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Growth of striated muscle in an Australian marsupial (*Setonix brachyurus*)

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INTRODUCTION

Marchok & Herrmann (1967) describe three stages in the embryonic differentiation of muscle cells. Cells in the first stage are described as *presumptive myoblasts*, recognized as distinct from mesenchyme only by their site in the embryo. These cells show a moderate rate of replication. At a slightly more mature stage, *myoblasts* replicate very rapidly. Their labelling index with [^{3}H]thymidine is more than three times that seen in adjacent loose mesenchyme. In the third stage, immediately preceding myoblast fusion, there is no replication of myoblasts.

The earliest form of muscle fibre, the *myotube*, is so named because it consists of a hollow tube composed of a peripheral sleeve of contractile myofibrils enclosing a central cytoplasmic core along which the nuclei may migrate. Stockdale & Holtzer (1961) found that a fluorescein-labelled antimyosin would stain myotubes, but not mononucleated cells which were present in the same culture. Furthermore, DNA was synthesized by the mononucleated cells, but not by myotube nuclei. They concluded that muscle-specific protein is not synthesized until DNA synthesis is complete.

Protein synthesis proceeds continuously in myotubes. As a result, the central cytoplasmic core becomes packed with myofibrils, and nuclei are forced to the periphery of the fibre. During further growth the following processes occur simultaneously.

(i) *An increase in fibre numbers.* In rats, Chiakulas & Pauly (1965) state that the rapid postnatal increase in the number of fibres ceases after 3 weeks, although the total cross-sectional area of each muscle increases constantly. Hence, up to a certain age, the increase in the size of a muscle is partly due to an increase in the number of fibres. After this age, further growth is entirely due to the enlargement of individual fibres.

(ii) *An increase in fibre diameter.* The extent of the increase is largely dependent on the work-load of the muscle. (Bowden & Goyer, 1960.)

(iii) *An increase in fibre length.* Lengthening occurs by appositional rather than interstitial growth. (Kitiyakara & Angevine, 1963), which would imply that new sarcomeres are added to both ends of existing fibres.

(iv) *Nuclear replication.* The number of nuclei in a muscle increases markedly during postnatal growth. (Montgomery, 1962; Enesco & Puddy, 1964.)

The foregoing account poses certain problems. If differentiated muscle nuclei lose the ability to divide, where do all the new nuclei come from? How do the fibres increase in number? Is there a new population of small fibres?

These problems are accentuated in the animal studied, due to its unusual manner of reproduction. The newborn quokka (*Setonix brachyurus*) is extremely immature, weighing less than half a gram, but some features are precociously developed, as in other marsupials (Hill & Hill, 1955). The difference between the maturity of the forelimbs and hind limbs is particularly relevant (Fig. 1). At birth the newborn quokka climbs unaided up its mother's anterior abdominal wall to reach the pouch. This considerable feat is achieved using its well-developed forelimbs, which even have claws to assist grasping the mother's fur. In striking contrast, the hind limbs are minute, functionless buds.

These limb proportions are reversed in the adult, which resembles a tiny, squat kangaroo (Fig. 2). The hind limbs are large and powerful, adapted to the animal's hopping gait, whereas the diminutive forelimbs are used for grasping.

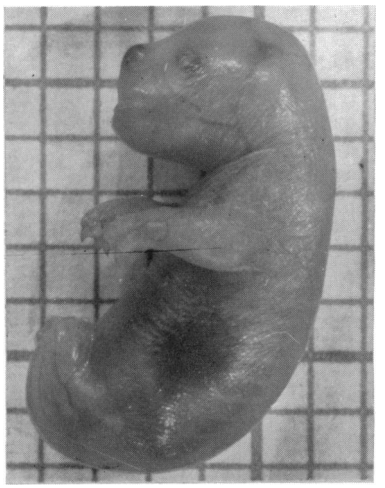

Fig. 1

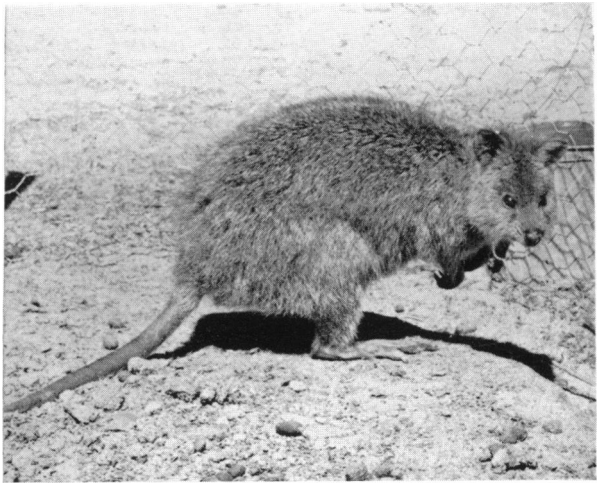

Fig. 2

Fig. 1. 6-d-old quokka on graph paper. The paper is ruled in tenths of an inch.
Fig. 2. Adult quokka. Compare the ratio of limb sizes with that in the 6-d-old.

MATERIALS AND METHODS

Selection of material

One muscle in the forelimb and one in the hind limb were selected as representative of the skeletal musculature of the respective limbs. The aim was to find a homologous pair, of relatively small size, easily distinguishable from surrounding muscles, with a simple internal fibre arrangement and a longitudinal axis parallel to that of the limb segment (to assist orientation). The most satisfactory pair was extensor digitorum communis (EDC) in the forelimb and extensor hallucis et digiti secundi (EHDS) in the hind limb (Fig. 3).

A total of nine animals was used (all that were available) with ages ranging from 4 d before birth to adulthood.

Histological techniques

Several fixatives were used: Bouin's fluid, Susa's fluid and formalin. Tissues were embedded in 'Paraplast' and serial sections cut at 7 μm. The sections were mounted in strict order for the purposes of cinephotomicrography, and stained with haematoxylin and eosin.

Parameters of muscle growth

The maturity of a muscle can be assessed from the myofibrillar density, nuclear position, fascicular pattern and amount of connective tissue, but these features are difficult to quantitate, and have therefore been omitted from this report. Distinctive measurable parameters were selected so that quantitative results could be given.

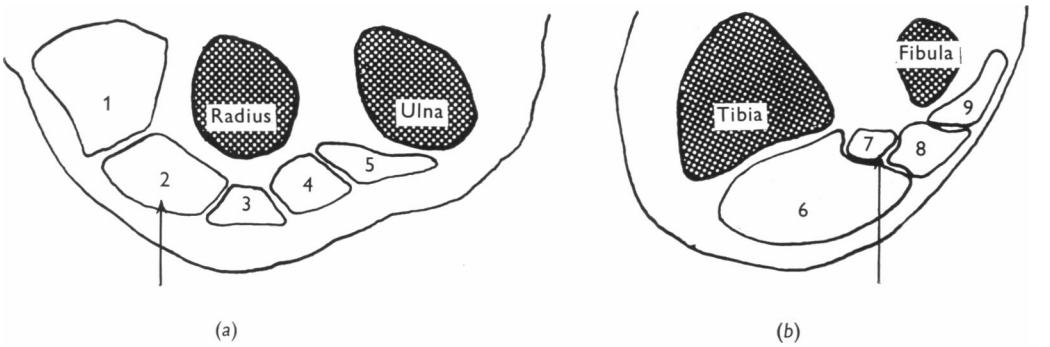

Fig. 3. Transverse sections through (*a*) forelimb and (*b*) hind limb to show the extensor musculature. Arrows mark the muscles studied.

(*a*) 1—Extensor carpi radialis
2—Extensor digitorum communis (EDC)
3—Extensor digiti minimi
4—Extensor carpi radialis
5—Extensor digitorum profundus

(*b*) 6—Tibialis anterior
7—Extensor hallucis et digiti secundi (EHDS)
8—Extensor digitorum medialis
9—Extensor digitorum lateralis.

Results given include gross measurements (length and cross-sectional area of belly), number of fibres and their diameter. Fibre counts and diameter measurements were made from photographs of the most appropriate sections, at the widest part of the muscle belly.

Cinephotomicrography

A basic problem in histology is the appreciation of a three-dimensional structure from a series of two-dimensional images. By using 16 mm cinefilm, and photographing serial sections in the correct sequence, a film can be produced in the same fashion as an animated cartoon. (Heard, 1954; Hayden, Husek & Sirotnik, 1967.) This was done with muscles from two quokkas (the foetus and the 41-d-old), so as to gain a better understanding of the internal fibre arrangement. For each section, ten frames of colour film were exposed, using a Bolex 16 mm camera mounted on a Zeiss microscope.

RESULTS

In order to make the results as clear and concise as possible, they are presented chiefly in the form of tables and graphs. These show the changes that occur in the selected muscles with increasing age. Because of the magnitude of the changes, Figs. 4–6 were drawn using logarithmic scales. In each graph, the horizontal axis shows the animal's age. In order to include negative (foetal) ages, the graphs were constructed by adding 20 d to the true ages, plotting on a normal logarithmic axis and then recalibrating.

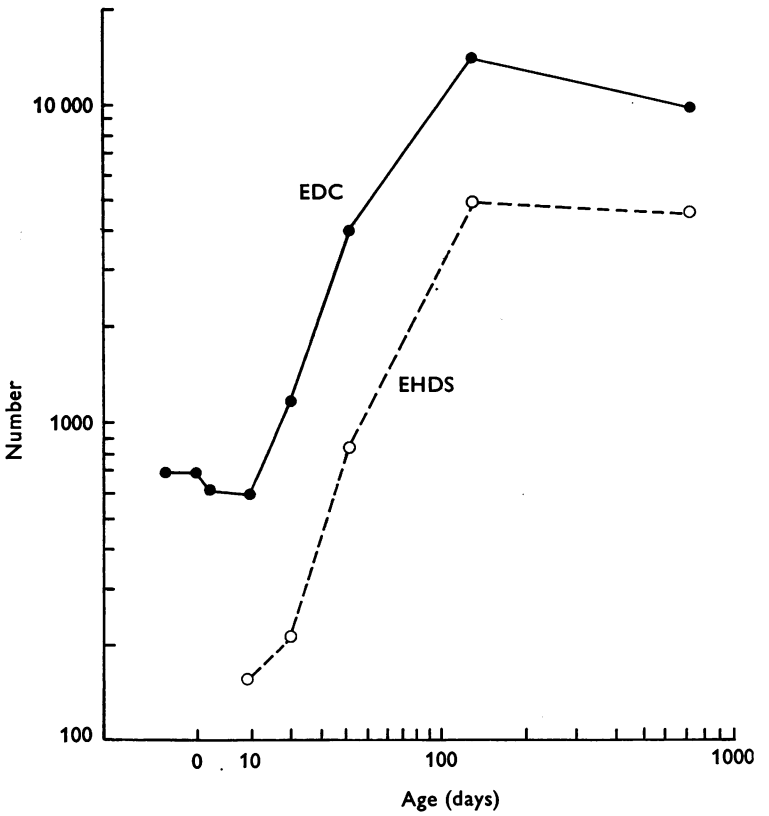

Fig. 4. Increase in the number of muscle fibres with age.

Weights and gross dimensions

Only in the adult quokka was it feasible to dissect out the individual muscles. In all younger animals, they were left *in situ*, and histological sections were made of the entire limb. Hence, only the adult muscle weights were obtained: EDC, 0·75 g; EHDS, 0·20 g.

The lengths of some of the smaller muscles were obtained by observing the number of sections in which they appeared, and multiplying by the thickness of the section. The adult muscles were measured directly. EDC increased in length from 1·5 mm

in the youngest animal (4 d prebirth) to 45 mm in the adult. The hind limb musculature was not sufficiently differentiated to enable EHDS to be distinguished until 9 d of age. In this animal, EHDS was 0·79 mm long, compared with 30 mm in the adult.

Increase in number of muscle fibres with age

In Fig. 4, the following points should be noted: (*a*) the enormous total increase in the number of fibres in both muscles; (*b*) the relatively greater total postnatal increase in the hind limb muscle. (29-fold for EHDS as compared with 14-fold for EDC); (*c*) the steady fibre number in EDC up to about 10 d. The major increase in number of fibres occurs between 20 and 50 d approximately.

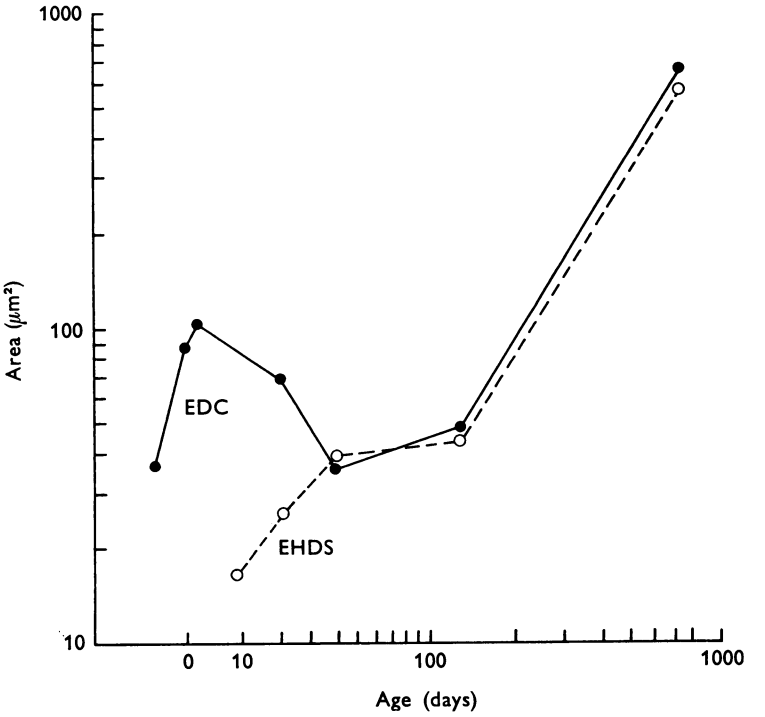

Fig. 5. Change in mean cross-sectional area of muscle fibres with age.

Change in mean cross-sectional area of muscle fibres with age

Figure 5 shows the following points: (*a*) the large overall increase in mean fibre size; (*b*) in the adult, the forelimb muscle fibres are similar in size to those of the hind limb; (*c*) the curves for forelimbs and hind limbs have similar shapes, but that of the hind limb is displaced to the right, indicating its delayed development; (*d*) the fibres of EDC decrease in size between the ages of about 10 and 40 d.

Increase in maximal cross-sectional area of muscle belly with age

The information for Fig. 6 was obtained by tracing on graph paper the outline of a projection of the muscle belly at its widest point, then measuring the area of the

tracing. Hence this area includes not only that of all the muscle fibres, but also the connective tissue elements between and surrounding the fibres.

In both the forelimb and hind limb, the increase in area is continuous and remarkably constant, *in contrast* to the growth of the component fibres (Fig. 5).

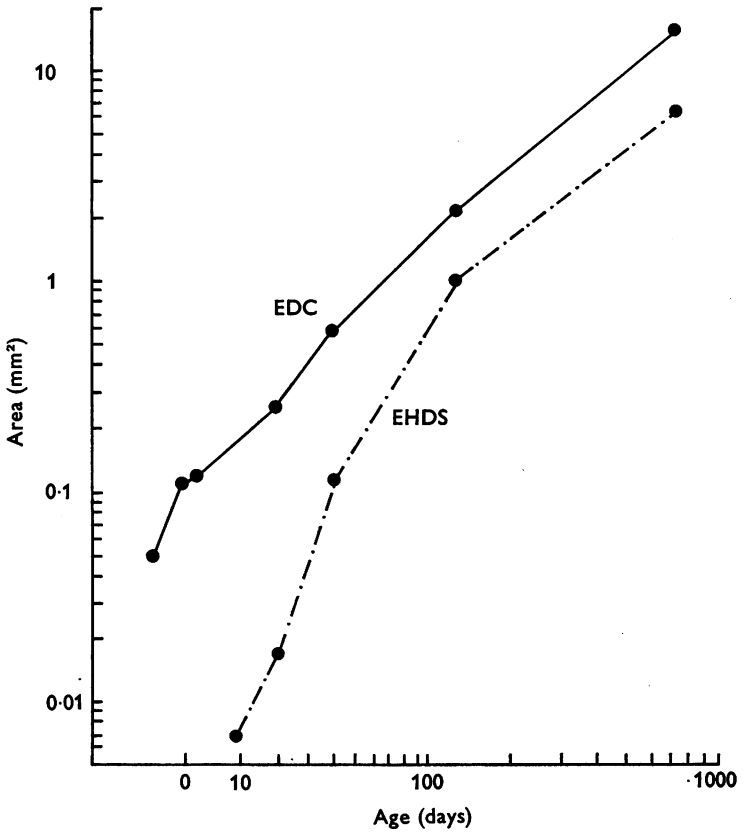

Fig. 6. Increase in the maximum cross-sectional area of the muscles with age.

Table 1. *The mean diameter* (μm) *of the muscle fibres in EDC and EHDS at different ages*

Age (days postnatal)	EDC	EHDS
−4	6·7±1·0	—
0	10·4±1·4	—
2	11·4±1·2	—
9	—	4·5±0·7
20	9·0±2·6	5·7±0·9
41	6·4±1·5	6·2±1·3
128	7·8±0·8	7·4±0·7
Adult	28·7±7·4	26·5±4·6

Change in the distribution of fibre diameters with age

The means and standard deviations of fibre diameters are shown in Table 1. In addition, the distribution of fibre diameters is presented in the form of histograms for the forelimb muscle at several different ages (Fig. 7). The original narrow unimodal distribution of the young animals becomes a broad bimodal distribution at 20 d of age, then resumes a unimodal pattern in the older animals.

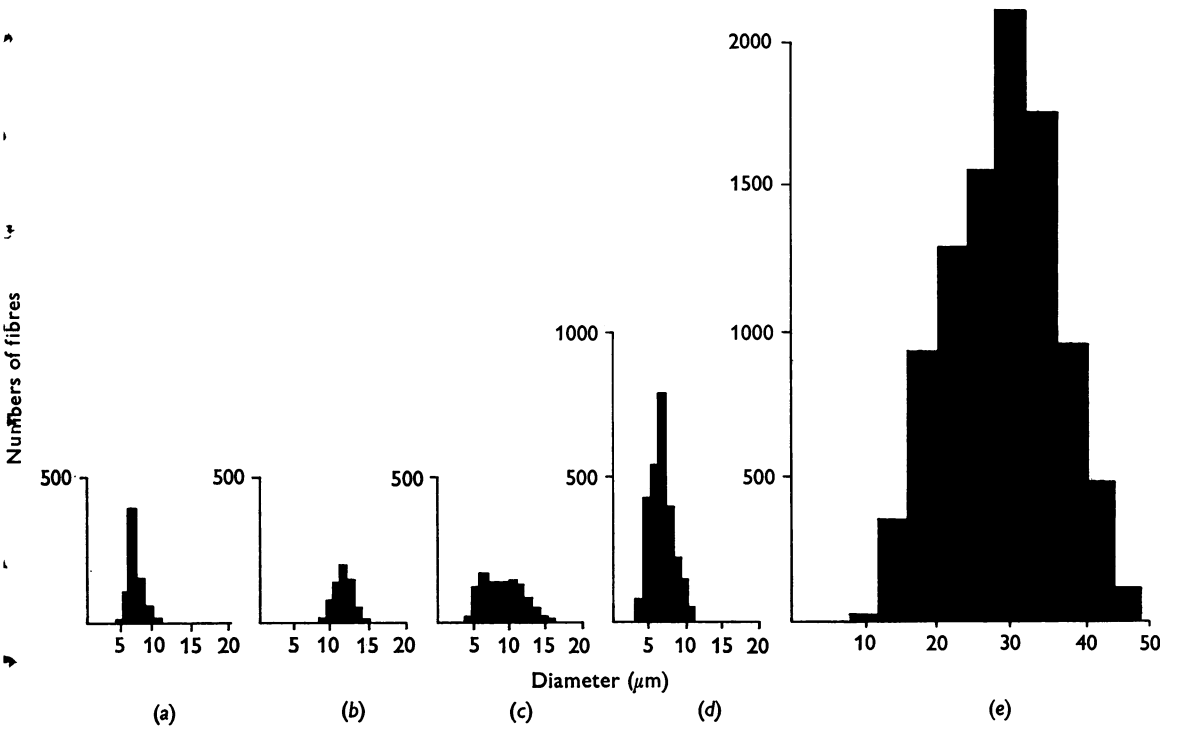

Fig. 7. Histograms of muscle fibre diameters in the extensor digitorum communis muscle at different ages: (*a*) 4 d before birth; (*b*) 2 d; (*c*) 20 d; (*d*) 41 d; (*e*) adult.

Cinephotomicrography

The extensor digitorum communis muscle was filmed at two different ages, and the results compared. The first muscle was in the foetal quokka, 4 d before birth. At this age, the muscle fibres were very uniform in size and appearance, all at the myotube stage (Fig. 8). The other muscle was from the 41-d animal, at the period of maximal proliferation of fibres, with a wide variation in diameters (Fig. 9). Most of the myonuclei are peripheral in position. The portion filmed covered 15% of the total length of the muscle.

The processed film was projected several times forwards and backwards, then examined in more detail with the aid of a manually-operated film editor. The following observations were made:

(i) *Length of muscle fibres.* Individual fibres in EDC of both ages could be traced

with ease from section to section. The fibres usually extended throughout the entire length of the muscle.

(ii) *Fascicular pattern.* The fibres in the 41-d EDC were in well defined groups of three to fifteen fibres separated by strands of connective tissue (Fig. 9). The relative positions of these groups remained constant throughout the muscle. In the 22-d gestation EDC, the fibres existed as individual entities rather than in groups (Fig. 8). However, they too maintained a constant spatial relationship to each other throughout the muscle belly.

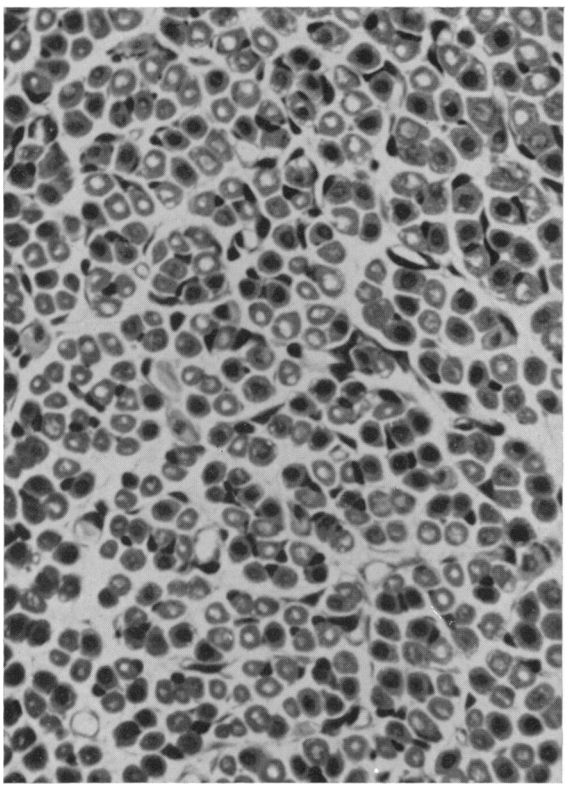

Fig. 8

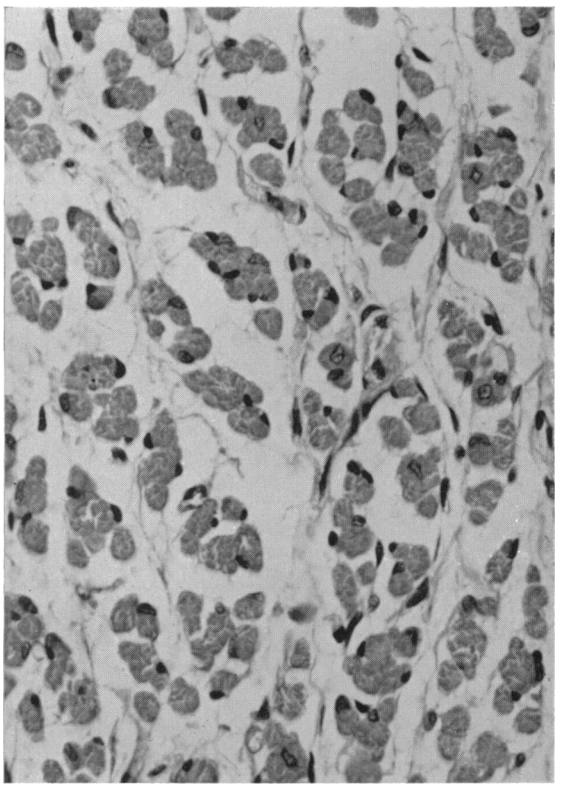

Fig. 9

Fig. 8. Transverse section through extensor digitorum communis, age 4 d before birth. (× 500.)

Fig. 9. Transverse section through extensor digitorum communis, age 41 d. (× 500.)

(iii) *Branching of muscle fibres.* From the examination of a single photograph or single section of the 41-d EDC, it was not always possible to state exactly how many fibres were in a given fasciculus. The identification of every individual fibre required the inspection of serial sections. In this way many very small fibres could be identified nestling alongside larger fibres. If a fasciculus was followed on the film through a large number of sections, the number of component fibres often appeared to change. Some of the small fibres, clearly identified for a distance, seemed to disappear. Some of these reappeared as the fibres were traced to another level in the belly.

(iv) *Nuclear frequency*. The number of nuclei within or associated with a given fibre was counted in some of the fibres at both ages. At 22-d gestation, there was an average of one nucleus every 19 μm along the fibre. There were approximately equal numbers of central and peripheral nuclei. At 41-d of age, there was an average of one nucleus every 20 μm.

The results are subject to considerable error, owing to the difficulty of deciding which nucleus belonged to which fibre, and also distinguishing between peripheral myonuclei and adjacent fibroblast nuclei.

DISCUSSION

In the quokka, postnatal growth of striated muscle involves both hypertrophy and hyperplasia. Figure 4 leaves no doubt about the enormous increase in the number of fibres. The problem is to explain their origin. Several possibilities merit consideration. MacCallum (1898) suggested that there might not be any actual fibre multiplication, but merely an increase in the length of short fibres. This would cause them to grow past one another, so that a greater number would be cut in any one cross-section. From the observations made in the cinefilm, this is clearly not so in the quokka. Even at the early myotube stage, fibres traverse the entire length of the muscle.

A second possibility is that there could be a germinative centre serving as a constant source of myoblasts. If this were the case, one would expect to find evidence of it in the form of a distinct group of embryonic cells surrounded by a zone of myotubes and very small fibres. In actual fact no such region exists. Large and small fibres are distributed evenly throughout the muscle belly.

Finally, new fibres could arise throughout the muscle. Whilst this is obviously so, the mechanism involved is more difficult to elucidate. The following relevant information should be considered before any speculation is made.

In the course of their growth, the muscle fibres increased to something like twenty times their original length. In spite of this, the nuclear concentration remained approximately the same. Therefore, a large number of new nuclei are required simply to satisfy the demands of the existing fibres, let alone to supply all the new fibres. All told, the number of myonuclei must increase to several hundred times that present at the early myotube stage (cf. Enesco & Puddy, 1964). Nuclear replication ceases once myonuclei become incorporated in a myotube (Stockdale & Holtzer, 1961). Where then is the source of these new nuclei? Labelling with [^{3}H]thymidine has shown that most mitoses occur at the ends of the muscle (Kitiyakara & Angevine, 1963). This accounts for the nuclei which are incorporated into the ends of existing fibres. What of the new small fibres arising within the depths of the muscle? Their nuclei could be derived from either the muscle fibres or from undifferentiated cells lying in the connective tissue between fibres. Using the electron microscope, Shafiq, Gorycki & Mauro (1968) found mitoses in both the free undifferentiated cells and the satellite cells of skeletal muscle, but never in normal myonuclei.

It is reasonable to suggest that some new myotubes form independently by the fusion of free myoblasts. Others may develop from satellite cells associated with older fibres. Muir, Kanji & Allbrook (1965) found myofibrils in cells which re-

sembled satellite cells in position, that is they were included within the basement membrane of the parent fibre. In the 3-d-old mouse, they found that 20% of the nuclei in striated muscle fibres were satellite cells. It is easy to postulate that some of these might differentiate into myoblasts, fuse together to form a myotube, and finally break free of the basement membrane in which they were originally enclosed, thereby forming a new fibre. A final possibility is that the larger fibres may split longitudinally into two or more small fibres (Boyd, 1960; van Linge, 1962).

Figure 5 suggests that muscle growth occurs in three stages. In stage 1, the original myotubes grow rapidly in both size and functional capacity. In stage 2, a new population of small fibres appears (Fig. 7*c*), and the growth of the older fibres is temporarily suspended. In the third stage, no new fibres form, and all fibres grow together to reach the adult diameter. In the adult, the two populations are indistinguishable. The overall size of the muscle belly increases constantly in all three stages (Fig. 6).

These three stages have also been described in the foetal sheep (Joubert, 1955). In the quokka they are made prominent by the unusual circumstances of its birth. The ratio birth-weight to adult-weight is about 1:7000. In spite of its very small size, the newborn quokka requires precociously developed forelimb muscles in order to survive. Hence, in quokkas, the first stage of muscle growth involves the rapid development of a small number of fibres, just sufficient to enable the newborn to climb to its mother's pouch.

In man, the gestation period is much longer, and the birth weight is about one-twentieth of the adult weight. Furthermore, there is no premature demand for function. Hence the three stages of muscle growth are not as obvious, although they are still present. Wohlfart (1937) described thin '*a*-fibres' grouped around thick '*b*-fibres' in the sartorius up to two years of age. Montgomery (1962) counted the fibres in the human sartorius at different ages, but, due to the small number of specimens, was unable to state whether or not fibre multiplication was complete at birth.

The apparent decrease in fibre diameter seen in Fig. 5 can be interpreted in several ways. It could be entirely an artefact of fixation. However, if this were so, it is likely that it would show up as a decrease in the growth rate of the whole muscle (Fig. 6). Some shrinkage may be caused by loss of fluid from the fibres as they mature and fill with myofibrils; or the decrease could be due to smaller fibres splitting off the larger ones. All three factors may contribute to the final result.

The present investigation has answered some questions concerning muscle growth, and indicated the particular suitability of the quokka as an experimental animal; but the precise mechanism by which muscle fibres multiply is still uncertain. An electron microscopic study of quokka muscle at about 40 d of age should provide the required information, especially with regard to the role of satellite cells and the extent of fibre splitting.

SUMMARY

The growth and development of striated muscle was studied in two limb muscles of a marsupial, the quokka (*Setonix brachyurus*), using animals with ages ranging from 4 d prebirth to adult. Measurements made included the number of muscle

fibres, diameter of muscle fibres, number of nuclei, and the cross-sectional area of the whole muscle belly.

Muscle growth occurred in three stages. During the second stage the mean diameter of the fibres decreased or remained constant, whilst the number of fibres increased rapidly (up to 30-fold). The roles of undifferentiated cells, satellite cells, and splitting of fibres were considered in relation to the above changes.

The three-dimensional structure of two muscles was studied using the technique of cinephotomicrography. Embryonic muscle fibres (myotubes) were seen to extend the full length of the muscle belly from the earliest age.

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