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Growth Changes in Skeletal Muscle Histochemistry of Thoroughbreds and Other Horses

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ABSTRACT. The development of histochemical fibre types in m. semitendinosus, m. diaphragma and m. pectoralis transversus of Thoroughbreds and other horses from early prenatal life to adulthood was investigated in an attempt to explain differences in the properties of respective adult muscles. The myosin ATPase reaction differentiates fibres at the earliest ages investigated. Glycogen phosphorylase and succinate dehydrogenase activities gradually develop from an amorphous staining pattern in the young to the appropriate adult type. The proportions of fibre types in different muscles and parts of muscles anticipate those of adults at the earliest stages. While the proportional area of myosin ATPase low-reacting fibres increases with liveweight in the three muscles of the other horses, the increase is significant only in m. diaphragma of Thoroughbreds. When the prenatal horses of both types are compared with one another the percentage area of myosin ATPase low-reacting fibres is not significantly less in Thoroughbreds than in the other horses, in contrast to the distinct differences between the two types of adults.

Key words: Muscle; growth; horses; histochemistry.

INTRODUCTION

It has been recognised for some time that the proportions of skeletal muscle fibre types differ between horses which are noted for high speed running and those which are not.[11,24] Fibre type proportions in horse muscle vary in a number of ways. Thus, those in foetal horse muscle differ from those in adults.[9] Furthermore, variations exist in the proportions of fibre types in different parts of individual limb muscles of adult horses.[12,17] Such variations may influence comparisons in samples of similar muscles between immature and mature horses.[2]

The purpose of this study was to investigate changes in fibre type proportions with growth in comparable areas of similar muscles of Thoroughbreds and other horses.

MATERIALS AND METHODS

Foetuses and postnatal specimens less than two years of age were obtained from post mortem room sources. All horses used were fresh and non-debilitated. The number of animals of each type from which samples of the three muscles investigated; m. semitendinosus, a predominantly propulsive muscle; m. diaphragma, a predominantly respiratory muscle; and m. pectoralis transversus, a predominantly postural muscle, are shown in Table 1. The present study also uses material analysed in a previous investigation,[12] and the number of samples also appear in Table 1. Blocks were taken from the superficial part of the pectoralis and the costal part of the diaphragm. The complete cross section of the semitendinosus of horses was taken from the part of the muscle that plays over the tuber ischiadicum. A 0.5 cm thick slice of this complete cross section from young horses was cut with a brain knife and, as for samples for the other muscles, mounted on pieces of cork 0.5 cm thick which were then frozen to a cryostat chuck. Samples were then frozen rapidly by plunging into

Table 1. *The numbers of the types of animals from which samples of each muscle were obtained for histochemical analysis*

Figures in brackets refer to the numbers of samples obtained from adult animals. Thoroughbred X = Thoroughbred cross

Type of animal	Muscle		
	Semiten-dinosus	Diaphragma	Pectoralis transversus
Thoroughbreds	24 (9)	17 (6)	20 (10)
Other horses	9 (15)	7 (11)	9 (16)
Thoroughbred X	3 (5)	2 (3)	3 (6)

dichlorodifluoromethane (Arcton 12, I.C.I.) cooled to its melting point of −158°C by liquid nitrogen. Serial sections from samples were cut 10 μm thick in a cryostat at −20°C and mounted directly onto coverslips and allowed to thaw and dry rapidly at room temperature. Fibre outlines were demonstrated by fixing sections for 10 min in 4% formaldehyde, which were then washed and stained for 20 min in Ehrlich's haematoxylin. Enzyme reactions for succinate dehydrogenase (SDHase), glycogen phosphorylase (GPase) and myosin adenosine triphosphatase (myosin ATPase) pH 9.5 were demonstrated as described previously.[6] Outlines of approximately 400 fibres in two areas lying about 20 fibre breadths deep to the surface of the muscle and about 200 fibre breadths apart were drawn on transparent paper placed on a glass screen, by back projecting a haematoxylin stained section onto the screen. The histochemical reaction (assessed as being either high or low) was indicated on tracings and the areas of paper representing each fibre type were weighed to measure both the proportional area occupied by each fibre type and the mean area of each fibre type. The mean of estimates from the two areas was used to represent the muscle. These data together with those obtained from similar samples of the three muscles from adult animals which were reported previously[12] were used to investigate changes during growth. The total number of fibres having a low staining intensity for myosin ATPase in complete transverse sections of m. semitendinosus of young horses were estimated by sampling the muscle at 0.5 mm intervals across its entire transverse section. Fibres exhibiting an intermediate reaction to the myosin ATPase technique during stages of rapid growth were of necessity individually described as high or low reacting relative to the overall level of activity of fibres in each section. Computer facilities and statistical methods[7,8] were used to test for differences between the two types of animal.

Fig. 1 Fresh frozen transverse sections of muscles. (*a, b*) Serial sections from the superficial caudal region m. semitendinosus of a 24 kg, 318 foetal female Thoroughbred, demonstrating myosin ATPase (Fig. 1 *a*) and SDHase (Fig. 1 *b*) activities. Myosin ATPase low reacting (light staining) fibres occur individually among the high reacting (dark staining) fibres. Low reacting fibres for myosin ATPase have a high reaction for SDHase while high reacting fibres may have a high or low SDHase reaction. (*c, d*) Serial sections from m. diaphragma of the same animal as *a* and *b*, demonstrating myosin ATPase (*c*) and SDHase (*d*) activity. All fibres have a uniformly high activity of SDHase. (*e*) Section from the deep medial region of m. semitendinosus of the same animal as *a–d*, demonstrating myosin ATPase activity. (*f–h*) Serial sections from the superficial region of m. pectoralis transversus of a 32 kg, 317 day female foetal Thoroughbred, demonstrating myosin ATPase (*f*), SDHase (*g*) and GPase (*h*) activity.

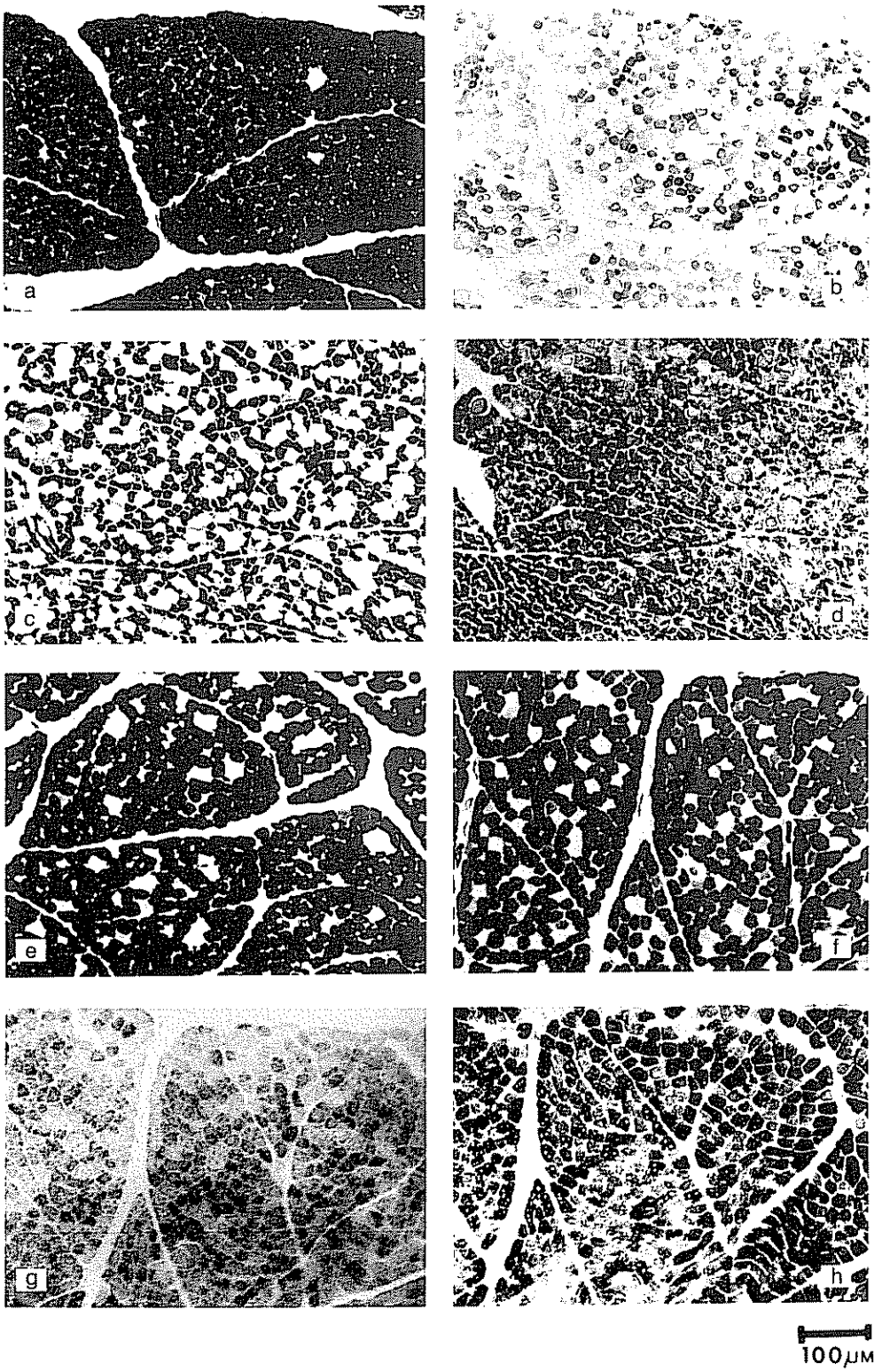

a
b
c
d
e
f
g
h
100μm

Table 2. *Percentage of fibres sampled low in succinate dehydrogenase activity (SL), glycogen phosphorylase activity (PL), and myosin ATPase activity (AL), and percentage of area of samples occupied by AL fibres, in m. semitendinosus, m. diaphragma and m. pectoralis transversus of young horses*

Type of horse	Body-weight (kg)	Sex	Age[a]	M. semitendinosus				M. diaphragma			
				SL	PL	AL	AL area	SL	PL	AL	AL area
Thoroughbred	11	F	338	0	0	2.2	2.4	0	1	41	41
Thoroughbred	13	F	274	89	0	1.7	1.6	**	**	**	**
Thoroughbred	16	M	337	54	0	0.60	0.70	0	0	23	24
Thoroughbred	19	F	326	47	0	0.81	0.71	0	2	35	43
Thoroughbred	24	M	314	83	0	3.9	2.5	0	0	32	51
Thoroughbred	24	F	318	33	0	1.9	1.2	0	0	26	33
Thoroughbred	28	M	285	72	0	2.3	3.2	**	**	**	**
Thoroughbred	29	M	326	46	0	1.7	2.2	0	2	36	64
Thoroughbred	31	F	311	66	0	0.87	0.71	0	0	36	45
Thoroughbred	32	M	321	62	0	1.4	1.0	**	**	**	**
Thoroughbred	32	F	317	65	0	2.0	1.4	0	0	41	57
Thoroughbred	34	M	330	53	0	4.2	2.6	0	0	41	49
Thoroughbred	35	F	274	50	0	3.7	2.2	**	**	**	**
Thoroughbred	35	F	341	62	0	2.4	6.8	**	**	**	**
Thoroughbred	39	M	339	46	0	6.1	2.4	0	0	49	33
Thoroughbred	39	M	338	35	0	1.9	1.4	**	**	**	**
Thoroughbred	41	F	340	61	0	6.6	3.6	0	0	43	56
Thoroughbred	42	M	338	**	**	1.7	1.5	**	**	40	41
Thoroughbred	43	F	338	64	0	7.4	8.0	0	0	45	41
Thoroughbred	50	F	338	52	0	1.3	0.7	0	0	41	42
Thoroughbred	59	F	346	**	**	3.6	3.4	**	**	**	**
Thoroughbred	337	F	703	46	4	3.7	1.9	0	0	69	76
Thoroughbred	420	M	896	51	0	7.2	3.7	0	1	79	84
Thoroughbred	433	M	886	49	0	2.3	1.2	0	1	67	63
Connemara X	2	M	160	0	0	5.7	5.8	0	0	10	10
Welsh Mountain	3	M	158	0	0	3.7	3.4	0	0	8.8	13
Dartmoor	22	F	339	**	**	3.5	2.5	**	**	**	**
Thoroughbred X	39	M	326	62	0	0.78	0.4	0	42	42	33
Thoroughbred X	62	M	348	67	0	1.8	1.1	**	**	**	**
Welsh Mountain	109	M	855	63	0	15	5.2	0	4	56	61
Welsh Mountain	115	F	703	50	0	8.7	4.2	0	6	60	65
Welsh Mountain	118	M	703	50	0	25	8.8	0	70	70	72
Welsh Mountain	154	M	703	51	0	7.2	3.7	0	0	66	70
Welsh Mountain	178	F	703	**	**	10	4.3	**	**	**	**
Thoroughbred X	203	M	460	64	0	13	8.5	0	0	62	64
Fell X	207	M	703	46	0	11	4.8	0	3	65	68

[a] Age is calculated in days from conception. (338 days is added to postnatal ages.)
[b] Thoroughbred X = Thoroughbred cross.
** No data available.

M. pectoralis transversus			
SL	PL	AL	AL area
0	0	7.5	8.7
**	**	**	**
0	0	19	22
0	0	21	25
47	0	8.9	8.6
0	0	13	12
0	0	7.5	7.1
0	0	18	22
17	0	10	8.5
0	0	22	18
0	0	24	19
0	0	22	18
**	**	**	**
**	**	**	**
0	0	14	11
0	0	15	16
0	0	24	18
**	**	21	19
0	0	42	41
0	0	34	28
**	**	31	21
0	0	24	9.1
**	**	**	**
0	0	43	29
0	0	10	10
0	0	8.4	6.4
**	**	22	18
0	0	24	16
0	0	37	32
17	45	46	39
0	0	41	35
0	0	51	23
0	0	29	19
**	**	52	42
0	44	44	33
0	0	37	27

RESULTS

Fibre type differentiation in young horses

The myosin ATPase reaction differentiated fibres at all stages of growth studied, i.e. from 158 days *in utero* onward, into high (AH) and low (AL) reacting fibres. The percentages of fibres which have a low reaction for myosin ATPase (AL), succinate dehydrogenase (SL) and glycogen phosphorylase (PL) are listed in Table 2. At the earliest stages investigated, AL fibres occurred singly among numerous smaller AH fibres in m. semitendinosus and m. pectoralis transversus (Fig. 1 *a*, *e*, and *f*). However, in m. diaphragma there was a greater incidence of AL fibres (Fig. 1 *c*). From about 300 days *in utero* it was possible to differentiate fibres in m. semitendinosus with SDHase reaction (Fig. 1 *b*), into high (SH) and low (SL) reacting fibres. When sections from the three muscles were incubated together, it was apparent that the overall density of the reaction products due to the SDHase reaction was greater in m. diaphragma (Fig. 1 *d*) than in m. pectoralis transversus (Fig. 1 *g*) which in turn was slightly greater than in m. semitendinosus (Fig. 1 *b*).

The overall colour of the reaction products due to the GPase reaction—dark blue—became darker with increasing live weight. It was not possible to distinguish fibre types in m. semitendinosus or m. pectoralis transversus of young horses by means of this reaction, since almost all fibres exhibited a high activity (PH) of the enzyme (Fig. 1 *h*). In m. diaphragma, some AL fibres had a low activity for GPase (PL) (Table 2). In all three muscles, AL fibres frequently had a lower GPase activity than AH fibres as indicated by the darkness of staining.

With increasing age and bodyweight the adult patterns of fibre type differentiation were gradually assumed, i.e. AH, SH, PH and AL, SH, PH in the three muscles and additionally the AH, SL, PH fibre type in m. semitendinosus.[12]

Table 3. *Logarithmic regression equations comparing the percentage areas of samples of m. semitendinosus, m. diaphragma and m. pectoralis transversus occupied by myosin ATPase low-reacting (AL) fibres with bodyweight in horses (non-significant regressions are excluded)*

Dependent variable	Type of animal	Number of observations	Regression coefficient (b)	Se b	log a	r
% AL area in m. semitendinosus	Other horses	32	0.186	0.089	−0.267	0.356
% AL area in m. diaphragma	Thoroughbreds	23	0.180[c]	0.030	0.832	0.790
	Other horses	23	0.343[c]	0.044	−0.020	0.862
% AL area in m. pectoralis transversus	Other horses	34	0.250	0.046	0.142	0.696

SE b: Standard error of regression coefficient (all values of b are significantly greater ($p<0.05$) than 0.
c: Values of b bearing superscript c are significantly different ($p<0.01$) from one another.
r Correlation coefficient.

Changes in the number and in the area occupied by AL fibres during growth

Differences between Thoroughbreds and other horses. Data for the three muscles are given in Table 2. The different proportions of fibre types in similar muscles at different ages are evident on comparing Figs. 1 and 2. Changes in the proportional areas occupied by AL fibres with increasing bodyweight and the corresponding regression equations, computed using data from this study and adults[12] are shown in Fig. 3 and Table 3. Since there was no significant difference between them, the Thoroughbred crosses and the other young horses are grouped together.

M. semitendinosus. The distribution of AL fibres across the transverse sectional profile of m. semitendinosus varied in both types of horses. The proportion of AL fibres was highest in the deep medial region of the muscle and become progressively less in its caudal superficial region (Fig. 1 *a, e*). Comparisons were made from data derived from the caudal superficial region of the muscle. In Thoroughbreds the graph relating the percentage AL area with bodyweight shows a considerable scatter of data points (Fig. 3). As a result, the regression equation (log $y=0.101 \log X-0.192$; $r=0.190$, $F=1.157$ with 31 degrees of freedom) does not significantly describe the relationship. Nevertheless it indicates a tendency for an increase in AL area with bodyweight as occurs in the other horses (Table 3). Furthermore in prenatal animals, although the values for both the percentage number of, and area occupied by AL fibres in Thoroughbreds were less than those for the other horses, the differences were not significant (Table 2).

M. diaphragma. The percentage area of samples occupied by AL fibres increased with increasing bodyweight in both Thoroughbreds and other horses over the entire range of bodyweight investigated but to a significantly greater extent ($p<0.01$) in the latter than in the former (Table 3, Fig. 3). At prenatal stages the numerical proportion ($p<0.02$) and proportional area ($p<0.05$) of samples occupied by AL fibres were both significantly greater in Thoroughbreds than in the other horses.

In young postnatal animals, PL fibres occurred in m. diaphragma in 5 of the 16 young Thoroughbreds and 5 of the 9 other horses (Table 2). The values for the Thoroughbreds were less than those for the others.

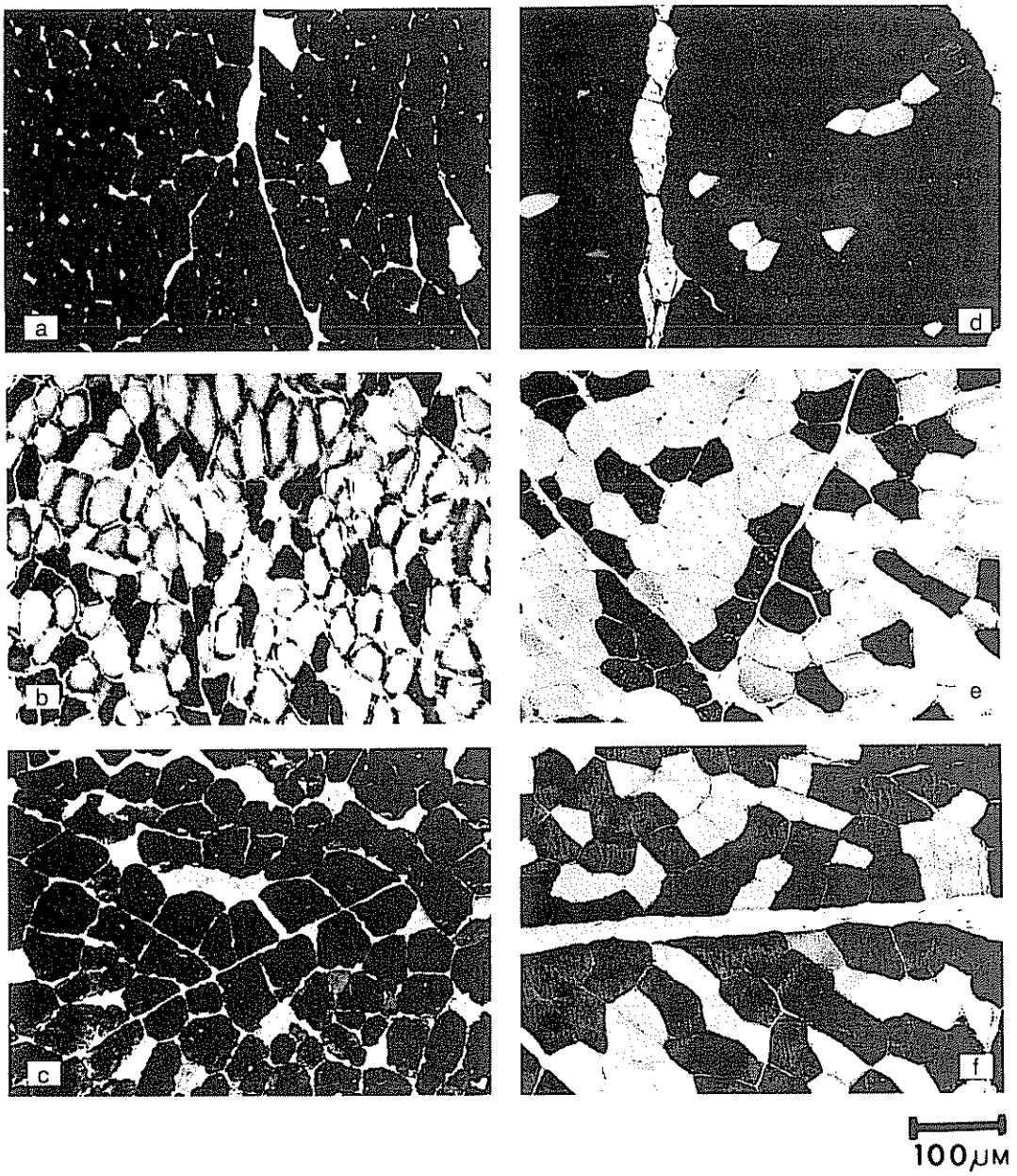
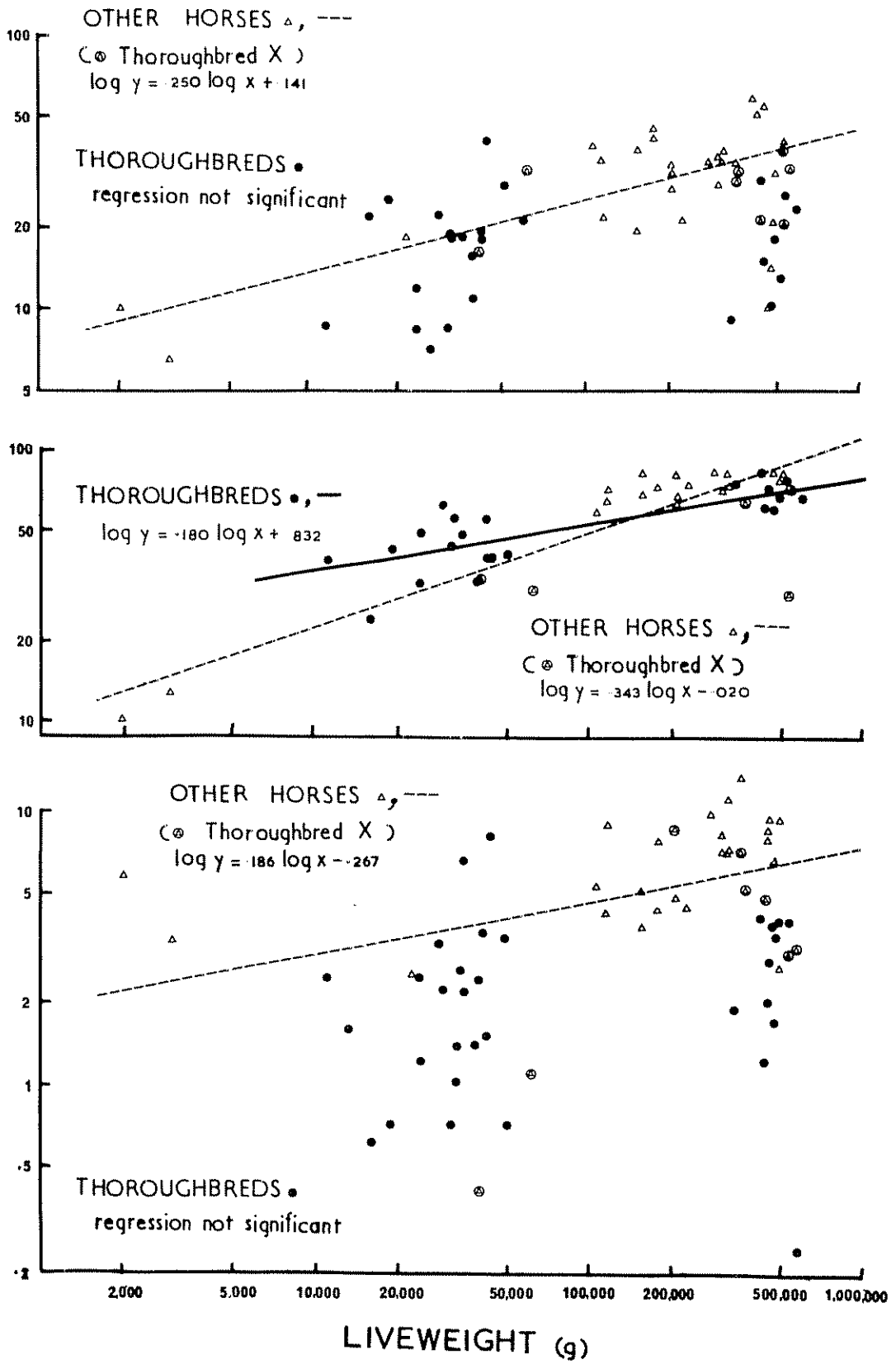


Fig 2 Fresh frozen transverse sections of muscles (*a–c*) Sections from the caudal superficial region of m. semitendinosus (*a*), m diaphragma (*b*) and m. pectoralis transversus (*c*) of a 337 kg yearling Thoroughbred female demonstrating myosin ATPase activity. (*d–f*) Sections from the caudal superficial region of m. semitendinosus (*d*), m diaphragma (*e*) and m pectoralis transversus (*f*) of 115 kg yearling female Welsh Mountain pony, demonstrating myosin ATPase activity There are fewer low reacting fibres in m semitendinosus than in m pectoralis transversus, which in turn has fewer than in m diaphragma in both horses


OTHER HORSES △, ---
(⊛ Thoroughbred X)
log y = ·250 log x + ·141
THOROUGHBREDS •
regression not significant
100
50
20
10
5
THOROUGHBREDS •, —
log y = ·180 log x + ·832
OTHER HORSES △, ---
(⊛ Thoroughbred X)
log y = ·343 log x − ·020
OTHER HORSES △, ---
(⊛ Thoroughbred X)
log y = ·186 log x − ·267
THOROUGHBREDS •
regression not significant
10
5
2
1
·5
·2
2,000
5,000
10,000
20,000
50,000
100,000
200,000
500,000
1,000,000
LIVEWEIGHT (g)

M pectoralis transversus There was an increase in the proportion of the cross sectional area of m. pectoralis transversus occupied by AL fibres with growth in the non-Thoroughbreds (Table 3, Fig. 3). The non-significant regression equation describing the scattered data for Thoroughbreds (log $y=0.063$ log $X+0.920$; $r=0.185$; $F=0.992$, with 28 degrees of freedom) suggests an increase in AL area in the Thoroughbreds which is less than that for the other horses. Although the values for Thoroughbreds were the greater, there was no significant difference in percentage numbers or areas of AL fibres between the two types of horse in the prenatal period. SL fibres were present in samples of m. pectoralis transversus of two Thoroughbreds and one of the other horses. Two other horses had PL fibres (Table 2).

Differences between muscles within each type. In all the young Thoroughbreds (Table 2) the numerical proportion of AL fibres in m. semitendinosus (mean 2.98, SD 2.03) was less ($p<0.001$) than that in m. diaphragma (mean 43.8, SD 14.9), or in m. pectoralis transversus (mean 21.1, SD 10.2; ($p<0.001$)). Similarly, the percentage area occupied by AL fibres in m. semitendinosus (mean 2.4, SD 1.87) was less ($p<0.001$) than that in m. diaphragma (mean 49.6; SD 15.6) or in m. pectoralis transversus (mean 18.1, SD 8.51). Both values for m. pectoralis transversus were significantly ($p<0.001$) greater than those for m. diaphragma.

In all the young non-Thoroughbreds the numerical proportion of AL fibres in m. semitendinosus (mean 8.8, SD 6.69) was less than that in both m. diaphragma (mean 48.9, SD 23.7; $p<0.001$) and m. pectoralis transversus (mean 33.5, SD 14.9; $p<0.01$). Similarly, the percentage area occupied by AL fibres in m. semitendinosus (mean 4.39, SD 2.65) was less ($p<0.001$) than that in m. diaphragma (mean 50.7, SD 25.0) and in m. pectoralis transversus (mean 25.0, SD 11.5). The value for the percentage AL area was significantly less ($p<0.01$) in m. pectoralis transversus than in m. diaphragma.

Fig. 3 Growth changes in the proportional area of myosin ATPase low reacting fibres in samples of (*a*) m. pectoralis transversus, (*b*) m. diaphragma and (*c*) m. semitendinosus of Thoroughbreds and other horses.

DISCUSSION

Ideally comparisons of growth changes in different types of animal should be carried out over similar ranges of liveweight and equivalent developmental periods in both types. However, in this investigation, sample availability depended on submission rates from post mortem examinations which differed considerably between the two types. Furthermore, young members of breeds of horse having a mature body weight similar to that of Thoroughbreds (e.g. draft horses) were also unavailable for examination. Nevertheless the mixed breeds used for comparison with Thoroughbreds and the inclusion of Thoroughbred cross horses with the other group, allow for identification of possible overriding features which differentiate Thoroughbreds from other breeds of horse.

Analyses in this investigation have related data with liveweight, as was done when comparing adults of each type.[12] This procedure was used because, with increasing mass during growth, functional adaptions may be necessary within skeletal muscles to cope with the greater postural and propulsive demands, gross structural adaptions with growth being theoretically inadequate in horses.[13,15]

Evidence has been presented that the myosin ATPase high reacting fibres at pH 9.5 in adult muscle correspond to fast-twich fibres and myosin ATPase low reacting fibres correspond to slow-twitch fibres.[6] The relative alkali stability of adult horse muscle, as demonstrated by histochemical methods, concurs with their having fast and slow isotypes as distinguished by immunocytochemical methods based on laboratory animal prepared immune sera.[23] However, it is recognised that during growth in laboratory animals changes in myosin isotypes may occur

within individual fibres and that these may be associated with either production of both forms of isotype, changes in patterns of synthesis associated with innervation of developing muscle or that alternatively that embryonic forms of myosin may exist.[25] As the relationship of the histochemical reaction for myosin ATPase at pH 9.5 with myosin isotypes as identified by immunological methods in developing horse muscle has not definitely been established, fibres are classified according to whether they give a high or low reaction to histochemical procedures. However, it is assumed that fibres having an alkali labile myosin ATPase reaction in foetal muscle are the presumptive alkali labile fibres of adults. The system of fibre classification used in this report has been used previously in comparing horse muscle with that of other mammals[6] and in comparing different types of adult horse.[12] Fibres designated AH.SH.PH., AH.SL.PH. and AL.SH.PH. correspond to such classifications as Type 11a, Type 11b and Type I[3]; FOG, FG and SO[20]; and FTH, FT and ST[19] respectively, used by other authors.

It appears from the findings that the differences between muscles in both the numerical proportions and in the proportions of muscle cross sectional area occupied by AL fibres are established early in life in both Thoroughbreds and other horses. However, the differences between the two types of horse, i.e. fewer AL fibre numbers and smaller proportional areas in muscles of Thoroughbreds,[12] apparently develop with growth.

Differences in fibre type proportions between muscles at the earliest stages of growth investigated in this study and in similar studies in pigs[5] and dogs[10] suggest an anticipation of proportions in adult muscles. However, anticipation of breed differences is not conspicuous in this study.

A pattern of fibre type development where individual myosin ATPase low-reacting fibres occur among a number of high-reacting fibres at early stages of development and increase in proportion during growth has been recognised in a number of species.[5,22] The timing of the increase may vary between muscles within a given animal, depending on the 'maturity' of the muscle. This pattern has been postulated as being an adaptation of skeletal muscle to meet the increased isometric and postural demands of increasing body size,[5,22] since alkali labile myosin ATPase fibres are most suitable for isometric function.[1]

The enhanced acceleration potential of the Thoroughbred may be indicated by the greater cross sectional area of its m. semitendinosus[13] and the greater development of its pectoral and femoral regions in comparison with other horses.[15] This may be related to the lesser postural adaptions of fibre types in limb muscles of Thoroughbreds as indicated by the lack of a significant increase in myosin ATPase low reacting fibres in these muscles with growth. This matter needs further investigation.

The contrasting results of investigations into changes in aerobic and anaerobic capacity of horse muscle with growth[2] may be related to the differing age range, breeds and training status of the animals investigated. The capacity of skeletal muscle to produce ATP aerobically or anaerobically with increasing body size may be influenced by a number of factors. For example, the ability of the equine circulatory system to supply oxygenated blood to skeletal muscle is constrained by the lesser rate of increase in heart weight relative to total muscle weight during growth.[16] Therefore it might be predicted that the ability of the muscular system to produce ATP aerobically would be gradually curtailed in the growing animal. Biochemical assays in material used in the present study indicated that the SDHase activity was greater in adults than in young horses.[10] An interspecies adaptation of skeletal muscle to produce ATP anaerobically with increasing body size has been reported.[6] The proportion of aerobic fibres having an aerobic capacity increases in m. semitendinosus of the horse and although quantitative extrapolations cannot be made, the intensity of

SDHase and GPase staining increases during growth in the other two muscles. Thus it is probable that both the aerobic and anaerobic capacities of horse muscle increase during growth over the age ranged investigated in spite of possible inadequacies in the circulatory system.

As a result of an investigation into fibre type proportions in human twins[18] it has been suggested[24,21] that proportions of fibre types in adult horse muscle are genetically determined. However, the probable similarity in liveweight and lifestyle of the monozygous twins was not considered.

The results of the present investigation indicate that, while differences occur in the proportion of fibre types in muscles of prenatal animals, such proportions alter during growth. This indicates that there may not be a simple genetic control over fibre type proportions in adult horses. The complexity of genetic expression in skeletal muscle is recognised.[4]

Although many factors are associated with enhanced running capacity,[14] an appreciation of the mechanism of the control of muscle fibre type proportions in adults should be of paramount interest to the breeder of potential fast running horses.

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