Postnatal Development of the Masseter Muscles in the Japanese Field Vole Microtus montebelli, with Special Attention to Differentiation of the Fast-Twitch Oxidative Fiber

Authors: Sugasawa, Katsumi, and Mōri, Takayuki

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Postnatal Development of the Masseter Muscles in the Japanese Field Vole *Microtus montebelli*, with Special Attention to Differentiation of the Fast-Twitch Oxidative Fiber

Katsumi Sugasawa and Takayuki Mōri*

Zoological Laboratory, Faculty of Agriculture, Kyushu University 46-06, Fukuoka 812-81, Japan

ABSTRACT—Postnatal development and differentiation of the masseter muscles consisting only of fast-twitch oxidative (FO) fibers in the adult Japanese field vole *Microtus montebelli* were studied using histochemical and electron microscopic techniques. The masseter muscles were composed of myotubes and muscle fibers at day 0 (birth day). Most muscle cells showed the strong reaction for myosin ATPase after both alkaline and acid preincubations. For NADH-dehydrogenase (NADH-DH), small granular diformazan deposits were recognized in the sarcoplasm. Afterwards, the masseter muscles consisted of myofibers and satellite cells at day 5. For myosin ATPase, weakly-reactive fibers after acid preincubation (fast-twitch fibers) increased in number. For NADH-DH, granular diformazan deposits in all the myofibers increased in size. Since all the myofibers had numerous sarcoplasmic reticula, and they reacted strongly after alkaline preincubation and weakly after acid preincubation for myosin ATPase at day 10 when the young start to take solid food, it seems that the masseter muscles become contractive fast. At day 15 (before weaning), all the myofibers showed the adult-like strong reaction for NADH-DH and had numerous well-developed mitochondria, thus they acquired the ability of the fast and sustained contraction. It is accordingly considered that the masseter muscles of the vole mature in a short time after birth because of adaptation for herbivorous food habit.

INTRODUCTION

Histochemically, the mature masseter muscle in the Japanese field vole *Microtus montebelli* is unique in that it is composed of only fast-twitch oxidative (FO) fibers (Sugasawa *et al.*, 1997) as in highly specialized flight muscles in the little brown bat *Myotis lucifugus* (Armstrong *et al.*, 1977; Powers *et al.*, 1991; Schutt *et al.*, 1994). The differentiation of the FO fiber has been studied histochemically, biochemically and immunocytochemically on the pectoral muscles of the bat (Powers *et al.*, 1991; Schutt *et al.*, 1994). Furthermore, the histochemical changes of the masseter muscle with development of feeding behavior are well known in the rat (Hiraiwa, 1978; Maeda *et al.*, 1981; Miyata *et al.*, 1996). However, there has been no study on enzyme-histochemical and ultrastructural characteristics of the muscles consisting only of FO fibers.

The purpose of the present study is to clarify the histochemical and ultrastructural differentiation of the FO fiber of the masseter muscle in the vole during postnatal development.

* Corresponding author: Tel. +81-92-642-2948; FAX. +81-92-642-2804.

MATERIALS AND METHODS

The newborns of the vole *Microtus montebelli* were kept with their mothers in cages in an environment controlled room (23 ± 1°C, LD 14:10). All the animals were given *ad libitum* a herbivorous diet (ZF, Oriental Yeast Co., Ltd., Tokyo), commercial mouse diet (NMF, Oriental Yeast Co., Ltd., Tokyo) and water.

The histochemical analyses of the muscles were performed at days 0 (birth day), 3, 5, 7, 10, 13, 15 and 20 after birth, and two to five voles were used for each age group. Masseter muscles were taken from ether anaesthetized animals. The muscle tissues were rapidly frozen in isopentane solution cooled with dry ice. Thick cross-sections of the muscles (8 μm) were stained for myosin adenosine triphosphatase (ATPase) (Padykula and Herman, 1955) after alkaline (pH 10.5) or acid (pH 4.3) preincubation (Brooke and Kaiser, 1970a, b; Suzuki, 1977), for reduced nicotinamide adenine dinucleotide dehydrogenase (NADH-DH) (Burstone, 1962) and for phosphorylase (Takeuchi and Kuriaki, 1955) activities.

The electron microscopic observations were performed at days 0, 5, 10, 15, and three voles were used for each age group. The skin covering the head was removed, and then the entire head was decapitated and fixed for 20 min in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.2, subsequently the masseter muscles were removed and placed in the same fixative for 2 hr. The muscles were washed briefly in the same buffer and further fixed for 2 hr in 1% osmium tetroxide buffered with sodium cacodylate at pH 7.2. The tissues were dehydrated in an alcohol series and embedded in Epon 812. Thin sections (~60 nm) were cut on a Porter-Blum MT-1 microtome using a glass knife, and doubly stained with lead and uranyl acetate before examination in an Hitachi-H600A electron microscope (75 kV).

In this paper the term “myotube” refers to a muscle precursor cell with centrally located multiple myonuclei and a few myofibrils. A “muscle fiber” refers to a muscle cell confirmed by the presence of many myofibrils and multiple nuclei beneath the sarcolemma.

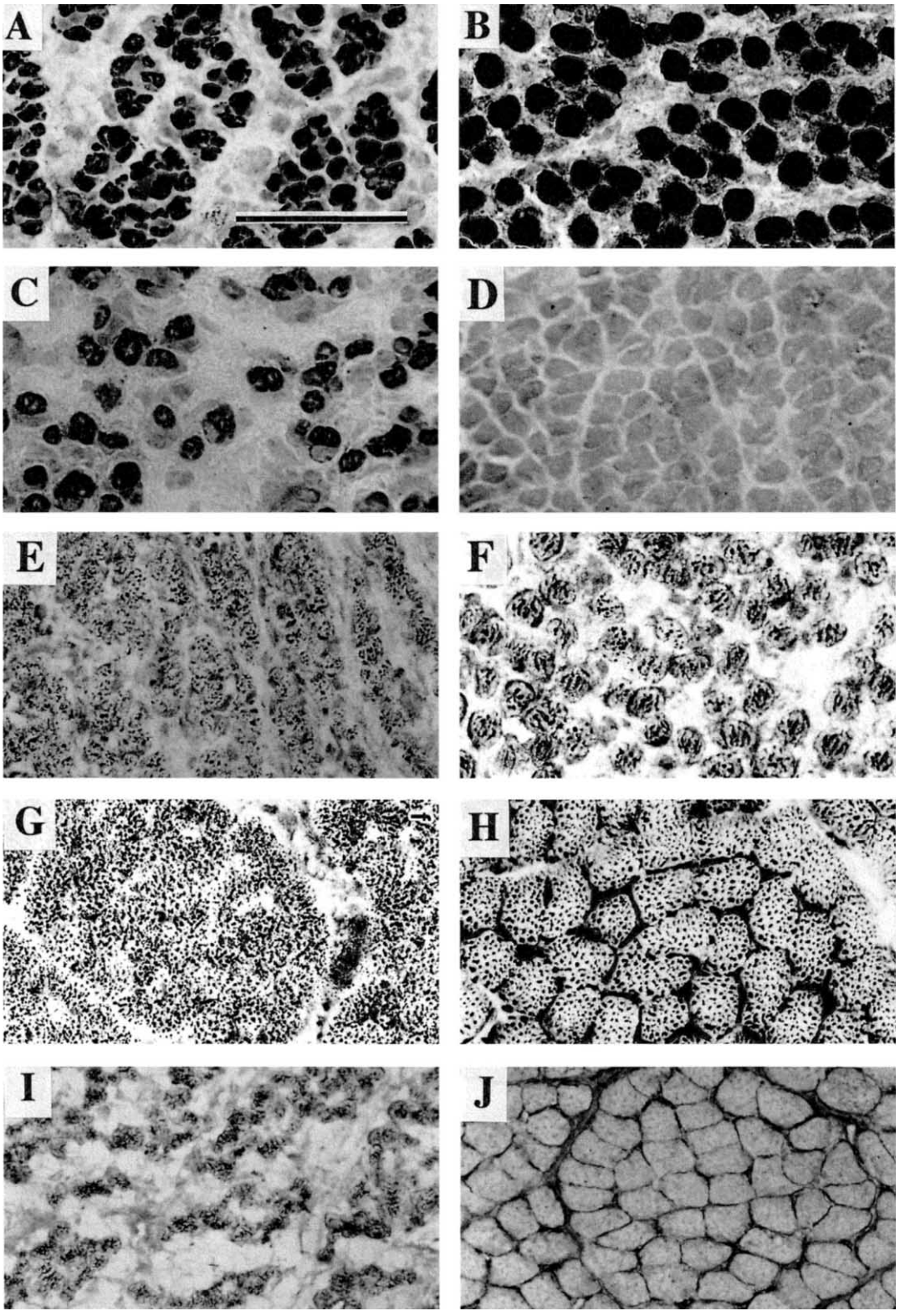

Fig. 1. Histochemical changes of the masseter muscles in *M. montebelli* during development. **A** (day 0) and **B** (day 5); myosin ATPase activity at pH 10.5. **C** (day 0) and **D** (day 10); myosin ATPase activity at pH 4.3. **E** (day 0), **F** (day 5), **G** (day 10) and **H** (15 day); changes of NADH-DH activity. **I** (day 0) and **J** (day 10); changes of phosphorylase activity. Bar = 50 μm.

RESULTS

Histochemical analysis

Relatively young muscle samples (days 0~7) were difficult to characterize histochemically.

As for myosin ATPase staining property, cells containing central vacuoles consistent with the presence of central nuclei seemed to be myotubes. For myosin ATPase after alkaline preincubation, the myotubes and myofibers strongly reacted at day 0 (Fig. 1A), and then the myofibers increased in number with age. At day 5, only myofibers strongly reacted for it (Fig. 1B). For myosin ATPase after acid preincubation, although almost all the myotubes and myofibers strongly reacted at day 0 (Fig. 1C), and then the weakly-reactive myofibers increased gradually in number with age. All the myofibers weakly reacted for it at day 10 (Fig. 1D).

NADH-DH activity of the muscle cells was very low at day 0 (Fig. 1E). There were scattered small diformazan deposits produced by the reduction of nitro-blue tetrazolium. Afterwards the granular diformazan deposits became large with age (Fig. 1F, G). At day 15, strong reactions were recognized at the subsarcolemmal region as in adult specimens (Fig. 1H).

For phosphorylase, the myofibers of the masseter muscles stained homogeneously light at day 0 (Fig. 1I), although they somewhat increased the activity until day 10 (Fig. 1J).

Electron microscopic observation

At day 0, the developing masseter muscles consisted of myotubes (58%) and muscle fibers (42%) (Fig. 2A). These muscle cells were often found in basal-lamina-surrounded "clusters" (Fig. 2A, B). The myotubes had centrally located euchromatic myonuclei, a few peripheral myofibrils, a Golgi apparatus and some rough endoplasmic reticula (Figs. 2A, B, 3). The myofibers contained many fascicular arrangements of myofibrils and subsarcolemmal located myonuclei (Fig. 2A, B). Many myofibers possessed mitochondria which were arranged between the bundles of myofibrils (Fig. 2B). The interdigitating processes (i.e., peg in socket arrangements) and gap junctions were often found between the myotubes or between the myotube and myofiber, and there were vesicles beneath the sarcolemma of each muscle cell (Figs. 2B, 3 and inset).

At day 5, the masseter muscles were composed of mostly polygonally shaped myofibers with subsarcolemmal flattened nuclei (Fig. 4A). The myofibers exhibited the characteristic structural organization seen in the adult muscle and contained the small mitochondria (Fig. 4B). Fusiform-shaped mononucleated cells were wedged between the basal lamina and sarcolemma of the myofibers. Their heterochromatic nuclei, ultrastructural appearance and sublaminal location suggest that some of them are myosatellite cells (Fig. 4C).

At day 10, the myofibers were clearly bundled by the perimysium (Fig. 5A) and found as independent cells enclosed in their own basal lamina (Fig. 5B), though they were still small (6 μm in diameter) (Fig. 5A). The sarcoplasmic reticula were well developed, while mitochondria were undeveloped (Fig. 5B).

At day 15, the changes were recognized as an increase in number of myofibrils within an individual muscle fiber. As the result, the muscle fibers increased considerably in size (10 μm in diameter) (Fig 6A and inset). Another change was a substantial increase in number of mitochondria with a conspicuously dense matrix (Fig. 6B). The mitochondria with dense matrix were disposed at subsarcolemmal regions (Fig. 6A). Many pale lipid droplets were frequently associated with the mitochondria (Fig. 6B).

DISCUSSION

At day 0, the masseter muscles in the vole were developing as a hole, because they were composed of myotubes and myofibers which are classified histochemically as undifferentiated type 2C fibers according to Dubowitz (1985). Thus, the contractile activity of the masseter muscles in the vole seems low at this time as in those of the neonatal rat (Miyata *et al.*, 1996) and mouse (Hurov *et al.*, 1992) whose masseter muscles consist only of fast-twitch (fast-twitch oxidative glycolytic and fast-twitch glycolytic) fibers in their adults.

Afterwards, all the myotubes became myofibers by day 5, and the fast-twitch fibers, which histochemically react strongly for myosin ATPase after only alkaline preincubation (Brooke and Kaiser, 1970b; Peter *et al.*, 1972), increased gradually in number as the 2C fibers decreased. The staining intensity for both oxidative and glycolytic enzymes in the muscles gradually increased with age. It is suspected that these developmental changes of the masseter muscles before starting to take solid food in the vole are induced by a predetermined developmental program rather than the contractile activity of the muscles as pointed out by Maeda *et al.* (1981), because the contractile activity of the muscles seems insufficient at this phase (Herring, 1985).

The masseter muscle of the vole seems to acquire the fast contractile activity until starting to take solid food (day 10), because the masseter muscles were composed of only fast-twitch fibers with many sarcoplasmic reticula. On the other hand, the masseter muscles of the rat consist only of fast-twitch fibers about 10 days after starting to take solid food (day 25) (Maeda *et al.*, 1981). Thus, myosin ATPase of the masseter muscles in the vole seem to differentiate relatively rapidly. Since oxidative enzyme activity of the masseter muscles in the vole strengthened afterwards as in that of the rat (Miyata *et al.*, 1996), oxidative enzyme of the masseter muscles seems to be activated by the mastication activity with biting. As to glycolytic enzyme in the fibers, since the masseter muscles of the vole showed weak phosphorylase activity as in that of the adult vole (Sugasawa *et al.*, 1997), the powerful or sudden contraction activity of the vole seems low at this time (day 10).

It is known that the vole is weaned at about day 18 (Obara, 1975). All the masseter muscles in the vole had many and

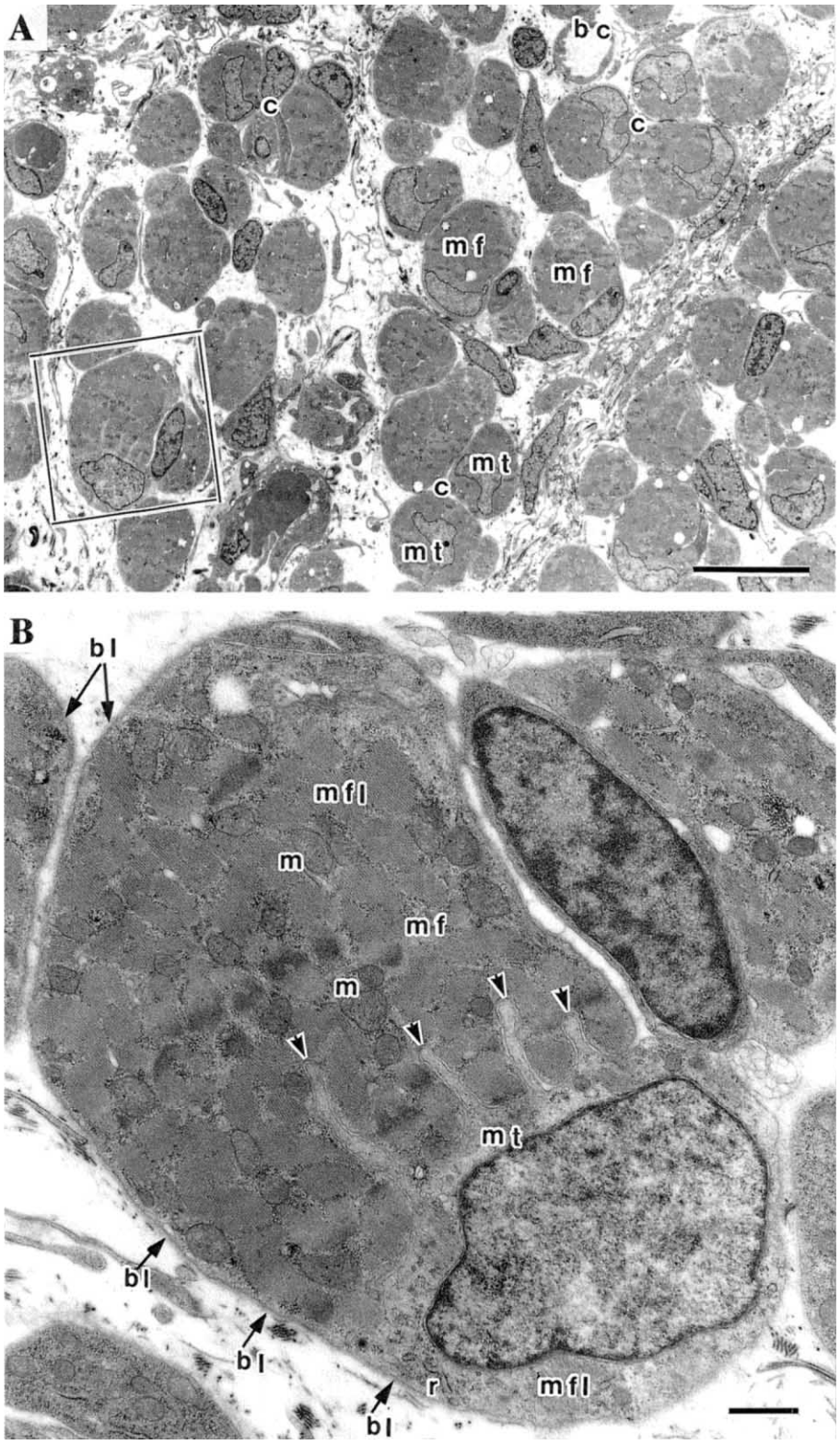

Fig. 2. Electron micrographs of the masseter muscles in the vole at day 0. (**A**) Transversal section of the masseter muscle consisting of myofibers and myotubes. (**B**) Higher magnification of the area in the rectangle on **A**. The large muscle fiber and small myotube, associated in cluster, are surrounded by a common basal lamina. Arrow-heads indicate peg and socket interdigitations between the myofiber and myotube. bc, blood capillary; bl, basal lamina; c, cluster; m, mitochondrion; mf, myofiber; mfl, myofibril; mt, myotube; r, rough endoplasmic reticulum. Bar = 10 μm (**A**), 1 μm (**B**).

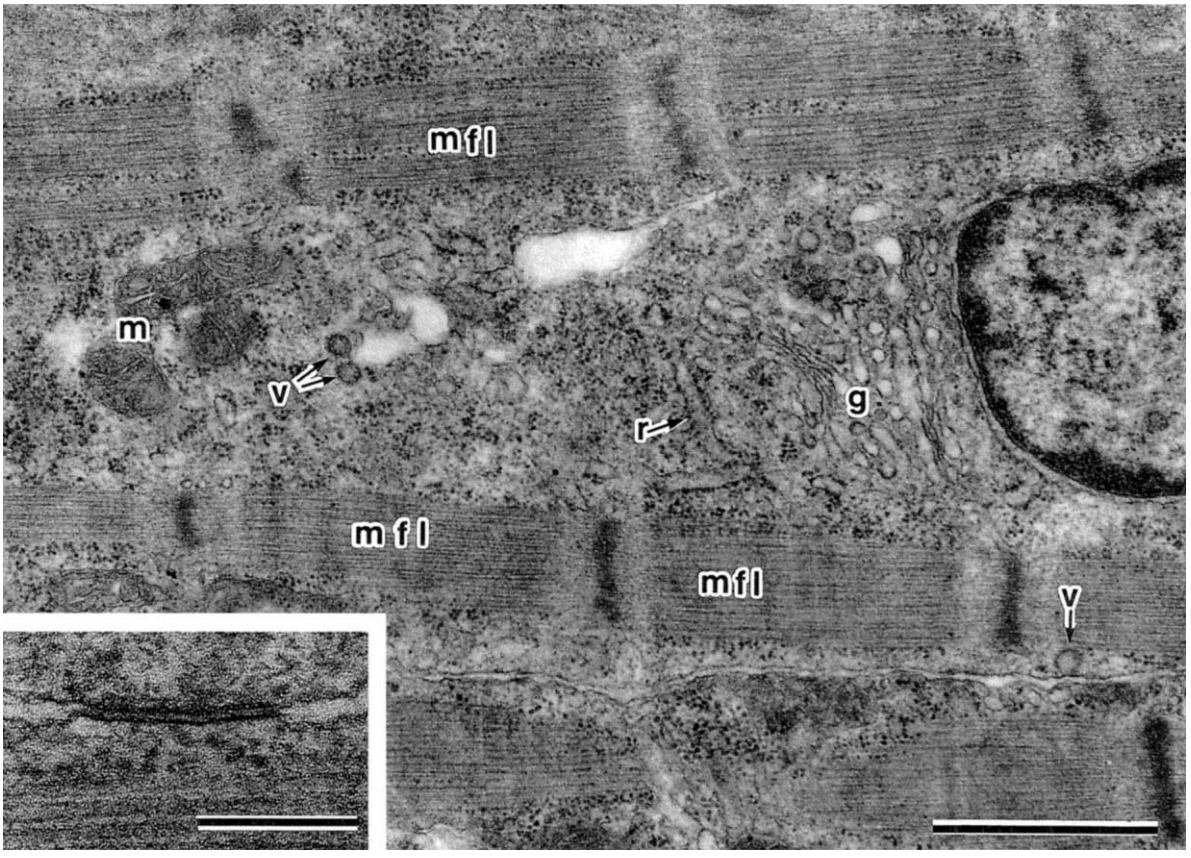

Fig. 3. Electron micrograph of the masseter muscle in the vole at day 0, showing scattered myofibrils, a Golgi apparatus, rough endoplasmic reticula and vesicles in the myotube. Inset: A gap junction between myotubes. g, Golgi apparatus; m, mitochondrion; mfl, myofibril; r, rough endoplasmic reticulum; v, vesicle. Bar=1 μm (main picture), 0.2 μm (inset).

large well-developed mitochondria with strong oxidative enzyme activity at day 15; thus, they have been already composed of only FO fibers at this time. Accordingly , the masseter muscles of such young acquired the fast and sustained contractile activity as in the adults; that is, they have already matured before weaning. From the above-mentioned fact, it seems that the masseter muscles of the vole develop relatively rapidly rather than the rat (Maeda *et al.*, 1981; Miyata *et al.*, 1996) and mouse (Hurov *et al.*, 1992) whose masseter muscles continue to develop by days 40-60 (10-30 days after weaning). Furthermore, it is assumed that oxidative enzyme of the masseter muscles in the vole is also activated by other factors rather than the mastication activity, because the vole masseter muscles have already matured before weaning. We are now studying the effects of other factors on the development of the masseter muscles in the vole.

As for the relationship between food habit and disparity of the growth stage of young at birth (precocity or altricity), many herbivores such as ungulates which consume grass that is easy to find or to catch may produce precocial young, while most omnivorous or herbivorous myomorphic animals are altricial (Case, 1978). Of course, the vole is also altricial in spite of herbivorous food habit (Obara, 1975). It has been shown histochemically and electron microscopically that the masseter muscles of the vole can contract fast and enduringly, and consequently that the muscle well adapt for herbivorous food habit (Sugasawa *et al.*, 1997). The present study, in which the masseter muscles were known to differentiate in a short time after birth, adduced ontogenetically for the above finding.

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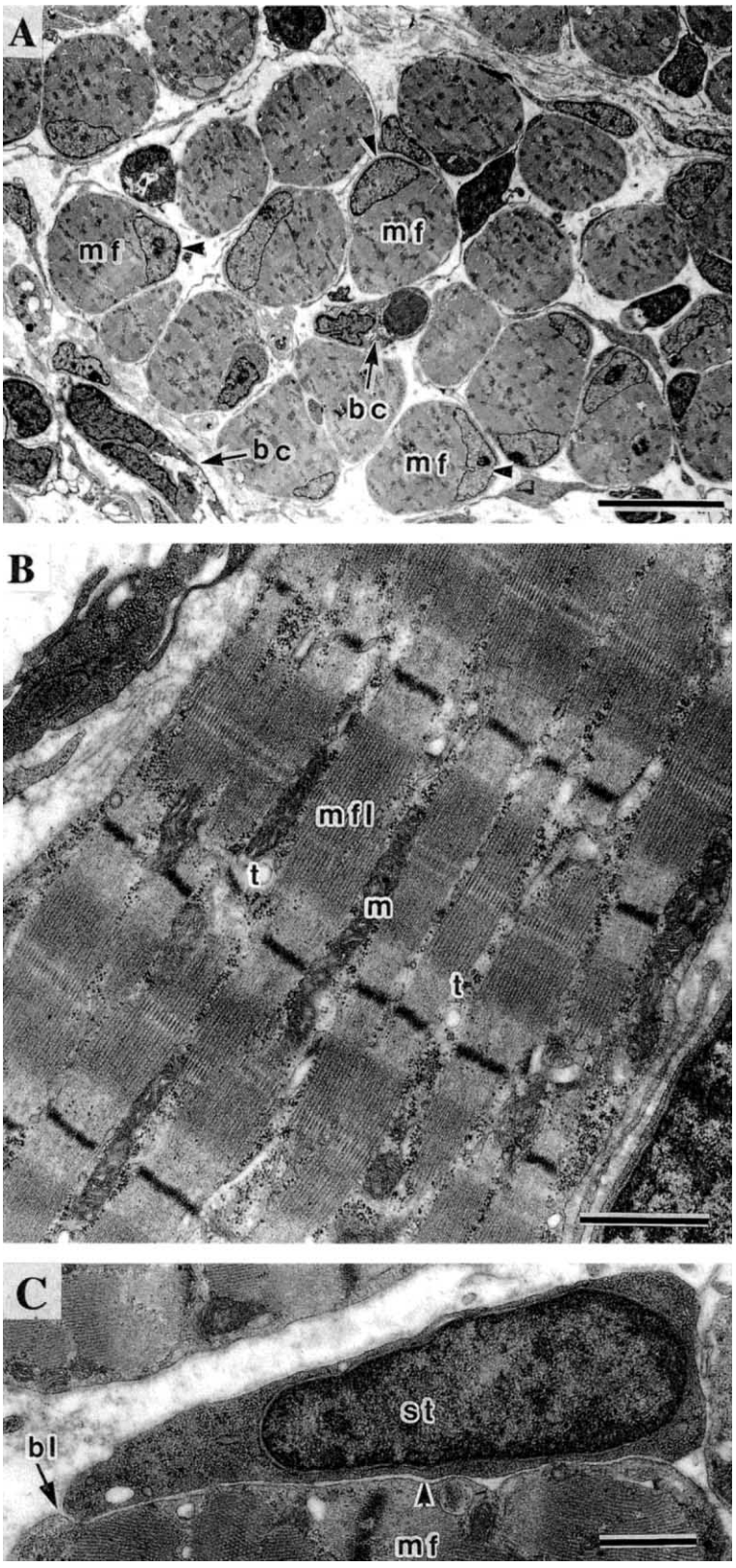

Fig. 4. Electron micrographs of the masseter muscles at day 5 in the vole. (**A**) Transversal section showing myofibers with nuclei (arrow-heads) beneath the sarcolemma. (**B**) Longitudinal section of a myofiber showing well organized myofibrils and small mitochondria. (**C**) An undifferentiated mononucleated (probably satellite) cell wedges between the basal lamina and the sarcolemma (arrow-head). bc, blood capillary; bl, basal lamina; m, mitochondrion; mf, myofiber; mfl, myofibril; st, satellite cell; t, transverse tubule. Bar=10 μm (**A**), 1 μm (**B**, **C**).

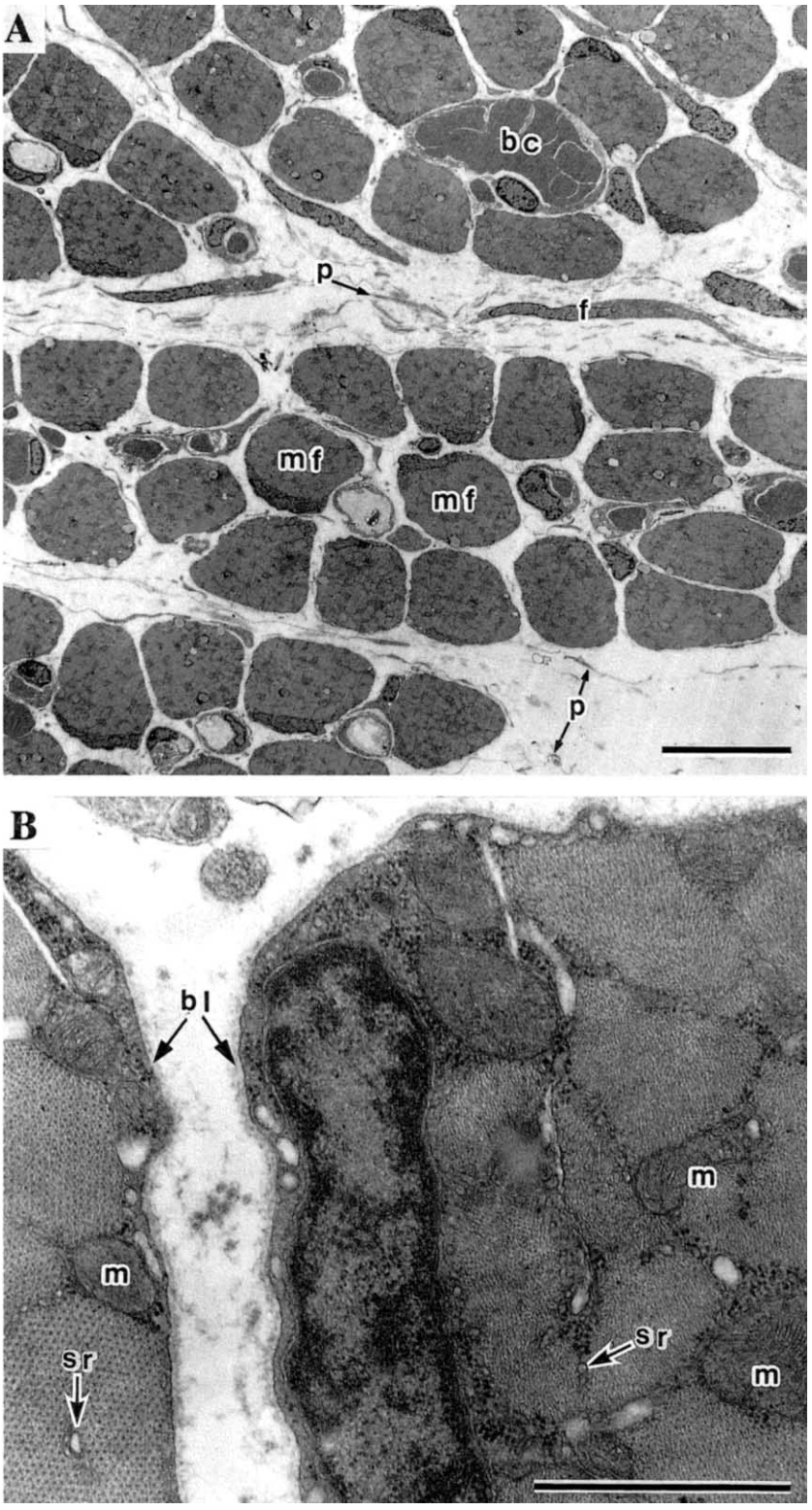

Fig. 5. Electron micrographs of the masseter muscles at day 10 in the vole. (**A**) Transversal section showing myofibers clearly bundled by the perimysium. (**B**) Higher magnification of the myofibers with their own basal lamina and many sarcoplasmic reticula. bc, blood capillary; bl, basal lamina; f, fibroblast; m, mitochondrion; mf, myofiber; p, perimysium; sr, sarcoplasmic reticulum. Bar=10 μm (**A**), 1 μm (**B**).

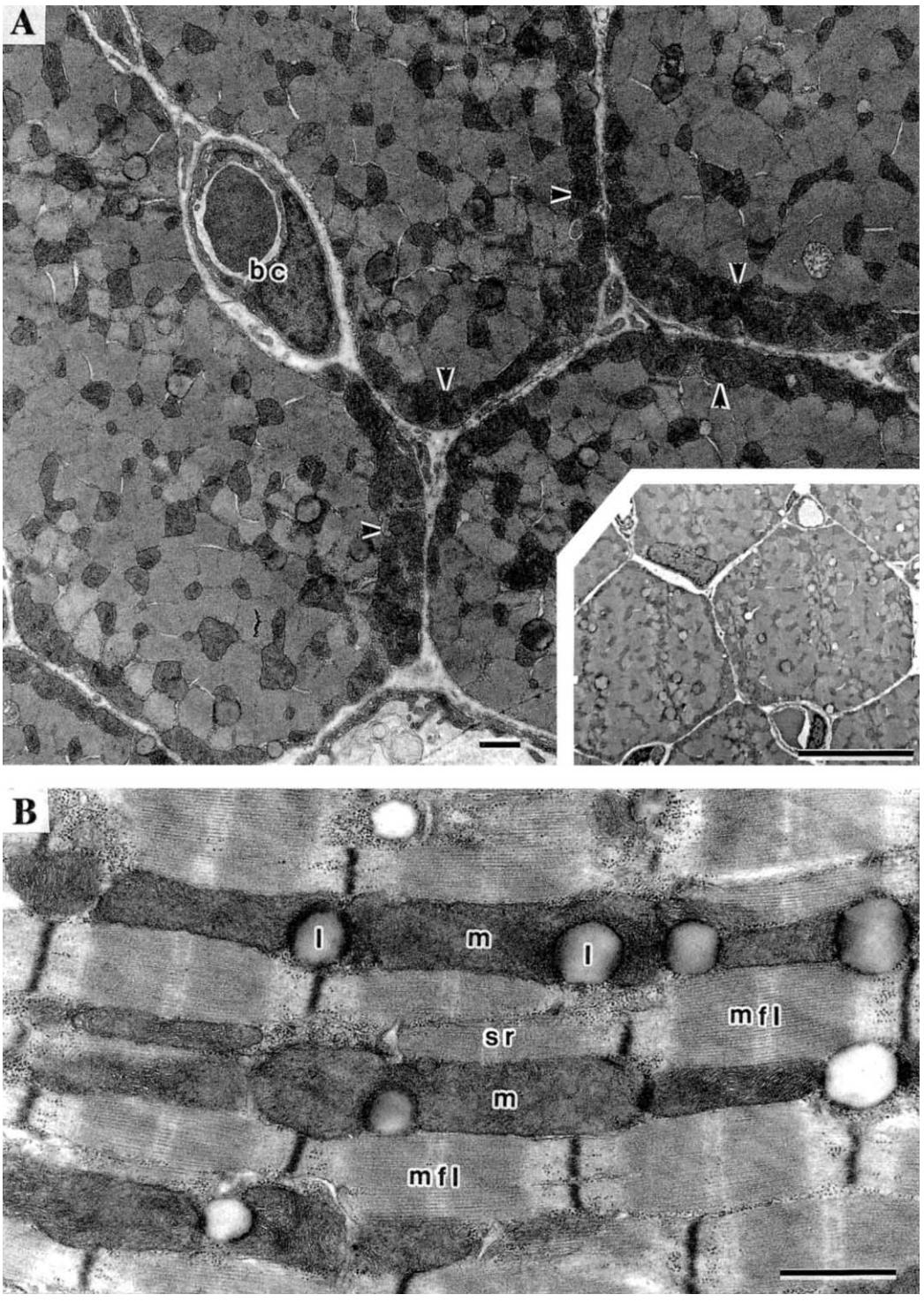

Fig. 6. Electron micrographs of the masseter muscles at day 15 in the vole. (**A**) Transversal section of myofibers showing subsarcolemmal aggregation of mitochondria (arrow-heads). Inset: Lower magnification of myofibers showing an increase in their size compared with Fig. 5A (at day 10). (**B**) Longitudinal section of the myofiber showing large mitochondria, associated with large lipid droplets, between the myofibrils. bc, blood capillary; l, lipid droplet; m, mitochondrion; mfl, myofibril; sr, sarcoplasmic reticulum. Bar=1 μm (**A**, **B**), 10 μm (**A**, inset).

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