The Effects of Zero Gravity

On Rat Skeletal Muscle

Submitted by John J. Maleš for
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Department of Physiology,
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Abbreviations

AEM	Animal Enclosure Module. A cage housing five rats.
D0	Day zero
D9	Day 9
D18	Day 18
DFPT	Delayed Flight Profile Test
EDL	Extensor Digitorum Longus
EMG	Electromyogram
HRP	Horse Radish Peroxidase
MHC	Myosin Heavy Chain
RAHF	Research Animal Holding Facility. A cage housing a single rat.
SLS-1	Space Life Sciences Mission - 1
Tm	Tropomyosin
Tn	Troponin
V_{max}	Maximum contractile velocity

Summary

It is well known that 0-G conditions can alter skeletal muscle. This has been established from the knowledge gained from 0-G experiments conducted using rats, as well as some data from human subjects also exposed to 0-G. Further information has been gained from experiments which model the effects of 0-G on skeletal muscle, both in animals and in humans. Some of the changes observed include atrophy, particularly of postural muscles, and a change in the fibre types present in the muscle. To further elucidate these changes, particularly the changes in MHC expression, an experiment involving the exposure of rats to 0-G was performed.

Rats were exposed to 0-G conditions for 9 days aboard the Space Shuttle Colombia in the Space Life Sciences Mission 1 (SLS-1) of June, 1991. Of the rats exposed to 0-G, one group was killed within hours of the Shuttle returning to earth, whilst another group was kept on earth for 9 days postflight. The rats were flown in two cage types, one group being in single cages, the others caged in groups of 5. Thus, the rats that were exposed to 0-G could be divided into 4 groups, depending on what type of cage they were housed in, and on whether they were kept alive or not upon return to earth. Various control groups mimicked the time course and cage types of the flown rats, except that they were not exposed to 0-G. The soleus and EDL muscles were removed from the rats, frozen in freon 12 cooled in liquid nitrogen, and transported to Sydney in a liquid nitrogen shipper.

The rat muscles were analysed immunocytochemically using a series of antibodies specific to various MHC isoforms. A quantitative analysis of fibre type proportions, and average fibre diameters was carried out on some of the muscles in the control and flight groups.

It was found that changes in the muscles occurred between the D0 and D9 controls, more so in the soleus. In the soleus, the number of fibres expressing fast MHC decreased significantly, and the average fibre size had increased, consistent with the known increase in muscle weight over that time. In contrast to this, the flight muscles showed a significant increase in the number of fibres expressing fast MHC, such that a shift towards fibres expressing both fast and slow MHC isoforms was observed. Also, substantial muscle atrophy had occurred. After the 9 days of recovery, there was still a high level of fast fibre staining and a high number of transitional fibres present, with the fibre proportions not differing from the values found in the flight muscles. The atrophy in the recovery muscles was in the process of being reversed, although muscle weight data suggests the reversal was not complete.

The changes in the EDL were relatively slight. In the flight group, there was no evidence of a shift in fibre type proportions, such as a population of transitional fibres. Further, 0-G induced muscle fibre atrophy was not observed in the EDL. The IIB fibres maintained the same cross-sectional area, whilst the slow and type IIA fibres displayed a slight, but statistically significant hypertrophy.

Thus the changes observed were largely confined to the soleus, with shifts in fibre type proportions and fibre size being evident. The changes in the EDL were relatively minor. The difference in the effects of 0-G on these two muscles suggests it is the usage pattern of the muscle, and how it is changed in 0-G, that is important in determining how a particular muscle will react to 0-G.

Chapter 1: Introduction

1.1 Skeletal Muscle Structure

Skeletal muscle is a contractile apparatus under voluntary control. It consists of long, thin myofibres containing many nuclei, formed by the fusion of many small cells called myoblasts. Myofibrils are rods of contractile proteins which are aligned serially to form sarcomeres. The sarcomeres contain thick and thin filaments, arranged in a parallel, overlapping array. The thick filament is made up primarily of myosin, while the thin filament is made up of actin and the regulatory proteins, troponin and tropomyosin. It is the interaction of myosin and actin, with the thick and thin filaments sliding past one another, using the energy stored in ATP, which causes contraction of the muscle.

1.1.1 The Motor Unit

A single neuron supplies many muscle fibres, and these together form a single unit known as a motor unit. Each muscle contains many motor units. All muscle fibres within a motor unit contract simultaneously when the neuron is activated. Varying the level of force produced by a skeletal muscle can be accomplished by changing the frequency at which a given motor unit is being stimulated, or by varying the number of motor units activated. Different motor units have different patterns of activity. Motor units in the soleus, for example, are continuously active at low frequency, whether the animal is standing or walking. These are known as tonic motor units. Those in the extensor digitorum longus (EDL), for example, are known as phasic motor units, because they fire at high frequencies for relatively brief periods when the animal is moving (Hennig & Lømo, 1985; Hennig & Lømo, 1987; Hutchison *et al.*, 1989).

Muscle fibres in single motor units appear to be homogenous biochemically (Nemeth *et al.*, 1981), histochemically (Burke *et al.*, 1971), and in their isomyosin content (Gauthier *et al.*, 1983), suggesting the nerve plays a part in their characteristics.

1.1.2 Myosin

Myosin makes up most of the thick filament. Myosin has a molecular weight of approximately 470 kd, consisting of two heavy chains and four light chains. The proposed shape consists of a rigid α helical rod, with two globular heads, each attached by a hinge-like region. Two light chains are found in each head, as is the N-terminus of one myosin heavy chain (MHC). The rod region is made up of the two MHCs.

The MHC has actin activated ATPase activity, and through its cleavage of ATP, converts chemical energy into mechanical energy which is used to make the muscle contract. MHCs exist in at least four distinct isoforms in mature skeletal muscle, as well as several others in developing and regenerating muscle. These vary in their rate of ATP cleavage.

There is no significant difference in the force per cross-sectional area produced by different muscle fibre types. There are, however, differences in the maximal contractile speed (V_{max}), and this has been the basis for the physiological classification of different muscle fibre types. Power is the product of force and velocity. Thus, muscle power is proportional to V_{max}. Barany (1967) demonstrated that V_{max} was proportional to the myosin ATPase activity of the muscle. Since it is the MHC that contains the ATPase activity, this suggests that it is the MHC that largely determines V_{max}.

Muscle fibres, therefore, can be conveniently classified by the differences in their MHCs, since this is a phenotypic difference between muscle fibres which can be correlated to the physiological difference in contraction rate. Two broad classifications exist under this schema: "slow" or type I muscle fibres (expressing type I MHCs), and "fast" or type II fibres. The fast fibres can be sub-classified into IIA (also known as fast, fatigue resistant and fast red), IIB (fast fatigable, fast white), and the only recently characterised IIX (Schiaffino *et al.*, 1989; Gorza, 1990). IIX in some ways appears to be intermediate between IIA and IIB. The shortening velocity of IIX fibres, however, is similar to that of IIA fibres (Schiaffino *et al.*, 1990). IIX is also known as IId (Takahashi, 1991; Bär & Pette, 1988). These three fast fibre types each express unique MHCs. To complicate matters, fibres with mixed MHC isoforms do exist, such as IIB/IIX and I/IIA (Schiaffino *et al.*, 1990). Embryonic and foetal

forms of myosin are also expressed (for review see Swynghedauw, 1986), but only briefly during normal development in muscle. It must also be noted that this classification system applies only to limb muscles; other muscle groups can have different classification systems. For instance, cat jaw muscles express a "superfast myosin" which is not found in the limb.

The use of monoclonal antibodies specific to various MHC isoforms found in skeletal muscle has allowed the relatively easy characterisation of muscle fibres into different categories, related to their differing speeds of contraction. Another method used for classification of muscle fibre type is histochemistry, which relies on the difference in susceptibility of fast and slow ATPase activity to inactivation by acid or alkali preincubation (Barnard *et al.*, 1982).

The various muscle fibre types possess different isoforms of myosin and other contractile proteins. There is, also, variation in cell metabolism, with some fibres relying mainly on fatigue-resistant oxidative phosphorylation (types I and IIA); the cells containing a large number of mitochondria. Other fibre types rely mainly on glycolytic metabolism (IIB), which is fatigable. IIX fibres are intermediate between IIA and IIB in their fatigability (Schiaffino *et al.*, 1990). Thus, adult vertebrate muscles are a heterogenous group which differ in their physiological, histochemical, and biochemical properties. Not only are the fibre types different, but muscles contain varying distributions of these fibre types. For example, in the rat, a slow twitch muscle such as the soleus contains mainly type I fibres, with a smaller proportion of IIA (Schiaffino *et al.*, 1989; Sugiura *et al.*, 1992). A fast twitch muscle such as the EDL contains mainly types IIA, IIX, and IIB, with a small number of type I fibres (Schiaffino *et al.*, 1989; Sugiura *et al.*, 1992).

Another protein present on the thick filament is C-protein. C-protein has an approximate molecular weight of 140 kd. It is present in transverse stripes across the A band, and forms part of the thick filament. C-protein exists as different isoforms in fast and slow muscle, although some fibres that appear to be fast by histochemical criteria contain both fast and slow C protein isoforms (Dhoot *et al.*, 1985). The function of C protein is under debate, but it does, however, bind to myosin (Moos *et al.*, 1975), and is thought to modify the ATPase rate in the actin-myosin interaction (Moos & Feng, 1980).

1.1.3 The Thin Filament

The thin filament is made up of actin, troponin (Tn) and tropomyosin (Tm) in the ratio of 7:1:1 respectively (Zot & Potter, 1987). Actin polymerises to form a double stranded helix, along which the Tm molecule is polymerised head to tail. Actin exists in the same α-skeletal isoform throughout all skeletal muscle cells (Swynghedauw, 1986). Tn is found at intervals along the thin filament in association with Tm.

Tn, consisting of three subunits (TnC, TnI, TnT), is an important protein in skeletal muscle contraction (Zot & Potter, 1987). Contraction is brought about by a rise in free intracellular calcium. Tn binds this calcium at the TnC subunit, inducing a conformational change in Tn, affecting not only Tn but also Tm and actin. This change allows the actin to form cross bridges with myosin, increasing the rate of ATP consumption and causing the fibre to contract. TnC binds calcium, TnI inhibits the actomyosin ATPase, and TnT links the Tn complex to Tm.

The subunits of Tn have been shown to exist in different isoforms in different skeletal muscle cell types. Dhoot *et al.* (1978, 1979a,b) in her various papers, has shown that TnC, TnI, and TnT each have an isoform specific to type I, and an isoform specific to type II fibres. Thus Tn could be a useful marker for determining muscle fibre isoforms. It does, not, however, offer as high a level of differentiation as the MHCs provide. It has also been found that these subunits, such as TnT, can exist in forms specific to cardiac muscle. However, Tn isoforms can be expressed in a somewhat more irregular manner than this categorisation suggests. For example, Saggin *et al.* (1990) found that cardiac TnT isoforms are expressed in the skeletal muscles of embryonic and foetal rats; the isoforms disappearing in the first few weeks after birth. These isoforms can be subsequently re-expressed in mature muscle fibres after denervation, or in regenerating fibres after an injury.

Tm is a dimeric protein consisting of α and β subunits of molecular weight 33 and 37 kd respectively (Cummins & Perry, 1973). γ and δ isoforms also exist (Heeley *et al.*, 1985). Tm has a regulatory role in the actin-myosin interaction, and probably has a role in stabilising the thin filament. It is found in both grooves of the double stranded thin filament structure, polymerised head to tail. Tm is involved in the regulation of contraction. The conformational change in Tn upon binding calcium moves Tm, allowing actin and myosin to interact. As a result, contraction occurs.

The proportions of the different isoforms vary in different muscle types; the αα form predominating in fast muscle, the αβ form predominating in slow (Swynghedauw, 1986).

1.2 Regulation of Fibre Type

1.2.1 Neural Regulation of Muscle Fibres

In 1960 significant breakthroughs were made into understanding the plasticity of skeletal muscle fibres by Buller *et al.* (1960a,b). It had been observed previously that different muscles had different contractile properties; some muscles had slow contraction rates and others had fast contraction rates. To test the relationship between nerve and muscle, they cross innervated a fast muscle of a cat with the nerve of a slow muscle (a "slow" nerve) and vice versa. They found that the slow muscle became fast contracting and the fast muscle slow contracting. These classic experiments showed that neural regulation was an important influence on skeletal muscle. These experiments spawned the idea that muscle fibres are all fundamentally the same, but "plastic", able to change their phenotype depending on the neural stimulus. These results encouraged substantial research into this area (Pette & Vrbova, 1985).

Two hypotheses emerged as to how the nerve influenced the muscle. Firstly, Buller *et al.* (1960b) proposed that some trophic substance or substances passing through the nerve and into the muscle influenced muscle type. The other hypothesis stated that the specific pattern of impulses received from the nerve influenced the muscle type, and as such the different frequency and duration of firing of slow and fast nerves determined fibre type. Since then the evidence has supported the impulse pattern hypothesis. For example, Gorza *et al.* (1988) found that chronic high frequency stimulation of the rat soleus, a slow twitch muscle, produced a decreased twitch time, to somewhere between the normal soleus and normal EDL (fast twitch muscle) times. Immunocytochemistry revealed that stimulated fibres expressed fast myosins while the unstimulated controls did not. Similar results were obtained by Lømo *et al.* (1974, 1980). Complementary to this, chronic low frequency stimulation

of fast limb muscles can slow their contraction (Salmons & Sreter, 1976), increasing the expression of IIA and IIX isoforms at the expense of IIB (Termin *et al.*, 1989). Similar results were obtained in nerve cross union experiments (Buller *et al.*, 1960b; Close, 1969), suggesting the impulse pattern hypothesis is correct.

1.2.2 Myogenic Regulation of Muscle Fibres

Evidence has emerged that the nerve is not the only regulator of muscle fibre type. It appears that different muscles, and even fibres of the same type can behave differently when conditions change, depending on their myogenic origin, or their cell lineage. The neural regulatory hypothesis cannot explain these differences. For example, it cannot explain why the transformation of muscle fibres in stimulation and cross union experiments is not complete. It has been found that no matter what the stimulation pattern, the soleus cannot become the same as the EDL and vice-versa.

The cross innervation of the EDL with the soleus nerve performed by Close (1969) resulted in a twitch time only half as slow as the normal soleus. Westgaard & Lømo (1988) found the same difference in twitch times when electrically stimulating both a denervated EDL and soleus at the same low frequency of 10 to 15Hz (as the soleus would normally receive). Similarly, the soleus could not become completely fast-like under the same stimulation patterns as a fast muscle. Chronic high frequency stimulation of the soleus resulted in a V_{max} half of that of the EDL (Gorza *et al.*, 1988), as does cross innervation of the soleus with the nerve to the EDL (Close, 1969). The high and low frequency stimulation patterns used above are known to be similar to the normal stimulation patterns in fast and slow muscles respectively (Hennig & Lømo, 1985).

Further evidence of these muscle specific differences can be seen by examining the changes in MHC expression which occur in different muscles undergoing stimulation. The soleus will express IIA and IIX MHC when stimulated at high frequencies, or in denervation, but will not express significant amounts of IIB MHC, even when the same stimulation would produce large amounts of IIB in the EDL (Ausoni *et al.*, 1990; Schiaffino *et al.*, 1989). In this situation, 90% of the fibres of the soleus also continue to co-express slow MHC along with type II MHC, unlike the EDL (Gorza *et al.*, 1988). The reverse is also true, an EDL cannot become identical

to a soleus. Low frequency stimulation of the EDL or tibialis anterior will result in a shift from IIB towards IIA/IIX MHC, but slow MHC never makes up more than 2% of the total, whereas slow MHC would be in the majority in the soleus under the same conditions (Ausoni *et al.*, 1990; Gorza *et al.*, 1988; Termin *et al.*, 1989). It appears from this evidence that muscles differ in their behaviour to the same stimuli; that is, the muscles and their fibres are intrinsically different, having different "adaptive capacities", only being able to compensate for changes in activity within that range. The above examples show the soleus cannot express IIB fibres, the EDL cannot express type I or slow fibres in any great amounts.

It is now known that muscle fibres are a developmentally heterogenous group. Myotubes are formed by the fusion of myoblasts, which soon become innervated, and eventually develop into mature muscle fibres. Firstly the larger, primary myotubes form, and then later, the smaller secondary myotubes form around the primary myotubes. Even if these myotubes are expressing the same phenotype when mature, they have developed from different cell lineages and can thus behave differently when exposed to altered neuronal stimuli such as denervation, hindlimb suspension or 0-G, as discussed later. There are at least four patterns of myosin gene expression in the myotubes of mammalian limb muscles (Hoh, 1991; Hoh *et al.*, 1988). Slow primaries express embryonic and slow myosins (but not foetal) in early development and continue to express slow myosins as mature fibres. Fast primaries initially express embryonic, foetal and slow myosins, replaced by fast myosin in mature muscle. Secondary fibres can also be divided into slow and fast groups. Both initially express embryonic and foetal myosins, replaced by slow myosin in slow secondaries, and fast myosin in fast secondaries. Hoh *et al.* (1989) proposed that within the muscle allotypes of which limb is one, the various isotypes of primary and secondary fibres are myogenically determined. Later in development, these fibre types can change in accordance to the neural stimulation they are receiving, within the limitations of fibre phenotype that their developmental origin allows them to express. For example, the fast fibres in the soleus, the fast secondaries, are gradually converted into slow fibres under the influence of the nerve (Boreham *et al.*, 1988; Maltin *et al.*, 1990; Kugelberg, 1976) - as the rat gains weight, a greater need for non fatigable slow fibres exists. Even though these fibres have been "forced" to

express the slow fibre type, the myogenic origin or "program" of the fibre can reassert itself if the conditions allow.

Evidence of myogenic regulation has emerged from studies of the development of avian muscle. Similar to mammalian muscle, avian muscle has primary and secondary fibres which develop separately, and both groups have been shown to be heterogenous (Crow & Stockdale, 1986; Miller & Stockdale, 1986a and 1987). Also, the formation of different primary fibre types can occur without innervation (Crow & Stockdale, 1986; Phillips & Bennet, 1984; Fredette & Landmesser, 1991). Even stronger evidence for the myogenic lineage theory is that an individual myoblast from a chicken embryo cultured *in-vitro* can divide, and then fuse to form muscle fibres (Miller & Stockdale, 1986a). Embryonic myoblasts were found to develop into myotubes of one of the three fibre types, but any one particular myoblast only ever gave rise to one type of muscle fibre; foetal myoblasts only ever gave rise to one class of fibre, that is, fast (Miller & Stockdale, 1986a,b). Studies of avian muscles have thus provided evidence that a myogenic lineage exists, and that the nerve is not necessary for the differentiation of the three different fibre types present in the bird. It is therefore the myoblasts that carry the information required for differentiation.

Further evidence of muscle specificity has been gained through the study of specialised muscles containing specialised contractile proteins. Hoh & Hughes (1988) showed that a cat jaw muscle, which homogenously expresses a myosin not found in limb muscles, known as superfast myosin, when transplanted into a limb muscle bed and reinnervated by a fast limb nerve, still expressed superfast myosin rather than limb fast myosin. This shows it is not a difference in the innervation between the limb and jaw muscle beds that caused a difference in the myosin expression, and suggests again that it is the fibre class or allotype (Hoh *et al.*, 1989) that determines which myosins can be expressed. Hoh *et al.* (1989) suggests that the allotype restricts the phenotypic options of the myofibre. Thus limb and jaw muscles belong to different allotypes.

Denervation of muscles has proved to be a useful way of studying the regulation of fibre type. Under a neural regulatory hypothesis, it would be predicted that the effect of denervation on fibres of the same type or phenotype would be the same, and this has been shown not to be the case. Denervating the rat soleus, for example,

results in approximately half the fibres continuing to express slow MHC exclusively, with others shifting to expression of fast or a combination of slow and fast (Gorza *et al.*, 1988; Hoh *et al.*, 1989; Schiaffino *et al.*, 1989). All of the phenotypically same slow fibres of the soleus (making up the majority of the muscle) thus did not undergo the same changes once the influence of the nerve was gone. Some remained slow, others shifted towards the fast fibre type. It seems likely that it is the fast secondary fibres, which have converted to the slow fibre type under the influence of the nerve later in development, that are re-expressing the fast fibre type in the absence of neural stimulation. So, even though the mature fibre type is the same (slow), the difference in the developmental or myogenic origin of the fibre (slow primary or fast secondary) has determined how the fibre will behave when the neural stimulus was removed in denervation. It has also been shown that the expression of fibre type in developing rat muscles is independent of the presence of the nerve (Condon *et al.*, 1990).

The importance of the developmental origin can also be seen in other limb muscles. In the rat EDL, during development, there are approximately 200 slow fibres, almost all of slow primary origin, and as the muscle matures, there is a gradual "loss" of these slow fibres as they are converted to IIA fibres by the influence of the nerve. If the limb is denervated, these fibres revert back to their original phenotype, and there is an increase in the number of slow fibres, with the same topographic distribution as that of the slow primaries in the developing EDL (Hoh *et al.*, 1989). Further, these slow primaries (including those which were phenotypically IIA prior to denervation), hypertrophy whilst the other fibres in the muscle atrophy (Hoh *et al.*, 1989). So the myogenic origin of the fibre determined the behaviour of the fibre, rather than the fibre phenotype, which in this case could have been either slow or fast.

Therefore, the myogenic origin of the fibre plays an important role in determining how the fibre will behave if the conditions to which it is exposed, such as neural stimulation, are altered. The neural stimulation pattern can change the muscle fibre type, but only within the limitations placed upon it by it's cell lineage or myogenic origin. That is why a limb muscle fibre will never express superfast myosin, or why

most fibres from the EDL will not express slow myosin, no matter how the neural stimulus is changed.

1.3 Zero Gravity and Models of Zero Gravity

1.3.1 Earth Bound Animal Models of the Zero Gravity Condition

Because of the limited opportunities and expense of investigating the effects of 0-G on muscle through space missions, various earth-bound models that simulate 0-G conditions have been developed, especially for the rat. Some of these include the hindlimb suspension model, developed by Morey (1979), whole body suspension, and hindlimb immobilisation.

Hindlimb suspension is by far the most popular, judging by the volume of literature. This model consists of removing the weight carried by the hindlimb by placing a harness around the proximal half of the tail, suspending the hind quarters, such that the hindlimbs do not touch the ground. All weightbearing is removed from them (Morey, 1979; Figure 1.1). Whole body suspension similarly involves removing weight bearing from the hindlimbs, by placing a harness around the abdomen of the animal, again so the hindlimbs do not touch the ground (Figure 1.1). The rats can move relatively freely in both these models, using their forelimbs. Hindlimb immobilisation is relatively self explanatory, being a method whereby the hindlimb is held in a fixed position such that it cannot conduct the normal range of motion. However, the limb bears weight in this model. Since hindlimb suspension is the most commonly used, and perhaps the best of the models (as discussed later), it is the one which will mostly be elaborated upon.

Some attempt has been made to determine which model has effects on the hindlimb which are most similar to those observed in 0-G. For example, Fitts *et al.* (1986) compared the effects of hindlimb suspension and hindlimb immobilisation. It was discovered, as it was in other studies, that both models primarily affected slow twitch muscles such as the soleus. As such, comparisons of the two models were restricted to this muscle. Both models showed decreases in muscle mass and peak tetanic tension. The V_{max} of the soleus was increased in hindlimb suspension, but not

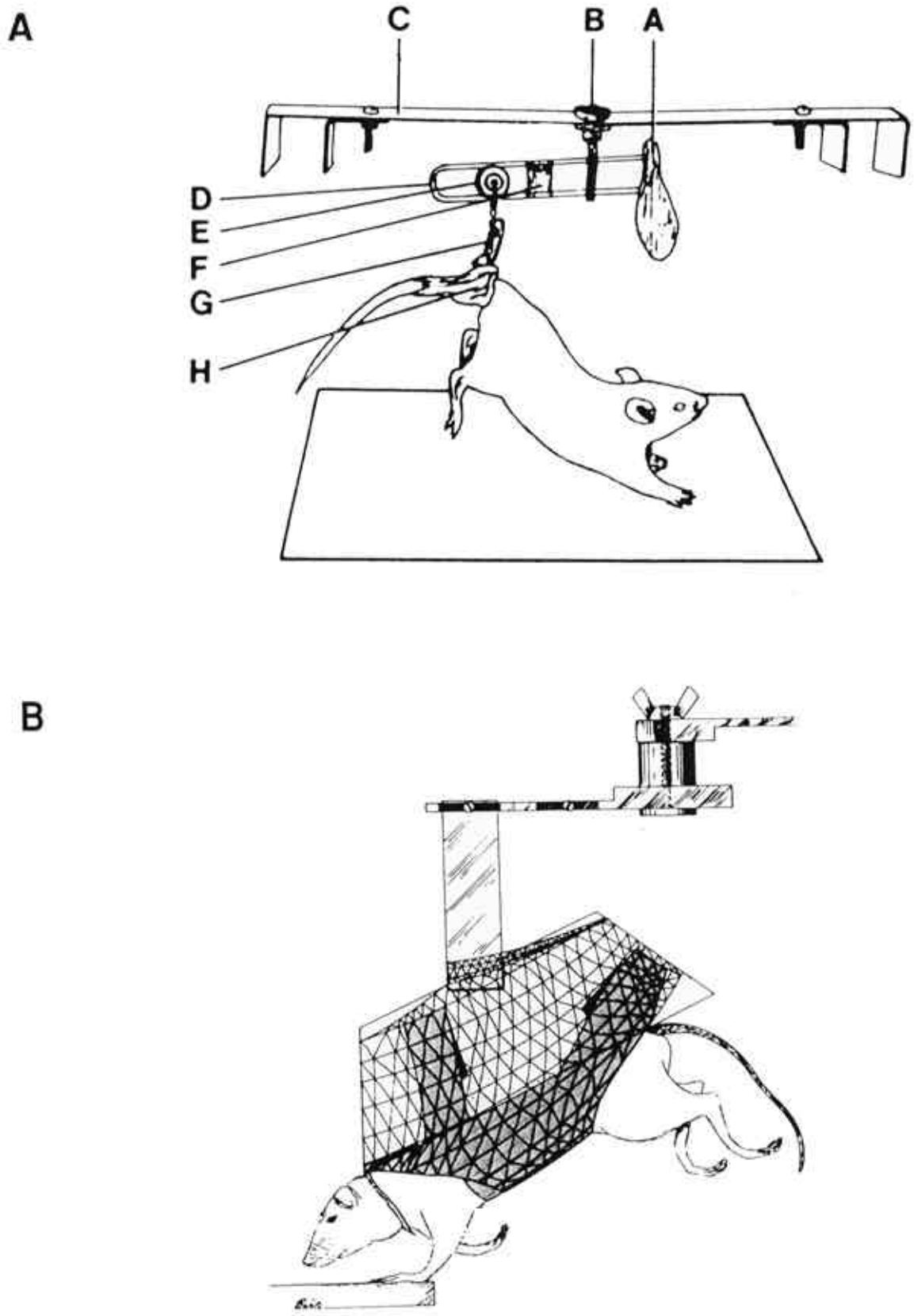

Figure 1.1 Diagrammatic representations of hindlimb suspension (A - from Jaspers & Tischler, 1984) and whole body suspension (B - from Mussachia *et al.*, 1980). The rats are free to move using their forelimbs, but their hindlimbs play no weightbearing role.

in hindlimb immobilisation. This, along with electrophoretic studies, suggested an increase in type II MHC in hindlimb suspension, but not in hindlimb immobilisation. These and other factors showed that while these models had similarities in their effects on slow muscles, such as the decrease in muscle mass, there were also differences. It was concluded that hindlimb suspension may better mimic the weightless condition, because this model completely removes the load from the limb, whereas in immobilisation, load bearing is still present despite the inability of the limb to move.

The changes that occur in hindlimb suspension have been studied at many levels. Reported almost universally is a preferential protein loss and therefore, atrophy in slow twitch muscles, particularly the soleus (Chi *et al.*, 1992; Desplanches *et al.*, 1987a; Fell *et al.*, 1985; Goldspink *et al.*, 1986; Haida *et al.*, 1989; Hoh & Chow, 1983; Jaspers *et al.*, 1985; Steffen & Musacchia, 1984; Thomason *et al.*, 1987a,b, 1989; Winiarski *et al.*, 1987). This atrophy is associated with a decrease in the cross-sectional area of the fibres. Some disagreement exists as to the effect on fast twitch muscle weight, in muscles such as the EDL. Although all agree the changes are small in comparison to those in the soleus, some studies report no change in mass (Chi *et al.*, 1992; Hoh & Chow, 1983; Roy *et al.*, 1987; Winiarski *et al.*, 1987), some a decrease in mass (Desplanches *et al.*, 1987a; Marsh *et al.*, 1992), and yet others an increase (Ohira *et al.*, 1992; Steffen & Musacchia, 1984). Some of the inconsistency may be due to different ways of expressing muscle mass; some studies measure muscle mass relative to body weight (the suspended animals tend to have lower body weights than the controls), whereas others do not.

Changes in myosin isoform expression in the soleus have also almost always been reported. The changes are a shift towards faster myosin isoforms, that is from type I to type II (Bonen *et al.*, 1988; Desplanches *et al.*, 1987a; Diffee *et al.*, 1991; Elder & McComas, 1987; Gardetto *et al.*, 1989; Reiser *et al.*, 1987; Templeton *et al.*, 1984, 1988) Others have more recently attempted to establish which type II isoforms have increased. Takahashi *et al.* (1991) used improved electrophoretic methods to separate the three type II MHC isoforms, IIA, IIX, and IIB. They show that the normal soleus expresses mainly type I and some type IIA (being the only fast isoform present), while rats suspended for 21 and 28 days show progressively larger amounts of IIA

expression as well as some expression of IIX. Campione *et al.* (1990 and 1992) discovered the same changes in the hindlimb suspended soleus using electrophoresis as well as monoclonal antibodies with immunoblotting techniques. Neither of these studies, however, found any IIB MHC. None of the above studies have reported shifts in myosin expression in fast twitch dorsiflexors such as EDL.

Accompanying this change in myosin isoforms (as well as changes in other contractile proteins which will not be discussed here), is an increase in the speed of contraction of the soleus. The findings in the soleus have been that while the maximum tension developed is reduced, (as would be expected with atrophy reducing the amount of contractile protein and the cross-sectional area of the muscle), the twitch contraction time decreases (Elder & McComas, 1987; Fell *et al.*, 1985; Fitts *et al.*, 1986; Hoh & Chow, 1983; Templeton *et al.*, 1984; Winiarski *et al.*, 1987), and the V_{max} increases (Diffee *et al.*, 1991; Fell *et al.*, 1985; Fitts *et al.*, 1986; Gardetto *et al.*, 1989). Some of these studies have reported no change in the speed of fast twitch muscles (Diffee *et al.*, 1991; Hoh & Chow, 1983; Winiarski *et al.*, 1987). Diffee *et al.* (1991) also found that muscle tension fell not only in a direct comparison with the control muscles; the tension per cross-sectional area also fell. This suggests a more than proportional decrease in the expression of contractile proteins, as opposed to other cellular proteins, within muscle fibres. Other studies have reported a decrease in the myofibril protein concentration (μg/mg of muscle), both in 0-G (Baldwin *et al.*, 1990) and in hindlimb suspension (Thomason *et al.*, 1987a), in agreement with the above suggestion.

It is of interest to see whether the changes seen in hindlimb suspension are reversed after its cessation. The period after suspension (or after 0-G exposure) is known as the "recovery" period. It seems that muscles eventually return to normal, but that a substantial period of time is required for them to do so. Desplanches *et al.* (1987b) found a complete reversal of MHC isoforms to control values, after a recovery period. However, this recovery is quite long, with Marsh *et al.* (1992) showing that 28 days was not enough time for MHC isoforms to return to control levels in the soleus, following 28 days of hindlimb suspension. In a related study, Fitts & Brimmer (1985) found that the return of all mechanical and biochemical

parameters to control values following 90 days of hindlimb immobilisation required a recovery period of 90 days.

1.3.2 Mechanisms Causing Change in Hindlimb Suspension

Studies of the mechanisms causing the changes induced by hindlimb suspension have also been undertaken. It has been proposed that alterations to the neural impulse pattern directed to the muscles in hindlimb suspension is one of the major causes of change. This is particularly important in postural muscles such as the soleus. It has been proposed that one of the major causes of change in hindlimb suspension is due to the change in neural activity to the muscles, particularly to the soleus and other postural muscles. Normally, gravity pushing down on the hindlimb produces a gravitationally-induced stretch reflex of the soleus (Figure 1.2), which in turn leads to a constant low frequency neural excitation of it. In hindlimb suspension, as the hindlimb does not bear weight, the soleus is not continuously stretched by gravity as is normally the case. It is proposed that the absence of this gravitationally-induced stretch reflex means that the soleus does not receive the constant low frequency stimulation that it normally does. To test this idea, two studies have recorded electromyograms (EMG), the impulse patterns received by muscles, in hindlimb suspended animals. Both studies agree that EMG activity in the soleus falls to very low levels immediately following hindlimb suspension (Alford *et al.*, 1987; Riley *et al.*, 1990b). However Alford *et al.* (1987) found that EMG activity recovered quickly, returning to 81% of normal by 7 days, while Riley *et al.* (1990b) found no recovery in activity, with activity being 13% of normal at 7 days. In either case, there was an initial decrease in activity. Only Alford *et al.* (1987) analysed a fast twitch muscle, the tibialis anterior, which was found to have increased EMG activity. EMG experiments, however, have the problem that electrodes can shift to inappropriate positions, and can record signals from muscles other than those from which they are supposed to.

The regulation of protein expression in the soleus during hindlimb suspension has been described as having a triphasic time course (Booth & Kirby, 1992). This model refers to the major influence on changing protein levels during each phase, but in all

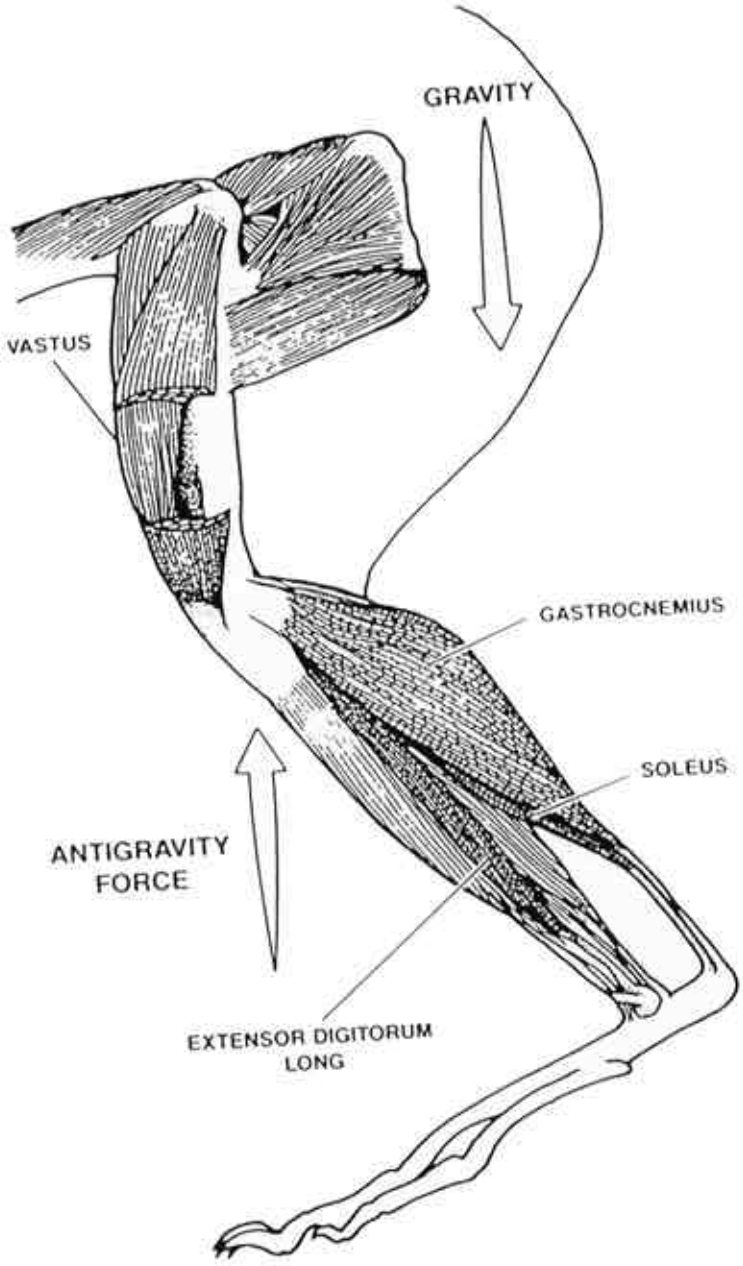

Figure 1.2 Diagramatic representation of the muscles of a rat hindlimb. The force of gravity on the hindlimb normally causes the soleus to be stretched.

phases complex interactions are taking place, all of which play a role in the regulatory process. Phase I, lasting for approximately the first day, shows a rapid decrease in protein synthesis rates, particularly of myofibrillar proteins. This decrease can be detected within 6 hours of commencing hindlimb suspension (Booth & Seider, 1979). The decrease in protein synthesis may be because of down-regulation of the translation of these proteins, as the mRNA levels are initially normal. Phase II, occurring from days 2 to 14, involves two major mechanisms. Firstly, an increase in proteolysis, with an increased turnover of myofibrillar protein, without the increase in protein synthesis that would be necessary to maintain myofibrillar protein levels. Secondly, pretranslational controls may play a part, with the amount of mRNA for at least some of the myofibrillar proteins being reduced (Howard *et al.*, 1989). Phase III shows a large reduction in the rate of myofibrillar protein degradation, with a steady state of myofibrillar protein synthesis and degradation being reached by day 24. Along with these changes, the mRNAs specific for fast isoforms of MHC are expressed in relatively greater amounts in slow twitch muscles, to produce the increased amounts of these isoforms that are present.

It is known that the hindlimbs of rats in hindlimb suspension adopt a position of plantar flexion, as a consequence of the unloading, where the foot droops down into a lower position than it would normally adopt (Loughna *et al.*, 1986; Riley *et al.*, 1990b). This position has the effect of stretching some muscles (such as the EDL and tibialis anterior), whilst shortening others (soleus - Figure 1.2). Some studies consider that muscle length, or the tension of the muscle is important in whether or not a muscle atrophies, for example, Loughna *et al.* (1986). It was found that if the hindlimb suspension model was modified such that one hindlimb was held in a dorsiflexed position, so the soleus was stretched rather than shortened, the amount of atrophy was reduced, and the rate of protein synthesis was increased (Loughna *et al.*, 1986; Goldspink *et al.*, 1986). In the EDL the muscle suffered slightly greater atrophy, supposedly as a result of it being in a shortened position in this model. However, this, and other studies do not seem to have adequately addressed the difference between the role of stretch *per se*, and the role of increased neural stimulation while the muscle is stretched.

As has been seen, the effects of hindlimb suspension vary depending on the muscle in question. Michel & Gardiner (1990) explored this further. As well as having control and hindlimb suspended groups of rats, a third group was hindlimb suspended along with having their sciatic nerve chronically perfused with tetrodotoxin (TTX). TTX selectively blocks sodium ion channels, preventing the transmission of action potentials past the point of perfusion. TTX, however, does not affect other neural factors that influence the muscle, such as spontaneous ACh release, fast axonal transport, and so forth. So the use of TTX leads to complete muscle disuse. It was found that TTX, as well as hindlimb suspension, had no added effect on the soleus over hindlimb suspension alone. This suggests that the removal of weightbearing function alone causes a maximal atrophic response, as the muscle is already suffering disuse in hindlimb suspension alone. However, in other, fast, muscles (gastrocnemius and plantaris), minimal change was observed in hindlimb suspension alone, whereas a dramatic change in morphology, with a large amount of atrophy followed superimposed TTX perfusion. This suggests that hindlimb suspension is an environment relatively close to normal (or with at least enough activity to maintain the muscles in a normal state) for some muscles, whilst other muscles, namely the soleus, suffer almost complete disuse.

There has been some suggestion that the changes observed in hindlimb suspension are due to stress on the animals in this model. In particular, it was thought that increased levels of circulating glucocorticoids may have an atrophic effect on the muscles of the hindlimb. However, it has been found that removing the adrenal gland from rats did not abolish the atrophy caused by hindlimb suspension, although glucocorticoids did play a modifying role (Jaspers & Tischler, 1986). Stress levels in hindlimb suspended rats are not thought to be very high, with only a transient increase in adrenal gland wet weights in the first few days, which, for example, have returned to normal by the fourth day of suspension (Thomason *et al.*, 1987b). Similarly, circulating levels of glucocorticoids are transiently increased, returning to normal by day 7 (Steffen & Musacchia, 1987).

1.3.3 Similarity of Changes in Zero Gravity and its Models

Hindlimb suspension appears to be relatively similar to spaceflight, at least in a qualitative way. The most valid comparisons have been where conditions for the rats such as food, caging environment, as well as the strain and the age of the rats has been similar in the control, suspended and flight groups. Such experiments have shown that the changes in hindlimb suspension are similar to those in found 0-G, but that the changes observed in suspension are somewhat greater. For example, in one closely matched experiment from the Cosmos 2044 mission, fibre cross-sectional areas increased in the tibialis anterior of both flight and suspended animals, but the increase was 5% greater in the suspended animals. Ohira *et al.* (1992) also investigating the Cosmos 2044 flight, reported a 14% greater decrease in cross-sectional area of fibres in the suspended solei compared to the 0-G exposed muscles. It appears, from these two studies and others, that the changes in fibre type proportions, if any, are similar in suspension and in spaceflight. The whole body suspension model, while not extensively used, has similar effects on the hindlimb, and has also been shown to model 0-G fairly well, at least in slow twitch muscles. Musacchia *et al.* (1990) for example, compares muscle mass of spaceflight rats and rats in whole body suspension. The soleus, in whole body suspension, lost 30% of its mass, in comparison to the spaceflight muscles which showed a 20% loss. However, the EDL showed some differences in the two systems. In whole body suspension the EDL showed a 20% increase in muscle mass, whereas the space flown rats showed a 15% loss in muscle mass.

To summarise, hindlimb suspension has substantial effects on the muscles of the hindlimb. It has the ability to change contractile properties, muscle size, mRNA, and protein isoform expression. However, the magnitude of these effects depends on the muscle in question. It is the slow twitch postural or anti-gravity muscles such as the soleus and the adductor longus which are the most greatly affected. However, the smaller effects on other muscles of the hindlimb cannot be ignored. The low expense of this model and the similarity to 0-G on the hindlimb musculature means that valuable experimental data can be gained from it, especially with experiments that would be difficult or too costly to be performed in 0-G.

1.3.4 Earth Bound Human Models of the Zero Gravity Condition

Some attempts have been made to model the effects of 0-G on humans. A variety of techniques have been used to unload the lower limb. One is the "head down bedrest" model, (as used by Convertino *et al.*, 1989; Duvoisin *et al.*, 1989; Dudley *et al.*, 1989; Hargens *et al.*, 1983; Hikida *et al.*, 1989). In this model people are confined to a bed with a tilt (usually at an angle of approximately 6°) such that the head is the lowest part of the body. The bed rest reduces the load on the limb to a minimum, and the head down tilt causes a fluid shift in the body away from the lower limbs and towards the head and chest, as is known to occur in 0-G (Leach *et al.*, 1991). Some studies have been carried out on people with immobilised lower limbs due to some misadventure, for example Lindboe & Platou (1982) investigated the lower limb muscles of people following knee surgery. However, these studies have the problem that the trauma and associated stress of the misadventure can affect the muscles as well as the actual immobilisation itself. A recently devised model is that of "unilateral lower limb suspension" (Berg *et al.*, 1991; Hather *et al.*, 1992). In this model, a platform shoe is worn on one foot, and the other foot is held off the ground using a strap. The person walks using the aid of crutches, and in this way the leg with the strap is free to move but does not bear weight. All of the above studies using the various models have reported the presence of atrophy, which is greater in postural muscles as has been reported in the rat, but the rate of atrophy is much slower. For example, Desplanches *et al.* (1987a) reported a 63% reduction in cross-sectional area of the rat soleus following 35 days of hindlimb suspension, while Hather *et al.* (1992) found a 17% decrease in the human soleus after 42 days of unilateral lower limb suspension.

However, the data from these human studies is rather often limited; the cross-sectional area data reported is often for the whole muscle, rather than for single fibres. This whole muscle measure is often used because the data can be obtained using non-invasive imaging techniques.

In contrast to the rat model, no change in fibre type proportions has been detected in humans, in the few such studies attempted (Hargens *et al.*, 1983; Hather *et al.*, 1992; Lindboe & Platou 1982). This may be a genuine species difference, but it is also possible that any change in fibre type has not been detected because of the use

of the relatively insensitive histochemical techniques. The small sample of muscle obtained by biopsies may also be a problem. Another possibility is that since the rate of change is slower in humans, the changes observed in rats would take a longer period of time to detect.

1.3.5 Spaceflight

The atrophy that has been widely reported in hindlimb suspension is also found in spaceflight. As in hindlimb suspension, slow twitch muscles, in general, atrophy more than fast twitch muscles. For example, after 7 days of spaceflight on the Cosmos 1667 mission (Desplanches *et al.*, 1990), the mass of the soleus fell by 23%, while that of the EDL fell 11%. Cross-sectional areas similarly decreased more in the soleus than in fast twitch muscles. Miu *et al.* (1990), in examining rats from the Cosmos 1887 mission of 12.5 days duration, showed a decrease in the cross-sectional areas of 40% in the soleus and a decrease only half as great in the fast twitch medial gastrocnemius. The 7 day Spacelab-3 mission (Martin *et al.*, 1988) reported a decrease of 36% in soleus cross-sectional area, and only a 16% decrease in the EDL.

Selective atrophy and decreases in cross-sectional area have also been reported by Baldwin *et al.* (1990), Desplanches *et al.* (1991), and Riley *et al.* (1987). One disagreement is given by Manchester *et al.* (1990), who states that both the soleus and tibialis anterior atrophy by the same amount, losing 35% of their mass.

Disagreement as to the effect on fast twitch muscles is seen here as it is in hindlimb suspension. Jiang *et al.* (1992) reports a rise in the cross-sectional area of the fast twitch tibialis anterior following the 14 day Cosmos 2044 mission, while Riley *et al.* (1992) reports a statistically significant increase in the cross-sectional areas of only the slow fibres in the tibialis anterior, whilst the other fibre types remain unchanged.

The use of wet muscle weight data may not be ideal as a measure of muscle atrophy. Damage can occur to the weakened muscles on landing, and in the recovery period until the rats are killed, as will be discussed later. During this time, oedema can form, increasing the wet muscle weights. This increase in wet muscle weight is due to changes in the amount of fluid in the muscle, rather than changes in the

amount of muscle protein. For example, Riley *et al.* (1992) found that wet weights following the Cosmos 2044 mission were near identical to the control values, even though muscle cross-sectional areas were much reduced. This anomaly was due to the presence of oedema in the 0-G exposed muscle.

Another trend to emerge is the progressive nature of the changes, which become greater the longer the duration of exposure to 0-G. In some cases only muscle weight data is available, but it can serve as a useful, though limited, comparison. For example, 7 days of spaceflight decreased soleus weight by 23% (Cosmos 1667), 18.5 days decreased it by 38% (Cosmos 782), and 21.5 days (Cosmos 1129) caused a decrease of 55% (Desplanches *et al.*, 1990). The progressive nature of the changes occurring can also be seen in the shorter term missions, but not as clearly, as the 0-G exposure times are relatively similar. Unfortunately, no other useful data is available from the longer term space missions.

The shifts in fibre type proportions witnessed in suspension are also seen in 0-G. Not a great deal of success was attained in determining any changes in fibre types using histochemistry, with Martin *et al.* (1988) being able to show a statistically significant increase of 11% of fast fibres in the soleus, with no change in the EDL. Desplanches *et al.* (1990) reported a 10% increase in fast fibres in the soleus from Cosmos 1667 using histochemistry. Others, (Desplanches *et al.*, 1991; Miu *et al.*, 1990 - both analysing Cosmos 1887 data; Riley *et al.*, 1987 - Spacelab-3) failed to show any difference with histochemistry. Miu *et al.* (1990) showed that this difference in ability to detect changes in fibre types was due to the limitations of the histochemical technique, by simultaneously analysing fibre type proportions with histochemistry and immunocytochemistry (antibody techniques). While histochemistry showed no significant change, immunocytochemistry detected that mixed fast-slow fibres (or transitional fibres) increased from 8% to 31% in the soleus. Of additional note in this study is also that the mixed fibres make up a significant proportion of the control fibre population. Other immunocytochemical studies have also shown significant changes. Ohira *et al.* (1992) showed a 13.7% increase in mixed fast-slow fibres in the soleus after the Cosmos 2044 mission. Baldwin *et al.* (1990) showed that it was the slow myosin isoform which was selectively lost in the 0-G environment. The above studies showed no change in fibre

proportions in fast twitch muscles, as was reported in hindlimb suspension, with one exception. Desplanches *et al.* (1990) reported a decrease in slow fibres from 10% to 5% in the EDL. This has not been reported elsewhere.

Since the results from space flown and hindlimb suspended rats appear similar, the purpose of exposing rats to the 0-G environment may be questioned, especially considering the expense involved. Several answers emerge. On a practical level, there are other systems which need investigating in 0-G conditions for which it is not so simple to produce a satisfactory model, and since those areas need to be examined, material in the form of the muscles from the 0-G exposed rats will be available. Although there are some differences between the unloading models and the results obtained from 0-G, it is not certain the changes are occurring through the same mechanism or mechanisms. Another reason is that the muscles of space-flown rats have been shown to sustain damage which they do not in the earth-based models. This could be due either to something associated with the 0-G conditions, or possibly due to damage caused to the muscles in either landing or re-entry. Riley *et al.* (1990a) found that extensive muscular damage occurred after Cosmos 1887. Segmental necrosis, interstitial oedema, presence of increased numbers of inflammatory cells such as macrophages and neutrophils, and evidence of damage to the microcirculation, are all features that are not present in models such as hindlimb suspension. There was an increased presence of the protein ubiquitin, this protein possibly having a role in "tagging" proteins for proteolysis, and thus playing a role in atrophy and necrosis. However, with this particular mission there was the problem that the rats were not killed until 2 days after landing due to a change in the landing site, and this long duration re-exposed to 1-G would have caused, or added to, muscle injury. In another Cosmos mission, Cosmos 2044, a 14 day mission as discussed by Riley *et al.* (1992) damage was also found, with increased ubiquitination, damaged microcirculation, lesions suggestive of hyperextension of the sarcomeres, and tearing of the connective tissue. It was suggested that part of the damage could be due to the exposure of the animals to high G forces during re-entry (3-G to 4-G for 5 minutes), and the landing impact, estimated at 20-G for 10 milliseconds. It is thought that this, along with the re-exposure of the weakened muscles to 1-G for 8-11 hours before being killed caused the muscle damage. Other

means of spaceflight which involve less stressful take-off and landing, such as the Space Shuttle in which the maximum G force during take-off and landing does not exceed 3-G, may provide some answers.

Other differences between the models and space flight may also be present. It has been suggested that the increased level of exposure to cosmic radiation of animals in space leads to an increased level of damage to their slow antigravity muscles than would occur due to weightlessness alone (Ilyina-Kakueva & Portugalov, 1979). Exposure to an added dose of radiation while in space may aggravate the atrophic response of the slow muscles, while having little effect on fast muscles. Further, Ushakov *et al.* (1978) suggested that irradiation inhibits protein synthesis in muscle.

Only one study on the effects of 0-G on a primate, the rhesus monkey, has been undertaken. In contrast to the rat data, they found no atrophy in the soleus, and slight atrophy in the fast twitch muscle tibialis anterior. They also found no change in fibre proportions. However, the study is rather flawed. The sample size was small (approximately 40 fibres per muscle from 2 flight and 4 control muscles) and pre-flight muscle biopsies were taken 107 days before the flight. The monkeys were strapped into chairs with very limited movement, and these chairs allowed them to generate large isometric forces in their limb muscles which may have countered the effects of 0-G. They were also trained to sit in these chairs for several hours every day for 2 years before the flight which may have led them to adapt to the reduced levels of limb muscle activity. In short, while there may be species specific differences, it appears difficult to accept a lack of change on the basis of one problematic experiment.

In summary, 0-G can bring about large changes, particularly in slow twitch muscles. Some of these effects can be adequately simulated, while some aspects of the changes in 0-G are not seen in the hindlimb suspension model.

1.4 Aims of the Project

In a similar vein to the spaceflight experiments discussed above, this project will involve the analysis of muscles from rats exposed to 0-G for 9 days aboard the Space Shuttle during the Space Life Sciences-1 (SLS-1). The muscles available are the soleus and EDL, from both rats exposed to 0-G and various ground based controls. Analysis will involve immunocytochemistry, or the use of monoclonal antibodies to determine fibre types. Specifically, the analysis will involve how fibre types and fibre sizes are altered by 0-G. The range of monoclonal antibodies used will hopefully allow a specific indication of how MHC isoforms are changing in 0-G. It is expected that a shift towards one or more fast MHC isoforms will be observed in the soleus, accompanied by atrophy. The changes that will occur in the EDL appear to be less predictable, but it seems likely that any changes which do occur will not be as great as those that are likely to be detected in the soleus.

Chapter 2: Methods

2.1 Pre-Flight and Initial Post-Flight Procedures

The initial part of this experiment was conducted in the USA, at the Kennedy Space Centre. Pathogen free male Sprague Dawley rats of 5 weeks of age and of weights 90-100g were obtained from a colony at Taconic Farms, Germantown, New York, and were shipped to the Space Centre. An excess of rats (181) was obtained for the launch, this number being progressively reduced, some rats being required for pre-flight experiments, other unsuitable rats also being removed. Of the 181 rats initially received, 10% were used for necropsy and microbiological experiments. So 163 rats were shipped to the Kennedy Space Centre. Once the rats were received, a further 10% of them underwent microbiological examinations. In the time before the launch, the rats were housed individually in clear plexiglass cages. The rats weighed approximately 90-110g at receipt.

The rat selection process involved certain criteria in an attempt to produce a homogenous population of rats suitable to the many experiments which would be conducted on them. Among the selection criteria were the following (as described by Meylor, 1991):

1) Animal health - as determined by daily checks by the Ames Research Center veterinarians;

2) Body weight and weight gain history;

3) Haematological criteria, with evaluation of the usefulness of each rat for injections and blood draws;

4) Comments made on injection of bone markers; and

5) General animal behaviour or anomalies.

Once the population of rats which were to be used for the experiment was established, through the consideration of the above criteria, rats were randomly placed into the various experimental groups described below.

There were two experimental groups of rats involved, each requiring an initial population of 181 rats. The first group which will be called the SLS-1 group,

included the rats flown on the space shuttle (or flown rats) and various controls. The second group, named the "Delayed Flight Profile Test" (DFPT) group, contained another set of rats which were selected in exactly the same way, and exposed to exactly the same conditions as the SLS-1 group except that they were all ground based controls. As the name implies, the DFPT experiment was performed after the actual shuttle flight.

Two cage types were used. The Research Animal Holding Facility (RAHF - Figure 2.1) contained small "shoe box" cages in which single rats were housed. The other cage type was the Animal Enclosure Module (AEM - Figure 2.1), which housed rats in groups of 5. The cages were designed so that food and water were provided *ad libitum*. A small, positive inward air pressure was maintained at all times to stop the escape of particulate matter from the cages and into the general shuttle environment.

Rats were divided up into various control and flight groups, and a summary of these complex classifications follows. In both the SLS-1 and DFPT groups, rats were killed at three different times:

1) "Launch" day rats (ie. launch plus zero days, or L+0d). This group contains only control rats not exposed to 0-G. Rats were 58 days old (just over 8 weeks) at launch;

2) "Mission Length" rats (L+9d) killed 9 days after launch, ie. as soon as possible (several hours) after the return of the shuttle to earth. This group contains both control groups and rats exposed to 0-G. Control rats were kept in AEM and RAHF cages as were the flight rats, for the duration of the flight. The rats were killed when they were 67 days old; and

3) "Mission Length Plus Recovery" rats (L+9d+9d), killed 18 days after launch, with the flown rats being exposed to the 9 days of 0-G and a subsequent 9 days of "recovery" period at 1-G before being killed. Control rats in this group were killed 18 days after launch as well. In the 9 day recovery period (as well as during the flight), both the flown and control groups were kept in their AEM and RAHF cages as appropriate. These rats were 78 days old at death.

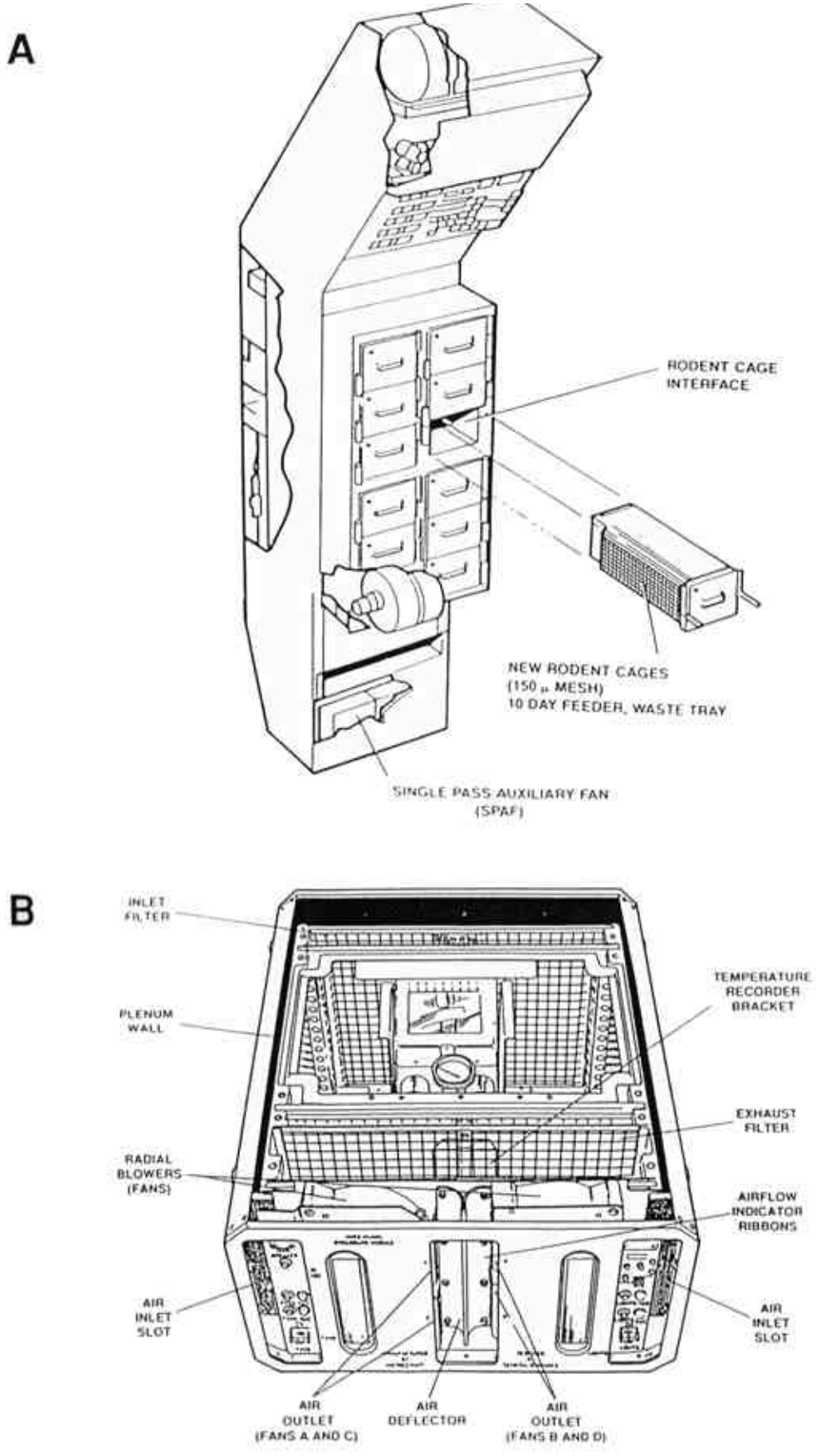

Figure 2.1 Cages in which rats were housed. A - Research Animal Holding Facility (RAHF), caging rats individually. B - Animal Enclosure Module (AEM), caging rats in groups of 5.

The flight and recovery controls were exposed to the same environment as the flight animals, including caging, food, lighting, and so forth. The launch of the space shuttle occurred on the 5th June, 1991, whilst the commencement of the DFPT group experiment occurred on the 10th of July, 1991.

Once the rats were killed, their soleus and EDL muscles were removed (amongst many muscles, and other organs used for other experiments), individually attached to labelled pieces of cardboard with Tissue Tek (Miles Scientific), frozen in freon 12 cooled in liquid nitrogen, and then stored in liquid nitrogen. The muscles were transferred to a liquid nitrogen shipper, then shipped to this laboratory, arriving in December, 1991.

Not all of the above groups of muscles have been analysed as yet. Those control groups which have been analysed to date are the DFPT launch controls (L+0d) for both soleus and EDL, which will be denoted D0 (Day zero) controls henceforth, and the SLS-1 mission length controls (L+9d) for soleus and EDL, which will be denoted D9 controls (Day 9). Of the flown muscles, both the mission length (L+9d) soleus and EDL muscles have been analysed, and will be denoted "flight" muscles henceforth, whilst only in the soleus have the mission length plus recovery muscles (L+9d+9d) been analysed, to be called "recovery" muscles henceforth. 10 soleus and 10 EDL muscles were available for analysis in each group, except for the recovery group where 9 muscles of each type were available. In each experimental group, 5 rats were group housed (AEM cages), 5 individually housed (RAHF cages) except for the recovery group where 4 individually housed rats were available.

2.1.1 Discussion of Pre-Flight and Initial Post-Flight Procedures

Experiments of this type are by their nature very expensive and rare. Because of this, it was necessary to maximise the number of experiments that could be performed on a limited population of rats. As such, conflicting demands from the various experiments existed as to the most suitable species of rat and the age at which they were to be used. Further, because each possible launch required a group of 181 rats to be prepared in the manner described, it was undesirable for the rats to be of too mature an age, as this would require longer term planning for what was already a daunting organisational task.

After all these factors were considered, our laboratory had little input into the exact design of the experiment, past the initial experimental idea. For example, for our purposes it may have been better if Wistar rats had been used rather than Sprague-Dawleys, and it probably would have been desirable for the rats to be older, as will be discussed later. These compromises have to be expected in such a group experiment, and have hopefully not detracted greatly from the knowledge gained.

2.2 Tissue Preparation

Muscles were received in a liquid nitrogen shipper, and were transferred to a standard liquid nitrogen container. Preparation of muscle blocks suitable for cutting on a cryostat was performed in the cryostat (at -20°C) to prevent thawing of the tissue.

The midbelly of each muscle was cut out using a scalpel, and attached to a cork block using Tissue-Tek (Miles Scientific), with a ring of Tissue-Tek around the muscle. These blocks were again stored in liquid nitrogen, as were the left over end regions of each muscle. The end portions are to be used for mRNA analysis by another laboratory.

2.3 Slide Preparation

Microscope slides were gelatinised to enhance their adhesion characteristics. Slides were dipped for 1-2 minutes into a warm solution of 30g gelatin, 0.3g chromic potassium sulfate made up to 500ml with distilled water.

Semi-serial cross sections of 4 to 10μm thickness were cut from the muscle blocks on a Leitz cryostat (model 1720), and the sections were placed onto the gelatinised microscope slides. The slides were then air dried with a fan for at least 20 minutes.

2.4 Immunocytochemical Techniques

Three techniques can be used to visualise the binding of an antibody to tissue. All

involve the binding of a secondary antibody which binds to the Fc region of the primary antibody. The first involves a fluorescently labelled secondary antibody. Here the binding of the primary antibody can be visualised by the use of a fluorescent microscope. This technique has the problem of a rapid fading of the fluorescence. Secondly, the secondary antibody can be conjugated to horse radish peroxidase (HRP). When hydrogen peroxide and diaminobenzidine are added to this, the HRP produces an insoluble brown reaction product, visible under the light microscope. This technique has high sensitivity, as each HRP molecule can produce a large amount of reaction product; the reaction product is very stable, allowing the slides to be used for at least several years. This HRP technique is well established in this laboratory. Thirdly, a biotin-streptavidin system can be used. Here the secondary antibody is conjugated to a molecule called biotin, and then streptavidin is added. Streptavidin has a high affinity to biotin, binding to it strongly. The streptavidin may be either conjugated to HRP or fluorescently labelled. This technique is thought to have a similar sensitivity to the HRP method (Kiernan, 1990), but requires a greater number of steps. The technique used was the HRP technique, as it has been well established in our laboratory in the past, providing good sensitivity and contrast.

Sections were exposed to one of the various isoform specific anti-MHC antibodies (approximately 60µL/section), known as the primary antibody, either overnight at 4°C or for 3 hours at 37°C. The slides were then washed 6 times in phosphate buffered saline (0.01M PO_4, pH 7.2) at 5 minute intervals. Next, the slides were exposed to one of two HRP conjugated secondary antibodies (DAKO products - 60µL/section) for 1.5 hours at 37°C. This secondary antibody was either anti-sheep IgG specific, in the case of STE, or anti-mouse IgG in all other cases, as all the other primary antibodies (with one exception) used were mouse IgG antibodies. After another set of 6 by 5 minute washes in PBS, the slides were incubated for 10 minutes in a HRP reaction mix (0.06% diaminobenzidine, 0.03% H_2O_2, 0.05M Tris, pH 7.6) producing a brown pigment in the cells which had reacted with the primary antibody. The slides were again washed in PBS, dehydrated with 70% ethanol for 1 minute, 95% ethanol for 1 minute, and finally with 100% ethanol for 2 minutes. They were then immersed in xylene for at least 2 minutes, before being mounted in DePeX (BDH Chemicals) with a coverslip placed over the section.

Black and white photographs of the muscles were taken with a Zeiss MC100 camera connected to a Zeiss Axioplan microscope.

A number of primary antibodies were used, all monoclonal antibodies raised in mice, with the exception of STE, which is a polyclonal antibody raised in sheep. All the mouse monoclonal antibodies were IgG, except for BF-F3 which was an IgM antibody. Some of the antibodies used were hybridoma supernatants, while others were grown as ascites in mice to increase the antibody titre. A summary of the primary antibodies is presented in Table 2.1.

5-4D (with the complete name NOQ7.5.4D) was used at a dilution of 1:20 and is specific for slow or type I MHCs (Narusawa *et al.*, 1987) and displayed excellent sensitivity and staining characteristics.

Many antibodies which stain either all or a subset of the type II fibres were used. 1A10 (Everett, 1986) and MY-32 (Havenith *et al.*, 1990) both stain all type II (IIA, IIX and IIB) fibres. 1A10 was used at a dilution of 1:500, MY-32 at 1:2000. 1A10 and MY-32 (Sigma Immunochemicals) both stain foetal MHC as well.

5-2B (with the complete name NOQ7.5.2B), specific to type IIA and IIX (Draeger *et al.*, 1987), was used at a dilution of 1:1000, displaying a lower level of staining sensitivity, and a higher level of background staining. This antibody is known to stain all type II fibres in the cat including the IIB isoform, unlike in the rat (Hoh *et al.*, 1988). It had previously been determined empirically (Hoh *et al.*, 1988) that it was necessary to incubate the sections with an acidic phosphate buffered saline solution (pH 2.8) for 15 minutes prior to incubating with 5-2B to obtain a successful reaction. Presumably this acid pre-incubation altered the epitope on the MHC in such a way that it allowed the 5-2B to bind to it.

STE, a cross-absorbed polyclonal antibody raised in sheep, stained foetal myosins (Hoh *et al.*, 1988). A relatively high level of background staining was observed with this antibody. It was used at a 1:300 dilution.

The following antibodies were kindly donated by Stefano Schiaffino (Schiaffino *et al.*, 1989; Kucera *et al.*, 1992). BF-G6 reacts with IIB and Embryonic MHCs, and was used at a dilution of 1:1000. SC-71 reacted positively with IIA MHC, and was used at a dilution of 1:2000 in EDL and 1:10000 in soleus. BF-35, used at a dilution of 1:200, stained type I, IIA, and IIX fibres. That is, it reacted positively for all adult

Name	Ig Class	Specificity	Reference	Comments
5-4D	Mouse, IgG	I	Narusawa *et al.*, 1987	Supernatant
1A10	Mouse, IgG	II, Foetal	Everett, 1986	Ascites
MY-32	Mouse, IgG	II, Foetal	Havenith *et al.*, 1990	Sigma Immunochemicals, ascites
5-2B	Mouse, IgG	IIA, IIX	Draeger *et al.*, 1987	15 min acid pre-incubation . Low sensitivity, ascites
STE	Sheep, Polyclonal	Foetal	Hoh *et al.*, 1988	High background
BF-G6	Mouse, IgG_1	IIB, Embryonic	Kucera *et al.*, 1992	Donated by Schiaffino, all ascites
SC-71	Mouse, IgG_1	IIA	Schiaffino *et al.*, 1989	
BF-35	Mouse, IgG_1	I, IIA, IIB		
BF-F3	Mouse, IgM	IIB		Not successfully used, donated by Schiaffino, ascites
RT-D9	Mouse, IgG_1	IIB, IIX		

Table 2.1 A summary of the characteristics and properties of the primary antibodies used in this study.

MHC isoforms except IIX, providing a "negative" stain of fibres possessing IIX myosin.

Two other antibodies were donated by Schiaffino, these being BF-F3 and RT-D9. BF-F3 is supposed to stain IIB fibres, and RT-D9 is supposed to stain IIB and IIX fibres. However, these were unable to be used successfully as discussed later.

As the number of antibodies is large and their names not very descriptive, a summary of the staining characteristics of the various primary antibodies is presented in Appendix A, which folds out for easier reference.

2.5 Quantitation of Fibre Proportions and Diameters

This quantitation was performed using computer-aided morphometric analysis, with the aid of an Olympus BH-2 microscope attached to a National video camera model WV-1500E/A. The image from the camera was displayed on a monochrome Kaga Denshi monitor. Also attached to the monitor was an Apple II compatible computer, so that both the image from the computer and the microscope could be visualised on the monitor simultaneously (Haslasz & Martin, 1984). Using a software package named "Magellan", the proportion of fibres stained with a particular antibody, as well as the average fibre diameters for a population of fibres could be determined. Cross-sectional area is related to the square of the diameter, and as such the average fibre diameter was able to be used to detect changes in the cross-sectional areas of muscle fibres.

2.5.1 Soleus

In the soleus, the proportion of fibres staining with 5-4D (type I fibres), 1A10 type II), and 5-2B (types IIA, IIX) was examined in detail, in all available muscles. Fasicles of fibres, which could be identified on the sections stained with all three antibodies, were examined for the proportions of fibres staining with these three antibodies. Three to five such fasicles from the cross section of each muscle were randomly chosen for analysis. Approximately 500 fibres per muscle were counted in the D0 control and in the flight muscle. In the D9 control and recovery muscles, approximately 160 fibres per muscle were counted. From these counts, the proportion of fibres stained with each antibody was calculated for each muscle.

Fibre diameters in the soleus were determined on a number of muscles from each of the above groups. Approximately 160 fibres from each of these muscles was measured using Magellan, with the computer calculating average fibre diameters for each fibre, and subsequently, for the population of fibres from a muscle or group of muscles.

Analysis of BF-35, STE, and BF-G6 involved counting the number of fibres differing from the majority across the whole muscle cross section, in each case a very small minority of fibres. That is, the number of fibres unstained with BF-35, and the number of fibres stained with STE and BF-G6.

SC-71 and MY-32 were used in confirmatory roles, and were not used to stain all the muscles.

2.5.2 EDL

Fibre type proportions were not examined in detail in the EDL, for reasons discussed later. However, the number of slow fibres (5-4D positive) present in the whole muscle cross section was counted for the flight muscles, as well as the D0 and D9 controls.

Fibre diameters were examined for a number of the muscles in the abovementioned groups. The fibre diameters of all 5-4D positive fibres in a number of muscles were measured. Also, using the antibodies SC-71 (type IIA) and BF-G6 (type IIB) approximately 150 fibres per muscle, found in 3 to 5 fasicles, were measured for their average fibre diameters. This was carried out as it was in the soleus with the antibodies 5-4D, 5-2B and 1A10, as explained previously.

2.6 Statistical Analysis

All statistical analysis was performed using the unpaired Student's t test, with $P<0.05$ being considered a statistically significant difference.

2.7 Discussion of Methods

Various methods were attempted to enhance the staining characteristics of the various antibodies used, that is, enhance the signal to noise ratio. This included adding 2% Cobalt Chloride (w/v) to the HRP reaction mix, in an attempt to enhance the staining characteristics (Kiernan, 1990). However, this was not successful, with a background precipitate forming over the entire area of the section. Also attempted was the use of an anti-IgM specific HRP conjugated secondary antibody for BF-F3 (an IgM antibody), which improved the staining characteristics marginally, but still not enough to be consistently usable. Also attempted was the used of a biotin-streptavidin system to stain the sections, a system with a similar sensitivity to the HRP conjugated secondary antibody system. Again, improvements were marginal or

non-existent. In summary, it was found that those antibodies with poor staining characteristics (namely, BF-F3 and RT-D9) were unable to be improved usefully by using modified approaches to staining, whilst the other antibodies already possessed adequate or good staining characteristics which did not require the added complications the modified techniques involved. Since the other antibodies donated by Schiaffino were successfully used with no difficulty, it may be that these two antibodies for some reason had lost their activity - perhaps in transit, or in the process of preparing them for transit.

The quantitative procedures for the soleus were changed between the D0 controls, flight muscles and the D9 controls and recovery muscles. The change was that the number of fibres counted for determining fibre type proportions was reduced for the D9 control and recovery groups. This was done for several reasons. The various fibre types are evenly distributed across the muscle as will be seen later in the micrographs, and so the smaller sample size was adequate to get a representative sample. Thus the added work led to little or no gain. Further improvements in efficiency were gained by measuring fibre type proportions in the same fasicles with the diameters, as was carried out in the D9 controls and recovery soleus groups.

Some technical difficulties were encountered with the first few EDL D0 controls - this was the first group of muscles which were analysed, and as such, several of the early muscles were lost in the attempts to devise a way of placing the muscle on a block so that it could be sectioned effectively. Normally, muscles are prepared for sectioning before being frozen. In this case, the muscles arrived frozen, making it relatively difficult to prepare the muscle for sectioning without thawing, and thus ruining the muscle.

Chapter 3: Results

3.1 The Soleus

Micrographs of representative soleus muscles from each experimental group stained with 5-4D, 1A10 and 5-2B are seen in Figures 3.1 to 3.4. Figure 3.1 shows low power micrographs of the cross section from a representative soleus muscle from the D0 control and flight groups stained with the abovementioned antibodies. Figure 3.2 shows a region from the above muscles, viewed under high power. Figures 3.3 and 3.4 show low power and high power micrographs, respectively, of representative solei from the D9 control and flight groups prepared in the same way as Figures 3.1 and 3.2. Positively stained fibres appear black in these micrographs.

In all of the muscle groups, the majority of fibres are stained with 5-4D (slow), with a fairly even distribution of unstained fibres. The proportion of stained fibres appears to be little different between the groups as determined by this antibody. A smaller number of fibres are positively stained with 1A10, and the proportion of fibres stained with this antibody varied quite considerably amongst the groups. The most striking variation in the number of 1A10 stained fibres can be seen by comparing the D9 control in Figure 3.4 with either the recovery group (Figure 3.4) or the flight group (Figure 3.2). The D9 control has far fewer 1A10 positive fibres than either the flight or recovery groups. The flight and recovery groups also appear to have more 1A10 positive fibres than the D0 control group.

If the number of fibres stained with 5-4D appears unchanged or to change relatively little, and the number stained with 1A10 appears to change, then the number of fibres stained with both antibodies must change (assuming all fibres are stained by at least one of the antibodies). The fibres stained with both 5-4D and 1A10 are termed transitional fibres, as will be discussed later. Many more transitional fibres are seen in the flight and recovery groups than in the D0 and D9 controls. Several transitional fibres are noted in Figures 3.2 and 3.4.

5-2B (IIA/IIX) appears to stain a subset of the fibres stained by 1A10. It often does not stain fibres which are lightly stained by 1A10, and therefore is said to have

Figure 3.1 Low power view of serial sections from representative solei of the D0 control (A-C) and flight (D-F) groups. A,D - 5-4D; B,E - 1A10; C,F - 5-2B. Scale bar represents 500μm.

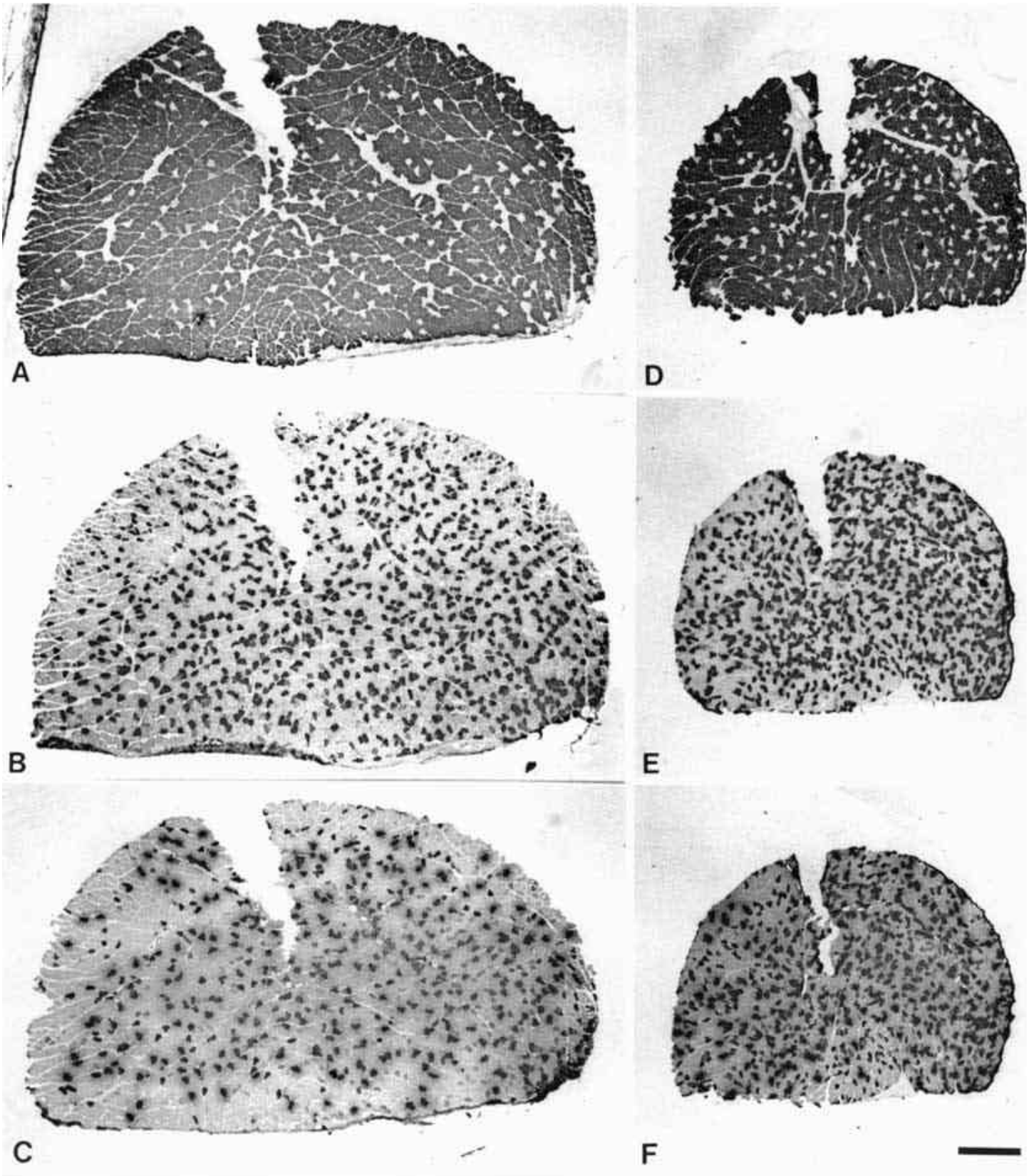
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Figure 3.2 High power view of serial sections from representative solei of the D0 control (A-C) and flight (D-F) groups. A,D - 5-4D; B,E - 1A10; C,F - 5-2B. Arrows denote transitional fibres. Circles denote fibres stained with 1A10, but not with 5-2B. Scale bar represents 80μm.

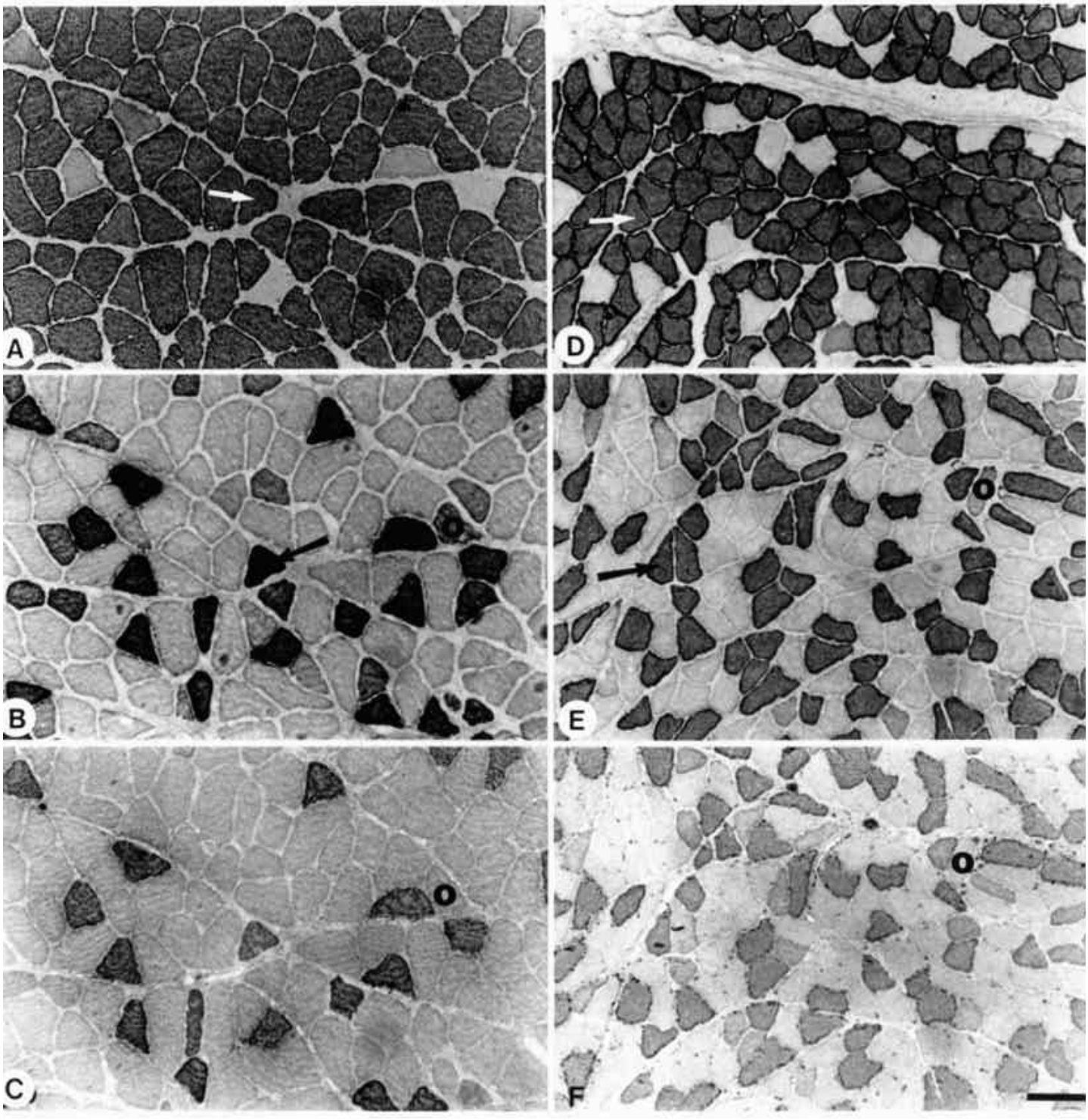
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Figure 3.3 Low power view of serial sections from representative solei of the D9 control (A-C) and recovery (D-F) groups. A,D - 5-4D; B,E - 1A10; C,F - 5-2B. Scale bar represents 500μm.

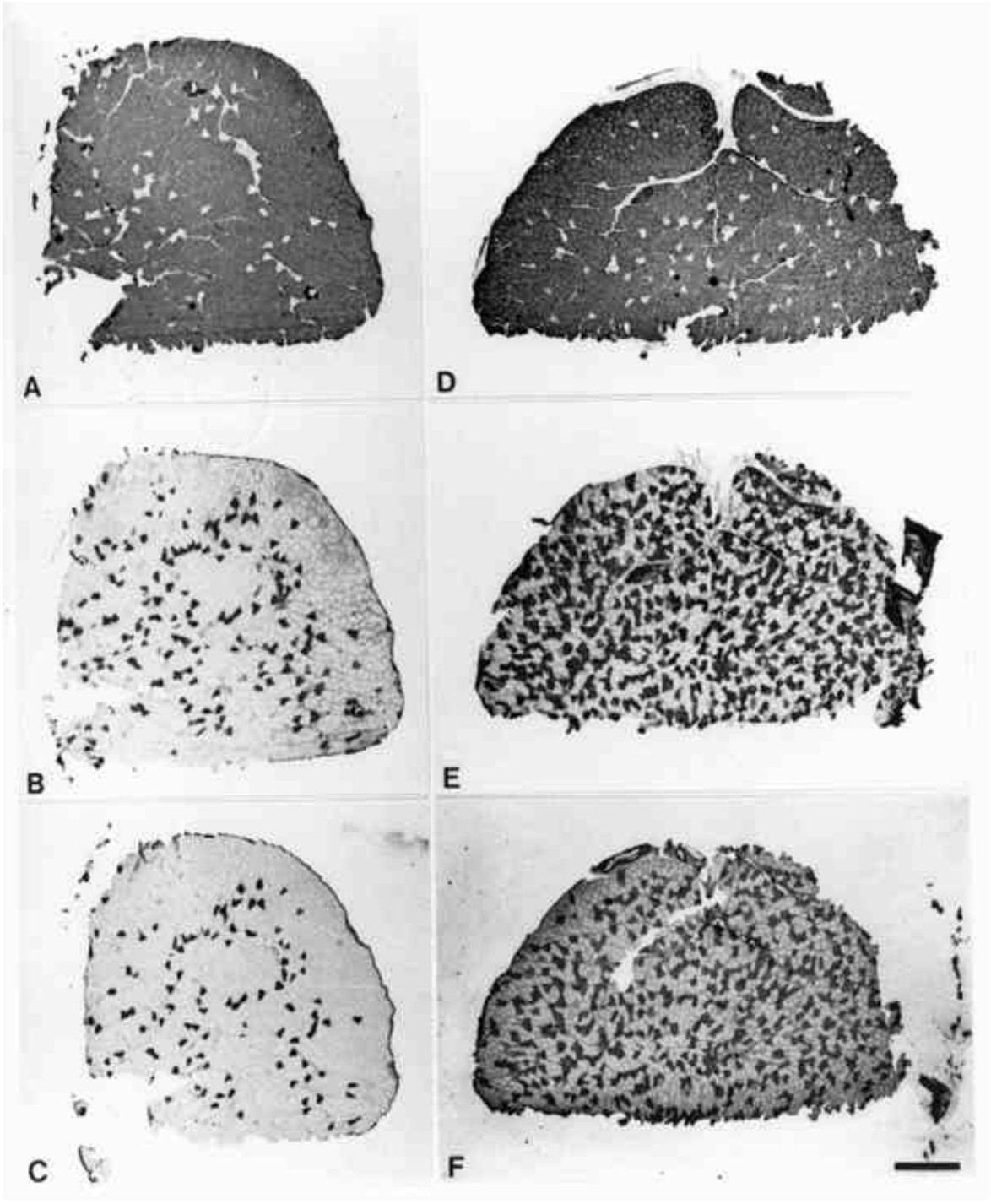
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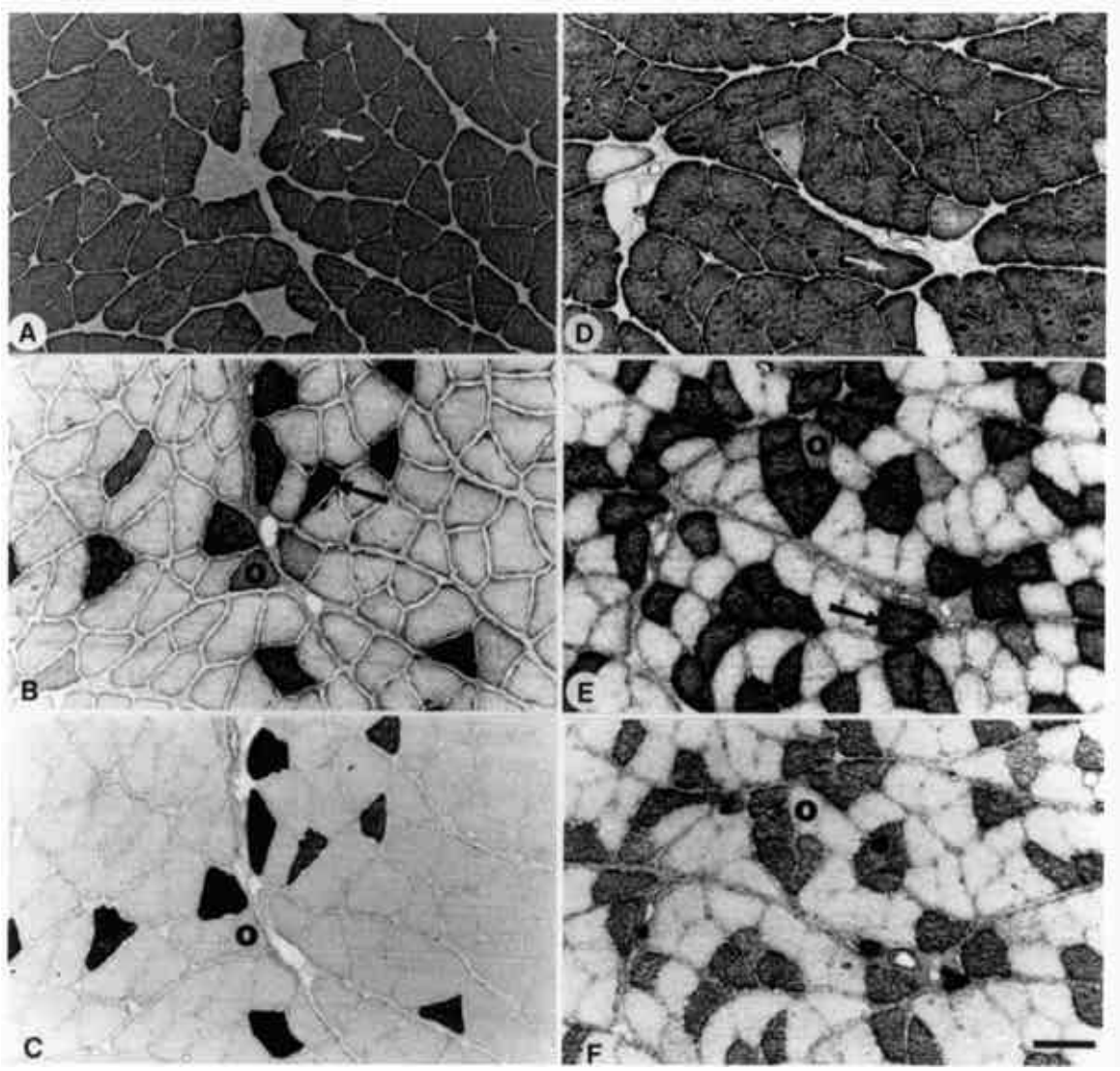
Figure 3.4 High power view of serial sections from representative solei of the D9 (A-C) and recovery (D-F) groups. A,D - 5-4D; B,E - 1A10; C,F - 5-2B. Arrows denote transitional fibres. Circles denote fibres stained with 1A10, but not with 5-2B. Scale bar represents 80μm.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a lower sensitivity than 1A10. Fibres containing small amounts of fast MHC, which are stained by 1A10, tend not be stained with 5-2B. Several fibres stained by 1A10 but not stained by 5-2B are noted in Figures 3.2 and 3.4. However, the proportion of 5-2B stained fibres seems to vary in a similar way to that of 1A10 between the various experimental groups.

Perhaps the most striking differences are seen in the average cross-sectional areas of the fibres in the different groups. This can be most easily judged by determining the number of fibres present in the high power micrographs in Figures 3.2 and 3.4, which all show an area of the same size, and at the same magnification. The flight group has fibres with the smallest cross-sectional area (and hence the largest number of fibres visible in the area), followed by the D0 controls. The D9 and recovery groups have the largest fibres, and appear to be fairly similar in size to each other.

The micrographs provide a useful comparison, but they only show one muscle from each group. A comprehensive analysis of the fibre proportions and diameters in the soleus, using data from many muscles, follows.

3.1.1 Fibre Type Proportions Using 5-4D, 1A10, and 5-2B

The proportions of fibres stained with 5-4D, 1A10, and 5-2B (staining slow, fast and IIA/IIX respectively) and their average fibre diameters were examined in detail, as described previously. Figure 3.5 compares the fibre proportions of the D0 and D9 controls using the above 3 antibodies. A fourth category appears on the graph, this being the "transitional fibres" category. This category refers to those fibres which have both slow staining properties (positively stained by 5-4D) and fast staining properties (positively stained by 1A10). Since all the fibres found in the soleus are stained with either or both of these antibodies, the proportion of fibres displaying both fast and slow characteristics was able to be determined. By adding the proportion of fibres positive with 1A10 and 5-4D, the amount that this sum exceeded 100% was the proportion of fibres displaying transitional fibre characteristics.

Figure 3.5 compares fibre proportions in the D0 and D9 control groups. As Figure 3.5 shows, the majority of fibres in the soleus are slow fibres, staining positively with 5-4D. A smaller population of fibres displays the fast fibre type as determined by 1A10 and 5-2B, and an even smaller population are transitional between the two

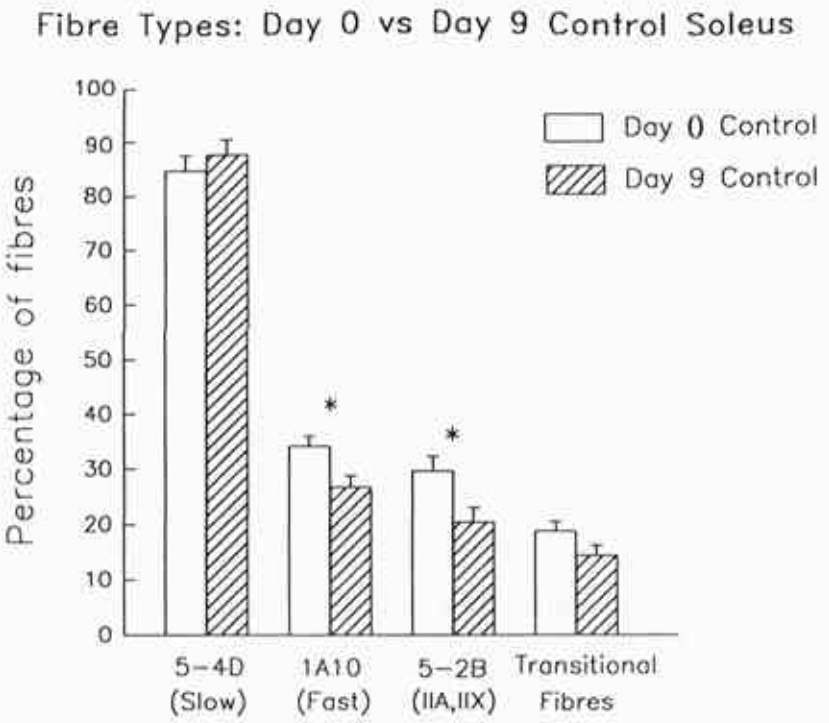

Figure 3.5 Comparisons of fibre type proportions in 0 day control and 9 day control soleus groups. Bars represent means±SE. n=10 for all groups. * $P<0.05$ in day 9 control group with respect to day 0 control group.

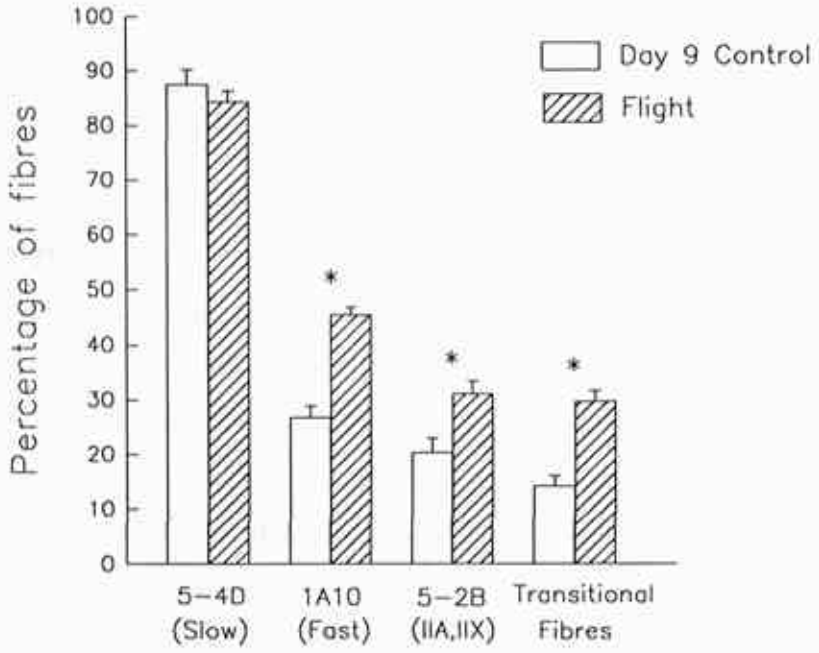

Figure 3.6 Comparisons of fibre type proportions in day 9 control and flight soleus groups. Bars represent means±SE. n=10 for all groups. * $P<0.05$ in flight group with respect to day 9 control group.

types. Comparing the D0 and D9 controls, the proportion of slow fibres is unchanged, as is the proportion of transitional fibres. However, the percentage of fast fibres decreased, as detected by both 1A10 (the more sensitive antibody) and 5-2B (less sensitive).

The comparison between the flight group and the appropriate age-matched control, the D9 control, is seen in Figure 3.6. Again there is no significant change in the proportion of fibres showing slow properties as determined by 5-4D, in contrast to the other categories, which do show changes. An increase in the number of fast staining fibres is detected by both 1A10 and 5-2B. Also, there is an increase in the proportion of transitional fibres.

The changes between the flight and recovery groups of soleus muscles can be observed in Figure 3.7. As can be seen from this figure, the only statistically significant change from the flight (soleus) group was an increase in the number of fibres stained with 5-4D. The proportions of fast fibres (as detected by 1A10 and 5-2B) as well as transitional fibres stayed at the high levels which do not differ from the flight group. The comparison between the recovery muscles and the D9 control group can be seen in Figure 3.8. Similar to the D9 control versus flight comparison, an increased proportion of 1A10, 5-2B and transitional fibres was observed with no change in the proportion stained with 5-4D.

The number of fibres analysed to determine the fibres type proportions in the soleus are shown in Table 3.1.

3.1.2 Fibre Diameters

The fibre diameter analysis in the soleus is shown in Figure 3.9. Average fibre diameters for slow and fast fibre types are compared in the four experimental groups. In the slow fibres (5-4D positive), an increase in average fibre diameter of 18.4% occurred between the D0 and D9 controls. During that same period, the slow fibres of the flight group atrophied, or decreased in diameter by 26.6%, with respect to the D9 control. In the recovery group, the slow fibres had increased to a size larger than the D9 control, having increased in diameter by 41.3% with respect to the flight group. All these changes are statistically significant, as are the following changes in the size of the fast fibres. Similar changes were shown to have occurred in the fast

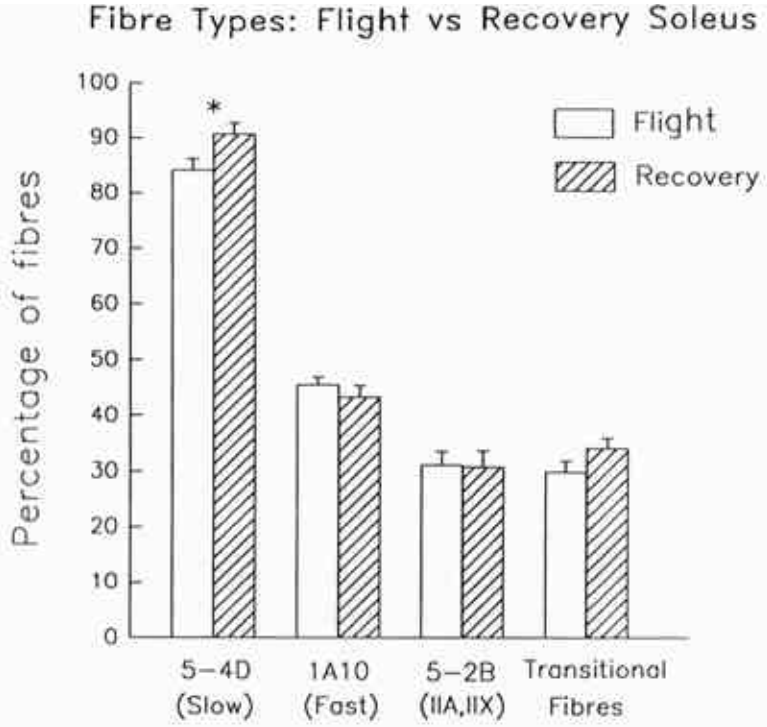

Figure 3.7 Comparisons of fibre type proportions in flight and recovery soleus groups. Bars represent means±SE. n=10 for flight group, n=9 for recovery group. * $P<0.05$ in recovery group with respect to flight group.

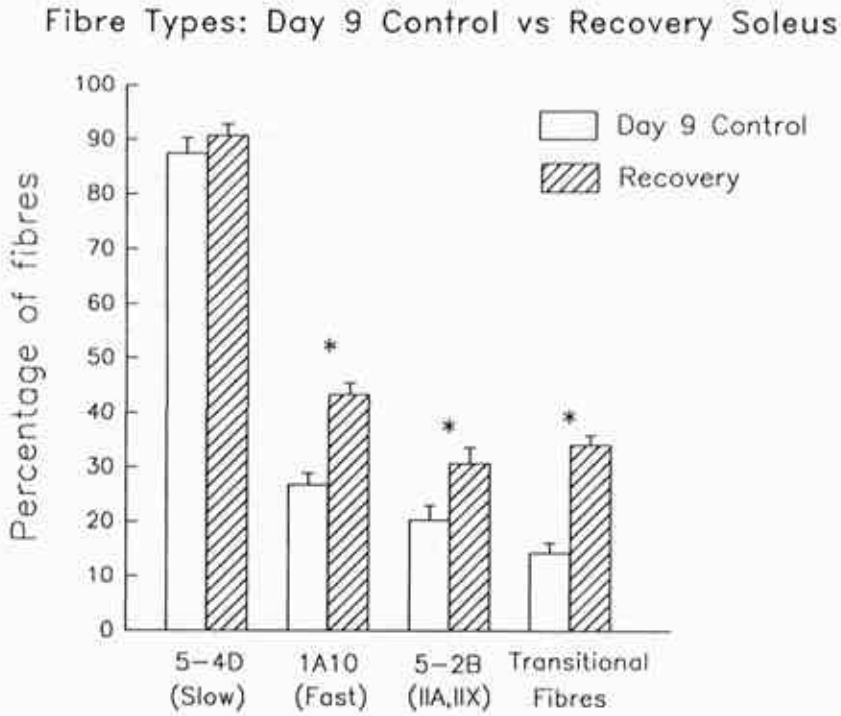

Figure 3.8 Comparisons of fibre type proportions in day 9 control and recovery soleus groups. Bars represent means±SE. n=10 for control group, n=9 for recovery group. * $P<0.05$ in recovery group with respect to flight group.

Experimental group	Number of muscles analysed	Total fibres counted	Average numbers of fibres counted
D0 Control	10	5180	518
D9 Control	10	1627	163
Flight	10	6143	614
Recovery	9	1460	162

Table 3.1 Numbers of fibres counted for analysis of fibre type proportions in the various experimental groups in the soleus (Figures 3.5 - 3.8). This number of fibres was counted three times, once each with 5-4D, 1A10 and 5-2B.
Number of muscles analysed: This reflects exactly the number of muscles available in each experimental group to be analysed.
Total fibres counted: The total number of fibres counted in a particular group, once with each antibody.
Average numbers of fibres counted: The average number of fibres counted per muscle.

Experimental group	Muscles analysed	5-4D fibres analysed: total	5-4D fibres analysed: average	1A10 fibres analysed: total	1A10 fibres analysed: average
D0 Control	6	664	111	311	52
D9 Control	7	938	134	318	45
Flight	6	984	164	523	87
Recovery	6	887	148	425	71

Table 3.2 The numbers of fibres which underwent fibre diameter analysis to produce Figure 3.9.
Muscles analysed: the number of muscles from each experimental group from which fibre diameters were measured.
5-4D and 1A10 total fibres analysed: the total number of 5-4D positive (slow) and 1A10 positive (fast) fibres, respectively, measured in each experimental group.
5-4D and 1A10 average fibres analysed: the average number of 5-4D positive (slow) and 1A10 positive (fast) fibres, respectively, measured per muscle in each experimental group.

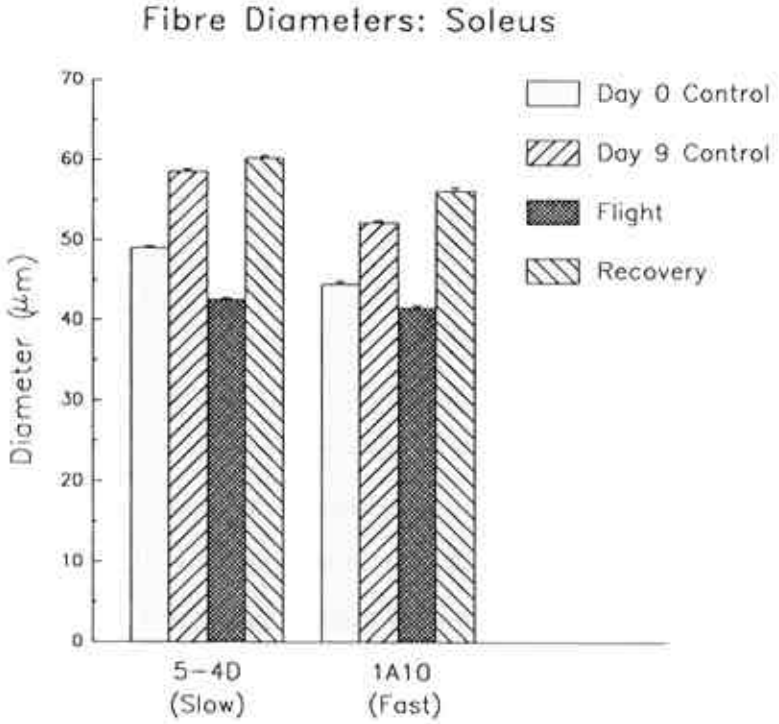

Figure 3.9 Comparisons of average fibre diameters in the soleus. Bars represent means±SE.

fibres (1A10 positive), as also seen in Figure 3.9, but the percentage changes were a little smaller. That is, the fast fibres increased 16.9% between the D0 and D9 control groups; the flight fibres atrophied 20.2% with respect to the D9 controls, and the fibres in the recovery group increased 35.4% relative to the flight group. The numbers of fibres analysed to determine fibre diameters is shown in Table 3.2.

The recovery group wet muscle weights in the soleus were 13% below those of their age-matched control (Riley, 1991), suggesting that the soleus muscle cross-sectional areas would still have been smaller than control values after 9 days of recovery.

3.1.3 Other Immunocytochemical Data

The other antibodies were not so rigorously quantified for various reasons. Figure 3.10 shows a comparison between 3 antibodies, 1A10 (fast) MY-32 (fast) and SC-71 (IIA) as they stain in the soleus. MY-32 and 1A10, as would be expected, appear to have very similar staining characteristics, with 1A10 perhaps being a little more sensitive, picking up some fibres having fast characteristics that MY-32 does not. Similarly, SC-71 had very similar staining characteristics to the above two antibodies in the soleus, but picking up fewer fibres than MY-32.

STE was used to determine the presence of any foetal MHC, and in general, no foetal MHC was detected. No fibres from any of the soleus muscles in any of the groups displayed any foetal staining, except for one recovery muscle which showed a small number of positively stained fibres, displayed in Figure 3.11. A representative soleus muscle from each group stained with STE can be seen in Figures 3.11 (low power) and 3.12 (high power). Table 3.3 quantifies these characteristics. It should be noted that the small number of fibres stained with STE in the recovery group as quantified in Table 3.3 was due to the abovementioned recovery muscle containing a small number of positive fibres.

BF-35, which stained all adult skeletal fibre types other than IIX, was found to stain virtually all fibres in the soleus. As Table 3.3 shows, on average, fewer than 5 fibres from the whole muscle section (containing several thousand fibres) were found to be unstained. The actual staining characteristics of representative muscles can be viewed in Figures 3.12 (high power) and 3.13 (low power).

Figure 3.10 High power view of serial sections of a soleus stained with 1A10 (A); SC-71 (B); MY-32 (C). Note the similarity in staining patterns. Scale bar represents 80μm.

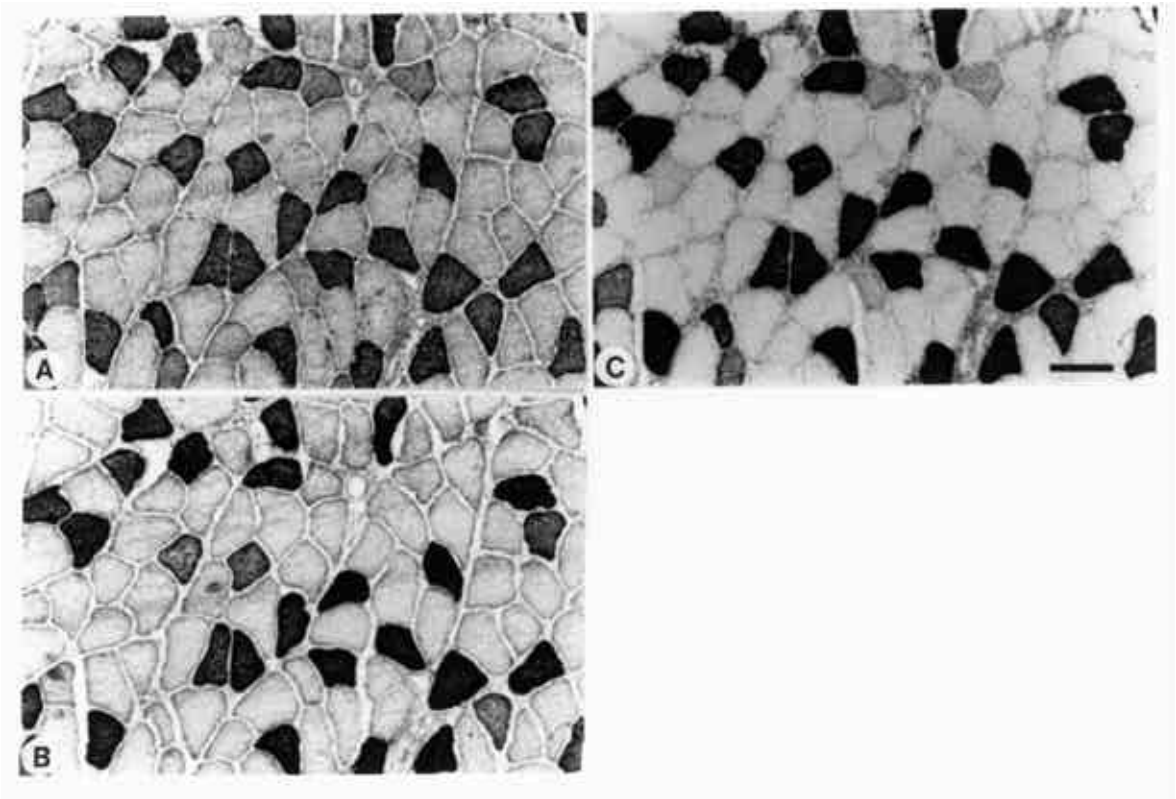
A
B
C

Figure 3.11 Low power view of representative solei stained with STE. A - D0 control; B - Flight; C - D9 Control; D - Recovery. Note the small number of positively stained fibres in the lowest part of the recovery muscle. Scale bar represents 500μm.

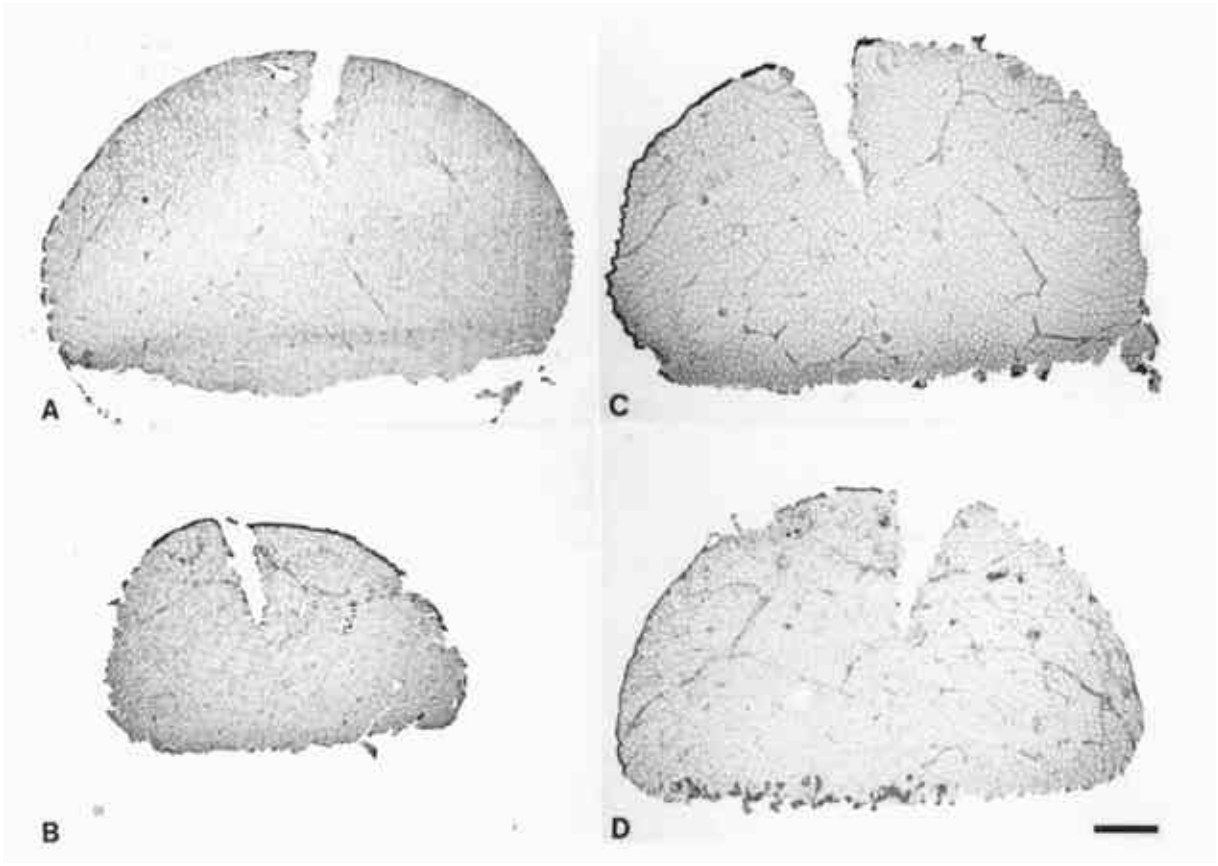
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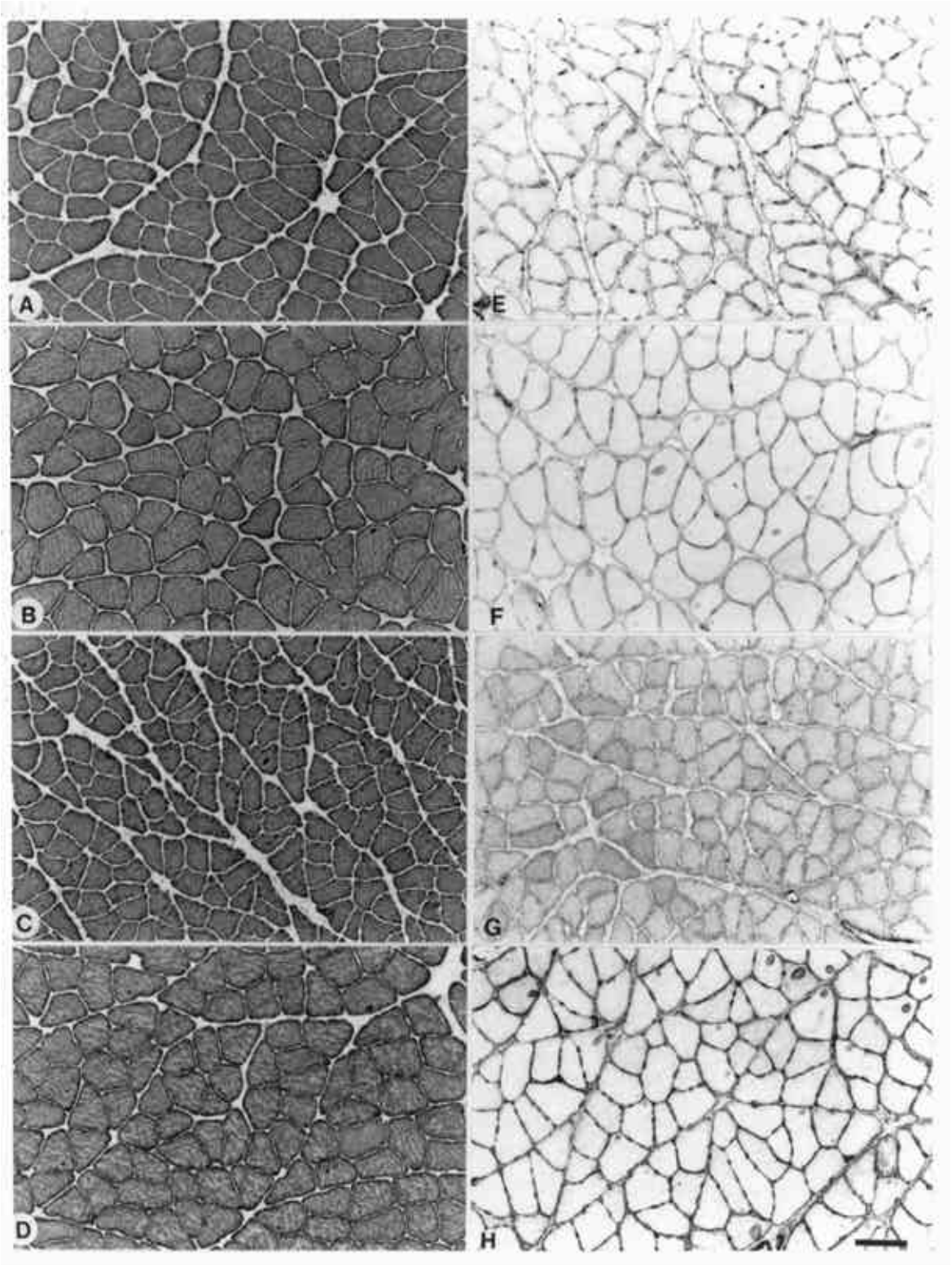
Figure 3.12 High power view of representative solei stained with BF-35 (A-D) and STE (E-H). A,E - D0 control; B,F - D9 control; C,G - Flight; D,H - Recovery. Scale bar represents 80μm.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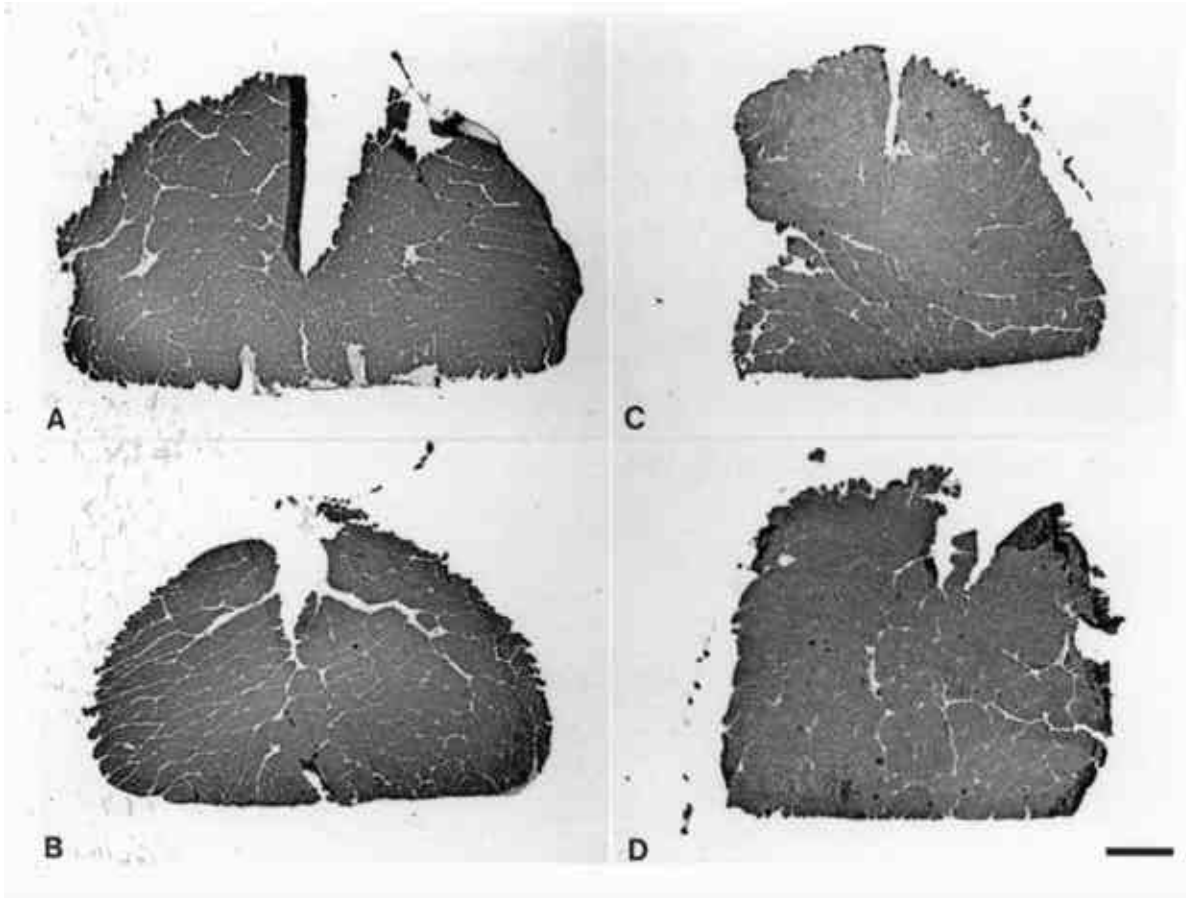
Experimental group	Number of fibres unstained with BF-35	Number of fibres stained with STE	Number of fibres stained with BF-G6
D0 Control	0.0±0.0, n=10	0.0±0.0, n=10	2.7±1.1, n=6
D9 Control	0.0±0.0, n=5	0.0±0.0, n=10	0.4±0.4, n=5
Flight	4.1±2.5, n=10	0.0±0.0, n=10	2.4±1.4, n=10
Recovery	0.0±0.0, n=8	3.6±3.6, n=9	5.8±3.0, n=4

Table 3.3 Supplementary data for the soleus, describing the number of stained, or unstained fibres (as specified) *across the whole muscle section.* Values are expressed as means±SE. N values refer to the number of muscles analysed.

Antibody	Day 0 Control	Day 9 Control	Flight	Recovery
5-4D	0.042*	0.87	0.97	0.53
1A10	0.15	0.35	0.24	0.97
5-2B	0.012*	0.91	0.21	0.09
Transitional	0.12	0.46	0.46	0.41

Table 3.4 Comparisons of fibre proportions between group caged (AEM), and individually caged (RAHF) rats. This table shows *P* values comparing the fibre proportions of the 2 cage types, in each of the control and flight experimental groups.
* $P<0.05$.

Figure 3.13 Low power view of representative solei stained with BF-35. A - D0 control; B - Flight; C - D9 Control; D - Recovery. Scale bar represents 500μm.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A very small number of fibres were found to be stained with BF-G6 (IIB) in the soleus, as shown by Table 3.3, and Figures 3.14 (high power) and 3.15 (low power). The few stained fibres, and muscle spindles which were also found to be positively stained, acted as a useful positive control to the specific staining characteristics of this antibody.

3.1.4 Effect of Caging Environment on Fibre Type Proportions

The rats were flown in two cage types, the AEM (group caged) and RAHF (individually caged). Table 3.4 examines whether there were differences in fibre type proportion between the different cage types, within the same experimental condition. As the probability values show, only in the D0 control were any significant differences found. The number of slow fibres (5-4D positive) and the number of fast fibres, as determined by 5-2B, were found to be statistically different - the group caged animals having fewer slow and more fast fibres than the individually caged animals.

3.2 The EDL

3.2.1 Fibre Type Proportions

Proportions of different fibre types were not examined in detail in the EDL. Such an analysis proved to be a problem, because of the uneven distribution of the different fast fibre types across the muscle. Figure 3.16 displays low power micrographs of a D9 control and flight EDL, each stained with SC-71 (IIA) and BF-G6 (IIB). The distribution of the IIA fibres tends to be greater at one end of the muscle (the "red" region), with IIA fibres gradually decreasing towards the other end (the "white" region) where IIB fibres predominate. Because of this uneven distribution, results of a fibre type proportions analysis would be dubious unless the sample was very large, and such an analysis was not undertaken.

There is a complementarity between the IIA and IIB fibres in the EDL, as can be seen by viewing a region of each of these muscles at high power in Figure 3.17. IIA fibres are stained by SC-71 and unstained by BF-G6. IIB fibres have the reverse

Figure 3.14 High power view of representative solei stained with BF-G6. A - D0 control; B - Flight; C - D9 Control; D - Recovery. Arrows denote positively stained fibres. Scale bar represents 80μm.

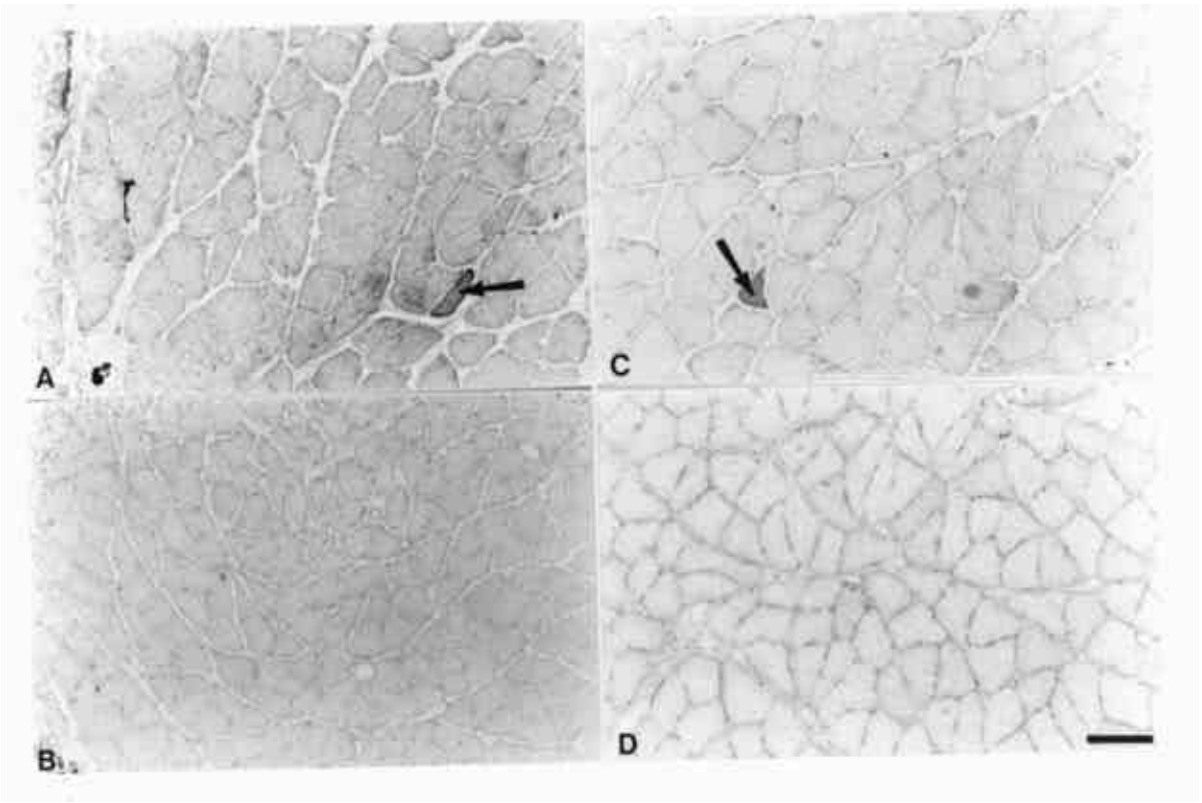

A
C
B
D

Figure 3.15 Low power view of representative solei stained with BF-G6. A - D0 control; B - Flight; C - D9 Control; D - Recovery. Scale bar represents 500μm.

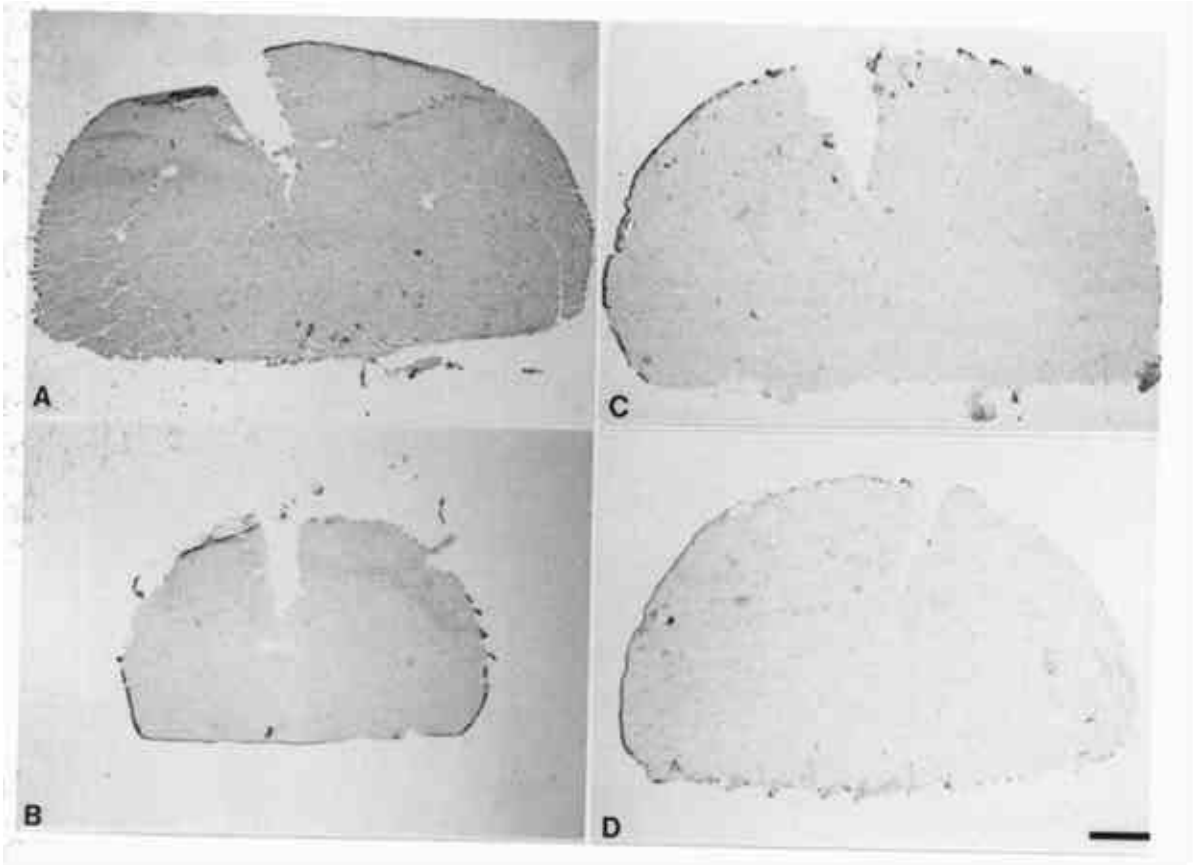

A
B
C
D

Figure 3.16 Low power view of representative EDLs from the D9 control (A-C) and flight (D-F) groups. A,D - SC-71; B,E - BF-G6; C,F - 5-4D. Note A,B and C,D are serial sections, whereas C,F are not; they come from another muscle within their respective experimental groups. Scale bar represents 500μm.

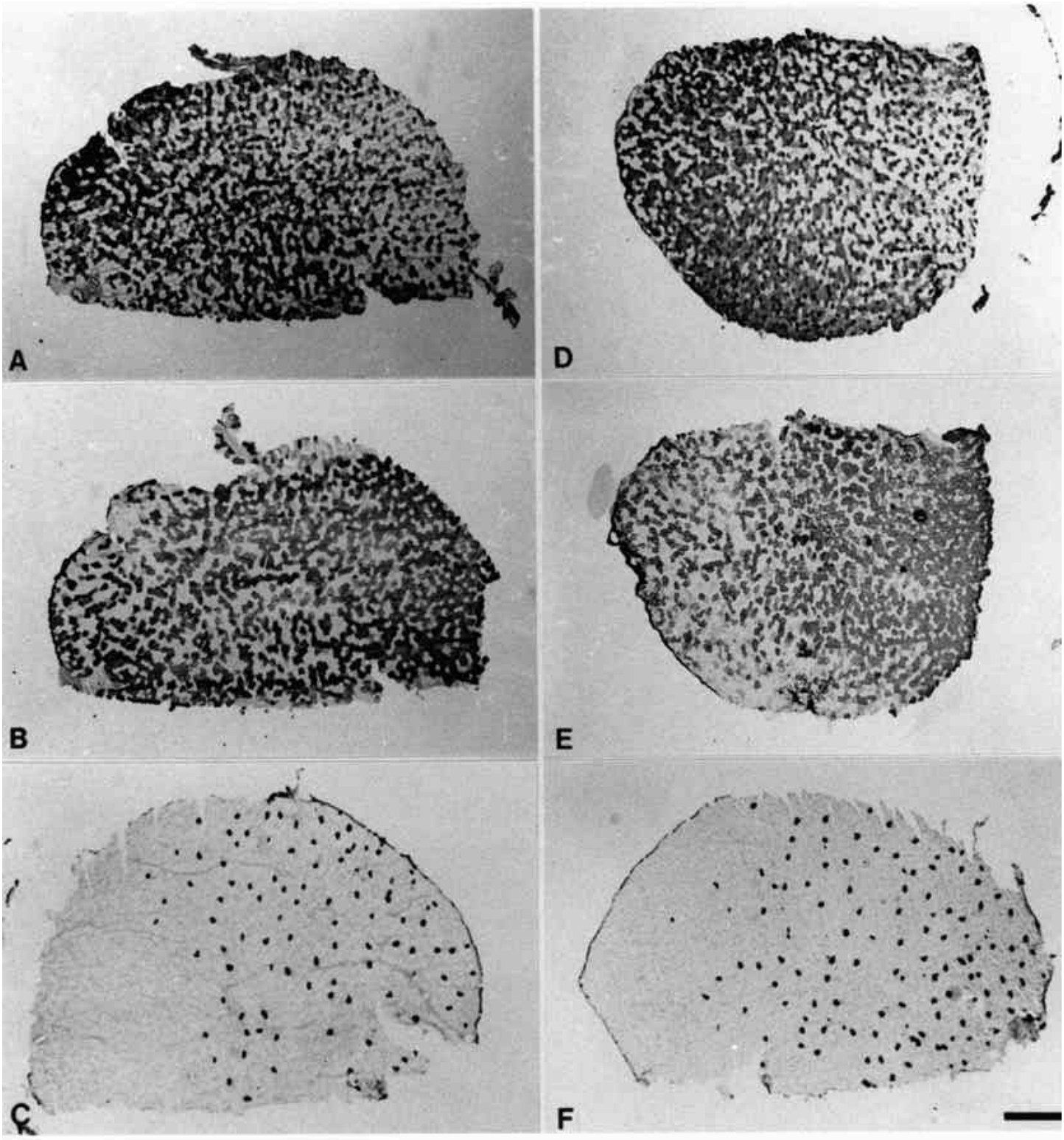
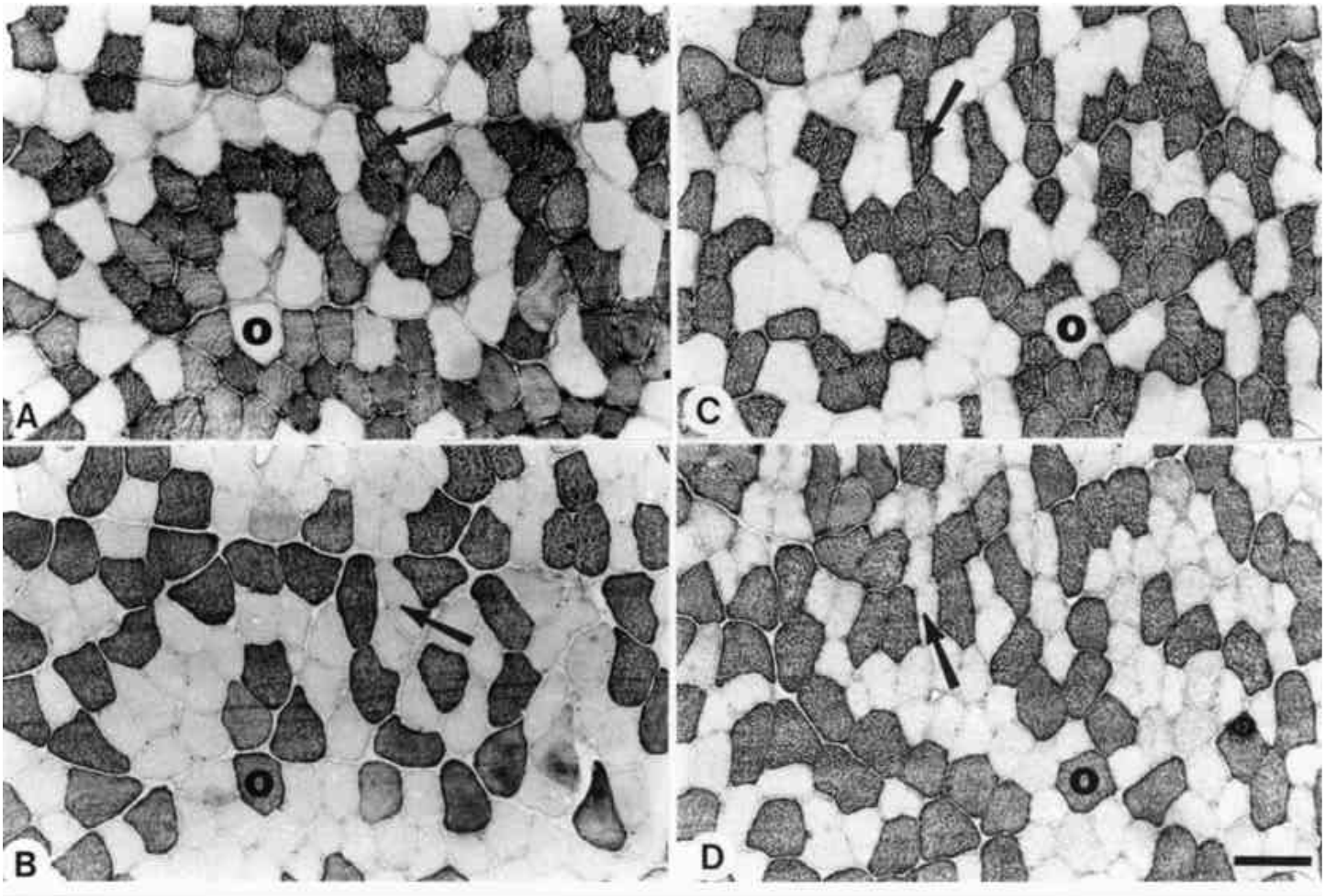

A
B
C
D
E
F

Figure 3.17 High power view of representative EDLs from the D9 control (A,B) and flight (C,D) groups. A,C - SC-71; B,D - BF-G6. Arrows represent IIA fibres. Circles represent IIB fibres. Scale bar represents 80μm.


A
B
C
D

staining pattern, and several IIA and IIB fibres are marked in Figure 3.17. No evidence of a transition between IIA and IIB fibres was observed. Such a transition would be evident if fibres stained with both BF-G6 and SC-71, in a similar fashion to the simultaneous slow and fast staining found in the flight and recovery solei.

Analysis of the total number of slow fibres present across whole cross-sections of EDL muscles was made. It was found that no statistical difference in their number could be observed between the D0, D9 and flight groups. The distribution of slow fibres in the EDL can be seen in Figure 3.16. This figure shows that the number of slow fibres in the EDL is small, and fairly evenly distributed across approximately two thirds of the muscle. Some variability in the number of slow fibres in the EDL existed, with anywhere from 27 to 194 slow fibres in any one muscle, making it difficult to detect if there was any difference according to the experimental group. The variation was found in all the groups however, and the mean values were not significantly different, as seen in Figure 3.18.

3.2.2 Fibre Diameters

Figure 3.19 shows the comparison of the fibre diameters in the D9 control and the flight groups for the EDL. The slow fibres in the flight group undergo a small, but statistically significant hypertrophy, with the average fibre diameters increasing 4.5%. Similarly, the IIA fibres have a small significant increase of 3.8%. The IIB fibres have no significant change. Figure 3.17 provides a visual confirmation that the fibre sizes in the D9 control and flight muscles are similar, not having the large decrease in size observed in the soleus. Figure 3.17 also shows that the IIB fibres are larger than the IIA fibres. The number of fibres analysed to determine the average fibre diameters is shown in Table 3.5 (fast fibres) and Table 3.6 (slow fibres).

The fibre diameters of the IIX fibres were not measured, as this would involve measuring the diameters of non-stained fibres (BF-35 stains all fibre types except for IIX, and so provides a "negative stain" of the IIX fibres). It was found difficult to measure the average diameter of non-stained fibres with an adequate degree of accuracy. It was also difficult to distinguish the exact outline of the fibre, and quite possible to mistake two fibres as being one, thus increasing the average diameters incorrectly. As such, analysis of IIX fibre diameters was not undertaken.

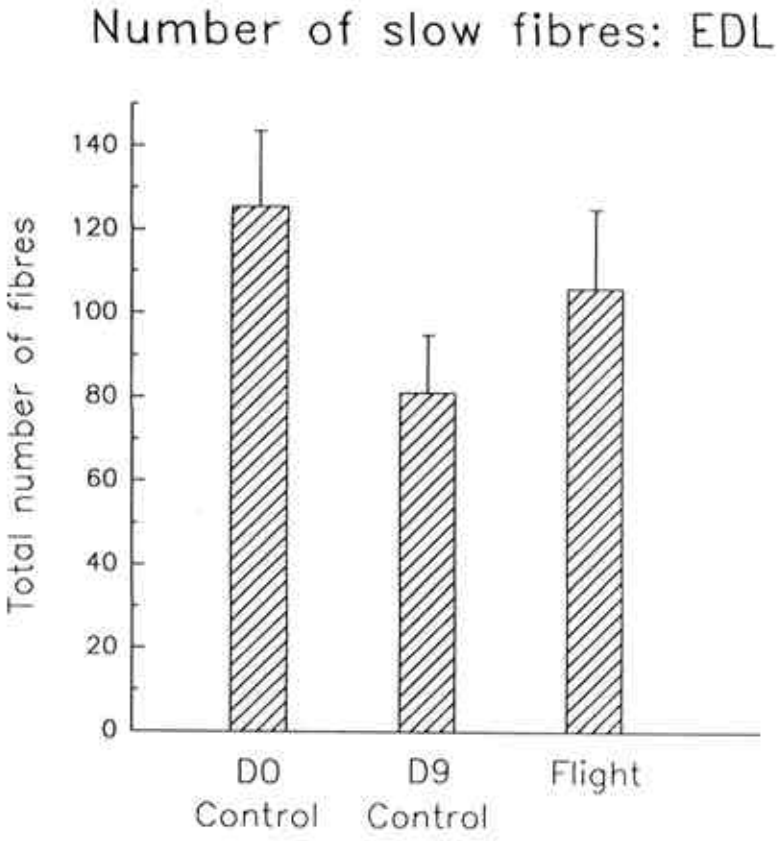


Figure 3.18 Comparisons of the number of slow fibres in the D0, D9 and flight groups across the whole cross section of the EDL. Bars represent means±SE. No statistically significant differences.

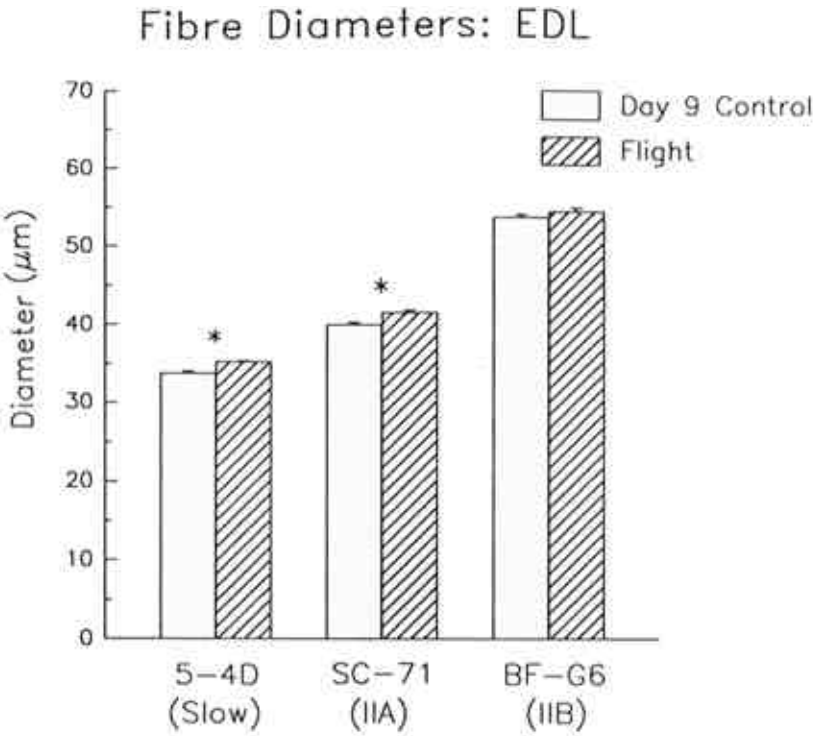


Figure 3.19 Comparisons of average fibre diameters in the day 9 control and flight groups in the EDL. Bars represent means±SE. * $P<0.05$ in control group with respect to flight group.

Experimental group	Muscles analysed	SC-71 fibres analysed: total	SC-71 fibres analysed: average	BF-G6 fibres analysed: total	BF-G6 fibres analysed: average
D9 Control	4	245	61	222	56
Flight	4	191	48	184	46

Table 3.5 The numbers of fast fibres which underwent fibre diameter analysis in the production of the above histogram.
Muscles analysed: the number of muscles from each experimental group from which fibre diameters were measured.
Total/Average fibres analysed: the total/average number of fibres positive with each antibody in each experimental group

Experimental group	Muscles analysed	5-4D fibres analysed: total	5-4D fibres analysed: average
D9 Control	6	486	81
Flight	6	694	116

Table 3.6 The numbers of slow fibres which underwent fibre diameter analysis in the production of the above histogram. Note all of the slow fibres in the muscles analysed were measured

Chapter 4: Discussion

4.1 The Soleus

It must be noted that when examining the results of this, and other similar experiments, the changes observed are not only as a result of 0-G, but are the result of a combination of the launch, 0-G, landing, and recovery periods. Of particular importance are the landing and recovery periods, as the weakened limb muscles of the rats are firstly exposed to greater than 1-G during the landing, and then 1-G for a period of some hours before they are killed. This period of landing and recovery before the animal is killed allows muscle damage to occur (Riley *et al.*, 1990a, 1992). In short, the changes observed here are due to launch, 0-G, and the landing/recovery phase.

4.1.1 Changes in Fibre Type Proportions

The first change of note in fibre types in the soleus is that between the D0 and D9 control groups (Figure 3.5). The findings here suggest a general trend towards greater slow fibre expression and reduced fast fibre expression in the soleus as the rat gets older. This trend has been observed elsewhere (Boreham *et al.*, 1988; Maltin *et al.*, 1990; Sugiura *et al.*, 1992; Kugelberg, 1976), and is thought to be due to the neural influences on the muscle as the rat develops. As rats age, their weight increases, and the gravitationally induced stretch reflex on postural muscles, such as the soleus, becomes greater. Since a greater chronic load is placed on the soleus, greater continuous demands are placed on the muscle fibres. The fibre type most suited to this constant load is the relatively non-fatigable slow fibres. Whilst there is no significant increase in the proportion of slow fibres between the D0 and D9 controls, there is a significant decrease in the proportion which have fast fibre properties.

In contrast to the fast to slow transition normally occurring during this phase of rat development, a much greater change in the opposite direction occurs in the 0-G exposed rats (Figure 3.6). The proportion of fibres staining with the anti-fast antibodies increases, but the proportion of fibres staining with the anti-slow antibody

does not change. This can be explained by the fact that the proportion of transitional fibres (having a positive staining reaction with both 5-4D and 1A10) increases by an amount similar to the increase in the proportion of fast fibres, around 17%. This data suggests that a sub-population of fibres which are normally slow are beginning to express fast fibre characteristics, whilst still not having lost their slow characteristics, and thus are in a state of transition between the slow and fast states. The fibres are in this transitional state presumably because of the short duration of the 0-G conditions. How this slow to fast fibre type transformation would continue, if left for a longer period of time, cannot be judged by this experiment. One possibility is that a new steady state will be reached, with a higher proportion of fast fibres. Another possibility is that eventually, all fibres will become fast, if enough time is allowed. Of these possibilities, the former seems more likely, as there is no evidence that all fibres change their fibre type, even after long term unloading (Elder & McComas, 1987; Desplanches *et al.*, 1987a; Bonen *et al.*, 1988; Templeton *et al.*, 1988). This evidence comes from long term ground based unloading studies, as long term 0-G missions with rats have not been attempted.

Following the 9 day recovery period, the proportion of fast fibres as well as transitional fibres remained at the high post-flight levels. This suggests that the recovery period was not a sufficient length of time for the soleus to recover to the control levels of fast and transitional fibres. This agrees with studies in suspension and immobilisation models where a return to control values for various parameters took a long period of time (Fitts & Brimmer, 1985; Kandarian *et al.*, 1991; Marsh *et al.*, 1992). However, these studies investigated longer term hindlimb unloading, with the minimum length being 28 days of suspension. No other studies to date have investigated recovery from a short period of 0-G or hindlimb suspension exposure, but this study suggests that a period longer than the exposure time is necessary for the soleus to return to normal, assuming it does so.

The comparison of the recovery group with the D9 controls is not ideal. The ideal comparison would be with the D18 controls. This D18 control group is the age-matched control for the recovery group, which experienced a similar environment to the recovery rats for the 9 days of flight and 9 days of recovery, except for the 0-G. This group has not been analysed as yet due to time constraints. As was seen when

comparing D0 and D9 controls, a change occurred in which the general trend was towards fewer fast fibres and more slow fibres. If this trend were to continue to the D18 controls, the high levels of fast and transitional fibres in the recovery muscles would appear as even higher, relative to the control. Further, the D18 control may offer an explanation as to why there is a statistically significant increase in the proportion of slow fibres when comparing flight and recovery groups (Figure 3.7). It may be that the increase in slow fibres seen in the recovery muscles is a part of normal rat development, and this has been superimposed upon the increase in the number of fibres expressing the fast MHC caused by the 0-G environment.

It seems clear that in the soleus there is a shift towards the expression of a fast MHC isoform or isoforms, but several provisos have to be met. Firstly, since 1A10 (and MY-32) cross react with foetal MHC, it was necessary to assure that the increase in 1A10 positive fibres was not due to the presence of foetal MHC. It is known that muscle fibres regenerating after muscle damage, as can be caused by the recovery period (Riley *et al.*, 1990a, 1992), can lead to the transient expression of foetal myosins (Sartore *et al.*, 1982). Table 3.3, and Figures 3.13 and 3.14 show this lack of staining, with the single exception of one muscle in the recovery group having a small number of STE positive fibres. The fact that 5-2B, which does not cross react with foetal MHC, shows similar changes in fibre type proportions as 1A10 is further confirmatory evidence. Whilst not affecting the results of the antibodies used with the EDL, it is also true that no foetal MHC was detected in the EDL either. The smaller proportion of fast fibres detected by 5-2B in the soleus can be attributed to the lower sensitivity of this antibody. The lack of BF-G6 (IIB) staining (Figures 3.14, 3.15) in the soleus also rules out the difference between 1A10 and 5-2B staining patterns being due to the presence of IIB myosin, which 1A10 can detect, but 5-2B cannot.

Further detail on the nature of the MHC expression in the soleus can be gained from other antibodies used in this study. Firstly, BF-35 stained virtually all fibres in the soleus, as can be seen in Table 3.3, and visually in Figures 3.11 and 3.12. This shows that virtually no fibres are IIX fibres, or, more correctly, virtually no fibres express only IIX myosin. With this antibody, it is impossible to rule out that fibres are expressing IIX concurrently with other MHCs, as such fibres would also appear

positively stained. It would be possible, for example, for IIX to be co-expressed in IIA fibres, in which case the presence of IIX MHC would not be detected by an absence of staining. Takahashi *et al.* (1991) and Campione *et al.* (1990) did detect IIX MHC after hindlimb suspension. Nevertheless, on the basis of BF-35, it seems unlikely that IIX fibres or IIX MHC would be present in very large amounts or numbers. To be certain of the IIX staining pattern, an antibody staining IIX positively would be necessary. Further, BF-G6, which stains IIB fibres positively, stained very few fibres in the soleus, showing that large numbers of IIB fibres are not present. Fast and transitional fast/slow fibres form a significant minority population in the soleus, even more so in the space flown muscles. By a process of elimination it can be seen that the great majority of fast fibres in the soleus are not IIB, nor IIX, and therefore must be IIA. Therefore, the shift in fibre type occurring in the space flown rat solei is a shift towards the IIA fibre type. Direct evidence of the MHC shift being towards IIA can be seen in Figure 3.10, where 1A10 and SC-71 (the IIA specific antibody) are shown to have very similar staining patterns. SC-71, however, was not used extensively due to very limited amounts of this antibody, as well as time constraints.

4.1.2 Changes in Fibre Size

Accompanying the changes in fibre types are perhaps the more obvious changes in average fibre diameter, or cross-sectional area (Figure 3.9). Comparing the D0 and D9 controls, we can see that fibre diameters have increased. This is due to muscle growth, which is a part of normal rat development. During that period of time, when the muscle fibres should have been growing, flight muscle fibres atrophied significantly, due to the lack of use they suffered. The lack of the gravitationally induced stretch reflex stimulation caused a decrease in protein synthesis, with a simultaneous increase in protein degradation. In the recovery group, it was found that the average fibre diameters increased, becoming greater in size than the D9 controls. However, in light of the wet muscle weight data (Riley, 1991), it appears likely that the fibre diameters in the recovery group would not have reached the levels of the D18 (age-matched) controls, as the weight of the recovery solei are 13% lower than

the D18 control group. Again, the best comparison for the recovery group would have been with the D18 control group.

It is the slow fibre diameters that are a more reliable indicator of changes in cross-sectional area. Because there is little change in the proportion of fibres staining with 5-4D (anti-slow), it is likely that the same population of fibres is being analysed in all the different muscle groups, when considering the slow fibres. The fast fibre population however, is changing, as some fibres normally only in the slow fibre group are also in the fast group following 0-G exposure. That is, the increased transitional fibre population following 0-G means that a changing population of fibres is analysed in the fast fibre category. It would be technically difficult to measure the transitional fibres and fast only fibres groups separately given the equipment on hand. In spite of the problems, the measurements of fast fibre diameters showed similar changes to the slow population, although of a smaller magnitude. This may be due to fast fibres being less susceptible to atrophy in 0-G, but it may be due to an anomaly caused by the problems mentioned above.

The changes in cross-sectional area of the individual fibres do not always appear to be reflected by the cross-sectional area of the entire muscle in the various groups. The most likely explanation is that the entire cross section of the muscle has not been sectioned, either due to only part of the muscle being dissected out in the first instance, or part of muscle cross section being lost in the sectioning process. It is also possible that there is a variation in the number of muscle fibres between different animals. Therefore, the average size of individual fibres is a better indication of the changes in fibre size than the size comparisons between the whole cross sections of any two muscles.

Table 3.4 investigated if differences in fibre proportions existed between the different caging environments. Surprisingly, the only change in fibre proportions between the cage types occurs in the least likely candidate, the D0 controls. This group only spent a matter of hours in the different cage types, the amount of time the flight rats spent in their cages while on the ground, and this would appear to be an inadequate time to cause any changes. Therefore, it seems likely that the differences between these two groups were due to the rats being different as they were selected, especially since none of the other experimental groups, where differential caging

occurred for a period of 9 or 18 days, showed any cage based differences. 5 rats were present in each group in each cage type (except for the recovery RAHF group, with 4), and this relatively small number could allow a spurious difference between the groups to be present.

4.2 Changes in the EDL

The dramatic changes in fibre proportion and fibre size of the soleus was not emulated in the EDL. In stark contrast to the obvious atrophy in the soleus, little change in fibre size occurred in the EDL (Figure 3.19). The slow and IIA fibres of the EDL actually reversed the trend in the soleus, with some hypertrophy. The IIB fibres were unchanged in size. Although the EDL muscles in the recovery group were not analysed, it seems likely that the cross-sectional areas of the recovery muscles would be normal or near-normal, as wet muscle weight data for this experiment shows that EDL weights had returned to control values by the end of the recovery period (Riley, 1991). Further, the lack of change during the 0-G exposure, and therefore, the lack of anything to recover from, adds further weight to the argument that the EDL is likely to have fibre cross-sectional areas the same as that of the age-matched control group by the end of the recovery period.

No evidence of changes in fibre proportions, such as a transition of fibre types, was detected in the EDL. Figures 3.16 and 3.17 show that no IIA/IIB transition is occurring in the flight muscle. Such IIA/IIB fibres are also not found in the D9 control group. It is difficult to answer the question of whether there is a transition between the IIX fibre type and any other with the antibodies available. BF-35, which stains all adult skeletal fibre types except IIX, provides a "negative stain" of the IIX fibres. By definition, if a fibre is unstained by BF-35, it contains only IIX MHC and is not in transition. If a fibre is stained with BF-35, all that can be said is that it contains at least one, and possibly more than one of the adult MHCs, not including IIX. A stained fibre may also contain IIX MHC, as long as it is in combination with another adult MHC. So whether, for example, IIA/IIX or IIB/IIX fibres are present cannot be determined using this antibody. Further, the total number of slow fibres per

muscle did not change either, as it can, for example, in denervation (Hoh *et al.*, 1989).

No change in fibre type was detected using immunocytochemistry. It may be possible that changes could be detected using other means, and in particular by separating the MHC isoforms using using gel electrophoresis (as performed by Bär & Pette, 1988). However, on the basis of the immunocytochemical evidence of this study, it would appear unlikely that a change has occurred, and therefore, likely that no change would be detected using this method.

Experiments involving gel electrophoresis would also be useful in the soleus. Immunocytochemical methods have shown that a subpopulation of soleus fibres are expressing fast MHC. However, the proportion of the total MHC that has changed after 9 days of 0-G is unclear using this method. The immunocytochemical classification of "transitional fibres" (in the soleus, a slow to fast transition) indicates that a single fibre contains both fast and slow MHC. The relative proportions of slow and fast MHC could vary over a considerable range in a fibre, with the fibre still being considered transitional. The changes in the absolute proportion of MHC isoforms after 0-G exposure could be determined on a whole muscle level using gel electrophoresis and subsequent densitometric analysis.

4.3 Differential Effects of Zero Gravity on Skeletal Muscle

It is obvious that not all muscles behave the same way under the influence of 0-G. The differences between the soleus and EDL illustrate this well. Also true is that not all fibres, or fibre types behave in the same way in 0-G. For example, a sub-population of the slow fibre type in the soleus begins transforming into the fast fibre phenotype after 9 days of 0-G. The question arises as to why only part of the slow fibre population transforms into this fast fibre type. If it was the fibre phenotype alone that determined behaviour under 0-G conditions, it would be expected that all fibres of the same type would behave in the same way, and would either all be unchanged, or would all start to show this transitional state. A likely explanation is that the change, or lack of change in fibre type is related to the developmental or myogenic origin of the fibre. 0-G appears to have an effect on the soleus which is in

some ways similar to denervation, judging by the atrophy, and EMG recordings of ground based rats (Alford *et al.*, 1987; Riley *et al.*, 1990b). In the presence of this atrophy-like state in the soleus, it seems likely that the fast secondary fibres, which are converted into slow fibres by the influence of the nerve during normal rat development, revert to their original fast phenotype in 0-G, as they do in denervation (Hoh, 1991; Hoh *et al.*, 1989). As such, 0-G, and in particular, the altered neural stimulus caused by 0-G, is further evidence that fibre type or phenotype alone is not enough to predict fibre behaviour under altered conditions. The developmental origin, or lineage of the fibre must also be taken into account.

That fibre type alone is not enough to predict the changes that occur in 0-G can also be seen by comparing the changes in cross-sectional area in slow fibres. In the soleus, slow fibres atrophy significantly. In contrast, the slow fibres in the EDL actually hypertrophy. Similarly, the IIA fibres in the EDL hypertrophy slightly, whilst the soleus IIA fibres (since most of the fast fibres in the soleus are IIA fibres) atrophy, although not to as great an extent as the slow fibres of the soleus. This difference in behaviour of the fibres in these two muscles suggests the changes are muscle specific.

4.3.1 Mechanisms of Zero Gravity Induced Muscle Changes

It has been reported many times that the muscles that change the most in 0-G or 0-G models are the slow postural muscles, such as the soleus and adductor longus, whilst the fast twitch, phasically contracting muscles such the EDL and tibialis anterior change relatively little. This seems to be related to the usage of the muscle, or more particularly, the change in the usage of the muscle. The changes to the usage of the soleus have been discussed at length. The changes to the usage of the EDL however, would probably be relatively small. It would be thought that the EDL would still contract phasically. As such, the changes in muscle usage in the EDL would not be as great, as the EDL normally contracts phasically. In other words, the phasic muscles receive a neural stimulation pattern somewhat similar to that which they would normally receive, as has been shown in the EMG study in hindlimb suspension (Riley *et al.*, 1990b). As such, the changes would be predicted to relatively small, and this appears to be so. It may also be true that the neural impulse

pattern is more important to keep slow fibres in their slow state than it is to maintain the properties of fast muscles, as has been seen in denervation studies. To summarise, it appears to be the usage pattern of the muscle in space, relative to the normal usage pattern of the muscle in 1-G conditions, that plays an important role in determining what changes occur to the muscle as a whole, and at the single fibre level.

As a consequence of the unloading caused by 0-G and its models, the hindlimb adopts a position of plantar flexion. This finding has been reported in rats both in spaceflight and in unloading models (Riley *et al.*, 1990b). It has also been seen in humans in 0-G. In this position, the soleus is shortened, whilst the EDL is stretched. The shortening of the soleus would further decrease the amount of neural stimulation it received, hence being a contributor to the atrophy of the muscle. The stretching of the EDL in this position may be part of the explanation as to why it hypertrophies. It is known in hindlimb unloading models that protein synthesis rates in the EDL can increase, and this may be due to the increased neural stimulation which is the result of this stretching. However, other factors are also at work here, since the protein degradation rates also increase at the same time.

The soleus would be expected to change rapidly in 0-G because of the dramatic changes in usage that occur to postural muscles in space. Changes to the EDL, if they do occur, would be expected to be more subtle, because the muscle is undergoing relatively subtle changes in activity; a fast muscle in 0-G would presumably be still contracting occasionally (phasically) as it always does, so it may require a longer term space mission to see whether changes do occur. It seems unlikely, that in the presence of such a drastic change in the environment affecting many body systems, that no change to the EDL would occur. Whether or not this is the case, the contrast between the EDL and soleus reveals that there is a great deal of muscle specificity in the changes that occur in 0-G.

4.4 Significance of the Changes Observed in Zero Gravity

0-G is not a physiological stimulus which is found in the normal environment to which animals and people have been exposed. Nor is it a stimulus to which there has been exposure in the past, so that some evolutionary mechanisms to adapt to it could have been developed. Yet significant changes occur to muscles in the 0-G environment. The question arises as to why this adaptation occurs. The answer lies in the fact that the muscles are not responding to the stimulus of 0-G itself, but rather to the altered demands placed on the muscles. All biological systems have conflicting demands placed on them; they must complete the task at hand, but with the minimum of energy and resource expenditure, as all systems are competing for these same resources. Thus all systems must adapt, as far as is possible, to complete their tasks with maximum economy. Such adaptations can be clearly seen in skeletal muscle. The amount and type of muscle usage can to a large extent determine the characteristics of a muscle. The muscles of a weightlifter, a long distance runner differ greatly, as do the leg muscles of a person with a broken leg. Adaptation not only occurs to cope with the usage patterns of each particular person or animal of a species, but also occurs on an evolutionary scale. Under evolutionary pressure, different muscles have specialised to cope with their "normal" usage pattern. Within this specialisation, a flexibility or plasticity exists to cope with differing usage patterns, which can alter both the fibre size, and fibre type, as the need arises. However, the developmental origins of the fibres can limit this plasticity.

Thus, 0-G is recognised by the muscle as a change in the usage pattern demanded of it, and it must adapt to this change. The reason for the atrophy observed in 0-G is fairly obvious; the force levels and duration of force production necessary in 0-G are much lower in muscles such as the soleus than they are in 1-G. The muscle adapts to the decreased demands on it by decreasing its cross-sectional area, to which force is proportional. The larger muscle (normal in 1-G) is not necessary to function effectively in 0-G, and so the muscle reduces its size for maximum economy. Similarly, the observed changes in fibre type are likely to be an adaptation for increased economy. Slow fibres have a greater rate of protein turnover than fast fibres (Garlick *et al.*, 1990; Bates & Millward, 1983), and therefore a greater rate of protein synthesis (Goldberg, 1967; Lewis *et al.*, 1984). This higher metabolic rate

associated with slow twitch fibres, in comparison to fast twitch fibres is an "expense" that is only necessary if the muscle will be contracting tonically. The lack of such tonic, anti-gravity usage in 0-G means that muscles adapt by expressing the less "costly" fast fibre types, with their lower metabolic rates.

The same adaptations in fibre type and size seen in 0-G are seen on earth in 1-G conditions, as a functional adaptation to differing patterns of usage. Thus, although 0-G itself is a unphysiological stimulus, muscles are able to adapt, not to the 0-G itself, but to the altered usage pattern in 0-G. The adaptation that occurs is the same no matter what the conditions. That is, the muscles attempt to adapt in such a way that they will be able to complete the tasks demanded of them, as economically as possible.

4.5 Comparisons with Other Studies

The findings presented here are in general very similar to those of other 0-G experiments. Only recently has immunocytochemical analysis of muscles exposed to 0-G been undertaken, and in fact it was only when the current experiment was heading towards completion that much of the data became available.

As an example, the 14 day Cosmos 2044 mission had similar findings to the present study (Jiang *et al.*, 1992; Ohira *et al.*, 1992). After the 14 day Cosmos 2044 mission (Ohira *et al.*, 1992), it was found that the percentage of transitional fibres increased 13.7% in the soleus (compared to 15.5% here), while the proportion of fibres expressing slow MHC did not change. In the detection of fast fibres immunocytochemically, the Cosmos 2044 studies are very comparable to this study, as they used MY-32 as their anti-fast antibody, which has been shown to have a very similar staining pattern to 1A10 used here (Figure 3.10). They too found the expected atrophy and decrease in fibre cross-sectional area in the soleus, with the slow fibres decreasing in size more than the fast fibres. Other studies have been unable to show, due to a lack of appropriate antibodies, anything more than a shift to fast (type II) myosin in the soleus, whereas this study showed that this was in fact a shift towards type IIA. No change in fibre type proportions in a fast twitch extensor muscle, the tibialis anterior (similar in function to the EDL) was seen in this study (Jiang *et al.*,

1992). Other studies finding similar results were discussed in Section 1.3.2. What has become apparent from this and previous studies is that immunocytochemical fibre typing is much more effective at detecting changes in fibre types than histochemical fibre typing.

While atrophy of the soleus has been found universally, there appears to be some difference in the changes reported in the EDL. Some findings report no atrophy (Jiang *et al.*, 1992), whilst others report atrophy of the EDL, although always to a lesser extent than the soleus (Martin *et al.*, 1988; Riley *et al.*, 1987). Riley *et al.* (1992) reported a 17% hypertrophy of the slow fibres in the EDL (compared to 4.5% here) from the Cosmos 2044 mission, but no change in the size of the fast fibre types, unlike the findings here.

The use of the Sprague Dawley strain of rats in this experiment may have not been ideal for the examination of changes in muscle fibre type. It was surprising to find a high level of transitional fibres in the control animals, as previous work in this laboratory, using Wistar rats had not shown a large transitional fibre population in normal rats (less than 1%, from Hoh & Hugh, unpublished observations). Miu *et al.* (1990) also found a significant transitional fibre population in their control Sprague Dawley rats (8%). The presence of a such a transitional fibre population in the control rats of this study made the increases caused by 0-G less immediately obvious. In comparison, the Cosmos 2044 mission (Jiang *et al.*, 1992), which used Wistar rats, also found an increase in the proportion of transitional fibres in the soleus. In this study, the change in fibre types was somewhat clearer, as the control solei had virtually no transitional fibres. However, the fact that similar changes have been shown to occur in both Wistar and Sprague Dawley rats shows that the 0-G effects are not specific to one particular strain of rat.

Using older rats may have been more ideal, as it may have minimised any normal changes in fibre type and size between the D0 and D9 controls as was seen in this study. However, similar qualitative changes have been reported in young (1.5 month) and adult (5 month) rats in both hindlimb suspension and 0-G (Steffen *et al.*, 1990), with only a slower rate of atrophy in the adult rats separating the two groups.

4.6 Applicability of the Rat Model Data to Humans

With an increasing amount of knowledge being gained into the effects of 0-G on rats (in skeletal muscle and in many other systems), the question of the applicability of these findings to humans has to be asked. Humans and rats share similar regulatory mechanisms in skeletal muscle fibres. They both show similar patterns of hypertrophy, atrophy and shifts in fibre type given appropriate stimuli. Regulatory mechanisms in both humans and rats are similar, both attempting to providing skeletal muscles which can complete all tasks required of them as economically as possible. Given the similarity in regulatory mechanisms, it would appear likely that human skeletal muscle would undergo similar changes to those observed in the rat model.

Only limited studies have been undertaken in humans on earth modeling the effects 0-G, and even more limited studies on astronauts exposed to 0-G. Often the data collected has been less than ideal. There seems to be no doubt, however, that atrophy occurs in human skeletal muscle in a similar way to that in the rat in 0-G, albeit at a slower rate (Leach *et al.*, 1991; Vorobyov *et al.*, 1983). Similarly, atrophy has been observed in the human earth-bound models of 0-G (see introduction). The slower rate of muscle atrophy in humans exposed to 0-G, is probably due, at least in part, to the differences in metabolic rate between rats and humans, and partly due to the extensive exercise programs the astronauts undertake in 0-G to prevent muscle atrophy. One study on the effects of 0-G on rhesus monkeys has been undertaken, as a primate model of 0-G (Bodine-Fowler *et al.*, 1992), but this was rather flawed, as was discussed in the introduction. Future work, as well as work in progress, will be studying biopsies from both rhesus monkeys and from human subjects exposed to 0-G.

While it is known that atrophy occurs in humans in 0-G, it is only now that studies on changes in fibre types are being contemplated or undertaken, with results not yet available. The limited studies on human models of 0-G which have been undertaken have not shown changes in fibre type, but because of the limited nature of these studies, it is difficult to draw solid conclusions from this result.

4.7 Future Studies

Much work remains in this field. The obvious limiting factor is the expense involved. One soon to be attempted experiment will involve rats which will be killed in space, so that the effects of 0-G without the landing and recovery phases can be studied. Also, as longer term space missions are being contemplated, the necessity for longer term 0-G experiments increases. Such experiments could be conducted aboard the proposed American space station Freedom.

Another important study would be to determine the EMG pattern of different muscles whilst in space, both on animals, and humans if possible. This would allow a better understanding of the changes in neural activity that occur in the 0-G environment.

All of this research is leading to a better understanding of the effects of 0-G on humans, and as such, the work in the rhesus monkey and as much as possible on humans needs to be carried out. Muscle biopsies on humans may provide information on whether the animal models are substantially different to humans. Such experiments, taking muscle biopsies from astronauts, are now being considered.

It seems more and more clear that exercise alone will not prevent 0-G induced changes such as atrophy. For example, Vorobyov *et al.* (1983) found that astronauts aboard the Salyut-6 space station could not prevent muscle atrophy and loss of muscle strength despite 2.5 hours of exercise per day. Rats which have undergone exercise prior to hindlimb suspension have not slowed the atrophy of postural muscles in suspension (Guezennec *et al.*, 1990), while exercise during suspension could only reduce atrophy by approximately 50% (Hauschka *et al.*, 1988). So, the research in this field is aiming to develop ways to minimise the effects of 0-G on the musculature. As such, experiments to determine the amount of gravity, or some equivalent force to maintain muscles in a better state, are of importance. Such experiments are being considered for the proposed American space station Freedom, using a centrifuge to simulate differing levels of gravity for various amounts of time, and the ability of such methods to prevent or slow the deleterious effects of 0-G. One earth based study in a similar vein already has been completed (Daunno *et al.*, 1992). It found that exposure of rats to 1-G for 15 minutes, 4 times per day, was enough to prevent atrophy the soleus in hindlimb suspension. Interestingly, exposure

to 1.2-G using a centrifuge for the same time period per day did not completely prevent atrophy, suggesting some undefined stress associated with centrifugation.

As people have ventured into space for the first time, many problems have arisen, and some of these have been overcome. It is hoped that this, and studies undertaken in the future, will allow for a better understanding of what is required for people to function efficiently in the 0-G environment.

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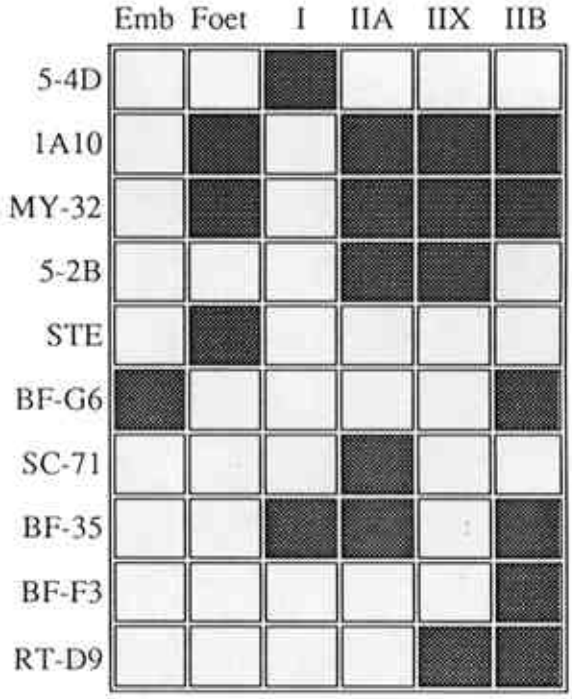
Appendix A: Antibody Specificity



A summary of the staining characteristics of the primary antibodies used in this study. Shaded squares indicate a positive staining reaction with Embryonic (Emb), Foetal (Foet), types I, IIA, IIX or IIB myosin heavy chain.