Muscle Fibre Size in the *Palmaris Longus* and Anterior Head of the *Biceps Femoris* During Growth: Effects of Hypophysectomy, Food Restriction and Muscle Function

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Abstract

Muscle fibre growth is regulated by hormonal and dietary factors. The purpose of this study was to determine the possible role of functional differences between the forelimb and the hindlimb in muscle growth. The forelimb is concerned largely with feeding behaviour, a movement function, while the hindlimb involves both movement and postural functions. The effects of hypophysectomy (hypox) and food restriction (FR) begun at 60 days on muscle growth over 120 days was compared in a forelimb muscle, the *palmaris longus*, and a hindlimb muscle, the anterior head of the *biceps femoris* in the Wistar rat. Between 60 and 180 days body weight increased by 65%, the weight of the forelimb muscle by 52% and the hindlimb muscle by 78%. These changes were prevented by both hypophysectomy and food restriction. The size of type 2 or movement muscle fibres in the forelimb increased by 19% and in the hindlimb by 42%. The size of type 1 or posture fibres in the forelimb muscle did not increase significantly with age and was not affected by either hypophysectomy or food restriction. Thus, in the forelimb where movement is so important, there is growth of type 2 (movement) fibres, but no growth of the type 1 (posture) fibres.

Key words: Morphometry, muscle fibre size, type 1, type 2, growth, hypophysectomy, food restriction, fibre size variability, fast muscle.

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Changes in muscle fibre size and composition throughout life are controlled by neural, myogenic, endocrine, dietary, functional and age factors. Neural factors from the motor nerve supply determine the biochemical and morphological characteristics of muscles [20]. Within the muscle itself the contractile properties of individual fibres, which determine fibre type, are affected principally by myosin heavy chains [12].

Endocrine influences such as hypothyroidism, have been shown to decrease heavy myosin chain development in fast fibres [10, 21, 27] and thus inhibit fast fibre (type 2) development. However, hypothyroidism increases sedentary behaviour, also resulting in a shift towards slow (type 1) fibres.

The principal dietary factor determining the rate of muscle growth is food intake [24]. Food acts partly by mediation of the endocrine system and food restriction is known to inhibit the secretion of hormones from the hypothalamus, pituitary, and other endocrine organs [6]. In fact food restriction induces what is commonly referred to as dietary hypophysectomy [18].

The effects of ageing in hindlimb muscles have been studied by Shorey et al. [22, 23, 24]. Their work showed that muscle fibre size and weight, increased with growth until middle age and then declined with old age. In old age, fibre sizes became more variable due to fibre atrophy and compensatory hypertrophy. These age changes were minimised by hypophysectomy and food restriction.

The role of the endocrine system in the growth of skeletal muscle has also been explored by Shorey et al. [24]. Hypophysectomy and food restriction were shown to inhibit the growth of both types 1 and 2 fibres in the *gastrocnemius* muscle in the hindlimb. Very small amounts of hormone are still secreted during food restriction, thus the effects of hypophysectomy were greater than those of food restriction, causing cessation of growth and even atrophy of type 2 fibres.

The function of an organ influences muscle development, as in the case of jaw closing muscle [13] where a

superfast fibre is developed which is twice as fast as limb fast muscle. The limb muscles of the rat have different functions, which may affect the development and growth of muscle fibres. The forelimb of the rat is concerned largely with the process of feeding, with posture assuming only a secondary role. The hindlimb is required both for body movement and for posture maintenance, the latter function being especially important during feeding from a raised food source.

The aim of this study was to determine the possible role that functional differences between forelimbs and hindlimbs may play in the development and growth of muscle fibres in two fast muscles from these limbs. The techniques of hypophysectomy and food restriction were used to assess the contribution of hormonal and dietary factors to these functional differences.

Materials and Methods

Rats were housed in wire bottomed cages in pairs (except for the food restricted animals which were housed individually) in a temperature controlled room at 25°C, with 12 hours of artificial light per day. Rats were fed pelleted food (Youngs Stockfeed, Pty Ltd, Young, NSW; Composition: fat 4.7%, protein 21.6%, starch 32.2%) which was available *ad libitum*, except to the food restricted rats. Water was available *ad libitum* to all groups.

Hypophysectomy was performed on 60 day old rats, with a corresponding weight of between 220-280 g, under general anaesthesia with 1% halothane. The pituitary gland was removed using Koyama's transauricular technique [16], as modified by Everitt and Wyndham [7]. Post-operative care included 3 tablets of 5 mg prednisolone dissolved in the 600 ml of water. The animals were weaned off the prednisolone over a period of 10 days.

Food restriction was begun when the animals were approximately 50 days old, and over the following 3 weeks the rats were fed decreasing amounts of food to bring about an accommodation of approximately 9 g per day. This food intake is half that of a control rat, but similar to the daily intake of a hypophysectomised (hypox) rat.

The rats used were all male, and of the conventional non-inbred University of Sydney Wistar strain. There were 5 groups; 60, 120, and 180 days old controls, and 180 days old food restricted (FR) and hypox animals. Each group consisted of 4 animals. After the pre-determined experimental period, the rats were sacrificed with an overdose of sodium pentobarbitone. Dissections were performed on both the left and right forelimbs and hindlimbs. The muscles chosen for analysis were predominantly composed of type 2 (fast) fibres; the *palmaris longus* from the forelimb and the anterior head of the *biceps femoris* from the hindlimb. The *palmaris longus* was analysed within the whole flexor compartment of the forelimb. These muscles were taken as representative of fast muscles, the anterior head of the *bicepsfemoris* as a homogeneously fast muscle and the *palmaris longus* as a fast muscle with only a small component of type 1 (slow) fibres.

Following dissection, the muscles were weighed, frozen in super cooled isopentane and stored in liquid nitrogen. Prior to sectioning, the blocks of tissue were removed from liquid nitrogen storage, coated in OCT embedding medium (Tissue Tek 4583, Miles Inc, Elkhart, Indiana, USA), placed in a cryostat chamber and maintained at -25°C.

From each muscle, 9 transverse serial sections were cut from 3 levels, 1 mm apart, in the midbelly of the muscle. Three levels were cut to determine variability between levels within muscles. Muscle sections were placed on chrome-alum gelatine coated slides at room temperature and stored at -25°C until stained, a maximum of 24 hours later. Sections were stained to demonstrate myofibrillar ATPase activity at pH 9.4 [11, 19]. Type 1 fibres stain lightly and type 2 fibres stain dark.

Morphometric analysis of the stained sections using a fully automated image analysis system, the MD30 Plus (manufactured by Leading Edge Pty Ltd, Adelaide), was undertaken to determine the cross-sectional areas of individual type 1 and type 2 muscle fibres in three random areas of each section. The cross-sectional area was then converted to an equivalent circle diameter (ECD) which is computed as $2\sqrt{(\text{area}/\pi)}$. The ECD measurements were analysed using a full nested multiway analysis of variance (ANOVA). This was a hierarchical testing: within and between levels in muscles, within and between muscles in rats, between rats within the groups, and between the 5 groups of rats. *Post-hoc* analysis of differences between individual groups was made with the Tukey and Scheffe tests.

Results

Table 1 shows body weights and muscle weights from the anterior head of the *biceps femoris* (ABF) and the whole flexor compartment of the forelimb. The increase in body and muscle weights with age between the control groups, from 60 days of age to 180 days of age, is an indication of growth. The hypox and FR animals showed large reductions in both body and muscle weights when compared with control animals of the same age, indicating that growth ceased to occur after the age of 60 days when the experimental treatments were implemented.

Characterisation of muscle fibre size and proportion in a hindlimb muscle: the anterior head of the biceps femoris (ABF)

The ABF comprises only type 2 (fast) muscle fibres which are dark staining. In this study no specific method was used to distinguish type 2A from type 2B, although the intensity of staining did differ between them (Fig. 1). Muscle fibre size in all groups exhibited a normal distribution. Figures 1-4 demonstrate the effects of age, hypox and FR. Growth of type 2 fibres occurred between 60 days (Fig. 1) and 180 days (Fig. 2). Type 2 fibres were noticeably smaller in hypox (Fig. 3) and FR rats (Fig. 4). Type 1 fibres were absent in the ABF of rats from all 5 groups.

Table 2 details the mean equivalent circle diameters (ECD) or size of type 2 muscle fibres in the ABF in each

Table 1. Age, body weight, and hindlimb (ABF) and forelimb (whole flexor) muscle weights in the 5 groups of rats.

Group	Age (days)	Body weight (g)	Mean anterior biceps femoris weight (g)	Mean whole flexor weight (g)
Control Rats	60	282.8 ± 29.90	0.41 ± 0.093	0.60 ± 0.044
Control Rats	120	418.5 ± 12.37	0.67 ± 0.055	0.77 ± 0.063
Control Rats	180	466.3 ± 53.23	0.73 ± 0.075	0.91 ± 0.081
Hypox Rats	180	213.0 ± 22.85	0.35 ± 0.075	0.52 ± 0.068
FR Rats	180	206.0 ± 21.60	0.37 ± 0.073	0.58 ± 0.077

There were 4 rats in each group. Means ± standard deviation were calculated using values from both left and right muscles.

of the 5 groups. Analysis of variance (ANOVA) revealed highly significant differences between age and treatment groups ($p < 0.01$). *Post-hoc* analysis of these results showed that significant differences ($p < 0.01$) in fibre size existed between all the groups except between the 180 day hypox and FR rats ($p > 0.05$).

The mean muscle fibre ECD of hypox rats at 180 days, and to a lesser extent in FR rats, was smaller ($p < 0.01$) than that of 60 day controls. Thus, muscle fibres in the ABF of hypox and FR animals exhibited no growth and atrophy was a significant ($p < 0.01$) feature of these fibres.

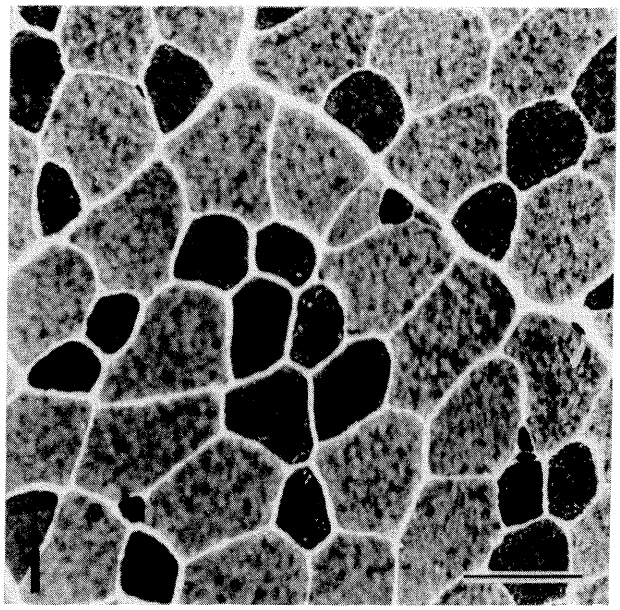

Figure 1. Transverse section of an area from the anterior head of the biceps femoris (ABF) muscle from the hindlimb of a 60 day old control rat. The section contains type 2 fibres (dark) only. No attempt was made to distinguish between the type 2A fibres and the type 2B fibres even though they stained at different intensities. Scale = 250 μm.

Variability of type 2 fibres within the 5 groups is also shown in Table 2. Within the 60 day and 120 day control groups, and the experimental groups, only a small amount of variability existed. Within the 180 day control group significantly larger variation was apparent ($p < 0.01$). More importantly the coefficient of variation which allows comparison of variation in populations with different means, was much greater in the 180 day controls. This increased variability indicates atrophy and compensatory hypertrophy in these muscle fibres, a feature of ageing muscle fibres that is more pronounced in middle aged and particularly older rats. Variability was much lower in hypox and FR rats than controls of the same age (180 days) and controls 4 months younger (60 days), indicating that atrophy and hypertrophy did not occur in these experimental groups.

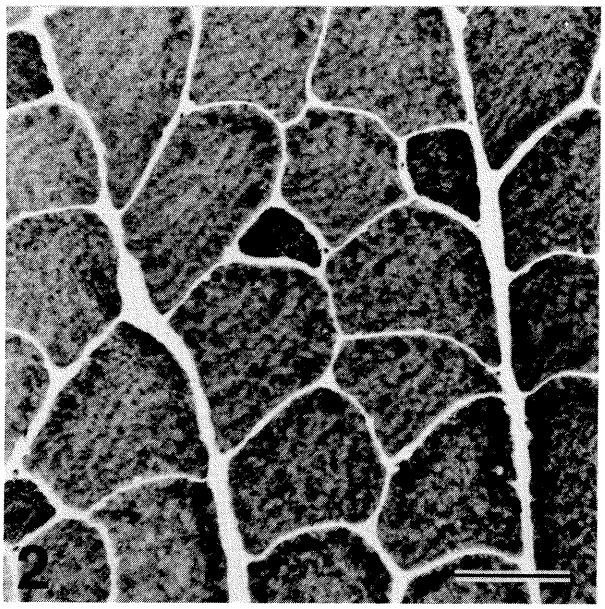

Figure 2. Transverse section of an area of the ABF muscle from a 180 day old control rat. The fibres are larger than in 60 day controls (Fig. 1). Scale = 250 μm.

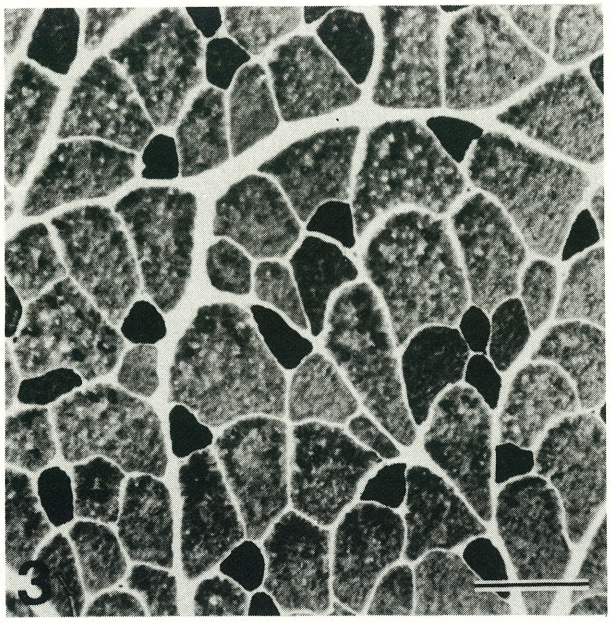

Figure 3. Transverse section of an area of the ABF muscle from a 180 day old hypophysectomised rat. The fibres are distinctly smaller than those of 180 day controls (Fig. 2). Scale = 250 μm.

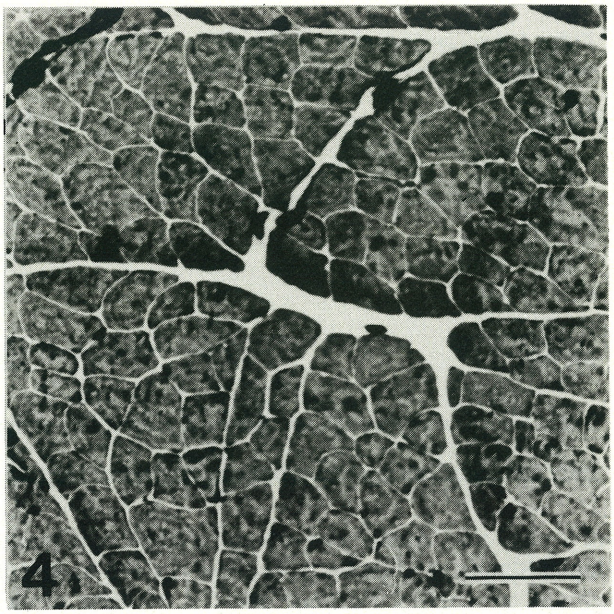

Figure 4. Transverse section of an area of the ABF muscle from a 180 day old food restricted rat. The fibres are much smaller than those of 180 day controls (Fig. 2). Scale = 250 μm.

Characterisation of muscle fibre size and proportion in a forelimb muscle: the palmaris longus (PL)

The *palmaris longus* is comprised of both type 1 fibres (which did not stain) and type 2 (dark staining) muscle fibres. Stratification of fibre types is obvious in these figures, but sampling for image analysis overcame this problem. Figures 5-8 illustrate the effects of age, hypox and FR. Table 3 details the mean fibre sizes (ECD) and percentages of type 1 fibres in the 5 groups of rats.

The sizes of both type 1 and type 2 fibres exhibited normal distribution. There were no significant changes due to age, hypox or FR ($p > 0.05$) in the fibre size of type 1 fibres (Table 3). However significant differences existed between the 5 groups regarding fibre type proportions ($p < 0.01$). *Post-hoc* analysis showed that 180 day controls and 180 day hypox rats had significantly more type 1 fibres than 60 day controls ($p < 0.01$). The 180 day controls were not significantly different from the 180 day hypox rats ($p > 0.05$). Thus, the proportion of type 1 fibres increased by a similar amount between 60 and 180 days in both control and hypox rats. The 180 day FR rats did not significantly differ from the 60 day controls ($p > 0.05$).

Table 2. Equivalent circle diameter and fibre size variability of type 2 muscle fibres in the anterior head of the biceps femoris in the rat hindlimb. A minimum of 9 000 muscle fibres were measured from each group for estimates of fibre size. The mean fibre sizes of all control groups are significantly different from each other ($p < 0.01$). Hypox and FR groups are significantly smaller than the 60 day control group ($p < 0.01$). Hypox and FR groups are not significantly different from each other ($p > 0.05$).

Group	Mean (μm)	Variance	SD	SE	CV
60 Day Controls	41.40	14.42	3.80	0.45	0.09
120 Day Controls	54.73	38.69	6.22	0.73	0.11
180 Day Controls	59.16	60.55	7.78	0.92	0.13
180 Day Hypox	36.10	18.53	4.30	0.51	0.11
180 Day FR	36.51	9.72	3.12	0.39	0.08

SD = standard deviation; SE = standard error; CV = coefficient of variation

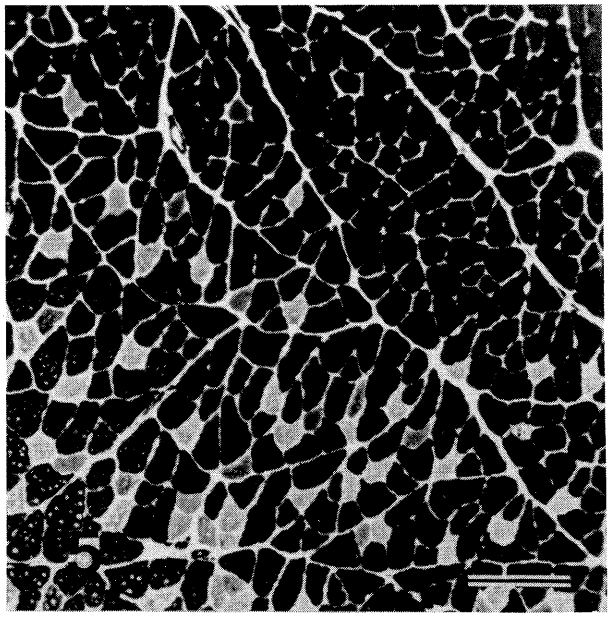

Figure 5. Transverse section of an area of the palmaris longus (PL) muscle from a 60 day old control rat. This section illustrates the fibre gradient of the unstained type 1 fibres within the muscle, with type 1 fibres more concentrated in the deep portion of the muscle. Scale = 500 μm.

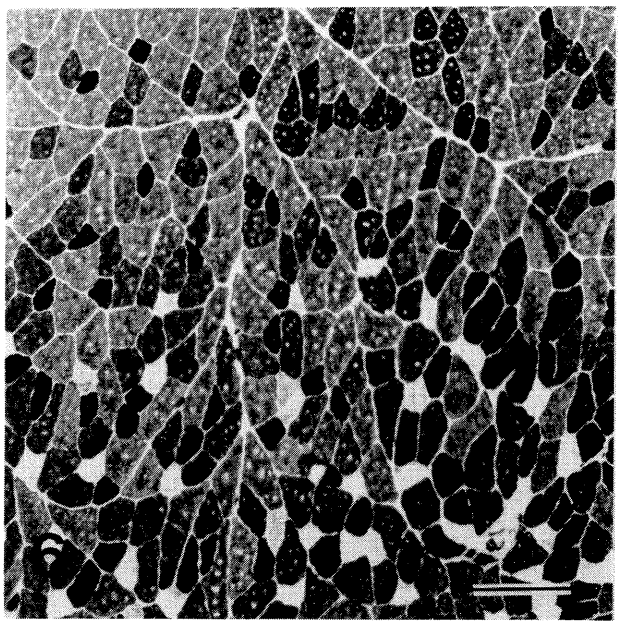

Figure 6. Transverse section of an area of the PL muscle from a 180 day old control rat. Scale = 500 μm.

Significant differences in mean type 2 fibre size (ECD) were found between all 5 groups ($p < 0.01$). The 19% increase ($p < 0.01$) in the mean ECD of the muscle fibres of control rats from 60 days to 180 days, again indicated normal growth. The muscle fibre sizes in hypox rats were significantly smaller ($p < 0.01$) than in all other groups (Table 3). The mean ECD of the FR group was significantly smaller ($p < 0.01$) than the 60 day control group. Thus, in the hypox and FR groups fibre growth was arrested and followed by significant atrophy, this effect being more pronounced in the hypox group.

Analysis of variance showed that the size of type 2 fibres in the *palmaris longus* exhibited significant variation ($p < 0.01$) between rats within groups and between legs within rats, in all groups except the hypox group. This is illustrated by the measurements of variability presented in Table 3, in particular the coefficient of variation. The ABF by comparison (Table 2) showed no significant differences in type 2 fibre size within and between rats of the same group.

The amount of variance within and between rats in the same group as revealed by ANOVA, was investigated using the multiple r statistic. This quantity is also known as the coefficient of determination and is a measure of the variation explained by ANOVA. The 60 day control animals were significantly different ($p < 0.01$) from each other with a multiple r of 64%. Thus only 64% of the variance could be attributed to differences between animals in the group. The remaining variance was the result of differences between the left and right forelimb.

The 180 day FR group also had significant differences ($p < 0.01$) in the fibre sizes of the PL within the group. However the multiple r was 0.17, hence only 17% of the variance found within the group was the result of variance between the rats. The remaining 83% of the differences within the groups arose from differences between the left and right forelimb.

In contrast, the multiple r for the 180 day control rats was 0.78 [where significant differences ($p < 0.01$) were found within and between rats within groups]. Thus, 78% of the variance found was the result of variation between rats.

An age related increase in fibre size variability in controls between ages 60 and 180 days occurred in both type 1 and type 2 fibres as indicated by the increase in the coefficient of variation (CV) and other measures of variability shown in Table 3.

Discussion

This paper is a continuation of work previously published [22, 23, 24] investigating the long and short term effects of hypophysectomy and food restriction on the morphometry of skeletal muscle fibres in the rat. The present work, now including a forelimb muscle, further contributes to the understanding of the hormonal and histological basis of muscle function in the forelimb. In control rats between the ages of 60 and 180 days the forelimb muscle, the *palmaris longus*, exhibits growth of type 2 (movement) fibres, but not type 1 (posture) fibres. Furthermore hypophysectomy prevents growth of type 2 fibres but not type 1. These findings are consistent with the major function of the forelimb, which is feeding, a behaviour that is largely

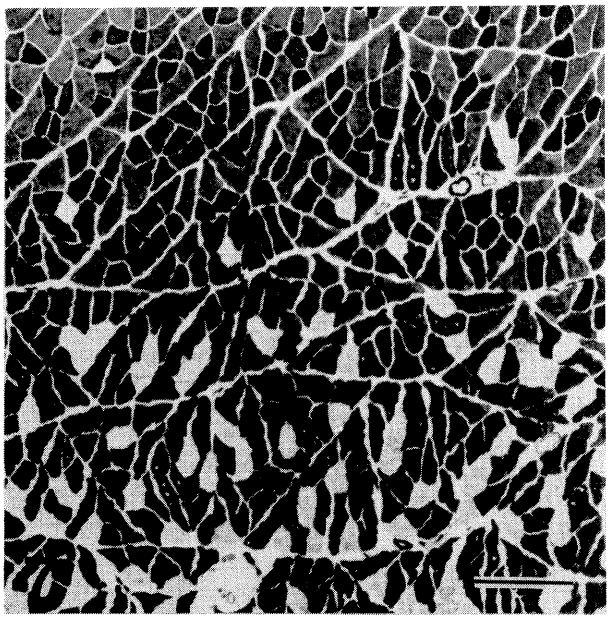

Figure 7. Transverse section of an area of the PL muscle from a 180 day old hypophysectomised rat. There is an increased number of type 1 (unstained) fibres in this section. Scale = 500 μm.

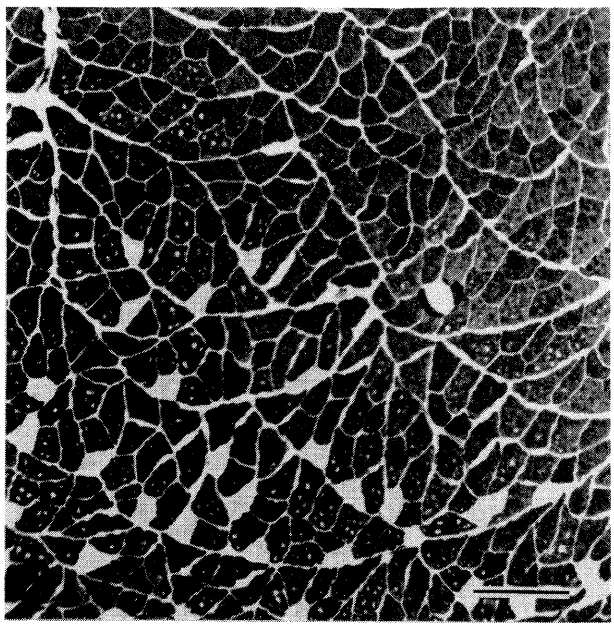

Figure 8. Transverse section of an area of the PL muscle from a 180 day old food restricted rat. Scale = 500 μm.

movement oriented. These effects are achieved mainly by hormonal action.

There are a number of reasons why results from this work show that fast muscle in the forelimb and hindlimb of the rat is directly and indirectly regulated by hormones. Firstly, previous studies on rat *gastrocnemius* [24] and *soleus* [22] in the hindlimb showed that hypophysectomy and food restriction inhibited the growth of whole muscle and individual type 2 muscle fibres. Similar effects were observed in this study of the *palmaris longus* (forelimb) and the anterior head of the *biceps femoris* (hindlimb). Growth failure and muscle atrophy in such rats is due to a lack of pituitary hormones, a consequence of hypophysectomy and food restriction. The sensitivity of skeletal muscle fibres to pituitary hormones ensures that growth and development in muscles from hypophysectomised animals are affected, particularly those muscles with a large component of fast fibres. Armario et al. [1] demonstrated that suppressed secretion of growth hormone and thyroid stimulating hormone occurred during food restriction, which is restored following resumption of normal food intake [6].

Secondly, fast muscles were particularly affected due to their greater component of type 2 fibres. Type 2 muscle fibres in hypophysectomised and food restricted animals at 180 days were characteristically much smaller and less variable in size than in 60 day and 180 day control animals. Absence of thyroid hormone causes poor muscle development and function [8]. This is particularly the case for type 2 fibres, as thyroid hormone has been reported to affect fast fibre development [10, 21], as a result of the expression of fast myosin heavy chains [15, 27].

Thirdly, type 2 fibres are also particularly affected by growth hormone [4, 17]. Following hypophysectomy and food restriction, type 2 fibres atrophy to a smaller size than at the time of hypophysectomy, that is they were smaller than type 2 fibres of 60 day control rats. The inhibition of growth can be partly attributed to lack of growth hormone which reportedly increases muscle size [5, 25, 26] by increasing cellular proliferation and thus protein synthesis in muscle fibres [9, 25, 26, 27]. An increase in the proportion of type 1 fibres has also been attributed to growth hormone [2, 3].

Previous studies of hindlimb muscles, *gastrocnemius* [24] and *soleus* [22], showed that the proportion of type 1 muscle fibres remained constant with age in control animals but increased with age following hypophysectomy. In the present study of a forelimb muscle the percentage of type 1 fibres increased from 11% to 15% in hypox animals. However, the percentage of type 1 fibres in the control muscle also increased from 11% at 60 days to 16% at 180 days. Unpublished data (Armstrong RA, Everitt AV, Kofod MG, Shorey CD, Stark EJ, 1993) from a similar study, between 60 and 180 days, of forelimb and hindlimb postural muscles (a slip of the *flexor digitorum superficialis* and the *soleus*) have results consistent with this study, where the proportion of type 1 fibres increased in both hypox and 180 day controls in the forelimb and the proportion of type 1 fibres in the hind limb increased in hypox rats only. Thus, type 1 muscle fibres in the forelimb differ in some way to those in the hindlimb. This difference

Table 3. Equivalent circle diameter, fibre size variability and fibre type proportion of type 1 and type 2 fibres in the palmaris longus in the rat forelimb. A minimum of 9 000 muscle fibres were measured from each group for estimates of fibre size. Significant differences were present in the % of type 1 fibres, but not type 1 fibre size. Type 1 muscle fibres were distributed along a gradient from superficial to deep across the PL (Fig. 5). The PL was visually divided into three and fibres were measured randomly from an area in each third. Muscle fibre proportion was based on the total number of type 1 and type 2 fibres measured across the entire section. For type 2 fibres all of the means between all groups were significantly different ($p < 0.01$).

Type 1	Mean (μm)	Variance	SD	SE	CV	% of Type 1
60 day controls	39.22	12.23	3.50	0.41	0.09	11.25%
120 day controls	40.38	16.95	4.03	0.48	0.10	14.52%
180 day controls	42.01	39.35	6.27	0.74	0.15	16.25%
180 day hypox	38.42	9.45	3.07	0.36	0.08	15.07%
180 day FR	40.11	11.87	3.44	0.41	0.09	11.61%
Type 2	**Mean (μm)**	**Variance**	**SD**	**SE**	**CV**	
60 day controls	47.28	14.21	3.77	0.44	0.08	
120 day controls	49.78	8.00	2.83	0.33	0.06	
180 day controls	56.04	42.80	6.54	0.77	0.12	
180 day hypox	39.70	7.27	2.70	0.32	0.07	
180 day FR	45.56	5.31	2.30	0.27	0.05	

SD = standard deviation; SE = standard error;
CV = coefficient of variation

could be intrinsic, but the likely explanation is that extrinsic factors account for the differences, such as the function of the muscle. Hindlimb muscles are used predominantly for posture by more sedentary rats. Sedentary behaviour increases with age and even more so after hypophysectomy, thus hypox rats have hindlimb muscles with a significantly higher proportion of type 1 fibres. However, the forelimb is used predominantly for feeding, a more movement oriented behaviour, thus the proportion of type 1 fibres in hypox animals did not exceed that found in the 180 day controls.

Type 1 muscle fibres were absent from the ABF in the hindlimb. Hypophysectomy did not induce the development of type 1 fibres between the ages of 60 and 180 days in this muscle. Myogenic factors probably limit the extent to which hypophysectomy can alter fibre type proportion. The phenotype of fibres is limited according to constraints imposed by the allotype of that fibre. Fibres from different allotypes express unique forms of myofibrillar proteins. The extent to which a fibre is affected by the absence of hormones during a vital stage of growth is limited by the developmental origins of that fibre [13, 14].

An age related increase in fibre size variability was evident in all control groups. Variance has been shown to be greater in middle (500 days) and old (1000 days) age rats [22, 23], however in the present study a significant rise was apparent between 60 and 180 days. This increased variance is typically caused by fibre atrophy which is often accompanied by compensatory hypertrophy of other fibres. These effects are the consequence of an increase in sedentary behaviour, probably the result of a reduction in both basal metabolic rate and pituitary activity.

In conclusion, the growth of type 2 (fast or movement) fibres in both forelimb and hindlimb muscles of the rat is inhibited by hypophysectomy or food restriction. Movement is an important function in both forelimbs and hindlimbs. However, in the forelimb postural function is less important and less frequent than movement behaviour, especially during feeding behaviour. Thus in the forelimb type 1 (postural) fibres in the *palmaris longus* did not grow significantly between 60 and 180 days and were not affected by hypophysectomy. Also the proportion of type 1 fibres in *palmaris longus* increased with age from 60 to 180 days but this increase was not affected by hypophysectomy. Therefore type 1 (posture) fibres in the forelimb muscle react differently to hormones and age than type 2 (movement) fibres.

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