Bennington, 1978).

Of further interest in the control group was the greater incidence of individual nerve fibre changes in the plantar and plantar digital nerves, which were sampled closer to the neuronal cell body, when compared to those found in the distal end of the longer left recurrent laryngeal nerve. A feature of distal axonopathies is the close correlation between the length of the nerve and the severity of changes. However, some authors believe that total axonal volume, which is greatest in long and large diameter fibres, is of equal importance in determining the susceptibility of nerves to distal axonopathies (Spencer et al, 1979; Sumner, 1978). In the horse an increased susceptibility of long and large myelinated fibres has been demonstrated by Duncan (1975) and Cahill (1985). Therefore the apparent increased susceptibility to change of some nerve fibres in the hindlimb may be related to their size, rather than length alone. Although it is difficult to compare different techniques of measuring fibre size, it would appear that the range of fibre size in the limb nerves is greater than the recurrent laryngeal nerve, which is composed mostly of medium size fibres. Therefore, some larger fibres in the hindlimb may be affected prior to evidence of peripheral nerve disease being apparent in the recurrent laryngeal nerves. This may explain the presence of similar changes to those seen in control horses in the limb nerves of the two ponies examined, particularly as evidence of abnormalities in the

recurrent laryngeal nerves were not found in smaller horses in this present study, or in previous reports (Duncan and Griffiths, 1973; Duncan and Hammang, 1987).

The individual nerve fibre changes seen in the clinical group of horses were similar in type and distribution to those observed in control horses, although a greater frequency of these changes were found. The type of abnormality observed and their increased frequency in distal parts of the nerves and in laryngeal hemiplegic horses, seemingly indicates that the pathological process which results in this disease also involves the hind limb nerves. This finding supports the observations of Cahill (1985).

It may be argued that some of changes observed in the clinically affected animals were related to the higher average age of these horses compared to the controls. However the presence of changes in both young control and clinically affected horses indicates that aging alone is not responsible for the changes observed.

The effect of age on the incidence of nerve fibre abnormalities has been well documented in man (Lascelles and Thomas,1966; Ochoa and Mair, 1969; Arnold and Harriman, 1970; Dyck, 1975; Griffiths and Duncan, 1975; Braund et al, 1982), but is not known in horses. The nerve fibre changes associated with ageing, and their increased frequency in the more distal parts of long nerves (Cottrell,1940; Takahashi, 1964), is similar to that observed in older horses in this present study. The cause of such age-related changes is not clear. The problem is one of determining whether the changes are purely as a result of ageing or are related to disease processes which are common in old age.
An obvious difference in the frequency and distribution of individual nerve fibre changes exists

between the hind limb and left recurrent laryngeal nerves of laryngeal hemiplegic horses. In the left recurrent laryngeal nerve the extent of individual nerve fibre changes present at the proximal level was often more obvious than that found at the distal. The reason for this was that the distal samples contained such a large degree of fibre loss so that few individual nerve fibres, on which observations could be made, were present. In contrast, in the limb nerves, the highest incidence of individual nerve fibre changes was usually present in the more distal samples of the nerves examined.

A difference in the distribution of nerve fibre damage could be explained if such damage resulted from a disease process which preferentially affected long nerves. The left recurrent laryngeal nerve, since it is the longest nerve in the horses' body, would be expected to show the most destructive effects. The next longest nerve, the plantar digital, would be more severely affected than shorter nerves, for example, the peroneal. The incidence of nerve damage found in this present study closely paralleled this distribution. A complicating observation is, however, that similar changes, albeit less severe, are present in control animals. These changes, as mentioned, may also result from the effects of a distal axonopathy. This raises the questions;
a) whether the severe changes found in laryngeal hemiplegic horses are due to a single disease process affecting long nerves in the majority of horses; a process which manifests its greatest effects in laryngeal hemiplegics
or b) whether the changes in laryngeal hemiplegics are superimposed on nerves in which a disease process causing a similar distribution of lesions already exists.

Nerve fibre density

The wide variation in fibre density found between fascicles in the peroneal nerves during the individual nerve fibre study was confirmed morphometrically during nerve fibre density analysis. Such a variation in density of nerve fibres greatly increases the possibility of experimental error when assessing subtle degrees of fibre loss. For this reason it seems likely that fibre density analysis is of little value in determining small degrees of fibre loss.In contrast, the more consistent fibre density found throughout the tibial nerve and its branches allowed a more accurate evaluation of fibre loss using this technique.

The decrease in fibre density from proximal to distal in both major hind limb nerves of laryngeal hemiplegic horses and the tibial nerve and its branches may arise from branching of the nerve, or pathological loss of fibres, or a combination of both these processes. The distal decrease in fibre density in some control horses in which few or no individual nerve fibre changes could be identified, indicates that some branching of the nerves is occurring between the two sampling sites. In the laryngeal hemiplegic horses individual nerve fibre changes were observed much more frequently, indicating that pathological loss of fibres was also occurring.

The greater loss of fibres in the most distal sampling sites of the plantar and plantar digital nerves in the clinical group compared to control animals, is similar to the situation found in the left recurrent laryngeal nerve. Again this is consistent with the possibility of a similar disease process affecting both laryngeal and limb nerves. As the clinical group was, on average, older than the control animals, age related changes need to be considered as a possible cause of this fibre loss. There is, however, some disagreement in the

literature as to the effects of age on fibre density. Stevens *et al*, (1973) and Tohigi *et al* (1977) reported a decrease in fibre density with increasing age in humans, while Griffiths and Duncan (1975) and Braund *et al* (1982) could find no such association in horses and dogs respectively. The severe fibre loss seen in some young laryngeal hemiplegics suggests age is probably not the major cause of the greater decrease in fibre density in this group.

It is of interest to note in the common and deep peroneal and tibial nerves where samples were taken above the hock joint, that in most instances there was little evidence of increased fibre loss in the clinical group.

Mean individual cross-sectional axonal area

Most studies investigating peripheral nerve changes have relied on the measurement of the diameter or circumference of nerve fibres to determine fibre size. Where these techniques have included the myelin sheath, a considerable experimental variable can result from the variation in myelin thickness which occurs at various sites along the nerve (Arnold and Harriman, 1970). The use of sophisticated image analysis techniques in this present study allowed the accurate measurement of the cross-sectional axonal area of a large number of individual nerve fibres without this complication.

The proximal to distal decrease in mean cross-sectional axonal area that was identified in each group of horses may have resulted from the selective loss of larger fibres or from the tapering of individual fibres, which has been shown to occur in other species (Sunderland *et al*, 1949; Thomas, 1970; Williams and Wendell-Smith, 1971; Toft *et al*, 1988).

Loss of the larger diameter fibres could have arisen due to branching of the nerve or a disease process

which preferentially affects larger fibres. In view of the proximal to distal decrease in fibre density which was found in all groups in the present study, and the presence of individual nerve fibre pathology in many animals, even in control horses, at least part of the decrease in fibre size was probably the result of a selective damage to larger size nerve fibres. Nevertheless, the possibility exists that some tapering of fibres occurs.

The significantly smaller mean cross-sectional axonal area in the clinical animals, indicates that loss of large fibres has preferentially occurred in this group. The increased loss of larger fibres in the clinical, compared to the control group, supports the hypothesis that the disease process causing idiopathic laryngeal hemiplegia, which preferentially affects larger fibres(Duncan, 1975; Cahill, 1985) also affects the hind limb nerves.

Histographic analysis

Histographic analysis of cross-sectional fibre size clearly illustrates the progressive increase in small diameter fibres which occurs as the hindlimb nerves proceed distally. It also highlighted the relatively larger percentage of small fibres in laryngeal hemiplegic horses when compared with controls. This difference is consistent with the hypothesis that the disease process which results in laryngeal hemiplegia also affects long hindlimb nerves.

Some of the increase in percentage of small diameter fibres in clinically diseased animals may also have resulted from attempts at regeneration of nerves. Evidence of this was found during observations on individual nerve fibre pathology, particularly in the deep peroneal nerves, where regenerating clusters, each containing several small diameter fibres, were frequently observed.

Total cross-sectional axonal area

The measurement of total cross-sectional axonal area demonstrated that there is a loss of axonal substance as nerves progress distally in the hindlimb of horses. It also showed that this loss is greatest in laryngeal hemiplegic horses in the distal portions of their nerves. Possible reasons for this difference between control and clinical horses has already been discussed.

The errors in the individual measurement of fibre size or density, for the purpose of detecting overall or selective loss of nerve fibres, are greatest in nerves in which wide variations in these are found between fascicles. Using measurement of total cross-sectional axonal area, however, these errors can be minimised. For this reason the usefulness of this measurement is greatest in nerves where considerable variability in fibre size and density exists. This present study established that calculation of total cross-sectional area was of greatest value in such nerves and confirmed that a proximal to distal decrease, which was greatest in laryngeal hemiplegic horses, was present. A similar method was used by Quilliam (1956) to evaluate the possibility of tapering of nerve fibres, but has not previously been reported in studies of equine nerves.

Calculation of total cross-sectional axonal area in the tibial nerve and its branches confirmed the previous results of a decrease in both nerve density and mean fibre area from proximal to distal sites of sampling.

In both the common peroneal and tibial nerve trunks, the greater decrease in total axonal area in clinical compared with control horses confirms previous findings regarding the effect of idiopathic laryngeal hemiplegia on limb nerves.

Teased fibre examination

(a) Morphology

Although teased fibre analysis was undertaken in only a small number of horses, it provided further insight into the nature of the abnormalities observed in transverse sections of control, subclinical and clinical animals.

The presence of pathological abnormalities in teased fibre preparation of distal limb nerves in control animals in this study is in agreement with the findings of Wheeler (1987), who demonstrated that approximately 9% of fibres in the palmar nerve of apparently normal horses had signs of pathology. This level is slightly higher than that found at most levels of nerves examined in the one control horse in this present study, except for the tibial and plantar nerve samples, which showed obvious pathological changes on both teased fibre and transverse sections. The higher incidence of abnormalities observed by Wheeler (1987) may have been a result of sampling older horses, or the presence of a peripheral nerve disease, particularly idiopathic laryngeal hemiplegia, as the horses were not examined for this.

The predominant changes of segmental demyelination and remyelination, variability in myelin thickness and the actively degenerating fibres in clinical and subclinical horses, and an increase in the severity of these changes from proximal to distal, is consistent with primary axonal damage, and secondary segmental demyelination. The increased incidence of these teased fibre abnormalities in the two clinical horses further confirms the previous findings in this study of the effects of the disease causing laryngeal hemiplegia on the limb nerves.

(b) Mean internode length

The results of morphometric analysis of individual teased fibres indicated a proximal to distal decrease

in internode length, which is in agreement with the finding of Cahill (1985),who suggested in an examination of equine recurrent laryngeal nerves, that this may result from either tapering of nerve fibres; the presence of intercalated internodes; regenerated fibres with uniformly short internodes; or the selective loss of larger diameter fibres. The results of morphological and morphometric examination of transverse sections of these nerves in this present study indicates the most likely cause is a combination of loss of larger fibres, as well the presence of regenerated fibres.

PART IV CONCLUSIONS

Muscle Morphology

1. As previous researchers have found, the left dorsal and lateral cricoarytenoid muscles in idiopathic laryngeal hemiplegic horses show obvious pathological changes indicative of neurogenic muscle disease.

2. The deep digital flexor muscle frequently contains morphological evidence of pathological change. These changes are more common in the distal areas of the muscle and are of increased frequency and severity in laryngeal hemiplegic horses.

3. The morphological appearance of the short digital extensor muscle is different to other muscles examined. It contains a smaller number of fibres in each fascicle compared to more proximal muscles, and a higher frequency of muscle spindles. Pathological changes are frequently seen in this muscle. These changes are seen more commonly and are more severe in laryngeal hemiplegic horses.

4. Morphological evidence of pathological change is seldom observed in the cranial tibial, long digital extensor and lateral digital extensor muscles of normal horses or those with laryngeal hemiplegia.

Muscle Morphometry

5. In the deep digital flexor muscle there is a proximal to distal increase in the proportion of type 1 fibres.
6. In the cranial tibial muscle there is a proximal to distal decrease in the proportion of type 1 fibres. There is also wide variation in the

percentage of each fibre type in different areas of this muscle.

7. The short digital extensor muscle contains mostly type 1 fibres, and in some horses no type 2 fibres are present. There is a wide variation in the percentage of fibre types between horses in this muscle.

8. The fibre type proportions in the long and lateral digital extensor muscles are not influenced by the disease process which causes laryngeal hemiplegia. In fact very little variation in fibre type proportions exists between horses in these muscles. However, a lower percentage of type 1 fibres is present in the caudal and lateral areas compared to the medial area of the long digital extensor muscle.

Muscle Fibre Diameter

9. In all muscles examined, laryngeal hemiplegics have more smaller and larger fibres than control horses, indicating an increased number of atrophic and hypertrophic fibres. This is most evident in the deep digital flexor muscle.

Individual Nerve Fibre Pathology

10. There is minimal evidence of nerve fibre abnormalities in the common peroneal nerve. However, changes are observed more frequently in the distal continuation of this nerve, the deep peroneal. These changes are more common in clinical laryngeal hemiplegic horses, most of which have abnormalities of increased severity and frequency compared to normal horses, in the distal portion of this nerve.

11. Abnormalities are present in the proximal portions of the tibial, plantar, and plantar digital nerves in most horses. In the plantar and plantar digital nerves these changes are most severe and include the presence of regenerating clusters, degenerating fibres, onion bulb formation, and thinly myelinated fibres. In the plantar digital nerve these changes are more severe in clinical than control horses.

Nerve Fibre Density

12. The common and deep peroneal nerves contain two distinct populations of nerve fibres. Although consistent throughout single fascicles, adjacent fascicles often contain different sizes and densities of fibres.

13. The tibial nerve and its continuation is consistent in its fibre density appearance. It has a proximal to distal decrease in fibre density.

Nerve Fibre Size

14. In control horses there is a proximal to distal increase in the proportion of smaller fibres in the common and deep peroneal nerves. A similar, but lesser increase is present in the tibial nerve and its continuation.

15. There is a selective loss of larger myelinated fibres in the common and deep peroneal nerves in clinical laryngeal hemiplegic horses.

16. There is a selective loss of larger myelinated fibres in the tibial nerve in clinical laryngeal hemiplegic horses. A similar, but less obvious, loss is also present in the plantar and plantar digital nerves.

17. There is a proximal to distal decrease in the total cross-sectional axonal area in both the peroneal and tibial nerve trunks. The total axonal volume at all levels in each nerve was less in clinical than control horses in this study.

Teased Fibres

18. Teased fibre abnormalities are often seen in the distal limb nerves of subclinical and clinical laryngeal hemiplegic horses, but an insufficient number of control horses for comparison were examined.

General Conclusions

19. Abnormalities indicative of neurogenic disease are present in the hindlimb muscles, particularly the deep digital flexor and short digital extensor, in many horses which appear free of clinical signs of disease.

20. Considerable variation exists in the proportion of type 1 fibres in some limb muscles, and these proportions are affected by the site of sampling, the age of the horse and the muscle sampled. The greatest variation is found in the deep digital flexor and cranial tibial muscles, while lesser degrees of variation is present in the long digital extensor. The variation in proportion of type 1 fibres is least in the lateral digital extensor.

21. Abnormalities are present in the distal limb nerves in many apparently normal horses. These abnormalities include the presence of thinly myelinated fibres, regenerating clusters, onion bulbs, degenerating fibres, and signs of demyelination and remyelination.

22. Abnormalities are also present in the distal limb nerves in laryngeal hemiplegic horses, and are similar to those identified in normal horses. However, the frequency and severity of the changes observed in laryngeal hemiplegics was much greater than in controls.

23. The nature and distribution of the abnormalities in the distal hindlimb nerves of horses with idiopathic laryngeal hemiplegia are similar, but less severe, than those in their recurrent laryngeal nerves. Therefore, it is possible that the distal axonopathy preferentially affecting the large nerve fibres which results in this disease involves the distal hindlimb nerves as well as the recurrent laryngeals.

24. It could not be resolved from this study if a single disease process was affecting both normal and laryngeal hemiplegic horses. It may be that the abnormalities in apparently normal horses have the same aetiology as in laryngeal hemiplegics, although because of a combination of factors, which may include the size of the horse, its sex, nutritional status and genetic predisposition, the disease does not progress to become clinically apparent.

    Alternatively, peripheral nerve disease in normal horses may have a different aetiology to that which causes the idiopathic form of laryngeal hemiplegia. As such, the abnormalities which were identified in the hindlimb nerves in laryngeal hemiplegic horses, may be in addition to those identified in normal horses.

25. The effect on performance of these abnormalities in the distal hindlimb muscles and nerves could not be accurately determined from this study.

In view of the changes observed, it would seem unlikely they would result in clinical abnormalities in younger horses. However, as idiopathic laryngeal hemiplegia is a chronic, progressive disease, it may be that older horses could possible show some clinical evidence of this disease process.

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APPENDIX 1

STAINING METHODS FOR MUSCLE TISSUE

Myosin ATPase
(according to the technique of Padykula and Herman, 1955, as modified by Davies and Gunn, 1972).

SOLUTION1

Substrate

1.0M tris (hydroxymethyl) aminomethane[1]	12ml
0.18M calcium chloride (hexahydrate)[2]	6ml
ATP disodium salt[3]	90mg
distilled water	to 45ml
Adjust the pH to 9.5 with 0.1N hydrochloric acid	
distilled water	to 60ml

SOLUTION 2

Cacodylate buffered formaldehyde pH 7

2.14g sodium cacodylate (trihydrate) in 50ml distilled water	
0.2M hydrochloric acid	6.3ml
40% formaldehyde	20ml
distilled water	to 200ml

SOLUTION 3

2% cobalt chloride[4]

SOLUTION 4

1% ammonium sulphide[5]

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1. Tris (hydroxymethyl) aminomethane, Sigma Chemical Co.,, St Louis, USA.
2. Calcium chloride, hexahydrate, BDH Chemicals Ltd, Poole, England.
3. Adenosine-5'-triphosphone acid, BDH Chemicals Ltd, Poole, England.
4. Cobalt chloride, Koch-Light Laboratories Ltd, Buckinghamshire, England.
5. Ammonium sulphide 10%, Ajax Chemicals, Sydney, Australia.

Method

1. Place sections in solution 2 for two minutes
2. Wash in two changes of distilled water.
3. Incubate in solution 1 for 20 minutes at 37°C.
4. Wash in two changes of distilled water.
5. Place the sections in solution 3 for three minutes.
6. Wash in two changes of distilled water.
7. Place the sections in solution 4 for 30 seconds.
8. Wash and mount in glycerine jelly.

Haemotoxylin and eosin (Culling, 1974)

1. Fix in 70% alcohol.
2. Wash.
3. Stain in Ehrlich's haematoxylin[1] for 10 minutes.
4. Rinse in tapwater to remove excess stain.
5. Differential in acid alcohol for 10 sconds.
6. Regain blue colour and stop decolourisation by washing in Scott's tapwater for 2 minutes.
7. Rinse in tapwater.
8. Stain in eosin[2] for 40 seconds.
9. Rinse rapidly in water.
10. Dehydrate in alcohols.
11. Clear in xylol.
12. Mount.[3]

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1. Haematoxylin, May & Baker Ltd, Dagenham, England.
2. Eosin, Harleco, Philadelphia, USA.
3. DPX mountant, BDH Chemicals Ltd, Poole, England

APPENDIX 2

COMPOSITION OF SOLUTIONS FOR PROCESSING NERVE SAMPLES

0.1M cacodylate buffer

21.4g sodium cacodylate trihydrate[1]
986ml distilled water
14ml 0.2M hydrochloric acid

Adjust pH to 7.35 - 7.4 with hydrochloric acid or sodium hydroxide.

2% glutaraldehyde

80ml 25% glutaraldehyde[2]
920ml 0.1M cacodylate buffer

Adjust pH to 7.35 - 7.4 with 0.1M hydrochloric acid or 0.1M sodium hydroxide.

1% osmium tetroxide

1g osmium tetroxide[3]
100ml 0.1M cacodylate buffer

1% phenylenediamine

0.1g p-phenylenediamine[4]
10ml 70% alcohol

66% glycerol

165ml 99% glycerol[5]
85ml distilled water

---

1. Sodium cacodylate, BDH Chemicals Ltd, Poole, England.
2. Glutaraldehyde, 25% solution in water, Koch-Light Laboratories Ltd, Buckingham, England.
3. Osmium tetroxide, Sigma Chemical Co., Missouri, USA.
4. P-phenylenediamine grade 2, Sigma Chemical Co., Missouri, USA.
5. Glycerol, Ajax Chemicals, Sydney, Australia

APPENDIX 3

COMPOSITION OF EMBEDDING RESIN USED IN PROCESSING NERVE SAMPLES

SOLUTION A

polarbed 812 resin[1]	62ml
dodecenyl succinic anhydride (DDSA)[2]	100ml

SOLUTION B

polarbed 812 resin	100ml
methyl nadic anhydride (MNA)3	89ml

RESIN FOR EMBEDDING

solution A	25ml
solution B	75ml
2,4,6-tri (dimethylaminomethyl) phenol[4] (DMP-30)	2ml

Solutions A and B, and the final resin were all prepared in advance and stored frozen. When required for processing, the polar bed was removed from the freezer 2-3 hours before it was required.

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1. Epon 812 resin or Polarbed 812 resin, Polaron Equipment Ltd, Watford, England.
2. DDSA, Polaron Equipment Ltd, Watford, England.
3. MNA, Polaron Equipment Ltd, Watford, England.
4. DMP-30, Polaron Equipment Ltd, Watford, England.

APPENDIX 4

Morphological grading of muscle samples from the laryngeal muscles and various sites in the limb muscles of all horses examined

Horse No.	1		2		3		4		5		6		7	
Leg Examined	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Muscle and Site														
Lateral cricoarytenoid	+	0	++++	+	+	++	++++	0/+	+	0	++/+++	+	++++	0
Dorsal cricoarytenoid	+	0	-	+	+	-	+++	0	0/+	0	+	+	+++	0
Deep digital flexor,														
proximal	0	0	0	0	0	0	0	0	0	0	0	+/++	+	0
middle	0	0	0	0	0	0/+	0	-	0/+	0		0	0	0
distal	0	++	0	0	0	0	+/++	++	0/+	+	+	-	+	+
lateral					0	0/+			0	0				
cranial					+	0/+			0	0				
caudal					0	0			0	-				
medial					0	0			0/+	0				
Cranial tibial,														
proximal	0	0	0	0	0	0	0	0/+	0	0	0	0	0/+	0/+
middle	0	0	0	0	0/+	0/+	0	0(++)	0/+	++	0	0	0	0
distal	0	0	0	0	0	0	0	0	0/+	0	-	0	0	0
lateral					0	0			0	0				
cranial					0	0			0	0				
caudal					0	0/+			0	0				
medial					0	0			0	0				
Long digital extensor,														
proximal	0	0	0	0	0	0/+	0	0	0	0	0	0	0/+	0(++)
middle	0	0	0	0	0	0	0	0	0	0	0	0	0	0
distal	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lateral					0	0			0	0				
cranial					0	0			0	0				
caudal					0	0			0	0				
medial					0	0			0	0				
Lateral digital extensor,														
proximal	0	0	0(+)	0	0	0/+	+	0	0	0	-	0	0	0
middle	0	0	0	0	0	0	0	0	0	0	0	0	0	0
distal	0	0	0/+	0	0	0	-	0	0/+	0	0	0	0	0
lateral					0	0			0	0				
cranial					0	0			0	0				
craudal					0	0			0	0				
medial					0	0			0	0				
Short digital extensor	+	+	0	0	0	++	+++	++	+	0	0/+	0	0	0

(table continued over page)

Key

0	No pathology
+	Mild pathology
+ +	Moderate pathology
+ + +	Marked pathology
+ + + +	Severe pathology
0(+,++)	No pathology in most of the sample, but some changes confined to a small area of muscle

Appendix 4 (continued)

Horse No.	8		9		10		11		12		13		14	
Leg Examined	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Muscle and Site														
Lateral cricoarytenoid	0	0/+	++++	++	+	0/+	0	0	0	0	0	0	++++	+
Dorsal cricoarytenoid	0	0/+	+++	0	0/+	0/+	0	0	0	0	0/+	0		
Deep digital flexor														
proximal	0	0	+	0/+	0	0	0	0	0	0	0	0		
middle	0	0	+	++/++	0	0	0(+)	0	0	0	0	0	0	0
distal	+	+	++	+++	0/+	0/+	0	0/+	0	0	++/+++	+++		
lateral					0	0	0	0			0(+)	0		
cranial					0/+	0/+	+	+			++	+		
caudal					0	0	0	0			0	0		
medial					0	0	0	+			0	0		
Cranial tibial,														
proximal	0	0	0	0(+)	0	0	0	0	0	0	-	0		
middle	0	0	+/++	+	0	0	+	++	0	0	0	0	0	0
distal	0	0	0	0	0	0	0	0	0	0	0	0(+)		
lateral					0	0	0/+	0			0	0		
cranial					0	0	0	0(+)			0	0(+)		
caudal														
medial														
Long digital extensor,														
proximal	0	0(++)	0/+	0	0	0	0	0	0(+++)	0	0	0		
middle	0	0	0	0	0	0	0	0	0(+++)	0	0	0	0	0
distal	0	0	0	0	0	0	0	0	0	0	0	0		
lateral					0	0	0	0			0	0		
cranial					0	0	0	0			0	0		
caudal					0	0	0	0			0	0		
medial					0	0	0	0			0	0		
Lateral digital extensor,														
proximal	0	0	0	0	0	0/+	0	-	0	0	0	0		
middle	0	0	0	0	0	0	0	0	0	0	0	0	0	0
distal	0	0	0	0	0	0	0	0	0	0	0	0		
lateral					0	0	0	0			0	0/+		
cranial					0/+	0	0	0			0	0		
caudal					0	0	0	0			0	0		
medial					0	0	0	0			0	0		
Short digital extensor	0	0(+++)	++/+++	+++	+	0	+	0/+	0	0	0	0	0	++++

(table continued over page)

Appendix 4 (continued)

Horse No.	15		16		17		18	
Leg Examined	Left	Right	Left	Right	Left	Right	Left	Right
Muscle and Site								
Lateral cricoarytenoid	++++	+++	++	+/++	+	0	0	0
Dorsal cricoarytenoid	++	0/+	+/++	0	0	-	0	0
Deep digital flexor,								
proximal	0(+)	0/+	0	0	0	0	0	0(+)
middle	++	++	0(+)	0	0	0	0	0
distal	++	++/+++	+	+	+	++	0	0
lateral							0/+	0
cranial							+/++	0
caudal							0	-
medial							0	+/++
Cranial tibial,								
proximal	0	0	0	0	0(+)	0	0	0
middle	0(+)	0	0	0	0	0	0	0
distal	0	0	-	0	0	0/+	0	0
lateral							0	0
cranial							0	0
caudal							0	0
medial							0	0
Long digital extensor,								
proximal	0	0	++	0(+)	0	0	0	0
middle	0	0	0	++	0	0	0	0
distal	0	0	0	0	0	0	0	0
lateral							0	0
cranial							0	0
caudal							0	0
medial							0	0
Lateral digital extensor,								
proximal	0	0	0	0	0	0	0	0
middle	0	0	0	0	0	0	0	0
distal	0	0	0	0	0	0	0	0
lateral							0	0
cranial							0	0
caudal							0	0
medial							0	0
Short digital extensor	0(+)	0	+/++	+	0	0	+/++	0

Horse No.	19		20		21	
Leg Examined	Left	Right	Left	Right	Left	Right
Muscle and Site						
Lateral cricoarytenoid	++++	0	0	0	++++	+
Dorsal cricoarytenoid	+++/++++	0	0	0	+++	+
Deep digital flexor,						
proximal	0	0	0	0		
middle	0	0	0/+	-		0
distal	+	++/+++	+/++	0		
lateral			0	+		
cranial			-	++		
caudal			0	0		
medial			0	0		
Cranial tibial,						
proximal	0	0	0	0		
middle	0	0	0	0		0
distal	0	0	0	++		
lateral						
cranial						
caudal						
medial						
Long digital extensor,						
proximal	0(+)	0	0	0		
middle	0	0	0	0		0
distal	0	0	0	0		
lateral						
cranial						
caudal						
medial						
Lateral digital extensor,						
proximal	0	0	0	0		
middle	0	0	0	0		0
distal	0	0	0	0		
lateral						
cranial						
caudal						
medial						
Short digital extensor	0	0	0	0		0/+

APPENDIX 5

The difference in the percentage of type 1 fibres when either 200 or 1000 muscle fibres were counted

	Percentage type 1 fibres							
Horse Number	1				2			
Number of fibres counted	200		1000		200		1000	
Leg Examined	Left	Right	Left	Right	Left	Right	Left	Right
Muscle								
Deep digital flexor								
proximal	11	9	12	19	1	7	6	9
middle	15	33	17	31	39	47	35	38
distal	26	32	27	31	34	44	37	54
Cranial tibial								
proximal	52	72	52	74	70	70	69	74
middle	69	34	69	30	61	47	60	57
distal	43	15	37	16	24	35	28	31
Long digital extensor								
proximal	20	15	19	15	26	38	30	35
middle	23	23	23	24	30	25	25	27
distal	21	23	23	22	20	31	28	28
Lateral digital extensor								
proximal	19	21	21	18	13	21	12	30
middle	-	30	-	32	21	29	20	34
distal	34	25	31	25	21	42	18	47
Short digital extensor	86	88	91	92	100	95	97	93

APPENDIX 6

The mean percentage of type 1 fibres in the laryngeal muscles and in various sites of the limb muscles in all horses examined

Horse No.	1		2		3		4		5		6		7	
Leg Examined	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Muscle and Site														
Lateral cricoarytenoid	20	17	4	19	25	10	28	29	29	28	29	36	-	23
Dorsal cricoarytenoid	32	27	-	44	30	-	47	44	21	34	44	28	27	32
Deep digital flexor,														
proximal	11	9	1	7	5	33	13	2	21	23	16	42	44	14
middle	15	33	39	47	15	33	26	5	25	31	-	38	35	33
distal	26	32	34	44	26	3	22	63	-	37	40	-	42	47
lateral					44	49			40	42				
cranial					48	52			19	-				
caudal					11	12			39	-				
medial					31	33			28	35				
Cranial tibial,														
proximal	52	72	70	70	51	59	69	43	-	60	49	65	62	60
middle	69	34	61	47	12	15	58	61	29	33	48	64	40	57
distal	43	15	24	35	5	13	29	31	21	18	-	38	33	6
lateral					39	50			40	50				
cranial					60	79			66	69				
caudal														
medial														
Long digital extensor,														
proximal	20	15	26	38	15	11	37	28	23	19	29	13	21	15
middle	23	23	30	25	16	16	29	34	10	21	28	16	16	19
distal	21	23	20	31	7	10	25	28	24	24	24	18	15	20
lateral					16	13			21	23				
cranial					23	15								
caudal					20	14								
medial					23	23			25	23				
Lateral digital extensor,														
proximal	19	21	13	21	15	18	45	28	20	-	30	29	35	25
middle	—	30	21	29	30	12	30	27	28	-	30	33	28	29
distal	34	25	21	42	21	14	35	25	34	27	24	-	28	25
lateral					25	25			22	30				
cranial					21	21								
craudal					31	24								
medial					31	32			27	40				
Short digital extensor	96	88	99	98	54	56	79	74	70	64	47	54	75	42

(table continued over page)

Appendix 6 (continued)

Horse No.	8		9		10		11		12		13		14	
Leg Examined	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Muscle and Site														
Lateral cricoarytenoid	37	18	22	6	16	22	22	24	20	24	27	42	15	31
Dorsal cricoarytenoid	20	30	58	33	25	28	30	31	21	24	29	51		
Deep digital flexor														
proximal	20	25	21	19	13	34	23	40	5	9	45	42		
middle	25	27	45	26	31	47	35	47	10	37	42	42	24	46
distal	42	38	36	45	33	38	39	39	43	43	41	39		
lateral					40	56	43	37			46	44		
cranial					56	47	48	42			49	54		
caudal					7	38	16	19			24	30		
medial						13	26	48			32	37		
Cranial tibial,														
proximal	62	66	71	61	60	75	70	67	17	29	68	78		
middle	55	64	52	50	69	35	65	68	21	39	70	66	38	39
distal	44	29	25	35	33	26	33	17	16	13	28	31		
lateral					58	33	71	62			36	-		
cranial					66	70	73	78			60	74		
caudal														
medial														
Loong digital extensor,														
proximal	20	-	6	14	18	17	24	21	17	24	17	19		
middle	24	26	17	23	30	22	37	33	25	26	24	33	15	23
distal	34	21	20	17	22	28	25	21	26	25	21	26		
lateral					25	17	16	31			24	21		
cranial					22	26	32	22			22	22		
caudal					12	16	25	23			27	22		
medial					30	26	24	30			33	28		
Lateral digital extensor,														
proximal	29	44	19	22	23	28	37	-	18	26	36	26		
middle	23	30	17	32	21	30	26	27	16	24	29	29	21	21
distal	23	34	12	32	30	29	23	31	31	30	32	27		
lateral					26	24	26	25			30	33		
cranial					34	23	31	25			26	39		
caudal					29	25	31	19			28	30		
medial					15	29	31	46			27	20		
Short digital extensor	59	58	78	85	100	97	64	71	99	100	59	72	75	48

(table continued over page)

Appendix 6 (continued)

Horse No.	15		16		17		18		19		20		21	
Leg Examined	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Muscle and Site														
Lateral cricoarytenoid	47	39	29	28	31	25	32	67	-	20	20	24	-	24
Dorsal cricoarytenoid	46	23	30	24	18	31	47	61	79	64	21	30	39	41
Deep digital flexor,														
proximal	31	37	17	39	44	22	47	46	23	36	41	35		
middle	37	39	43	25	39	34	48	57	34	40	43	-		46
distal	59	48	46	52	49	34	45	44	48	32	43	34		
lateral							46	39			41	55		
cranial							61	-			-	51		
caudal							21	18			13	12		
medial							43	15			2	43		
Cranial tibial,														
proximal	79	87	63	52	60	64	47	48	65	57	65	62		
middle	73	39	57	48	30	59	32	28	59	46	61	46		46
distal	16	26	-	28	22	64	31	17	22	13	32	33		
lateral														
cranial														
caudal														
medial														
Long digital extensor,														
proximal	17	13	22	30	32	17	34	18	27	21	24	23		
middle	20	21	34	34	20	24	27	23	21	32	28	22		23
distal	21	15	25	16	-	27	31	20	18	26	31	23		
lateral														
cranial														
caudal														
medial														
Lateral digital extensor,														
proximal	27	20	44	25	28	30	21	24	25	26	27	33		
middle	23	20	27	21	26	23	23	23	27	15	32	15		19
distal	19	25	36	19	-	27	33	30	29	27	30	23		
lateral														
cranial														
caudal														
medial														
Short digital extensor	57	46	75	62	48	62	92	68	63	54	67	53		41

APPENDIX 7

Mean muscle fibre diameter of type 2 and type 1 fibres in the laryngeal and limb muscles examined

Muscle		LCAM		DCAM	DDF		CT		LGDE		LTDE		SDE	
Leg Examined		Left	Right	Left	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Horse No.	Fibre Type													
2	Type 2	54	40	-	-	43	53	48	42	48	54	48	-	-
	Type 1	74	39	-	-	40	38	41	34	35	36	35	-	-
3	Type 2	47	41	47	46	41	43	47	40	46	48	56	36	34
	Type 1	44	45	48	48	41	39	68	43	44	40	45	35	35
4	Type 2	35	44	35	48	-	49	48	59	58	65	43	33	-
	Type 1	62	44	52	34		43	44	42	39	46	37	31	-
5	Type 2	42	53	45	46	46	43	45	48	45	52	-	31	40
	Type 1	48	47	51	52	44	46	42	43	41	33	-	37	34
6	Type 2	57	35	39		37	47	41	44	44	46	40	30	30
	Type 1	55	35	49	-	36	42	41	34	34	39	31	32	30
7	Type 2	-	43	28	50	52	60	-	54	50	62	52	32	37
	Type 1	-	39	47	54	49	47	57	48	42	49	37	32	33
8	Type 2	43	35	43	57	63	48	44	61	61	53	48	38	40
	Type 1	47	37	42	54	55	46	43	47	53	38	45	34	33
9	Type 2	23	47	30	49	50	52	48	52	49	52	62	50	14
	Type 1	31	34	29	46	33	47	49	30	36	18	32	35	22
10	Type 2	48	41	44	47	44	44	44	55	50	50	51	47	-
	Type 1	43	40	46	51	51	46	46	45	43	44	42	42	-
11	Type 2	43	41	50	40	-	53	48	54	53	52	44	45	44
	Type 1	35	35	49	48	-	52	46	41	41	38	33	41	36
12	Type 2	28	31	42	32	34	39	33	32	42	35	-	-	
	Type 1		20	19	26	28	29	29	23	33	25	23	51	45
13	Type 2	47	16	40	47	50	47	52	53	56	53	52	30	27
	Type 1	38	49	48	44	51	39	36	42	-	46	44	27	27
14	Type 2	21	42	48	48	50	48	46	51	53	50	50	38	36
	Type 1	42	43	46	49	55	46	43	46	44	45	39	39	31
15	Type 2	23	49	27	42	49	63	63	57	60	57	60	51	45
	Type 1	40	50	53	61	65	58	63	49	57	49	57	42	38
16	Type 2	41	44	47	40	45	45	43	40	42	45	43	36	-
	Type 1	45	46	47	44	37	36	-	34	-	25	28	35	-
17	Type 2	46	47	38	45	41	50	43	44	57	-	-	38	41
	Type 1	51	41	41	53	47	52	46	38	43	-	-	41	40
18	Type 2	55	57	48	39	48	63	64	66	64	65	45	51	
	Type 1		45	50	51	51	52	49	50	45	51	43	41	44
19	Type 2	12	49	25	54	52	46	52	50	54	58	-	31	42
	Type 1		50	59	57	56	46	45	44	45	43	-		
20	Type 2	46	43	-	-	55	51	57	53	50	53	55	39	
	Type 1		39	37	-	-	45	46	42	44	38	35	47	31
21	Type 2	5	47	32	43	-	49		43		51			
	Type 1	-	35	40	45	-	42		33		43		33	

KEY: LCAM - lateral cricoarytenoid; DCAM - dorsal cricoarytenoid; DDF - deep digital flexor; CT - cranial tibial; LGDE - long digital extensor; LTDE - lateral digital extensor; SDE - short digital extensor

APPENDIX 8

Atrophy and hypertrophy table: The multiplication factors and mean fibre diameters from control horses used to calculate the degree of atrophy and hypertrophy of muscle fibres using the method of Brooke and Engel (1969).

TYPE 2 FIBRES

Multiplication Factors	x4	x3	x2	x1	0 Normal	x1	x2	x3	x4
Muscle									
Deep digital flexor	15<	20	25	30	35 - 55	60	65	70	>75
Cranial tibial	15<	20	25	30	35 - 55	60	65	70	>75
Long digital flexor	15<	20	25	30	35 - 65	70	75	80	>85
Lateral digital flexor	20<	25	30	35	40 - 60	65	70	75	>80
Short digital extensor	5<	10	15	20	25 - 50	55	60	65	>70

TYPE 1 FIBRES

Multiplication Factors	x4	x3	x2	x1	0 Normal	x1	x2	x3	x4
Muscle									
Deep digital flexor	20<	25	30	35	40 - 60	65	70	75	>80
Cranial tibial	15<	20	25	30	35 - 55	60	65	70	>75
Long digital flexor	15<	20	25	30	35 - 55	60	65	70	>75
Lateral digital flexor	10<	15	20	25	30 - 50	55	60	65	>70
Short digital flexor	5<	10	15	20	25 - 50	55	60	65	>70

APPENDIX 9

Atrophy and hypertrophy factors for the limb muscles and calculated using data from Appendix 8

Muscle		DDF				CT				LGDE				LTDE				SDE			
Fibre Type		Type 1		Type 2		Type 1		Type 2		Type 1		Type 2		Type 1		Type 2		Type 1		Type 2	
Leg Examined		L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
Horse No.																					
2	A	35		17		16	17	0	4	37	27	10	10	5	10	2	15				
	H	1		4		1	4	34	20	0	0	0	3	0	0	8	0				
3	A	7	25	5	12	13	1	8	11	10	13	52	2	1	4	10	12	1	3	2	3
	H	3	0	8	0	1	111	1	17	2	4	9	0	4	11	0	27	4	4	2	0
4	A	59		7		5	1	1	1	7	16	0	0	0	6	0	35		4		2
	H	0		12		3	3	8	11	0	1	7	10	8	1	69	2		0		0
5	A	2	17	10	4	1	13	7	5	6	11	4	8	15		1		0	2	10	0
	H	0	1	9	3	3	2	0	7	3	1	3	1	0		3		7	2	2	3
6	A	46		1		9	7	5	6	30		4	4	0	18	10	30	1	9	7	9
	H	0		0		2	0	6	2	0		0	0	0	1	0	1	0	1	0	0
7	A	0	9	2	1	1	0	0	0	5	2	0	0	1	1	0	2	6	3	5	1
	H	5	9	17	26	2	44	26	65	8	1	4	1	23	2	47	9	4	1	0	4
8	A	7	2	0	0	0	3	3	5	2	1	1	1	5	0	10	15	4	18	0	0
	H	17	17	49	93	2	1	12	2	11	24	33	30	1	13	23	6	5	6	0	3
9	A	13	76	9	1	3	4	1	4	60	24	0	9	16	124	6	0	4	39	0	101
	H	2	0	19	15	7	12	25	2	0	0	3	6	0	0	24	49	7	0	49	0
10	A	6	4	7	10	5	9	7	7	1	7	0	2	0	3	0	7		0		0
	H	8	12	12	6	9	9	4	4	1	2	9	6	8	6	6	15		14		20
11	A	9		18		2	2	4	6	7	8	4	1	5	20	0	18	0	3	1	0
	H	1		1		30	4	46	18	2	2	0	2	0	1	5	3	5	8	18	0
12	A	173	121	17	46	62	63	42	18			44	61								
	H	0	0	0	0	0	0	0	0			0	0								
13	A	16	3	11	8	18	23	14	3	7	0	6	0	2	1	3	5	17	16	7	13
	H	1	2	15	27	1	0	12	24	1	5	4	5	5	9	15	9	0	0	0	0
14	A	4	0	0	10	9	13	4	5	3	3	1	0	0	3	8	9	0	13	2	2
	H	3	15	5	24	5	3	11	5	9	5	2	3	6	3	8	8	9	2	3	0
15	A	15	5	23	13	1	0	1	1	5	0	3	0	0	1	5	0	1	4	0	2
	H	61	73	8	27	58	82	92	90	18	48	12	18	82	36	27	41	36	6	46	15
16	A	18	46	9	6	24		4	6	40		12	14	54	34	16	24		4		4
	H	2	0	1	6	0		8	23	0		0	3	0	0	1	2		3		0
17	A	8	19	11	24	0	0	4	13	16	8	3	0					0	3	2	2
	H	16	3	8	4	18	6	24	7	0	1	0	11					28	30	0	9
19	A	0	2	5	1	5	4	4	6	3	4	4	1	6		1		4	0	7	0
	H	18	22	50	29	6	1	8	23	4	5	0	8	0		30		1	26	0	6
21	A	12		7		14		0		41		9		7		24		0		3	
	H	1		6		6		8		0		0		10		17		3		5	

Key
DDF - deep digital flexor; CT - cranial tibial; LGDE - long digital extensor; LTDE - lateral digital extensor; SDE - short digital extensor; L - left; R - right; A - atrophy; H - hypertrophy

APPENDIX 10

The frequency of individual nerve fibre changes in the laryngeal and limb nerves of all horses examined

Groups of Horses / Leg Examined		Clinical Left	Clinical Right		Subclinical Left	Subclinical Right		Control Left	Control Right
Nerve	Horse No.			Horse No.			Horse No.		
Recurrent laryngeal, proximal	2	++/+++	+	1	++	+	3	0/+	0
Recurrent laryngeal, distal		+	++		++	++		+	0
Common peroneal		+	0		0/+	+		0	0
Deep peroneal, proximal		+/++	0/+		+	0		0	0
Deep peroneal , distal		+	0		-	+/++		0	0
Tibial		+	0/+		0	0		+	+
Plantar		-	-		-	-		-	-
Plantar digital		+	++		+	0		0	0
Recurrent laryngeal, proximal	4	++	-	6	++	0	5	+	0
Recurrent laryngeal, distal		+	-		++	0		0/+	0
Common peroneal		0	0		0	0		0/+	0
Deep peroneal, proximal		+	+		0	0		0	0
Deep peroneal, distal		++/+++	+		0	0		0	0
Tibial		0/+	0/+		0	0		0	0
Plantar		-	-		-	-		-	-
Plantar digital		++	+/++		+	0/+		0	+
Recurrent laryngeal, proximal	7	++	+	16	+	0	8	+	+
Recurrent laryngeal, distal		+++	++		+	+		+	+
Common peroneal		0	0		+	-		+	+
Deep peroneal, proximal		+	0		0	-		0/+	0/+
Deep peroneal , distal		+	+		+	+		0/+	0
Tibial		0	0		-	0		0	0
Plantar		++	++		-	-		++	+/++
Plantar digital		++/+++	+++		+	0/+		+/++	+/++
Recurrent laryngeal, proximal	9	++++	+++	17	++/+++	-	10	+	0
Recurrent laryngeal, distal		+	++++		++	+		+	0
Common peroneal		+	0		-	0/+		0	0
Deep peroneal, proximal		+	+++		0	+		+	0
Deep peroneal, distal		+	++/+++		+	+		0/+	0/+
Tibial		0/+	0		0	0		0/+	0
Plantar		++	++/+++		0	0		+	++/+++
Plantar digital		+++	++/+++		0	+		+	++/+++

(continued over page)

Key
0 - No individual nerve fibre changes + - Rare change, ++ - Occasional change, +++ - Frequent changes,
++++ - Numerous changes

APPENDIX 10 (continued)

Recurrent laryngeal, proximal	15	+++	+++	11	0	0
Recurrent laryngeal, proximal		++	++/+++		0/+	0
Common peroneal		+++	++		0	0
Deep peroneal, proximal		++	++		+	+
Deep peroneal , distal		++/+++	++		+/++	+
Tibial		+/++	++		0/+	0
Plantar		+/++	+++		++	++
Plantar digital		++/+++	++		++	+/++
Recurrent laryngeal, proximal	19	+++/++++	++++	13	+	-
Recurrent laryngeal, distal		++	+++		0/+	-
Common peroneal		0	0		+	0
Deep peroneal, proximal		0/+	0		0	0
Deep peroneal , distal		0	+		+	0
Tibial		0	0		+++	+
Plantar		+	0		+/++	++/+++
Plantar digital		+/++	+/++		++	++
Recurrent laryngeal, proximal	21	+	-	18	+	-
Recurrent laryngeal, distal		-	-		+	-
Common peroneal		-	0/+		+/++	-
Deep peroneal, proximal		-	++		0	-
Deep peroneal, distal		-	+		-	0
Tibial		-	+		0/+	-
Plantar		-	+		++/+++	++/+++
Plantar digital		-	++/+++		++	++
Recurrent laryngeal, proximal				20	0	0
Recurrent laryngeal, distal					0/+	0
Common peroneal					0	0
Deep peroneal, proximal					0	+
Deep peroneal, distal					+	0
Tibial					0	+
Plantar					+	-
Plantar digital					++	++

APPENDIX 11

Density (no./mm^2) of nerve fibres in the limb nerves of horses examined in the clinical, subclinical and control groups

Groups of Horses		Clinical			Subclinical			Control	
Leg Examined		Left	Right		Left	Right		Left	Right
Nerve	**Horse No.**			**Horse No.**			**Horse No.**		
Common peroneal	2	10,626	11,793	6	5,042	6,209	3	5,084	3,167
Deep peroneal proximal		9,334	11,543		7,542	6,251		4,667	6,376
Deep peroneal distal		8,542	7,417		9,084	7,626		6,292	6,125
Tibial		-	6,501		6,459	-		5,709	7,292
Plantar		-	-		-	-		-	-
Plantar digital		6,959	5,209		-	5,917		5,875	4,667
Common Peroneal	4	-	-	16	4,542	-	5	7,751	4,917
Deep peroneal proximal		9,167	6,376		-	4,792		7,334	-
Deep peroneal distal		5,834	8,251		7,792	5,459		11,293	10,042
Tibial		6,583	7,917		-	6,209		6,667	8,001
Plantar		-	-		-	-		-	-
Plantar digital		5,125	-		6,626	4,834		8,542	5,083
Common peroneal	7	3,667	-	17	-	-	8	8,167	9,501
Deep peroneal proximal		3,917	8,751		3,416	-		6,667	6,084
Deep peroneal distal		6,834	8,334		-	8,709		-	6,709
Tibial		5,917	8,334		7,084	-		6,209	5,584
Plantar		8,042	5,292		5,834	-		5,209	7,000
Plantar digital		5,167	2,709		4,834	-		5,375	9,209
Common peroneal	9	12,959	7,292				10	7,626	8,292
Deep peroneal proximal		5,375	6,083					9,334	6,542
Deep peroneal distal		11,876	8,209					5,292	6,667
Tibial		7,834	6,917					7,667	8,542
Plantar		5,792	4,917					8,334	7,126
Plantar digital		4,583	5,083					5,417	5,625
Common peroneal	15	-	-				11	-	7,292
Deep peroneal proximal		7,834	4,334					9,125	4,959
Deep peroneal distal		-	8,417					5,792	7,084
Tibial		8,667	8,084					8,709	8,626
Plantar		4,500	4,709					8,376	6,417
Plantar digital		5,375	4,000					5,709	5,917
Common peroneal	19	-	8,001				13	-	-
Deep peroneal proximal		4,417	8,126					-	7,334
Deep peroneal distal		5,542	8,251					6,334	8,334
Tibial		9,084	-					4,167	6,917
Plantar		5,917	5,625					5,917	-
Plantar digital		4,792	3,959					5,000	5,250
Common peroneal	21	-	8,001						
Deep peroneal proximal		-	5,625						
Deep peroneal distal		-	7,376						
Tibial		-	5,709						
Plantar		-	5,125						
Plantar digital		-	5,792						

APPENDIX 12

Mean cross-sectional axonal area (μm^2) in the limb nerves of all horses examined

Groups of Horses		Clinical			Subclinical			Control	
Leg Examined		Left	Right		Left	Right		Left	Right
Nerve	Horse No.			Horse No.			Horse No.		
Common peroneal	2	8	8	6	23	30	3	34	44
Deep peroneal proximal		14	10		16	24		23	32
Deep peroneal distal		12	13		7	11		24	22
Tibial		-	14		21	-		24	17
Plantar		-	-		-	-		-	-
Plantar digital		8	9		-	10		17	16
Common peroneal	4	-	-	16	29	-	5	21	34
Deep peroneal proximal		11	20		-	14		19	-
Deep peroneal distal		10	13		13	24		11	14
Tibial		20	18		-	24		20	17
Plantar		-	-		-	-		-	-
Plantar digital		12	-		9	11		9	14
Common peroneal	7	52	-	17	-	-	8	22	22
Deep peroneal proximal		42	14		57	-		21	24
Deep peroneal distal		20	16		-	14		-	26
Tibial		24	18		25	-		23	22
Plantar		12	15		20	-		19	15
Plantar digital		10	6		17	-		21	10
Common peroneal	9	11	24				10	20	21
Deep peroneal proximal		15	9					10	21
Deep peroneal distal		9	8					23	14
Tibial		20	20					19	13
Plantar		13	20					11	9
Plantar digital		17	12					11	6
Common peroneal	15	-	-				11	-	20
Deep peroneal proximal		12	35					13	28
Deep peroneal distal		-	15					20	14
Tibial		13	13					14	14
Plantar		19	17					12	13
Plantar digital		17	21					15	9
Common peroneal	19	-	19				13	-	-
Deep peroneal proximal		43	19					-	15
Deep peroneal distal		19	13					16	15
Tibial		16	-					14	20
Plantar		23	17					18	16
Plantar digital		11	13					12	20
Common peroneal	21	-	13						
Deep peroneal proximal		-	25						
Deep peroneal distal		-	11						
Tibial		-	19						
Plantar		-	18						
Plantar digital		-	12						