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Muscle Development in Squid: Ultrastructural Differentiation of a Specialized Muscle Fiber Type

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ABSTRACT The ultrastructural differentiation of two muscle fiber types of the squid *Sepioteuthis lessoniana* was correlated with development of prey-capture behavior. Transmission electron microscopy was used to document the differentiation of the fast-contracting cross-striated muscle cells of the tentacles and the obliquely striated muscle cells of the arms of specimens sampled at one week intervals from hatching to 5 weeks. By using high-speed video recordings, the ultrastructural differentiation was correlated with changes in prey-capture behavior that occur during development and growth. The ultrastructural analysis focused on the muscle cells of the transverse muscle of the tentacles and the transverse muscle of the arms. For the first 2 weeks after hatching, the tentacle transverse muscle fibers do not show the adult ultrastructure and are indistinguishable from the obliquely striated fibers of the transverse muscle of the arms. Transverse striation of the tentacle muscle cells appears at approximately three weeks and adult ultrastructure is present by 4–5 weeks after hatching. The high-speed video recordings show correlated behavioral changes. During the first 2–3 weeks after hatching, the animals use a different prey-capture mode from the adults; they jet forward and capture the prey with splayed arms and tentacles rather than employing the rapid tentacular strike. © 1996 Wiley-Liss, Inc.

Decapod cephalopods, the squids and cuttlefishes, differ from other cephalopods in possessing ten appendages including a pair, the tentacles, whose primary function is prey capture. The tentacles differ from the remaining four pairs of appendages, the arms, which function in prey handling, behavioral displays, stabilization during locomotion, and reproduction. The arms are equipped with suckers along their entire length, whereas the tentacles have suckers only on the expanded distal club. During prey capture in the adults, the arms are flared, the tentacles are elongated with remarkable rapidity, and the suckers on the tentacular clubs attach to the prey. The prey is then withdrawn by the tentacles and transferred to the arms which subdue and manipulate it for ingestion using bending movements and adhesion by the suckers (Kier, '82; Messenger, '68, '77). Phylogenetic studies (Naef, '21, '23; Boletzky, '93, in press) suggest that this condition is derived from a primitive condition in which 10 arm-like appendages exist. Octopuses retain the primitive condition but have lost one pair of appendages.

Both the arms and tentacles are characterized by a tightly packed, three-dimensional array of muscle fibers. Previous studies (Kier, '82; Kier and Smith, '85; Smith and Kier, '89) have identified the transverse muscle of the tentacles to be the musculature responsible for the extremely rapid tentacular stalk extension. The homologous transverse muscle of the arms provides the skeletal support required for the slower bending movements (Kier, '82). Despite the dramatic difference in function, no difference in the gross arrangement of the transverse muscle of the tentacles or arms has been found (Kier, '82, '91). Further, a comparison of the biochemistry of the proteins of the myofilament lattice of the cross-striated tentacle cells and the obliquely striated arm cells revealed remarkably little

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biochemical heterogeneity (Kier and Schachat, '92). It is at the level of the ultrastructure of the muscle cells of the transverse muscle masses that specialization has occurred. In the tentacle, these cells are short-sarcomere, cross-striated cells and are thus unlike the obliquely striated muscle cells of the transverse muscle mass of the arms and of virtually all other cephalopod musculature (Fig. 1). The characteristics of the ultrastructure of the cross-striated muscle cells of the tentacle are indicative of high shortening velocity and rapid excitation–contraction coupling (Kier, '85, '91). Thus, evolution of the muscle cells of the transverse muscle of the tentacle for the rapid strike in prey capture involved a different pattern of specialization from that observed in vertebrate muscle fiber types, which show little ultrastructural differentiation and significant biochemical heterogeneity (Kier and Schachat, '92).

In the present study, the ontogenetic development and differentiation of the obliquely striated muscle fibers from the arms and of the cross-striated muscle fibers from the tentacles of the squid *Sepioteuthis lessoniana* are examined at the ultrastructural level and compared with the use of the arms and tentacles during prey capture.

There are a number of reasons why such a

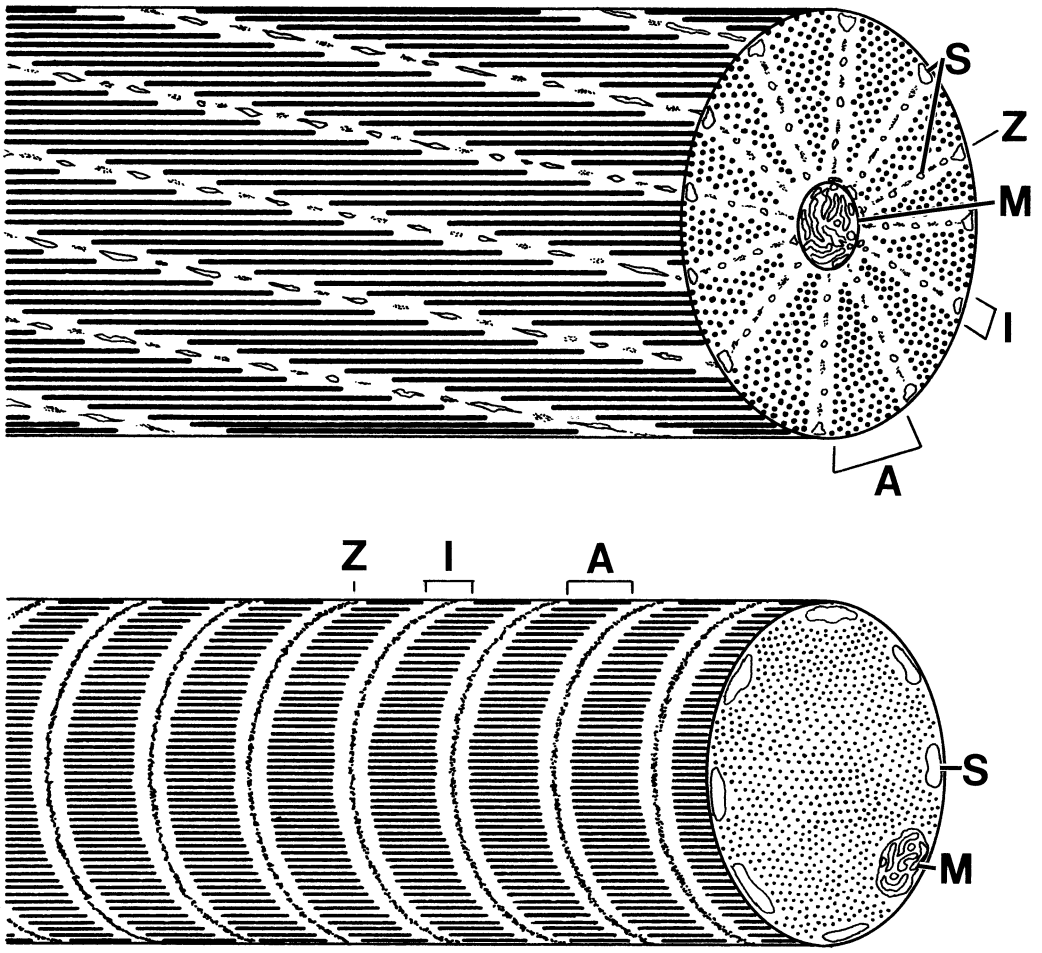

Fig. 1. Schematic diagram of an obliquely striated (top) and a cross-striated (bottom) muscle fiber from squid. Note that a cross-section of an obliquely striated muscle cell shows an analogous sequence of bands to those seen in a longitudinal section of the cross-striated fiber. The A band (A), I band (I), Z elements (Z), sarcoplasmic reticulum (S), and mitochondria (M) are labeled for each fiber type. The thin filaments have been omitted from the diagram for clarity.

study is of interest. First, it is likely that the morphology of the specialized tentacles is derived from the morphology seen in the arms. It is possible that the means by which these muscles arise in ontogeny may provide clues to the ways the specialization arose. Second, previous studies suggest that newly hatched squids do not exhibit the specialized prey capture behavior. Boletzky ('74) describes the prey capture of young squids and states that the tentacles cannot be elongated in the hatchlings. Instead a young squid "shoots forward when attacking" and captures the prey with the arms (Boletzky, '74). Chen et al. ('96), in a study of the ontogeny of copepod capture by *Loligo opalescens,* observed that the tentacles were not used initially by hatchlings; rather, the prey was captured with the arms. The tentacular strike was not observed until approximately four weeks after hatching (development at 17–18°C). In a study of the early life history of *Loligo pealei,* Vecchione ('81) observed a notable increase in variance in the tentacle length of preserved specimens of >4.5-mm dorsal mantle length (DML), while a similar increase in arm length variability was not observed. The variation in tentacle length in the older juveniles resembles the situation in preserved adult squid and Vecchione ('81) suggested that this variability indicates that the tentacles are extensible only in animals of >4.5-mm DML. These observations on prey-capture behavior in young squids are particularly interesting in the context of an analysis of ultrastructural differentiation of the muscle cells because the lack of adult prey capture behavior in young animals might be the result of incomplete differentiation of the tentacle musculature. Third, the two muscle cell types occur in serially homologous organs in a single individual. Thus, comparison of the details of muscle specialization and development can be made while controlling for phylogeny, drift, and general physiological adaptation. Finally, the results are of interest in the context of vertebrate muscle fiber specialization because the pattern of evolution of muscle specialization in decapod cephalopods is entirely different from the vertebrate pattern. An understanding of specialization of muscle performance in groups other than vertebrates provides a broader understanding of the evolution and significance of muscle form in animals in general.

MATERIALS AND METHODS

Experimental animals

Specimens of the oval squid, *Sepioteuthis lessoniana* Lesson, 1830 were obtained from the Marine Biomedical Institute of the University of Texas Medical Branch (Galveston, TX). This species was chosen for study because it has been successfully reared in culture since 1987 through multiple generations at the Marine Biomedical Institute and thus hatchlings and juveniles of known age are available (Segawa, '87; Lee et al., '94). Specimens examined in this study included hatchlings from egg strands collected in Japan and from laboratory-spawned eggs. To obtain specimens of known age, all animals that hatched on a given day were isolated in a plastic mesh enclosure in the culturing tank. Individuals were then removed, anesthetized, and fixed (see below) at hatching and then at 1-week intervals ending with 5-week-old juveniles. The temperature in the rearing tanks was maintained at 23°C and the animals were fed crustaceans and fish six times per day (see Lee et al., '94 for details of the culturing procedures).

Histology

Specimens were anesthetized with a 1:1 mixture of 7.5% $MgCl_2 \cdot 6H_2O$ and seawater (Messenger et al., '85) and fixed in 10% formalin in seawater for 24 h. The tissue was then cut into blocks. For the hatchling, week 1, and week 2 specimens, the head was removed and bisected longitudinally into right and left arrays of the arms and tentacle. For the week 3, week 4, and week 5 specimens, a single tentacular stalk and single arm (arm III) were removed. Both the left and the right tentacle and left and right arm (arm III) were examined from two specimens from each stage. The tissue blocks were cut so that samples of tissue could be examined from the base, middle and terminal portion of each appendage. The tissue blocks were then partially dehydrated in an ethanol series to 95% ethanol and then infiltrated with unpolymerized glycol methacrylate plastic (Reichert-Jung Historesin, Leica Instruments GmbH, Heidelberg, Germany). Following polymerization, the blocks were sectioned with glass knives at 0.5–3.0 μm in planes perpendicular to the long axis of the appendage. The sections were stained with Lee's methylene blue-basic fuchsin stain (Bennett et al., '76) and were examined with brightfield and polarized light microscopy.

Transmission electron microscopy

Specimens were anesthetized with a 1:1 mixture of 7.5% $MgCl_2 \cdot 6H_2O$ and seawater (Messenger et al., '85) and fixed at 4°C in 3.0% glutaraldehyde, 0.065 M phosphate buffer, 0.5% tannic acid, and 6% sucrose for 6–8 h. A minimum of seven specimens from each stage of development were examined. Following fixation, the specimens were transferred into 0.065 M phosphate buffer, 1% glutaraldehyde, 6% sucrose, and stored at 4°C until the entire developmental series was fixed. Small blocks of tissue from the tentacular stalk and arm number 3 were cut and rinsed in chilled 0.065 M phosphate buffer overnight. The tissue was postfixed for 40 min at 4°C in a 1:1 mixture of 2% osmium tetroxide and 2% potassium ferrocyanide in 0.13 M cacodylate buffer. The tissue was rinsed in chilled 0.065 M cacodylate buffer for 15 min, dehydrated in a graded series of ethanols, cleared in propylene oxide, and embedded in epoxy resin (Epox 812, Ernest F. Fullam, Latham, NY). The blocks were sectioned in planes perpendicular to, or parallel with, the long axis of the appendage, yielding longitudinal and transverse sections, respectively, of muscle fibers of the transverse muscle mass. The number of blocks examined for each appendage type at each stage of development ranged from 13 to 29. In order to standardize sampling, the blocks were trimmed to include sections of the trabeculae of the transverse muscle mass and associated longitudinal muscle bundles. Sections of silver interference color were stained with saturated aqueous uranyl acetate and Reynolds ('63) lead citrate and examined with a Zeiss EM 10CA transmission electron microscope.

Morphometrics

Dimensions of muscle fiber components and relative areas of muscles were measured on transmission electron and light micrographs. The micrographs were placed on a digitizing tablet (GTCO Digi-Pad 5) interfaced with a microcomputer and traced using Sigma-Scan software (Jandel Scientific, San Rafael, CA). Since the muscle cells are frequently irregular in cross-sectional shape, their diameters were estimated by measuring the average cross-sectional area of a sample of fibers and calculating the diameter of a right circular cylinder of the same cross-sectional area. For fiber diameter and thick filament diameter, a minimum of 25 measurements of each parameter were made from electron micrographs of each stage and appendage type and means and standard deviations calculated. Measurements of thick-filament length reported here are of the longest thick filaments measured from each stage of development. In the obliquely striated cells in particular, it is difficult to be certain that the section plane is exactly longitudinal and thus that an individual thick filament remains in the section plane along its entire length. Because of this problem, there is the possibility that the thick-filament lengths reported here may underestimate the actual filament length.

High-speed video recordings

High-speed video recordings of *Sepioteuthis lessoniana* juveniles during the first five weeks of life were made at the Marine Biomedical Institute of the University of Texas Medical Branch (Galveston, TX). A NAC Visual Systems HSV-1000 high-speed color S-VHS video system was mounted above culturing tanks containing *S. lessoniana* individuals of known age. Lighting was provided by eight 300-W dichroic mirror bulbs (GE-ELH) mounted in two fan-cooled pallets of my own design. An initial attempt was made to film prey capture from above in a specially constructed 60-cm × 60-cm × 15-cm deep glass aquarium into which the animals were transferred. Either as a result of handling during the transfer to the filming tank or because of some aspect of the tank itself (various masks and backgrounds were tried), the animals would not feed in the glass aquarium. All recordings were therefore made in the culturing tanks. Field-collected mysid shrimp (*Mysidopsis almyra*) and hatchery-reared guppies (*Poecilia reticulata*) were offered in the center of the recording field and prey capture was videotaped at 500 fields per second. Various attempts were made to offer tethered prey so as to permit higher magnification and thus greater image resolution recordings, but the animals would not feed on tethered prey. Approximately 50 prey-capture sequences were recorded. The major features of the ontogeny of prey-capture behavior were identified by viewing the recordings field-by-field on a Panasonic AG-1960 videocassette recorder.

RESULTS

Gross anatomy of the developing arms and tentacles

At hatching, the four pairs of arms and one pair of tentacles that characterize adult decapod cephalopods are present. Arm pair one

(numbering from dorsal to ventral) is the shortest of the arms (dorsal mantle length = 6.5 mm), measuring < 1 mm in length. Arm pair number three is the longest, measuring approximately 2.5 mm in length. Miniature suckers are present on all arms at hatching. The tentacles are the longest appendages at hatching, measuring approximately 5 mm in length when retracted. The tentacular stalk represents only 30% of the length of the tentacle, the remainder consists of the expanded terminal club equipped with suckers. As the animals mature, the relative proportions of the tentacular stalk and the club change; by the third week of development, the stalk is approximately 40% of the retracted tentacular length, and by the fifth week it is approximately 50% of the retracted tentacular length.

Microanatomy of the developing arms and tentacles

Many of the components of the arms and tentacles that are present in adult animals are recognizable in newly hatched animals as well (Fig. 2). The central axis is occupied by the axial nerve cord, which at hatching occupies approximately 25% of the cross-sectional area of the arm and 20% of the cross-sectional area of the tentacular stalk. Although the nerve cord increases in diameter as the animals grow, its size relative to the arm or tentacle cross-section decreases (Table 1). The nerve cord itself consists of a central core of neuropile surrounded by a thick layer of nerve cell bodies with large nuclei. In hatchlings, the neuropile is surrounded by the nerve cell bodies. As the animals grow, the neuropile extends to the oral and aboral surface of the nerve cord so that it divides the collection of nerve cell bodies into groups on each side of the nerve cord. A greater number of large axons are observed on the aboral and especially the oral sides of the neuropile as the animals grow.

The axial nerve cord is surrounded by muscle fibers of the transverse muscle mass (Fig. 2). Muscle fibers in this mass are oriented in planes perpendicular to the long axis of the arm or tentacle and thus extend across the diameter of the appendage. In hatchlings, the transverse muscle mass occupies a relatively small proportion of the cross section. As the animals grow, the relative size of the transverse muscle mass increases until it occupies the largest proportion of the cross section (Table 1). Surrounding the transverse muscle are bundles of longitudinally oriented muscle fibers that interdigitate with trabeculae extending from the central transverse muscle mass. The term "trabeculae" was used by Graziadei ('65) to describe similar extensions of the transverse muscle mass of the arms of *Octopus* and the term is therefore adopted here (Kier, '82). Numerous large nuclei are visible in the central transverse muscle mass but are rarely located within its trabeculae. The longitudinal muscle bundles of the tentacle are, in turn, surrounded by two thin layers of helically arranged muscle fibers, one arranged as a left-hand helix and the other as a right-hand helix.

In the arms, the longitudinal muscle bundles are enclosed on the oral and aboral surface by a layer of connective tissue and on the surfaces in between by a pair of obliquely arranged muscle layers. As in the adult (Kier, '82) the oblique muscles have their origin and insertion on the oral and aboral connective tissue layers and thus form a composite of muscle and connective tissue fibers surrounding the transverse and longitudinal arm musculature. Examination of longitudinal sections that graze the oblique muscles and those that graze the connective tissue layers show that the combination of the connective tissue layers and the oblique muscles forms a composite system of right- and left-hand helices.

As in the adult, the helical muscle layers in the tentacles and the oblique muscle/connective tissue composite layer in the arms of the hatchlings are surrounded by an additional layer of longitudinal muscle fibers. Embed-

TABLE 1. Relative cross-sectional areas of nerve cord and muscle of arm and tentacle[a]

	Hatchling	Week 1	Week 2	Week 3	Week 4	Week 5	Adult
Axial nerve cord							
% cross-section of arm	26	25	16	11	11	10	4
% cross-section of tentacle	20	16	10	6	6	8	3
Transverse muscle							
% cross-section of arm	30	29	42	43	43	42	47
% cross-section of tentacle	32	39	43	45	48	47	58

[a]The remainder of the cross-sectional area is occupied primarily by longitudinal muscle.

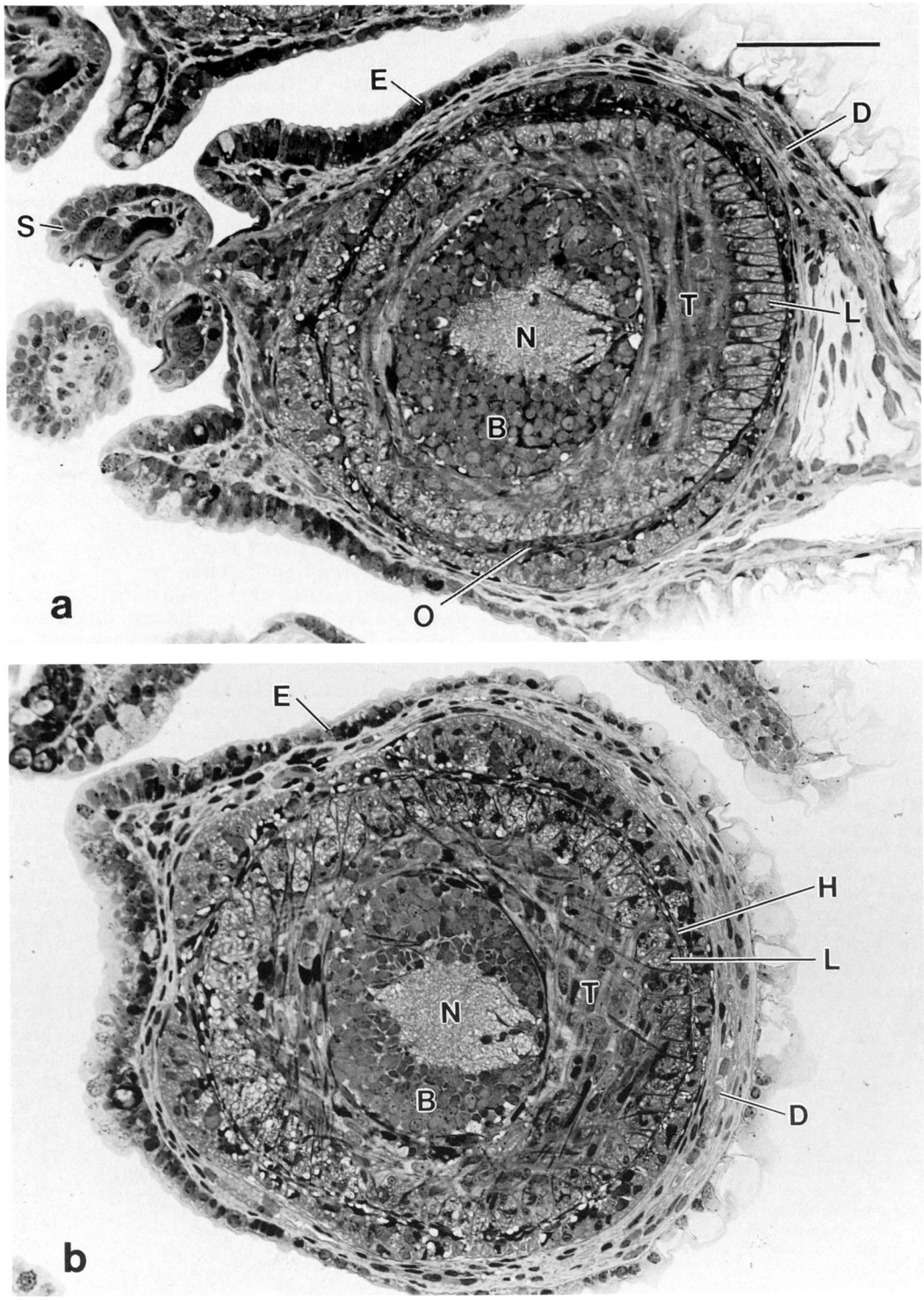

Figure 2

ded in this longitudinal musculature are six intramuscular nerve cords (not visible in the figure) that lie adjacent to the helical muscle layers in the tentacle and to the oblique muscle/connective tissue composite in the arms. The outer longitudinal musculature is surrounded by the dermis and a simple epithelium.

Differentiation of arm and tentacle fibers of the transverse muscle mass

One week

In striking contrast to the situation in adult animals, in hatchlings and 1-week-old animals, the ultrastructural appearance of the muscle fibers of the transverse muscle mass of the arms and tentacles is identical (Fig. 3). The transverse muscle fibers of both appendage types have the ultrastructural characteristics of regular, obliquely striated muscle; the thick and thin myofilaments are arranged in a staggered array and the Z elements in an oblique pattern. The cells are circular or polygonal in cross-sectional shape and are smaller in diameter than those of the adult (Table 2). Mitochondria are frequently observed in the core of the fibers, surrounded by the myofilaments. An amorphous electron-dense extracellular material is located between the fibers.

Tubules of the sarcoplasmic reticulum are evident within the myofilaments and also immediately beneath the sarcolemma. The sarcoplasmic reticulum within the myofilament lattice (the 'intramyoplasmic zone' of Amsellem and Nicaise, '80) is localized in the planes of the Z elements and appears to consist of an anastomosing network of tubules that are elongated parallel to the plane of the Z elements. Since the planes of the Z elements are oriented obliquely to the long axis of the cell, the tubules of the sarcoplasmic reticulum in this zone are also aligned at a small angle to the long axis of the cell (Fig. 1). This oblique alignment is observed in longitudinal sections by following one plane of sarcoplasmic reticulum longitudinally in the cell until it intersects with the sarcolemma. At these points of intersection, enlarged tubules, or terminal cisternae, of the sarcoplasmic reticulum are most common. The outer portion (i.e., the portion facing the surface of the fiber) of the membranes of the terminal cisternae is arranged in parallel with the sarcolemma forming specialized contacts called "peripheral couplings" (Nunzi and Franzini-Armstrong, '81; Kier, '85). Regularly spaced, electron-dense junctional feet were sometimes observed in the space between the sarcolemma and the outer membrane of the terminal cisternae. Terminal cisternae of one fiber are often located adjacent to the terminal cisternae of neighboring fibers. Where the terminal cisternae of two cells appose, the amorphous, electron-dense extracellular material normally located between the sarcolemmal membranes is reduced.

Transverse sections through the fibers show an orderly variation in thick filament diameters within each trapezoidal area of myofilaments between the rows of Z elements. The smallest-diameter thick filaments are located along each side of the band of myofilaments delimited by the rows of Z elements, while the largest-diameter thick filaments are located down the center of the band. This is interpreted to be the result both of tapering of the thick filaments toward their ends and the stagger of thick filaments relative to one another due to the oblique striation of the muscle cell. Because of this stagger, a section passes through the tapered end of the thick filaments at the edges of the band and through the thickest center portion of the filaments in the middle of the band (Kier, '85). At this stage of differentiation, the thick filament diameter at the middle of the thick filaments is smaller than that of the adult (Table 2). The thick filament diameter of the tentacle cells is similar to that of the arm cells at this stage and is the same as that of the adult tentacle cells. Thick filament lengths of the transverse muscle fibers of the

Fig. 2. Photomicrographs of transverse sections of an arm (**a**) and a tentacle (**b**) of a *Sepioteuthis lessoniana* hatchling (dorsal mantle length = 6.5 mm). The arm and the tentacle are oriented in the figure with the oral surface facing left. A portion of a sucker (S) is seen in the section of the arm. The axial nerve cord is located in the center of each section and consists of a central neuropile (N) and surrounding nerve cell bodies (B) with large nuclei. Surrounding the axial nerve cord are the muscle fibers of the transverse muscle mass (T); peripherally they interdigitate with bundles of longitudinal muscle (L). The transverse muscle cells of the arm insert on a connective tissue sheet orally and aborally and on connective tissue associated with a pair of oblique muscles (O) on each side of the arm. The transverse muscle cells of the tentacle insert on connective tissue associated with helical muscle layers (H). Additional longitudinal muscle is present underneath the dermis (D) and epithelium (E). Scale bar = 100 μm. A 1-μm-thick glycol methacrylate section stained with Lee's methylene blue-basic fuchsin and photographed under brightfield illumination.

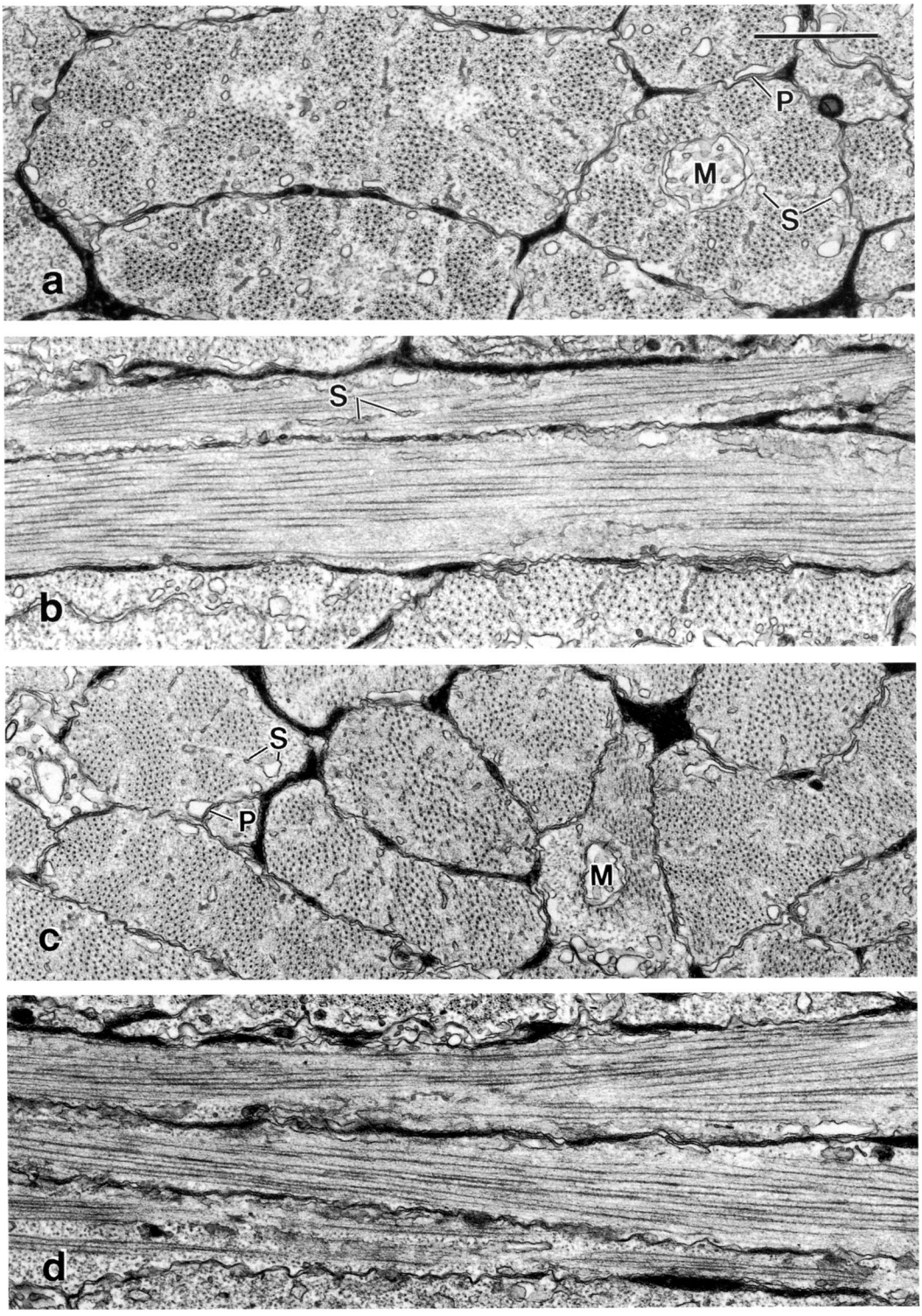

Figure 3

TABLE 2. Dimensions of ultrastructural components of developing arm and tentacle muscle cells

	Hatchling	Week 1	Week 2	Week 3	Week 4	Week 5	Adult
Fiber diameter (in μm)							
Arm, mean (±SD)	0.84 (0.38)	1.3 (0.40)	1.4 (0.33)	1.5 (0.45)	1.6 (0.44)	1.6 (0.30)	3.9 (0.39)
Tentacle, mean (±SD)	0.96 (0.36)	1.4 (0.27)	1.2 (0.36)	1.4 (0.40)	1.5 (0.39)	1.6 (0.32)	2.0 (0.35)
Thick-filament diameter (in nm)							
Arm, mean (±SD)	22 (2)	22 (3)	25 (3)	22 (3)	26 (3)	30 (2)	39 (5)
Tentacle, mean (±SD)	21 (3)	24 (3)	22 (2)	22 (3)	23 (3)	23 (2)	23 (3)
Thick-filament length (in μm)							
Arm, longest measured	2.2	3.2	3.1	3.8	4.4	4.4	6.4
Tentacle, longest measured	2.4	2.9	3.1	3.2/1.6[a]	1.1	1.2	1.2

[a]Since the week 3 tentacle muscle includes both obliquely striated cells and those showing approximate cross-striation, thick filament lengths for each type are given. The first value is for the obliquely striated cells and the second value is for the cross-striated cells.

arms and tentacles are similar in hatchlings and in 1-week-old animals (Table 2).

Two weeks

In animals two weeks old, most cells of the transverse muscle mass of the tentacles are obliquely striated and thus are still indistinguishable from those observed in the transverse muscle mass of the arms (Fig. 4). Transverse sections of the fibers at this stage show the typical obliquely striated pattern of zonation of the myofilaments that results from the stagger of the thick filaments and the oblique array of sarcoplasmic reticulum and dense bodies (Kier, '85) (Fig. 1). As in other cephalopod obliquely striated muscle cells, tubules of the sarcoplasmic reticulum are visible within the myofilament lattice, rather than being restricted to the zone immediately beneath the sarcolemma, as in the adult cross-striated tentacle cells. The cell diameter, thick-filament diameter, and thick-filament length of both the tentacle and arm cells are similar to that of the previous stage (Table 2).

Fig. 3. Transmission electron micrographs of muscle cells from a 1-week-old *Sepioteuthis lessoniana* (dorsal mantle length = 8 mm). Transverse (**a**) and longitudinal (**b**) sections of fibers from the transverse muscle of the arm (arm III), and transverse (**c**) and longitudinal (**d**) sections of muscle fibers from the transverse muscle of the tentacle. The fibers in both the arm and tentacle are obliquely striated. Mitochondria (M) are located in the central core. In transverse sections (**a,c**) trapezoidal areas of thick and thin myofilaments are delimited by rows of sarcoplasmic reticulum (S) and Z elements. The outer membranes of the terminal cisternae of the sarcoplasmic reticulum form specialized contacts or peripheral couplings (P) with the sarcolemma. Long thick filaments are visible in the longitudinal sections (**b,d**). An electron-dense extracellular material lies in between the closely packed cells. Scale bar = 1 μm.

Three weeks

By 3 weeks of age, a difference in ultrastructure between the transverse muscle fibers of the tentacles and of the arms is apparent (Fig. 5). In longitudinal sections of the tentacle muscle, many fibers show a striation pattern that appears to be intermediate between that of the adult cross-striated pattern and that of obliquely striated cephalopod muscle. In particular, the thick filaments of many of these fibers are shorter than of earlier stages (Table 2) and frequently show approximate lateral alignment across the cell. The dense bodies of these cells are also in approximate lateral alignment. In these transitional fibers, it is also less common to observe tubules of the sarcoplasmic reticulum within the myofilament lattice. The sarcoplasmic reticulum of these cells is primarily concentrated in the subsarcolemmal area of the fiber.

The cells of the transverse muscle of the arms of 3-week-old animals show ultrastructural characteristics of obliquely striated cells and are thus similar in appearance to those from earlier stages. Measurements of thick filament length in these cells reveal longer thick filaments compared with earlier stages (Table 2).

Four weeks

By 4 weeks of age, most muscle cells of the transverse muscle mass of the tentacle are cross-striated and appear similar to those of the adult animals (Fig. 6). Tubules of the sarcoplasmic reticulum are located almost exclusively peripherally in the cell, immediately beneath the sarcolemma. Thus, the cells lack invaginated tubules and are not divided into myofibrils (Kier, '85). The outer portion of the membranes of the tubules of the sarcoplasmic reticulum is arranged in parallel with

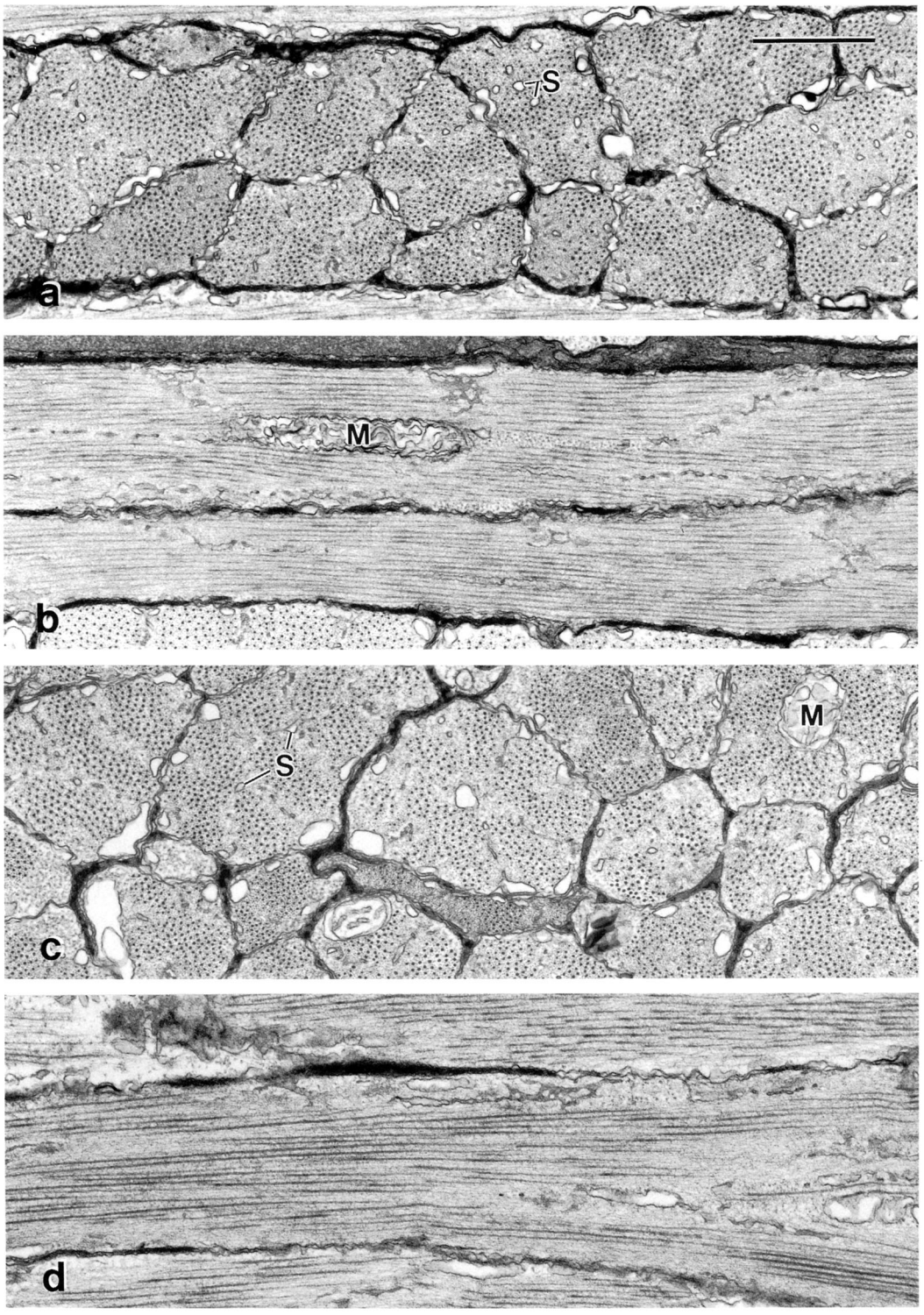

Fig. 4. Transmission electron micrographs of muscle cells from a 2-week-old *Sepioteuthis lessoniana* (dorsal mantle length = 11 mm). Transverse (**a**) and longitudinal (**b**) sections of fibers from the transverse muscle of the arm (arm III), and transverse (**c**) and longitudinal (**d**) sections of muscle fibers from the transverse muscle of the tentacle. The cells at this stage in both the arm and the tentacle are obliquely striated. The tentacle cells at this stage (**c,d**) show mitochondria (M) in the core and rows of tubules of the sarcoplasmic reticulum (S) extending into the center of the cells. Scale bar = 1 μm.

the sarcolemma, forming peripheral couplings that appear similar in morphology to those described earlier for the obliquely striated cells. Regularly spaced, electron-dense junctional feet are present in the space between the outer sarcoplasmic reticulum membrane and the sarcolemma of the peripheral couplings. Mitochondria are sparse and are present in the periphery of the cells adjacent to the sarcolemma. The thick filaments are short, and are in approximate lateral register in the cells (Table 2; Fig. 6). In transverse sections of the tentacle muscle fibers, the thick filaments appear hollow due to the presence of an electron-lucent core. This hollow appearance is particularly marked in sections that pass through the A band in regions of overlap between the thick and thin filaments and less obvious in sections that pass through the H region (see Kier, '85 for details). As in the adult, an M band is not visible, and the sarcomeres are sometimes sheared so that the Z discs, A bands, and I bands often follow a curved or angled course across the diameter of the cells. This lack of alignment can also be seen in transverse sections as the section plane passes in and out of the A band in a single cell. The Z disc of these cross-striated cells is not the highly organized network observed in vertebrate cross-striated cells (Kier, '85). Instead, the Z disc appears to be simply a lateral alignment of dense bodies. This is particularly evident in transverse sections that pass through the Z disc and show a loosely arranged grouping of electron-dense material.

The transverse muscle cells of the arms of 4-week-old animals are obliquely striated and appear similar to those of the adult. The thick filaments are longer than in the previous week (Table 2).

Five weeks

The ultrastructure of the fibers of the transverse muscle mass of the tentacles and of the arms of 5-week-old *Sepioteuthis lessoniana* is essentially unchanged from the previous week and very similar to that of the adult (Fig. 6). However, the thick filament length, thick filament diameter and cell diameter of the obliquely striated arm cells of 5-week-old animals are all smaller than in the adult (Table 2).

Summary of muscle cell differentiation

In summary, in hatchlings the ultrastructure of the fibers of the transverse muscle mass of the tentacles is indistinguishable from that of the arms. The fibers show the ultrastructural characteristics of cephalopod obliquely striated muscle, including oblique arrangement of long myofilaments, central mitochondrial core, and tubules of the sarcoplasmic reticulum in the core of the fibers in planes defined by the dense bodies. This oblique striation is retained throughout development into the adult in the fibers of the transverse muscle of the arms. The tentacle fibers are still obliquely striated after 2 weeks of development. By 3 weeks of development, the tentacle fibers begin to show some of the ultrastructural characteristics of the cross-striated adult tentacle cells. In particular, some of the fibers possess shorter thick filaments that show approximate lateral alignment across the cell. In addition, mitochondria are rarely observed in the core of the cells and instead are observed immediately beneath the sarcolemma. The location of the sarcoplasmic reticulum is also changed so that tubules of the sarcoplasmic reticulum do not appear in the core of the fibers but instead are restricted primarily to the surface of the cells under the sarcolemma. By 4 weeks of development, the tentacle fibers are cross-striated and resemble those of the adult.

Development of prey-capture behavior in hatchlings

The following is a summary of the major events of prey capture behavior in *Sepioteuthis lessoniana* from hatching to 5 weeks of age. A detailed analysis of the high speed video recordings of prey-capture behavior of developing *S. lessoniana* will be presented elsewhere.

Hatchling *Sepioteuthis lessoniana* show the same sequence of phases of attack during feeding that has been described previously for *Sepia officinalis* (Messenger, '68, '77) and for adult loliginid and ommastrephid squids (Kier, '82; Nicol and O'Dor, '85; Foyle and O'Dor, '88). The phases, in sequence, have been termed *attention, positioning,* and *strike.* When prey are introduced into the tank, the animals enter the attention phase of the attack, which involves first a rapid turning of the head so that the arms and tentacles are directed toward the prey and then a reorientation of the entire animal so that the long axis of the body is also aligned with the prey. This brief attention phase is followed immediately by the positioning phase, during which the animal swims toward the prey with the arms and tentacles forward. The arms are

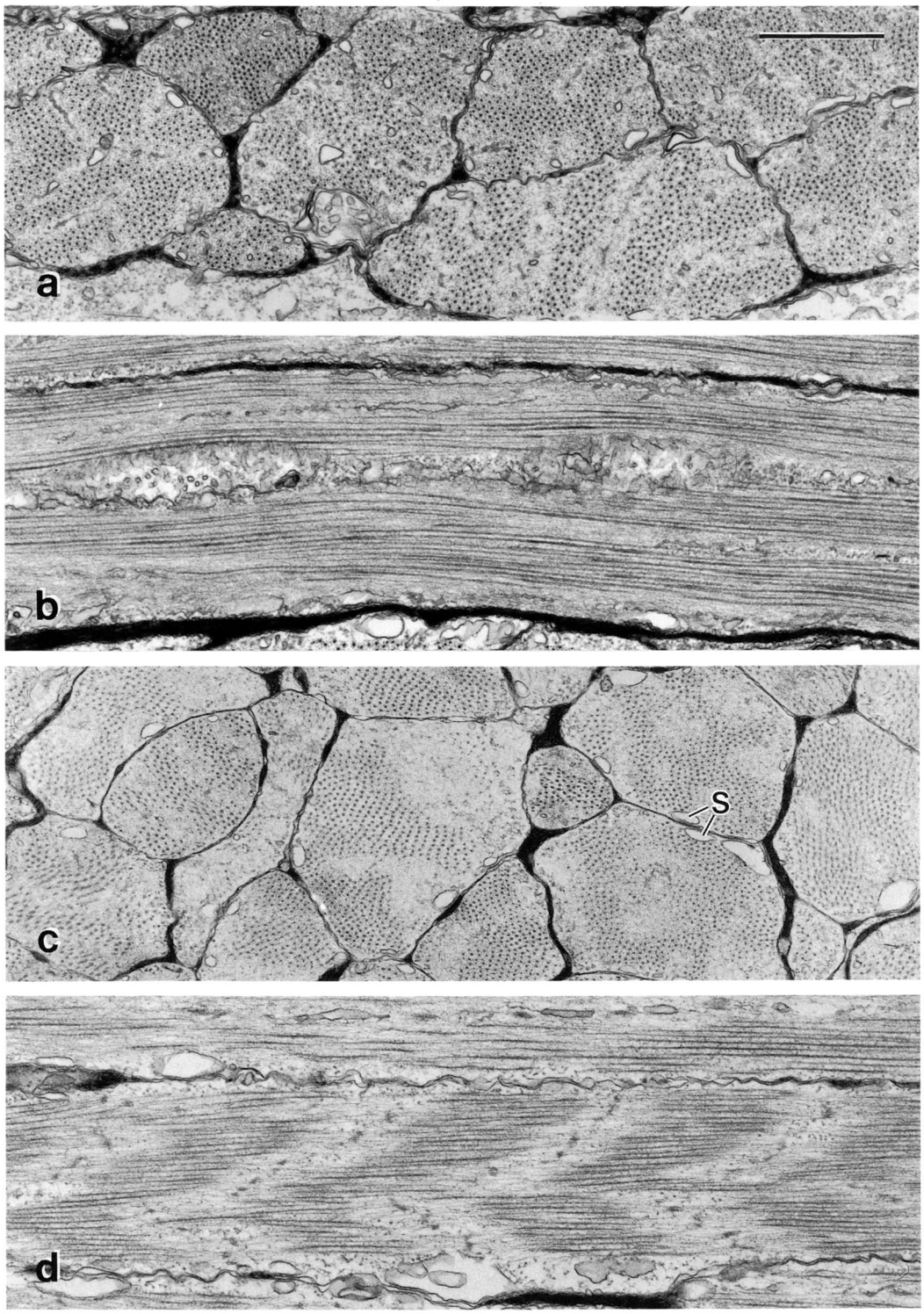

Figure 5

held in a tight cone-shaped arrangement aimed at the prey. If the prey is moving, the animal keeps the tips of the arms and tentacles pointed directly at the prey and alters course.

In animals ranging in age from hatching up to two to three weeks of age, feeding on mysid shrimp, the final phase of the attack, the strike, is markedly different from the strike of adult loliginid squid: it does not involve rapid elongation of the tentacles. Instead, the animals continue to approach the prey, then rapidly flare the arms and tentacles, jet forward, and thereby capture the prey directly within the flared arms and tentacles. The arms and tentacles are then closed around the prey, which is subsequently ingested. In spite of the relatively low resolution of the high-speed video tapes, this pattern of prey-capture behavior is easily recognized because the acceleration forward during the strike is much greater than that observed in the adult (Fig. 7).

By 4 weeks of age, and occasionally by three weeks of age, *Sepioteuthis lessoniana* show strike behavior typical of adult animals. The animals, in most cases feeding on guppies, continue to approach the prey until the distance to the prey is less than one mantle length. During the positioning phase, the tips of the tentacles are held together, projecting from the cone of arms. During the strike, the arms are flared, the tentacles are rapidly elongated, and the suckers on the tentacular club strike the prey. Flaring of the arms occurs in 60–80 msec and the tentacles are elongated in 20–40 msec. The tentacles are then retracted more slowly (approximately 200 msec), bringing the prey within reach of the flared arms. The arms are then used to manipulate the prey for ingestion. A strike with the tentacles was not observed in animals ranging in age from hatching to 2 weeks. In several instances, however, if 4-week-old animals were offered the small mysid shrimp prey normally fed to the younger animals, they did not strike with the tentacles and instead reverted to the strike behavior of younger animals.

Fig. 5. Transmission electron micrographs of muscle cells from a three-week-old *Sepioteuthis lessoniana* (dorsal mantle length = 12 mm). Transverse (**a**) and longitudinal (**b**) sections of fibers from the transverse muscle of the arm (arm III); the muscle cells are obliquely striated. Transverse (**c**) and longitudinal (**d**) sections of muscle fibers from the transverse muscle of the tentacle. Many muscle cells from the tentacle are no longer obliquely striated and instead show characteristics of the cross-striated cells of the adults. In transverse sections of the tentacle cells (**c**), the sarcoplasmic reticulum (S) appears to be restricted primarily to a zone immediately beneath the sarcolemma. Mitochondria are also less frequently observed in the core of the tentacle cells. In longitudinal sections (**d**), the shorter thick filaments are visible, and in many cells the dense bodies and thick filaments are in approximate lateral alignment across the cells. Scale bar = 1 μm.

DISCUSSION

Muscle cell differentiation and the ontogeny of behavior

This study of muscle development in the arms and tentacles of *Sepioteuthis lessoniana* reveals that during the first 2 weeks after hatching the muscle fibers of the transverse muscle of the tentacles show ultrastructural characteristics of obliquely striated cells and are indistinguishable from those of the transverse muscle mass of the arms. It is not until approximately 3 weeks after hatching that the cells begin to show the ultrastructural specializations that characterize the tentacle muscle of the adults. These observations are significant because previous studies (Kier, '85, '91) have hypothesized that the ultrastructural characteristics observed in the adult tentacle fibers are specializations for the unusually rapid tentacular strike observed in adult squid. In particular, the development of a short sarcomere, cross-striated cell type in the tentacle results in a significant increase in shortening velocity. Of primary importance to this discussion is the thick filament length. An approximate inverse correlation has been observed between the thick-filament length and the unloaded shortening velocity of molluscan muscle cells (Millman, '67) and of muscle cells in general (Josephson, '75). The increase in shortening velocity with decrease in thick-filament length is due to the fact that muscles with short thick filaments, and hence short sarcomeres, have more elements in series, per unit length of muscle. Because the shortening velocity of elements in series is additive, muscles with shorter sarcomeres show higher shortening speeds, assuming other factors are held constant (Huxley and Simmons, '72; Josephson, '75; van Leeuwen, '91, '92).

Since the transverse muscle cells of the tentacles do not show the ultrastructural specializations thought to be involved in high shortening velocity until 3 weeks after hatching, it is likely that the hatchlings do not have the capability to produce the rapid ten-

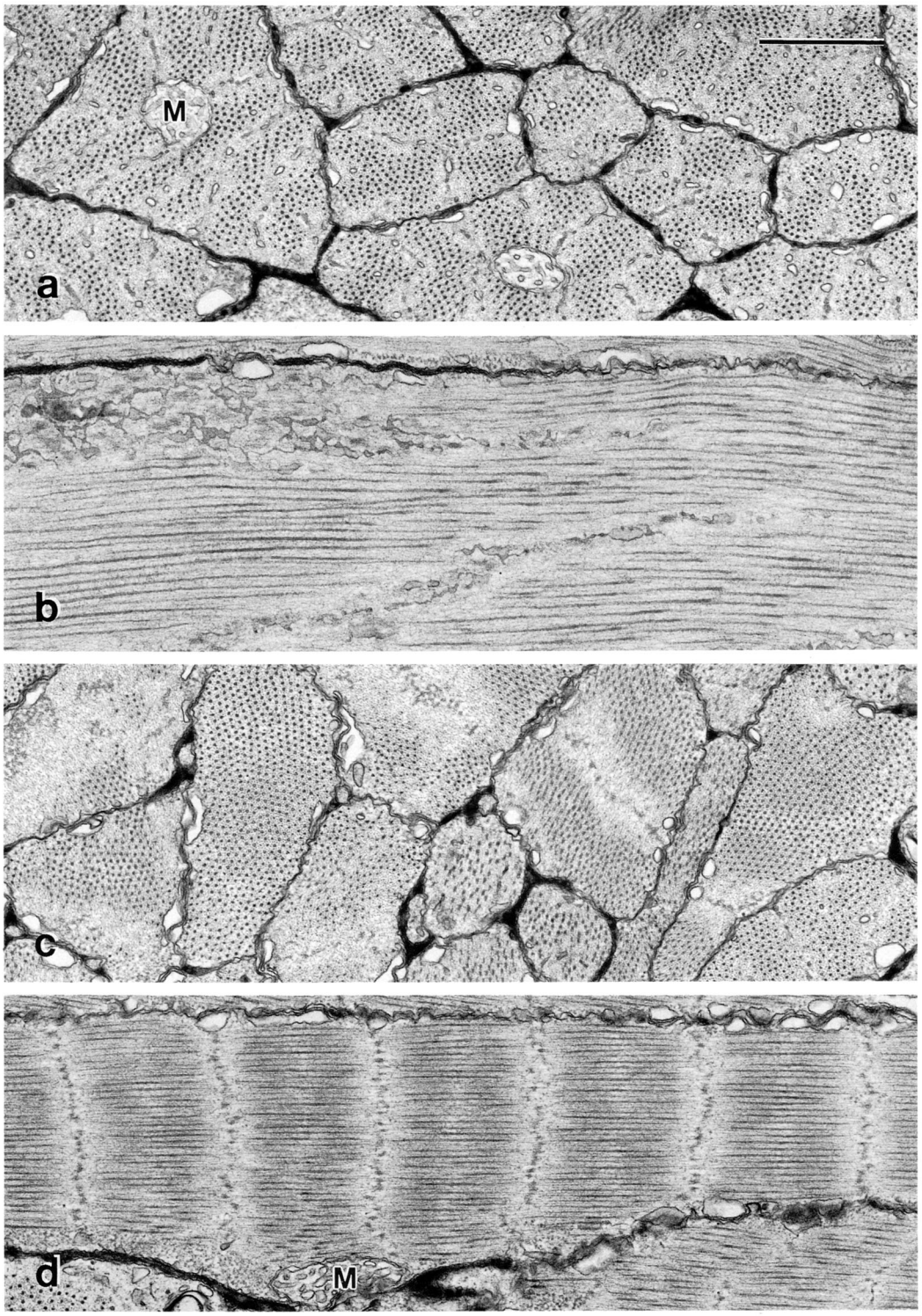

Figure 6

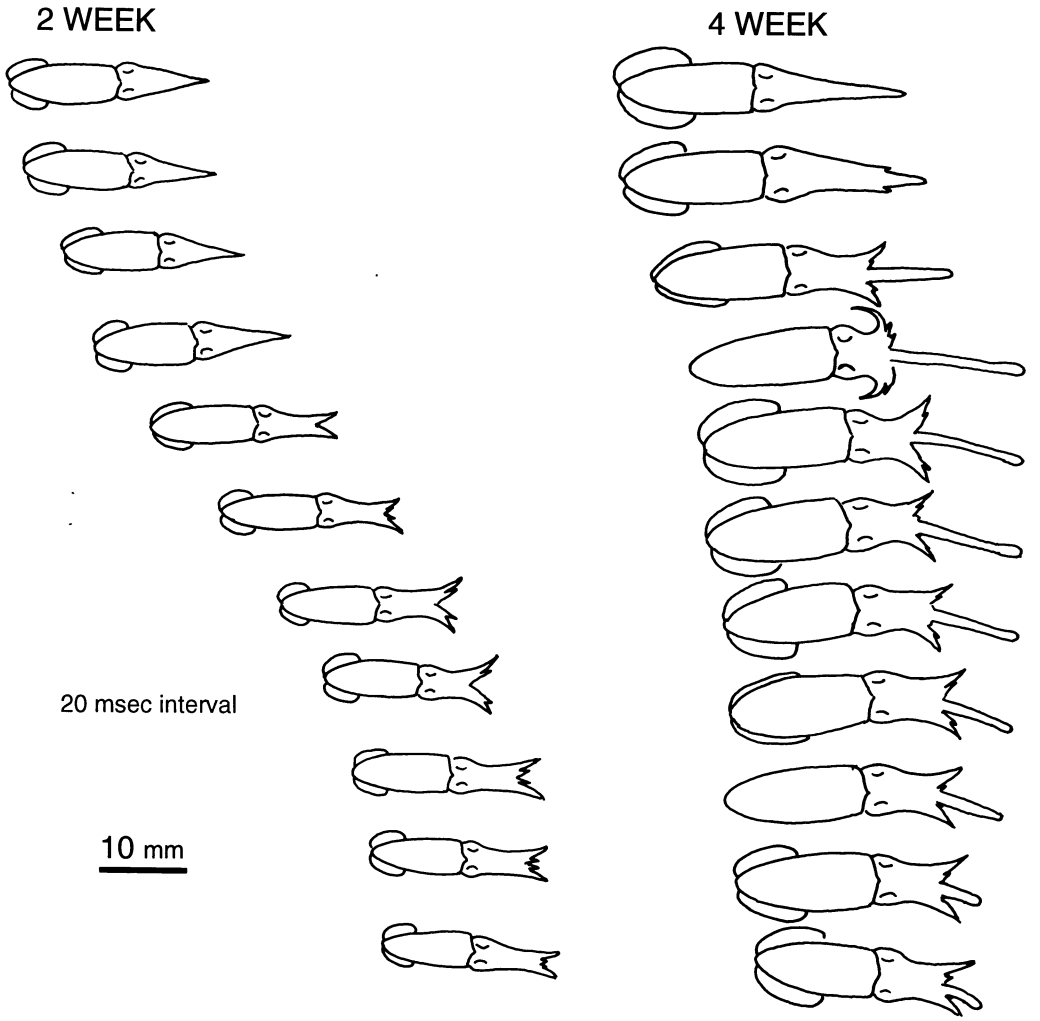

Fig. 7. Tracings from high-speed video sequences of prey capture in 2-week-old (left column) and 4-week old (right column) *Sepioteuthis lessoniana*. The sequence runs from top to bottom with an interval of 20 msec between frames. Note that the 2-week-old animal rapidly accelerates forward and captures the prey with the arms. By contrast, the 4-week old animal rapidly extends the tentacles and accelerates forward to a much lesser extent.

Fig. 6. Transmission electron micrographs of muscle cells from a 4-week-old *Sepioteuthis lessoniana* (dorsal mantle length = 14.5 mm). Transverse (**a**) and longitudinal (**b**) sections of fibers from the transverse muscle of the arm (arm III), and transverse (**c**) and longitudinal (**d**) sections of muscle fibers from the transverse muscle of the tentacle. Transverse muscle cells from the arm and tentacle at this stage show the characteristics of the adult ultrastructure. In the tentacle cells (**c,d**) the short thick filaments are aligned laterally in the cells and the Z-disc appears to be simply a lateral alignment of dense bodies. Mitochondria (M) are present in the periphery of the tentacle cells, compared with the location in the core of the arm cells and the sarcoplasmic reticulum does not extend into the center of the tentacle cells as it does in the arm cells. Scale bar = 1 μm.

tacular strike observed in the juveniles and adults. Such a constraint is suggested by the high-speed video recordings, which show different prey capture behavior in hatchlings. The mode of prey capture observed in hatchlings does not involve rapid elongation of the tentacles but instead, the animals jet forward and capture the prey directly with their arms and tentacles. Measurements of shortening velocities of developing tentacle cells are required in order to confirm that there is a morphological constraint on the behavior of hatchlings.

Evolutionary implications of observed developmental sequence

Based on developmental and comparative data, the ancestral coleoid (coleoids include the sepioids [cuttlefishes and sepiolids], teuthoids [squids], octopods [octopuses] and vampyromorphs [vampire squid]) is believed to have possessed ten similar, arm-like appendages (Naef, '21/'23; Boletzky, '93, in press). In the octopod clade, one of the arm pairs was lost (probably arm pair II, numbering from anterior to posterior), in the vampyromorphs one arm pair is reduced (arm pair II), and in the decapods (sepioids and teuthoids) arm pair IV was modified and elaborated to form the tentacles (Boletzky, '93). Thus, the most parsimonious hypothesis is that the cross-striated cells of the transverse muscle mass of the tentacles were derived from an obliquely striated muscle cell type. Indeed, obliquely striated muscle cells are characteristic of virtually all of the musculature of cephalopods including *Nautilus* (Kier, '85, '87, '89). The sequence of differentiation from obliquely striated to cross-striated fibers may therefore reflect the evolutionary history of these cells. Further study of the mechanisms of this reorganization may provide information on the way in which the derived and highly specialized cross-striated muscle evolved in decapods. In particular, it will be interesting to know if the observed changes in ultrastructure result from breakdown and replacement of the various components or their reorganization and redimensioning. For instance, is the change in thick filament length observed during differentiation the result of removal of the long thick filaments and assembly of short thick filaments, or are the existing myofilaments modified?

The sequence of development along with details of the structure of the cells support Prosser's ('82) contention that cross-striation, the transverse alignment of thick and thin filaments, has evolved many times in parallel. For example, although the cross-striated tentacle cells include an identifiable Z disc, it is not the organized, precise network that has been described for many invertebrate and insect cross-striated cells (see for example, Rowe, '71; Smith, '72; Hoyle, '83). Instead, the Z-disc appears to be simply a lateral alignment of dense bodies, and the development of this alignment is visible during differentiation of the cells.

Significance of observed developmental sequence

The sequence of differentiation in which the specialized tentacle muscle does not appear until three weeks after hatching limits the behavior of the young animals since they lack the muscle specialization required for the extremely rapid tentacular strike observed in the adults. Why is there a delay in differentiation of the cross-striated tentacle cells until 3 weeks after hatching? Further work is required to answer this question definitively, but there are several interesting possibilities, ranging from intrinsic morphological to externally imposed adaptive constraints. The first relates to the sequence of differentiation observed in this study. It is possible that the mechanism by which the cross-striated cells differentiate from an obliquely striated cell type imposes a constraint on how early the short sarcomere cross-striated pattern can develop. If this is true, the developmental mechanism and sequence constrain the evolution of behavior of the young hatchlings. It is also possible that neural development at hatching is not yet complete and the hatchlings, therefore, lack the neural control required for the behaviors associated with the strike (Chen et al., '96) and thus even if the appropriate muscle existed it could not be used. Other factors may relate to the size or the ecology of the animals at hatching. It is possible that for the young hatchlings the size of their prey or some aspect of their early life history make the tentacular strike used by the adults less effective for prey capture than the "lunge and grasp" observed in the hatchlings. Some possibilities include the effects of the relatively low Reynolds number environment of the early hatchlings, lack of effective adhesion by tentacle suckers at small size, or differences in the feeding ecology of young hatchlings, e.g., surface feeding of "paralarvae" (Vecchione, '81) or behavior of the prey consumed by hatchlings versus that of juveniles. If these factors are operating, then the relations between the timing of development of the cross-striated muscle and prey capture behavior are determined more by the functional demands than by developmental or structural constraints.

It is noteworthy that, unlike most hatchling teuthoids, most newly hatched sepioids capture prey using the adult mode of rapid tentacle elongation (Boyle, '83; Boletzky, '87). Although we have only limited early life-

history data, compared with the teuthoids the length of embryonic development of the sepioids is typically longer (Boyle, '83). One important exception is the pygmy cuttlefish, *Idiosepius,* which has an unusually short time of embryonic development, is small as an adult (6–8 mm dorsal mantle length), and has small hatchlings (Natsukari, '70). In *Idiosepius,* all the arms are formed prior to hatching as in other decapods, but the tentacle rudiments remain arrested in early bud stage (Natsukari, '70; Boletzky, in press). The tentacles develop after hatching and eventually serve in prey capture as in other decapods. Although more data are needed to resolve this issue, it appears that a longer developmental period may be required if the tentacles are used in the adult mode in newly hatched decapods. Like most interesting evolutionary problems, the exact sequence of factors that are responsible for the delay in differentiation of the cross-striated tentacle cells until three weeks after hatching is at present unknown.

In summary, at hatching, *Sepioteuthis lessoniana* does not possess the morphological specialization required for the specialized prey-capture behavior observed in the adults. The morphological specialization does not appear until after approximately three weeks of development, which correlates with the emergence of adult prey-capture behaviors. These specializations involve the differentiation of the transverse muscle cells of the tentacles from an obliquely striated precursor to a short-sarcomere, cross-striated muscle fiber type. The reasons for the delay in development of the ultrastructural specialization are as yet unclear and may range from constraints imposed by developmental mechanisms to functional requirements of the young.

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