Development of Craniofacial Musculature in *Monodelphis domestica* (Marsupialia, Didelphidae)

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ABSTRACT Development of craniofacial muscles of *Monodelphis domestica* (Marsupialia, Didelphidae) is described. In a period of 4–6 days all craniofacial muscles in *M. domestica* progress from myoblast condensation, to striated myofibers that are aligned in the direction of adult muscles and possess multiple, lateral nuclei. This process begins 1 to 2 days before birth and continues during the first few days after birth. Compared to other aspects of cranial development, muscle development in *M. domestica* is rapid. This rapid and more or less simultaneous emergence of craniofacial muscles differs from the previously described pattern of development of the cranial skeleton in marsupials, which displays a mosaic of acceleration and deceleration of regions and individual elements. Unlike the skeletal system, craniofacial muscles show no evidence of regional specialization during development. *M. domestica* resembles eutherian mammals in the relatively rapid and more or less simultaneous differentiation of all craniofacial muscles. It differs from eutherian taxa in that most stages of myogenesis occur postnatally, following the onset of function. The timing of the development of muscular and skeletal structures is compared and it is concluded that the relatively early development of muscle is not reflected by any particular acceleration of the differentiation or growth of skeletal structures. Finally, the difficulties in accounting for complex internal arrangements of muscles such as the tongue, given current models of myogenesis are summarized.

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INTRODUCTION

The marsupial neonate possesses specific adaptations required by the marsupial reproductive pattern. Marsupials are born in an embryonic condition relative to eutherians and must be able to perform a number of functions at a rudimentary state of development. They have been termed lactational specialists, because significant embryonic development occurs outside the uterus as the young animals are feeding (Renfree, '83). The evolutionary consequences of this specialization as a life history strategy have received wide attention (e.g., Hayssen et al., '85; Kirsch, '77; Lee and Cockburn, '85; Lillegraven, '75; Lillegraven, et al. '87; Parker, '77; Sharman, '73; Tyndale-Biscoe and Renfree, '87). Yet, only recently has attention been paid to the marsupial neonate (e.g., Clark and Smith, '93; Hall and Hughes, '87; Hill and Hill, '55; Maier, '93; Müller, '67); that the bones around the oral cavity are accelerated in their growth (e.g., Clark, '90; Clark and Smith, '93; Esdaile, '16; Hill, '11; Hill and Hill, '55; Nelson, '92); that the chondrocranium is particularly robust (e.g., Maier, '87a, '93; Müller, '68); and that there are specific adaptations that buttress the jaw articulation during its transition from one involving the malleus, incus, and otic region to the mammalian dentary-squamosal joint (e.g., Filan, '91; Lillegraven, '75; Maier, '87b; Müller, '68). However, there has been previously no detailed study of the development of craniofacial musculature in marsupials.

In this paper I describe the development of cranial musculature in the didelphid marsu-
pial, Monodelphis domestica. The paper has three goals. The first and most general is a discussion of the emergence of form and complexity of the major cranial muscles in a mammal. As pointed out by McClearn and Noden (‘88) such data are, for the most part, lacking for musculature and in particular for cranial musculature. The presentation of descriptive data in this paper follows the general form of McClearn and Noden (‘88) and includes the chronology of important stages in the differentiation of skeletal muscles. These stages include 1) the appearance and location of muscle condensations and the differentiation of promyoblasts into myoblasts; 2) the initiation of muscle fiber differentiation including the elongation and fusion of individual myoblasts into multinucleate myotubes, in which the synthesis of myofilaments begins; 3) the maturation of muscle fibers, including the migration of the cell nuclei from the center of the cell to a subsarcolemmal position and the organization of myofilaments into sarcomeres, evidenced by the appearance of striations; 4) the appearance and attachment to skeletal elements; 5) and finally, the emergence of tendons and intramuscular connective tissue structures. These and other aspects of muscle differentiation are reviewed in a number of papers (e.g., Bischoff, ‘78; Fischman, ‘70, ‘72; McClearn and Noden, ‘88; Ontell, ‘77; Ontell and Kozeka, ‘84a,b).

The second general goal is to compare the time course of the development of cranial musculature in Monodelphis domestica with the time course of the development of skeletal structures. Like other marsupials, Monodelphis domestica is born at a state of development that would be considered embryonic in placentals. The only cranial bones present at birth are the exoccipital and those surrounding the oral cavity (Clark and Smith, ’93). Ossification does not reach a state equal to most neonatal eutherians until approximately 3 weeks postnatal. The development of the bones surrounding the neural cavity is particularly slow. The brain is likewise rudimentary at birth. A newborn Monodelphis domestica has a level of central nervous system (particularly forebrain) development equivalent to a day 11 or 12 embryonic mouse (Cant, personal communication; Saunders et al., ’89). Differentiation of most central nervous system structures in Monodelphis domestica occurs during the 2–3 weeks following birth.

Clark and Smith (‘93) describe the development of the skull in Monodelphis domestica and other marsupials, and hypothesize that the cranial skeleton is plastic in development and in marsupials is influenced by two distinct gradients: one, the oral cavity is accelerated in development due to functional demands related to sucking, attachment, and respiration; and two, the cranial cavity is decelerated due to interactions with neural tissue and the very slow brain growth. In this paper I investigate whether similar gradients of development exist within the cranial musculature in response to functional specialization. At least two possible cases of heterochrony might be hypothesized. First, a number of authors (e.g., Müller, ’68; Lillegraven, ’75) have suggested that because the dentary-squamosal jaw joint forms postnatally, the joint must be stabilized and non-functional postnatally. If so, this hypothesis predicts that the jaw musculature may be delayed in development relative to the musculature of the tongue or pharynx. A second hypothesis concerns the ocular muscles. The eyes do not open until approximately 30–35 days after birth (Kraus and Fadem, ’87; unpublished observation), whereas the oral musculature is functional at birth. If oral muscles are accelerated in response to their early function, there should be a different relative time course of development of ocular muscles versus tongue or pharyngeal musculature.

A comparison of muscular and skeletal development is also of interest because numerous studies suggest that the mechanical environment has a significant influence on the formation of skeletal structures during development, including interactions between musculature and skeletal elements (e.g., Atchley and Hall, ’91; Atchley et al., ’84; Biewener and Bertram, ’93; Bjork, ’72; Carter, ’87; Carter, et al., ’87; Hall, ’84; Hall and Herring, ’90; Herring, ’93; Herring and Lakars, ’81; Vilmann, et al. ’85; and references therein). Clark and Smith (‘93) have demonstrated that some skeletal elements in Monodelphis domestica are accelerated in development whereas others are significantly delayed. What relations exist between the development of cranial muscles and their skeletal attachments?

The third general goal is to provide data and a framework that will allow a detailed comparison of the development of musculature, as well as other cranial elements, in other metatherians and eutherians. The cur-
 DEVELOPMENT OF CRANIOFACIAL MUSCLES

The present paper is a part of a series of studies on the relative development of craniofacial features in eutherian and metatherian mammals. Such comparisons are important because they illuminate the developmental specialization of metatherian mammals. Furthermore, the elucidation of the developmental events that are coordinated in all therian taxa and those that are independent in individual taxa is important in determining the integration of craniofacial development, and ultimately in understanding general patterns of developmental plasticity and constraint in cranial evolution.

MATERIALS AND METHODS

Specimens

*Monodelphis domestica* is a member of the family Didelphidae, the family generally considered to resemble most closely the ancestral marsupial condition (Clark and Smith, '93; Clemens, '79; Kirsch and Calaby, '77; Lee and Cockburn, '85; McCrady, '38). Adults of this species are relatively small (80–140 g), as are the neonates (75–100 mg). The mean litter size is approximately eight and both sexes are pouchless (*"Monodelphis"* refers to the "single uterus" or unpouched condition, as opposed to the pouch or "double uterus" *"Didelphis"*). *M. domestica* is one of 17 species of the genus, which is found throughout the northern two thirds of South America. *M. domestica* is relatively terrestrial, feeds on invertebrates, small vertebrates and fruit and appears to breed year round in both the wild and in the laboratory (Nowak, '91; Streilein, '82a-d; personal observation). The young are born after a gestation period of 14.5 days, begin to detach from the teat 10–14 days after birth and are weaned approximately 50 days after birth. Animals reach sexual maturity at an age of 4–6 months (Kraus and Fadem, '87; Stonerook and Harder, '92; Streilein, '82d; Trupin and Fadem, '82; unpublished observation).

The specimens used in this study include those that were serially sectioned and stained histologically, whole-mount cleared and double stained for cartilage and bone, and whole-mount prepared with antibodies to skeletal muscle (Table 1). The age of the specimens is indicated as follows: an E following the numerical age indicates the gestational age of prenatal specimens, a P following the numerical age indicates the postnatal age of the specimen. The day of birth is considered day 0P. The youngest age was from a 14 day intrauterine litter, approximately 0.5 day before birth. The oldest specimens examined were 30 days postnatal. The animals were obtained from a breeding colony at Duke University, Durham, NC, which was established with animals donated by Dr. B. Fadem (Fadem and Rayve, '85; Fadem et al., '82). Because females were generally checked once a day for the presence of a litter, time of birth was known within 24 hours. The pups were removed from the teats and were sacrificed with an injection of Nembutal (pups older than a month) or exposure to cold or carbon dioxide, and fixed as appropriate for the particular preparation.

**Preparation of specimens**

Most specimens were fixed in 10% phosphate buffered formalin, decalcified, embedded in paraffin, and serially sectioned in thickness of 10–12 μm. For most ages multiple specimens were prepared (see Table 1), and in most cases specimens were sectioned in multiple planes (transverse, sagittal and horizontal). These multiple views aided the visualization of three-dimensional orientation of muscle fibers. Alternate slides were stained with Milligan's trichrome or Weigert's hematoxylin counterstained with picropo-
ceau (Humason, ’72). In about a fifth of the specimens, every third slide was stained with Bodian’s silver stain, which reveals details on neural tissue (Bodian, ’36). Because marsupial neonates possess an extremely impermeable skin that limits infiltration, the outermost layer of epidermis was removed. In the larger specimens it was sometimes necessary to bisect the heads. Cleared specimens differentially stained for bone (alizarin red) and cartilage (Alcian blue) were prepared with modifications of the procedures of Wassersug (’76). Whole mount immunocytochemistry specimens were prepared following procedures developed by Dent and Klymkowsky (’89, Klymkowsky and Hanken (’91), and Hanken et al. (’92). Specimens for this procedure were prepared as follows. Specimens were fixed in Dent’s fixative [1 part dimethyl sulfoxide (DMSO): 4 parts methanol] for a period of a few days to several months. Specimens were either skinned or bisected sagitally, and then bleached several hours to overnight in a solution of 1 part 30% hydrogen peroxide: 2 parts Dent’s fixative. Specimens were incubated at room temperature with monoclonal antibodies to fast myosin (F59, donated by Dr. F. Stockdale, Stanford University), diluted into bovine calf serum (Sigma Chemical Co., St. Louis, MO) supplemented with 20% DMSO. After washing, specimens were incubated with an affinity-purified, peroxidase-conjugated goat antimouse antibody (BioRad), reacted with diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, MO), and dehydrated and cleared in BABB (1 part benzyl alcohol: 2 parts benzyl benzoate).

F59 is an antibody to fast myosin heavy chain protein that is found in all developmental ages of chick muscle. Around 99% of all primary myotubes express this protein (including most of those that also stain with antibodies to slow myosins, Stockdale, ’89). In addition this antibody reacts with all secondary (fetal) myotubes in chick (Stockdale, ’89; Stockdale et al., ’86). Although this antibody was initially studied in chick, it reacts with skeletal muscle in whole mount and sectioned immunological preparations of a wide variety of adult, juvenile, and embryonic mammalian taxa (personal observation). In mammalian embryos fast myosin appears to be expressed (i.e., is observable via immunological methods) before muscle can be identified by histological methods. Therefore, it is a useful marker for the early differentiation of muscle, however because virtually all myoblasts react to it at some time in their differentiation (Stockdale, ’89, ’92; Stockdale et al., ’86) it is not used here to demarcate early fiber types or muscle cell generations.

RESULTS

Cranial muscle groups

Cranial muscles may be categorized into developmental groups by their branchial arch origin and innervation, as done by McClearn and Noden (’88), or categorized into functional groups. In the presentation below the following six groups, reflecting a combination of developmental origin and function, will be described: 1) first arch muscles, largely jaw closing muscles, innervated by the trigeminal nerve (cranial nerve V); 2) second arch muscles, for the most part the facial muscles, innervated by the facial nerve (cranial nerve VII); 3) tongue muscles, which are innervated by the hypoglossal nerve (cranial nerve XII); 4) pharyngeal muscles, largely those considered to be derived from arches 4–6 and primarily innervated by the vagus nerve (cranial nerve X), but also by the muscles derived from the third arch and innervated by the glossopharyngeal nerve (cranial nerve IX); 5) laryngeal muscles, also derived from arches 4–6 and innervated by the vagus nerve; and 6) ocular muscles, derived from anterior somites and innervated by cranial nerves III, IV, and VI. In addition, there are brief comments on other muscles of the cervical and shoulder region.

First arch muscles

The first arch muscles of Monodelphis domestica include the external jaw adductor muscles, specifically the temporalis and the deep and superficial masseter and the pterygoideus muscles as well as a number of additional muscles. They are quite similar in form to the muscles in Didelphis marsupialis as described by Hiiemae and Jenkins (’69). The temporalis originates from the alisphenoid and parietal bones and the temporalis fascia. The deep masseter (termed the zygomatico-mandibularis muscle by some authors) is morphologically continuous with portions of the temporalis but is distinguished by its origin from the zygomatic arch. The superficial mas- seter is quite distinct from other portions of the adductor mass, with a clear anterior-posterior fiber course. It originates on the maxillary bone at the base of the zygomatic arch, and inserts on the angle of the dentary.
Morphological continuity exists between all adductor muscles, as some portions of the deep masseter fuse to the superficial masseter and others fuse with the temporalis. Additional first arch muscles include the internal (medial) and external (lateral) pterygoideus muscles, the tensor veli palatini and tensor tympani, the mylohyoideus, and the anterior belly of the digastic. All first arch muscles are innervated by the mandibular branch of the trigeminal nerve.

In the earliest available specimen (14E), most first arch muscles are present as condensations of promyoblasts and only a few myoblasts in the first arch region have begun to elongate (Fig. 1). At this time the temporalis and deep masseter form a single complex and are not yet distinguishable. Although the myoblasts of these muscles are slightly elongated and aligned in the general direction of the future muscle there appears to be no significant fusion of myotubes. The cells in these muscles are oriented towards a region of distinct mesenchymal tissue in the area of the future dentary, but there is neither chondrification nor ossification at either end of the muscle. At this time the superficial masseter is recognizable as a distinct condensation, in which the elongating myoblasts are aligned in a more anterior-posterior direction, at a right angle to the other portions of the adductors.

The condensation of myoblasts that will form the internal pterygoideus group is also present by day 14E, but the cells of this muscle group do not yet appear to have begun elongation or alignment. The mylohyoideus is clearly recognizable, with elongated and fused myotubes running between the rami of Meckel's cartilage. Fibers of the mylohyoideus attach to Meckel's cartilage, and this muscle is the only cranial muscle clearly attaching to a skeletal element at this age. The anterior digastric is also recognizable as a distinct condensation of myoblasts, external to the mylohyoideus. In all muscles, including those with elongated and oriented myoblasts, the nuclei are central and striations are absent.

By birth (day 0P) some myoblasts in the region of the adductor muscles have differentiated into muscle cells and exhibit striations under polarized light. Most fibers retain central nuclei. The temporalis and deep portions of the masseter form a continuous mass of fibers running more or less vertically and ending in regions that will be the internal and external surfaces of the dentary. This mass exhibits no division into subunits, because bony elements such as the zygomatic arch do not yet exist. The superficial masseter consists of fibers that run in a horizontal direction, between the maxilla (root of the zygomatic arch) and the posterior region of the dentary. There is no skeletal attachment, because ossification has not yet begun in the angle of the dentary. The external pterygoideus muscle, first apparent at this stage, differentiates from a condensation lying between the temporalis muscle and the internal pterygoideus, dorsal to the inferior alveolar branch of the mandibular branch of the trigeminal (V3). The myoblasts of the external pterygoideus are similar in their differentiation to the temporalis and run posterolaterally, gradually merging into a region of undifferentiated mesenchyme posterior to the dentary. The internal pterygoideus, tensor veli palatini, and tensor tympani form a continuous mass but are distinguished by the beginning of three distinct cell orientations. The cells of the internal pterygoideus are aligned in a mass running from the region of the ala temporalis to a region medial to Meckel's cartilage. Continuous with this mass are cells running medially to the region of the soft palate (tensor veli palatini), and posterior to the region of the differentiating middle ear (tensor tympani). At this stage the external pterygoideus, tensor tympani, and tensor veli palatini are all approximately the same size; the internal pterygoideus is only slightly larger. The mylohyoideus is well differentiated, with striations, and clear skeletal connections (Meckel's cartilage). The anterior digastic lies external to the mylohyoideus; the nuclei remain central.

The first arch muscles at day 1P are similar to those above, but denser, larger, and better differentiated. At this stage the external pterygoideus is a compact mass that terminates into a region of dense mesenchyme surrounding the posterior most extent of the dentary ossification. This region has not yet differentiated into the mandibular condyle. The internal pterygoideus, tensor veli palatini, and tensor tympani are distinct as individual muscles (Fig. 2A). The internal pterygoideus originates near the ossifications of the pterygoid bone, which first appears at this stage. The zygomatic bone also first appears at day 1P and fibers of the deep masseter align towards this bone. Both the temporalis and masseter insert into a distinct mass
Fig. 1. *Monodelphis domestica*. Transverse sections through the head of 14E (one day before birth). A: Overall view of first arch structures. B: Higher magnification of adductor muscles on a section approximately 200 µm anterior to A. Promyoblasts are just beginning to align in first arch region. Note that the palatal shelves have not yet elevated to separate the oral and nasal cavities. AT, ala temporalis; GG, genioglossus muscle; I, infundibulum; IP, internal pterygoideus; IT, intrinsic tongue muscle; MC, Meckel’s cartilage; MH, mylohyoideus; PS, palatal shelves; SM, superficial masseter; T, temporalis; V, trigeminal ganglion; V3, mandibular branch of trigeminal (inferior alveolar and lingual nerves). This and all subsequent sections are paraffin sections cut at 10 µm. Scale bars = 200 µm.
of connective tissue and undifferentiated mesenchyme that will form the coronoid process (Fig. 2B). This tissue contains a number of fibers running from the termination of the muscle into the region surrounding the ossifying dentary that appear to be collagen fibers by their histological stain and birefringence under polarized light (Kier, '92).

By day 3P all cranial muscles are clearly differentiated and striated, nuclei have begun to move laterally in many cells, muscles exhibit the general density of cells seen in more mature individuals, and connective tissue attachments and divisions appear. Further, ossification is proceeding so that many muscles are beginning to achieve skeletal attachments. The relation between skeletal growth and muscular division is reflected in the sub-division of the temporalis and deep masseter muscles. In previous stages this muscle formed a continuous set of fibers running more or less vertically and inserting into connective tissue on either side of the splints of bone lateral to Meckel's cartilage. By day 3P, with the further development of the coronoid process and zygomatic arch, the temporalis and deep masseter differentiate, with the deep masseter establishing skeletal connections with the zygomatic arch and inserting on the lateral surface of the dentary. The nuclei in the majority of cells have moved laterally and striations are present. The pterygoideus muscles, as well as the tensor tympani and tensor veli palatini are similarly developed. The hamulus of the pterygoid bone has appeared by day 3P and the fibers of the tensor veli palatini wrap around this bone (Fig. 3A). The internal pterygoideus inserts on the ossifying angular process of the dentary and the tensor tympani can now be seen as a distinct muscle running towards the differentiating malleus (Fig. 3B). There is no jaw condyle and the external pterygoideus continues to terminate in a region of undifferentiated mesenchyme.

The subsequent development of first arch muscles involves four major features. First, the remainder of the fibers develop into mature muscle cells, with the migration of nuclei into a peripheral (subsarcolemmal) location. Second, the muscles grow in relative size and density and develop internal divisions. Both the lateral migration of nuclei and the development of internal divisions is largely complete by day 6P. For example, the internal pterygoideus has three internal compartments that can be recognized at this time. Third, differential growth of various components produces the differential sizes of muscles seen in adults. For example, at birth all pterygoideus components are similar in size, but by day 6P differential growth has produced a relatively large internal pterygoideus and much smaller external pterygoideus, tensor veli palatini and especially tensor tympani muscles.

The final feature of muscle maturation involves the growth of skeletal connections. There is considerable variability within and among individual muscles in the timing of the establishment of bony connections. For example, the cartilaginous condyle of the jaw joint first appears at about day 7P (although it does not form the definitive jaw joint at this time, Clark and Smith, '93; Filan, '91). The joint capsule, however, does not appear until day 21P. As the condyle differentiates, the insertion of the external pterygoideus shifts from simply ending in the region of undifferentiated mesenchyme posterior to the mandibular ossification to extending into the connective tissues surrounding the secondary cartilage of the condyle. The postdental bones shift from participating in the support of the dentary to forming the middle ear ossicles. During this time the tensor tympani shifts from being a relatively large bundle of muscle fibers, to being a small bundle that inserts on the malleus, because its growth does not keep up with the growth of other cranial components. The bones of the sidewall of the braincase (e.g., the parietal, squamosal, and alisphenoid bones) grow particularly slowly and do not underlie most of the origin of the temporalis until approximately day 19P. The temporalis gains its posterior component as the bones of the cranial vault grow and ossify and the braincase elongates relative to the face.

The angle of the dentary appears on approximately day 4P and at this time the superficial masseter and the internal pterygoideus muscles attach together into a tendon that lies on the ventral margin of the dentary. The attachment of these muscles and the muscles themselves expand with the angular process as it grows posteriorly. Unlike rodents and many other mammals (Beresford, '81; Hall, '83; Herring and Lakars, '81; Moss and Moss-Salentijn, '83; Vilmann, '82) a prominent secondary cartilage does not develop at the angle. The coronoid process grows slowly into the masseter and temporalis muscles between days 3P and day 13P. This growth is
Fig. 2. *Monodelphis domestica*. Transverse sections through the first arch region of the head of day 1P. B is higher power photograph of right side of A. Note that all first arch muscles are distinct at this age. In B the thick region of connective tissue between the temporalis and masseter and the growing dentary can be seen. AT, ala temporalis; CT, connective tissue zone; D, dentary ossification; DM, deep masseter; GG, genioglossus; HG, hyoglossus; EP, external pterygoideus; IP, internal pterygoideus; IT, intrinsic tongue muscle; MC, Meckel’s cartilage; MH, mylohyoideus; SM, superficial masseter; T, temporalis; TT, tensor tympani; TVP, tensor veli palatini; V, trigeminal ganglion; V3, mandibular branch of trigeminal; Z, zygomatic ossification. Scale bar = 200 µm in A; 50 µm in B.
Fig. 3. *Monodelphis domestica*. Transverse sections through the head of 3P showing muscles of pterygoideus complex. B is approximately 340 μm posterior to A; both are at the same magnification. At this time no dentary-squamosal joint exists; note the robust jaw support provided by the incus and malleus (which is at this time still fused with Meckel’s cartilage) against the squamosal bone and otic region. AT, ala temporalis; D, dentary ossification; EP, external pterygoideus; IN, incus; IP, internal pterygoideus; H, hamulus of pterygoid bone; M, malleus; MC, Meckel’s cartilage; MH, mylohyoideus; OT, otic capsule; SQ, squamosal bone; TT, tensor tympani; TVP, tensor veli palatini; V₃, mandibular branch of trigeminal (inferior alveolar nerve). Scale bars = 200 μm.
quite slow and even at day 20P a thick region of undifferentiated mesenchyme separates the muscle and the still growing bone. Figure 4 shows sections through the area of first arch muscles at days 6P, 13P, and 19P to illustrate the maturation of muscles and their skeletal attachments.

Facial muscles

Muscles innervated by the facial nerve include the muscles of facial expression, such as the buccinator, the orbicularis oris and oculi, and several muscles inserting superficially in the nasal and auricular regions, and also the stapedius muscle and the posterior belly of the digastric. The facial muscles develop from the subcutaneous colli sheet of muscles, which in marsupials is divisible into two major sheets, the more superficial platysma and the deeper sphincter colli profundus. The deeper sheet gives rise to many individual facial muscles (Edgeworth, '35; Huber, '30). Because most facial muscles consist of sheets that are at most a few fibers thick, it is often difficult to document their emergence in sectioned material, and it is easier to see the development of facial muscles in the whole mount immunocytochemistry preparations.

Facial muscles are not recognizable in the 14E specimen and are barely visible in sectioned material even at birth (0P). At birth facial muscles are limited to a few slips of muscle in the oral, orbital, and auricular regions and the sphincter colli profundus sheet. This latter muscle does not extend dorsal to the lower border of Meckel's cartilage (Fig. 5A). By day P2, there has been considerable differentiation of facial muscles. The platysma is recognizable (Fig. 2A) and numerous bundles are present in the region of the future orbicularis oculi, buccinator and the maxillo-naso-labialis (Fig. 5B). The emergence of facial muscles, in particular the subdivision of sheets of muscles into distinct bundles and their extension into the anterior nasal region, continues over the next few days. By day 6P facial muscles are recognizable throughout the face even in sectioned material. The muscles around the eye and oral region are quite robust even though both the eyes and lips are still sealed with the peridermal seal and will not open for another 2–3 weeks.

The posterior digastric is present at day 0P and proceeds along a similar developmental course to the anterior belly of the digastric. The stapedius muscle is first distinct at day 3P. Subsequent development of the facial muscles is similar to that of the first arch muscles and reflects differential growth of the various components of the head. However, most facial muscles insert into connective tissues and not onto skeletal elements.

Tongue muscles

The arrangement of intrinsic tongue musculature in mammals is among the most complicated architectural arrangements seen in vertebrates. Bundles of fibers are aligned in three mutually perpendicular planes forming the verticalis, longitudinalis, and transversus muscles (Smith, '92; Smith and Kier, '89). The verticalis and transversus muscles are arranged in alternating sheets, each a few fibers thick, perpendicular to the long axis of the tongue. The extrinsic tongue muscles include the genioglossus (arising from the symphyseal region of the lower jaw), the hyoglossus (originating on the hyoid bone), and the styloglossus (attaching to the styloid process). All of these muscles receive innervation from the hypoglossal nerve, cranial nerve XII.

By 14E the myoblasts in the tongue have differentiated and are beginning to align (Fig. 6A). The fibers in the posterior portion of the tongue appear in advance of those in the anterior portion. At this point a few myoblasts have begun to fuse into myotubes and all orientations of tongue musculature are clearly present. Adjacent cells simultaneously orient into one of three mutually perpendicular planes, so that the three-dimensional arrangement of muscle fibers in the tongue is present at the earliest stages observed. Nuclei are central in the cells and many muscle cells remain globular in shape; no striations are visible (Fig. 6B). The genioglossus, hyoglossus, and geniohyoides muscles are at a similar stage of development to the intrinsic tongue muscles (Fig. 1). By the next day, at birth (0P), the maturation of myofibrils in the intrinsic tongue musculature is consider-

Fig. 4. Monodelphis domestica. Transverse sections through the first arch region of A, 6P; B, 13P; and C, 19P. Upper and lower arrowheads in each photograph represent the ventral-most extent of the ossification of the parietal and dorsal-most ossification of the alisphenoid bone respectively. Note the general increase in size of the dentary, the spread of the ossification in the cranial bones and the increase in the density of musculature. AT, ala temporalis; D, dentary; DM, deep masseter; IP, internal pterygoideus; SM, superficial masseter; T, temporalis muscle; Z, zygomatic ossification. Scale bars = 200 μm.
ably advanced. All intrinsic and extrinsic muscles are distinct: the muscle fibers are striated, nuclei are beginning to move laterally, and the general density of tongue fibers is high (Fig. 7A, B). The extrinsic muscles lag slightly behind the intrinsic muscles in the maturation of the myofibrils. For example, at day 1P, most intrinsic muscles possess lateral nuclei, while extrinsic muscles possess central nuclei (as evidenced by their hollow appearance in Fig. 8). Subsequent development of both extrinsic and intrinsic muscles involves increasing the density of fibers and maturation of myotubes. At all ages the intrinsic tongue muscles appear to be slightly advanced relative to other craniofacial muscles.

Pharyngeal muscles

The pharyngeal muscles in mammals are complicated and variable (Smith, '92 and references therein). In marsupials, there are two major sets of pharyngeal muscles, reflecting a primitive therian pattern (Edgeworth, '35). The first is the stylopharyngeus complex, which develops from muscles of the third arch and attaches to the styloid process (Reichert's cartilage in young individuals). The stylopharyngeus spreads from the styloid process into a broad, fan-shaped muscle extending into the nasopharynx and the upper part of the pharynx. A portion forms a superior constrictor, while other portions of this muscle become the longitudinally oriented stylopharyngeus muscle, which functions as a pharyngeal elevator or dilator. This complex is innervated by the glossopharyngeal nerve (cranial nerve IX). The second set of pharyngeal muscles is the constrictor pharyngeus proper, which forms from the remaining arches, and is innervated by the vagus nerve (cranial nerve X). This mass arises in two portions, one from the hyoid (forming the medial constrictor or hypopharyngeus) and the other from the thyroid and cricoid cartilages (forming the inferior constrictor or laryngopharyngeus) and runs dorsally to meet its opposite in a midline raphe. Marsupials differ from placentals in that there is no levator veli palatini and that the functional superior constrictor is formed by the expanded stylopharyngeus, rather than from the same mass of muscles giving rise to the middle and inferior constrictors (Edgeworth, '35).

The pharyngeal muscles, like the tongue muscles, appear as distinct masses and are well differentiated in the earliest available specimen (14E). For example, in the region of the larynx it can be seen that even before the cartilages of the larynx and hyoid apparatus (including the styloid process) have differentiated, myoblasts are aligned in distinct bundles representing the stylopharyngeus complex (Fig. 9). Again, like the tongue, the pharyngeal muscles develop rapidly. At day 0P the broad fan formed by the stylopharyngeus is apparent (Fig. 10), and all muscles are distinct. By days 4-6P muscles are indistinguishable from the adult condition (Fig. 11).

Laryngeal muscles

The laryngeal muscles are relatively simple, consisting of the dilator of the larynx and two constrictors, which control the laryngeal opening. In the earliest specimen available (14E) these muscles are not yet distinct and it is difficult to distinguish condensing muscle masses from the condensations of the cartilaginous skeleton (Fig. 9). However, by day 0P the intrinsic muscles of the larynx have differentiated, fused, and are striated. The cartilages are well developed and the epiglottis lies above the palate, a general mammalian trait. Like the pharyngeal muscles, the muscles of the larynx mature rapidly (Figs. 10, 11).

Ocular muscles

Muscles that move the eye include the four rectus and two oblique muscles. In Monodelphis domestica, the ocular muscles are not distinguishable as clear condensations in the 14E specimen, but are beginning alignment by day 0P. Figure 12 shows the eye muscle and temporalis muscle of a day 1P specimen. The superior and inferior rectus muscles are distinct, with aligned and striated myotubes. These muscles do not appear to be as ad-

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Fig. 5. Monodelphis domestica. Whole mount immunocytochemistry of superficial muscles of the facial and throat regions illustrating the differentiation and spread of this musculature during the first week of life. A: 0P, lateral view. B: 0P, ventral view. C: 2P. D: 6P. The eye is visible due to pigmentation in the retina. Note that the first facial muscles to appear are ventral and that those on the dorsal regions of the face are the last to emerge. The form of the adductor musculature is also of interest, as it can be seen in this view that these muscles arise as a single fan of fibers, which spiral to produce the final fiber directions. This spiral is present before any skeletal attachments emerge. AD, anterior digastric; DM, deep masseter; F, frontalis muscles; GG, genioglossus; GH, geniohyoideus; MH, mylohyoideus; NL, nasal and labial muscles; OC, ocular muscles; P, platysma; PAO, posterior auricular and occipital muscles; PD, posterior digastric; SC, sphincter colli; SM, superficial masseter; T, temporalis muscle. Scale bars = 1 mm.
Fig. 6. *Monodelphis domestica*. Transverse sections through the head of 14E showing precocious development of tongue muscles (this is the same specimen illustrated in Fig. 1). A and B are of the same section; B is a magnification of the central tongue region. Note that although many myoblasts remain globular and apparently undifferentiated, alignment of the three intrinsic muscle groups has already started. IT, intrinsic tongue muscles; LTM, longitudinalis tongue muscle; MX, maxillary ossification; PS, palatal shelf; TTM, transversus tongue muscle; VTM, verticalis tongue muscle. Scale bar in A = 200 μm; in B 50 μm.
Fig. 7. *Monodelphis domestica*. Horizontal section through the tongue of day 0P. B is a higher power photograph of A and is from the same section shown in Figure 10B (see Fig. 10A for section plane). Note clusters of myofibers each with multiple myofibrils. Although in many fibers the nuclei are lateral (as indicated by the presence of hollow cores), in some cells nuclei have moved to a lateral position and striations can be seen in transverse and longitudinal fibers. The label “LTM” is in the same position in both magnifications for orientation. LTM, longitudinalis tongue muscle; MC, Meckel’s cartilage; TTM, transversus tongue muscle; VTM, verticalis tongue muscle. Scale bar in A = 200 μm; in B 50 μm.
Fig. 8. *Monodelphis domestica*. Transverse section through extrinsic tongue muscles (plus others) of 1P. This is the same section as is illustrated in Figure 2; see that figure for orientation. Note the presence of very thin facial muscles and also the fact that virtually all muscle cells appear to have hollow cores in cross-section, indicating the presence of central nuclei. At this time most intrinsic tongue muscle cells are mature with lateral nuclei. AD, anterior digastric; GG, genioglossus; GH, geniohyoideus; HG, hyoglossus; MH, mylohyoideus; P, platysma; SC, sphincter colli. Scale bar = 100 μm.

Vanced as the temporalis muscle, as the myotubes are not as elongate and more nuclei appear to be central. However, as in the other craniofacial muscles, maturation of the ocular muscles occurs in the next 3–4 days.

**Other muscles**

In addition to the above cranial muscles, a number of additional muscles are of interest. These include muscles in the cervical region such as the sternomastoid and trapezius and large pre- and post-vertebral muscle bundles. These muscles are important in supporting the head and neck during the journey from the teat, and follow a similar time course to the muscles described above. They are, like the tongue and pharyngeal muscles, differentiated at birth, with elongated, striated myotubes. A particularly large prevertebral bundle, which runs from the occipital region to the cervical region, is present at birth (Fig. 10A). The largest muscle in the body at birth appears to be the pectoralis muscle, which like the tongue, is organized the day before birth and is well developed at birth.

**Summary of developmental timetable**

All craniofacial muscle groups are recognizable at birth, with the exception of some components of the facial muscles. Craniofacial muscles may be divided into three groups that exhibit slight differences in timing of maturation: the tongue, mylohyoideus, pharyngeal muscles, and some muscles of the neck and shoulder are the first to pass through the stages of elongation and striation; the first arch muscles and the majority of other craniofacial muscles follow these muscles by about a half day, and the facial and ocular muscles follow by another day. Fibers in the first group appear to be completely differentiated just before birth; the
second group are differentiated at birth. However, once differentiation begins, the rate of development is rapid in all the craniofacial muscles, with all muscles passing from the first appearance of condensations, to recognizable masses with mature myotubes (striated with lateral, multiple nuclei) in approximately 3–4 days.

**DISCUSSION**

*Regional specialization in cranial muscle development*

As emphasized earlier, cranial development in *Monodelphis domestica*, as well as other marsupials, is characterized by regional heterochrony (when a eutherian condition is taken for comparison; see Clark and Smith '93). In marsupials the oral region matures precociously, while structures associated with the neurocranium are extended in their period of development. The central nervous system, in particular the forebrain, is extended in its development in *M. domestica* (Saunders et al., '89), as well as in other marsupials such as the wallaby, *Macropus eugenii* (Nelson, '88; Renfree et al., '82; Reynolds and Saunders, '88) and the marsupial carnivore, *Dasyurus hallucatus* (Nelson, '92). In marsupials not only is most neurogenesis postnatal, but relative to eutherians of equivalent size it appears exceedingly slow. Correlated with this (and probably functionally related, e.g., Enlow, '68; Hall, '87; Herring, '93; Moss, '68; Moss and Salentijn, '69; Moss and Young, '60; Schowing, '68; Young, '59) is the exceedingly slow growth of the bony neurocranium. In *M. domestica* the bones of the skull continue to begin ossification over 2 weeks after birth (in *Macropus eugenii*, this is over a month after birth) and do not enclose the skull until 3–4 weeks after birth. The bones that ossify last are those surrounding the neurocranium. In mice, which are similar in size to *M. domestica*, the same events occur entirely prenatally, over a 4 to 6 day period, with no regional specialization.
Fig. 10. *Monodelphis domestica*. Laryngeal and pharyngeal muscles in OP. A: sagittal section; B: horizontal section. In B, anterior is at the top of the photo; the line in A shows approximate site of the section in B (B is the same section illustrated in Fig. 7). Note the development of muscle, in particular the fan-like stylopharyngeus and the very large prevertebral muscle. E, epiglottis; HY, portion of hyoid; NP, nasopharynx; O, oral cavity; PAL, soft palate; PV, prevertebral muscles; RC, Reicherts cartilage; SP, stylopharyngeus. Scale bar in A = 800 μm; in B 200 μm.
Muscular system development in *Monodelphis domestica* is in distinct contrast to the skeletal and central nervous systems. First, the entire muscular system appears accelerated as a whole relative to these systems. Myogenesis is well underway at a time when the central nervous system, in particular the forebrain, and the skeletal system are just beginning differentiation. Most major events of muscle differentiation occur in approximately 4–6 days (examination of prenatal specimens earlier than those available in the current study would be necessary to pinpoint the exact period) and there is no evidence of an accelerated or extended period of myogenesis of any particular muscle group. Finally, in marsupials the peripheral nervous system seems to be accelerated along with muscle development. In *M. domestica* and other marsupials, peripheral motor nerves and also sensory nerves such as the trigeminal are well developed at birth. It appears that in *M. domestica* the most significant differences in rate of neurogenesis involve the telencephalon and not structures of the hindbrain or peripheral nervous system.

The one muscle that is most often noted as being accelerated in development in marsupials is the tongue, and this is often assumed to be an adaptation for suckling. Most reports of cranial muscle development in eutherian mammals also note that the tongue is one of the earliest muscular elements to begin differentiation. Early development of the tongue has been noted in the mouse by Holt ('75) and in the rat by Rayne and Crawford ('71).
Fig. 12. *Monodelphis domestica*. Transverse section through the ocular muscles of 1P. Note the differentiation of ocular muscles and the fact that although they are at a less developed stage than the temporalis muscle, striations are present in these rectus muscles when they are viewed in polarized light. Eyes in *M. domestica* will not open until day 35P. IR, inferior rectus muscle; PB, palatine bone; SR, superior rectus muscle; T, temporalis. Scale bar = 100 μm.

In humans, tongue movements and the swallowing reflex are among the earliest muscle activities present, appearing during the tenth week of embryonic development (Doty, '68). Whether this early development in eutherians reflects phylogenetic history, or is retained because of functional constraints (tongue movements and palatal closure may be linked, Holt, '75) remains unexplored.

Thus, although muscles as a group appear early in development, there is little evidence of regional specialization or heterochrony within the craniofacial muscles of marsupials. A few muscles appear slightly accelerated in their development—the tongue and mylohyoid—while a few are somewhat slower in development—the ocular and facial muscles. However, the differences between these muscles are slight and similar differences among muscles in timing of development have been observed in both the quail and in eutherian mammals. In many cases the same muscles are early in development (i.e., the tongue) and late in development (i.e., facial muscles) in both eutherians and metatherians, and thus the patterns of timing differences are not unique to marsupials.

Previous studies of craniofacial muscle development

The pattern observed in *Monodelphis domestica* in the present study can be compared to the pattern described for the quail by McClearn and Noden ('88), who present the most detailed study of emergence of the form of craniofacial muscles in an amniote. In both taxa muscle groups begin myogenesis together in a narrow time period. In *Monodelphis domestica* the premuscle condensations are present on day 14E, and at birth, 1 day later, myotubes are present in virtually all muscles. By day 3 the form and fiber orientation of all craniofacial muscles are distinct. As in the quail, this early alignment is inde-
pended of the appearance of skeletal elements, and as noted by McClearn and Noden there is slight variation in the rate of development of individual muscles both within and between major groups of muscle. In the quail, although fiber orientations were established before any skeletal elements had appeared, subsequent muscle development for the most part was associated with skeletal elements. In *M. domestica* there appears to be no particular concentration of myogenesis around regions of osteogenesis. In some muscles growth is accompanied by skeletal growth, whereas in others, skeletal growth lags far behind muscle development. Muscle by muscle comparisons between *M. domestica* and the quail are difficult because, with the exception of the first arch muscles it is difficult to establish homologies between an avian (or reptilian) pattern and the mammalian condition (Smith, '92).

A few detailed studies exist on craniofacial muscle development in mammals (e.g., Gasser, '67; Reuter, 1897; Spyropoulos, '77). Rayne and Crawford ('71, '75) describe the development of four first-arch muscles, the masseter, temporalis, and the internal and external pterygoideus in the rat. Premuscle condensations appear at approximately day 14E. By day 15E, the muscle anlagen are characterized by a closely packed condensation of cells, many of which are fusiform and some of which are striated, and by day 16E all muscles are distinguishable. At birth (about 4 days later) the topography of muscles was "scarcely different" from the adult, and the origin and insertion areas of all muscles (except for portions of the internal pterygoideus) had for the most part ossified (Rayne and Crawford, '71). The absolute time course of development of craniofacial muscles in *Monodelphis domestica* and the rat is therefore similar. The 15E rat is roughly equivalent to the 14E *M. domestica* in both the state of muscle development as well as the general level of cranial development (e.g., the state of cranial cartilages and ossifications and the lack of a closed secondary palate). In both the rat and *M. domestica*, muscle histogenesis and morphogenesis proceeds relatively rapidly over the next five days, so that by day 4P in *M. domestica* and birth in the rat, most muscles contain a large number of striated muscle fibers that are aligned, subdivided, and resemble the adult condition.

Although the absolute time course of muscle development in *Monodelphis domestica* and the rat is similar, two important differences exist in the context in which this development occurs. First, the rate of development of other systems, in particular the central nervous system and the cranial skeletal system, is quite different in the two taxa. It takes *M. domestica* close to 3 weeks postnatal to reach the level of cranial skeletal or central nervous system development seen in the newborn rat or mouse (Clark and Smith, '93; Saunders et al., '89, personal observation). This means that muscle development is coincident with the ossification of skeletal attachments in the rat and other murid rodents such as mice, but in *M. domestica* they are far in advance of most skeletal development. Second, these events are embryonic in the rat and most other eutherians, but are postnatal in *M. domestica*. In *M. domestica* processes that are almost exclusively in utero in placentals—myoblast differentiation, and the formation of primary and secondary myotubes—are postnatal events (see also Bridge and Allbrook, '70). Although it is true that intrauterine mouth movements occur in eutherians, in marsupials, the oral muscles must be active and therefore are presumably subject to functional demands throughout the most critical period in myogenesis in a manner that is quite different from eutherian mammals.

Because all muscles develop together in marsupials, but bones demonstrate significant patterns of acceleration and deceleration, it means that some muscles are accelerated in development relative to their skeletal attachments. In contrast, in eutherians, and especially murid rodents, for which most information exists, bones and muscles develop during the same time period. The difference is particularly notable in the first arch muscles, as this group possesses the most distinct skeletal attachments (most facial, tongue and pharyngeal muscles attach only to cartilage or connective tissues) and spans the regions exhibiting skeletal acceleration and deceleration. The muscles or portions of muscles attaching to the dentary, maxilla and zygomatic arch are attached to bone relatively early in their development, while the portions of muscles inserting onto bones of the neurocranium develop late attachments. For example, the area of attachment of the temporalis muscle does not fully ossify until approximately 19 days after birth. This is a week after the young have begun to detach from and reattach to the teat.
One further difference is that unlike most mammals, Monodelphis domestica lacks a secondary cartilage at the angle of the jaw. Secondary cartilages are reported to grow in response to external stress such as muscle forces (Beresford, '81; Hall, '83; Herring and Lakars, '81; Moss and Moss-Salentijn, '83; Vilmann, '82), and it is interesting that a distinct secondary cartilage is not present at the angle in M. domestica, as virtually all growth of the dentary occurs after muscles have differentiated and are clearly functional.

The most important conclusion of these results is that although the muscular system has been shown to have significant effect on skeletal system development, presumably through mechanical effects, there is no necessary relation. Despite the fact that muscles insert and are functional and are therefore almost certainly exerting tensile forces on the neurocranium of Monodelphis domestica, cranial ossification is slow and still appears to be determined by other processes, most likely central nervous system differentiation and growth. This study thus supports numerous previous suggestions that there is a hierarchy of epigenetic effects determining cranial form (e.g., Bjork, '72; Enlow, '68; Hall, '87; Herring, '93; Moss, '68; Moss and Young, '60).

Muscle fiber alignment

One aspect of the morphology of cranial muscles is that they possess great spatial complexity. The mammalian tongue is of particular interest in this context because it is one of the most complex muscles—in its anatomy, function and development—in the vertebrate body. Any general model of muscle morphogenesis and alignment must be able to generate the internal pattern of muscle fibers, which run in three mutually perpendicular planes. At this time no firm model of the mechanisms determining the direction of myoblast alignment, elongation, and fusion exists, but, it has been suggested that alignment of myoblasts is mediated by molecular signals, including laminin (Ocalan et al., '88), fibronectin (Chiquet et al., '81) and collagens (e.g., Linsenmayer, et al., '73; Shellswell et al., '80). In addition, mechanical forces, perhaps mediated by such molecular gradients, have been proposed as being responsible for establishing the local environment in which myoblasts orient and align (Harris, '84, '87; Vandenburgh, '82). Bogusch ('86) has emphasized the role of fibroblasts in muscle alignment. Most of these hypotheses rely on spatial gradients, either mechanical or molecular to align myoblasts. For example, the mechanical model presented by Harris ('84, '87) or Vandenburgh ('82) presupposes a line of tension that could easily account for the alignment of the fibers in the transversus muscle, but could not account for the fact that cells immediately adjacent to these fibers simultaneously align either vertically or longitudinally, at right angles to the mechanical gradient producing alignment of the transversus fibers (Figs. 6, 7). Nor have the discussions of molecular signals accounted for this complex, three-dimensional arrangement in the tongue. Unlike other previously studied systems, it appears that either very small scale local environments or some intrinsic programming that causes individual premyoblast cells to respond differentially to external processes must be hypothesized in a muscle like the tongue.

Summary and conclusions

Several major conclusions can be drawn from the data presented in this study. First, and most specifically, while the skeletal system shows a distinct pattern of development in marsupials (Clark and Smith, '93), reflecting responses to the competing demands of neural growth and the requirements of a functional feeding system at an embryonic state of development, such a pattern is less distinct in the muscular system. All craniofacial muscles develop more or less in synchrony, as in eutherian mammals, and all muscles develop early relative to the central nervous system or skeletal system. It is unlikely that the synchronous development of muscles is simply in response to accelerated functional demands, as the ocular muscles, which are not functional until several weeks after birth, follow the same time course as other muscles.

Second, the relative timing of the development of skeletal and muscular tissues is different in Monodelphis domestica from that reported in eutherians. Most studies of craniofacial development in mammals have concentrated on murid rodents in which the significant events of skeletal and muscular development occurs simultaneously. The current study of marsupials demonstrates that muscular and skeletal development may be uncoupled, i.e., need not occur simultaneously. Because of this, marsupials may provide a useful model system in which to study the necessary, rather than possibly coinciden-
tial, relations between muscular and skeletal development.

Third, craniofacial muscles, and the tongue in particular, present specific conditions of internal morphology that are not yet explained by hypotheses on the mechanisms of muscle cell alignment and morphogenesis. These muscles may provide fruitful models for further study.

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LITERATURE CITED


