PRENATAL MUSCLE DEVELOPMENT

IN THE PIG

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DECLARATION

I hereby declare that this thesis embodies the results of my own work,and that it has been composed by myself.

Peter M.C.Wigmore

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I would particularly like to express my gratitude to Dr N Stickland, my supervisor, for his help and encouragement without which this project could not have been completed. John Strathearn gave a lot of help in the preparation of the thesis, particularly in the drawing of the graphs which were photographed by Kenny Thompson. Assistance was received from all the technical staff of the Anatomy Department, but particular thanks are due to Gordon Goodall and Stephen Mitchell for teaching me many of the techniques used here and for their help when animals came in. Mr Sandy Taylor of the pig unit Boghall Farm enabled the purchase of pigs of known gestational age and made available his farm records. I would also like to thank the typist, Carolyn Simpson for her patience and the speed with which she completed this thesis. The Biochemistry Department of the Veterinary Faculty kindly placed their laboratories and equipment at my disposal.

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CONTENTS

Summary

The basis for the project was a comparison of the prenatal growth of the largest and smallest pigs within litters. The differential growth rate of these two groups provides a natural experiment for determining factors important in limiting growth. The investigation concentrated on muscle growth because of its agricultural importance, but gross measurements of the fetus and its membranes were also made in an attempt to ascertain the cause of the size difference.

The material consisted of 12 gravid uteri of Large White x Landrace gilts sacrificed at approximately 10 days intervals from 38 days gestation until term, together with 2 new born litters. The project can be divided into 3 sections according to the techniques employed

1. Fetal and Placental growth

Gross measurements of fetal weight, length and position within the uterine horn were taken from each animal together with x-rays of the forelimbs of fetuses from two litters. The results showed that the small littermates, as well as being lighter, were shorter, had a lower ponderal index, had a less well ossified skeleton and tended to be found in the more crowded of the two uterine horns. All but the last characteristic is descriptive of small-for-dates babies in comparison to normal. Mean fetal weight was found to decline with increasing number in a uterine horn but the spacing between fetuses was always even. Examination of

farm records (247 pigs) showed a small (80gm.) but significant difference in the birth weights of male and female pigs.

The fresh weight, in situ length, circumference and area of all the fetal membranes were measured. All the above parameters were found to increase significantly during gestation except for placental length. This last parameter was found to show a strong position effect with the shortest placentae generally occurring in the middle region of the uterine horn. Macroscopic placental area was found to be an adequate measure of the exchange area as no difference could be demonstrated between placentae in terms of the surface enlargement due to their microscopic ridges. In general, fetal membrane weight and area correlated well with fetal weight, in contrast to placental length and circumference. The endometrium at the site of each fetus was thrown into macroscopic folds of up to 2 cm in height. These folds were found to be significantly greater in more crowded horns so that these placentae partly compensated for their shorter length.

2. Muscle histology and histochemistry

Frozen sections of the semitendinosus muscle stained with haemotoxylin and eosin were used to calculate fibre number and the mean sizes of primary and secondary fibres. Fibre number increased from 38 days until 85-90 days gestation when total fibre number was about 350,000. The time when fibre hyperplasia ceased was estimated from the time when total fibre number and the secondary/primary fibre ratio became

constant. From 60 days onwards the larger littermate had more fibres in the m. semitendinosus than its smaller sibling and the difference at birth was 17%.

Primary fibres were observed at 38 days and continued to form until 60 days. Equal numbers of primaries (about 18,000) formed in both large and small littermates. Primary fibre size increased from 38 to 70 days gestation after which these fibres declined in diameter. The initial increase in size was largely caused by the development of a region of myofibril free cytoplasm in the centre of these fibres giving them a tubular shape which was lost in the second half of gestation due to their collapse. Primaries in both large and small were of equal size at 38 days but a significantly greater maximum size was attained by primaries in large littermates (23 μ m) compared to the small (17 μ m). This size difference was due to differences in the extent of the myofibril free region, described above, which could account for 60% of the fibre diameter in large as opposed to only 30% in the small.

Secondary fibres were formed between 54 and 85 days gestation and there appeared to be no difference in the duration of hyperplasia between the large and small animal. This generation of fibres was found to be formed only on the surface of primary fibres. Secondary fibres showed little change in size until 100 days when hypertrophy started. There was a small but significant difference in their diameter between large and small (7 μ m and 6 μ m respectively for most of gestation). Comparison of the secondary/primary fibre ratio during gestation showed that the difference in fibre number mentioned above was due to fewer secondaries forming on each primary in the small as compared with the

large littermate.

A hypothesis was put forward that a difference in the available surface area of primary fibres, caused by their different diameters, had resulted in fewer secondaries forming on the surface of primary fibres in small littermates. Sections were taken of the m. semitendinosus of all 13 members of a litter of 70 days gestation. A significant correlation between fetal weight and primary fibre diameter was found, together with a significant correlation between primary fibre diameter and the secondary/primary fibre ratio.

Frozen sections of the m. semitendinosus were histochemically stained for Adenozine triphosphatase after acid preincubation. From 46 days until 102 days only primary fibres in the deep region of the muscle were stained, indicating that there were presumptive slow twitch fibres. After 102 days secondary fibres immediately adjacent to the above primary fibres also started to be stained so that by birth, characteristic bundles of slow twitch fibres were seen in the deep region.

3. Biochemistry

Assays for DNA, RNA and two fractions of protein (sacroplasmic and fibrillar) were carried out on the semitendinosus muscles of large and small littermates. All the above parameters increased during gestation but significantly lower total amounts were found in small littermates. No significant difference could however be found between large and small in the concentrations of these constituents. DNA concentration increased until 100 days after which it declined. The concentration of

nuclei (measured from sections) described a similar pattern but showed a significantly higher concentration of nuclei in small littermates. RNA concentration declined with age while protein concentration, after an initial decline, increased until term. Some of these results were discussed in terms of the histology reported in the previous section. Towards term smaller fetuses had significantly higher sacroplasmic/fibrillar protein ratios which is believed to be a sign of malnutrition. RNA/DNA ratios showed an initial decline but were constant for the rest of gestation. Protein/DNA ratios also showed a similar pattern with an initial decline followed by a constant level until it increased after 100 days. No significant difference could be demonstrated between large and small in the results for either ratio.

The conclusion of this project was that the smallest littermates were growth retarded due to prenatal malnutrition. The most important effect of this was the failure of small littermates to form as many secondary fibres as their large siblings. A theory linking this to their low primary fibre size was presented. Other factors affecting fibre number were discussed together with the effect this has on postnatal growth and performance.

General Introduction

Growth and development continue to provide one of the most interesting fields in which to work. They provide some of the most intransigent problems together with the possibility of some of the most exciting breakthroughs. To the clinician, with the conquering of so many diseases, congenital defects now form an increasing proportion of his cases. In agriculture, the object is to maximise growth rates and final size, but some agricultural animals now spend the majority of their life span in utero (Braude 1967) under the influence of factors which will determine their post-natal development. Of particular interest are the reasons behind size differences between individuals. The pig, which bears large litters provides a natural experiment whereby the growth of large and small pigs from within the same litter can be compared.

This project has concentrated on the prenatal development of muscle which, despite its obvious nutritional and commercial importance, has in some respects received little attention. This is despite the fact that it is known that fibre number and hence the potential for postnatal growth is determined before birth (Staun 1968; Stickland and Goldspink 1973). It has been shown that selection for larger animals has been selection for animals with an increased number of muscle fibres (Luff and Goldspink 1967) and the amount of meat on an animal can be correlated with the number of fibres in its muscle (Staun 1963; Stickland and Goldspink 1973). The basic mechanism of fibre formation by cell fusion has been known for some time and several excellent descriptions are available in

the literature (Kelly and Zacks 1969; Ashmore, Addis and Doerr 1973; Swatland 1973). However, there has been little quantitative work and no investigation into the origin of fibre number differences observed postnatally. Comparison of the postnatal growth of muscle in large and small porcine littermates has shown a large number of differences including differences in growth rate and final size (Widdowson 1971; Powell and Aberle 1980), DNA content (Widdowson 1971), fibre size and number (Powell and Aberle 1981, Hegarty and Allen 1978) and differences in the ratio between fibre types (Powell et al 1981). These differences constitute the major parameters of muscle growth and a comparative study of muscle development using large and small littermates should provide information on the onset and operation of factors influencing postnatal growth.

The project can be divided into three major sections according to the techniques used.

1. Gross measurements were taken of fetal and placental sizes to correlate with their position in utero. The distribution of fetal sizes within a uterine horn is claimed to show a decline in size from ovarian to cervical ends (Waldorf, Foote, Self, Chapman and Casida 1957; Perry and Rowell 1969) which if true implies an environmental cause for the size differences.

2. Histological measurements of fibre size and number were taken to provide a quantitative account of fibre development. This has not

been carried out in large and small littermates before. Histochemical staining enabled the differentiation of fibre types to be followed and this was aided by the unusual arrangement of the different muscle fibre types in the pig.

3. Assays for DNA, RNA and protein were carried out at most ages. These parameters and the ratios between them have been extensively used as a measure of growth in postnatal animals (Burleigh 1980). The biochemical parameters were also used to obtain a measure of growth independent of the histology.

To the farmer, small animals are a nuisance but by comparing their growth with that of their larger siblings some of the underlying mechanisms of growth may be revealed.

Part 1: Fetal and Placental Growth

Introduction

There have been few studies on the prenatal growth of the pig fetus and its membranes and virtually none except for Widdowson (1971) on the comparison of large and small littermates. However, extensive work has been done on human growth and the phenomenon of small-for-dates babies. The criteria used in the evaluation of these infants has proved invaluable in the interpretation of the results obtained here. The similarities between pigs and man during development and their response to environmental influences during pregnancy suggest that pigs may provide a useful model for work on human development (Cooper 1975).

At this point it would be useful to summarize what is known about reproduction in the pig so that the data presented later may be fully understood. After fertilization near the ovary, the eggs migrate by peristaltic motions of the uterine wall to distribute themselves within the uterine horns (Perry 1981). Eggs can migrate from one horn to the other via the body of the uterus and in this way grossly unbalanced pregnancies are avoided (Dziuk, Polge & Rowson 1964; Dhindsa, Dzuik & Norton 1967). Within the first week the blastocysts become 'sticky' and adhere to the uterine wall. Implantation only occurs along the side of the uterine horn attached to the mesometrial ligament (Perry 1981). No predetermined sites of implantation have been found but large numbers

of blastocysts are lost at this stage (Perry and Rowlands 1962). After attachment, the blastocysts greatly elongate by sending processes out in opposite directions up and down the uterine horn. Elongation stops when contact is made with the processes from adjacent conceptuses. The length of individual blastocysts is very variable but by the end of this period the length of the whole horn is occupied (Perry 1981). The fetal membranes then develop within the length of uterine horn occupied by the blastocyst. The placenta of the pig is described as diffuse (Steven 1975) and consists of a thin membrane of fetal tissue (the chorio-allantois) adhering to the uterine wall. In addition to macroscopic folds which follow corrugations in the endometrium, the outer surface of the chorion is covered by parallel rows of microscopic ridges (Amoroso 1952) among which are circular pits called areolae which fit over the uterine glands (Bramble 1933; Friess, Sinowatx, Skolek - Winnisem and Trautner 1981).

By mid term, when the youngest animals for this project were obtained, each fetus is enclosed within a chorionic sac, the avascular ends of which adhere to adjacent sacs (Ashdown and Marrable 1967). Each uterine horn has between 2 and 8 fetuses arranged linearly from ovary to cervix. At birth the membranes rupture and the animals pass down the horn, being expelled randomly from either horn (Marrable 1971).

As with other species which bear large litters there is considerable variation in birth weight within litters. The smallest liveborn animals

may be only one third the weight of their largest littermate (personal observation from farm records) but low birth weight correlates with poor postnatal survival (Pomeroy 1960) and about 25% of pigs die before weaning (Leman, Hurtgen and Hilly 1981). The cause of size variation within and between litters has been the subject of much speculation. Two effects which can be ascribed to the maternal environment have, however, been identified which appear to operate in all species which bear large litters. The first of these is called the position effect and describes the variation in size of fetuses within a uterine horn. In many species there is a gradient of fetal weight between different parts of the uterine horn. This results in the largest and smallest fetuses occuring in different, but characteristic locations. The other influence is the litter effect which describes the inverse relationship between the number of fetuses in a litter and their average weight. How these effects operate is unknown but nutritional differences between individuals are a possible explanation as will be discussed later.

The purpose of this section is to describe the changes during gestation in fetal and placental dimensions and to use this data in an attempt to identify the causes of the size difference between the largest and smallest fetuses used in the following sections on muscle growth.

Materials and Methods

All animals were obtained from the East of Scotland College of Agriculture farm at Easter Howgate and were gilts from a Large White, Landrace cross. The gilts were artificially inseminated when they reached between 100 and 120 kg. live weight (approximately 160 - 170 days old) and gestational age of the litter was taken from the date this took place. At the required gestational age the gilts were slaughtered at Gorgie abattoir by standard abattoir procedures and the gravid uterus removed and taken back to the R.(D.)S.V.S. for dissection.

The right and left uterine horns were identified from the orientation of the external genitalia of the gilt. The fetuses were removed through a small incision in the uterine wall adjacent to each conceptus. The umbilical cord of each fetus was ligatured before being cut at its fetal end and both fetus and membranes were labelled. The notation used in labelling took the form of a number after the letter S, which identified the gilt. This was followed by the letter R or L for the right or left uterine horn and a number giving the position of the conceptus within the horn with position one being adjacent to the ovary. The fetuses were weighed and sexed from their external genitalia immediately after removal and then wrapped in clingwrap to prevent dessication. In the younger litters, the largest and smallest fetuses were selected simply on the basis of weight while in older litters the largest and smallest males were used to avoid any possible influence of sex on growth.

The crown-rump length of each fetus was measured, as defined by Marrable and Ashdown, (1967) as a 'straight line beginning at the tail root and ending where it cuts the profile of the head after passing below the external ear'. To obtain accurate crown-rump lengths it was found necesssary to straighten the backs of fetuses against a rigid support. This corrected the tendency of fetuses to curl up, a posture which was more pronounced in smaller fetuses. Crown-rump length was not always easy to measure due to this arching of the back and neck, and similar difficulties have been noted by other authors (Ullrey, Spraghe, Becker & Miller 1965; Evans and Sack 1973; Cooper and John 1977) The crown-rump length was used to calculate the ponderal index. This is calculated from the formula ponderal index = weight/length^3 (Rohrer 1908; Lubchenco, Hansman & Boyd 1966) and is a measure of the 'leanness' or 'fatness' of an individual.

In prenatal litters, runt animals were defined as those whose weights fell more than two standard deviations below the mean litter weight (McLaren 1965). In newborn animals, any individual with a birth weight of less than 900 gms. was classified as a runt (Pomoroy 1960).

The day after the removal of the fetuses, the antimesometrial border of the uterus was opened along its length. The length of the chorionic sac of each conceptus was measured in situ as well as the length of any unoccupied space between conceptuses was also noted. The distance from the ovary to the origin of each umbilical cord on the placenta was measured on the assumption that this gave the spacing between the sites

of implantation. The distances between the fetuses were analysed both as raw data and as percentages of the total length of the uterine horn. The latter method was used to avoid the variation caused by differences in the uterine horn length in different gilts. The measurements from horns containing the same number of fetuses were combined to provide the average spacing between each site. This was done for horns containing 4, 5, 6 and 7 fetuses. A two-way analysis of variance was carried out to see whether there was any significant difference between the distances between fetuses in different parts of the uterus.

After measurement in situ, the fetal membranes were removed from the uterus, drained of their fluids and weighed before being put into 10% formalin. After fixation the chorionic sacs were cut so that they could be spread out over a flat surface to remove all macroscopic folds as described by Baur (1977) and a tracing was made of their area. The total surface area of each placenta was obtained by measuring the area of these tracings on a Reichart Videoplan image analyser. The transverse circumference of the placental sac was obtained by measuring the maximum width of the opened uterus at the site of each fetus.

From one litter (S2) pieces of the placentae of the largest and smallest fetuses were processed for wax embedding and sectioning. To obtain representative samples two regions from each placenta were taken, the first from immediately adjacent to the umbilical cord and the second

from mid way between this site and the avascular ends of the sac. Examination, under the dissecting microscope, of the external surface of the chorionic sac showed that the microscopic ridges described by Amoroso (1952) ran transversely to the long axis of the placenta and sections were cut at 90^{o} to their orientation. These sections were used to compare the enlargement of the placental area due to these ridges. This enlargement was estimated from camera lucida tracings using stereological techniques. A wareform grid was superimposed on the tracings and the number of intersections between the grid and the outline of the ridges was counted. The degree of enlargement S of the surface area due to these ridges was calculated using the equation $S = I \frac{1 \cdot 273}{36d}$ from Underwood (1970), where I is the number of intersections and d is the diameter of the hemicircles which make up the wareform grid.

In order to demonstrate the fetal position effect, fetal weights were expressed as a percentage of the total weight of fetuses in the horn. This was necessary because of the wide variations in mean fetal weight between different litters due to their different ages. Results from horns containing the same number of fetuses were then combined to provide an average relative size for each position in the horn from ovary to cervix. This was done for horns containing 4, 5, 6 and 7 fetuses.

In all, 12 prenatal litters were used. These were taken at approximately 6 day intervals from 38 until 108 days gestation. The average gestational age at parturition for this herd is 114 days. Animals from

two newborn litters were obtained within 12 hours of birth and put down with an injection of pentobarbitone. The birth weights of an additional 23 litters (all the farrowings for one month on the farm used) were obtained from the farm records.

The Edinburgh Regional Computing Centre was used to produce the polynomial regression curves of fetal weight against age using programmes developed by the Health Services Computing Facility of the University of California. The other statistics used were calculated using programmes developed by Texas Instruments.

Results

Changes in Crown-rump lengths and weight with age.

Taking all 14 litters the average number of living fetuses per litter was 9.9 with a sex ratio of exactly 1:1. Most litters contained at least one degenerating or partly resorbed fetus.

Fig. 1 shows the mean crown-rump length of each litter plotted against gestational age in days. The line through the points is a computed linear regression, the equation of which is given on the graph. The regression coefficient of the line is significant ($P < 0.001$).

Fig 2 shows the mean fetal weight for each litter plotted against gestational age in days. The line through the points is a third degree polynomial, the equation of which is given on the graph.

Fig 3 shows the weights of the largest and smallest males from each litter plotted against gestational age. Each line is plotted as a power curve, the equations of which are given on the graph. The regression coefficient for each line is significant (for both, $P < 0.001$). This graph shows that there is an approximately constant relationship between the weights of the largest and smallest male littermates with age. The smallest is always about 75% of the weight of the largest. This relationship is shown graphically in Fig. 4 where the weight of the

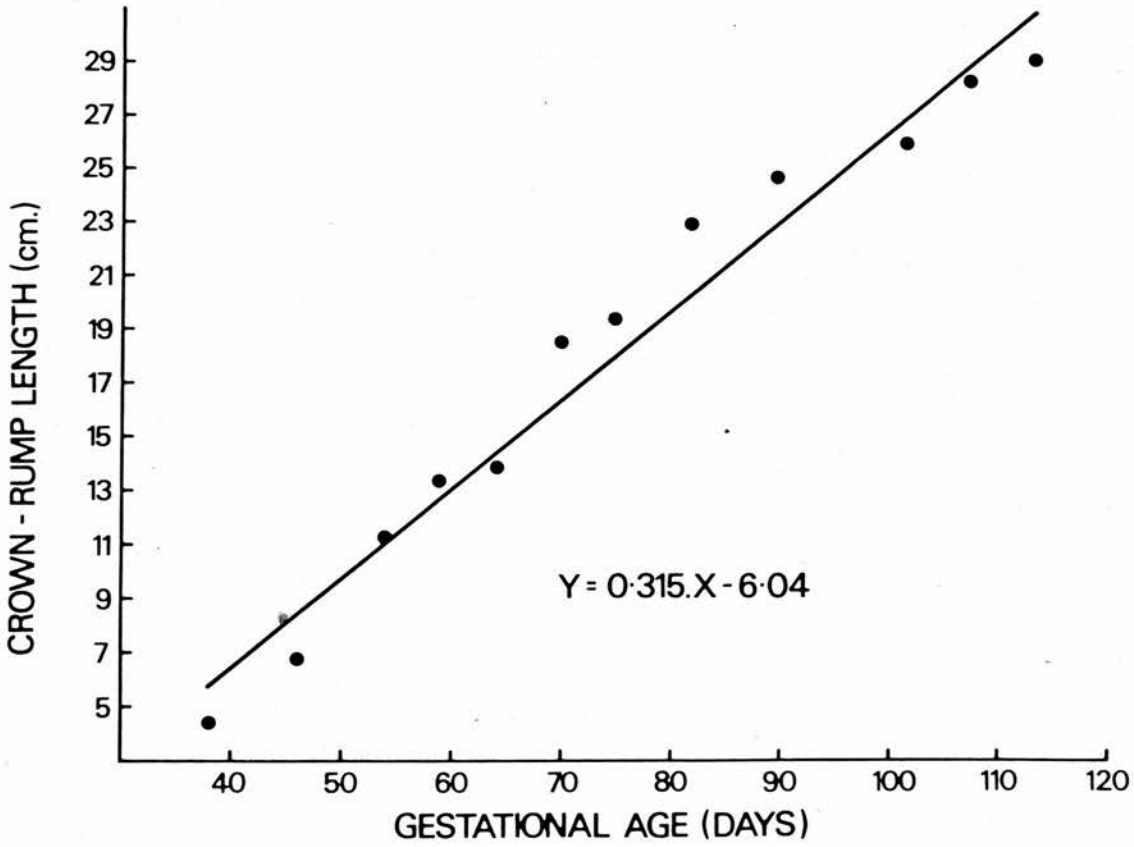


Fig.1 The mean crown - rump length of each litter plotted against gestational age.

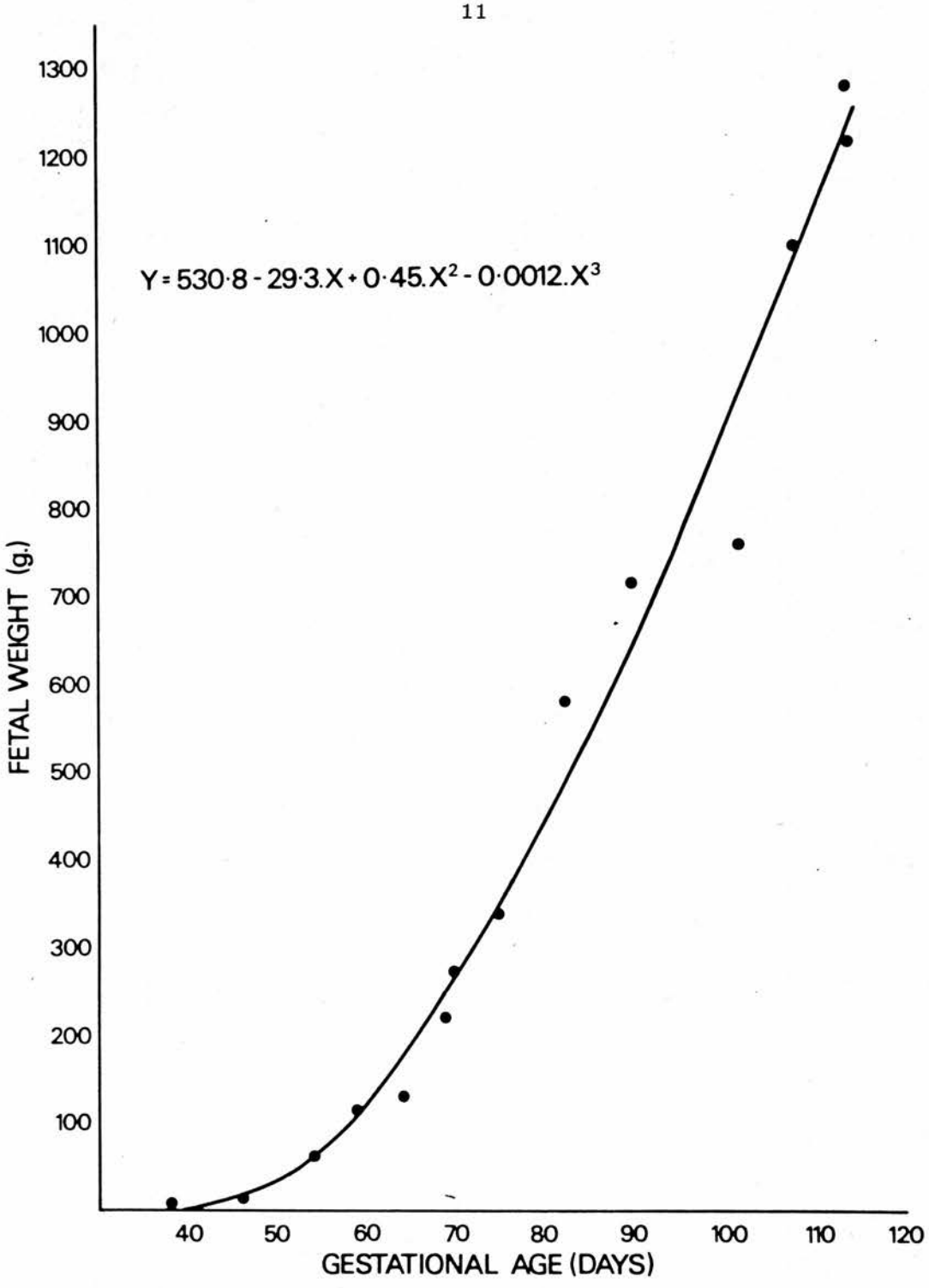


Fig.2 The mean fetal weight of each litter plotted against gestational age.

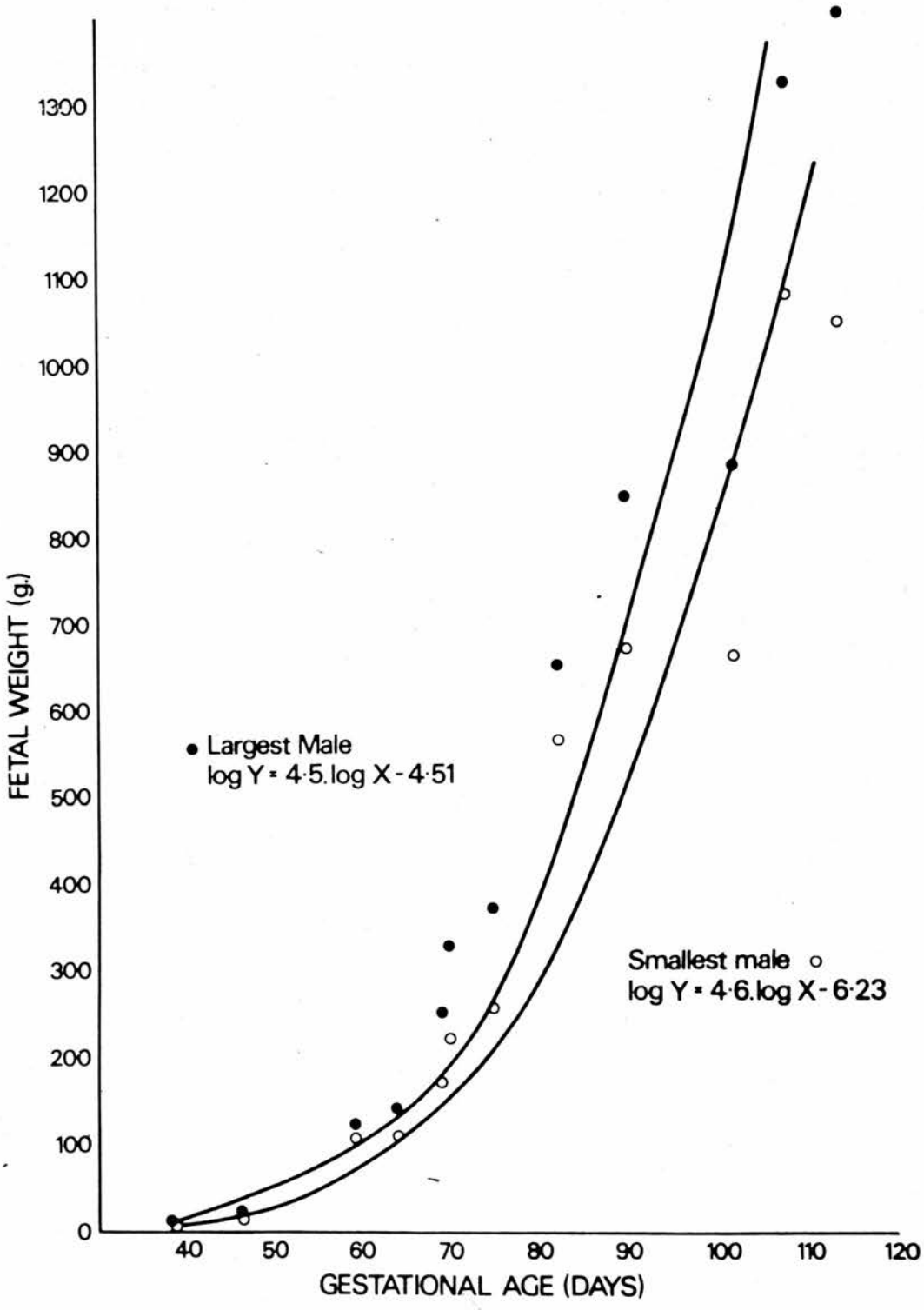


Fig.3 The weights of the largest and smallest males plotted against gestational age.

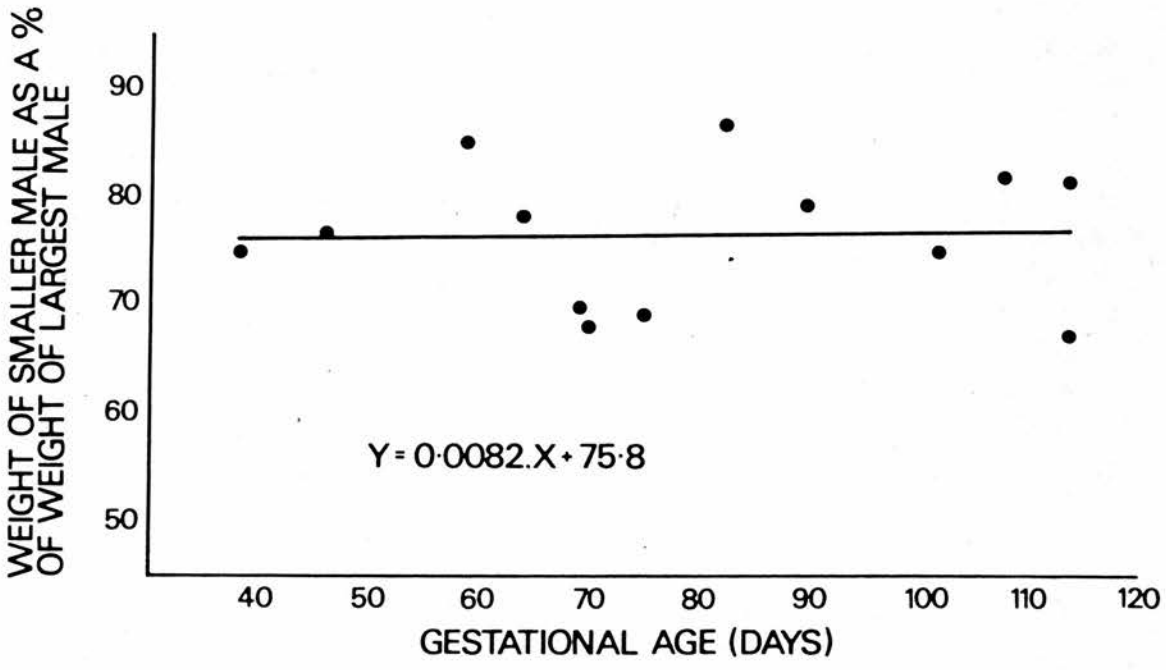


Fig.4 The weight of the smallest male expressed as a percentage of the weight of the largest male plotted against gestational age.

smallest is expressed as a percentage of the largest and is plotted against age. The line through the points is a computed linear regression, the equation of which is given in the graph. The slope of this line is not significantly different from zero ($P > 0.5$).

The occurrence of runts and the influence of sex on fetal or newborn weight

Using the definitions given in the materials and methods section, four runts were indentified from among the animals used in this project. This gives their incidence as 2.9% of live animals. The ages, weights and sexes of the four runts are given in Table 1. The position within the uterus of the three prenatal runts was identified and it was found that 2 (S3 and S5) occurred in the more crowded horn of their uterus and that all of them were found in the cervical half of the horn.

As can be seen from Table 1 all four runts were female and when the fetal or new born weights of all the litters used were examined, in 9 litters out of 13 (one litter too young to sex) the smallest animal was a female. In order to see if there was a significant difference between the weights of male and female pigs during development a paired t-test was carried out on the mean male and female weights for each litter. This showed no significant difference between the mean weights of males and females ($P > 0.05$). In order to obtain a larger sample, the farm records were used to obtain the birth weights and sexes of 23 litters which were all the farrowings at that farm in one month. This sample

Table 1 The ages, weights and sexes of statistically identified runts. Also shown are the mean weights of all fetuses (litter weight) in the litters in which they occurred.

Gestational age (days)	Mean Litter weight (g.)	Weight of runt (g.)	Sex of runt
64	132	84	Female
69	219	124	Female
102	756	553	Female
114 (new born)	1269	800	Female

contained 259 animals and a t-test was carried out on the mean male and female weight, the results of which showed a significant difference between the weights of male and female animals ($P < 0.05$) of 80gms.

Placental Measurements

As described in the materials and methods, four parameters of the fetal component of the placenta were measured. These were; fresh weight, macroscopic surface area, in situ length and transverse circumference. The first of these, placental fresh weight, is shown plotted against gestational age in Fig 5. The line through the points is a computed linear regression the equation of which is given on the graph. The regression coefficient is significant ($P < 0.005$). The line is not continued beyond 82 days gestation due to the increased variability of the points beyond this age; up until this age the placenta shows an increase in weight with age.

Fig. 6 shows the mean macroscopic placental area of each litter plotted against gestational age. The line through the points is a computed power curve, the equation of which is given on the graph. The regression coefficient is significant ($P < 0.01$)

One reason for measuring placental area was to examine the relationship between fetal weight and this parameter within litters. To do this, it was important to know whether differences in macroscopic placental area

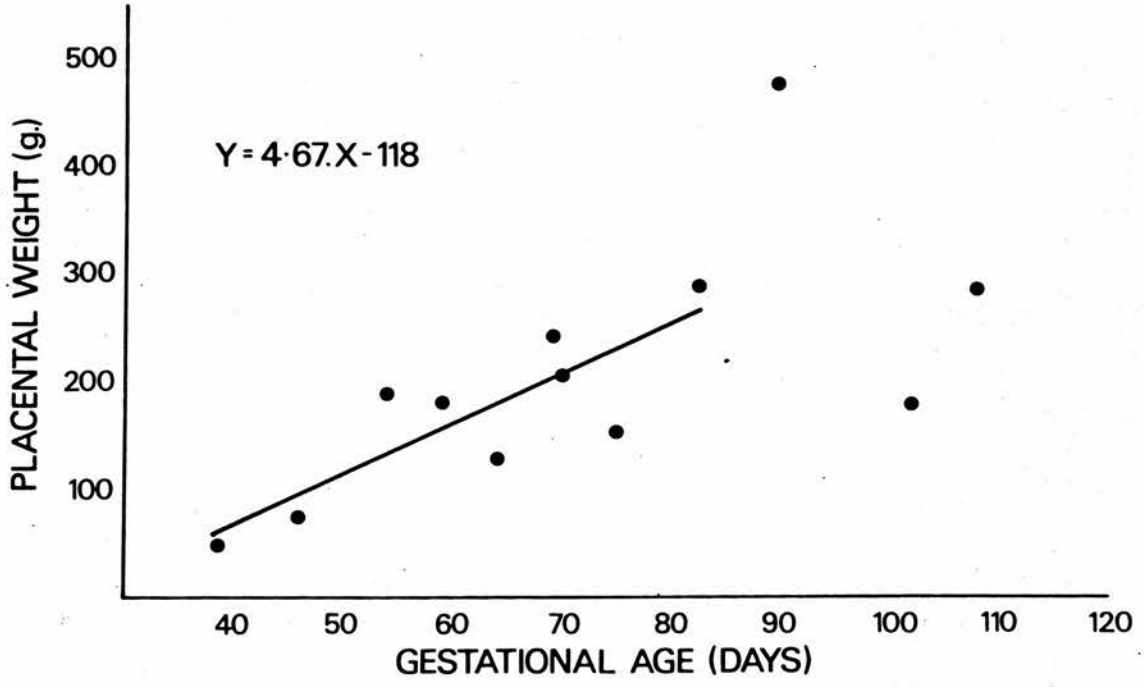


Fig.5 The mean feto – placental weight of each litter plotted against gestational age.

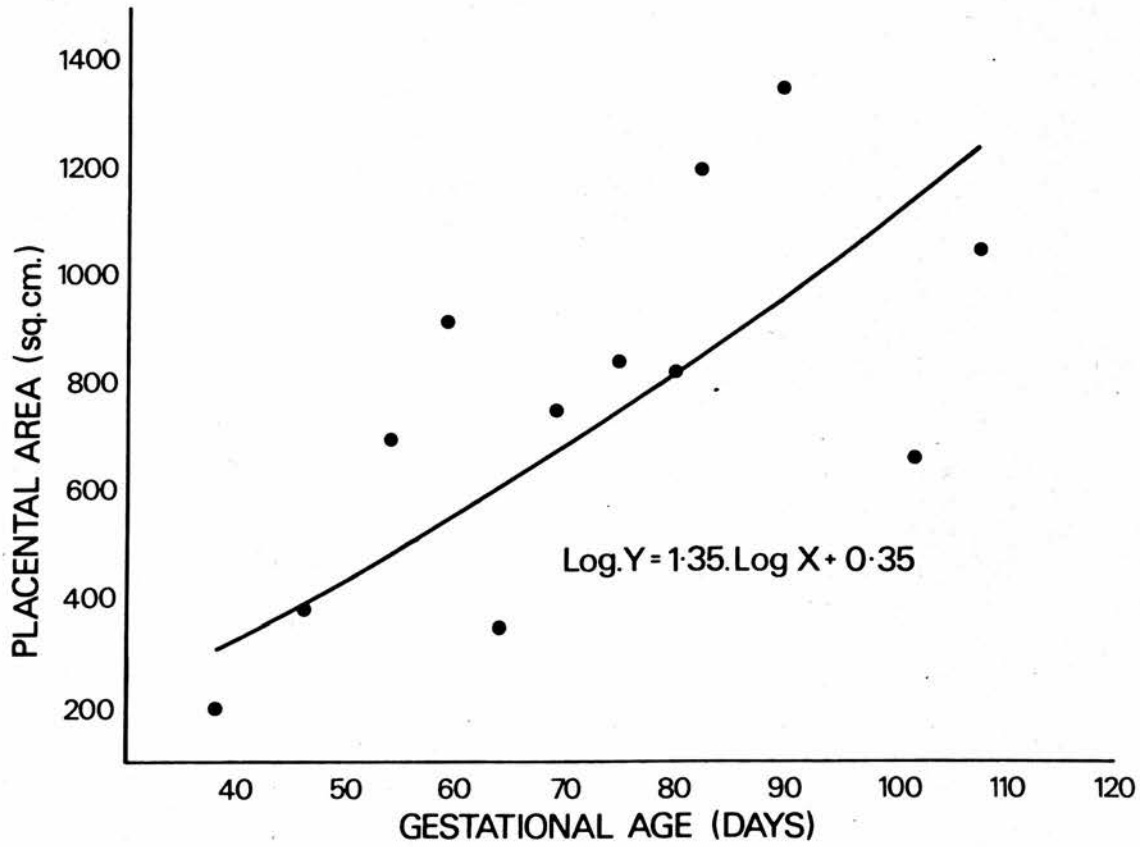


Fig.6 The mean placental area of each litter plotted against gestational age.

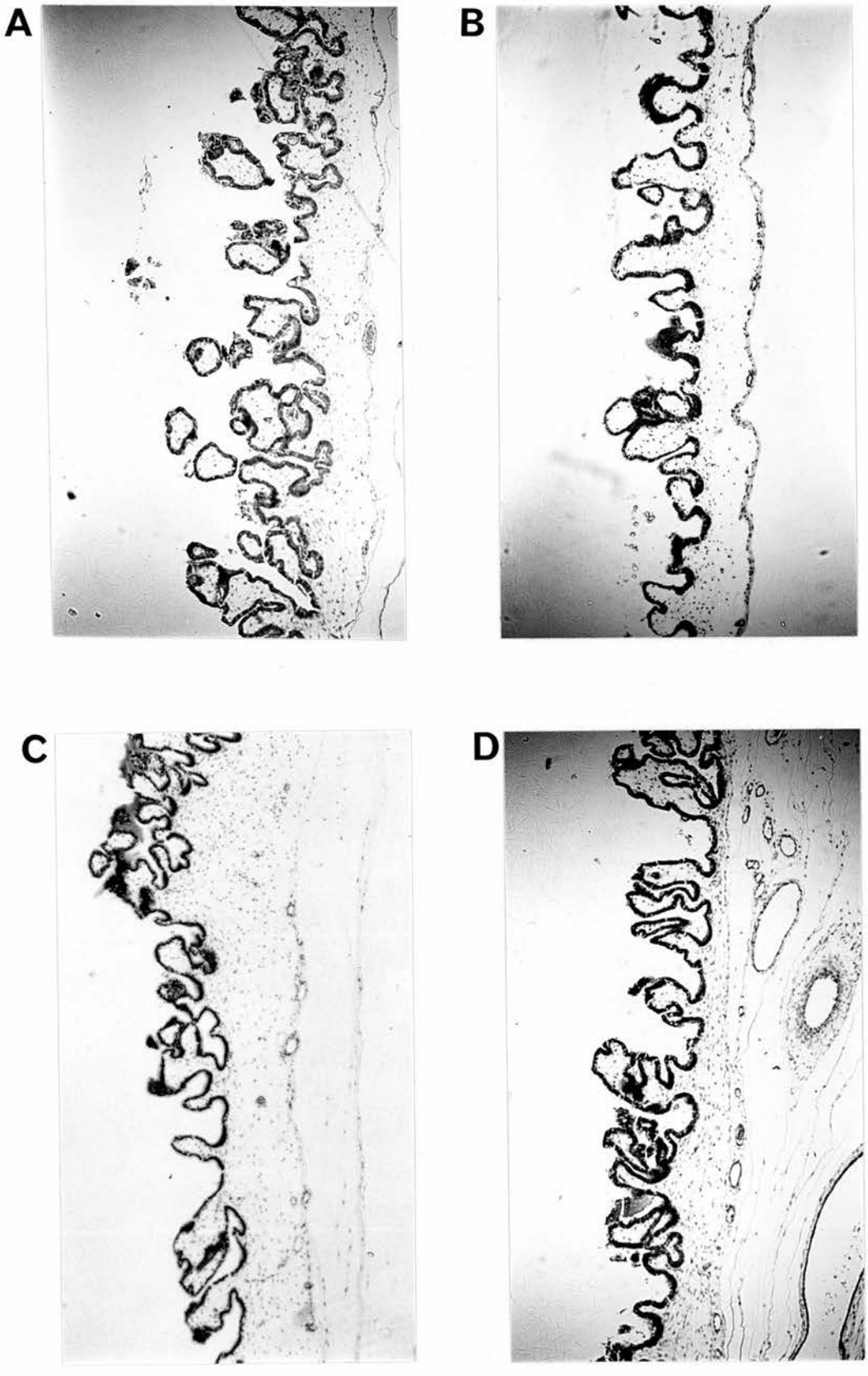
adequately described differences between placentae or whether they also differed in their microscopic structure. In the pig, placental surface area is increased by microscopic ridges which run parallel to one another across the long axis of the placenta. The degree of surface enlargement effected by these ridges was investigated in the placentae from the largest and smallest fetuses from one litter of 90 days gestation. As described in the materials and methods, wax sections were cut from two regions of the placentae, adjacent to the insertion of the umbilical cord and mid way between this region and the avascular ends of the placental sac. The sections were cut transversely across the ridges. Fig 7 shows photographs of these sections stained with haemotoxylin and eosin. Using the stereological techniques described in the materials and methods the surface enlargement of each region from both placentae was estimated from 10 positions on each section and the mean results are shown below in Table 2.

Table 2. The surface enlargement Sv produced by the microscopic ridges in the placentae from the largest and smallest fetuses from one litter. The surface enlargement from 2 regions of each placenta is shown.

Region	largest fetus	smallest fetus
Adjacent to the umbilical cord	6.8	7.7
Mid way between umbilical cord and avascular ends	4.1	4.6

Fig. 7 Sections of the placentae of two littermates of 90 days gestation. A and B are sections from the smallest fetus and C and D are from the largest fetus. The external surface of the placenta which was in contact with the uterus is on the left hand side of each section. Sections A and C were taken from regions adjacent to the umbilicus while those in B and D were taken from mid-way between the umbilicus and one of the avascular ends of the placenta.

Scale bar = 150 μm


A
B
C
D

A t-test showed that there was no significant difference in the surface enlargement between the largest and smallest, either in the umbilical region ($P \succ 0.4$) or mid way between this and the avascular ends ($P \succ 0.1$). There was however a significant difference ($P \prec 0.005$) between the 2 regions within the placenta from the largest fetus.

Fig. 8 shows the mean in situ placental length of each litter plotted against gestational age. The line through the points is a computed linear regression the equation of which is given on the graph. The shape of this line is not significantly different from zero ($P \succ 0.05$).

Fig. 9 shows the mean placental circumference for each litter plotted against gestational age. The line through the points is a power curve the equation of which is given on the graph. Placental circumference appears to increase with age although the rate of increase declines later in gestation. The slope of the line is significant ($P \prec 0.001$).

Spacing of fetuses within the uterine horn

As described in the materials and methods, the spacing between the implantation sites within a uterine horn was determined by measuring the distances between the placental origins of the umbilical cords of adjacent fetuses. The results were analysed by the method of least squares for horns containing 4, 5, 6 and 7 fetuses. No significant differences could be demonstrated in the spacing of fetuses in different parts of the horn either with the raw data or when expressed as a

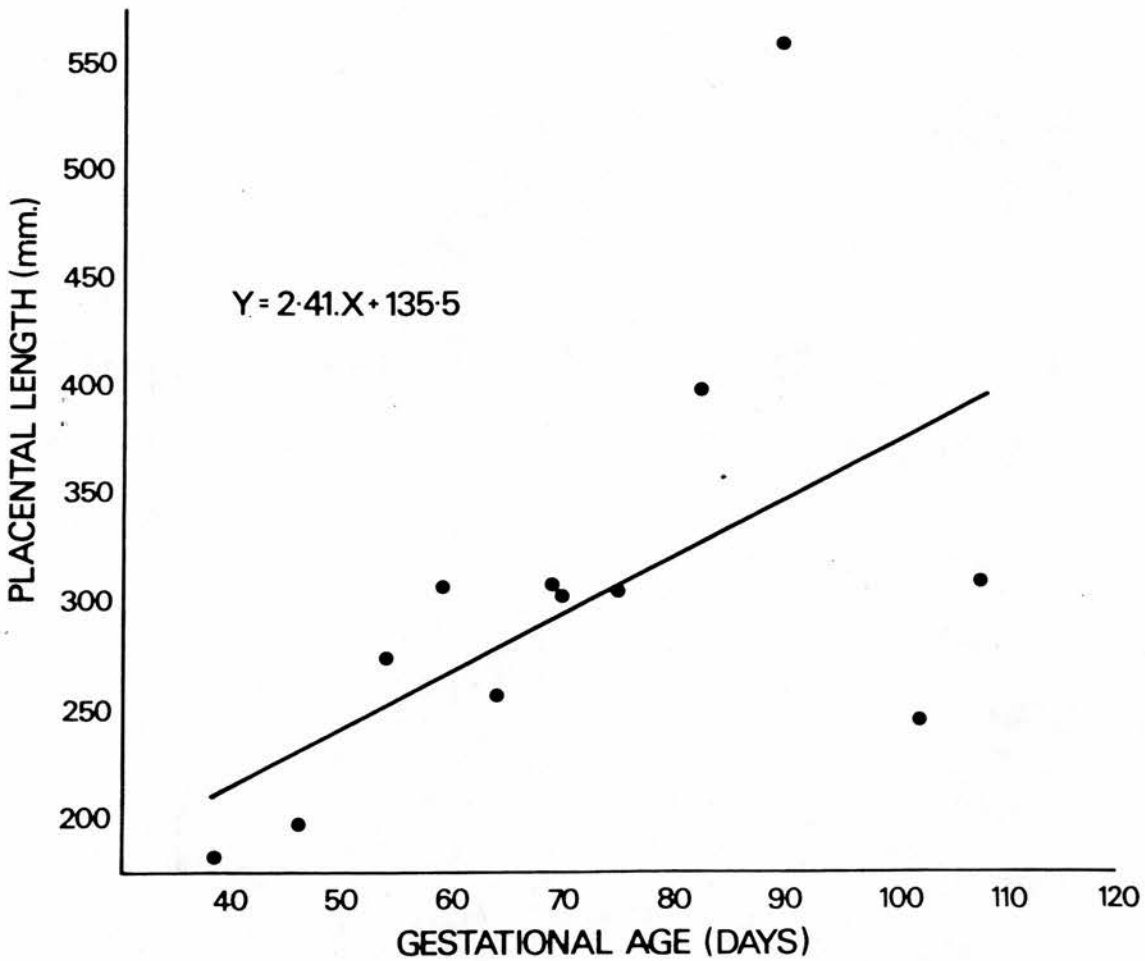


Fig.8 The mean in situ placental length of each litter plotted against gestational age.

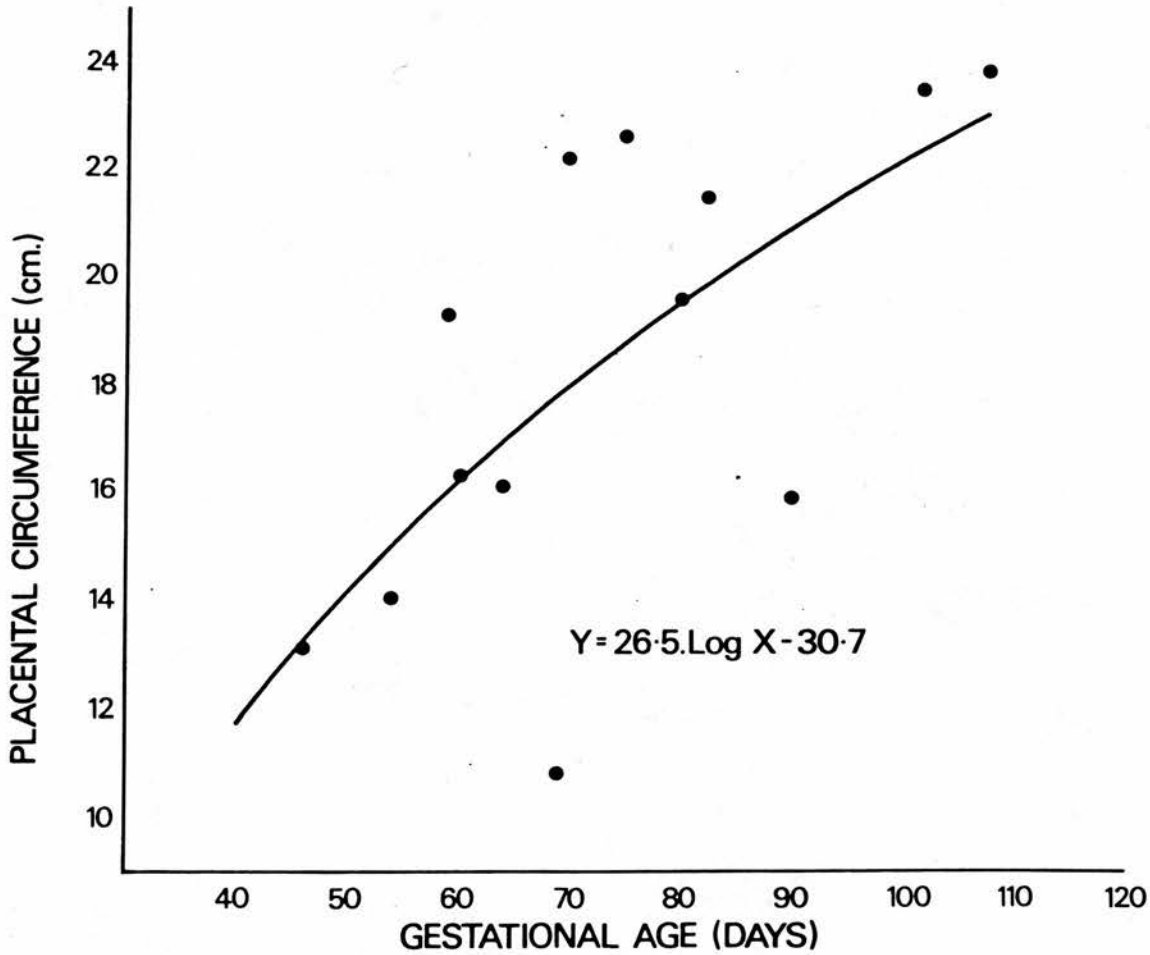


Fig.9 The mean placental circumference of each litter plotted against gestational age.

percentage of the horn length.

The effect of the number of fetuses in a uterine horn on the mean fetal weight

Owing to the effect of age on fetal weight, the effect of litter size on mean fetal weight could not be compared between the litters used in this project. Instead, the mean fetal weight from right and left horns of uteri containing unequal numbers of fetuses in each horn was used. These results in Table 3 show that, in all but one litter, the more crowded horn has a lower mean fetal weight.

The effect of the number of fetuses in a uterine horn on the mean placental length

As previously shown in Fig. 8, placental length does not significantly increase with age and so the results from different ages can be plotted on the same graph. Fig 10 shows the mean placental length of each litter plotted against the number of fetuses in the horn. The equation of the line is given on the graph and the linear regression coefficient is significant ($P < 0.005$). The graph shows a clear litter effect in that the average placental length is inversely proportional to the number of fetuses in the horn.

Table 3 The effect of the number of fetuses in a horn on the mean fetal weight in that horn.

Gestational age (days)	Right or left horn	No. in horn	Mean fetal weight
54	R	2	55.1
	L	1	79.2
64	R	8	121
	L	5	149
38	R	6	8.44
	L	8	7.69
69	R	5	228
	L	7	213
46	R	7	18.6
	L	6	19.2
59	R	4	111
	L	2	125
82	R	3	595
	L	5	573
102	R	7	762
	L	6	749
70	R	6	279
	L	7	269
114	R	2	1255
	L	4	1013

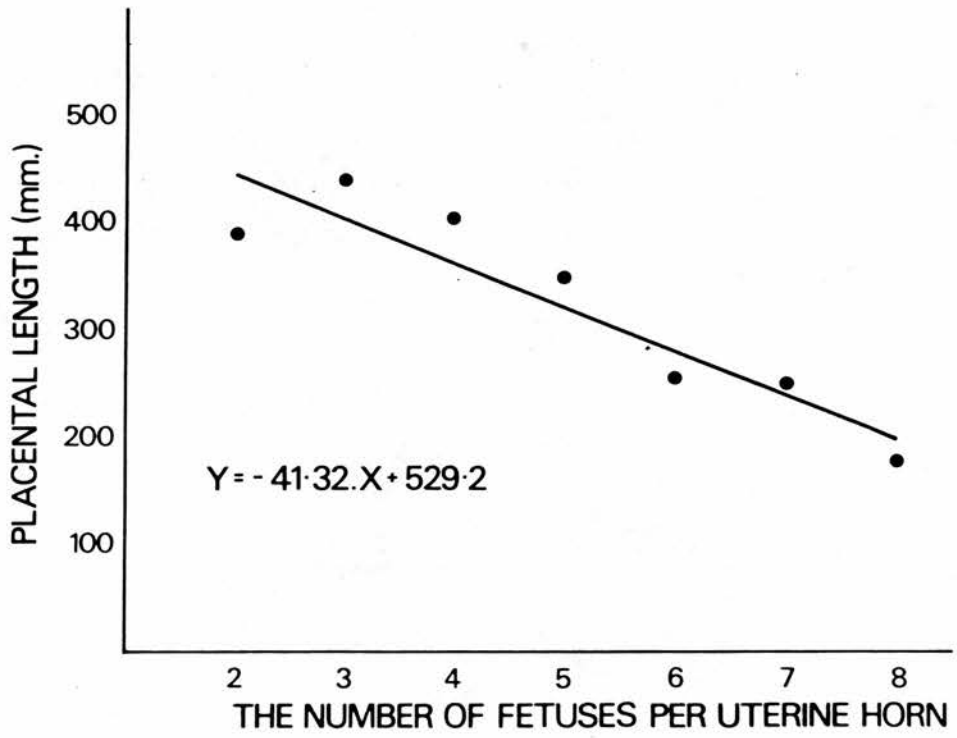


Fig.10 The mean placental length within a horn plotted against the number of fetuses present.

The relationship between the relative fetal weight and fetal position within the horn

The relative weights of fetuses in each position in horns containing 4, 5, 6 and 7 fetuses are shown in Fig 11. The positions of fetuses are shown evenly spaced in accordance with the previous results on spacing. No pronounced position effect is apparent.

The relationship between the relative placental length and fetal position within the horn

In Fig. 12 the percentage of the length of the uterine horn occupied by placentae in different position is shown for horns containing 4, 5, 6 and 7 fetuses. Each position is shown equidistant from its neighbours irrespective of the number of fetuses in the horn, as found previously. A much stronger position effect is apparent in these results than in those for fetal weight. Horns containing all numbers of fetuses show a characteristic U shaped distribution. The largest placenta occupies the most ovarian position and the shortest placenta occupies the penultimate position at the ovarian end for horns containing 4 or 5 fetuses or the penultimate position at the cervical end for horns containing 6 or 7 fetuses.

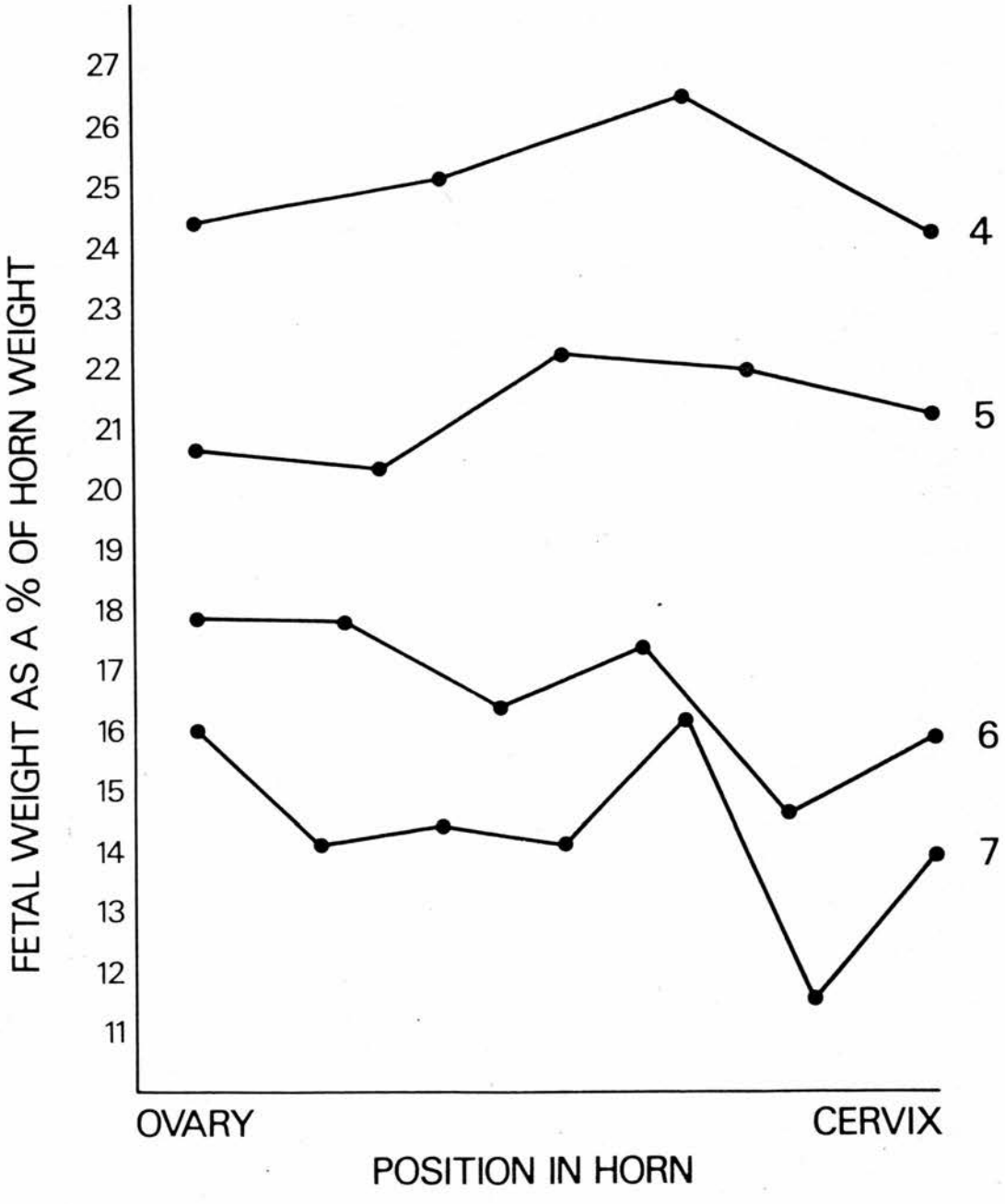


Fig.11 Fetal weight expressed as a percentage of the total weight of fetuses in a horn plotted against position within the horn for different numbers of fetuses.

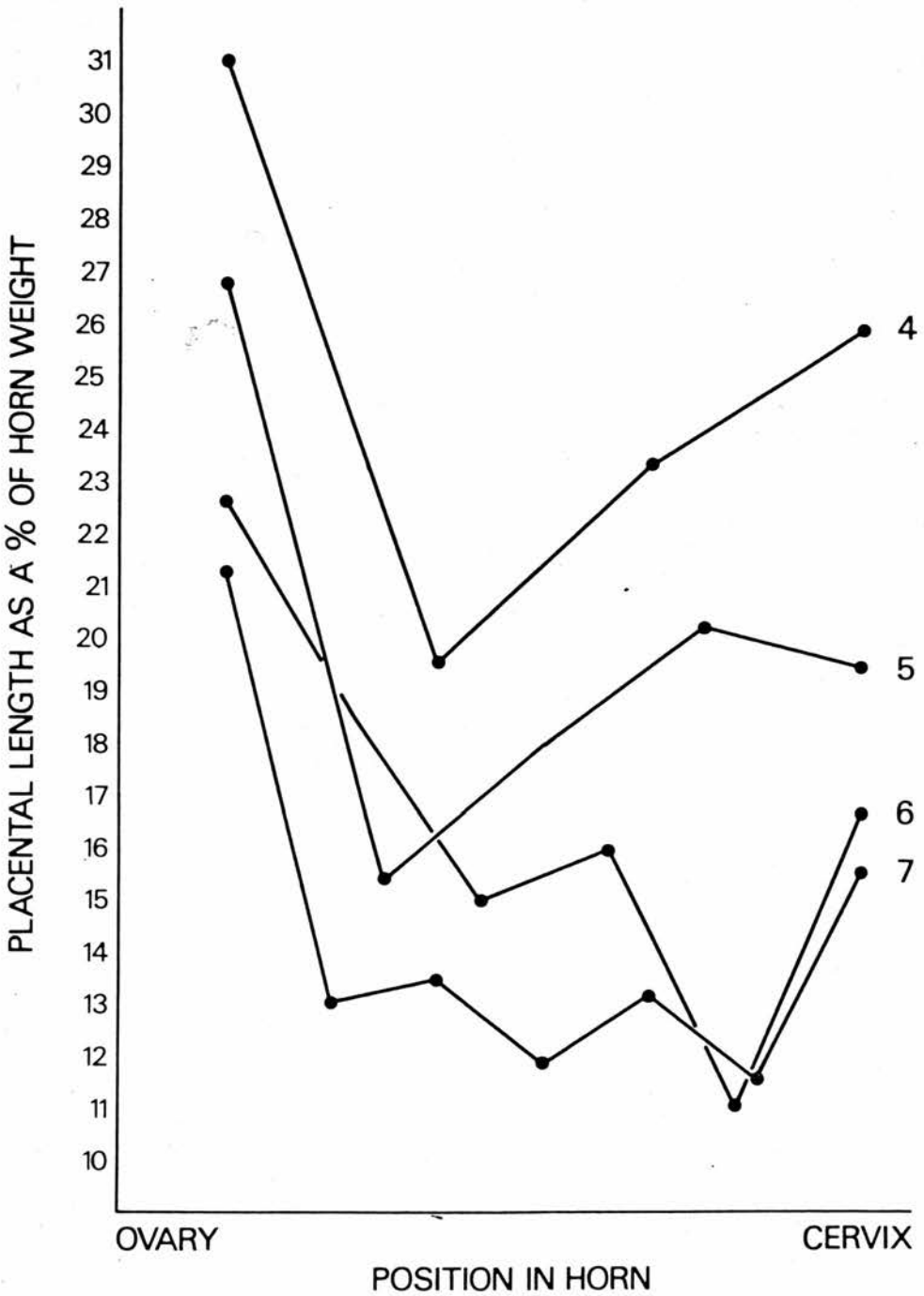


Fig.12 Placental length expressed as a percentage of the horn length plotted against position within the horn for different numbers of fetuses.

Table 4 Correlation coefficients (r) between placental parameters and fetal weights for each litter. * indicates significant correlation with fetal weight ($P < 0.05$). ** indicates a significant correlation with fetal weight ($P < 0.01$). N.S. indicates no significant correlation.

Gestational age (days)	No. of fetuses in litter	Placental weight r	Placental area r	Placental length r	Placental circumference r
38	14	0.662 **	0.711 **	0.541 *	
46	13	0.635 *	0.821 **	0.498 N.S.	0.147 N.S.
54	3	0.999 *	1.000 **		0.928 N.S.
59	6	0.308 N.S.	0.689 N.S.	0.400 N.S.	0.328 N.S.
64	13	-0.045 N.S.	0.405 N.S.	0.546 N.S.	0.572 *
69	12	0.119 N.S.	0.709 **	0.618 *	0.038 N.S.
70	13	0.744 **		0.674 *	0.646 *
75	7	0.826 *	0.823 *	0.372 N.S.	0.273 N.S.
82	8	0.753 *	0.787 *	0.370 N.S.	-0.106 N.S.
90	8	0.494 N.S.	0.739 *	0.281 N.S.	-0.143 N.S.
102	13	0.777 **	0.587 *	0.474 N.S.	-0.740 *
108	6	0.826 *	0.905 *	0.645 N.S.	0.799 N.S.

The relationship between placental parameters and fetal weight

Table 4 shows the correlation coefficients between fetal weight and the weight, area, length and circumference of placentae for each litter. The most consistent and highest correlations are those between fetal weight and placental area. Placental weight shows reasonable correlation but placental length and circumference in general correlate poorly with fetal weight.

The increase in placental area by folds in the uterine lining

The above results which show that fetal weight does not correlate with either placental length or circumference leave unexplained the good correlation between fetal weight and placental area. Examination of the inner surface of pregnant uteri showed that, at the site of each conceptus, large transverse folds run across the uterine horn. Fig. 13 shows a photograph of the internal lining of a pregnant uterus at 64 days gestation. The area shown is that occupied by a single conceptus and the maximum height of the folds is between 1 and 2 cms. In order to quantify the degree of folding, the ratio between placental area and placental length was calculated for each fetus.

Fig. 14 shows photographs of two sections of the uterine wall from the same uterus (S14, 108 days gestation). Each section has been cut from the centre of a region occupied by a fetus and close to the attachment

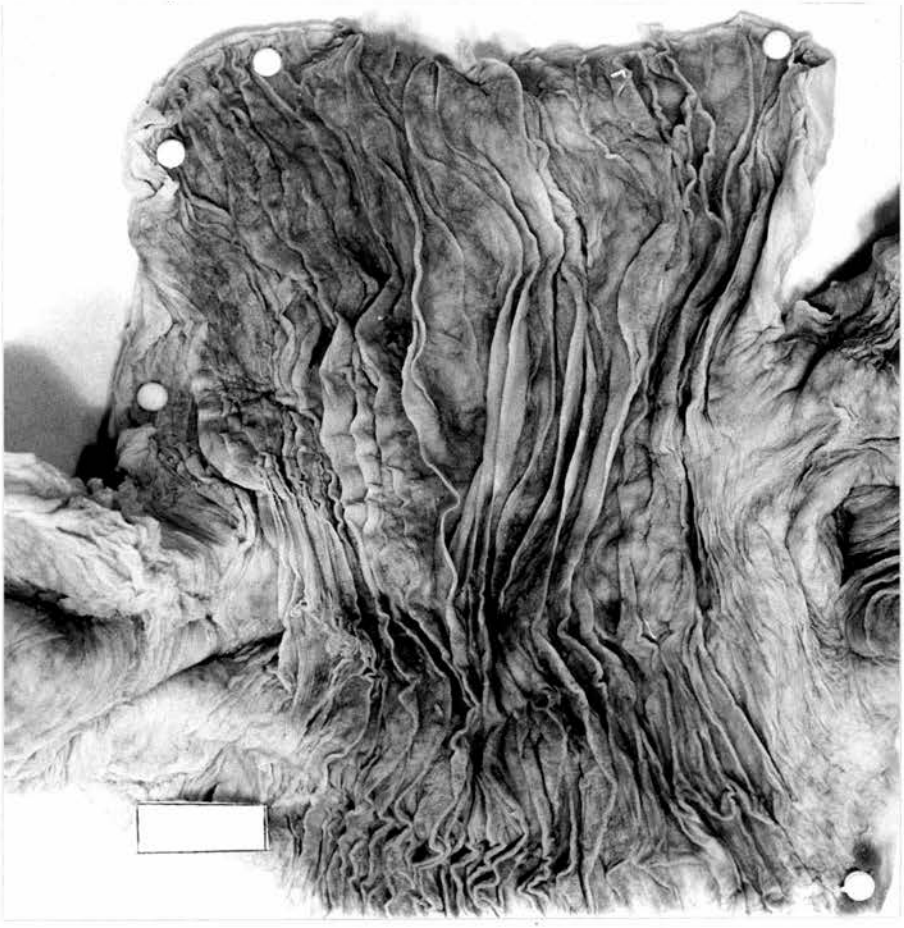


Fig.13 The inside lining of a pregnant uterus at the site of one conceptus with a gestational age of 64 days.Many deep folds can be seen running across the horn.
Scale bar = 2cm.

A

B

Fig.14 Sections of the uterine wall from the implantation sites of two conceptuses from the same litter. Both sections are cut at right angles to the transverse folds shown in Fig.13. The surface in contact with the placenta is uppermost in both sections. The placenta in contact with A. had an area/length ratio of 4·71 while that in contact with B. had a ratio of 2·48. The scale bars = 2 cm.

Table 5 The mean area/length ratio for the placenta from each horn of those uteri with unequal numbers of fetuses in each horn. Also shown is the mean placental area for fetuses in each horn.

Gestational age (days)	No. of fetuses in horn	Mean placental area/length ratio	Mean placental surface area (sq. cm.)
38	6	1.15	236.3
	8	1.09	177.2
46	6	1.93	407.5
	7	2.06	357.7
59	2	3.06	1052
	4	3.06	850.2
64	5	1.05	380
	8	1.83	320.3
69	5	2.24	744
	7	2.55	757
82	3	2.74	1174
	5	3.29	1224
102	6	2.45	599.5
	7	3.13	714.1
108	2	2.64	1125
	4	4.05	1012

of the mesometrial ligament. The area/length ratio of the placenta shown in the upper section is 4.71 and that for the lower section is 2.48. This photograph confirms the greater folding in those placentae with a higher area/length ratio than in those with a lower ratio.

The mean ratio for all the conceptuses in a horn was calculated for uteri containing unequal numbers of fetuses in each horn. Table 5 shows these results. In 6 of the 8 litters the placentae in the more crowded horn have a higher mean area/length ratio. A paired t-test on all 8 pairs of results showed a significant difference between them ($P < 0.005$). These results show that placentae in more crowded horns, which are shorter than those in less crowded horns (see Fig. 10), compensate by increasing their degree of folding and so increase their surface area.

Comparison of the largest and smallest littermates selected for muscle analysis

One reason for collecting the data presented in this section was to compare the results from the largest and smallest littermates used in the following sections to see if the cause of the size variation could be established. Table 6 shows the measured parameters for the largest and smallest animals from each litter. The only two parameters in the table which have not been discussed previously are the ponderal index and the placental weight/fetal weight ratio. The former, which is defined in the materials and methods, is a measure of the 'leanness' or 'fatness' of an individual, with lower values indicating a 'lean' animal.

Table 6 Comparison of the largest and smallest littermates from each litter.

Gestational age (days)	Large or small	Crown rump Length (cm.)	weight (g.)	Ponderal index	Placental area (sq. cm.)	Placental wt. fetal wt. ratio
38	Large	4.6	9.70	9.97	249	5.36
	Small	3.9	5.04	8.50	84	5.56
46	Large	6.8	21.2	6.75	431	3.78
	Small	6.8	16.3	5.18	287	4.79
54	Large	11.9	79.2	4.70	814	3.22
	Small	9.0	31.5	4.33	462	1.43
59	Large	14.0	125	4.56	948	1.38
	Small	13.2	107	4.65	951	1.68
64	Large	15.3	163	4.55	483	1.08
	Small	12.2	84	4.63	175	1.95
69	Large	17.6	253	4.73	946	1.15
	Small	14.5	124	4.07	641	2.39
70	Large	19.2	328	4.63		0.945
	Small	16.6	223	4.57		0.605
75	Large	20.3	398	4.76	1103	0.678
	Small	17.6	257	4.71	604	0.525
82	Large	23.1	650	5.27	1323	0.480
	Small	21.7	495	4.84	861	0.311
90	Large	24.0	845	6.11	1573	0.669
	Small	23.2	630	5.05	912	0.587
102	Large	26.2	878	4.88	902	0.236
	Small	24.5	660	4.49	713	0.240
108	Large	28.9	1315	5.45	1117	0.236
	Small	27.2	1075	5.34	957	0.251
114	Large	28.8	1326	5.55		
	Small	27.9	1080	4.97		
114	Large	29.0	1465	6.01		
	Small	26.9	1006	5.17		

The placental weight/fetal weight ratio was calculated to test whether smaller animals had a relatively smaller placenta than their larger siblings. In summary, the results in Table 6 show that the smaller animal is lighter in weight, shorter in crown-rump length, has a lower ponderal index and has a smaller placental area than its larger littermate. The only parameter which does not show a consistent difference between the largest and smallest is the placental weight/fetal weight ratio. The significance of these differences was tested using paired t-tests. These showed that there was a highly significant difference ($P < 0.001$) between large and small animals for crown-rump lengths, fetal weight and placental area and a significant difference ($P < 0.005$) for the ponderal index. The only parameter which was not significantly different between large and small littermates ($P > 0.5$) was the placental weight/fetal weight ratio.

In addition to the above parameters radiographs were taken of the right fore limbs of the largest and smallest littermates from two litters. This was done to provide information on the growth of hard tissues to compare with the soft tissue growth analysed in the following sections and as an additional measure of growth. Fig. 15 shows photographs contact printed from the radiographs. These pictures show that the carpal bones in the smaller animal are less well ossified, being smaller than those in the larger.

The position within the uterus of the largest and smallest fetus were recorded because, if either tended to be found in particular locations,

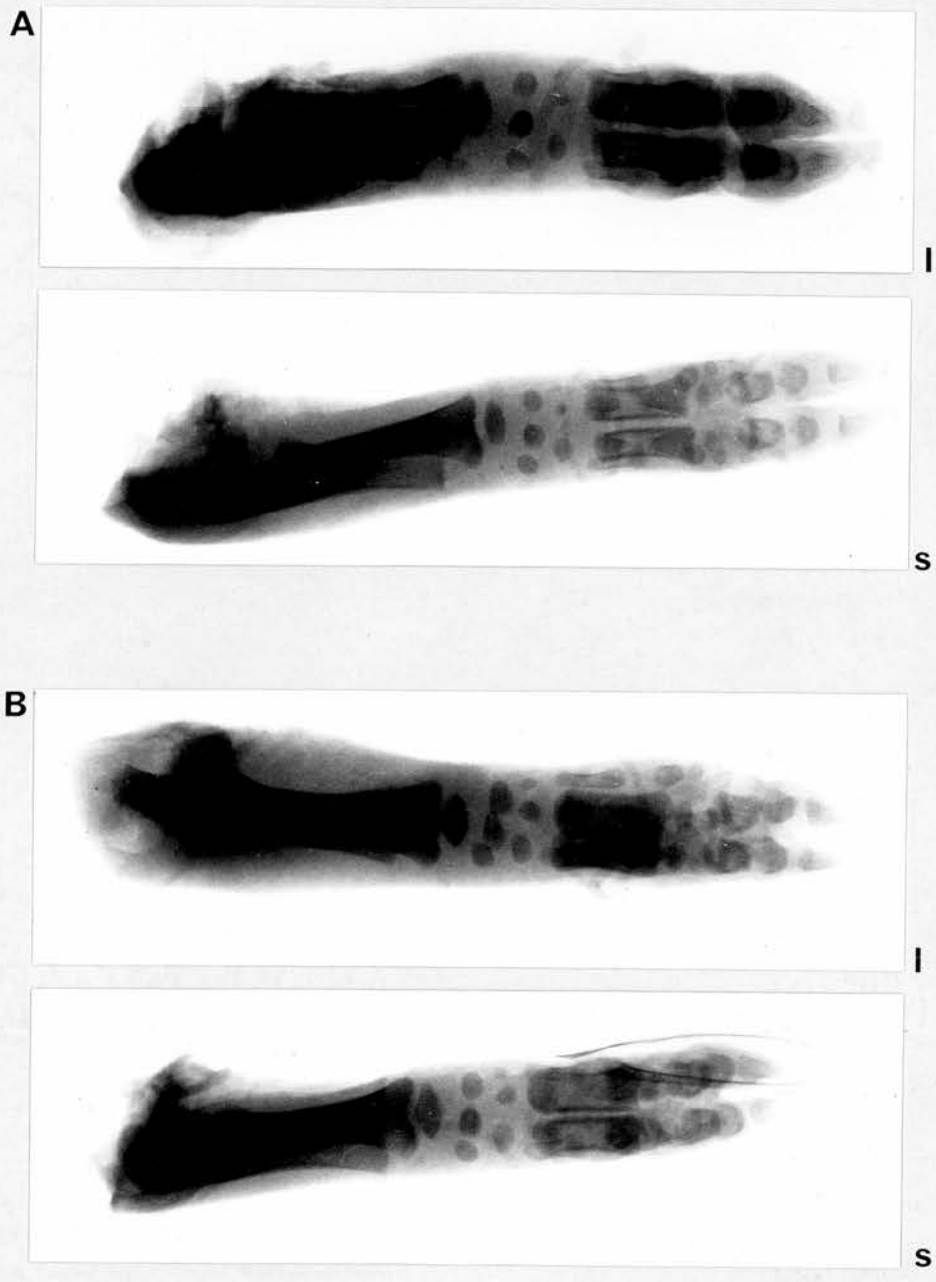


Fig.15 Contact prints from radiographs of the right forelimbs of four fetuses.Those in A.are the largest and smallest fetuses from a litter of 108 days gestation and those in B. are the same from a litter at birth.
l = largest s = smallest

this would be strong evidence for an environmental cause of the size difference. In the event, 5 of the largest fetuses and 2 of the smallest were found in the ovarian half of their uterine horns. Where the uterine horns contained unequal numbers of fetuses, 7 of the largest occurred in the less crowded horn compared with 2 of the smallest.

Discussion

Changes in Crown-rump length and fetal weight with age

The litter sizes and sex ratio found in the animals of this project are within the normal range described by Mount and Ingram (1973). The graph of crown-rump lengths against age (Fig. 1) is very similar to those shown by previous authors, (Ullrey, Spraque, Becker & Miller 1965; Marrable & Ashdown 1967; Evans & Sack 1973). These papers show a very slightly S shaped curve but over the period 38 to 114 days gestation covered by this project, the line is negligibly different from a linear regression. This graph was used to age one litter (S5) of unknown gestational age.

Fig. 2 shows the fetal weight increase with age with a polynomial regression line fitted to the points. This line is compared to the lines produced by Pomeroy (1960) and Ashdown and Marrable (1967) in Fig. 16. The equations used by these authors are given on the graph. All three lines are very close but both previous authors show a higher birth weight than was found in this project. This is possibly because sows rather than gilts were used by these authors. No test of the significance of the differences between the lines was possible due to the absence of the original data. Comparison of the weights of the largest and smallest males from each litter (Fig. 3 and 1) showed that there is a relatively constant relationship between their weights. The smallest is always

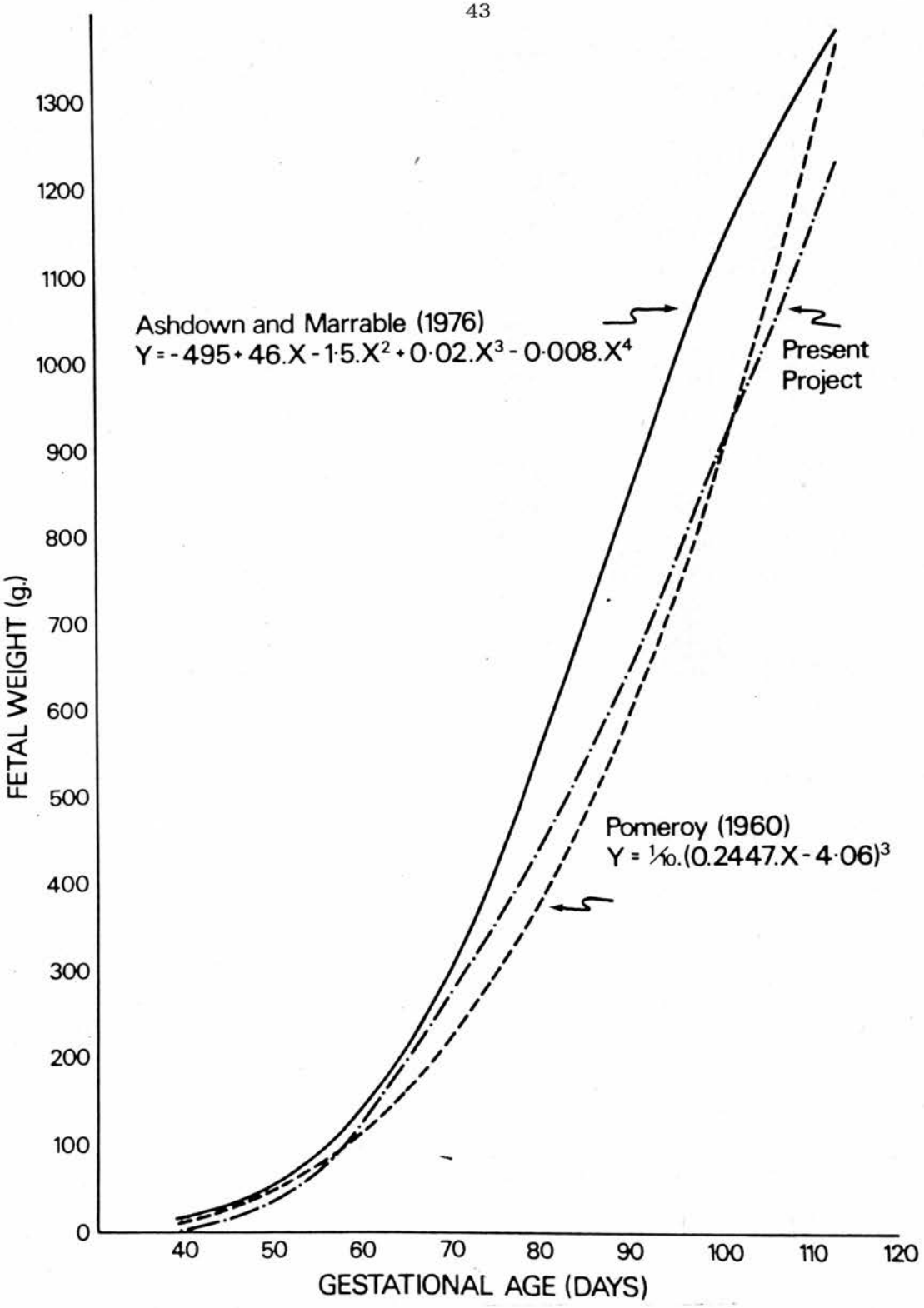


Fig.16 Fetal weight plotted against age. The line produced in the present project is compared with those of previous authors.

about 25% lighter than the largest. This result contrasts with the implication of some of the graphs which are given for human prenatal growth (see review Gruenwald 1973). In this review the prenatal growth of low birth weight population is described as being normal up until the last trimester of pregnancy. A weight difference between the high and low birthweight groups only appears late in gestation and gets progressively greater until birth. However, examination of the data supplied with some of these curves (Gruenwald 1973) shows that populations with a low birthweight have generally been below the weight of those with a high birth weight for the whole of the gestational period. The results obtained in this project are also similar to those given by Widdowson (1974) for the weights of growth-retarded human fetuses as compared to normal fetuses. In the graph shown in this paper (Widdowson 1974) the weight of growth-retarded fetuses show a constant relationship with the weights of normal fetuses.

Runts

Runts are an interesting phenomenon in species which bear large litters, as they are thought to represent individuals below the range of size expected from a normal population. Previous authors using much larger samples have found that runts constitute about 3% of fetuses in pigs (Perry and Rowell 1969). This would mean that about one litter in three should contain a runt and the identification of 4 runts in this sample of 14 litters is close to the expected frequency. One fetus (S1 R2), a male with a weight only 40% of the mean of the other two animals in the

litter is probably also a runt but due to the small litter size it cannot be identified statistically. Of the recognised runts, the earliest was found at 64 days gestation but runts have been identified as early as 44 days (Cooper, John, McFadyeen and Wootton 1978). The finding that all four definite runts were female is probably an artefact of the small sample size as no sex difference has been found in larger samples (Perry and Rowell 1969). These authors also state that the runt is not found in any particular location within the uterus in contrast to the results reported here where they all occurred in the cervical half of the horn, a distribution which agrees with the findings of Dziuk and Hentzel (1977).

Possible sex differences in weight

It is known that in man, males are slightly heavier at birth than females, (Lubchenko, Hansman and Boyd 1966; Woods, Malan and Hease 1980) and in pigs Widdowson (1974) reported that in her sample, the mean weight of 100 males was 40 gms. (3%) above that for 100 females at birth. However, when a t-test was applied to these results by the present author, the difference was found to be not significant. In the present study no significant weight difference could be demonstrated prenatally between males and females. From the farm records of birth weights the mean for 136 males was 80 gms heavier than the mean for 122 females and this difference was statistically significant.

Placental Growth

Four parameters of the placenta were measured, namely fresh weight, area, in situ length and maximum circumference. The fresh weight of placentae was measured by Pomeroy (1960) who showed that placental weight reaches a maximum at around 60 days and then remains constant for the rest of gestation. The results of the present investigation show an increase at least up until 75 days but thereafter the points are too variable to be certain of weight changes during the rest of gestation. For this reason no line is drawn through the points for older litters in Fig. 5.

Baur (1973) measured macroscopic placental area and obtained a similar increase and shape of curve to that shown in Fig 6. This shows that, placental area increases throughout gestation with a four fold increase in area between 38 days gestation and term.

The results from the measurement of the surface enlargement of placental area brought about by the presence of microscopic ridges on the external surface of the placenta showed that there was no difference in the degree of this enlargement between a large and a small fetus. This result indicates that differences in the macroscopic parameters are the only differences in area between placentae.

Placental circumference also shows an increase with age but placental

length shows no significant increase during gestation. Taken together, these results show that the placenta is not static but is a continuously growing organ which increases in every dimension except length during pregnancy. Placental length cannot increase due to the fixed length of the uterus (Pomeroy 1960) and the presence of adjacent fetuses. Placental length appears to be determined early in pregnancy by the extent of blastocyst elongation and is limited by competition for space between fetuses. This is illustrated by Fig. 10 which shows how placental length is negatively correlated with the number of fetuses in a horn.

With placental length fixed, macroscopic placental area increases during gestation by increasing the circumference of the placental sac (Fig. 9). This increase in the diameter of the uterine horn adjacent to each fetus is probably brought about by the secretion of amniotic and allantoic fluid within the chorionic sac. The volume of these fluids increases until 75 days gestation after which their volume declines (Pomeroy 1960) and this may explain the reduction in the rate of increase of placental circumference after this age (Fig. 9). Placental area is also increased by folds in the uterine lining and the effects of this will be discussed later.

Spacing of fetuses within the horn

The reason for investigating the spacing of fetuses within the uterine horn was to determine whether the variation in size within a litter could

be explained by the crowding of fetuses in some regions of the uterus. If this occurred it might be that the more crowded regions produced smaller fetuses. The spacing of pig embryos was measured by Anderson and Parker (1976) who were able to record the positions of embryos shortly after implantation. The animals used in this study were older and the position of implantation was assumed to be the point of insertion of the umbilical cord into the placenta. In agreement with Anderson and Parker (1976) no variation in spacing was apparent in any of the horns examined. All embryos had implanted at even intervals along the mesometrial side of each uterine horn. The interval between implantation sites is reduced as the number of fetuses in the horn increases but within litter size variations cannot be explained in terms of crowding in particular locations within a horn.

The effect of the number of fetuses in a uterine horn on the mean fetal weight

The reduction in mean fetal weight with increasing litter size is a well established phenomenon in all animals including man where twins have a lower mean weight that singletons. The result that more crowded horns contain lighter fetuses than less crowded horns (Table 3) is expected but it illustrates a strong environmental influence on fetal growth which probably operates by means of competition between fetuses for a limited resource.

The effect of the number of fetuses in a uterine horn on the mean placental length

Fig. 10 shows the strong effect of fetal number in reducing the mean placental length. The length of the uterine horn is only slighty affected by the number of fetuses present. Therefore the length of horn available for each placenta is correspondingly reduced as the number of fetuses in the horn increases.

The relationship between the relative fetal weight and fetal position within the horn

Several authors have reported a position effect on fetal weight in pigs (Waldorf, Foote, Self, Chapman & Cassida 1957; Perry & Rowell 1969; Dziuk and Hentzel 1977). These authors describe a distribution which has the heaviest fetus in the most ovarian position within the horn and a progressive decline in fetal weight with every position towards the cervix. This trend is reversed by the fetus in the most cervical position which tends to be heavier than its neighbour. When this weight distribution is plotted graphically against position it produces a skewed U shaped distribution. The absence of any pronounced position effect in the 12 prenatal litters available for the project is perhaps surprising but may be due to the small sample size. Perry and Rowell (1969) and Waldorf et al. (1957) demonstrated this weight distribution using much larger samples, (90 and 80 litters respectively) whereas Wootten,

McFadyen & Cooper (1977) were unable to demonstrate this effect with only 9 litters. The position effect is another example of an environmental influence on fetal growth and, since it becomes more pronounced as the litter size increases (Perry and Rowell 1969), it is probably due to competition between the fetuses. Similar position effects, with a U shaped distribution of fetal weights within the uterine horn, have been described in mice (McLaren & Mitchie 1960), rabbits (Rosaha & Greene 1936; Duncan 1969), guinea pigs (Ibsen 1928), and an inverted pattern in rats (Barr & Brent 1970; Ward, Karp & Aceto 1977). Numerous attempts have been made to determine the underlying cause and one explanation suggested has been the haemodynamic theory (McLaren & Mitchie 1960; McKeown, Marshall & Record 1976). This theory postulates a maternal arterial pressure gradient which parallels the differences in fetal weight with position. The haemodynamic theory predicts that a high arterial pressure will be associated with the development of larger fetuses. The validity of the theory has been questioned due to its failure to explain experimental results in several species (in mice, McLaren 1965; in rabbits, Duncan 1969; in rats, Barr et al, 1969, 1970a; 1970b). In the pig the vascular architecture, which consists of the largest arterial branches running to the middle portion of the horn. (Oxenreider, McLure and Day 1965; Perry and Rowell 1969), is such that it is hard to see how such an arterial pressure gradient could exist.

The relationship between the relative placental length and fetus position within the horn.

When placental length is plotted against position (Fig. 12) the U-shaped distribution previously described by other authors for fetal weight (see above) is found. This observation is particularly interesting as placental length is determined shortly after implantation by the extent of elongation of the blastocyst and thereafter appears to remain constant. If this is so, the position effect may be caused by factors operating within the first few weeks of gestation. In an attempt to test this, a diagram in the paper of Perry and Rowlands (1962) of blastocyst lengths and positions at three weeks of age was used to plot a graph of length against position. This appeared to show a clear position effect with a U shaped distribution, but the original data was unobtainable.

The relationship between placental parameters and fetal weight

A close relationship between placental weight and fetal weight is well established in man (Aberne and Dunhill 1966), sheep (Mellor & Murray 1981), mice (McLaren 1965) and pigs (Wootton, McFadyen & Cooper 1977; Waldorf et al 1957). The majority of prenatal litters used in this project showed a significant correlation between fetal weight and placental weight. Placental weight is often taken as a measure of placental function but in the pig placental area is easy to obtain as the placenta is very thin and its folds can easily be smoothed out.

Placental area, in most litters, shows a higher correlation with fetal weight (see Table 4) than does placental weight, probably because the latter is subject to variation in the amount of fluid retained after removal.

The much lower incidence of a significant correlation between fetal weight and placental length or circumference was surprising after the good correlation with area. It had initially been thought that placental length would correlate well with fetal weight, the hypothesis being that placental length would be the limiting parameter of placental area. Those fetuses which had only occupied a short length of uterine horn after implantation would in consequence have small placental areas and low fetal weights. If this had been the case, and a similar idea has been put forward by Marrable (1971), then a close relationship between fetal weight and placental length would have been expected.

The poor correlation between fetal weight and placental length and circumference in contrast to the good relationship between fetal weight and placental area suggests that placental area is increased by folds in the uterine wall. These folds are easily seen, particularly in older litters (see photograph Fig. 13). As previously described these folds which can be up to 2cm. high, run transversely across the uterine horn. The height and density of the folding are very variable but both tend to be largest on the mesometrial side of the horn near the position of umbilical cord insertion into the placenta. These folds considerably

increase the surface area of the placenta so that placental area would not be limited by the available length or circumference of the uterine horn. If this is so it is possible that fetuses which have only a short length of uterine horn available for placental growth would compensate for this by increasing the degree of placental folding.

The degree of folding in the placenta was quantified by calculating the placenta area/placental length ratio. Placental length rather than circumference was chosen because the folds ran at 90^{o} to the long axis of the placenta. If compensatory folding of the placenta occurs then fetuses in more crowded horns could compensate by having a higher area/length ratio than those in less crowded horns.

This hypothesis was tested by comparing the mean area/length ratio in left and right horns from those litters where there was an unequal number of fetuses in each horn (see Table 5). By comparing horns from the same uterus, the variation in placental area with age was avoided. The results in Table 5 show that in 6 out of the 8 litters there was a higher mean area/length ratio in the more crowded horn. A paired t-test showed this to be a significant difference ($P < 0.005$). These results indicate that compensatory folding of the placenta occurs in more crowded horns. This compensatory growth results in there being no significant difference between the mean placental areas in the more crowded and less crowded horns ($P > 0.200$) despite the significant difference in the mean placental lengths ($P < 0.025$). The ability of the uterine lining to produce folds enables the placenta to escape from the

restrictions imposed by the horn length.

Compensatory growth of the placenta, defined in terms of an enlarged placenta relative to fetal size, has been described in man when there have been deficiencies in the maternal circulation or in pregnancies at high altitudes (Gruenwald 1974) and in rats after ligation of a uterine artery (Bruce 1976). Enlarged placental cotyledons have also been described in malnourished ewes (Foote, Pope, Chapman and Cassida 1959; Everitt 1968). This, however, appears to be the first description of possible compensatory growth of the placenta in response to crowding and in terms of the space available to the placenta.

Comparison of the largest and smallest littermates

The major part of this project is concerned with muscle growth in large and small littermates and, in order for the comparison between these animals to be more meaningful, it was considered to be important to try to establish the cause of the weight difference between them. In order to do this Table 6 was compiled to show the similarities and differences between each pair of littermates for a variety of growth parameters. The results were fairly consistent for all pairs of animals used. The smaller animal is lighter and shorter and has a lower ponderal index and is associated with a smaller placenta though it has the same fetal weight/placental weight ratio.

These differences were tested statistically and all parameters except fetal weight/placental weight showed a significant difference between large and small. In addition, radiographs from the limbs of two litters (Fig 15) showed that the smaller fetuses had a less well developed skeleton. This observation has been made previously in pigs by Adams (1971) and Pomeroy (1960).

The reason these characteristics were chosen was because they have all been used to diagnose the cause of 'small-for-dates' babies. The differences between the large and small pigs in this project very closely parallel the differences observed between 'small-for-dates' and normal sized infants. These 'small-for-dates' babies have been shown to be not only lighter (Walther, Ramaekers & Van Engelshoven 1980; Davies 1981), shorter (Cassady 1970; Miller & Hassanein 1971; Walther et al 1980), have a lower ponderal index (Davies, Platts, Pritchard & Wilkinson 1979; Woods, Malan, de V. Meese and Van Schalkavijk 1980; Walther et al. 1980), smaller placentae (Younoszai & Maworth 1969) but the same placental weight/fetal weight ratio (Woods et al. 1980) as normal sized babies. It has also been shown that they suffer from delayed skeletal maturation as shown by X-rays (Scott & Usher 1964; Roord, Ramaekers & Van Engelshoven 1978; Walther et al. 1980). 'Small-for-dates' babies also show the phenomenon of 'catch-up-growth', (originally defined by Prader, Tanner & Von Marnach 1963) in the immediate post-natal period (Davies 1981). The phenomenon of 'catch-up-growth' is normally only associated with growing individuals, who after a period of starvation

received adequate nutrition and then exhibit above average growth rates.

On the basis of the above results and on skin fold thickness measurements, the authors quoted above conclude that 'small-for-dates' babies have suffered from prenatal malnutrition. This leads to a retardation in the growth of both soft and skeletal tissues to a degree dependent upon the duration of the nutritional insufficiency. The similarity of the small littermate pigs used in this project in their characteristics of weight, length, ponderal index, absolute and relative placental size to 'small-for-dates' babies, strongly suggests that both have a common cause. From the work that has been done on 'small-for-dates' babies this would appear to be prenatal malnutrition. Another feature of malnourished growing animals is that they have a nearly normal brain weight but a much lower liver weight than those better nourished individuals of the same age (Wigglesworth 1969). These organs were weighed in newborn pigs by Widdowson (1971) who showed that the runt had a brain weight 82% the size of its larger littermate but a liver weight only 26% the weight of that in its sibling. The implication of this result was that the difference in size between the two littermates was due to nutrition. Other examples of environmental influences on fetal weight, which have been mentioned previously, are the number of fetuses in a horn (litter effect) and the position of fetuses within the horn (position effect). Both these effects can cause a reduction in an individual's weight by competition with its littermates. The need for compensatory growth of the placentae in more crowded horns suggests that placental area may be a limiting factor in fetal growth but the

factors which influence the relationship between fetal and placental size have yet to be established.

It is not possible to be certain that every small individual used in this project had suffered from malnutrition and other possible causes for size variation within litters have been put forward. These include genetic variation, uterine pressure (McLaren 1965; Ward 1977; Barr et al. 1970b), immunological factors (Clark 1971; Beer, Scott & Ballingham 1975) and time of implantation (Hilberg & Rampalek 1974; Herbert & Bruce 1980). However, the evidence of growth retardation given in Table 5 and its similarity to 'small-for-dates' babies and the relative organ weights of Widdowson (1971), together with the existence of well established environmental effects, are not inconsistent with malnutrition being the major cause of the small size of the smallest littermates used in this project.

The duration of malnutrition experienced by individuals in different litters may not be the same but from work using ultrasound scans of human pregnancies, (Francourt, Campbell, Harvey & Norman 1976) it takes relatively long periods of malnutrition to stunt the length of a fetus and all the small fetuses used in this project are shorter than their larger littermates.

These results agree with the subjective appraisal of small or runt pigs which have always been regarded as malnourished or growth retarded (Widdowson 1971, Cooper 1975) and are known to have poor postnatal

viability (Pomeroy 1960; Leman, Hurtgen & Hilley 1979).

Part 2: Muscle Structure

Introduction

The commercial importance of muscle as meat has been mentioned in the general introduction. The postnatal growth of muscle can be improved by optimal feeding and environmental conditions but only within the growth potential of the tissue. Many factors affecting this potential appear to be determined prenatally, the most important of these being muscle fibre number. The importance of fibre number is its relationship to muscle mass (Luff and Goldspink 1967) seen in an extreme form in double 'muscled cattle', a condition thought to be due to increased muscle fibre hyperplasia (Swatland 1973a).

Comparison of the postnatal growth of large and small littermates (Widdowson 1971) has shown that the smaller individuals never attain the size of their larger littermates even after optimum feeding. This suggests that the prenatal events which have led these animals to be small at birth exert a permanent effect on their postnatal growth. Muscle fibre number was not measured in these experiments but it is likely that this tissue was growth retarded at birth. The absence of fibre hyperplasia postnatally (Staun 1970; Stickland and Goldspink 1973) would make this permanent.

All skeletal muscle fibres form by the fusion of mononuclear myoblasts

(see review by Kikuchi 1971) but fibre formation does not proceed uniformly. In mammalian and avian muscle, the fibres form in two successive generations. This process has been called the biphasic theory of muscle development (Kelly and Zacks 1968; Kikuchi 1971; Ashmore, Robinson, Rattray and Doerr 1972; Swatland and Cassens 1973). According to this theory a small population of primary fibres form by myoblast fusion in the region of the presumptive muscle. The surfaces of these fibres are then used as a substrate for the formation of secondary fibres. The second generation of fibres forms by myoblasts attaching to the surface of primary fibres and fusing to form fibres which initially lie on the surface of the primary fibres. After formation the secondary fibres detach from the surface of the primary and more secondary fibres then form on the surface of the same primary fibre so that each primary becomes surrounded by several secondaries. The relative numbers of primary and secondary fibres in a muscle varies between species (personal observation discussed later) and probably between muscles within an animal (Ashmore, Addis and Doerr 1973) but secondary fibre number seems always to be greater than primary fibre number.

Primary fibres can be recognised not only from their time of formation and central location within fascicles, but also because they are larger than secondaries. The size of the primaries in many species is enhanced by having a central region of myofibrillar free cytoplasm (rat - Kelly and Zacks 1968; pig - Thurley 1972; Human - Boyd 1960). This gives

them a tubular shape, hence the term myotube, which is distinguishable from the solid appearance of secondary fibres.

Only four groups of authors have examined prenatal pig muscle structure (Thurley 1972; Ashmore, Addis and Doerr 1973; Swatland and Cassens 1973; Swatland 1973b; Beermann, Cassens and Hausman 1978). These authors have confirmed the presence of primary and secondary generations of fibres in porcine muscle, an observation first made by Schwann in 1839 (cited by Swatland 1973). These generations are distinguishable by the above mentioned criteria and also, in certain locations, by their histochemical staining (Ashmore et al 1973; Beermann et al 1978). Most of the observations made by the above authors have been descriptive rather than quantitative apart from the following. Thurley (1968) measured fibre diameter and showed changes in the frequency distribution of fibre size with age. However, no distinction was made between primary and secondary fibres in the actual measurements, although he did show an increase in primary fibre size up to 55 days gestation followed by a decrease. Swatland (1973b) also reported a decline in primary fibre size after 55 days and relatively little change in secondary fibre size during the last two weeks of gestation. All authors concluded that fibre hyperplasia ceased before birth, which is in agreement with the finding that fibre number remains constant during postnatal growth (Staun 1972; Stickland and Goldspink 1973). This last point has however been challenged by Swatland (1973b; 1976) who, while agreeing that hyperplasia ceased prenatally, found that fibre number appeared to increase in the porcine sartorius muscle until

at least 160 days postnatally (Swatland 1976). This apparent increase was thought to be due to the intrafascicular growth of the muscle fibres. Ashmore et al (1973) and Swatland (1973b) give estimates of the ratio between secondary and primary fibre number during gestation. Both authors found an increase in the ratio until about 80 days.

In view of the previous work described above the following points were felt to be worth further investigation. Many of the above measurements were made only during the latter part of gestation and it was hoped to extend these to cover the whole period of muscle development. This applies particularly to primary and secondary fibre size. The rise and fall of primary fibre size reported by Thurley (1968) appears to be an interesting phenomenon, apparently unreported in other species. Both Ashmore et al (1973) and Beermann et al (1978) found that primary and secondary fibres could be differentiated histochemically but Beermann et al. (1978) only found this in the deep region of the muscle where fibre type conversion also took place prenatally. This last point is of interest as this phenomenon has been thought to be a weight dependent change in postnatal growth (Davies 1972). Confirmation and further investigation of these points was thought to be worthwhile.

In addition to investigating the general development of muscle, large and small littermates were compared. This has not previously been done for the above mentioned parameters and an investigation of these may explain the inequality of their postnatal performance. In particular, it

would be interesting to know if fibre number differences existed between these pairs of animals and if so the mechanisms which had brought this about.

Materials and Methods

As previously described in the materials and methods of the gross section, large and small animals were chosen by weight from each litter. All the work was done on the semitendinosus muscle. This muscle was chosen for the following reasons:- (1) it is of commercial importance, (2) some literature already exists on its growth (Ashmore, Addis and Doerr 1973; Beermann, Cassens and Hausman 1978; Hegarty and Allen 1978), (3) it is large enough to find in young fetuses, (4) it has well defined fast and mixed fibre type regions. The procedure used on this muscle was as follows. The semitendinosus of the left hind limb was exposed by removing the skin and a thin strip of blue latex was painted over the superficial surface of the muscle. This was retained on sections and enabled them to be orientated such that the deep and superficial regions were distinguishable. The semitendinosus muscles from both hind limbs were then removed, trimmed off their tendons and weighed. A thin slice was taken from the middle of the muscle and mounted on a chuck with water. If the muscle slice appeared to be an oblique section it was reorientated by mounting on cork and angling this to the chuck O.C.T. compound. The chuck and muscle sample were then frozen in a bath of Arcton ($C Cl_2F_2$, melting point - 158^{o}C) kept cool in a flask of liquid nitrogen. Muscle samples were in general not embedded in O.C.T. compound as this was found to slow their freezing. The muscles in the youngest animals used (30 days gestation) were too ill-defined to be dissected out. In these fetuses, latex was painted over the position of

the semitendinosus muscle and the whole limb was frozen and sectioned. Sampling of fibre size and number was done in the region between the bone and the latex.

The muscle samples frozen to the chuck were allowed to warm to -25°C inside a cryostat cabinet. 10 μm sections were cut on the cryostat and picked up on coverslips before being allowed to dry at room temperature. The orientation of sections was checked by staining with methylene blue and examining them under a microscope.

All histology was done on prerigor muscle. This was necessary because, due to the small size of the muscles, each muscle had to be used for several techniques. Many of these techniques (histochemistry, plastic embedding for electron microscopy and biochemical assays) needed to be done as soon after death as possible. It was also found that muscles left on the animal still showed contraction when removed 7 hours after death and it was impractical to wait this length of time.

Frozen sections were either stained by haemotoxylin and eosin or for the enzyme adenosine tryphosphatase (A.T.Pase) and mounted in D.P.X. Staining for A.T.Pase was done by the method of Guth and Samaha (1970) using an acid preincubation with no prior fixation. This technique was adopted as all previous work on the histochemistry of prenatal pig muscle has used it (Ashmore, Addis and Doerr 1973; Swatland 1975; Beerman, Cassens and Hausmann 1978). Tests on postnatal rat muscle showed that results obtained with an alternative technique (Brook and

Kaiser 1969) yielded different staining patterns at the same pH which emphasised the need to use an identical technique if comparison with previous authors was to be attempted.

Staining sections with haemotoxylin and mounting in D.P.X. was found to cause shrinkage of the fibres. The diameters of both primary and secondary fibres were reduced by about 22% due presumably to dehydration by the alcohol in the haemotoxylin and in the processing for D.P.X. Sections of all ages were treated in the same way and the degree of shrinkage appears to have been constant throughout gestation.

Sections stained with haemotoxylin and eosin were used to obtain the complete cross-sectional area of the muscle as well as mean fibre size and total fibre number. The cross-sectional area of these sections was obtained by using a camera lucida microscope attachment to trace the outline of the section. The area of the tracing was then measured on a Reichart Videoplan. Prenatal muscle fibres were classified as either primary or secondary fibres (Swatland and Cassens 1973). Primary fibres were recognised as those fibres which had peripheral myofibrils and a central nucleus or central myofibrillar free region. These fibres had a tubular appearance and were found in the middle of fascicles. Secondary fibres had a solid appearance, only occasionally having a central nucleus and were found surrounding the primary fibres. Recognition was made easier by the large size difference between primary and secondary fibres, primary fibres being two - three times the size of secondary fibres.

Several techniques for sampling fibre number and size from sections were tried using projection microscopes but the easiest method was found to be photomicrography. The technique used was to photograph a grid over the whole section at low power. Squares were picked at random from this grid and photographed. In this way, not only was a random sample obtained, but also the position of each sampling point was known. This enabled differences between regions within the muscle to be investigated. Sampling from all over the section is necessary due to the difference in fibre type ratios and fibre sizes in different regions of the muscle (Pullen 1977).

The sampling at each square took the form of a X200 and a X400 photograph. The lower power picture was used to obtain the number of fibres per unit area while the higher power was used to obtain the sizes of fibres. On average 12 squares were photographed from each section. To obtain the total fibre number in younger fetuses, where secondary fibres were very small, the mean number of primary fibres in X200 photographs were calculated and the ratio of secondary fibres to primary fibres was obtained from the X400 pictures. The product of these two figures gave the number of secondaries per unit area and the total number was obtained using the area of the whole section.

In slides from older animals where the primary fibres had started to collapse and secondary fibres were relatively large, the total number of primary and secondary fibres in the X200 photographs was counted

and used to find the total muscle fibre number.

Two main methods have been used to measure muscle fibre size; either cross-sectioned area or some measure of diameter has been employed. Recent authors have been fairly evenly divided over which parameter to measure with Davies (1973), Maier and Eldred (1974), Swatland (1975), Kelly and Zacks (1969) and Bendal and Voyle (1967) using area and Staun (1963), Luff and Goldspink (1967), Swatland and Cassens (1973), Swatland (1973), Thurley (1972), Ontell (1977) and Stickland (1978) using diameter. In this project the minor diameter, defined as the maximum width of a fibre at 90^{o} to its long axis was used. This diameter, discussed by Song, Shimada and Anderson (1963) and Schmitt (1976) in combination with the major diameter has the advantage of speed of measurement and unlike fibre area, is not subject to enlargement in obliquely cut sections (Brook 1970). In addition a comparison of area and diameter measurements from the same fibres showed that the diameters were more normally distributed compared to areas which showed a skewed distribution. Additional reasons for using diameters were that they showed differences in size between fibre types better than areas and the only two recent authors to measure prenatal pig muscle fibres (Thurley 1972 and Swatland 1973) have both used diameters.

Fibre diameters were measured from the photographs on a Reichart Videoplan.

In one litter (S13, 70 days gestation) frozen sections were cut from the semitendinosus muscles of every animal in the litter (13 individuals). These sections were mounted in glycerine to avoid shrinkage and the results are presented separately from those of other litters.

At the same time as samples were being taken for frozen sections, small pieces of muscle from the deep and superficial regions of the semitenenosus were removed and fixed in 2% glutaraldehyde in 0.1M. phosphate buffer (Ph 7.4) for 2 hours. After the initial fixation, the samples were post fixed in 1% osmium tetroxide in veronal acetate for 1 hour, dehydhrated in ascending concentrations of acetone and embedded in araldite resin. Thick (1μm) sections were cut from these blocks and stained with 1% toluidine blue in borax for examination under the light microscope. A small number of these sections were cut for the electron microscope and stained with uranyl acetate and lead citrate.

Semitendinosus muscles from animals which were not the largest and smallest in the litter were fixed in 10% buffered formalin. Some of these samples were used to tease out intact muscle fibres. This was done with needles under the dissecting microscope, the fibre ends being recognised by their rounded or conical appearance. Attempts were made to stain these fibres with haemotoxylin, eosin and methylene blue but they failed to take up the stain and so were viewed under a phase contrast microscope.

Three postnatal runt animals were obtained at the start of the project and serial frozen sections were taken from seven muscles from these animals. These serial sections were stained for succinic dehydrogenase (S.D.H.) glycogen phosphorolase (G.P.) and A.T.Pase. The techniques used were those of Nachlas, Tsou, de Souza, Cheng and Seligman (1957) for S.D.H. and Takeuchi's (1956) modification of Takenchi and Kuriaki (1955) for G.P. The fibres from these animals were classified according to the system of Peter, Barnard, Edgerton, Gillespie and Stempel (1972) which identifies three adult fibre types, namely, slow-twitch oxidative (S0), fast-twitch oxidative and glycolytic (FOG) and fast-twitch glycolytic (FG).

Statistical analyses were done using programmes written by Texas Instruments. S shaped curves were plotted using logit transformations given in Bliss (1970).

Results

Gross parameters of muscle

Fig. 17 and 18 show macroscopic parameters of the semitendinosus muscle plotted against fetal weight. Both these graphs were used when these parameters were not available for individual animals. This was the case in the youngest litters where it was not possible to dissect out the semitendenosus muscle and the whole limb was sectioned.

Fig. 17. shows m. semitendinosus weight for large and small littermates plotted against fetal weight. A double logarithmic curve is fitted to each set of points, the equations of which are given on the graph. The correlation coefficients for the lines of both largest and smallest was highly significant. (Largest $r = 0.993$, $P < 0.01$; smallest $r = 0.923$, $P < 0.01$). The double log. equations used to plot these curves are allometric growth equations. The slope (b) of the type of equation is the growth ratio of the two parameters being correlated. The value of b was 1.035 for the largest littermates and 0.833 for the smallest. The result for the largest littermate is not significantly different from 1.0 ($P > 0.5$) but that for the smallest littermates is significantly below 1.0 ($P < 0.05$). The two results are also significantly different from each other ($P < 0.05$).

Fig. 18 shows the square root of cross-sectional area of the m.

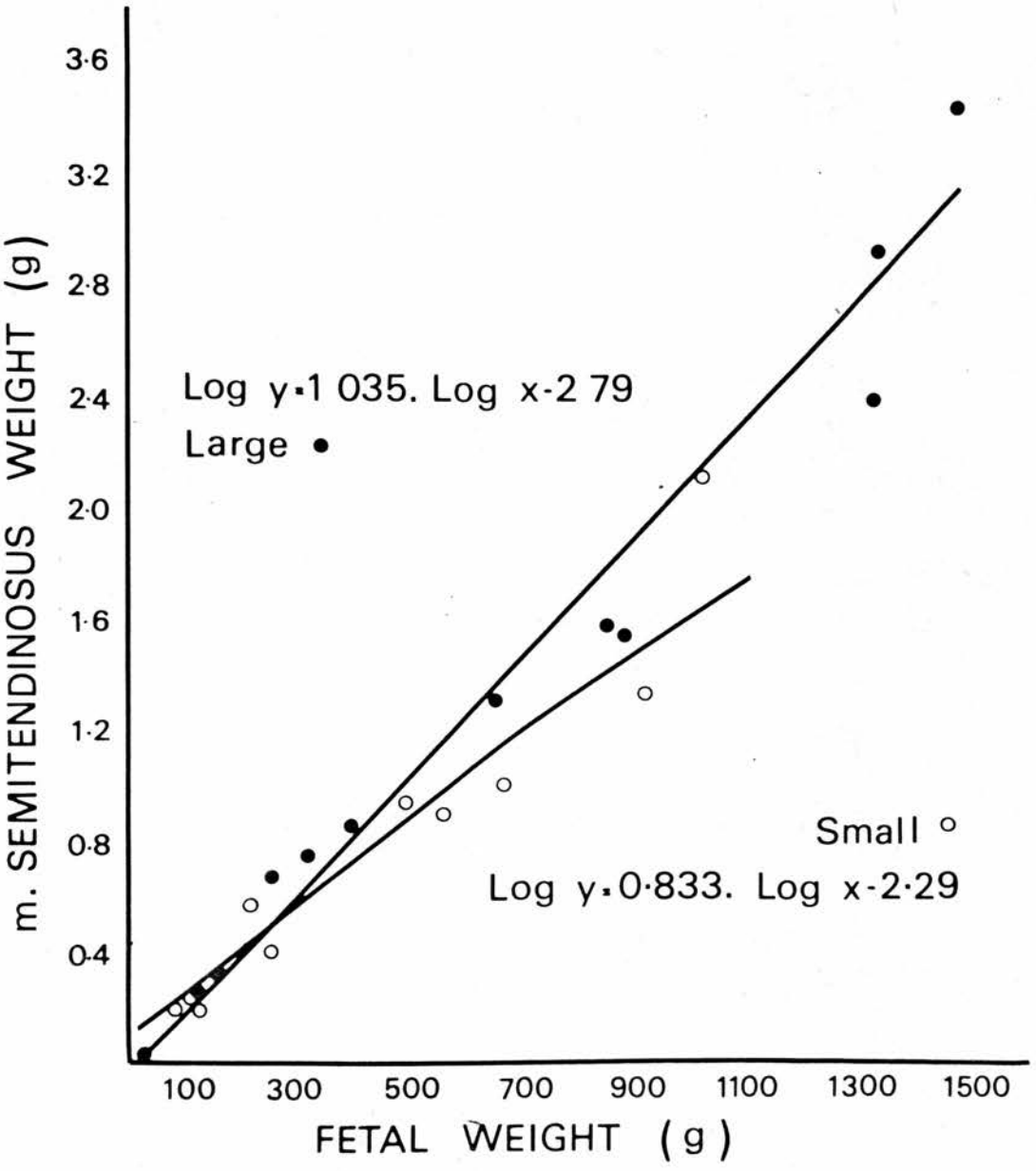


Fig.17 M.semitendinosus weight of the largest and smallest littermates of each litter plotted against fetal weight.

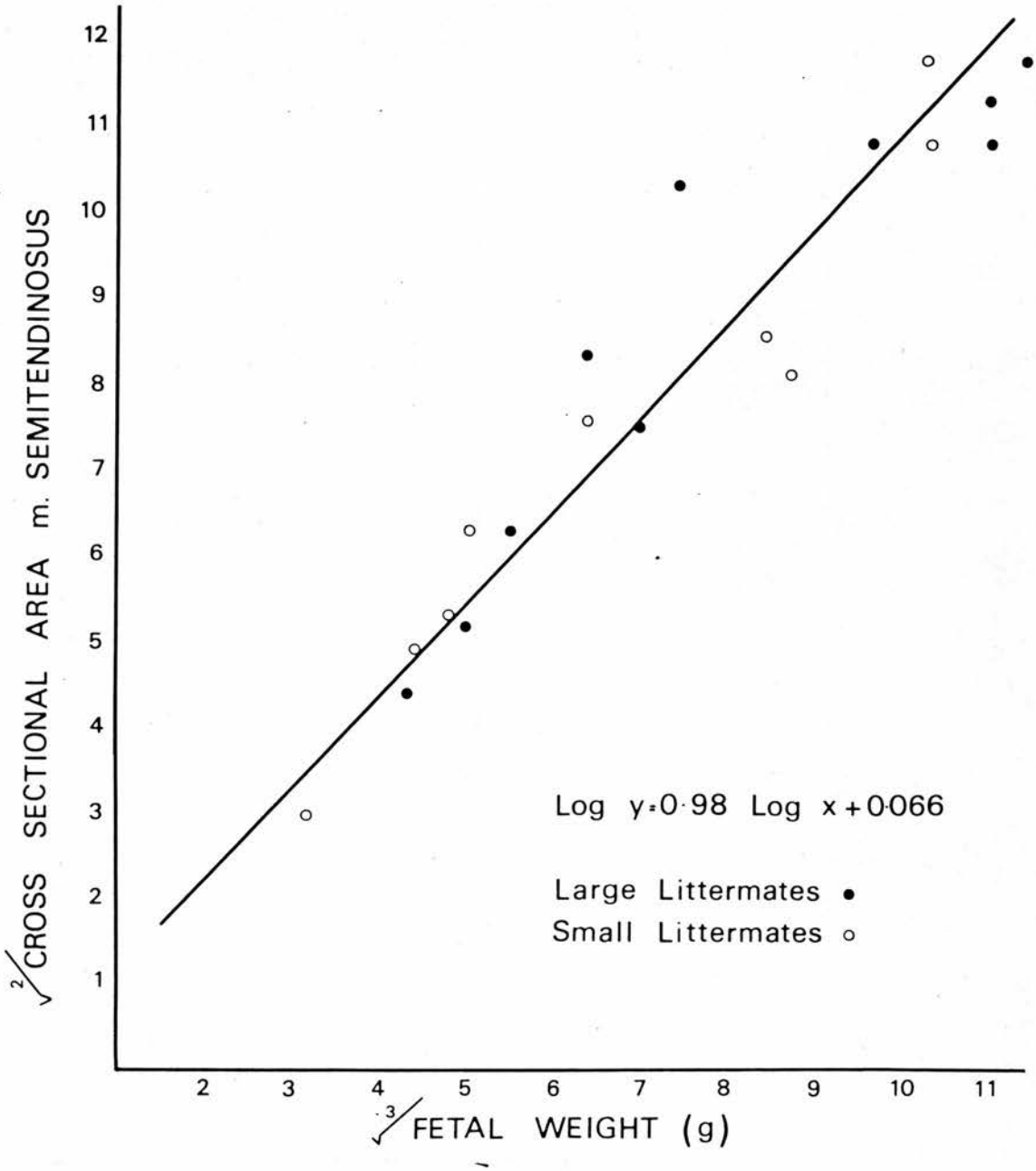


Fig.18 The square root of the cross sectional area of m. semitendinosus plotted against the cube root of fetal weight for the largest and smallest littermate from each litter.

semitendinosus plotted against the cube root of fetal weight. The equation of this curve is given on the graph and the correlation coefficient of this line is 0.962 ($P < 0.01$).

Description of muscle development

The description of muscle development which follows is largely based on frozen sections of the semitendinosus muscle of large and small littermates from 38 days gestation until term. To supplement the frozen sections some plastic embedded light microscope sections are included. The description is based upon three components of developing muscle, primary fibres, secondary fibres and mononuclear cells.

38 days gestation (Fig. 19 and 20)

Small primary fibres are the only type of fibre present at this age. These fibres are densely packed into bundles and have prominent central nuclei. Mononuclear cells can be seen lying between the fibres and some of these cells appear to be adhering to the surface of the fibres. There are no prominent differences between large and small littermates.

46 days gestation (Fig. 21 and 22)

Primary fibres are still the only type present, but their size has increased from that at 38 days gestation. The primary fibres are still densely packed but more mononuclear cells are now present between

them. Some of the primary fibres in the largest fetus have a 'myofibrillar free region' (M.F.R.) in the centre.

This causes large variations in the size of primary fibres. The myofibrils of these fibres are restricted to a subsarcolemmal location which gives the fibres a tubular appearance. This morphology is absent in the primary fibres of the smaller fetus.

54 days gestation (Figs. 23, 24 and 39)

The primary fibres have continued to increase in diameter and are now more widely separated. Secondary fibres can now be seen on the surface of a few primary fibres. Mononuclear cells are seen on the surface of primary fibres and are often found associated with secondary fibres. The primary fibres from the larger littermate now have a greater diameter than those in the smaller. This is due at least partially, to the large M.F.R. in the center of these fibres, a feature not found in the smaller littermate. The longitudinal section in Fig. 39 shows the central nuclei of the primary fibres and a large number of mononuclear cells on the surface of the fibre.

59 days gestation (Fig 25 and 26)

The primary fibres of both the large and small littermates have continued to enlarge and secondary fibres can be seen on their surfaces.

A few of these secondary fibres have detached from their primary fibre and are now lying free in the intercellular space. Large numbers of mononuclear cells are still seen adhering to the surfaces of the primary fibres. The appearance of primary fibres in the larger littermate is still different from that in the smaller animal due to the presence of M.F.R.s in the fibres of the former.

64 days gestation (Fig. 27, 28, 37, 38 and 40).

Each primary fibre in both large and small littermates has several secondary fibres surrounding it, many of which lie in the intercellular space. The number of mononuclear cells on the surface of primary fibres appears to have declined. The primary and some of the secondary fibres in the larger animal display a tubular appearance due to their M.F.R (Fig. 27). This feature is now seen in some of the primary fibres of the smaller animal (Fig. 28). These features can also be seen in the plastic sections shown in Figs 37 and 28. A longitudinal plastic section from the large littermate Fig 40, clearly shows the M.F.R., secondary fibres and mononuclear cells.

69 days gestation (Fig. 29 and 30)

The primary fibres of both large and small are now surrounded by secondary fibres of varying size, some of which have prominent central nuclei. There has been a reduction in the size of the primary fibres in the larger animal, a change brought about largely by a reduction in their

M.F.R. The muscle is now organised into fascicles consisting of 2 or 3 primary fibres surrounded by secondaries.

75 days gestation (Fig. 31 and 32)

The primary fibres of both large and small show little change from the preceding age. They retain their prominent central nuclei and occasionally have M.F.R.s. Secondary fibres now rarely have a central nucleus but rather have nuclei in a subsarcolemmal location. The increase in the number of secondary fibres has led to a decrease in the intercellular space. Connective tissue septa now divide the fibres into fascicles continuing several primary fibres and their surrounding secondary fibres.

82 days gestation (Fig. 33 and 34)

The histological appearance of the muscle is very similar to that in the preceding age. Primary fibre size has continued to decline and they are now only slightly larger than the surrounding secondaries. Primary fibres can still however be distinguished from secondary fibres by their prominent central nuclei.

114 days gestation (Fig. 35 and 36)

By this age primary fibres can not be consistently identified. A few

primary fibres retain a central nucleus but the majority of fibre nuclei have a subsarcolemmal location. The size difference between primary and secondary fibres no longer exists and there is little variation in size between secondary fibres. There are no obvious qualitative differences between large and small littermates.

Figs 19-36 are all transverse frozen sections of the semitendinosus muscle. These sections were stained with haemotoxylin and eosin and photographed at a magnification of X400.

Figs 37-41 are plastic embedded sections stained with toluidine blue and photographed at a magnification of X1000.

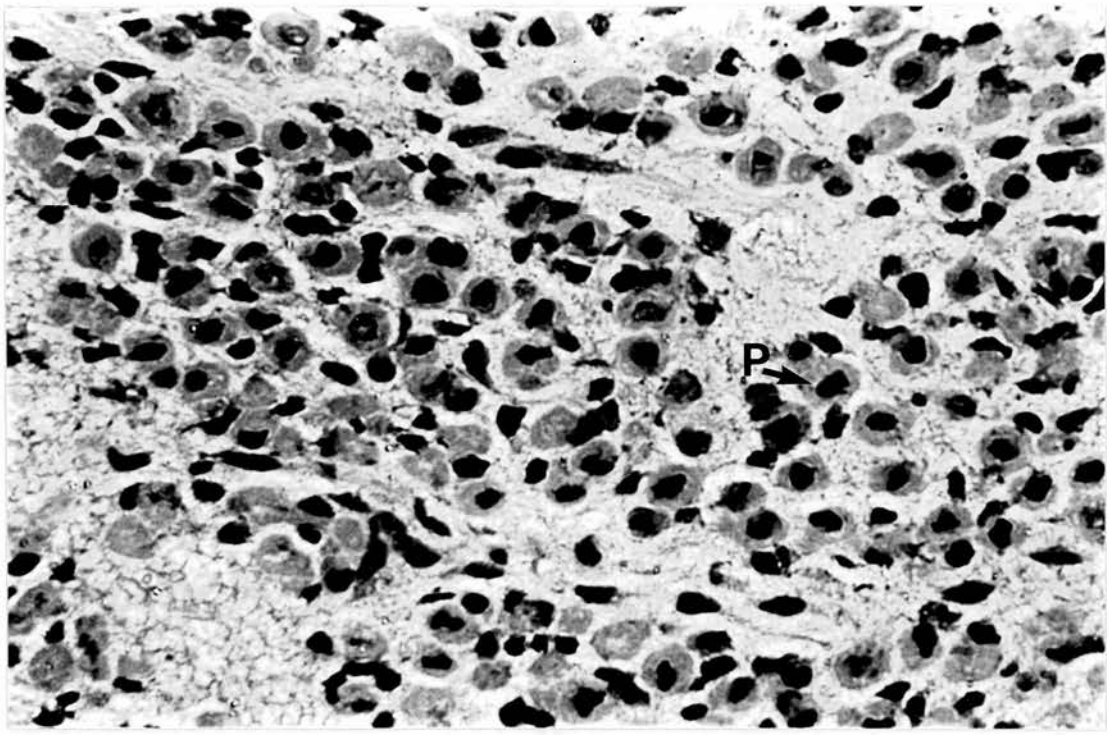


Fig.19 Largest fetus. 38 days. P = Primary fibre.

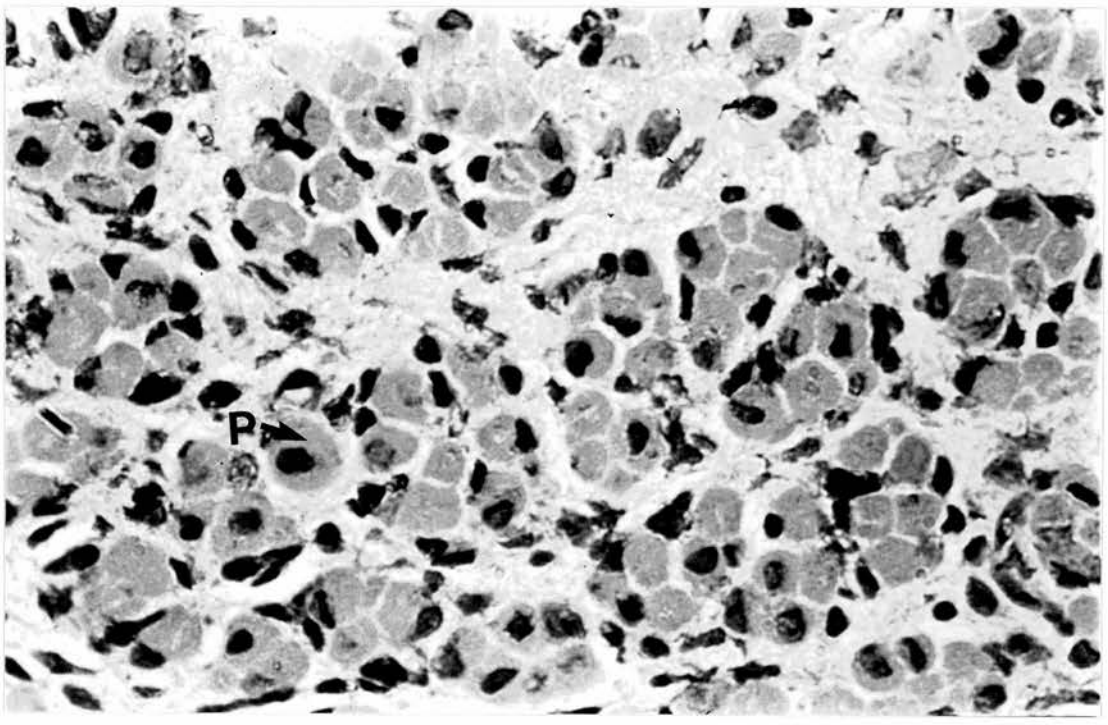


Fig.20 Smallest fetus. 38 days. P = Primary fibre.

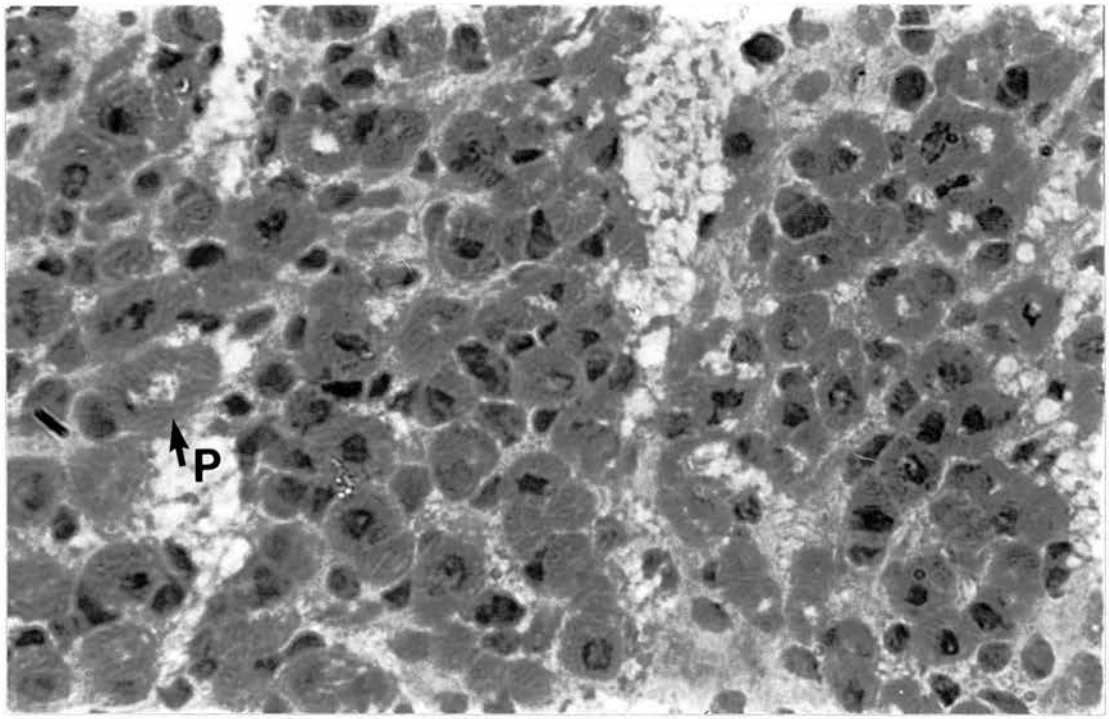


Fig.21 Largest fetus. 46 days. P = Primary fibre.

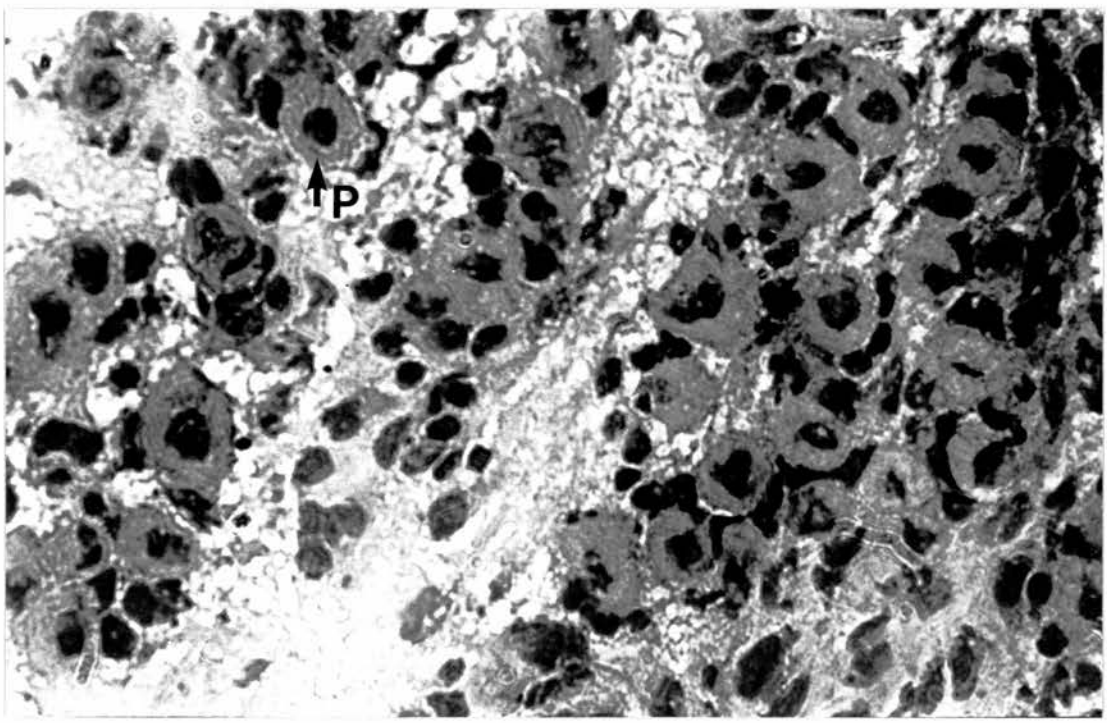


Fig.22 Smallest fetus. 46 days. P = Primary fibre.

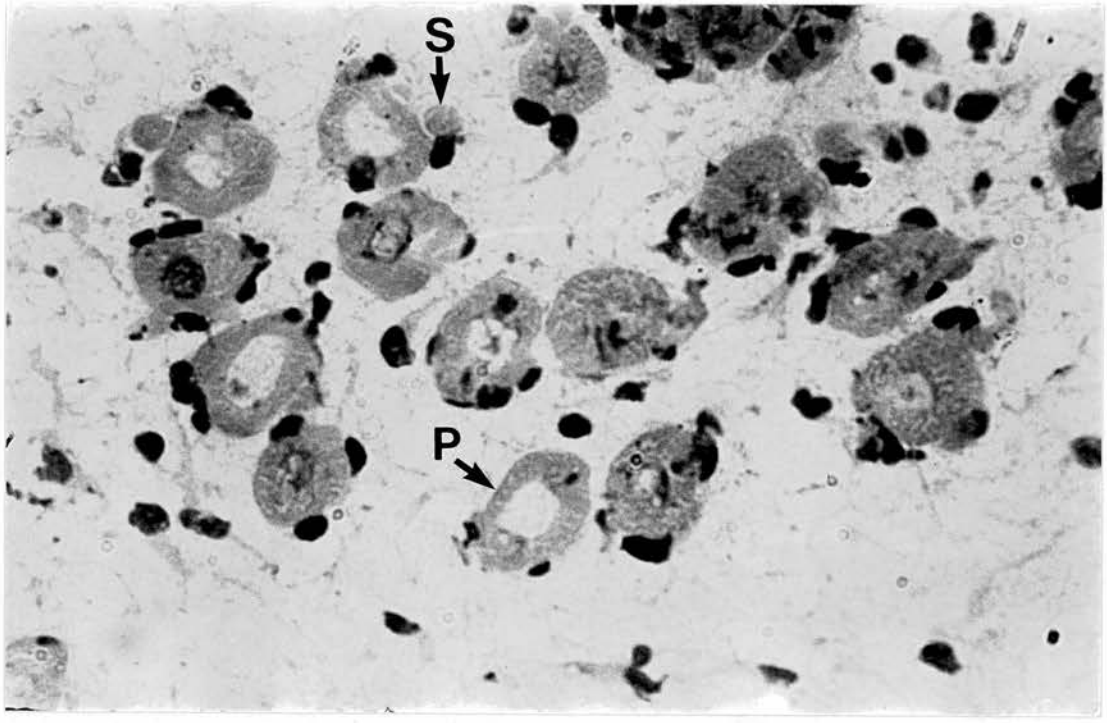


Fig.23 Largest fetus. 54 days. P = Primary fibre S = Secondary fibre.

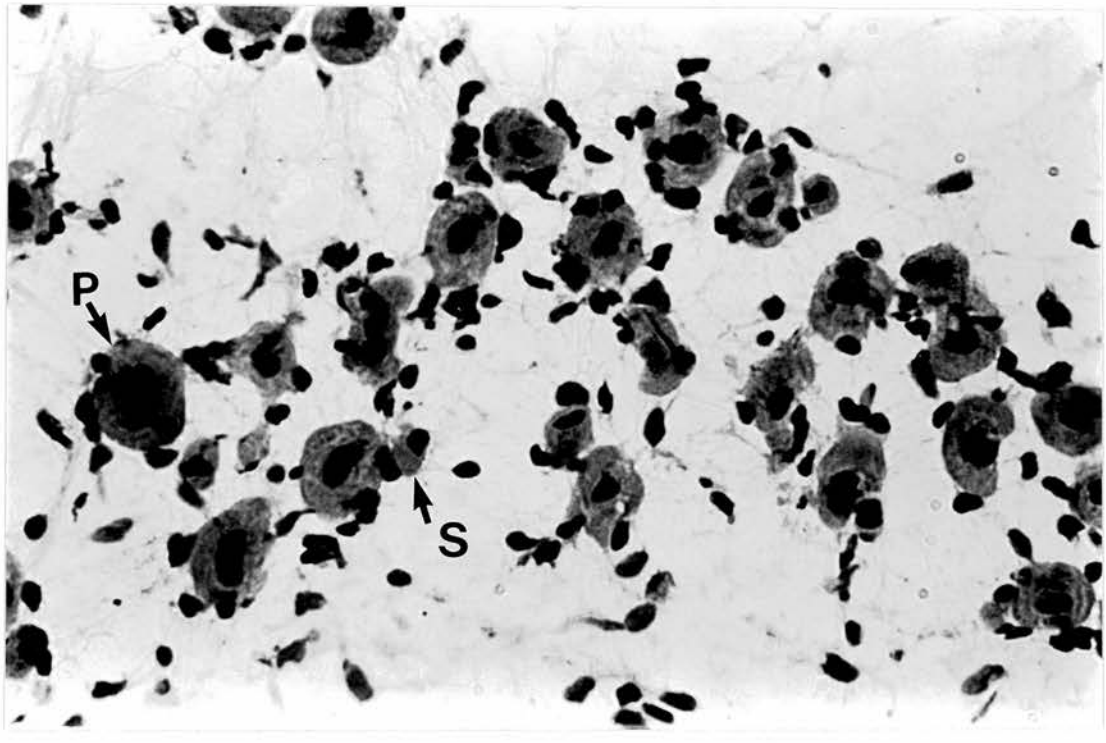


Fig.24 Smallest fetus. 54 days. P = Primary fibre S = Secondary fibre.

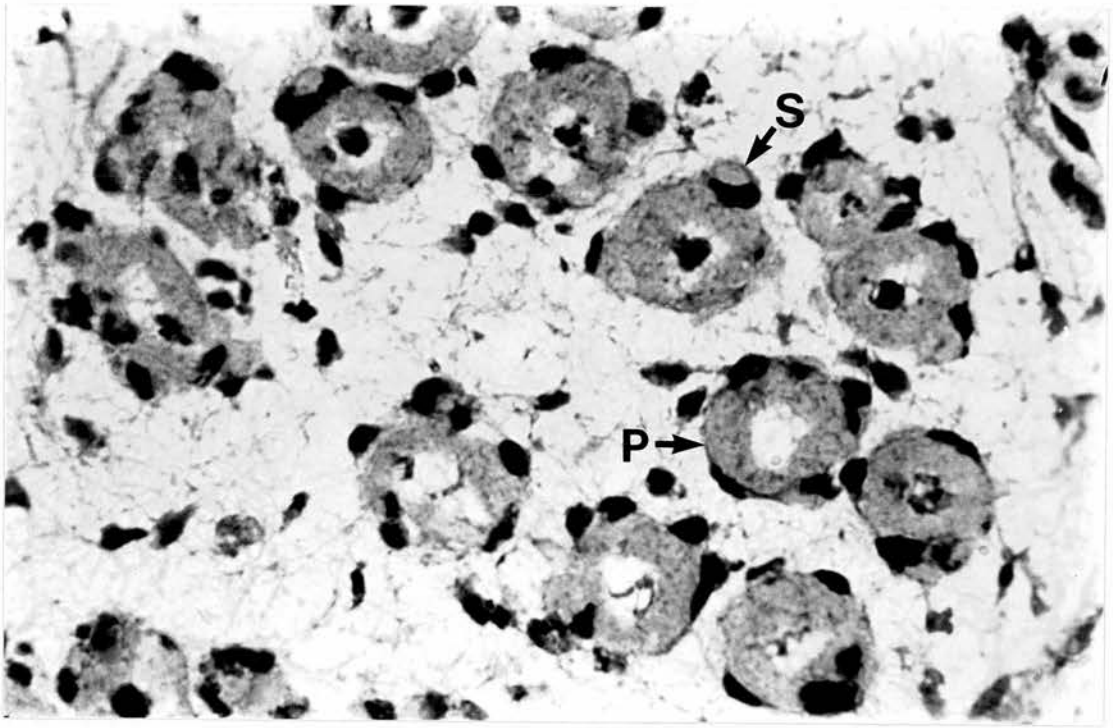


Fig.25 Largest fetus. 59 days. P = Primary fibre S = Secondary fibre.

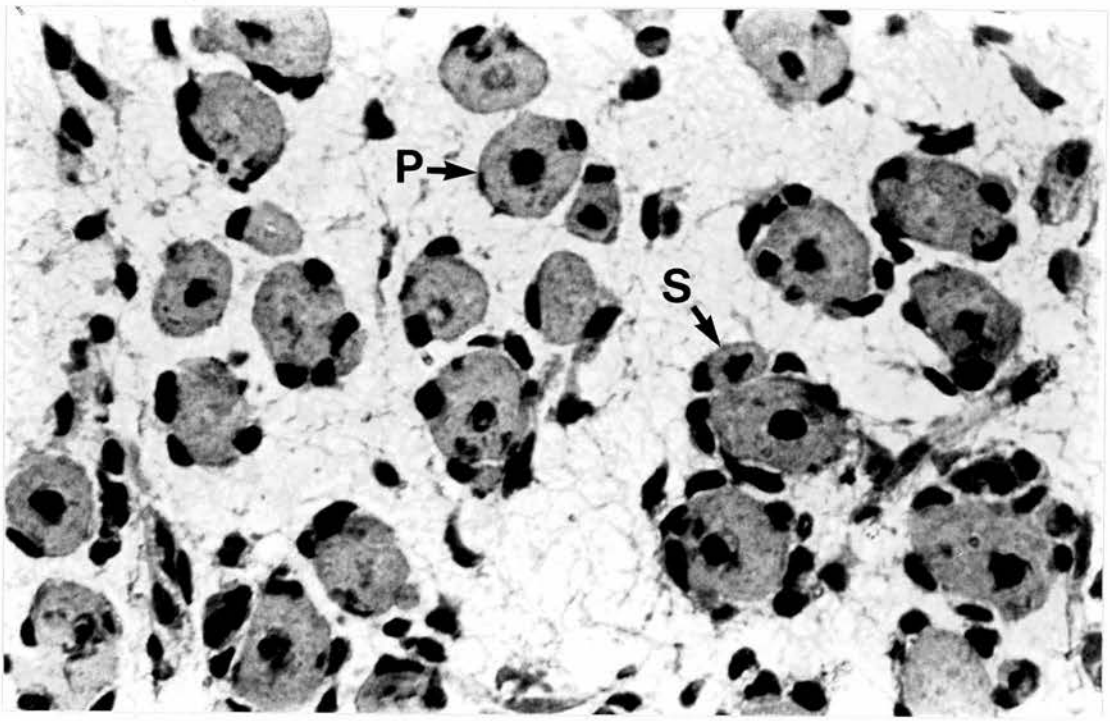


Fig.26 Smallest fetus. 59 days. P = Primary fibre S = Secondary fibre.

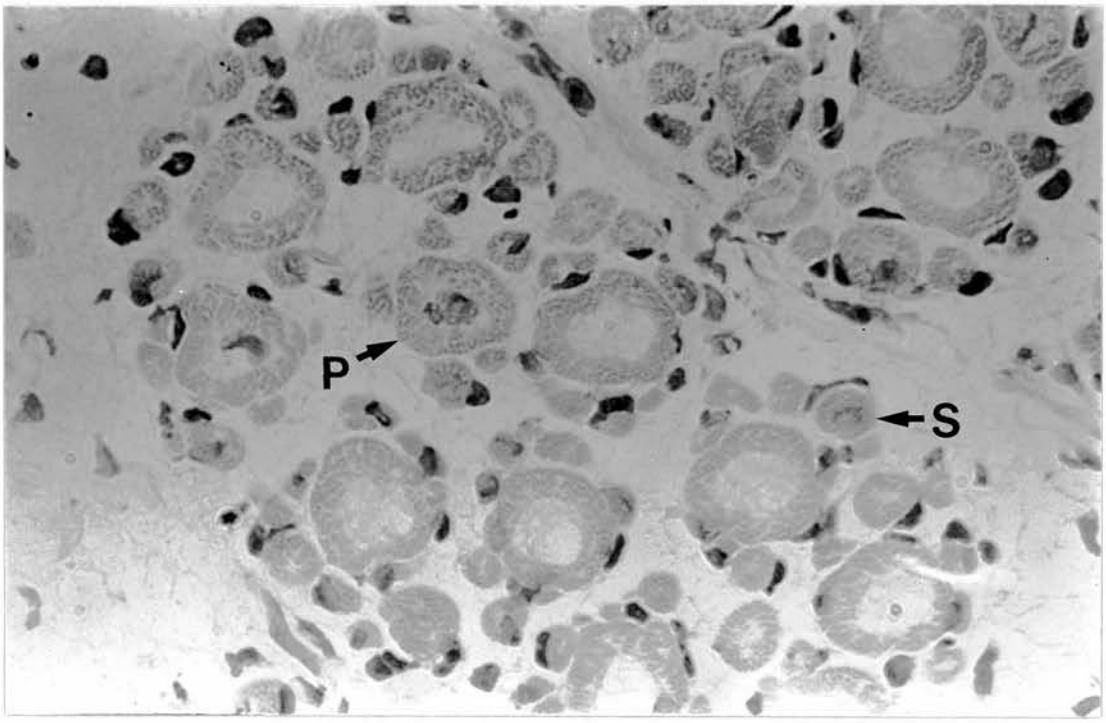


Fig.27 Largest fetus. 64 days. P = Primary fibre S = Secondary fibre.

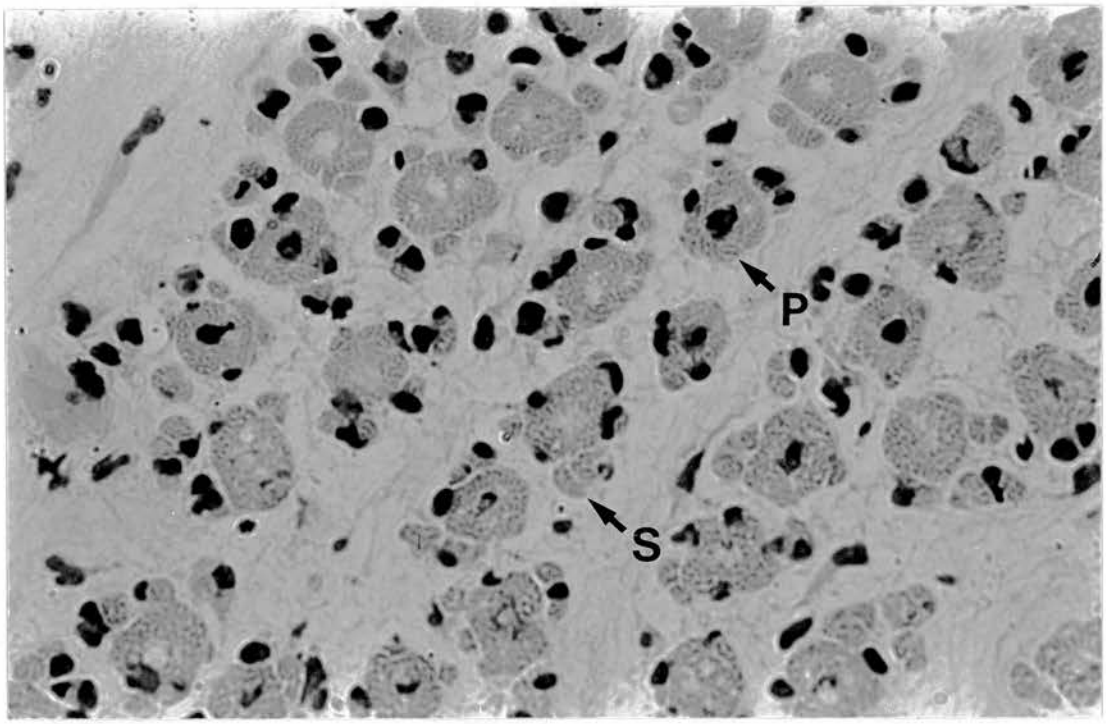


Fig.28 Smallest fetus. 64 days. P = Primary fibre S = Secondary fibre.

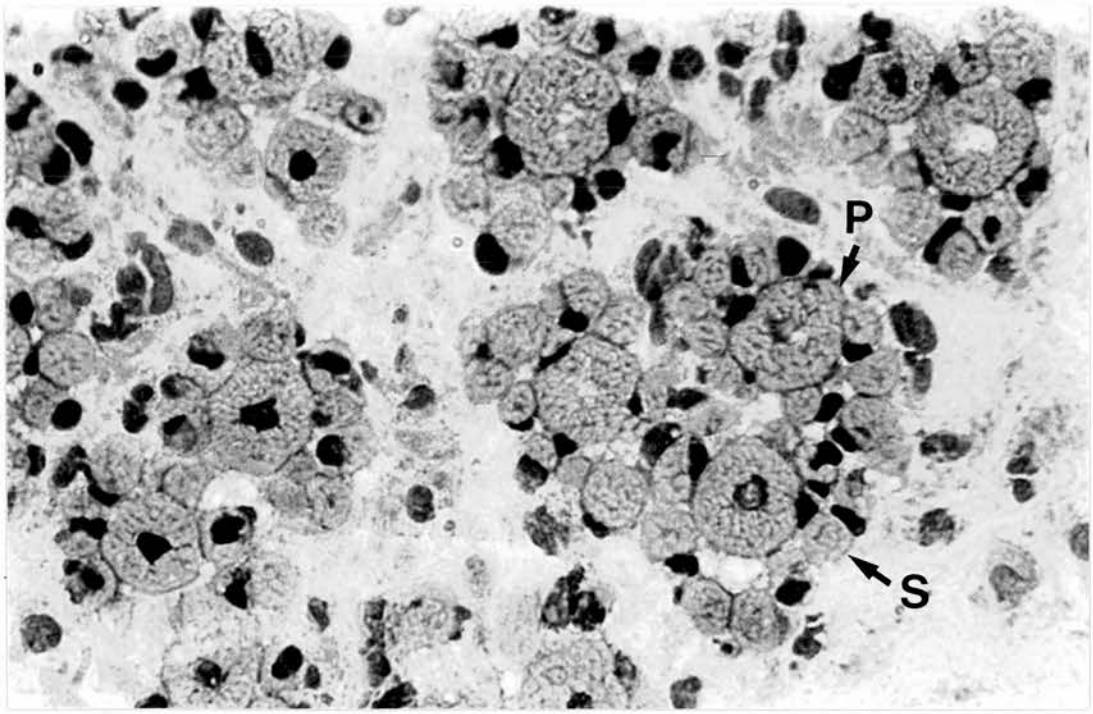


Fig.29 Largest fetus. 69 days. P = Primary fibre S = Secondary fibre.

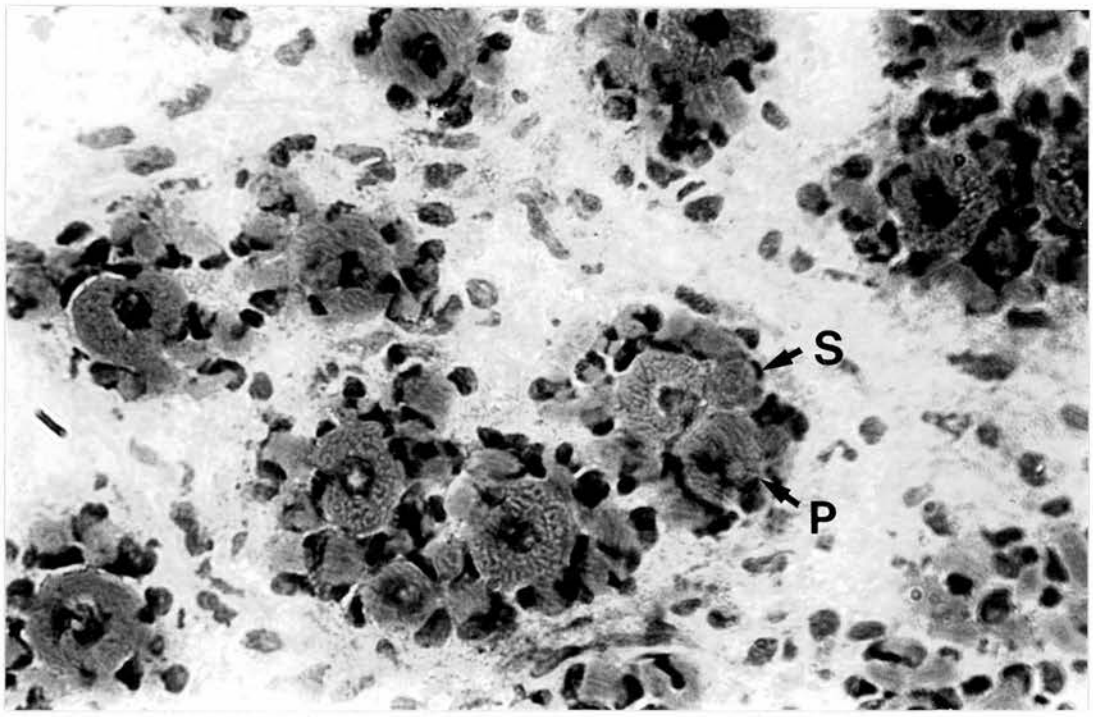


Fig.30 Smallest fetus. 69 days. P = Primary fibre S = Secondary fibre.

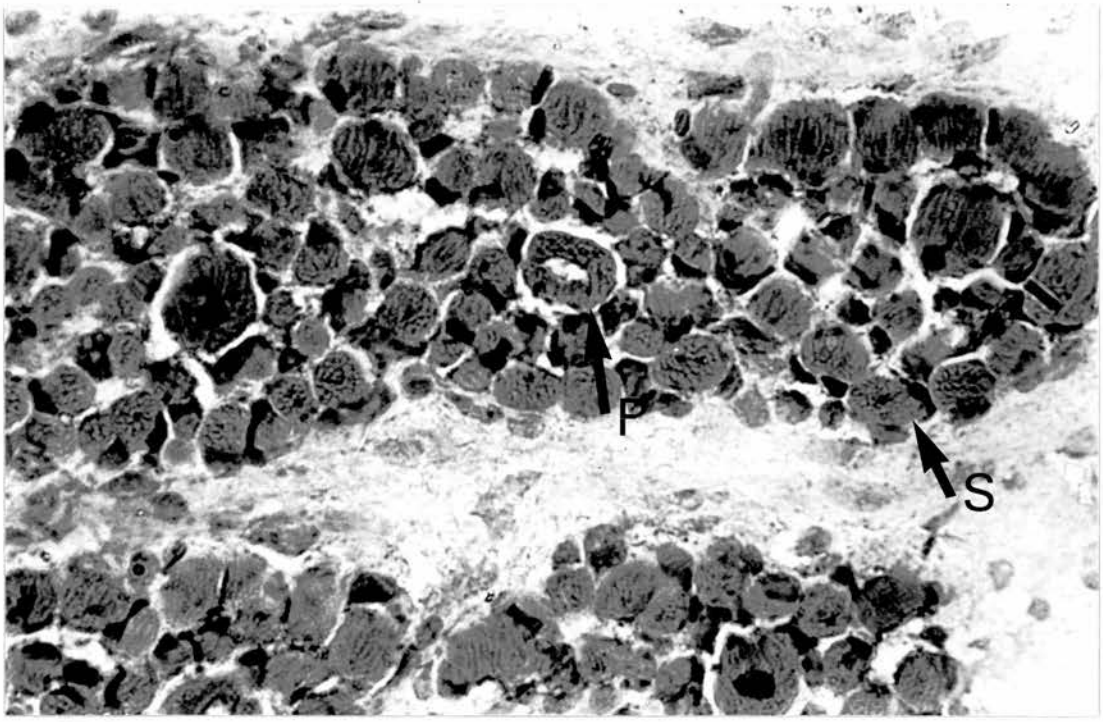


Fig.31 Largest fetus. 75 days. P = Primary fibre S = Secondary fibre.

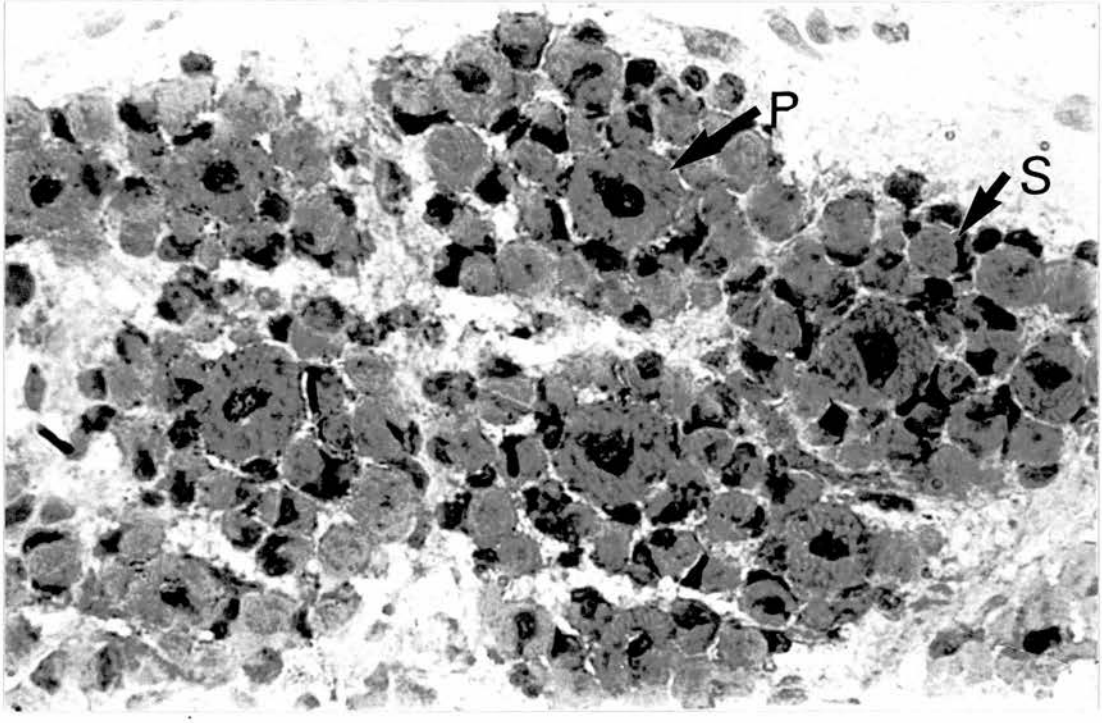


Fig.32 Smallest fetus. 75 days. P = Primary fibre S = Secondary fibre.

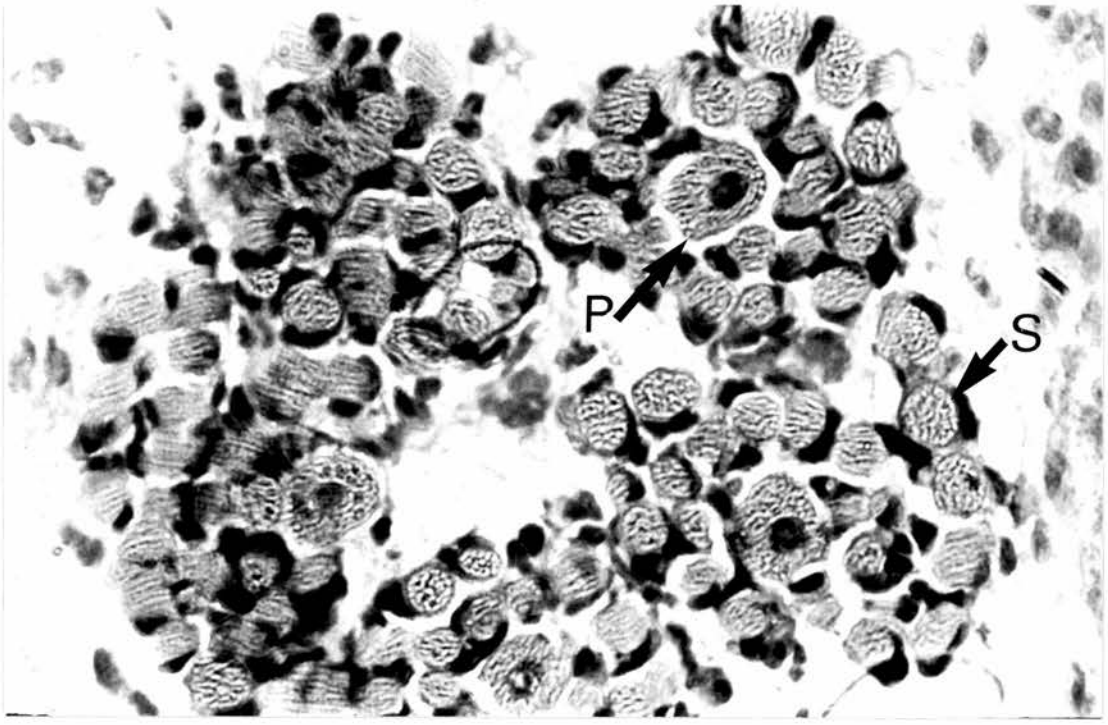


Fig.33 Largest fetus. 82 days. P = Primary fibre S = Secondary fibre.

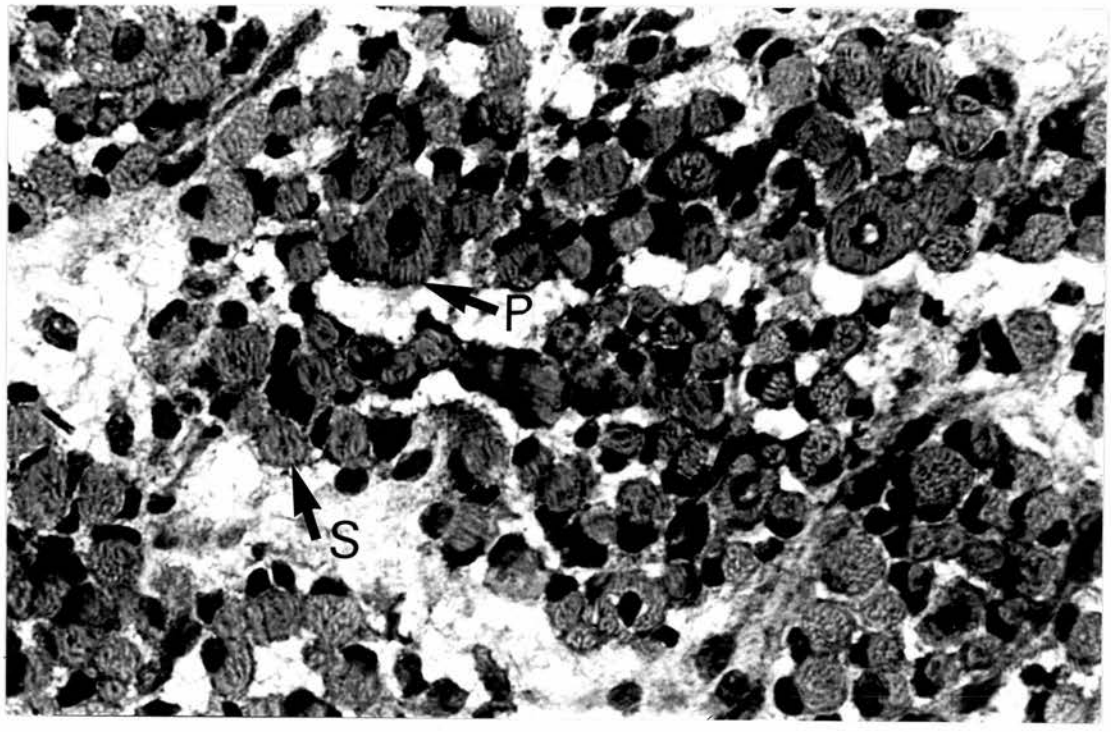


Fig.34 Smallest fetus. 82 days. P = Primary fibre S = Secondary fibre.

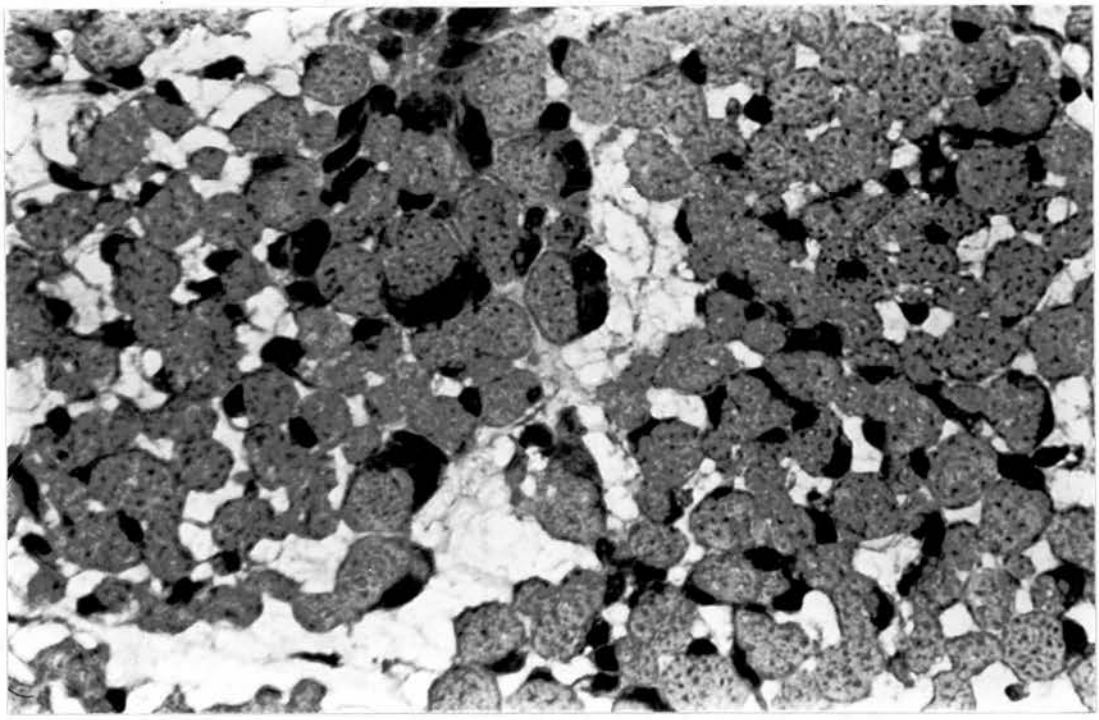


Fig.35 Largest fetus. 114 days (new born).

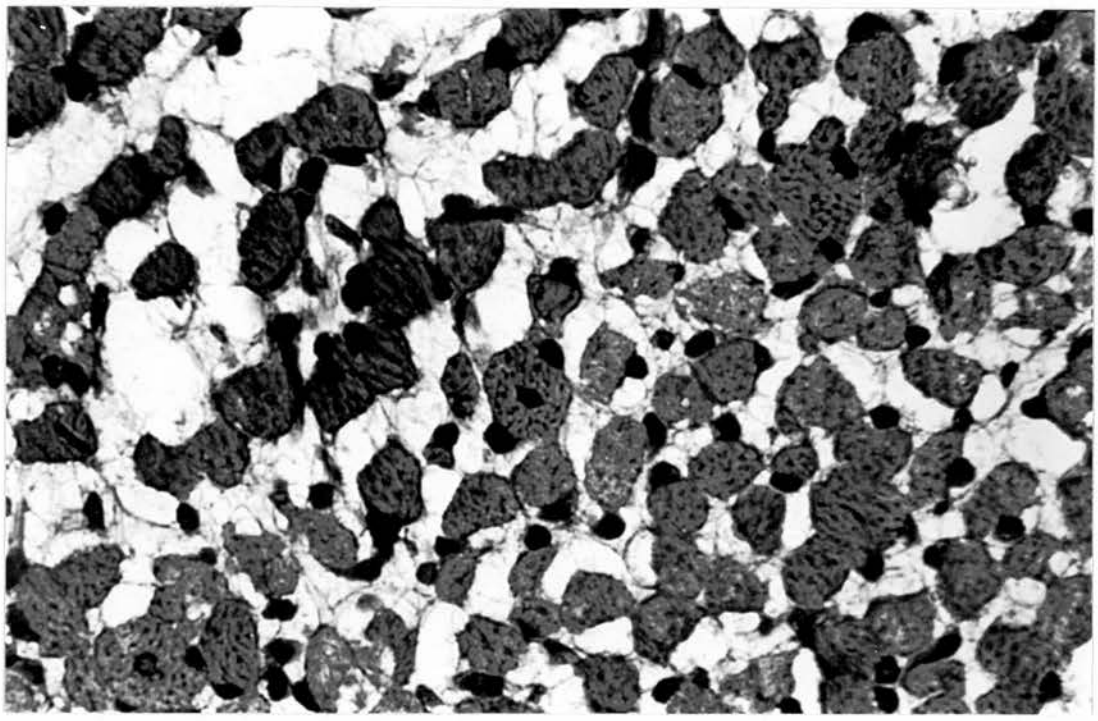


Fig.36 Smallest fetus. 114 days (new born).

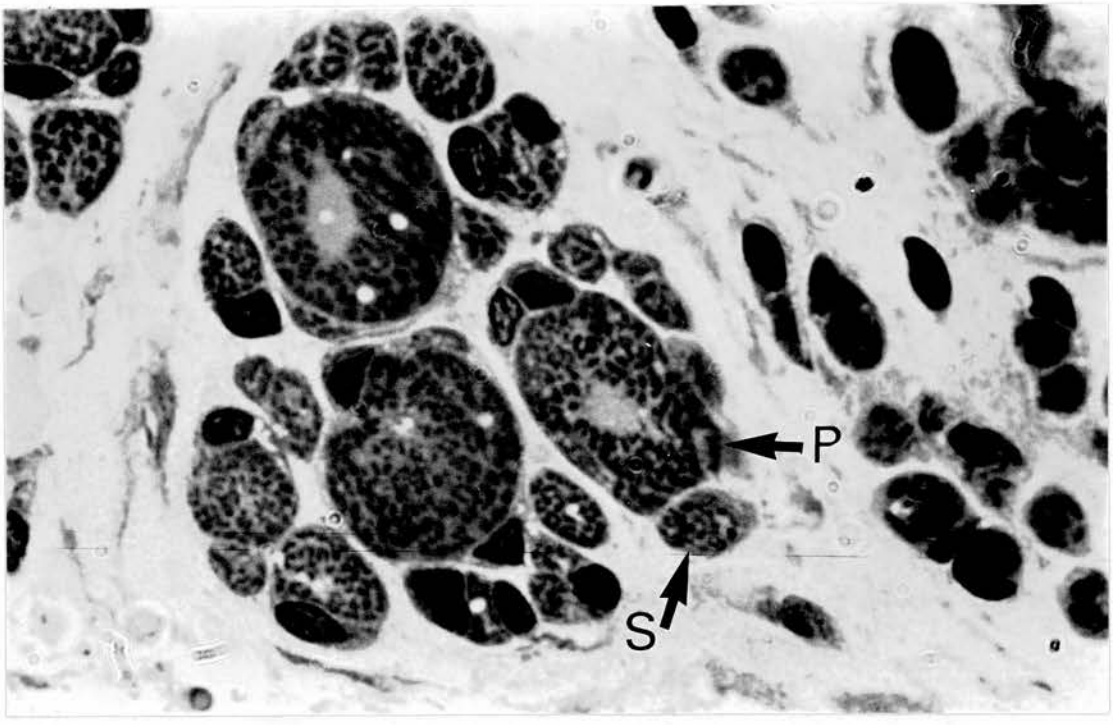


Fig.37 Largest fetus. 64 days. P = Primary fibre S = Secondary fibre.

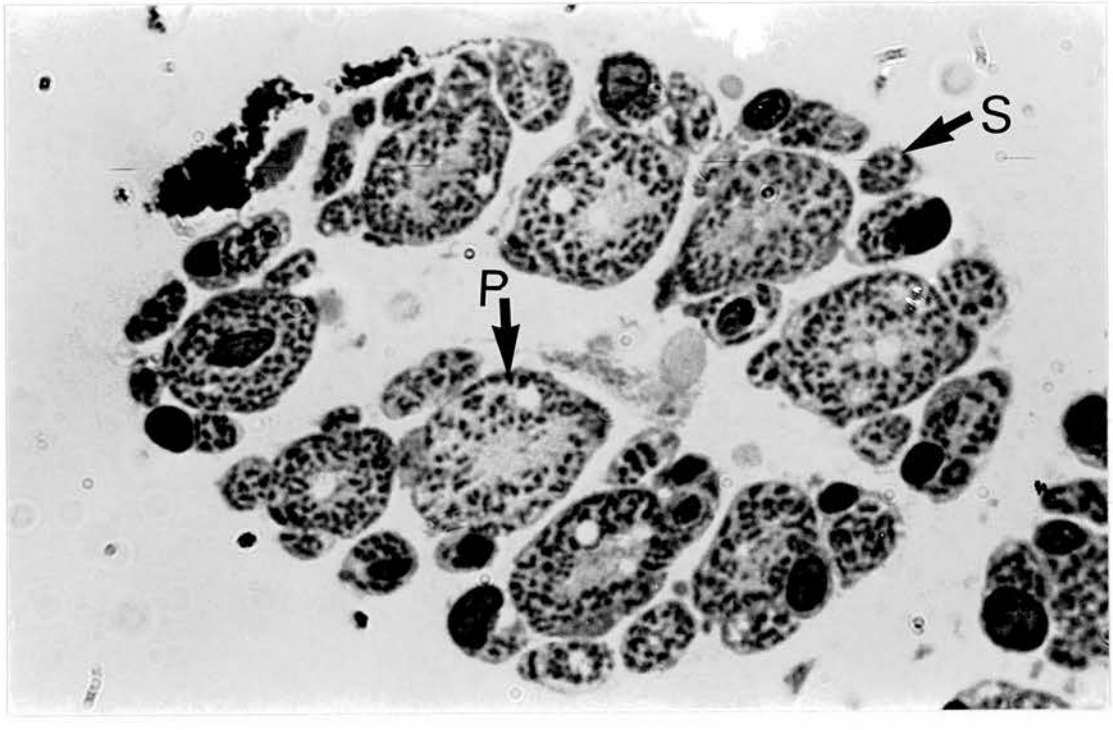


Fig.38 Smallest fetus. 64 days. P = Primary fibre S = Secondary fibre.

Longitudinal plastic embedded sections.

Fig.39 54 days.

Fig.40 64 days.

Fig.41 90 days.

P = Primary fibre.

S = Secondary fibre.

N = Nucleus of primary fibre.

C = Mononuclear cell on the surface of a fibre.

M = Myofibrillar free space in primary fibre.

Fig.39

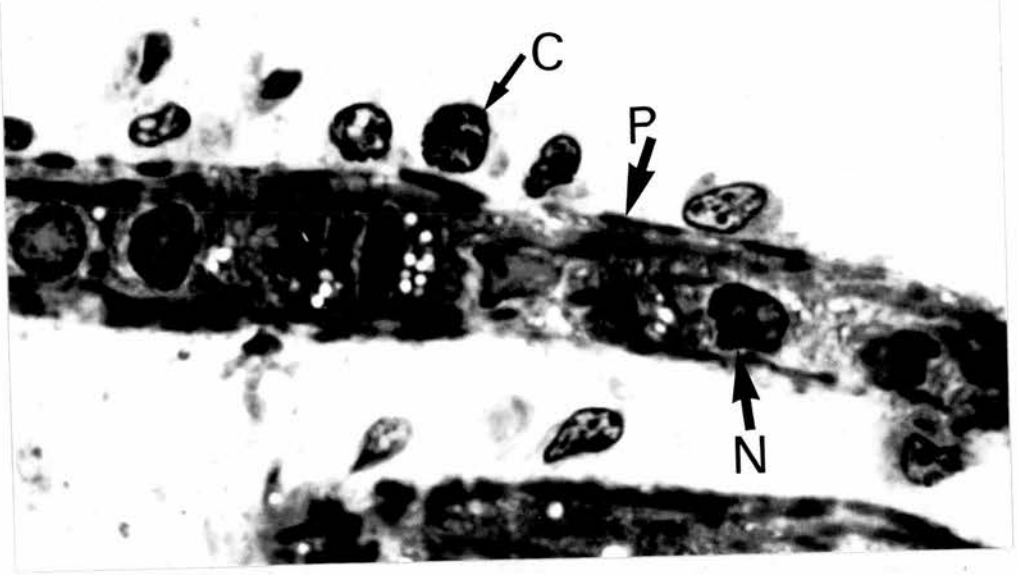


Fig.40

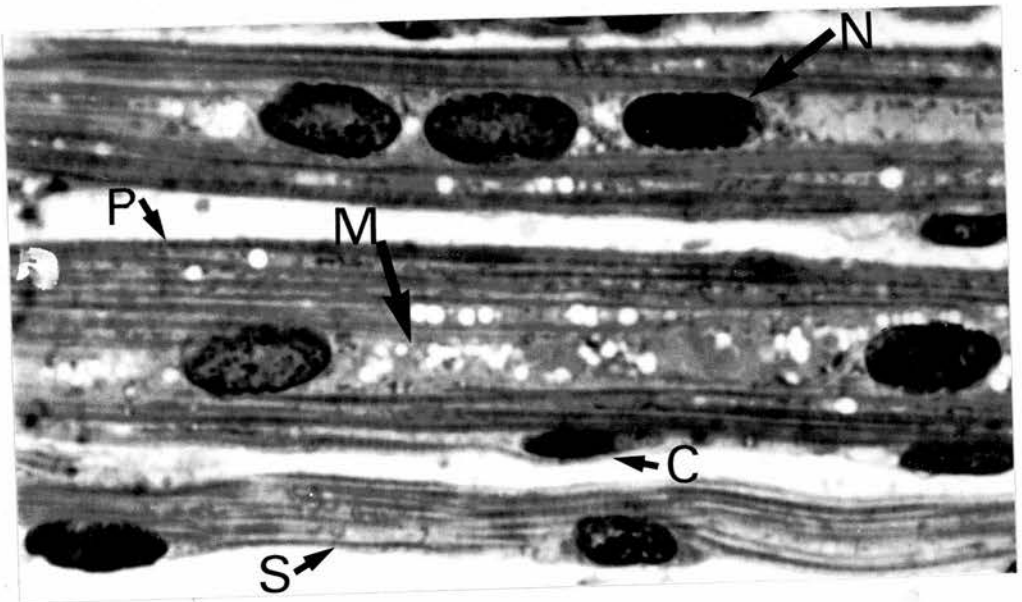
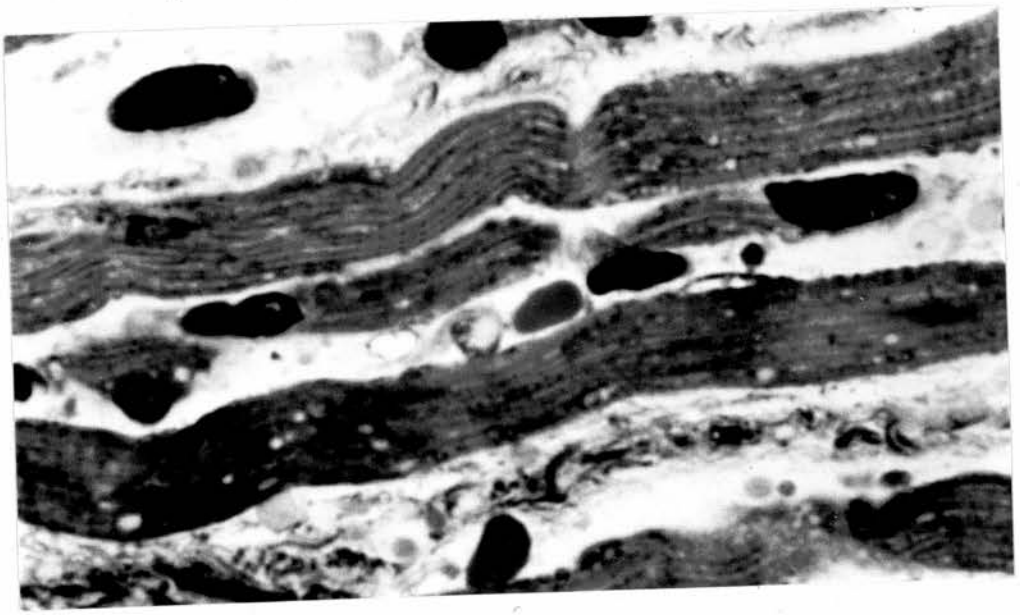


Fig.41



Quantification of muscle fibre parameters

Fig. 42 shows the changes in fibre diameter with age for pairs of littermates at each age. At the earliest age (38 days) both large and small had the same size of primary fibre. These fibres increased in diameter over the following weeks but the primary fibres in larger animals reached greater diameter than those in the small (22.8 μ m. as opposed to 17.4 μ m.). At their maximum size (64 days) it was noticeable that primary fibres in large animals varied in diameter depending on whether a nucleus was present in the plane of section (Fig. 27). In those parts of the fibre between nuclei, the fibre diameter was 10 per cent greater than when there was a nucleus present. This variation in diameter is caused by the expansion of the M.F.R. between nuclei. This 'swelling out' of primary fibres in large animals is responsible for most of the size difference between large and small. At their maximum sizes the M.F.R. can constitute up to 60 per cent of the diameter of primaries in large animals but only 30 per cent in small. The maximum primary size was reached earlier in the larger animals (64 days) than in the small (82 days). Towards the end of gestation primary fibre size decreased and many became indistinguishable from secondary fibres. A few relatively large primaries persisted until term, the size of these being the same in both large and small. The size difference between the primaries in the two groups of animals was statistically significant throughout gestation.

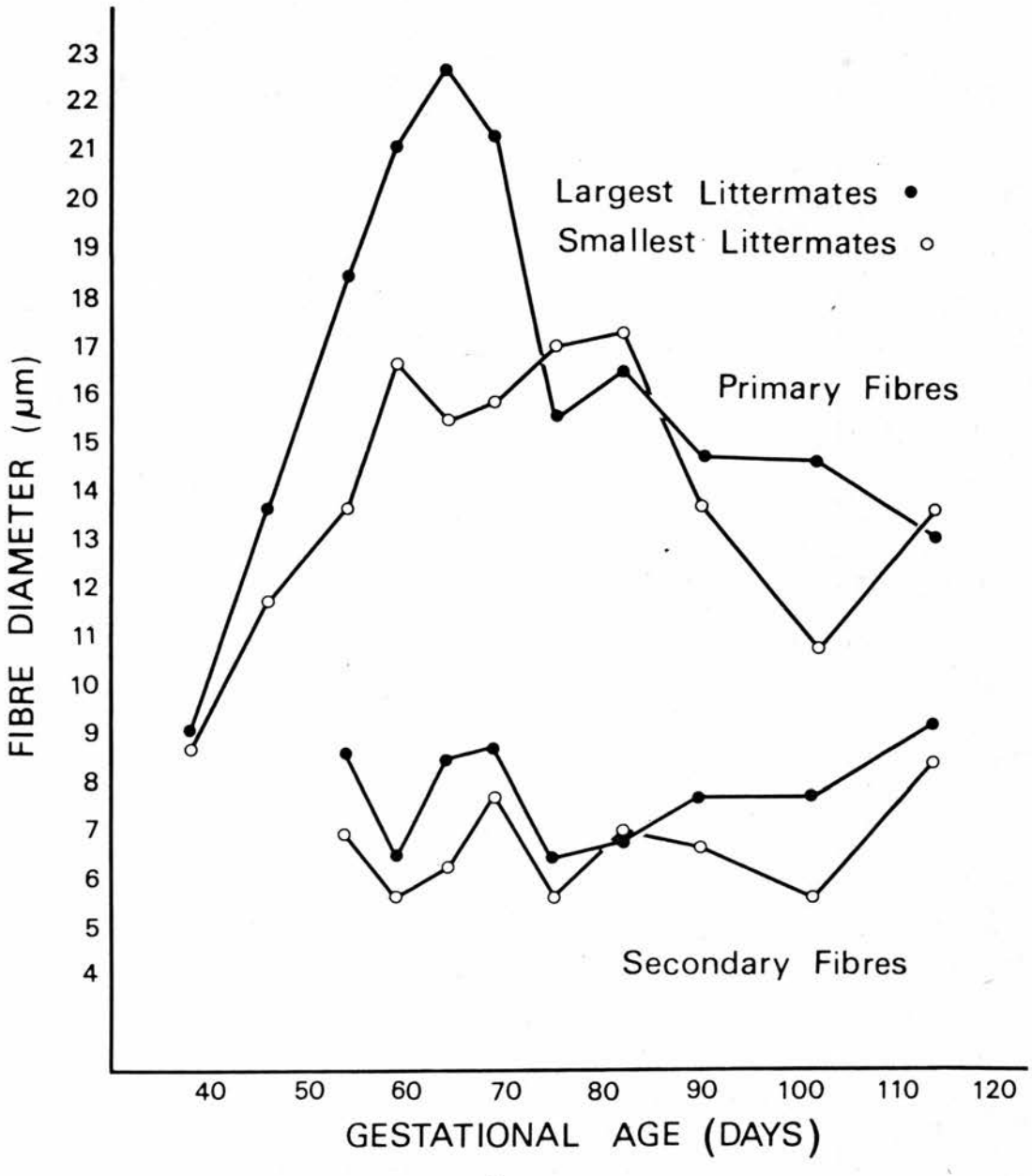


Fig.42 Fibre diameter plotted against gestational age. Primary and secondary fibres for both the largest and smallest littermates are shown.

Secondary fibres appeared at about 54 days and were significantly greater ($P < 0.005$) in diameter in the large animal throughout gestation. The mean size of secondaries in both large and small remained relatively constant until 102 days but hypertrophy may have started just before term. Measurement of the maximum secondary fibre size at each age, obtained from the two largest fibres in each sample square, also showed little change with age. Those in large animals had a maximum of about 9 μ m. compared to 8 μ m for those in small animals.

The graph of fibre number against age (Fig. 43) shows little initial difference in fibre number between large and small littermates. Fibre number increased slightly during the period 38 - 54 days when only primary fibres were forming. After 54 days fibre number increased rapidly due to secondary fibre formation, the maximum rate of increase being between 60 and 80 days. After this the rate of fibre formation declined and it appears that no new fibres were being formed by the time of birth. From 60 days onwards, the larger littermates had more fibres in its muscle than the smaller and at term this difference was approximately 17 per cent.

The points for both groups of animals in Fig. 43 appear to follow a sigmoid curve and so a logit transformation was used to fit curves (Fig. 44). The upper asymptote for each curve was estimated by taking the mean of the three oldest results for each group. The equations of these lines when log. X is plotted against the logit transformed values of Y are $Y = 22.8X - 41.9$ for large and $Y = 22.9X - 40.3$ for small animals.

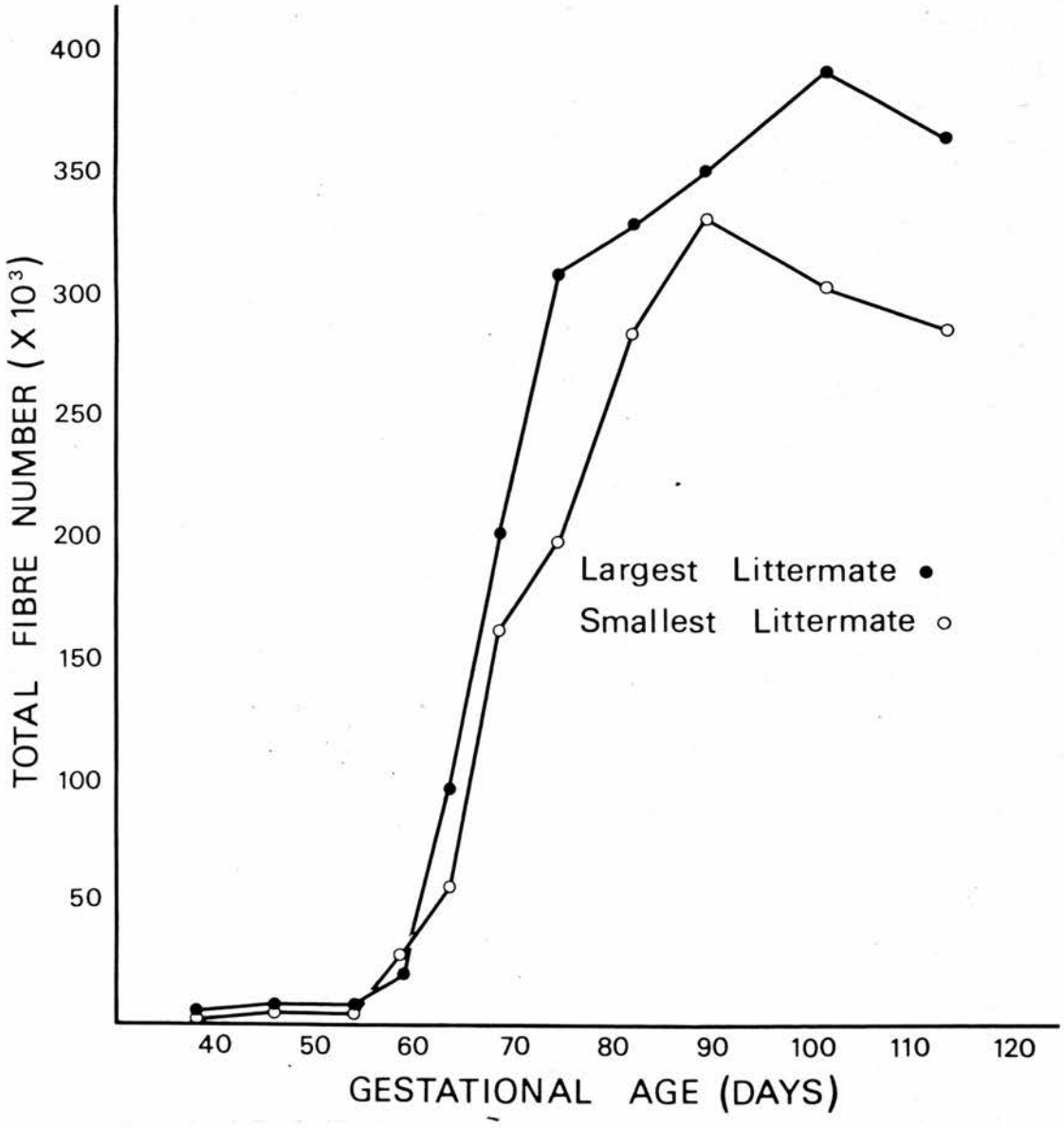


Fig.43 Total fibre number in the m. semitendinosus plotted against gestational age for both largest and smallest littermates.

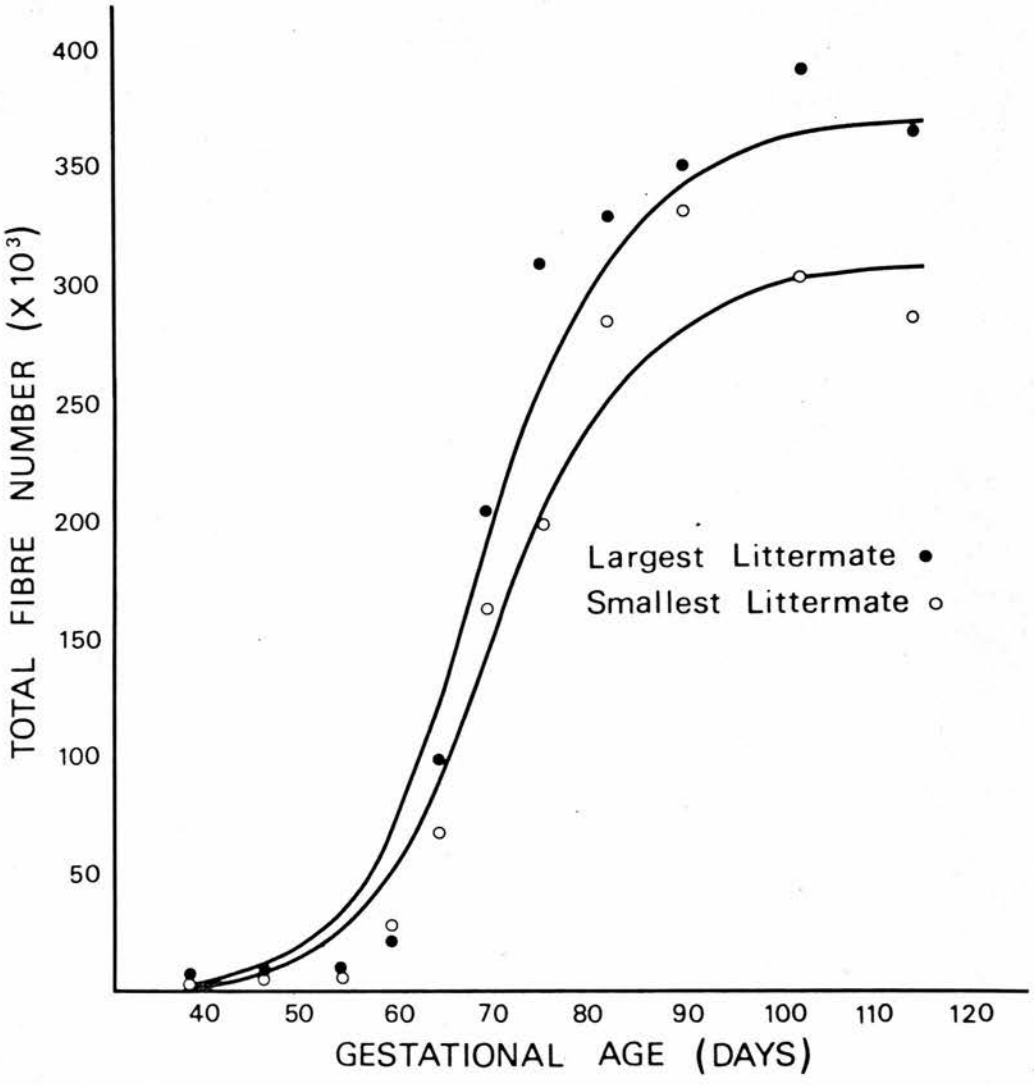


Fig.44 Total fibre number in the m. semitendinosus plotted against gestational age for both the largest and smallest littermates. Logit curves are fitted to the points.

The correlation coefficients for birth lines were highly significant ($P < 0.01$).

An alternative method of looking at changes in fibre number is to measure the number of secondaries surrounding each primary. The increase in this ratio (secondary no./primary no.) is shown in Fig. 45, where it is plotted against age. The apparent disappearance of primary fibres after 75 days (Fig 46) made calculation of the ratio difficult after this age. The final value can however be estimated from the total fibre number at term and the maximum observed number of primaries. These figures give a final ratio of 19.7 for large and 16.4 for the small. A different technique for calculating the ratio late in gestation, is to use histochemical staining (see later section) which can still differentiate primary and secondary fibres when they are morphologically similar. Only a few photographs for large animals were available and these give a ratio of 20 at 82 days and 21 at 102 days. The constancy of the ratio between these two ages strongly suggests that fibre hyperplasia has ceased by this time. This result is close to the assessment of the end of fibre formation from calculations of total fibre number (Fig. 43 and 44) where there is little increase in fibre number after 90 days. From the estimated ratio values at term for large and small given above (19.7 and 16.4) it is apparent that more secondaries form on each primary in large animals than in small animals. Using the figures shown in Fig. 45, this difference is statistically significant ($P < 0.05$).

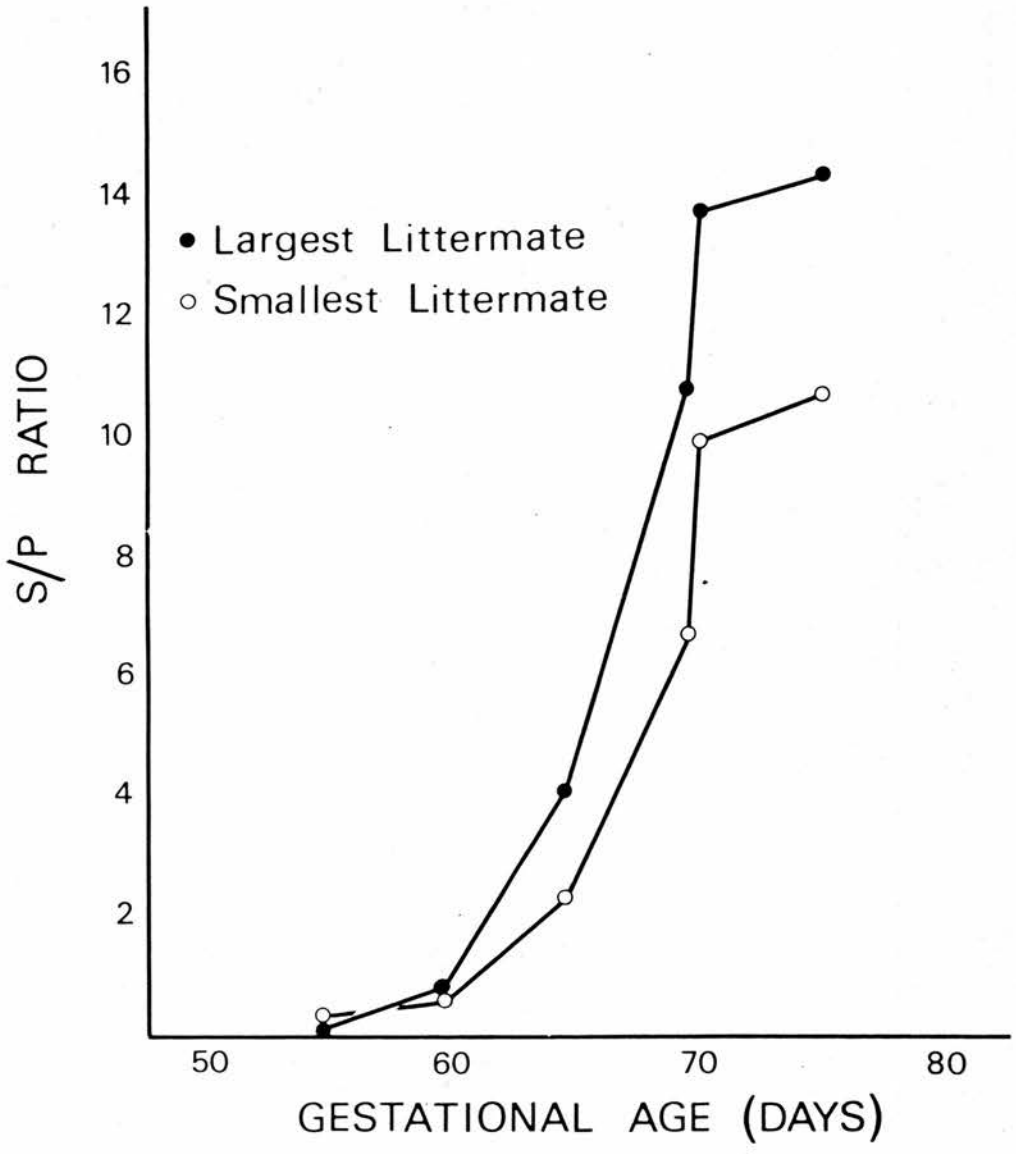


Fig.45 The secondary / primary fibre ratio (S / P Ratio) plotted against gestational age for both the largest smallest littermates.

When only primary fibre number is plotted against age (Fig. 46), new fibres are formed until between 59 and 64 days. After this, primary fibre number remains constant until 75 days when their number starts to decline. As previously described this is due to the difficulty in recognising primary fibres after they have cost their M.F.R. and have subsarcolemmal nuclei. The total number of primary fibres formed in the semitendinosus muscle is about 18,500. A paired t-test on primary fibre number during the period of secondary fibre formation (54 - 90 days) showed no significant difference between the number of primaries in large and small littermates ($P > 0.5$).

Summing up this section, it was found that, over the period studied (38 days until term) muscle fibre number in the semitendinosus muscle increased from about 3000 to about 350,000 fibres. Of this final total about 95 per cent were secondary fibres. Primary fibres were formed over a period of about 25 days during which approximately 700 fibres per day were forming. Secondary fibre hyperplasia lasted about 35 days and nearly 9500 fibres per day were formed. When large and small littermates were compared a difference in total fibre number was apparent throughout most of gestation (Fig. 43 and 44). This difference was not due to any difference in primary fibre number (Fig. 46) but rather to the fact that more secondaries form on each primary fibre in large animals. It was also noticeable that the time of maximum secondary fibre formation (60 - 80 days) coincided with the period when the greatest number of primary fibres was recognizable (60 - 75 days) and also the time when primary fibres were at their maximum size (60 - 75 days). The results of an investigation into the influence of primary

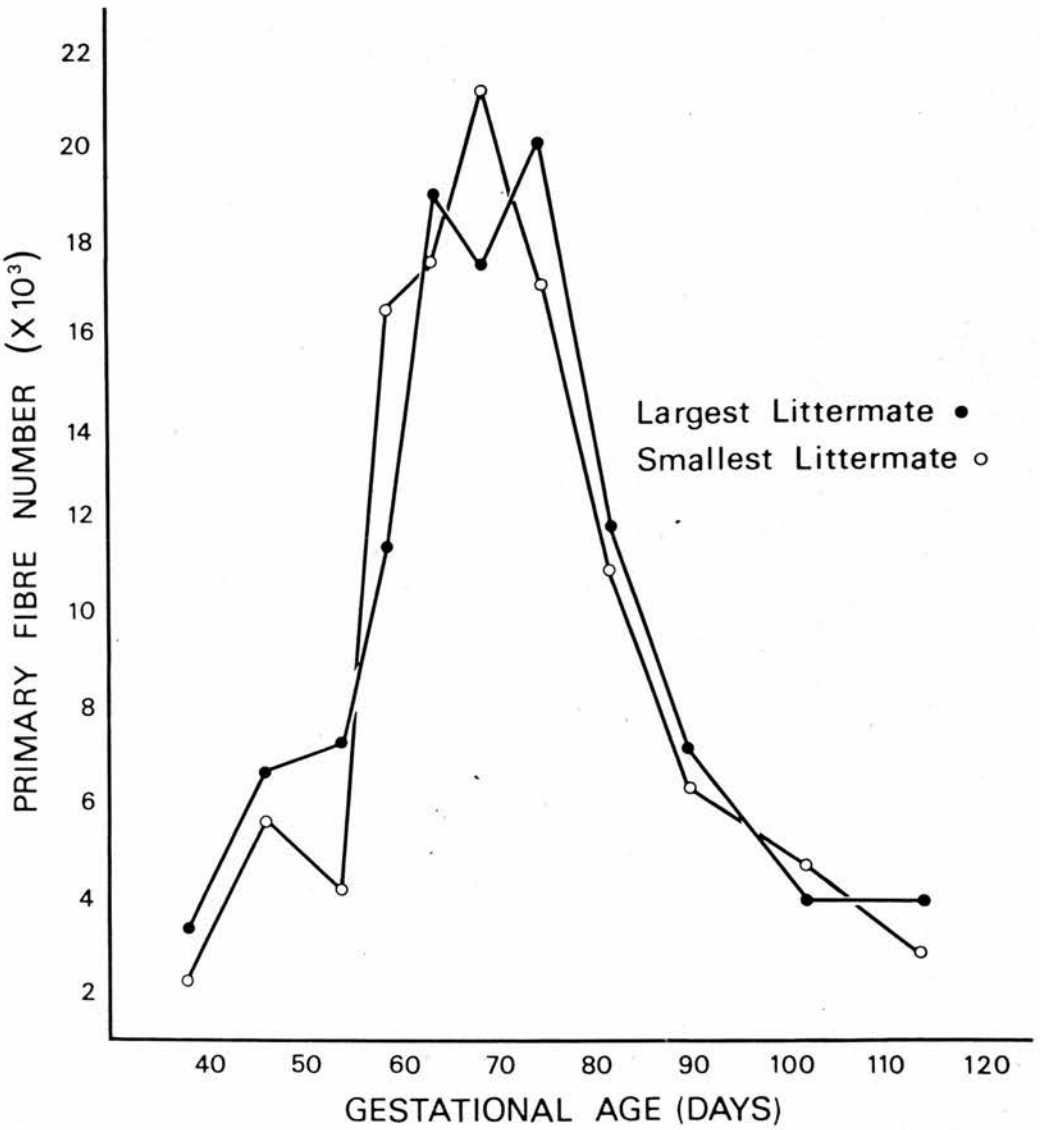


Fig.46 Total primary fibre number in m. semitendinosus plotted against gestational age for both the largest and smallest littermates.

fibre size on secondary fibre formation are given in the following section.

The relationship between fetal weight, primary fibre size and the number of secondaries surrounding each primary

As described in the materials and methods, one litter with a gestational age of 70 days was used to provide frozen sections of the semitendinosus of all 13 members of the litter. From these sections the mean primary fibre diameter and the mean secondary/primary ratio were obtained. These results are shown graphically in Figs. 47 and 52. Fig. 47 shows the relationship between primary fibre diameter and fetal weight, for which the correlation coefficient was significant ($P < 0.05$). This confirms the results given in Fig. 42 which shows that at this age smaller animals have small primary fibres. Figs. 48, 49, 50 and 51 show the difference in primary fibre size in large and small at 64 and 70 days. The graph in Fig. 52 also has a statistically significant correlation coefficient ($P < 0.005$), which indicates that the mean number of secondaries surrounding each primary is proportional to the mean primary size within the muscle. The link between primary fibre size and the secondary/primary ratio was also tested within muscles. This was done by calculating the correlation between these parameters in different regions of the same muscle. Five muscles were tested in this way (large and small 64 and 69 days, large 75 days) and a significant correlation was found in three of them.

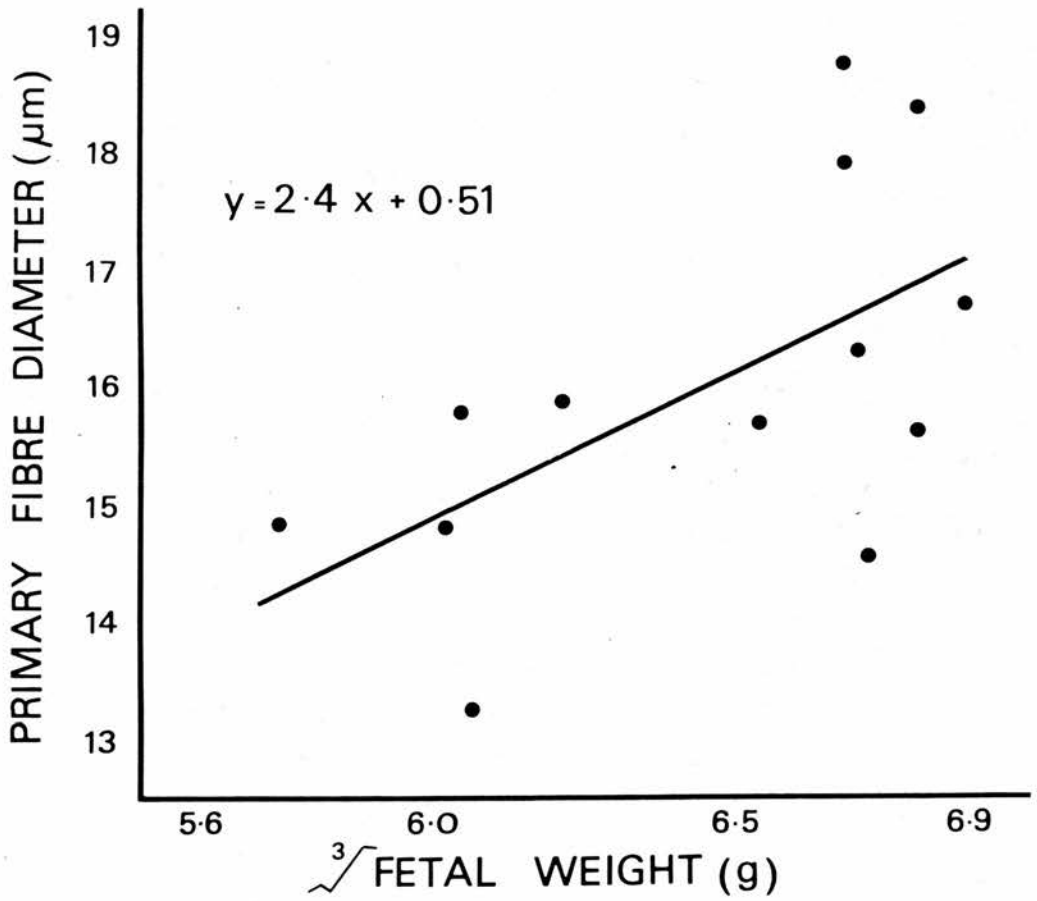


Fig.47 Primary fibre diameter plotted against the cube root of fetal weight for all the individuals of one litter of 70 days gestation.

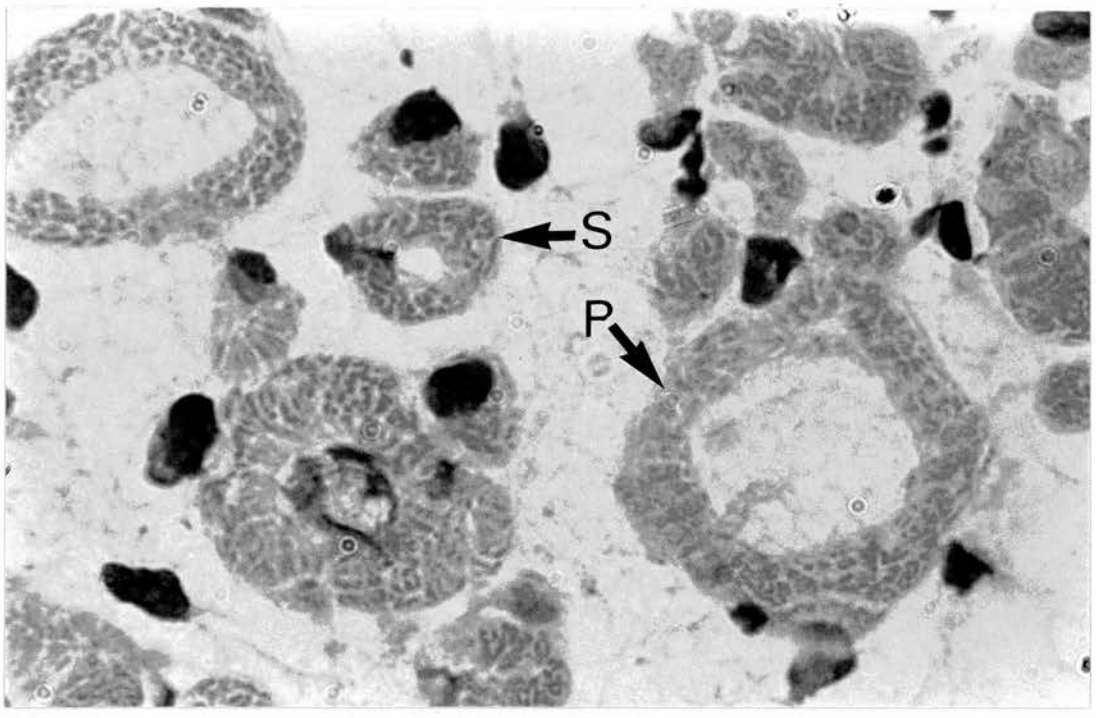


Fig.48 Largest fetus (163 gms). 64 days. P = Primary fibre.

S = Secondary fibre.

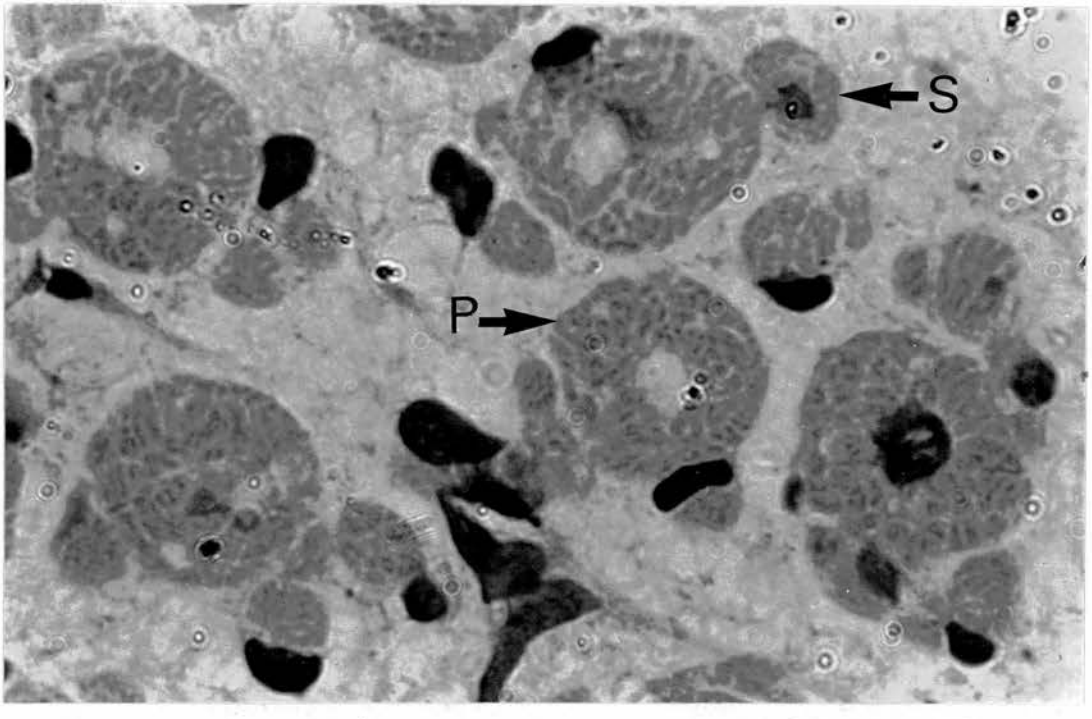


Fig.49 Smallest fetus (84 gms). 64 days. P = Primary fibre.

S = Secondary fibre.

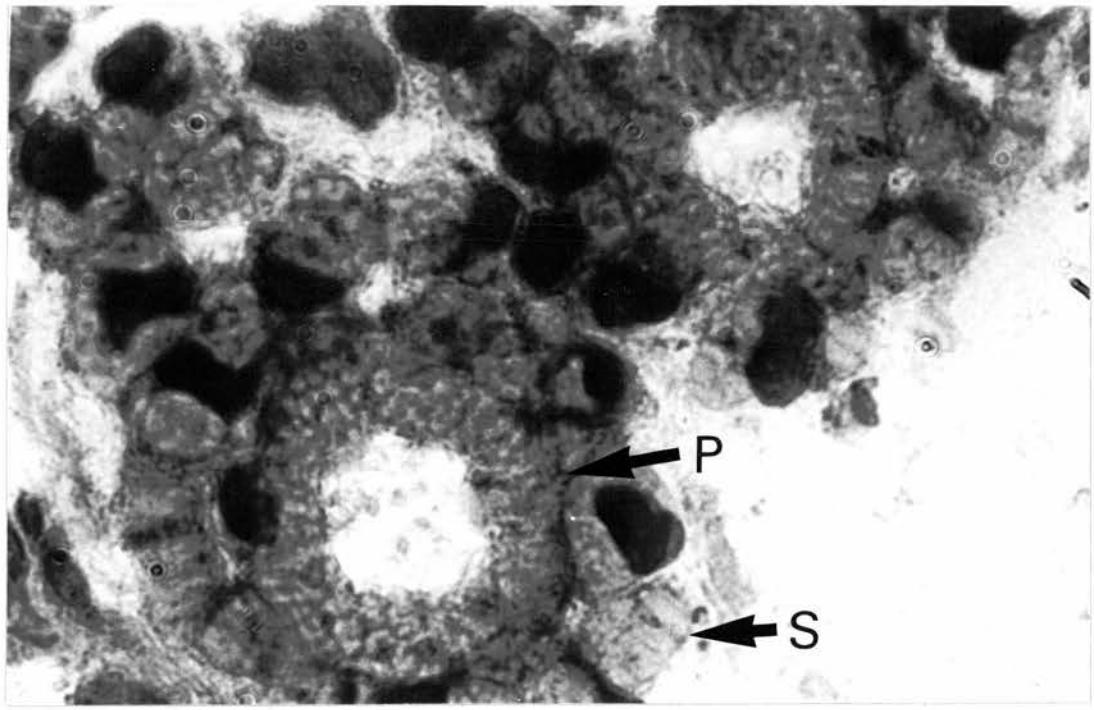


Fig.50 Largest fetus (318 gms). 70 days. P = Primary fibre.

S = Secondary fibre.

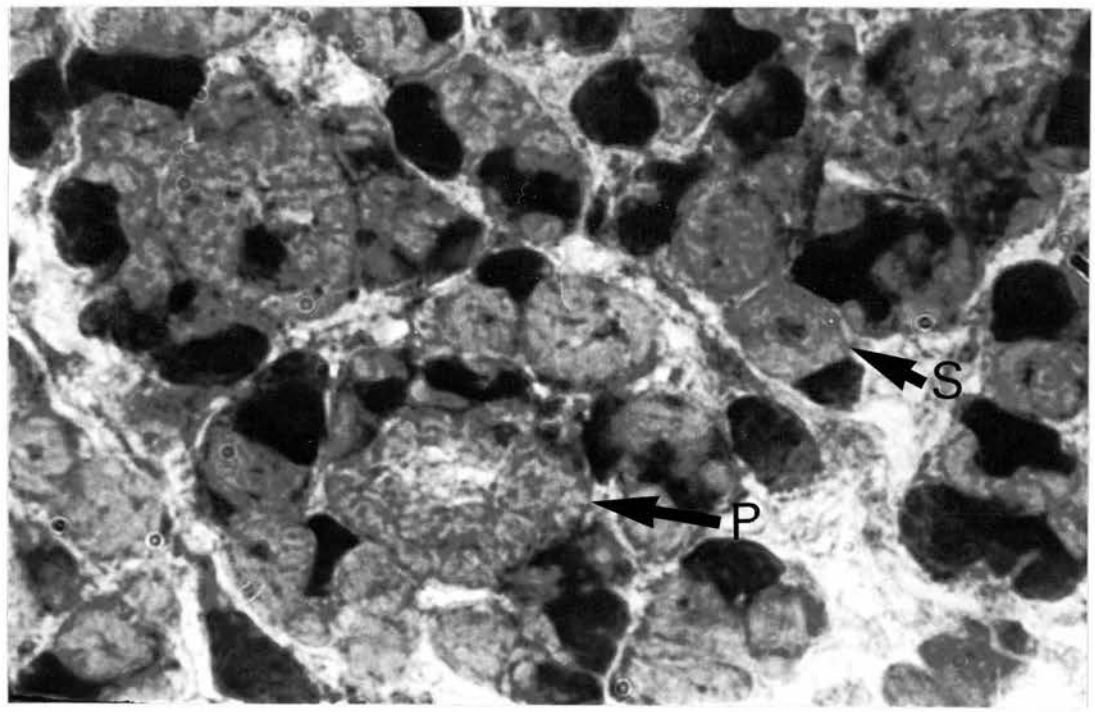


Fig.51 Smallest fetus (188 gms). 70 days. P = Primary fibre.

S = Secondary fibre.

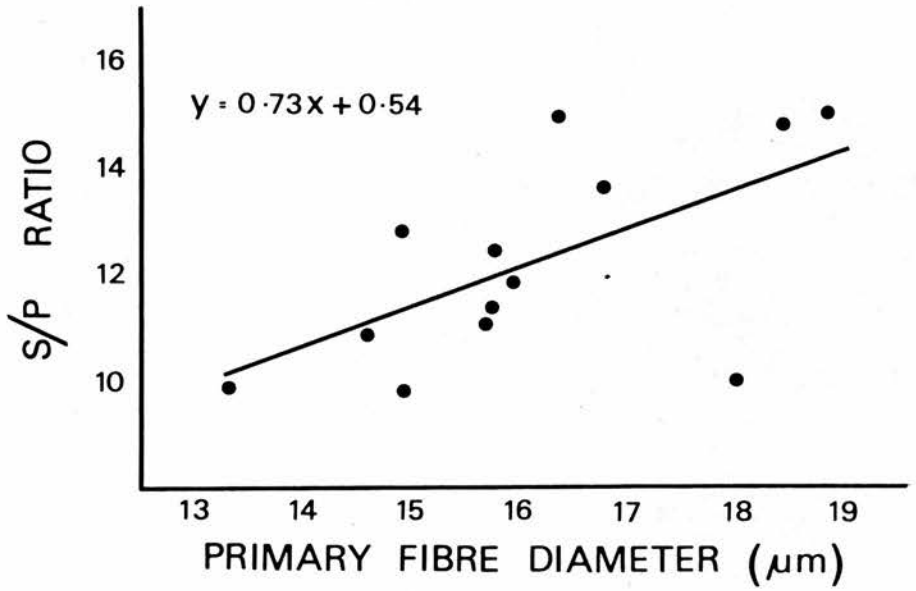


Fig.52 The secondary / primary fibre ratio (S / P ratio) plotted against primary fibre diameter for all the individuals of one litter of 70 days gestation.

Muscle fibre histochemistry

Fresh frozen sections of m. semitendinosus were stained for adenosine triphosphatase (A.T.Pase) after acid preincubation and the results are shown in Figs. 53 to 60. No qualitative difference could be detected between sections from large and small animals and so both are included in this description.

At 38 days gestation no fibres were stained by the A.T.Pase histochemical procedure. By 46 days however (Fig. 53), some primary fibres showed patchy staining. The fibres which did stain occurred in clusters. At 54 days a difference was apparent across the width of the muscle. Most primary fibres in the deep region stained darkly (Fig. 54) but none of those in the superficial region of the muscle showed any staining. At this age the secondary fibre generation has started to form, and those on the surface of primary fibres in the deep region of the muscle were also intensely stained (Fig. 54). At 70 days the differences between the deep and superficial regions were well established. All primary fibres in the deep region of the muscle were darkly stained while those in the superficial region remained unstained. Fig. 55 shows a region in the centre of the muscle, transitional between the deep and superficial regions. In this region only some primary fibres were stained. Secondary fibres in both deep and superficial regions remained unstained.

Sections taken at 75 days showed little change from those described at 70 days. Fig 56 is taken from the deep region of the muscle and shows darkly stained primary fibres surrounded by relatively unstained secondaries. Figs. 57 and 58 are lower magnification photographs between the deep and superficial regions. Fig. 57 is from the deep region and has darkly stained primary fibres surrounded by unstained secondaries. Fig. 58 is from the superficial region where neither generation of fibres is stained.

By 102 days some of the secondary fibres immediately adjacent to primary fibres, in the deep regions of the muscle were stained. This change which only occurs in the deepest region of the muscle leads to pairs of darkly stained fibres in the centre of fascicles (Fig. 59). Each pair of stained fibres consists of a primary and a secondary fibre. Fig. 60 is taken from an animal at birth and shows the deep region of the muscle. Additional secondary fibres in contact with primary fibres are now stained. This leads to clusters of up to four darkly stained fibres at this age.

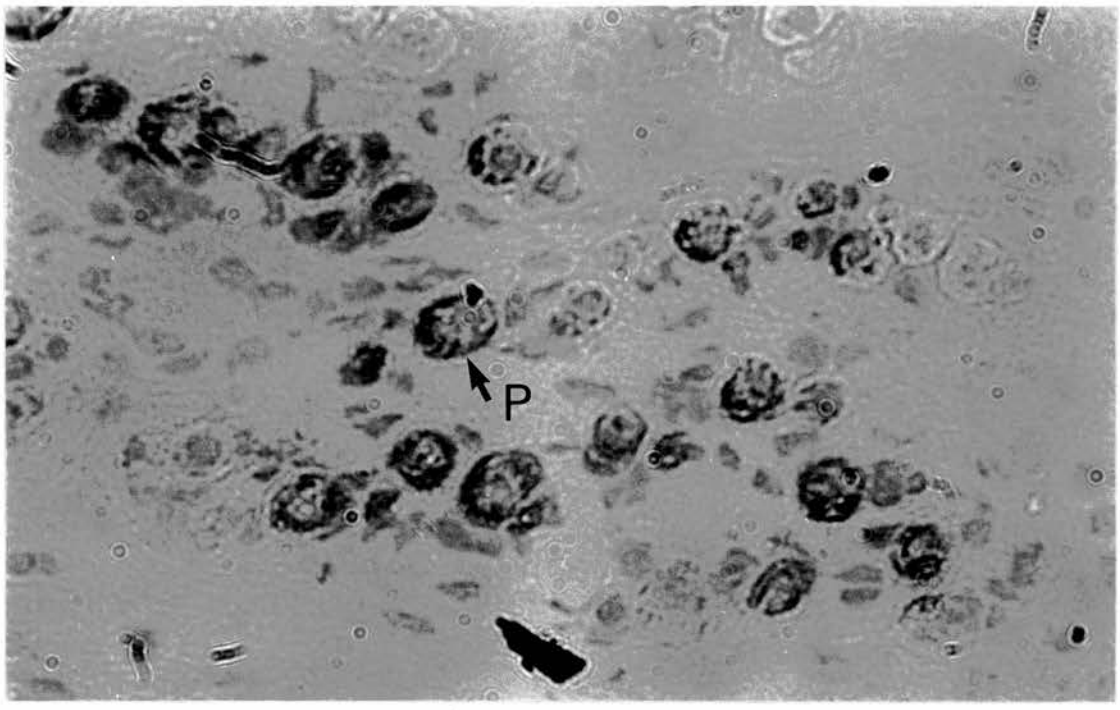


Fig.53 46 days. P = Primary fibre.

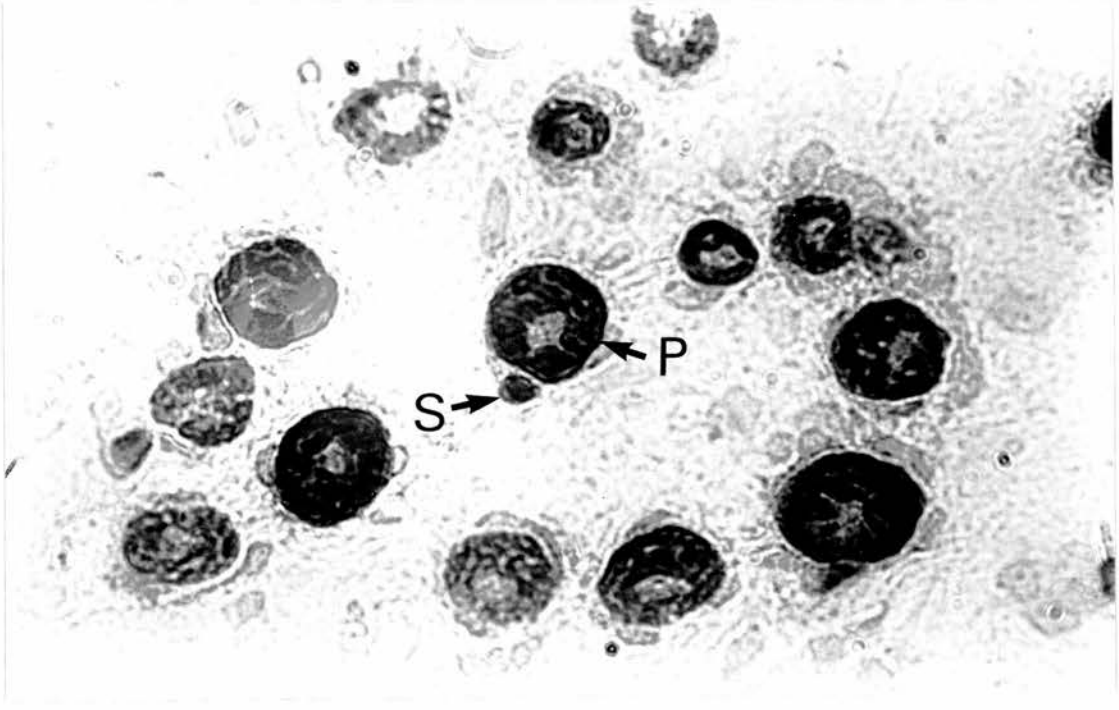


Fig.54 54 days. P = Primary fibre S = Secondary fibre.

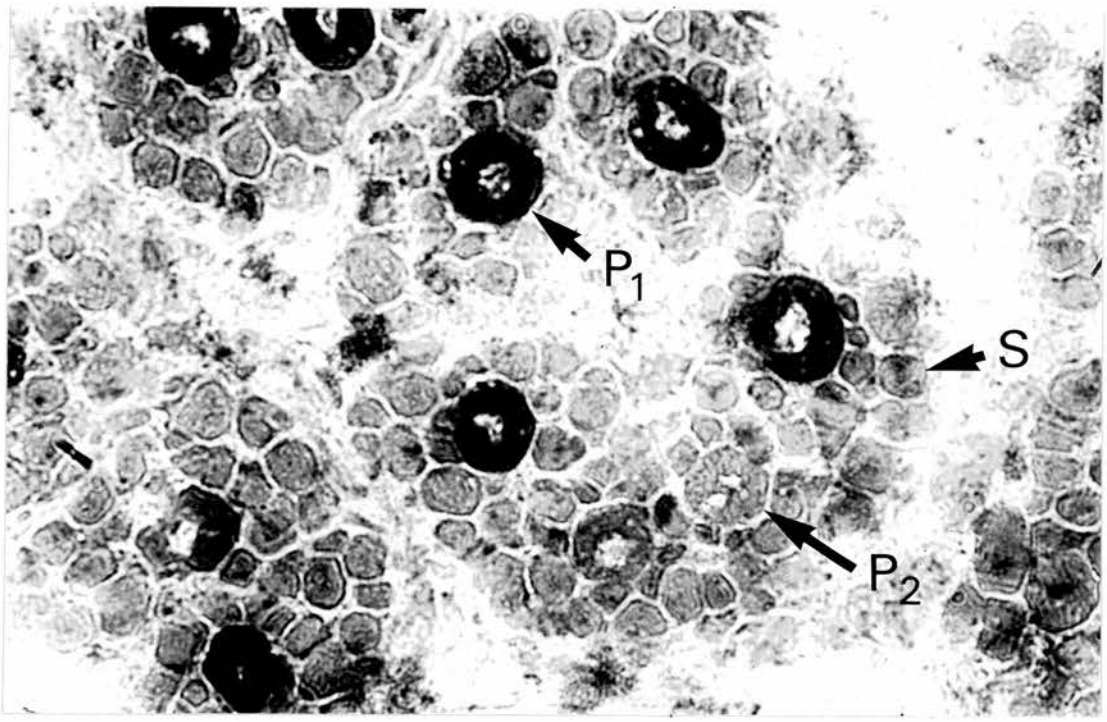


Fig.55 70 days. P_1 = Darkly stained primary fibre. P_2 = Unstained primary fibre. S = Secondary fibre.

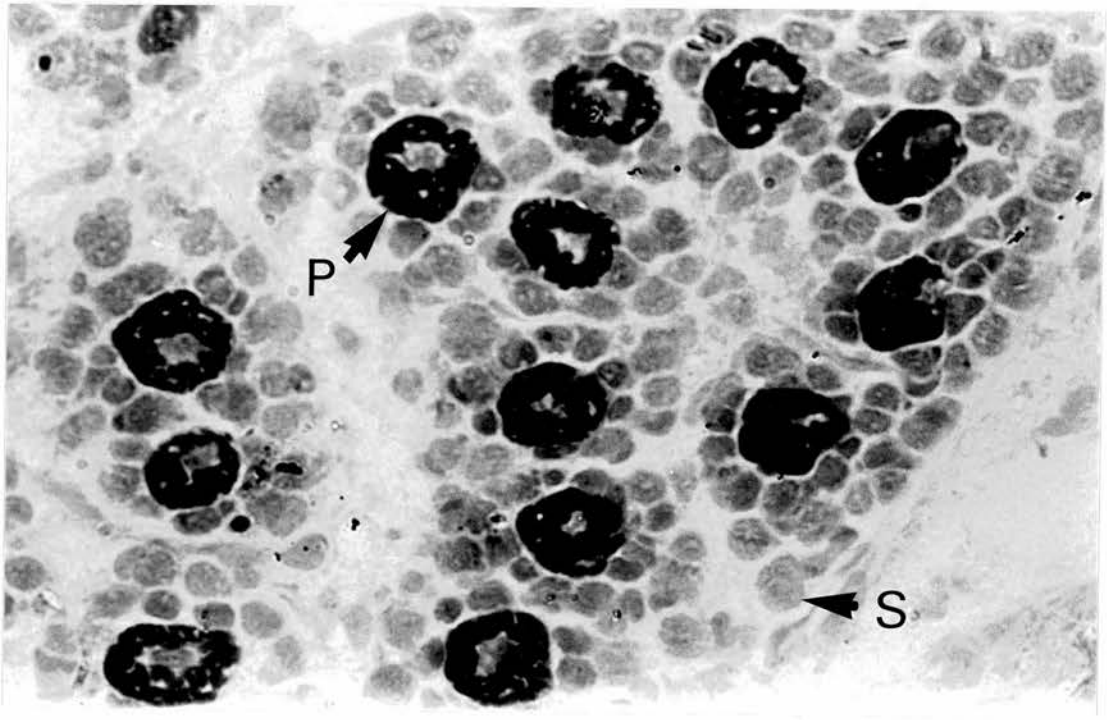


Fig.56 75 days. P = Primary fibre. S = Secondary fibre.

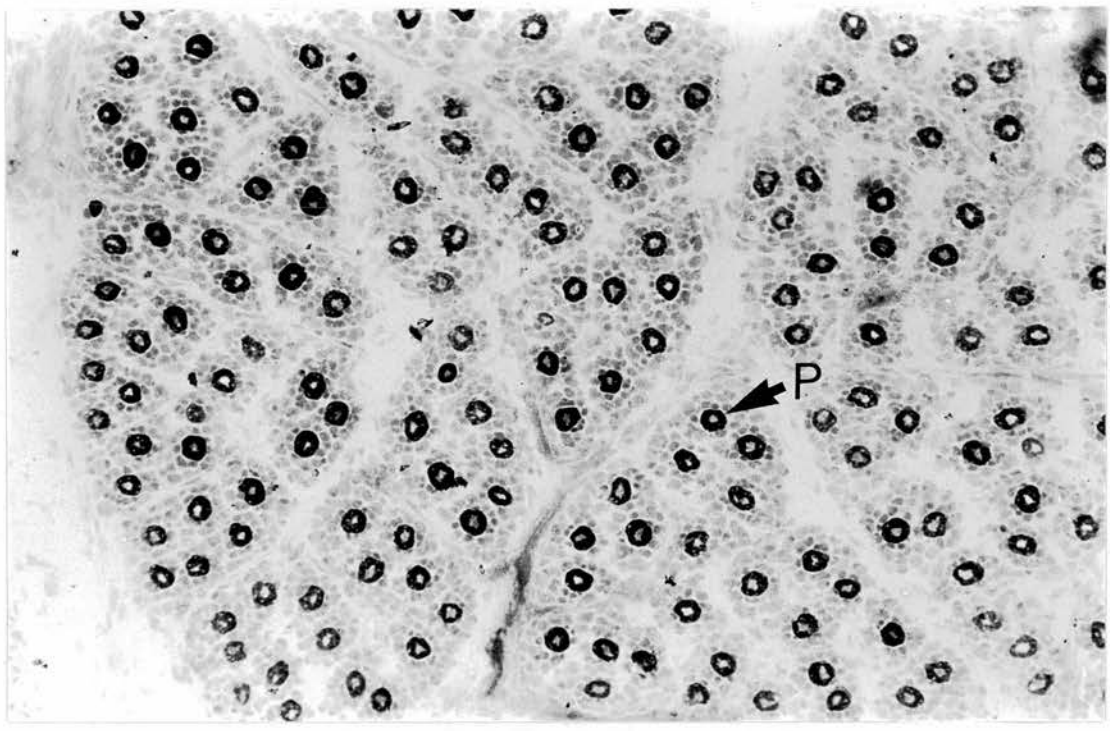


Fig.57 — 75 days. P = Primary fibre.

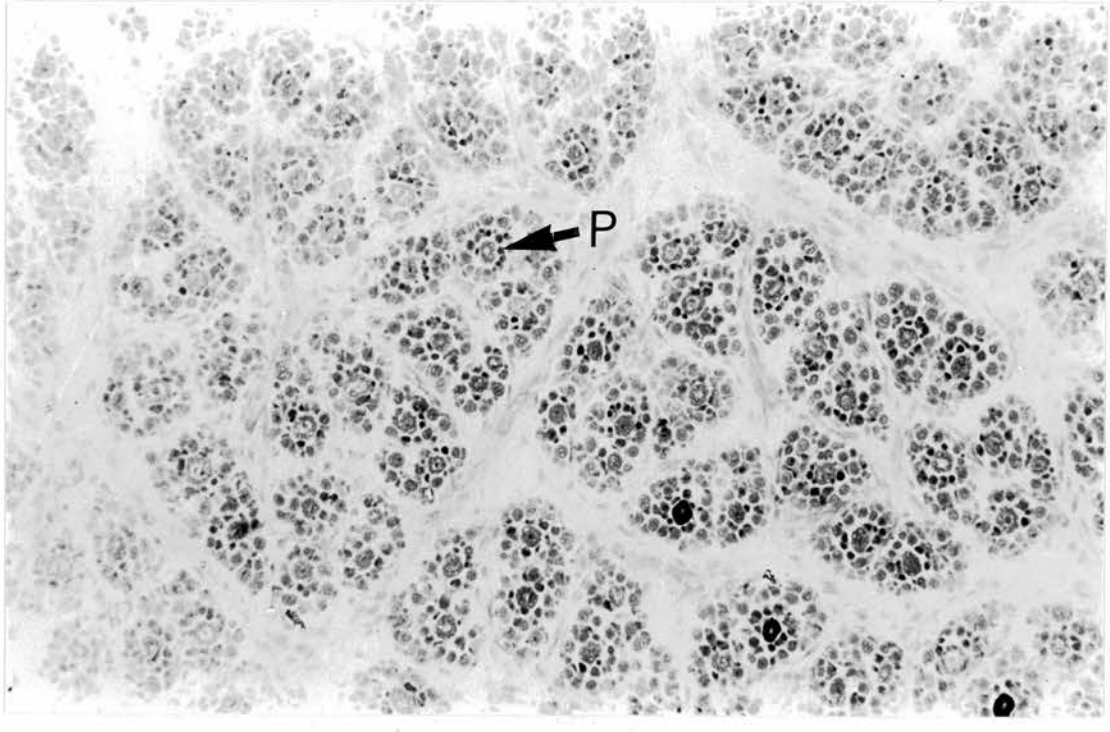


Fig. 58 75 days. P = Primary fibre.

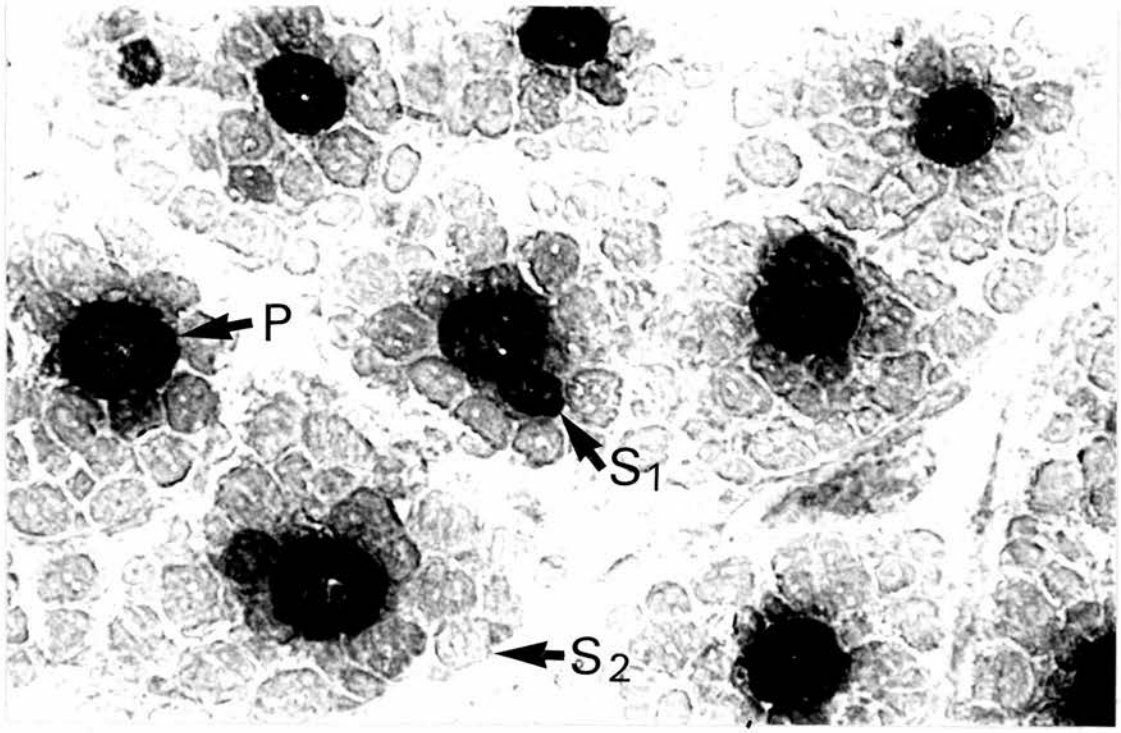


Fig.59 102 days. P = Primary fibre S_1 = Darkly stained secondary S_2 = Unstained secondary.

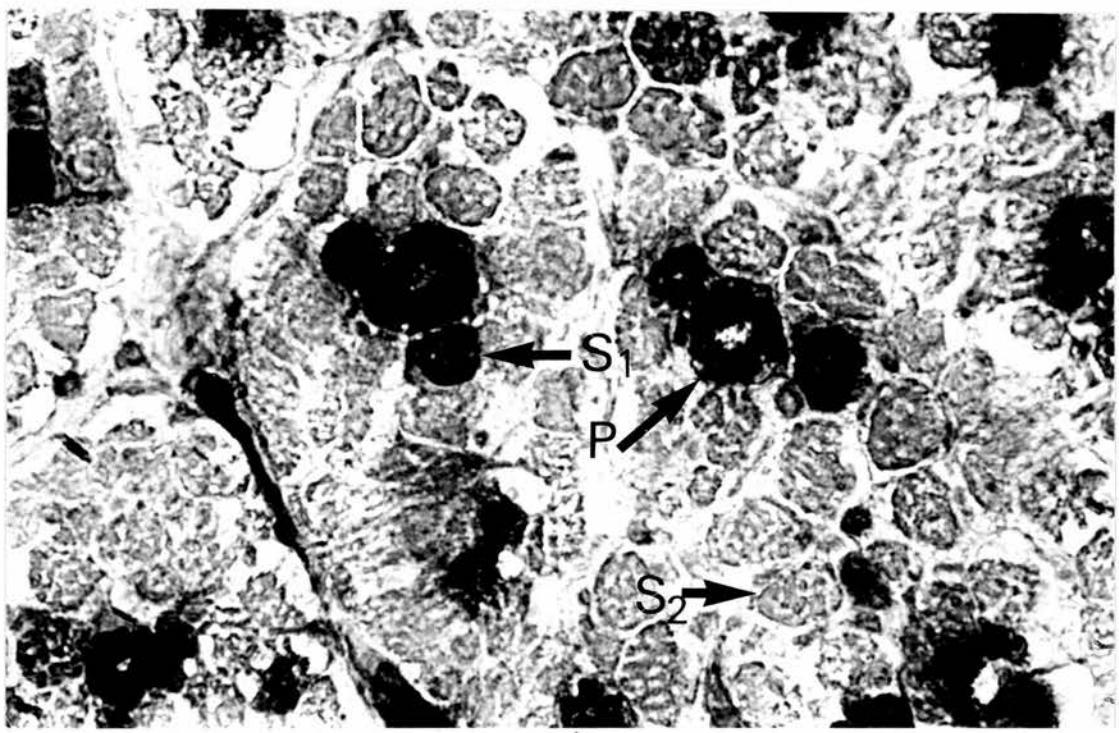


Fig.60 114 days (new born). P = Primary fibre S_1 = Darkly stained secondary S_2 = Unstained secondary.

Wavy fibres

Wavy or kinked fibres were initially observed in wax embedded sections and were investigated in material from a litter of 64 days gestation. Using muscles from the animals of this litter and also fetal mouse muscles, it was possible to demonstrate wavy fibres in frozen, wax embedded, plastic embedded and teased material (Figs. 61, 62, 63 and 64). Teasing and the sectioned material showed that both primary and secondary fibres were affected and that an individual wavy fibre could be found within a bundle of straight fibres (Figs. 61 and 62). When individual fibres were teased out, the kinking often appeared to be localised in a region a short distance (about 100 μ m) behind the fibre tip. The kinks extended over about 300 - 600 μ m of the fibre length and in sections could result in a shortening of up to 40% of this region of the fibre. Beyond the kinked region the fibre again became straight (Fig. 65). Electron micrographs were taken of wavy fibres and these showed that they were in a contracted state as shown by the short or absent I bands of their sarcomeres. Adjacent straight fibres were in a relaxed state and were not physically connected to the wavy fibres. (Fig. 66)

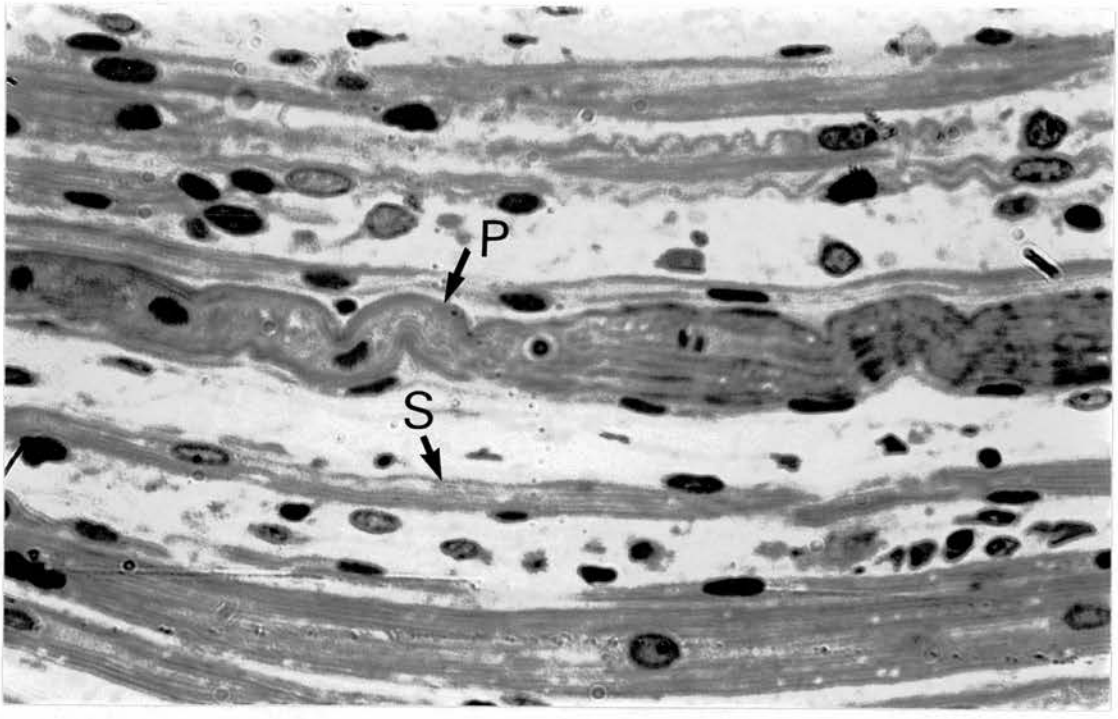


Fig.61 64 days. Longitudinal plastic embedded section. P = Kinked primary fibre S = Secondary fibre.

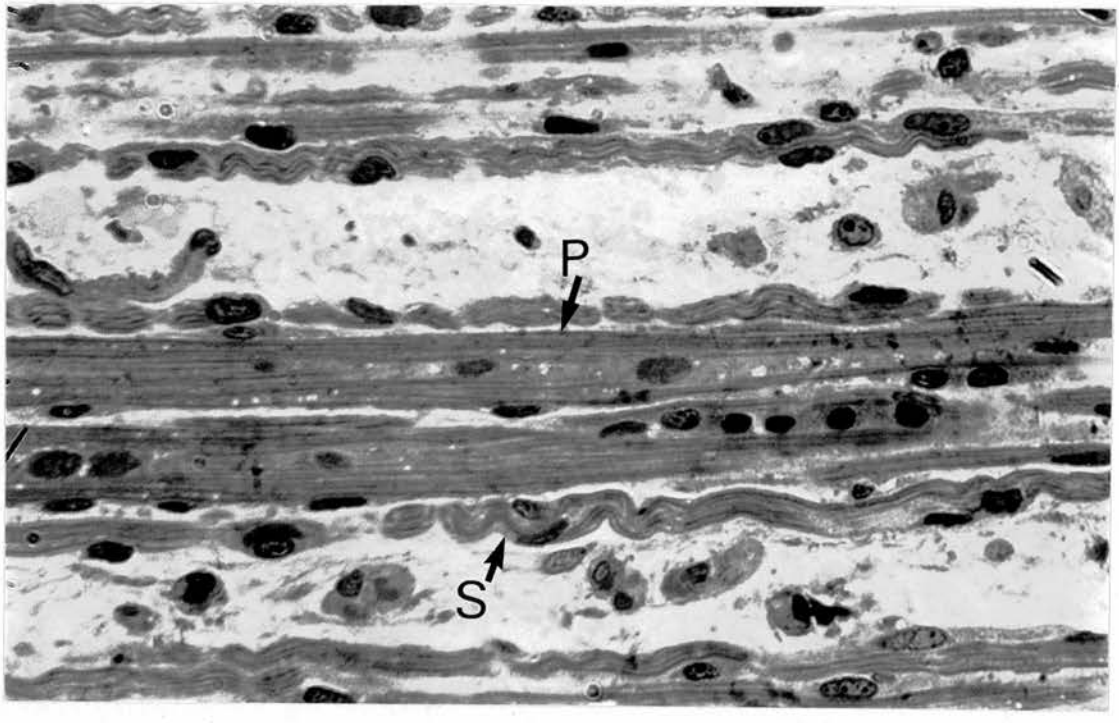


Fig.62 64 days. Longitudinal plastic embedded section. S = Kinked secondary fibre P = Primary fibre.

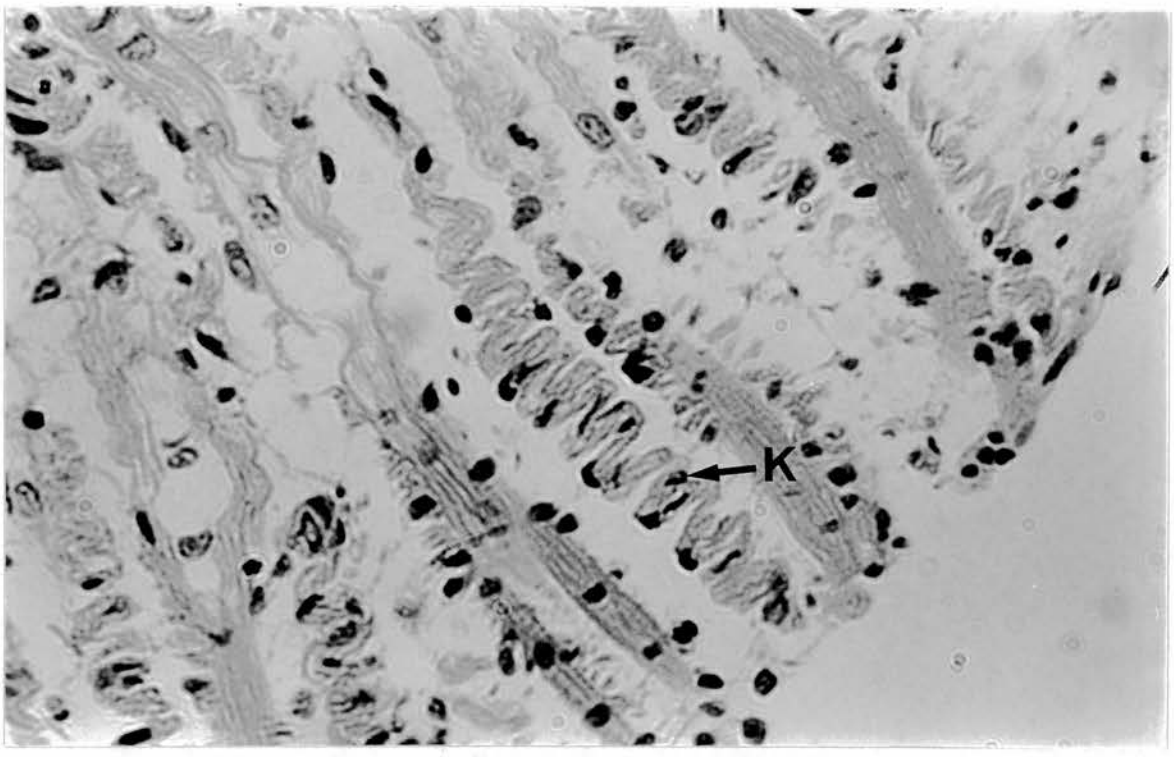


Fig.63 64 days. Longitudinal wax embedded section. K = Kinked fibre.

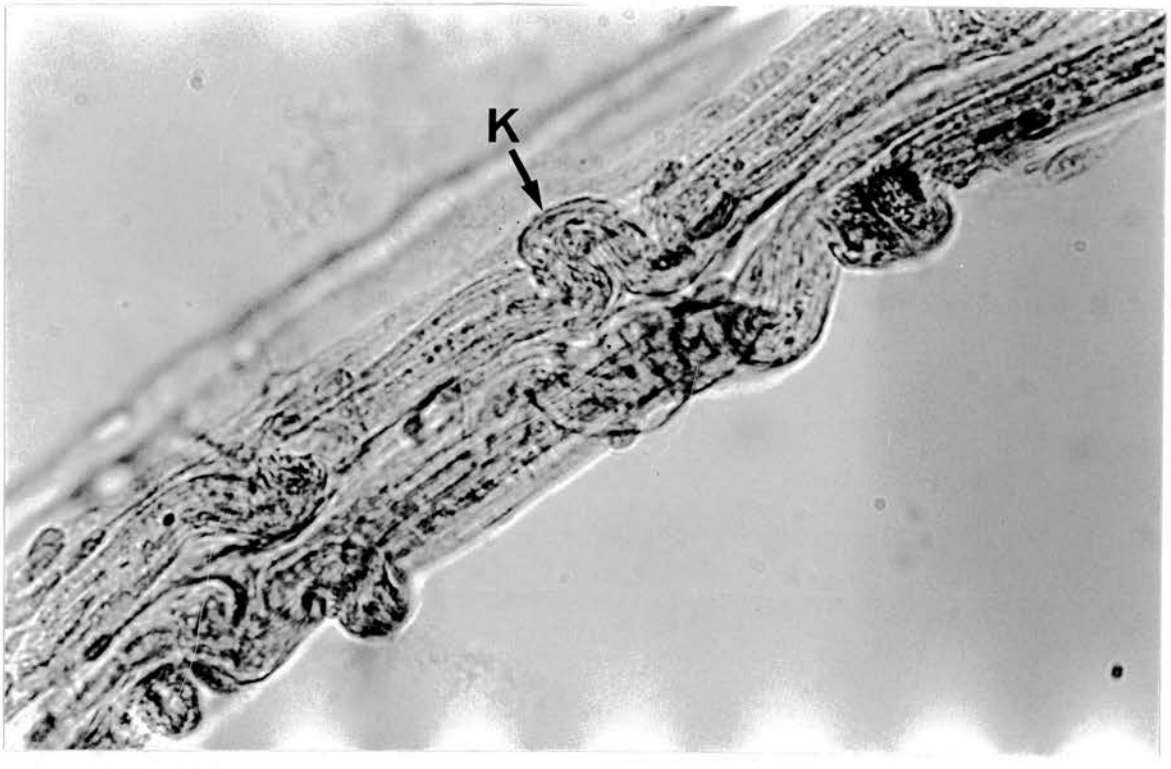
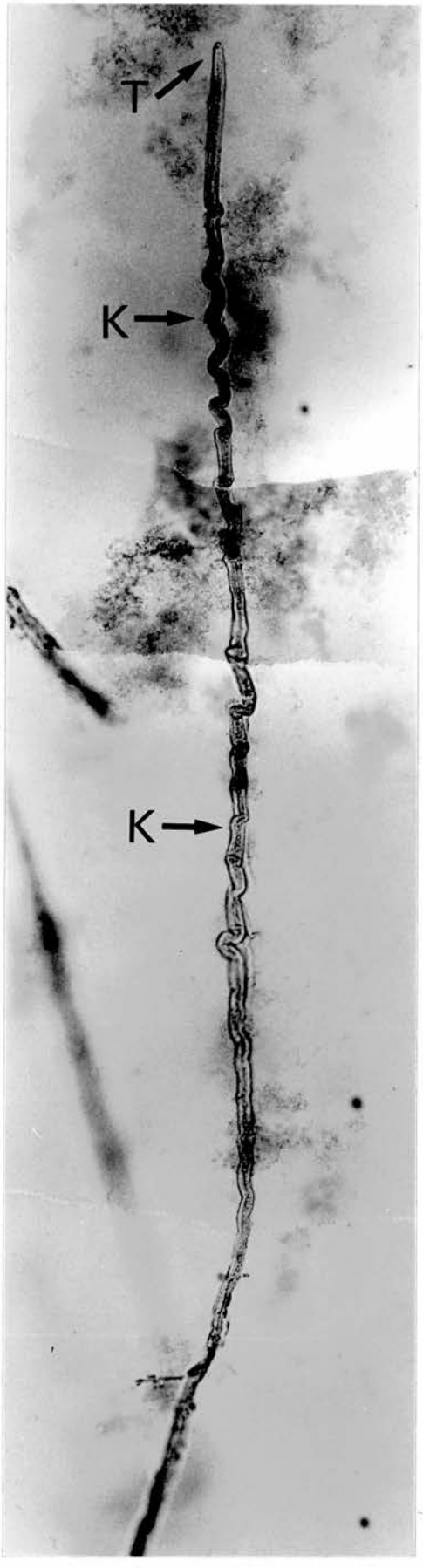


Fig.64 64 days. Teased bundle of fibres. K = Kinked fibre.

Fig.65 64 days. Single teased primary fibre.

T = Fibre tip.

K = Kinked region.



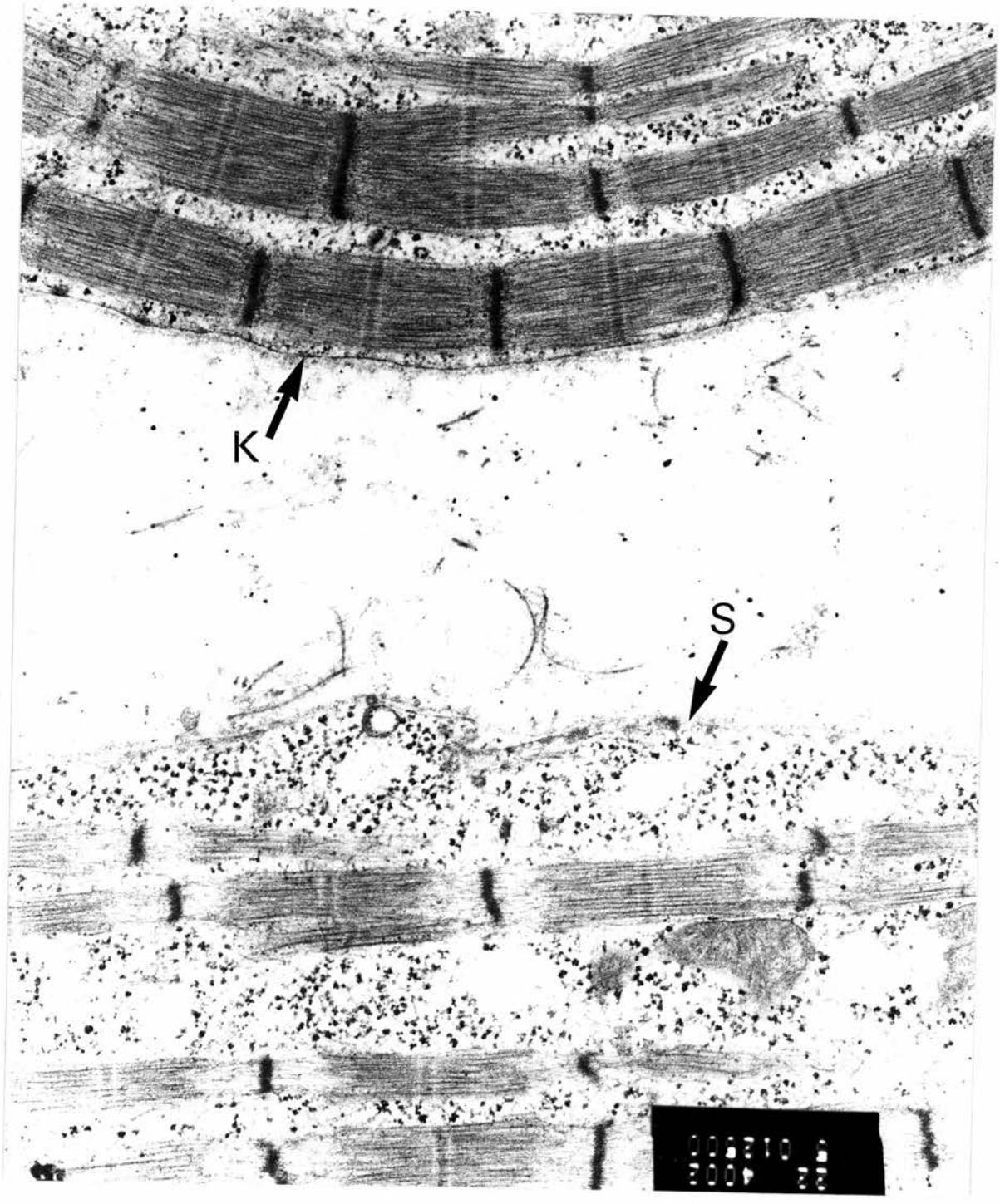


Fig.66 64 days. Electronmicrograph of a kinked fibre (K) and an adjacent straight fibre (S).

Postnatal Runts

Three postnatal runts, which were failing to put on weight, were obtained at the start of the project for practice purposes. Although sampling procedures and techniques were not fully developed, the conclusions from these results are presented here. Seven muscles were used, all of which contained a mixture of fibre types. Every muscle except the trapezius, abdominal cutaneus and the longissimus dorsi had a region of uniformly fast fibres which was located on the superficial side of the muscle. All the muscles showed the typical pattern of fibre types found in pigs. Bundles of several slow fibres (S0) were surrounded by fast fibres, the innermost of which were fast oxidative and glycolytic (FOG) and the outermost fast glycolytic (FG). (Fig. 67 and 68). The youngest animal (67 days old) showed a lack of glycolytic capacity, nearly all its fibres being oxidative. Up to 30 per cent of the fibres in some muscles of this animal were a fast oxidative (FO) type not found in adult muscle. Fast glycolytic fibres (FG) were confined to the superficial regions of muscles in this animal. In the older animals, glycolytic fibres were found throughout the muscle and the percentage of FO fibres had declined. Table 7 gives a summary of the fibre typing and the fibre diameter results from these three animals. The proportion of fast fibres and the sizes of the different fibre types varies greatly between muscles within an individual. Most muscles show a decline in the percentage of fast fibre types with increasing age. Poor sampling may explain the cases where some muscles fail to show this. When the

relative diameters of fibre types within a muscle were compared, both fast-twitch types (FOG and FG) had similar sizes but were smaller than the slow-twitch fibres (SO). On average the fast fibres were three quarters the size of the slow fibres.

Table 7. The proportion of fibre types and fibre sizes in three postnatal runts. DIAM = diameter. FG = fast-twitch, glycolytic fibres. FOG = fast twitch, oxidative and glycolytic fibres. SO = slow-twitch oxidative fibres.

AGE (days)	Wt. (kg.)	MUSCLE	% FAST FIBRES	DIAM. FG (μm.)	DIAM. FOG (μm.)	DIAM. SO (μm.)
64	4.1	m. semitendinosus	63	18	20	26
		m. biceps brachii	83	18	15	22
		m. trapezius	87		10	16
		m. ext. digiti quarti	70	29	26	29
		m. longissimus dorsi	88	15	13	17
		m. sartoruis	86	15	13	17
75	6.6	m. semitendinosus	53	18		28
		m. biceps brachii	81	23		35
		m. trapezius	43	10	17	17
		m. ext. digiti quarti	69	19		27
		m. abdominus cutaneus	44	9		17
144	16.8	m. semitendinosus	70	32	33	38
		m. longissimus dorsi	92	24	25	36
		m. sartorius	74	19		22

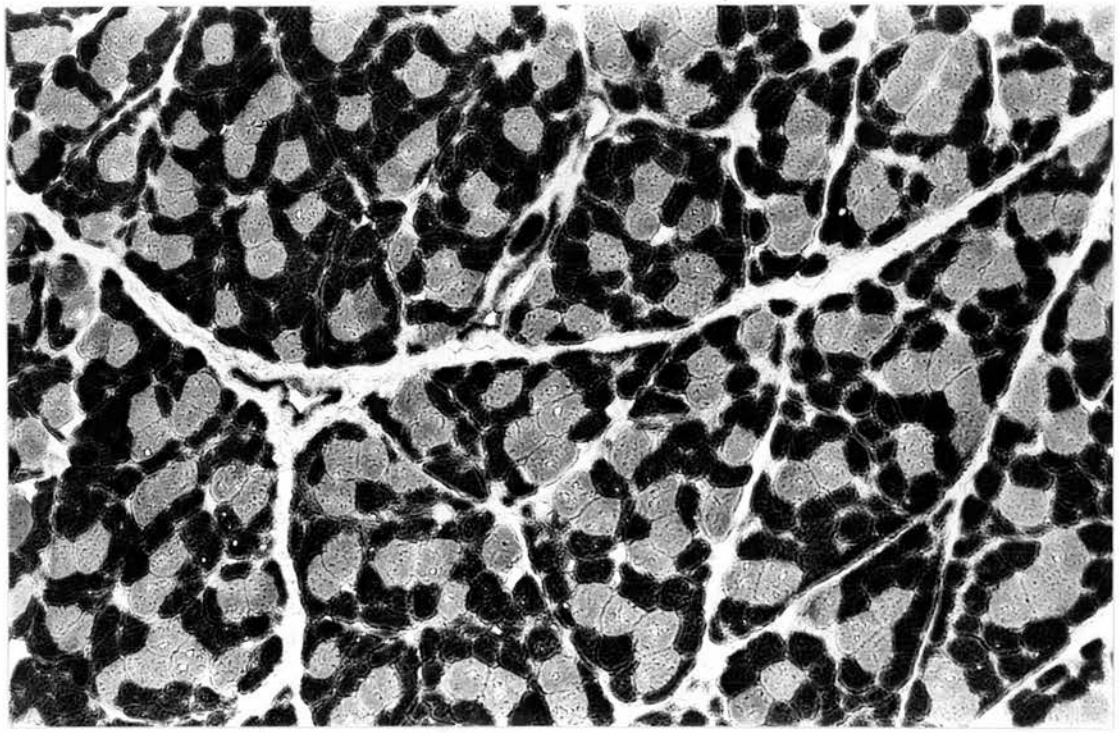


Fig.67 75 days postnatal. Extensor digiti quarti muscle stained for Adenosine triphosphatase after alkaline preincubation.

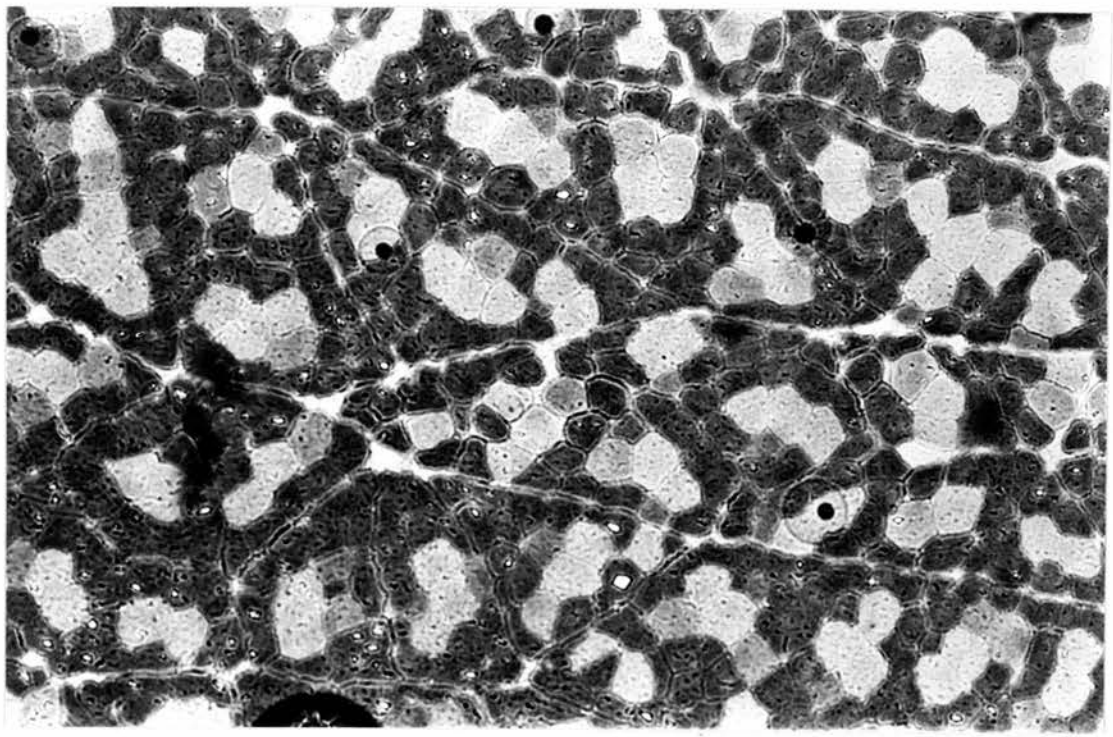


Fig.68 75 days postnatal. Biceps brachii muscle stained for Adenosine triphosphatase after alkaline preincubation.

Discussion

Gross parameters of muscle

The growth coefficient for the semitendinosus muscle of large littermates (Fig. 17) was not significantly different from 1.0, indicating that in these animals, the semitendinosus grows at the same rate as the whole body. The growth coefficient of this muscle, relative to body weight was calculated for postnatal well nourished large white pigs from the data of Davies (1973). Its value was found to be 1.037, which is very close to that obtained prenatally (1.035) for large littermates. This suggests that the optimum growth rate of the semitendinosus is the same in the pre-and postnatal periods. According to the classification of Davies (1973) this muscle has an 'average growth velocity' which means that it maintains a constant relationship with body weight.

Comparison of the prenatal growth coefficients for largest and smallest littermates showed a significant difference between them ($P < 0.05$), and the value for the smallest littermates was also significantly below 1.0 ($P < 0.05$). These results indicate that, whilst the semitendinosus in large littermates grows at the same rate as the rest of the body, the same muscle in small littermates grows more slowly than the rest of the body and will constitute a decreasing proportion of body weight. In agreement with this prediction, Widdowson (1974) and Powell and Aberle (1980) reported that, at birth, runt pigs have relatively smaller muscles

than their larger littermates. When post-natal growing pigs are malnourished during their first year of life (Widdowson 1974; Dickerson and Mcance 1964), muscle tissue suffers a greater degree of growth retardation than other body tissues. This leads to the malnourished animals having relatively smaller muscles than the well-nourished controls. This indicates that the probable cause of the retarded growth of the semitendinosus in comparison with the rest of the body was small prenatal littermates, is malnutrition.

General description of muscle development

This project confirms the biphasic nature of muscle development within the pig. From the ages of 54 and 90 days gestation there are clearly two populations of fibre, distinguishable by their size, shape and position within a fascicle and in some regions of the muscle by their histochemical staining. Swatland and Cassens (1973b) found no muscle fibres in pig limbs at 20 days gestation and so the onset of fibre formation must be between this age and 38 days when primary fibres were first observed in this investigation. Primary fibres were formed before the appearance of secondary fibres and during this period there was no evidence of primaries forming on the surfaces of existing fibres. No very small fibres were seen on the surfaces of other fibres and since primaries at this age (38 days) have a relatively uniform size many of them may have formed simultaneously. Their relatively low rate of formation as compared to the secondaries (700 as opposed to 9500 per day) may indicate the difficulty in forming fibres without the use of

a rigid substrate. It has been found by some authors working on cell culture (Holtzer, Jones and Yaffe 1975; Bignami, Dogliotti, Benigni and Brania 1982) that myoblasts will not fuse to form myotubes in semisolid media or in liquid suspensions nor on the surfaces of other mononuclear cells (Nameroff 1972). The requirement of a rigid substrate for fusion may explain the use of primary fibres as surfaces for the formation of secondary fibres.

The rise and fall of primary fibre size, first described by Thurley (1972) was confirmed in this study. These changes in fibres diameter were dependent upon the formation of a central myofibrillar free region (M.F.R.) and its subsequent collapse. It is probable that little myofibrillar proliferation takes place during the decline in primary fibre size as this has been found in developing human muscle (Stickland 1981).

The appearance of secondary fibres at 54 days is very similar to the times recorded by other authors (Thurley 1972, 55 days; Ashmore et al 1973, 50 - 60 days; Swatland and Cassons 1973, 50 days; Beermann et al 1978, 45 days). The smallest secondaries were always found in contact with primary fibres and they often appeared to be forming within a depression in the surface of the primary (Fig. 48). Longitudinal sections showed mononuclear cells on the surface of primaries with their long axis parallel to the primary (Fig. 40). The attachment of these cells to the surface of the primary is assumed to be the initial stage in secondary fibre formation. Secondaries larger than 2 - 3 μ m. across were detached from the primaries and lay free within the fascicle.

These observations confirm those of Thurley 1972; Swatland and Cassens 1973; and Ashmore et al 1973; that secondary fibres form by cell fusion in the surfaces of primary fibres. It is possible that secondary fibres themselves could act as a substrate for the formation of additional fibres which might then constitute a tertiary generation. The apparently random distrubution of secondary fibre sizes within a fascicle lends some support to this idea as small secondaries are sometimes found some distance from the nearest primary. As described above, however, the very smallest secondaries are only found on primaries and it would therefore appear that only two generations of fibres are formed. This is consistent with the observations of Kelly and Zacks 1969 that secondary fibres only form on the surfaces of primaries which are above a certain size. The constancy of the mean secondary fibre size during most of gestation (Fig. 42) was surprising as it was expected that the fibres would hypertrophy after formation. The lack of increase in the mean diameter is not due to the continual formation of new small fibres as the maximum secondary fibre size also remains approximately constant until 102 days. These results suggest that after formation and detachment from the primary fibres, secondary fibres initially triple their size but then stop enlarging until just before term.

The graphs of total fibre number (Figs 43 and 44) and the ratio between secondary and primary fibres were used to estimate the age at which fibre hypertrophy ceased. Both methods indicated that fibres stopped forming between 80 and 90 days. This age is similar to that given by

other authors working on pig muscce development and shown below.

Swatland (1973)	70 days
Thurley (1972)	before 100 days
Ashmore (1973)	75 days

The secondary/primary ratio obtained for large animals is shown compared to those of Ashmore et al 1973 and Beerman et al 1978 in Fig. 59. All three sets of results are similar and show that between 20 and 25 secondaries are formed around each primary in the semitendinosus muscle. This ratio is much higher than that found in laboratory animals where it is normally between 2 and 5 (Stickland personal communication on mice, Harris 1981 on rat diaphragm muscle).

The conclusion that fibre hyperplasia ceases before birth is in agreement with measurements of fibre number postnatally. The majority of authors, (McMeekan 1940; Staun 1963; Stickland and Goldspink 1973; Davies 1973) who have counted or estimated total fibre number during postnatal growth have found that fibre number remains unchanged during this period. Two authors have, however, claimed an increase in fibre number in the pig postnatally. Swatland (1973b and 1976) found that the number of fibres in the sartorius muscle increased linearly throughout gestation and showed an increase in the rate of appearance of new fibres in the immediate postnatal period. This result was explained by the growth of intrafascicularly terminating fibres (Swatland and Cassens 1972) into the plane of section to give an apparent hyperplasia and so

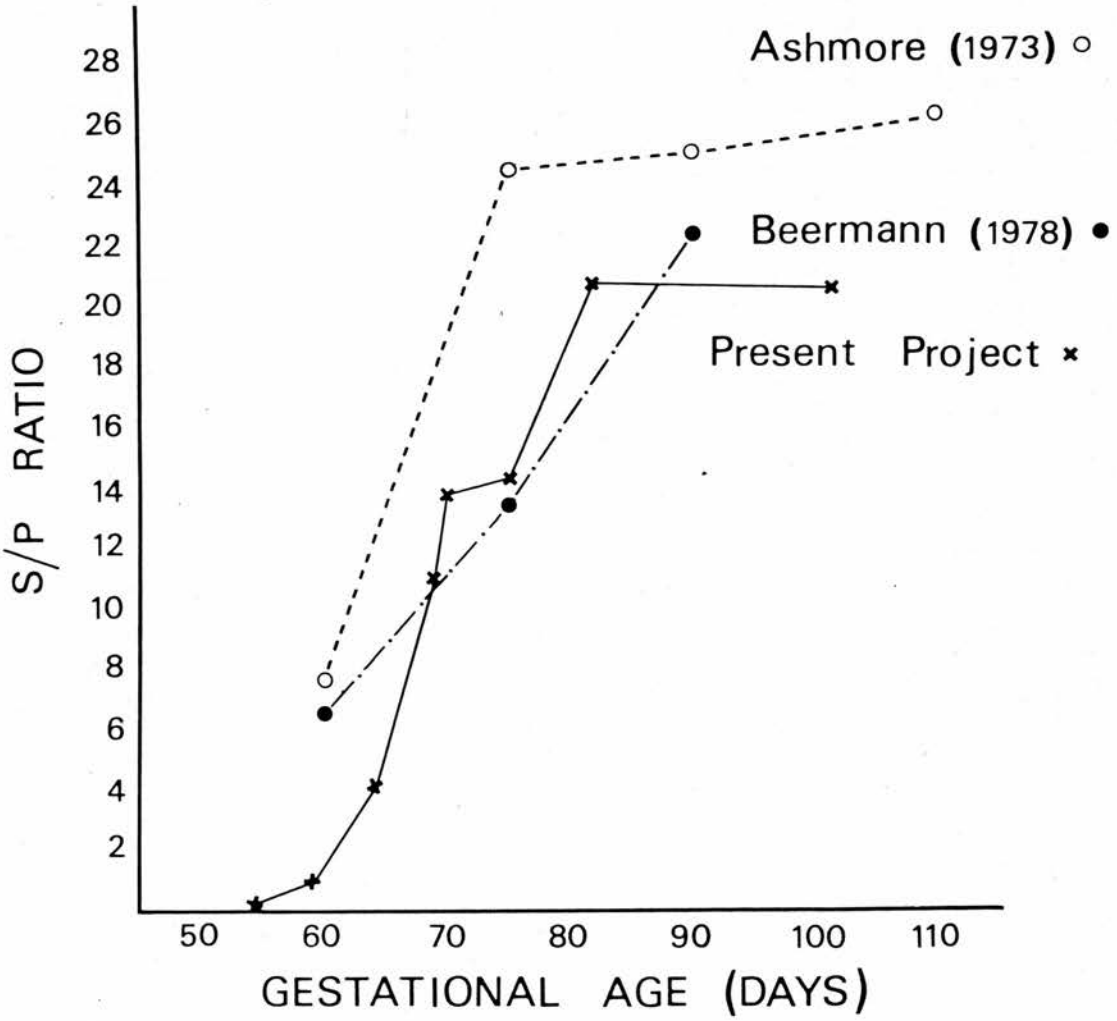


Fig.69 The secondary / primary fibre ratio (S / P ratio) plotted against gestational age. The present project is compared with the results from two previous papers (Ashmore,Addis, & Doerr 1973 and Beermann, Cassens & Hausman 1978).

does not contradict the result that real fibre hyperplasia ceases prenatally. Petrov (1976), however, has claimed that real fibre hyperplasia does continue during the first 10 years of postnatal life. He suggests that the mechanism of fibre number increase is fibre splitting of which he shows several photographs. This phenomenon has been found in disease, (Isaacs, Bradley and Henderson 1973) after extreme exercise (Ho, Roy, Tweedle, Heasner, Van Hass and Carrow 1980) and in regenerating fibres (Ontell and Hughes 1981) but is not generally regarded as a normal feature of muscle growth and so the results of Petrov (1976) remain unexplained. Neither the results of Swatland (1973b) or Petrov (1976) was confirmed in the present study. Fibre number reached a maximum at about 90 days gestation (Fig. 43) and thereafter showed no change for the rest of gestation.

The number of mononuclear cells observed between fibres appears to decline after about 64 days gestation. This result is in agreement with Campion, Richardson, Kraeling and O'Regan (1979) who found a decline in satellite cells from 95 days (their earliest observation) until term.

Differences between large and small littermates

There appears to be no difference in the time of formation of either primary or secondary fibres in large and small littermates. The most obvious difference between them is the presence of the large myofibril free region (M.F.R.) in the primary fibres of large animals which is

much reduced and later is forming in small littermates. This feature is largely responsible for the size difference between the primary fibres of the two groups shown in Fig. 42. The mode of formation and subsequent disappearance of the M.F.R. is unclear. Electronmicrographs of primary fibres (Fig. 66) show large numbers of glycogen granules interspersed within an irregular network of fine filaments and unstained droplets which were probably composed of lipid. These observations correspond to those of Boyd (1960) who reported PAS positive material and lipid droplets in the central cytoplasm of human primary fibres. Work on cultured myotubes has shown that they collapse when treated with colchicine (Holtzer, Sanger, Ishikawa and Strahs 1973). This suggests that microtubules may be involved in the maintenance of the M.F.R. and these filaments, together with intermediate and microfilaments have been observed within cultured myotubes (Stromer, Goll, Young, Robson and Parrish 1974; Ip and Fishman 1979). The presence of myofibril free regions within the secondary fibres of large littermates (Fig. 48) suggests that systemic factors may be involved in inducing the formation of this feature rather than factors localised to primary fibres. While the mode of formation of the myotube shape of primary fibres is unknown, it has been shown that their maturation into solid fibres requires the presence of motor neurones (Engel and Karpati 1968; Mcardle and Sansome 1977).

One of the most important results of this project was the reduced fibre number in small littermates as compared to their larger siblings. This result is in agreement with the findings of two recent papers on the

postnatal muscle growth of porcine littermates (Heggarty and Allen 1978; Powell and Aberle 1981). In neither of these papers is fibre number counted directly but from measurement of fibre diameter, muscle length and weight these authors concluded that the smaller littermate had fewer fibres and that this difference was present at birth.

This difference in fibre number was due to the formation of fewer secondary fibres around each primary in the smaller animals. This was shown by the difference in the secondary/primary ratio (Fig. 45) and the absence of any difference in primary fibre number between large and small (Fig. 46). There appeared to be no difference in the time available for secondary fibre formation between the two groups; rather it appeared that the rate of formation was greater in the larger fetuses. This led to the divergence of the lines for large and small shown in the graph of secondary/primary ratio against age (Fig. 45). There could be many causes for the reduction in the rate of formation of secondary fibres but the difference in primary fibre size was investigated as a possible mechanism.

The effect of primary fibre size on secondary fibre formation

As mentioned previously, maximum primary fibre size coincided with secondary fibre formation. Since secondary fibres only form on the surface of primaries, the function of this increase in size might be to provide a greater surface area for the attachment and fusion of the

myoblasts which form secondary fibres. Differences in primary fibre size at this age would then cause differences in the number of secondary fibres formed. This hypothesis was tested by using all the individuals in one litter. The results showed firstly that there was a significant correlation between fatal weight and primary fibre size and secondly that the number of secondaries surrounding each primary was also correlated with the mean size of the primary fibres (Fig. 47 and 52). These results support the hypothesis described at the beginning of this section and provide a statistical basis for the relationship between these parameters.

The effect of malnutrition on fibre number

One of the conclusions of the first part of this thesis was that the cause of the size difference between largest and smallest littermates was probably nutritional. In a literature search only two reports could be found of the effects of experimentally induced malnutrition during the time of fibre hyperplasia on fibre number. The first of these (Everitt 1968) involved starving pregnant ewes and examining the muscles of their offspring at 90 and 140 days gestation. The malnourished fetuses had 13% fewer muscle fibres at the earlier age and 31% fewer at the later age compared to well nourished controls. Aziz-ullah (1974) working with pregnant mice, showed a small non-significant difference in fibre diameter between malnourished and control fetuses at birth but a significant 17% difference in fibre number. Both these reports show that malnutrition when it occurs during the time of fibre hyperplasia can

reduce fibre number. Neither author unfortunately gives a description of the histological appearance of the developing muscle and so the relative size of primary fibres in the experimental and control animals is unknown.

Whilst malnutrition can reduce fibre number and it is the probable cause in the present investigation, it is worth considering two other factors known to effect fibre number, namely genetics and innervation. Several studies have shown that selection for body size (Luff and Goldspink 1967; Hanrahan, Hooper and McCarthy 1973) or growth rate (Ezekwe and Martin 1975) can increase fibre number in laboratory animals. Similar experiments have not been done on pigs but different genetic strains are, however, known to have different fibre numbers (Staun 1963) and total fibre number has a high degree of heritability within a strain (Staun 1970). The absence from the muscle during development of motor axons inhibits fibre formation (Mcardle and Sansone 1977; Betz, Caldwell and Ribchester 1980; Harris 1981). The fibres which fail to form are apparently secondary fibres (Harris 1981) but as long as some nerves are present the full fibre number is attained (Betz et al 1980). Because of this it is unlikely that lack of innervation normally limits muscle fibre formation. The lack of reduction in primary fibre number in the present project and in the above work on innervation suggests that primary fibres form relatively independently of external influences in contrast to secondary fibre formation which is much more susceptible to interference. Factors which affect the number of primaries are unknown

but the number of secondaries must depend upon the number forming upon each primary and the number of primaries. In the present project the low secondary/primary ratio in small littermates would appear to be due to a reduction in the number of secondaries that can form simultaneously on each primary. This assumes that the time taken for a fibre to form is the same in both large and small. The mouse biceps brachii also has a low ultimate secondary/primary ratio with only 2-3 secondaries forming on each primary (Stickland 1982). The probable cause of this is the short time available (2 days) for secondary fibre formation compared to the 35 days in the pig. Different muscles within the pig are reported to have different secondary/primary ratios (Ashmore et al 1973) and it would be of interest to know the factors responsible.

Histochemistry

As described by Beermann (1973), the semitendinosus muscle can be divided into deep and superficial regions which, although showing no difference in their morphological development, have different staining characteristics. The deep region is composed of both fast and slow fibres in the adult while the superficial region has only fast-twitch fibres. In the deep region of fetal muscle the primary myotubes stain darkly after 45 days and continue to do so throughout gestation. In contrast primary fibres in the superficial region remain unstained throughout the prenatal period. The age at which staining is first seen in primary fibres corresponds to the formation of motor end plates on these fibres (Szenstkuti and Cassens 1977; 1979; Beermann et al 1978).

Secondary fibres in both deep and superficial regions remain unstained until 102 days, apart from a transitory staining seen in some secondaries on the surface of primaries at 54 days (Fig. 54). This transitory staining appears to be previously unreported but no cause could be found for it. The age at which conversion of secondaries from fast to slow starts is similar to that given by Beermann et al (1978). The conversion is more extensive in the centre of the deep region compared to the periphery and presumably reflects changes in the characteristics of the motor neurones innervating these fibres (Kugleberg 1976). Conversion of fast to slow twitch fibres to form the characteristic bundles of slow fibres (Figs. 67 and 68), continues during postnatal growth (Davies 1972; Suzuki and Cassens 1980). This process has been thought to be an adaptation to the increasing weight of the animal (Davies 1972). The conversion of fibres in the prenatal animal suggests however that this is at least partly an age related process of maturation.

The results found here are very close to those described by Beermann et al (1978). Primary fibres in the deep portion are presumptive slow twitch fibres and stain accordingly from 46 days onwards. Secondaries adjacent to these primaries also become slow fibres after 102 days but the majority of secondaries in the semitendinosus become fast twitch fibres. Primaries and secondaries in the superficial region both become fast twitch fibres. The effect of a reduced fibre number on the relative proportions of different fibre types in adult muscle does not seem to have been investigated. Since only secondary fibres fail to form in

small littermates, it is possible that this would cause a reduction in the number of fast fibres in a mixed fibre type muscle. The constancy of primary fibre number in large and small animals would result in both sizes of animal having the same number of slow twitch bundles but the effect on the rate of fibre type conversion is unknown. A decrease in the proportion of fast fibres was found in some of the runt pigs used by Powell and Aberle (1981). It is also interesting to note that the increased fibre number found in double-muscle cattle (Ouhayoun and Beaumont 1968) is associated with an increase in the proportion of fast fibres (Holmes and Ashmore 1972; Hendricks, Aberle, Jones and Martin 1973; West 1974). This may indicate that the additional fibres are developed from secondaries.

The pattern of prenatal fibre type differentiation described here for the pig, where primary fibres form the first slow twitch fibres and additional slow twitch fibres are derived from the conversion of secondary fibres has also been found in other species (rat - Kelly and Schotland 1972); Kelly and Rubenstein 1980; Lamb - Ashmore, Robinson Rattry and Doerr 1972; Chick - Ashmore, Addis, Doerr and Stokes 1973; Man - Schloon, Schlottmann, Leonard and Grebel 1979). In these species however the different fibre types are randomly distributed within a muscle and do not form the slow fibre bundles seen in the pig.

Wavy fibres

Kinked or wavy fibres are a recognised phenomenon in adult muscle and have been observed previously in all the preparations used here (Lawrie, Gutherman and Hal 1958; Eagle and Henrickson 1970; Hooper and Hegerty 1973; Macnaughton 1974). All these authors regarded wavy fibres in postnatal muscle as an artefact of zigor contraction. The localisation of the kinking to near the fibre ends may indicate that this region is not strongly attached to adjacent fibres. This is certainly the impression gained from electronmicrogaphs (Fig. 66). The common occurrence of wavy fibres in fetal muscle has led Swatland and Cassens (1973) to ascribe a developmental function to them. These authors, working only with sectioned material, suggested that only secondary fibres attached to a primary fibre were kinked, the kinking being caused by the contraction of the primary and the lack of contraction in the secondary. The function of this was to 'throw' the secondary fibre off the surface of the primary on which it had formed. The present observations show that both primary and secondary fibres are kinked; that kinked fibres are contracted (Fig. 66) rather than relaxed and that the region of kinking does not appear to be in contact with adjacent fibres. These results make it unlikely that kinked fibres are anything other than an artefact.

Post-natal Runts

The small sample size of the post-natal runts means that only tentative conclusions can be drawn; however these animals appear to illustrate several interesting features of abnormal growth. At birth all muscle fibres in the pig are oxidative (Van den hende 1972; Ashmore, Tomkins and Doerr 1972, Davies 1972; Swatland 1975). All fast twitch fibres are a fast oxidative type not found in adult muscle. Fibre type differentiation takes place over the following few weeks when some fibres lose their oxidative capacity and gain glycolytic capacity. The majority of fibres in the youngest runt (67 days postnatal) used here, were uniformly oxidative. Glycolytic fibres could only be found in the superficial region and differentiation of glycolytic fibres may start earler in this region than in the deep region. There is no agreement in the literature on the age at which full glycolytic capacity is reached in the pig. In all the studies carried out, however, the adult pattern of three fibre types is attained within four weeks of birth (Cooper, Cassens, Kastenshmidt and Briskey 1970; Ashmore et al 1972; Davies 1972, Swatland 1975). The apparent delay in the differentiation of glycolytic fibres found here in the youngest runt may be due to malnutrition as has been observed in new born rats (Halita, Berlin, Schucht and Sourander 1978). When the relative sizes of the different fibre types was compared both fast fibre types (FG and FOG) were similar in size and smaller than slow twitch fibres. This is different from the normal size distribution found in pigs (Davies 1972) where the fast glycolytic fibres

are significantly larger than the fast oxidative glycolytic fibres and the slow fibres. The size distribution found here is similar to that described for malnourished postnatal rat muscle (Goldspink and Ward 1979) where poor nutrition reduced the size of the fast fibre types more than the slow. In conclusion, these runts appear to have suffered from postnatal malnutrition which has delayed the differentiation of their fibre types and altered their relative sizes.

Part 3: Biochemical Results

Introduction

Biochemical parameters have often been used to study muscle growth (see review by Burleigh 1980). The parameters measured here (DNA, RNA and Protein) can be used to provide the following information. Total DNA context is a measure of the number of nuclei in the tissue. This is because all muscle nuclei are diploid and contain the same amount of DNA (Cheek, Holt, Hill and Talbert 1971). Differences in total DNA therefore reflect differences in the rate of cell division. The amount of cytoplasm present is estimated by total protein and the ratio between protein and DNA gives a measure of cell size (Cheek et al 1971). This biochemical cell size is not equivalent to fibre size but provides a measure of the amount of cytoplasm controlled by each nucleus (Moss 1968). The capacity of muscle for protein synthesis per nucleus is given by the RNA/DNA ration (Needham 1964; Garlick, Burk, Swick 1976)

In addition to the above assays, total protein was divided into sarcoplasmic and fibrillar fractions (Helander 1957). This was done as an increase in the ratio between these fractions has been reported as a result of prenatal malnutrition (Azizullah 1973). If this result was found in the present study it would provide additional support for a nutritional origin of the size differences between large and small littermates. Total

DNA and the protein/DNA ratio also show characteristic changes after malnutrition at different stages of growth (Winick and Noble 1966). Finally it was hoped that it might be possible to relate some of the biochemical results to the histology described in the previous section. To this end nuclei concentration was also measured to compare with DNA concentration.

Materials and Methods

As described previously the largest and smallest fetuses were chosen by weight from litters ranging in age from 38 days gestation until term. The semitendinosus muscles were removed from these animals and weighed. Samples in the form of a slice which included both deep and superficial regions were taken from the belly of the muscle.

DNA and RNA extraction and assay

The extraction of DNA and RNA was based upon the modified Schmidt and Thannhauser (1946) technique given in Munro and Fleck (1966).

The muscle slice was minced with scissors and then a weighed sample of approximately 0.2 gm was taken and homogenised in 20X its weight of ice cold water. 2.5 ml of ice cold 0.6 N Perchloric acid (P.C.A.) were added to 5 ml of this homogenate. After mixing, this was allowed to stand for 10 minutes at $0^{o}C$.

1. Centrifuge at 10,000 rpm for 20 mins, supernatant discarded, precipitate washed 2x with 0.2w P.C.A.

2. 4 mls O.3 N KOH added and precipitate resuspended.

3. Solution incubated in oven for 1 hour at $37^{o}C$.

4. After cooling 2.5 mls of 1.2 N P.C.A. added and solution cooled on ice for 10 minutes.

5. Centrifuge at 9000 rpm for 20 minutes. The supernatant containing the RNA fraction was decanted and the precipitate was washed with 5 mls of 0.2 N P.C.A. which was added to the supernatant. (The precipitate contained the DNA fraction).

6. 10 mls of 0.6 N P.C.A. was added to the RNA fraction which was then made up to 100 mls with water. This was a solution of RNA in 0.1 N P.C.A. Using silica cuvettes samples were read at 260 n m. using the deuterium lamp of a Unicam Sp 1800 spectrophotometer against a 0.1 N P.C.A. blank. A linear standard curve was used which had an absorption of one with a solution of 32 μ gms RNA/ml.

7. 4 mls of 1 N P.C.A. was added to the DNA fraction and the precipitate dispersed.

8. The solution was heated in a water bath at 70°C for 25 minutes with occasional shaking. After cooling, the solution was centrifuged at 12,000 r.p.m. for 20 minutes.

9. The supernatant was collected in graduated tubes. The precipitate was washed with 3.8 mls of 1 N P.C.A. and the volume of

supernatant brought up to 8 mls with 1 N P.C.A.

10. 2 mls of Diphenylamine reagent (4% Diphenylamine in glacial acetic acid) was added to 2 mls of the DNA solution together with 0.1 mls of dilute Acetaldehyde (1.6 mg ml^{-1} Acetaldehyde in glacial acetic acid).

This was incubated over-night at 30°C and read at 595 nm against a 1W P.C.A. blank on the spectrophotometer.

Serial dilutions of a standard DNA solution (Calf thymus DNA) were incubated with the experimental samples and used to plot a standard curve.

Protein extraction and assay

The extraction of muscle proteins was based upon Helander (1957). In this technique 3 fractions of protein are extracted. Water soluble or sarcoplasmic proteins are extracted in a solution of phosphate buffer, myofibrillar proteins are extracted with 1·1 M KI and the remaining fraction is called stromal protein. This technique was not feasible due to the unavailability of nitrogen determining apparatus and the reactivity of KI with the folin reagent. A modified technique was therefore adopted. The initial extraction of sacroplasmic proteins with dilute buffer was retained but the remaining proteins were extracted with 1 N NaOH. This modification is similar to that employed by Hegarty

Bratsller and Pearson (1963) and Millward (1970).

Samples of muscle tissue were mounted on labelled chucks and frozen in liquid arcton cooled in a liquid nitrogen bath. These samples were kept frozen in the above solutions overnight and analysed the following day.

Frozen samples of muscle were ground using a cooled pestle and mortar. Weighed samples were then homogenised in X20 aliquotes of 0.03 M potassium phosphate buffer (pH 7.4).

The samples were left for 2 hours on ice and then centrifuged at 10,000 r.p.m.

After removal of the supernatant the above extraction was repeated and the supernatant combined to form the sarcoplasmic fraction.

The precipitate was resuspended in 6 mls of 1 N NaOH and heated in a water bath at 50°C for 1 hour. This solution was centrifuged at 10,000 r.p.m. and the supernatant formed the fibrillar fraction. If the extracts were cloudy due to the presence of lipid, this was removed by filtration through sintered glass. Proteins were assayed by the method of Lowery, Rosebrough, Farr and Randall (1951).

When the total of a biochemical component of the muscle was plotted against fetal weight, the relationship was expressed as an allometric

growth equation the slope of which gives the relative growth velocity of the two parameters. No curves were fitted to the points of other graphs as no additional information would be shown.

The concentration of nuclei was obtained by counting the number in X400 photographs of 10 m thick frozen sections. This number was corrected using the equation of Abercrombie (1946) $N = \frac{T}{T + D} \times n$ (where N is the real number of nuclei, T is the section thickness and D is the diameter of the nuclei in the long axis) to give the number of nuclei whose centres lay in the section.

Results

DNA

As shown in Fig. 70 total DNA is the semitendinosus muscle increases throughout gestation for both large and small littermates. Small littermates however had less DNA in their muscles than the large of the same age. A paired t-test of all the results for large and small littermates in Fig. 70 showed a significant ($P < 0.05$) difference between them. Runt littermates had even less DNA than the other 2 siblings.

When total DNA was plotted against fetal weight (Fig. 71), the lines for large and small were very close together and slopes were not significantly different from each other ($P > 0.5$) or from 1.0 ($P > 0.5$)

Since total DNA was proportional to fetal weight it follows that the concentration of DNA must be the same in both groups of animals. This result was confirmed by a paired t-test which showed no significant difference ($P > 0.5$) between the DNA concentration in large and small. DNA concentration did however vary with age (Fig. 72) reaching a maximum in both large and small at between 80 and 100 days gestation. At this age large animals were approximately 760 gms and small 580 gms. After this age DNA concentration declined. In conclusion, total DNA appears to be proportional to fetal weight while DNA concentration is proportional to fetal age.

Fig. 73 shows the concentration of nuclei plotted against gestational age. The shape of this graph is very similar to that for the concentration of DNA and peak in concentration was reached at about two days. Unlike DNA concentration however, small animals had a consistently higher concentration of nuclei than the large.

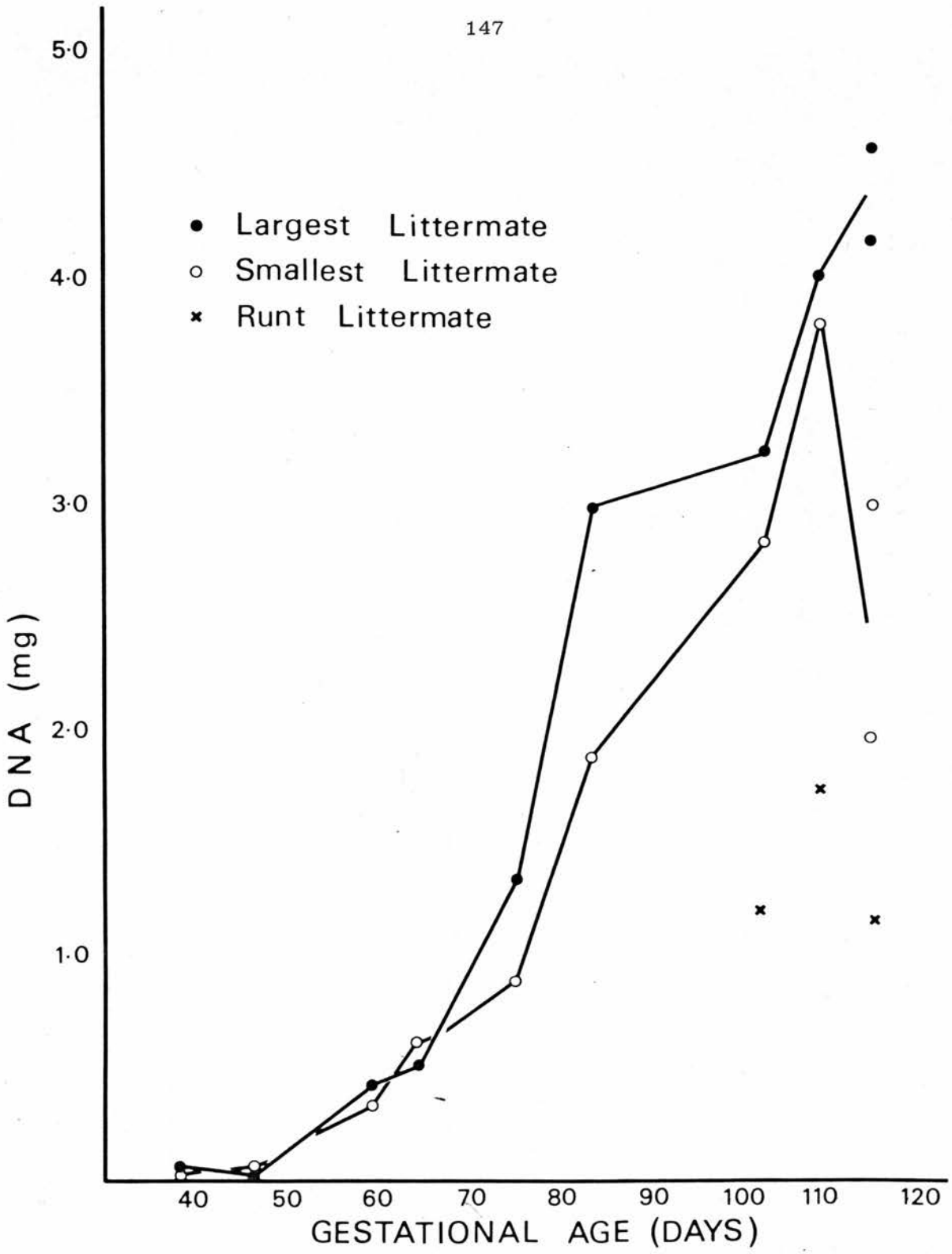


Fig.70 The total DNA content of m. semitendinosus plotted against gestational age. The largest, smallest and runt littermates of each litter are shown.

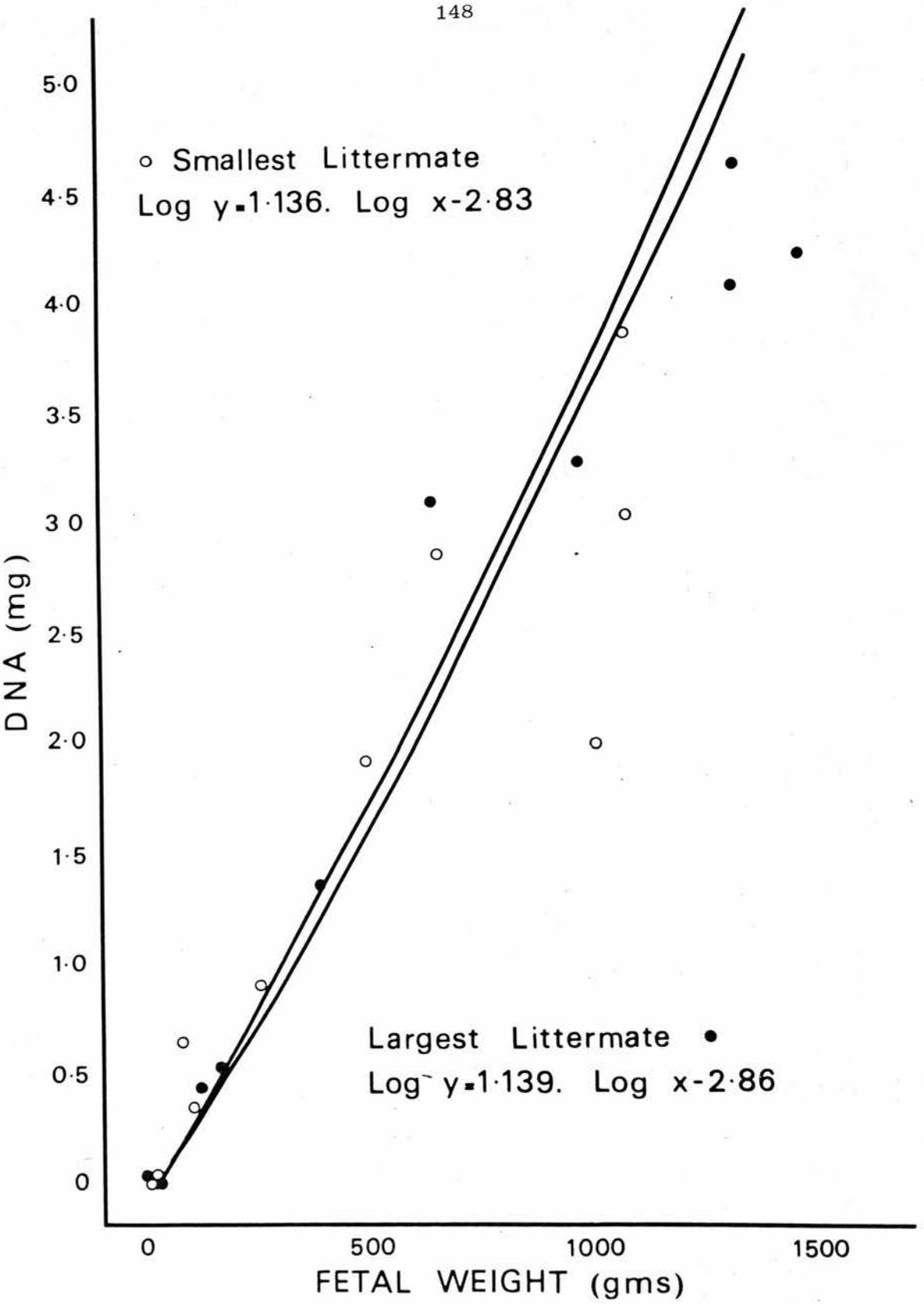


Fig.71 The total DNA content of m. semitendinosus plotted against fetal weight for the largest and smallest littermates.

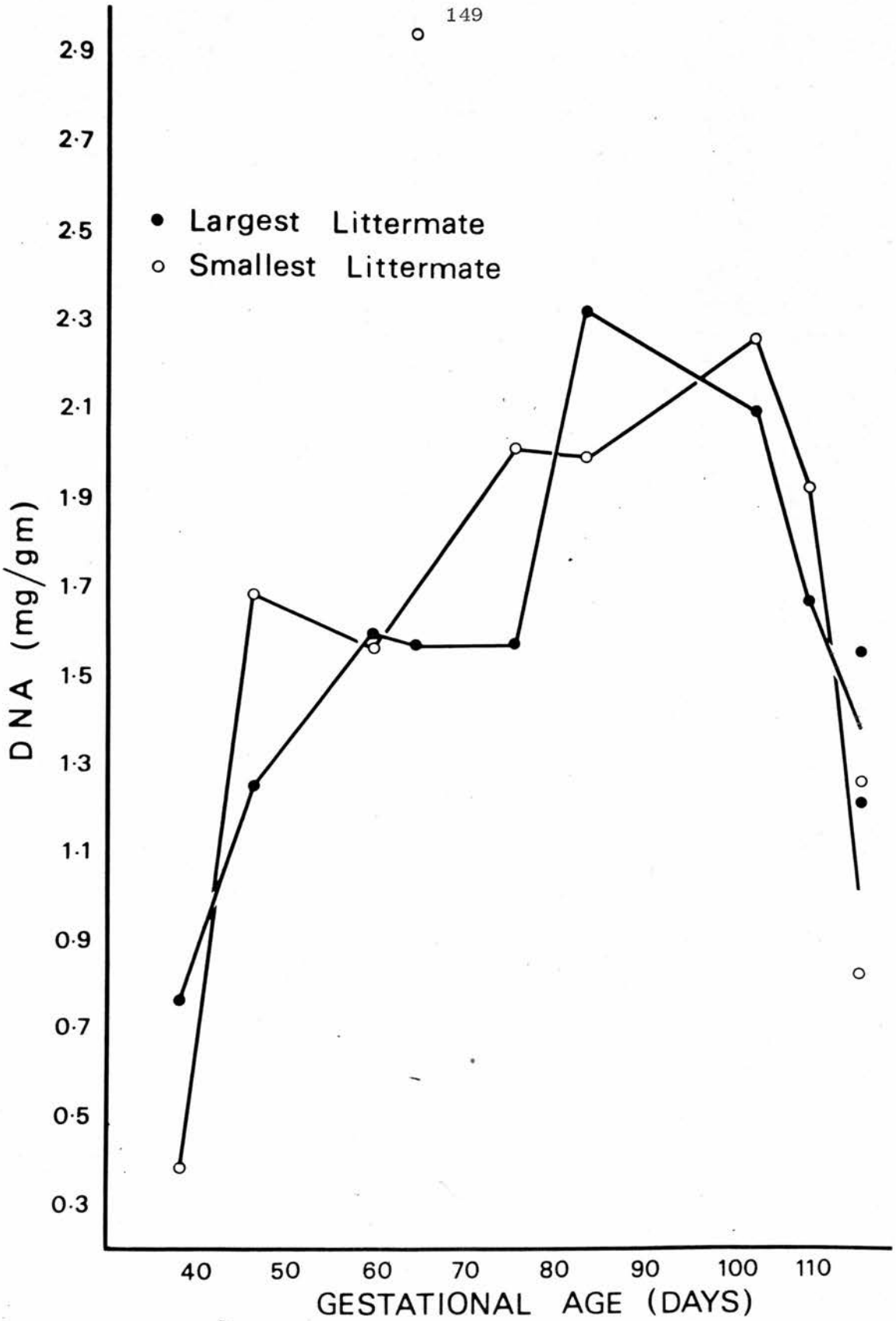


Fig.72 DNA concentration within m. semitendinosus plotted against gestational age for the largest and smallest littermates.

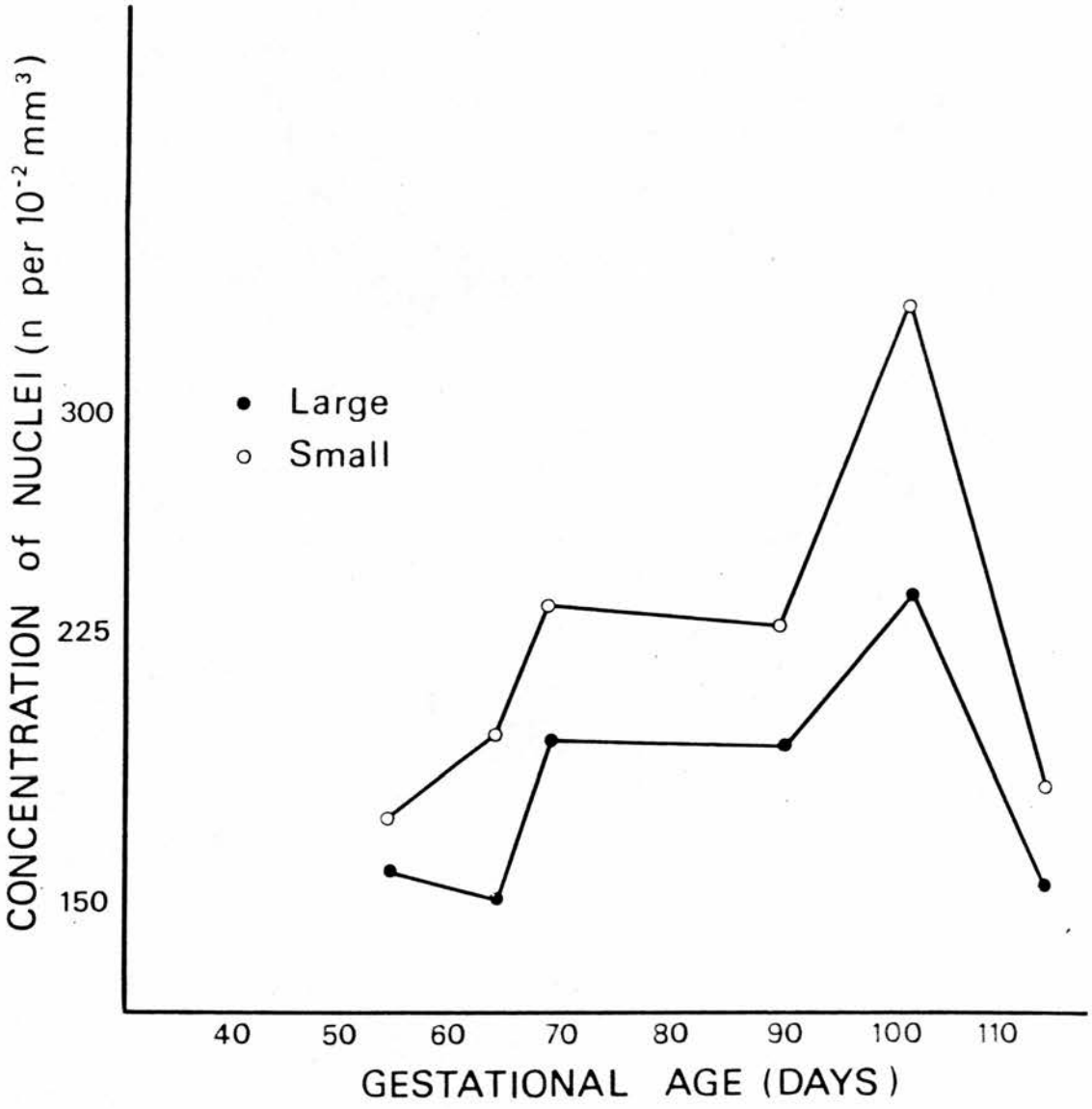


Fig.73 Nuclei concentration within m.semitendinosus plotted against gestational age for the largest and smallest littermates.

RNA

The results for RNA were similar to those for DNA. Fig. 74 shows how total RNA is the muscle increased during gestation. At any given age small littermates have less RNA than their larger siblings and runt littermates have still lower amounts. The differences between large and small are significant ($P < 0.025$) as demonstrated by a paired t-test.

When total RNA is plotted against fetal weight (Fig. 75) there is no significant difference in the slopes of the two lines ($P > 0.5$) and neither are the slopes significantly different from 1.0 ($P > 0.5$). Like DNA, a paired t-test showed no significant difference between the concentration of RNA during gestation. RNA concentration declines with age in both large and small littermates (Fig. 76). Total RNA in the muscle appears to be proportional to fetal weight while RNA concentration is inversely proportional to age.

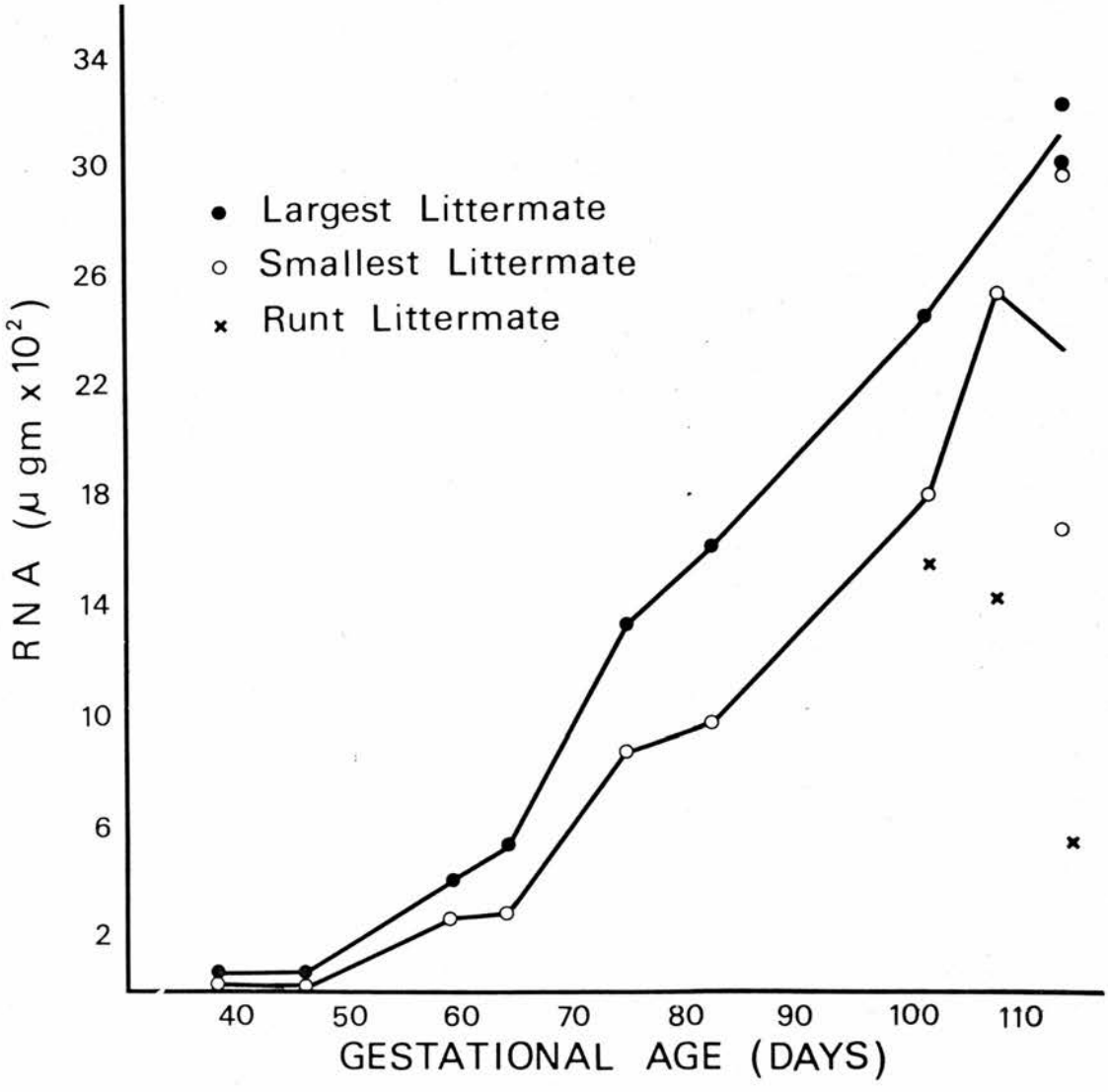


Fig.74 The total RNA content of m.semitendinosus plotted against gestational age. The largest, smallest and runt littermates of each litter are shown.

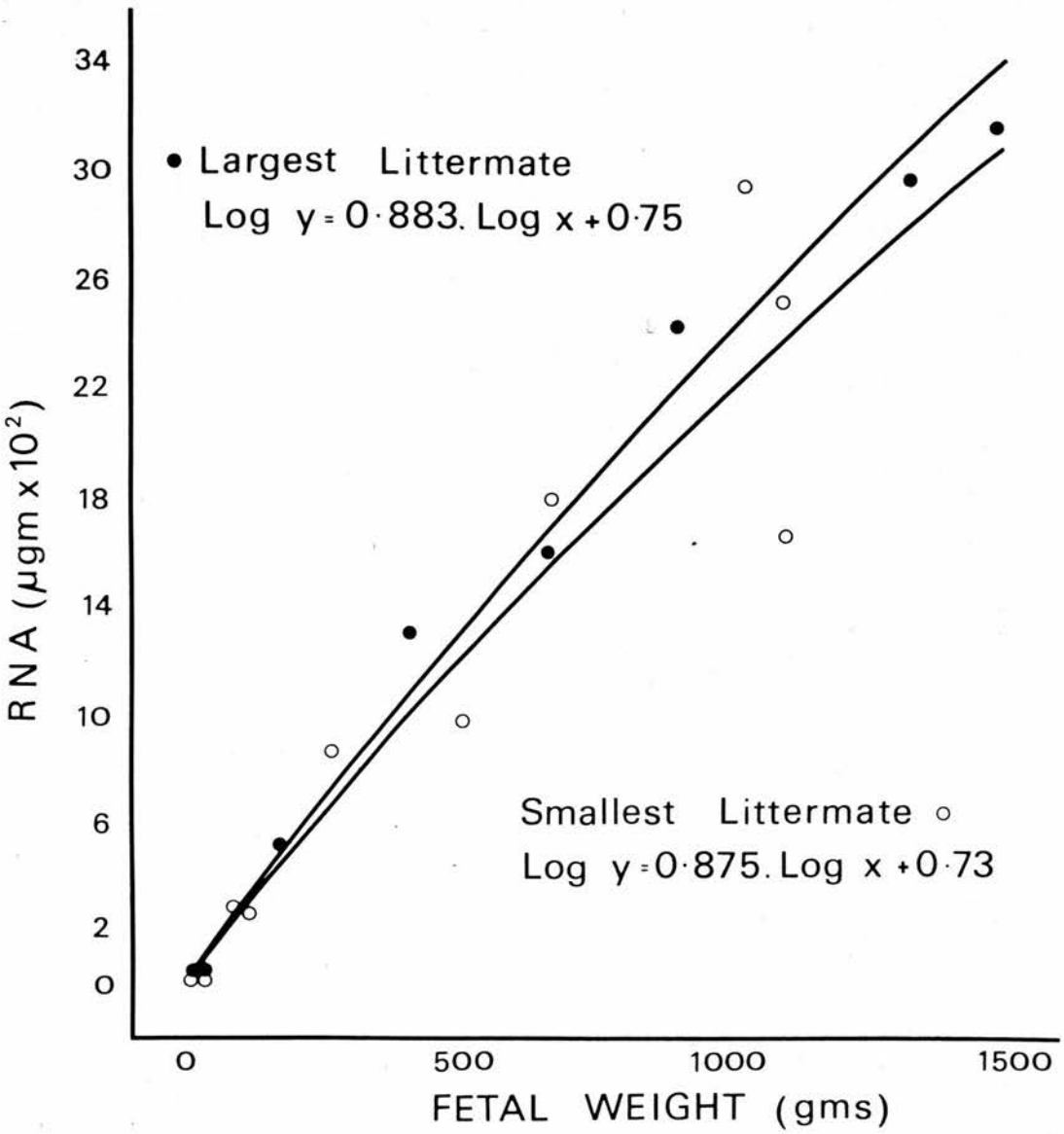


Fig.75 The total RNA content of m.semitendinosus plotted against fetal weight for the largest and smallest littermates.

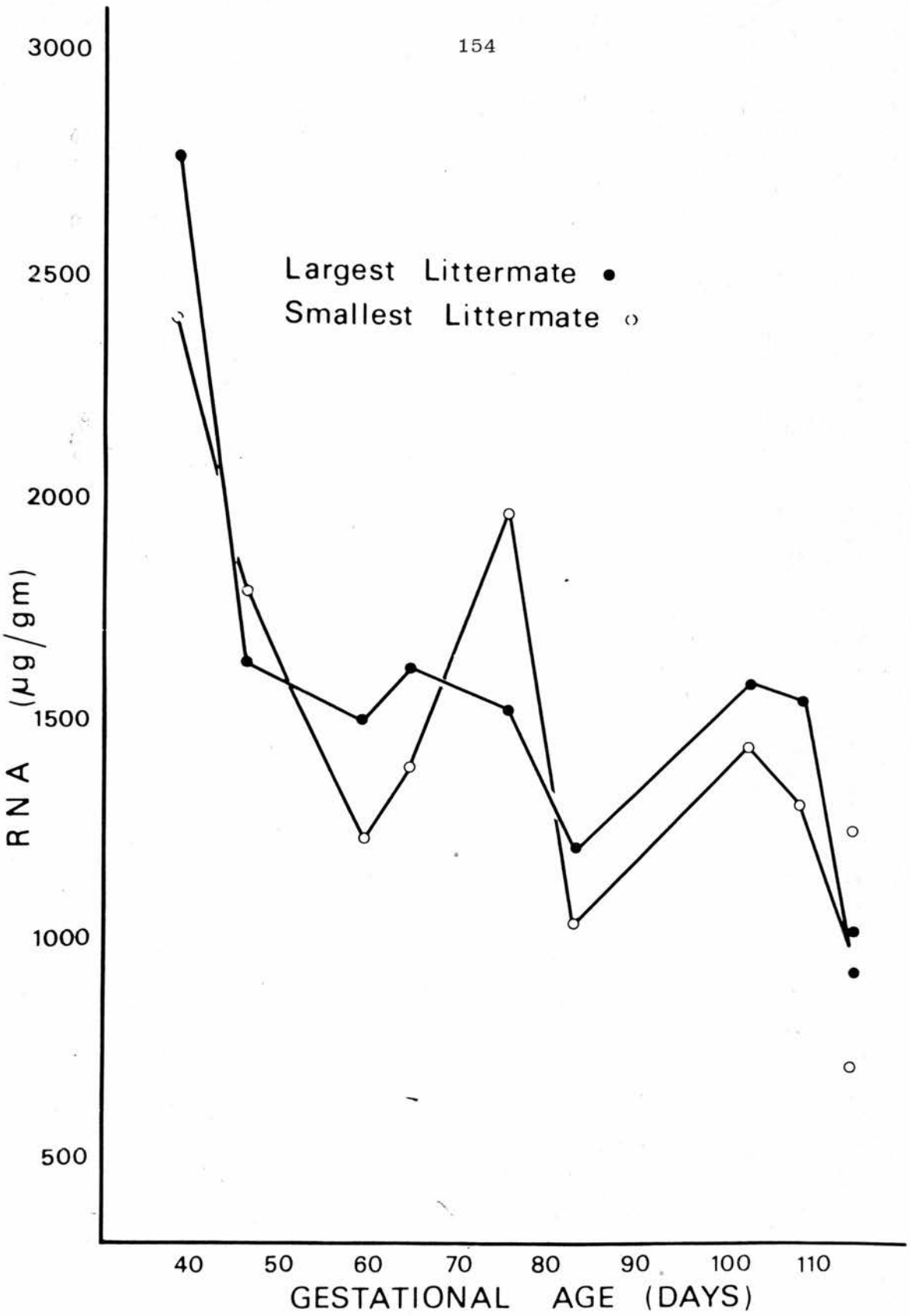


Fig.76 RNA concentration within m.semitendinosus plotted against gestational age for the largest and smallest littermates.

Protein

The total protein content of the semitendinosus muscle increases during gestation (Fig. 77). Small littermates have significantly ($P < 0.05$) less protein in their muscles at any given age as shown by a paired t-test. Runt littermates have the lowest amount of protein at the ages they were measured. Allometric equations of total protein content against fetal weight (Fig. 78) showed that there was no significant difference between large and small at any given body weight. The slopes of the two lines shown in Fig. 78 were not significantly ($P > 0.5$) different from 1.0. This graph appears to differ from the similar graphs shown for DNA and RNA (Figs. 71, 75) in that the four values obtained from new born animals are much higher than would be expected from the slopes of the computed lines.

Protein concentration (Fig. 79) declines from 38 until 59 days and then increases throughout the rest of gestation. No significant difference could be demonstrated between the total protein concentrations of large and small littermates ($P > 0.5$, paired t-test).

Sacroplasmic and fibrillar proteins from large and small animals are shown plotted against age in Fig. 80. Approximately equal amounts of these fractions of protein were present in the muscle at early ages but the amount of fibrillar protein increases more rapidly, so that by term 2/3 of the total protein is fibrillar. No significant difference could be demonstrated between large and small in either sarcoplasmic or fibrillar

proteins. Allometric equations were calculated for sarcoplasmic and fibrillar proteins against fetal weight. Large and small were combined for these calculations and the equations are given below.

Sacroplasmic protein; $\text{Log } Y = 1.20 \text{ Log } X - 1.96$

Fibrillar protein; $\text{Log } Y = 1.33 \text{ Log } X - 2.11$

No significant difference could be found between the regression coefficients of these equations, nor are they significantly different from 1.0.

Table 8 shows the ratio of sarcoplasmic to fibrillar protein for the last four ages before term. The ratio declines in both groups of animals but is consistently higher in the small.

Table 8. The ratio between sarcoplasmic and fibrillar proteins in large and small littermates.

Age (days)	Large or Small	Sarcoplasmic/ Fibrillar proteins
83	L	0.76
	S	0.87
102	L	0.49
	S	0.67
108	L	0.59
	S	0.65
114	L	0.53
	S	0.58

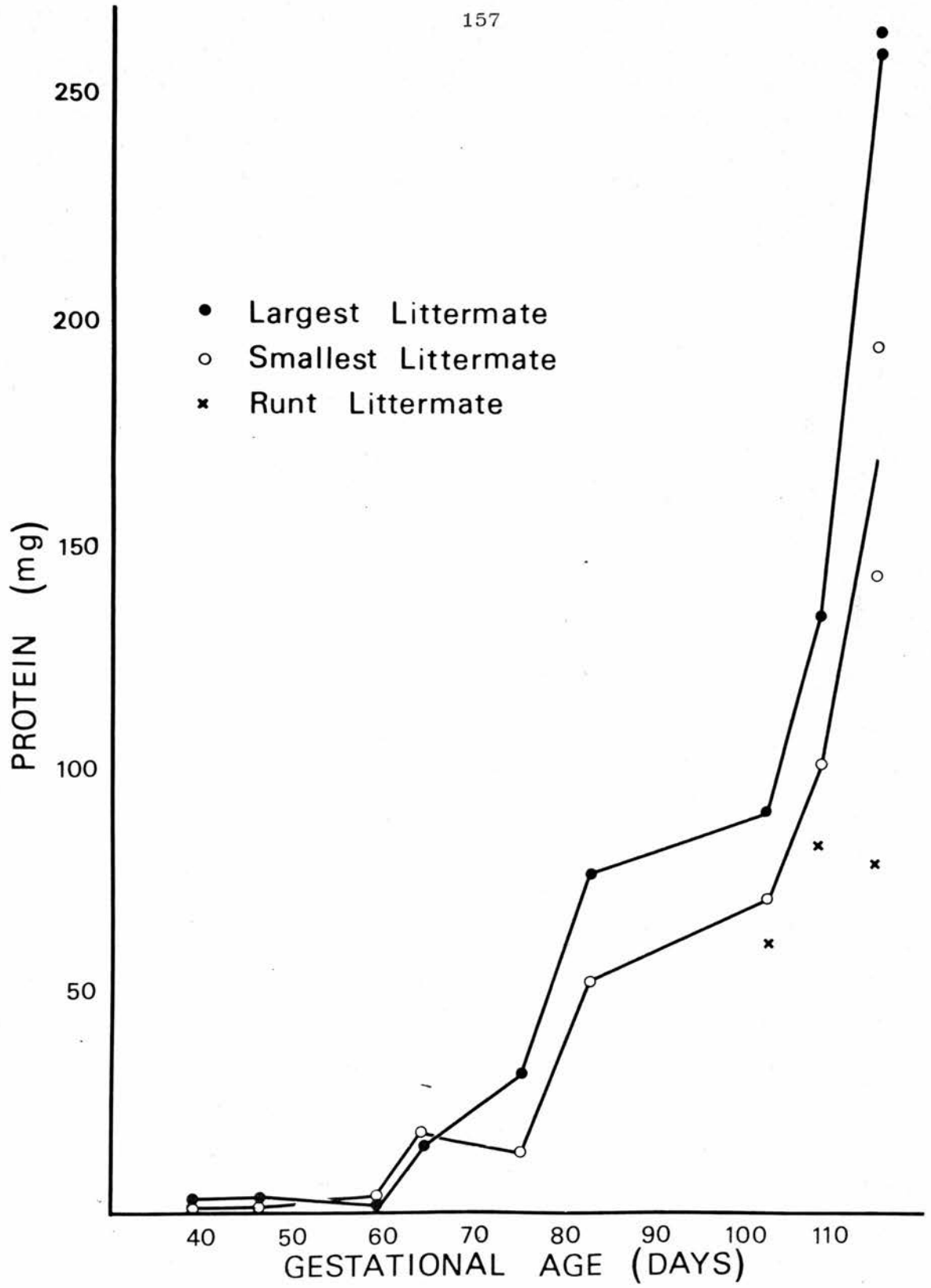


Fig.77 The total protein content of m.semitendinosus plotted against gestational age. The largest, smallest and runt littermates of each litter are shown.

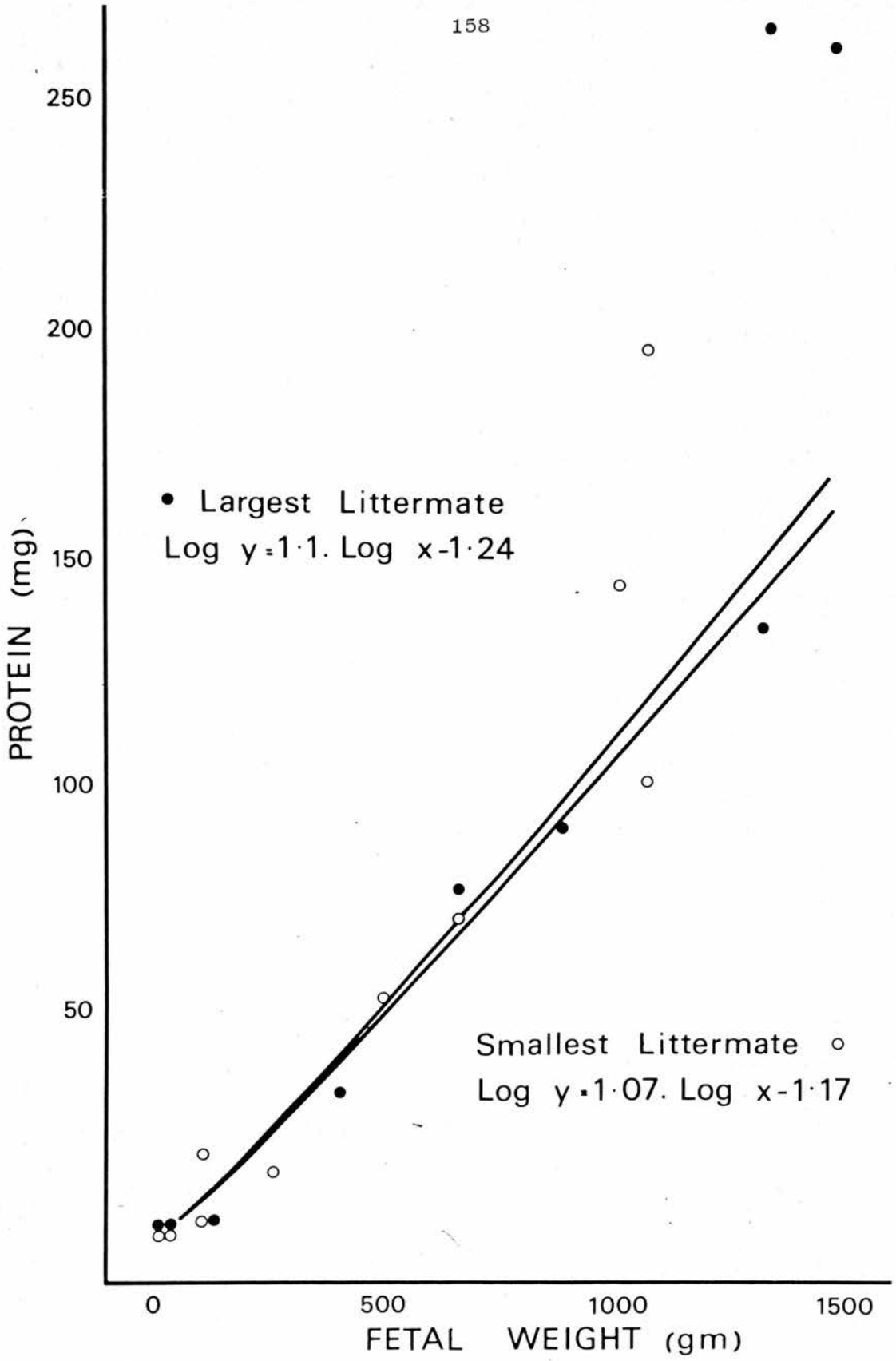


Fig.78 The total protein content of m.semitendinosus plotted against fetal weight for the largest and smallest littermates.

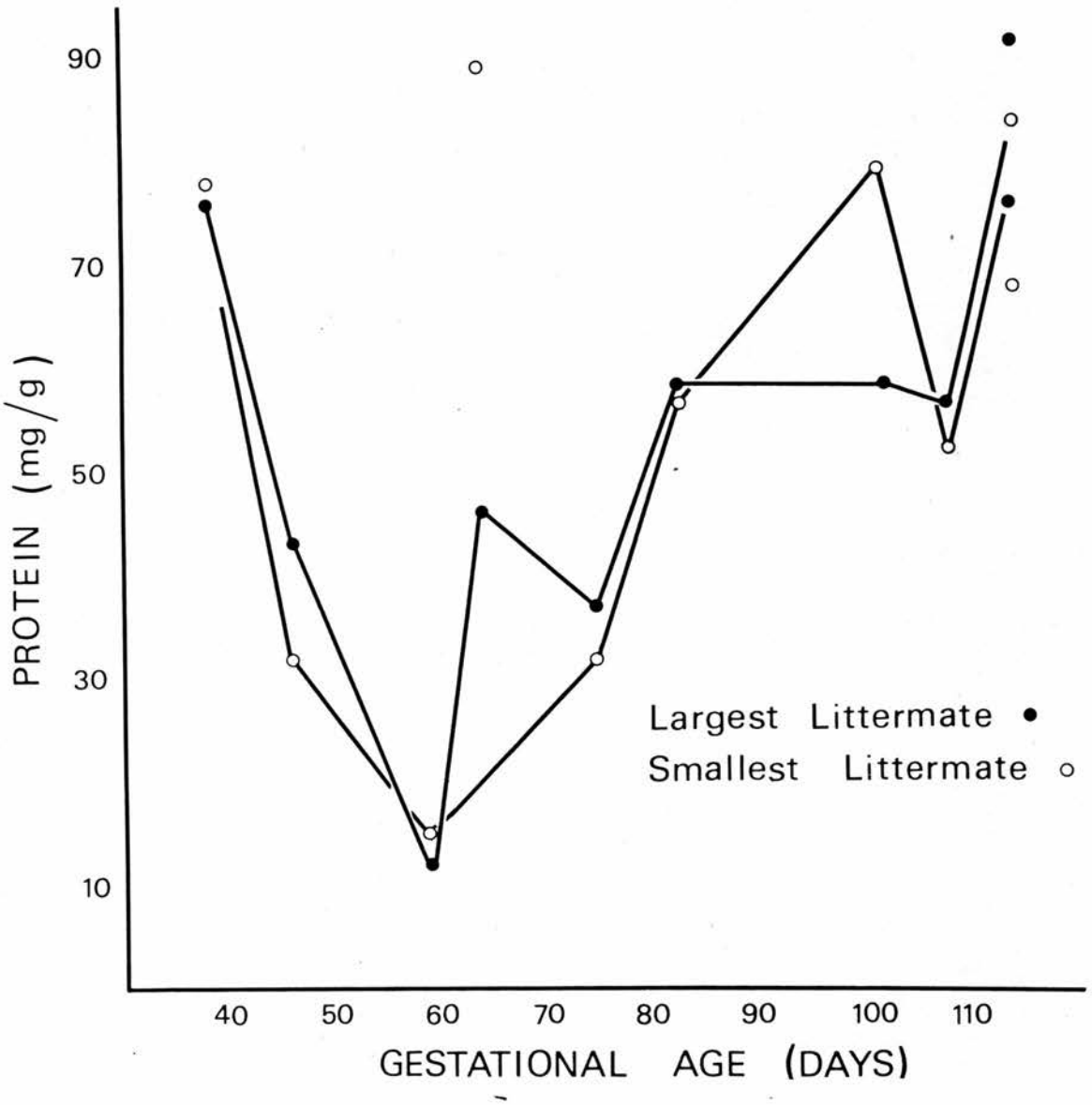


Fig.79 Protein concentration within m.semitendinosus plotted against gestational age for the largest and smallest littermates.

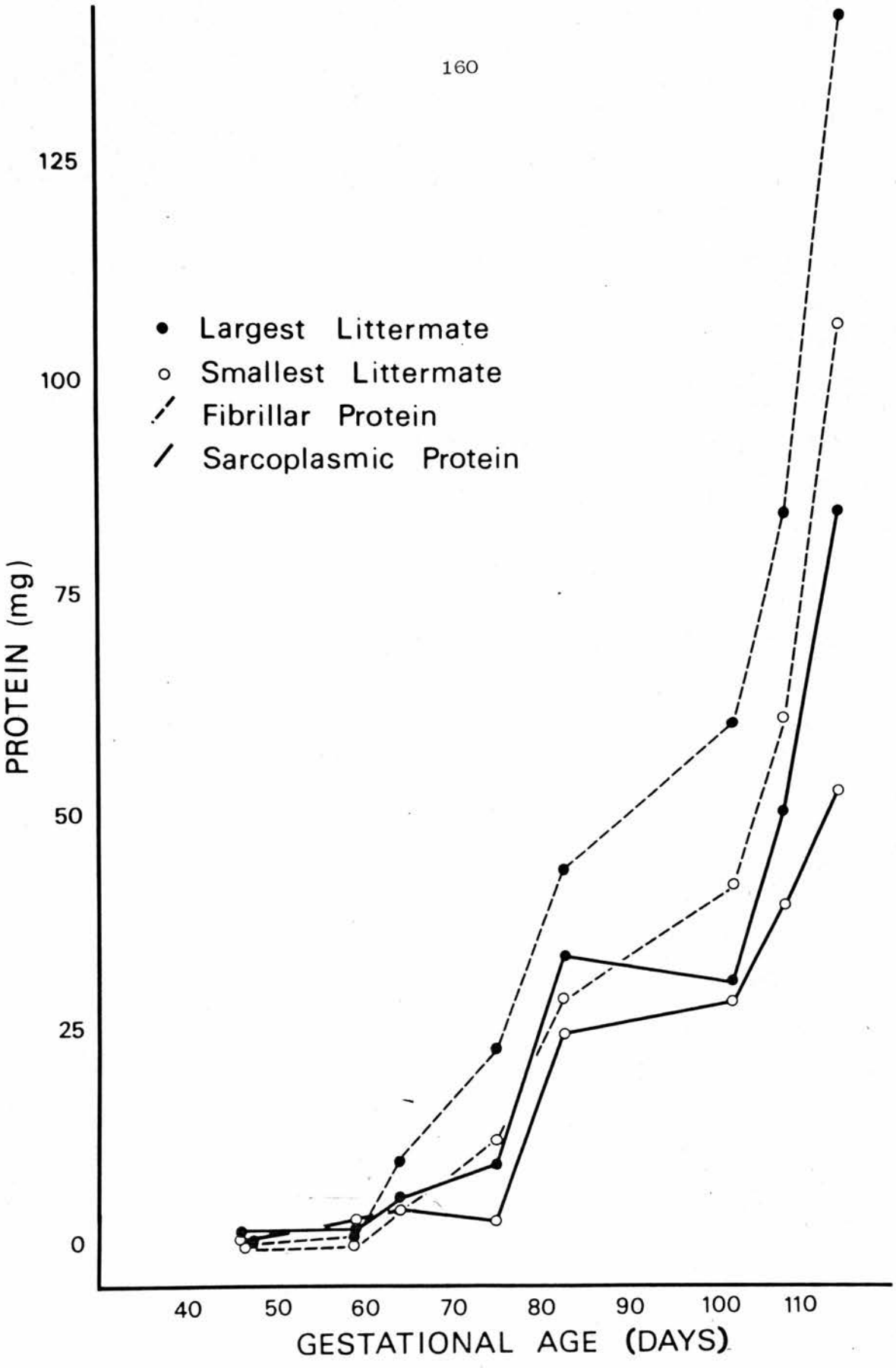


Fig.80 The total sarcoplasmic and fibrillar protein content of m.semitendinosus plotted against gestational age for the largest and smallest littermates.

RNA/DNA and Protein/DNA ratios

Fig. 81 shows the total RNA/total DNA ratio plotted against age. A paired t-test showed that there was no significant difference between the results of the largest and smallest littermates. Apart from the decline between 38 and 46 days, the ratio remains constant for the whole of gestation.

Fig. 82 shows the total protein/total DNA ratio plotted against age. A paired t-test again showed no significant difference between the largest and smallest littermates. This graph also shows a steep drop in the ratio between 38 and 46 days. The ratio then remains constant until 102 days after which it starts to increase.

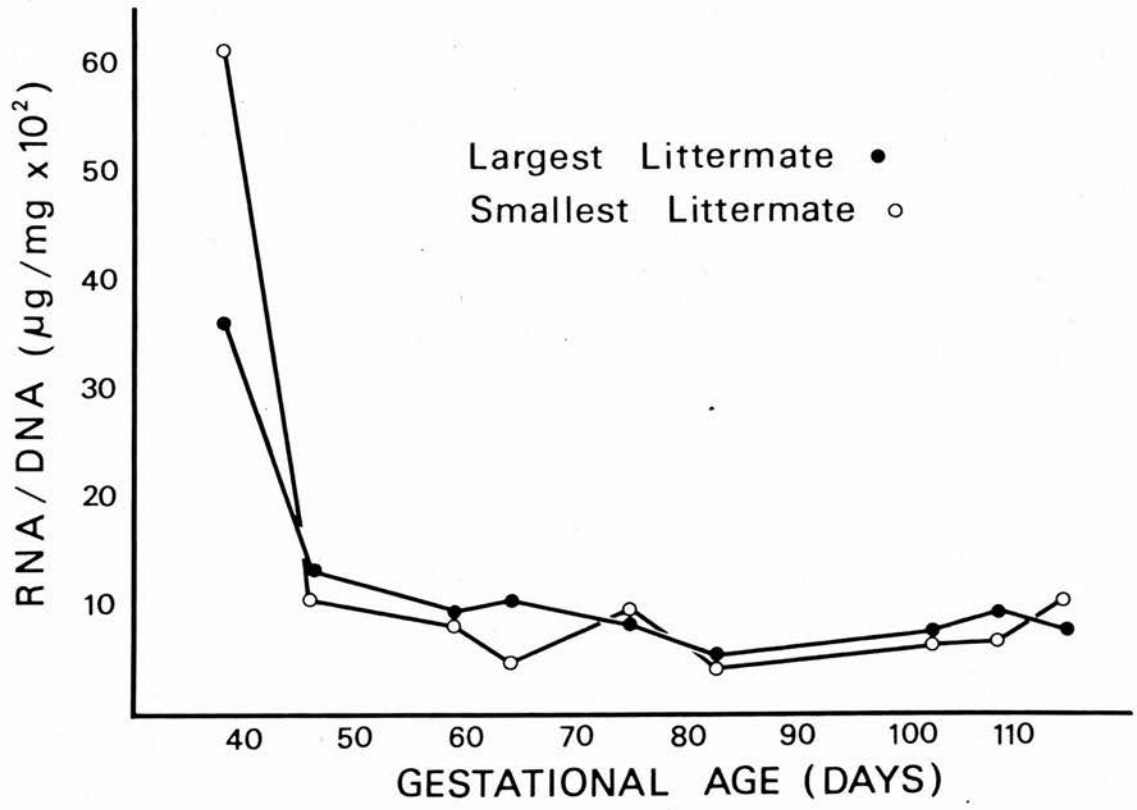


Fig.81 The RNA/DNA ratio of the largest and smallest littermates plotted against gestational age.

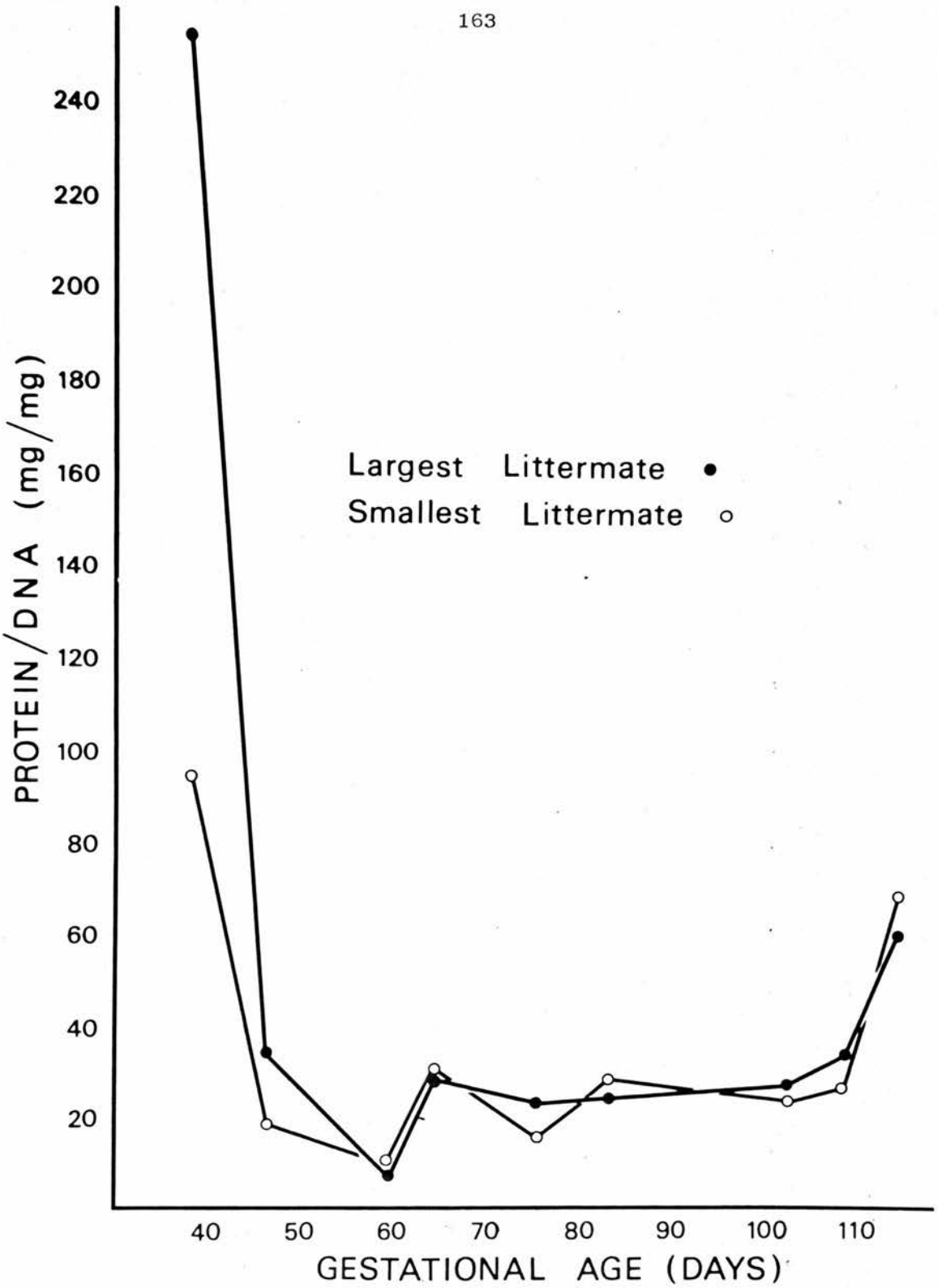


Fig.82 The protein/DNA ratio of the largest and smallest littermates plotted against gestational age.

Discussion

Total DNA, RNA and Protein

All the total amounts of the biochemical parameters increased with age (Figs. 70, 74 and 77) and large animals had significantly greater amounts than small animals at all ages. The most interesting of these results is the lower levels of total DNA found in small littermates which indicates a reduced rate of cell division in these animals. A lower total DNA, RNA and protein has been found after malnutrition during growth (OH and Guy 1971; Winick and Noble 1966; Nnanyelugo 1978) and in small-for-dates babies (Widdowson, Crabb and Milner 1972). It has also been found in genetically small animals (Robinson and Bradford 1969; Rucklidge 1981) The total amounts of these constituents in the above cases appear to be related to the weight of the animal, irrespective of the cause. When plotted against weight (Figs 72, 76 and 79) DNA and protein had regression coefficients above 1.0 and RNA below 1.0. None were however significantly different from unity indicating that all components increased at the same rate as the whole body.

The fibrillar and sarcoplasmic fractions of protein also increased with age (Fig. 80), fibrillar protein increasing faster towards the end of gestation. During this time there was a significant difference in the sarcoplasmic/fibrillar ratio between large and small (Table 8). The small animals had relatively less fibrillar protein, a result identical to that of Azizullah (1973) with the new born offspring of malnourished pregnant mice.

Unlike the total amounts which were weight related, the concentrations of DNA, RNA and protein were age related. The values obtained here were similar to those of other authors who have measured concentration at single ages in the prenatal pig (92 days - Widdowson 1971; 110 days - Campion 1981; at birth - Robinson 1969). The only author to provide results for the whole of gestation is Hakkarainen (1975). His result for DNA concentration also showed a peak at 100 days as was found here but he found that RNA and protein concentration increased throughout gestation. The reason for these differences is unclear. Hakkarainen (1975) uses his own technique which involves the sequential analysis of DNA, RNA, lipid, glycogen and protein from the same sample. The author himself admits that protein, which is the last analysis to be performed, is subject to losses during the extraction of the other components and this was confirmed by the present author. It is therefore possible that differences in technique can explain at least the differences in protein concentrations.

It is possible to relate some of the changes in concentration to the histology described in the previous section. The rise in DNA and nuclei concentration until 100 days, corresponds to the period of fibre hyperplasia and the subsequent decline is probably due to fibre hypertrophy. A similar decline in DNA concentration after the end of fibre formation has been described in bovine fetal muscle (Prior, Scott, Laster and Campion 1979). The difference between large and small found in the nuclei, but not in the DNA concentrations, may indicate greater accuracy in the former method. The decline in RNA concentration was also found by Prior et al (1979) but cannot be

related to structural changes. Protein concentration falls to a minimum during primary fibre formation, possibly reflecting their low rate of formation and low RNA content (Beerman and Cassens 1977) but increases during secondary fibre formation and fibre hypertrophy.

Apart from an initial decline both the RNA/DNA and protein/DNA ratios were constant until 102 days after which the protein/DNA ratio increased. These results indicate that the rate of protein synthesis per nucleus and the biochemical cell size are constant until fibre hypertrophy starts just before term, a result also found by Widdowson et al (1972) in human fetal muscle.

The absence of a difference between large and small in the protein/DNA ratio was also noted by Winick and Noble (1966) in rats malnourished during cellular hyperplasia; Widdowson et al (1972) in small-for-dates babies and Azizullah (1973) in malnourished new born mice. This result, taken together with the lower total DNA found in small littermates, means that these animals had the same size of cell but fewer of them compared to their larger littermates.

The permanence of the stunted growth caused by malnutrition depends upon the age at which it takes place. Animals malnourished early in development, during cellular hyperplasia are unable to grow to the normal size but the effects of malnutrition later in development are reversible (Winick and Noble 1966). Since pigs malnourished in the immediate postnatal period fail to attain their normal adult size (Lodge,

Sarker and Friend 1977), malnutrition during gestation would be expected to have an even greater effect.

General Discussion

This project has been concerned with the comparison of large and small littermates as it was hoped that the different rates of growth of these two groups would provide information on factors affecting development. The main part of the work concentrated on the growth of muscle because of its agricultural and commercial importance. Before dealing with this tissue however it was felt to be important to try and establish the cause of the size difference between the pairs of littermates. To this end, gross measurements of the fetus and its membranes together with muscle histology and biochemistry were compared. In nearly all parameters the small fetus had lower values than its larger sibling and so appeared growth retarded. It was thought that the two must likely candidates for the cause of this were genetics and nutrition or a combination of both. From the work of previous authors it is clear that nutrition and inheritance can operate to produce similar effects during growth. For example, both prenatal malnutrition and selection for small size have produced a reduced DNA content and lower fibre number in muscle (Aberle and Doolittle 1976; Everitt 1968; Luff and Goldspink 1970). No work appears to have been done on the prenatal growth of genetically different sized animals and only a small amount on the effects of undernutrition prenatally. The results obtained here closely agree with those obtained in experimentally induced malnutrition (Everitt 1968; Azizullah 1973) and this, together with the similarity of the small littermates to 'small-for-dates' babies (Davies 1981), has led the present author to believe that nutrition is the major cause of the size difference

between large and small littermates.

The most important difference between the muscles of large and small were the differences in fibre number and total DNA. Fibre formation only takes place during a short period prenatally and so a lack of fibres at birth can not be corrected by post-natal conditions (Staun 1963; Stickland and Goldspink 1973). The effects on muscle of undernutrition are therefore different before and after birth. Before birth undernutrition can affect fibre number while postnatally it only affects fibre size (Stickland, Widdowson and Goldspink 1975).

It is known that malnutrition during cellular hyperplasia can permanently reduce cell number (Winick and Noble 1966) and so it would be expected that the small littermates would have fewer nuclei throughout life. The apparent permanency of differences in these parameters helps to explain why runt pigs never attain normal adult size (Widdowson 1971).

The importance of fibre number on meat quality and quantity has already been mentioned. The difference in fibre number between both large and small in the present project provided an opportunity to look for the mechanism which had brought this about. It was found that although primary fibres appeared to be resistant to changes in their number, a reduction in their size affected their capacity to support secondary fibre formation. It is possible that other factors may have contributed to this. In particular, the effects of the reduced rate of cell division on fibre hyperplasia are unknown.

It would now be worthwhile to compare the prenatal growth of genetically different sized strains. Genetic selection for increased fibre number is also thought to have affected secondary fibre formation (Ashmore et al 1973). It would be interesting to use the parameters described in this project to see which of them operate to affect fibre number under these conditions.

It is probably not possible to change the nutritional status of the fetus beyond giving the mother adequate feeding. It is therefore unlikely that anything can be done to increase the prenatal growth of small littermates. It is hoped however that with an understanding of the parameters which influence muscle growth, it may be found that some are amenable to change which may have an effect on the current programmes of breeding and selection.

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APPENDIX ONE

Fetal and Placental Data

Age=Gestational age(days).Pos.=Position within the uterine horn. R=Right horn L=Left horn.Positions are numbered linearly from ovary to cervix.Weight=Fetal weight(gms).Crown R.=Crown rump length(cm.). Pl.Wt.=Placental weight(gms).Pl.A.=Placental area(sq.cm.)Pl.L.=Placental length(mm.)Pl.C.=Placental circumference(cm.).

Age	Pos.	Sex	Weight	Crown R.	Pl.Wt.	Pl.A.	Pl.L.	Pl.C.
54	R1	F	78.6	12.8	262	807.8		15
	R2	M	31.5	9.0	45	462.5	141	13
	L1	F	79.2	11.9	255	814.6	407	14.1
90	R1	F	660	24.9	575	1417.2	430	16.5
	R2	M	845	24.0	565	1573	495	17.5
	R3	F	778	23.6	525	1350	615	17.2
	R4	F	630	23.2	370	911.7	554	17.2
	L1	F	635	23.3	435	1071	560	16.5
	L2	M	670	23.4	350	1056	516	16.5
	L3	F	765	24.2	410	1834	712	17.0
	L4	M	735	22.9	585	1635	655	9.0
64	R1	M	143	14.5	155	385.3	269	14.2
	R2	M	116	13.6	93	198.3	255	17.4
	R3	M	129	13.8	155	619.0	188	18.0
	R4	F	104	13.2	165	205.8	215	15.0
	R5	M	112	13.0	109	395.2	180	15.0
	R6	F	125	13.o	53	150.1	130	16.0
	R7	F	84	12.5	164	174.9	155	11.9
	R8	F	158	14.4	116	434.7	117	19.0
	L1	F	163	15.3	177	482.9	555	16.5
	L2	F	137	14.2	101	292.3	301	17.0
	L3	M	140	14.0	109	330.2	230	17.5
	L4	F	153	14.4	136	430.0	358	16.0
	L5	F	154	14.3	107	364.4	464	16.0
38	R1		9.0	4.9	63	232.9	330	
	R2		9.1	4.5	74	297.4	238	
	R3		8.52	4.4	54	295.4	207	
	R4		9.7	4.6	52	249.1	207	
	R5		5.o4	3.9	28	83.5	73	
	R6		9.34	4.3	65	259.5	192	
	L1		6.90	4.1	55	257.0	282	
	L2		7.85	4.5	64	219.1	171	
	L3		9.00	4.8	55	184.3	167	
	L4		7.25	4.5	35	165.7	148	
	L5		7.40	4.1	33	139.0	135	
	L6		7.90	4.4	39	146.2	141	
	L7		7.40	4.2	24	116.5	105	
	L8		7.80	4.5	60	189.9	169	

Age	Pos.	Sex	Weight	Crown R.	Pl.Wt.	Pl.A.	Pl.L.	Pl.C.
69	R1	F	245	16.9	216	773	445	10.2
	R2	F	160	14.8	106	387)	204	11.0
	R3	F	245	16.1	251	937	344	13.2
	R4	F	253	17.6	291	946	353	11.0
	R5	F	236	16.7	226	677	310	11.7
	L1	F	240	16.3	296	1007	510	9.0
	L2	M	251	16.1	305	844	335	10.3
	L3	F	221	16.0	241	774	275	12.0
	L4	M	176	14.9	276	479	158	10.9
	L5	M	252	16.1	176	699	280	11.1
	L6	F	124	14.5	296	641	254	10.5
	L7	F	227	15.2	226	857	349	8.6
75	R1		240	Decomposed				
	R2		404	Decomposed				
	R3	M	345	18.2	165	653	360	22
	R4	F	323	17.9	141	702	248	18
	R5	F	361	17.8	171	1095	320	23,2
	L1	F	323	18.0	164	736	465	24
	L2	M	257	17.6	135	604	185	23
	L3	M	371	19.0	201	981	250	24.2
	L4	F	393	20.3	270	1103	342	24
46	R1	M	21.19	6.8	80	430.5	242	12.7
	R2	F	18.48	6.6	75	338.8	161	13.5
	R3	F	18.26	6.7	64	338.4	162	10.4
	R4	F	17.43	6.3	68	370.0	160	14.4
	R5	M	18.49	6.7	68	387.8	156	14.1
	R6	M	19.32	6.8	78	351.3	159	14.0
	R7	M	16.28	6.8	78	286.8	197	12.5
	L1	M	19.99	6.8	80	434.9	280	13.7
	L2	M	21.15	6.6	106	434.9	244	14.0
	L3	M	19.43	6.9	78	405.6	209	13.5
	L4	F	17.70	6.9	73	365.3	219	13.2
	L5	M	19.53	7.0	74	421.5	159	12.0
	L6	F	17.58	6.8	66	383.0	196	12.8
59	R1	M	107	13.2	180	950.7	410	17.2
	R2	F	109	13.2	163	647.2	172	18.2
	R3	M	107	13.0	178	816.2	238	20.8
	R4	M	120	13.1	203	986.7	360	19.8
	L1	M	125	14.0	171	948.1	360	21.8
	L2	M	125	13.6	184	1155.3	332	18.0
82	R1	M	585	22.0	297	1244	560	18.5
	R2	F	550	21.9	267	956	366	23.0
	R3	M	650	23.1	312	1323	392	20.5
	L1	F	570	21.9	317	1413	452	19.9
	L2	M	565	20.3	228	1189	334	21.5
	L3	M	645	23.5	353	1365	394	22.5
	L4	F	590	21.5	391	1290	385	24.0
	L5	F	495	21.7	154	861	290	22.0

Age	Pos.	Sex	Weight	Crown R.	P1.Wt.	P1.A.	P1.L.	P1.C.
102	R1	M	819	25.6	219	745.6	360	22.5
	R2	F	719	24.9	194	646.8	221	22.3
	R3	M	660	24.5	158	713.1	206	14.5
	R4	F	720	24.5	133	599.6	164	24.4
	R5	M	878	26.2	207	901.5	197	22.5
	R6	M	680	24.8	123	579.7	212	25.0
	R7	M	857	26.7	229	812.2	270	22.0
	L1	M	870	26.2	219	590.0	338	
	L2	F	760	24.5	233	752.3	304	
	L3	M	787	25.0	169	539.8	192	
	L4	F	771	24.5	148	547.4	190	24.4
	L5	F	553	23.1	112	468.3	180	
	L6	M	754	24.6	189	699.1	326	
114		F	1400		New Born			
		M	1200					
		F	1300					
		M	1200					
		M	1300					
		F	915	28.4				
		M	1080	27.9				
		M	1326	28.8				
114		M	1465	29.0	New Born			
		M	1006	26.9				
		M	1100					
		M	1300					
		M	1300					
		M	1400					
		M	1500					
		F	1200					
		F	800					
		F	1100					
		F	1100					
		F	1400					
		F	1200					
		F	1900					
70	R1	M	280	18.3	185		420	21
	R2	M	300	18.0	245		340	24
	R3	F	240	16.6	130		300	21
	R4	F	300	17.9			370	23
	R5	M	317	18.8	235		300	21
	R6	F	218	17.2	240		310	21
	L1	F	305	18.1	260		390	21
	L2	F	220	16.9	110		190	21
	L3	F	303	17.7	205		310	24
	L4	M	328	19.2	310		340	28
	L5	M	318	18.5	245		300	23
	L6	F	188	15.7	160		170	20
	L7	M	223	16.6	135		270	21
108	R1	M	1195	27.9	310	1140	460	22
	R2	M	1315	28.9	310	1117	400	25
	L1	M	1160	28.8	265	1146	420	23.6
	L2	F	1165	27.9	345	1177	250	28
	L3	M	1075	27.2	270	957	195	24
	L4	F	660	23.4	210	766	200	20.5

APPENDIX TWO

Muscle Data

Age=Gestational age(days).Weight=Weight of fetus(gms). M.Wt. = m.semitendinosus weight(gms).Area=Cross sectional area of m.semitendinosus (sq.mm.).Pri F.=Primary fibre diameter(m).Sec.F.=Secondary fibre diameter(m). No.Pri.=Total number of primaries. Total F.=Total number of fibres. S/P=Secondary/primary fibre ratio.

Age	Weight	M.Wt.	Area	Pri.F.	Sec.F.	No.Pri.	Total F.	S/P
54	31.53	(o.12)	9.24	13.7	6.95	4140	5589	0.35
	79.2	(o.o9)	19.57	18.51	8.6	7280	8663	0.19
90	845	1.6	(108.8)	14.86	7.77	7135	350455	
	630	(1.11)	(89.9)	13.81	6.66	6325	330905	
64	163	o.32	40.13	22.8	8.5	18982	97188	4.12
	84	0.21	24.47	15.6	6.3	17491	57720	2.3
38	9.34	(o.o17)	(5.94)	9.06		3393	3393	
	5.04	(0.02)	(3.88)	8.7		2349	2349	
69	253	0.60	70.72	21.38	8.76	17308	203888	10.78
	124	0.200	40.45	15.94	7.8	21112	162774	6.71
75	398	0.862	107.7	15.62	6.49	20131	308810	14.34
	257	o.444	58.1	17.14	5.68	17027	198024	10.63
46	21.2	0.032	(9.86)	13.78		6765	6765	
	16.28	0.054	(8.74)	11.87		5687	5687	
59	125	0.259	27.16	21.2	6.44	11470	21632	0.886
	107	0.235	29.05	16.70	5.67	16578	28713	0.732
82	650	1.322	(91.73)	16.61	6.89	11928	327781	
	495	0.949	74.04	17.42	7.04	10949	284236	
102	878	1.546	118.3	14.82	7.75	3946	390354	
	660	1.252	67.1	10.86	5.68	4773	301258	
114	1326	2.94	118.4	13.62	9.39	3891	362042	
	1080	2.35	118.7	12.66	7.72	2858	284296	
114	1465	3.47	140.2	13.01	9.24			
	1006	2.14		14.84	9.20			
108	1315	2.41	130.1					
	1075	1.93	141.8					
70	328	0.64	57.34			20458		

APPENDIX THREE

Biochemical Data

Age=Gestational age.Weight=fetal weight. T.DNA=Total DNA. C.DNA= Concentration of DNA. T.RNA=Total RNA. C.RNA=Concentration of RNA. T.Sar=Total sarcoplasmic protein. T.Fib=Total fibrillar protein. C.Pro.=Concentration of protein. All results relate to m.semitendinosus

Age	Weight	T.DNA	C.DNA.	T.RNA	C.RNA	T.Sar.	T.Fib	C.Pro.
64	163	0.507	1.573	522.9	1623	5.04	9.85	46.28
	84	0.617	2.95	291.9	1397	4.62	13.95	89.07
38	9.7	o.016	0.769	57.2	2773			76.0
	5.04	o.005	0.389	27.8	2403			77.8
75	398	1.35	1.57	1312	1533	9.20	22.72	37.03
	257	0.897	2.02	880	1982	2.59	11.98	32.82
46	21.2	0.04	1.25	52.2	1631	0.73	0.656	43.30
	16.2	0.041	1.69	43.1	1796	0.45	0.336	32.70
59	125	0.426	1.6	399	1500	1.92	1.28	12.00
	107	0.345	1.57	277	1259	2.10	1.21	15.06
83	650	3.087	2.335	1602	1212	33.5	43.8	58.45
	495	1.903	2.005	984	1037	24.8	28.65	56.29
102	878	3.265	2.112	2458	1590	30.0	60.87	58.79
	553	1.238	1.354	1547	1692	21.4	40.46	67.72
	660	2.856	2.281	1811	1447	28.3	42.41	79.34
114	1326	4.603	1.567	3007	1024	154.2	112.4	90.76
	1080	3.02	1.277	1681	714	73.2	122.9	83.33
	915	1.17	0.867	549	406	37.3	42.5	58.93
114	1465	4.2	1.21	3215	926	90.77	171.1	75.41
	1006	1.98	0.833	2975	1250	53.05	91.0	67.36
108	1315	4.05	1.681	3744	1553	50.34	35.32	56.2
	1075	3.85	1.94	2555	1317	39.89	31.61	52.17
	660	1.75	1.015	1418	1398	16.64	64.33	80.72

Scale:
Age in days.Weight in gms. T.DNA in mg. C.DNA in mg gm^{-1}. T.RNA in μgm. C.RNA in μgm gm^{-1}.TSar. and T.Fib. in mg. C.Pro in mg gm^{-1}

[*From the Proceedings of the Anatomical Society of Great Britain and Ireland*, 1–3 *April* 1981. J. Anat., (1981), **133**, 1, p. 132]

49. Prenatal muscle development in the pig: a comparison of the largest and smallest litter mates.

By P. M. C. WIGMORE and N. C. STICKLAND. *Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh*

The semitendinosus muscle was removed from both sides of the largest and smallest fetus in litters ranging from 38 days gestation to full term. One muscle was used to obtain frozen transverse sections for histological examination and the other muscle was used for biochemical analyses.

Measurements on the frozen sections showed that cell diameter in the small fetuses was smaller than that in the large, but this difference was largely correlated with the considerable fetal weight differences. The biochemical analyses showed that protein, RNA and DNA contents were also weight-related rather than age-related parameters.

Histological examination of the muscle sections revealed the expected biphasic development of muscle fibres, with primary myotubes being present at 38 days, and secondary fibres forming between 50 and 55 days gestation. In large and small fetuses the myotubes showed an increase in diameter of 144 % and 90 % respectively from the 38 days stage, before decreasing. As well as the difference in the amount of increase, it was noted that the maximum myotube diameter was attained significantly later in the small fetuses. Although myotubes from the large fetuses had the greater myofibrillar content they had also a significantly greater proportion of intracellular space (or non-myofibrillar content). The effect of these factors on myotube diameter may be important since secondary myofibres are known to form on the surfaces of myotubes. The reduced myotube surface area in the small fetuses may therefore be a factor contributing to their reduced fibre number at full term.

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