# Integration of Craniofacial Structures During Development in Mammals[1]

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SYNOPSIS. The integration of craniofacial elements during development in mammals is studied in a phylogenetic approach. Developmental series of four metatherian (marsupial) and five Eutherian (placental) taxa are examined, and the sequence of emergence of major characters of the central nervous system, the cranial skeleton and the cranial musculature is reconstructed. These sequences are transformed into a series of characters that are then mapped onto the phylogeny of the taxa. This phylogenetic approach makes it possible to distinguish between characters that are uniform across all mammals, and those that differentiate marsupials and placentals. The most significant difference between the two groups involves the relative timing of the development of the central nervous system and the somatic system. The central nervous system differentiates far in advance of the bones and muscles of the head in eutherians. In metatherians, somatic elements, particularly of the face, are accelerated. Additional other differences between the two groups of mammals are discussed. Many elements, however, are unchanged, and develop in a consistent relation despite overall shifts in development. These data are used to test several hypotheses on the ways that cranial development in mammals is integrated.

## INTRODUCTION

Integration, defined here as the association of elements through a set of causal mechanisms so that change in one element is reflected by change in another, has received significant recent attention. Commonly integration is studied through the statistical correlation of traits or growth, often combined with assessment of genetic correlation (*e.g.*, Atchley *et al.*, 1981; Cheverud, 1982; Cheverud, 1995; Olson and Miller, 1958; Zelditch, 1988; Zelditch and Carmichael, 1989). Developmental integration arises out of the association of events by morphogenetic processes such as regulatory genes, system-wide growth factors or hormones, or epigenetic interactions. In this study I explore developmental integration using a historical approach. My approach to the analysis of integration takes a

lead from Liem and Wake (1985) who advocate a historical analysis of functional integration to ask: "what happens to structurally adjacent elements when one of the components changes functionally and/or structurally? Can a level of functional integration be recognized that correlates with the rigid maintenance of character complex identity despite the change of adjacent elements?" (p. 371). Here I ask two analogous questions about historical patterns of developmental integration. First, what happens to developmentally adjacent elements, when one of the components changes its developmental rate, sequence or timing? Second, can patterns of developmental integration be recognized by documenting a rigid maintenance of developmental sequences or timing patterns despite changes in the development of adjacent elements?

In this study I compare craniofacial development in metatherian (marsupial) and eutherian (placental) mammals. These taxa are characterized by significantly different reproductive strategies (*e.g.*, Hayssen *et al.*,

1985; Kirsch, 1977a,b; Lee and Cockburn, 1985; Lillegraven, 1975; Lillegraven et al., 1987; Renfree, 1993; Tyndale-Biscoe and Renfree, 1987), which have led to significantly different developmental patterns (see Hall and Hughes, 1987; Hughes and Hall, 1988; Tyndale-Biscoe and Janssens, 1988 for recent reviews). Marsupials are born soon after the onset of organogenesis and most differentiation of tissues and structures occurs postnatally. In placentals, most organogenesis is intrauterine so that at birth most systems are well differentiated. Postnatal development of placental mammals largely consists of growth. As a result, in these two taxa the period that may be termed "embryonic"—that in which most organogenesis takes place—occurs in very different contexts and under very different functional constraints. At the time when basic processes of craniofacial morphogenesis are occurring, the marsupial young must be functionally independent, and cranial structures must at least be capable of holding the teat, suckling, swallowing and breathing. These functional requirements have led to different patterns of timing and sequence of developmental events in marsupial and placental mammals. However, both patterns of development lead to similar adult morphology.

Previous studies on the relative patterns of development in marsupials and placentals have focused on the functional or evolutionary significance of the differences for marsupials (e.g., Clark and Smith, 1993; Filan, 1991; Hall and Hughes, 1987; Hill and Hill, 1955; Hughes and Hall, 1988; Maier, 1993; Müller, 1968a,b; Smith, 1994). Here, rather than focus on the adaptations of either taxon, I use data on the different patterns of development to illuminate the way the head is constructed in mammals during development. The goal of this paper is to document which elements develop independently or differently in the two sets of taxa and which elements develop in a consistent relation to other elements across taxa. In other words, this paper attempts to use phylogenetic information to distinguish between developmental plasticity and developmental integration.

## MATERIALS AND METHODS

In the course of this study relatively complete developmental series of five eutherians, Mus musculus (Rodentia), Felis domestica (Carnivora), Sus scrofa (Artiodactyla), Manis javanica (Pholidota), and Tupaia javanica (Scandentia), and four metatherians, Monodelphis domestica (Didelphidae), Macropus eugenii (Macropodidae), Dasyurus quoll (Dasyuridae), and Perameles nasuta (Peramelidae), were examined. The collections of Mus, Sus, Monodelphis and Macropus were prepared in my laboratory (see Clark and Smith, 1993 and Smith, 1994 for details on preparation techniques, and on the specimens). The series of Felis was examined at the Cornell College of Veterinary Medicine comparative embryological collection (Ithaca, New York), and Manis, Tupaia, Dasyurus and Peremeles were examined at the comparative embryo collections at the Hubrecht Laboratory of the International Embryological Institute (Utrecht, The Netherlands). All specimens were serially sectioned embryos, originally embedded in paraffin and stained with common histological stains.

Each specimen was examined slide by slide to determine the state of a number of characters of the skeletal, muscular and central nervous systems. From these data, the sequence in which 28 specific events (Table 1) occurred was reconstructed. This sequence was then used to construct a matrix in which the timing of each of the 28 events relative to every other event was expressed. This produced a total of 378 characters. Three character states were defined: state 0 indicates that a given event occurred before a second event; state 1 indicates that the given event occurred in the same stage as the second; state 2 indicates that the given event occurred after the second event (this procedure is similar to ones independently developed by Mabee (personal communication) and Velhagen (1995). These characters were then entered into the data matrix in the MacClade computer program (Maddison and Maddison, 1992), and plotted onto independently determined phylogenies for both metatherians and eutherians (Marshall et al., 1989; Novacek, 1990; Sza-

TABLE 1. *Characters examined.*

| | |
|---|---|
| First ossification of | Dentary |
| | Premaxillary |
| | Maxillary |
| | Frontal |
| | Jugal |
| | Parietal |
| | Squamosal |
| | Alisphenoid |
| | Basioccipital |
| | Basisphenoid |
| | Exoccipital |
| | Periotic |
| Other skeletal structures | Dermal bones approach midline |
| | Cartilage in basioccipital region |
| | Closure of secondary palate |
| | First appearance of tooth buds |
| | Differentiation of malleus and incus |
| | Differentiation of condylar cartilage |
| | Joint capsule at dentary-squamosal |
| Muscle | First alignment of myoblasts (tongue) |
| | First appearance of striations |
| | Craniofacial muscles distinguishable |
| Central nervous system | Evagination of telencephalon |
| | Differentiation of pigment in retina |
| | Connections between olfactory nerve and epithelium |
| | Layering in cortex |
| | Swelling of thalamus and hypothalamus |
| | Primary lens cells fill lens vesicle |

lay, 1994). The maps were examined character by character to determine the distribution of characters within and between metatherians and eutherians.

The object of this study was to assess differences in developmental sequence among extant eutherian and metatherian mammals. Therefore, taxa were selected so that no higher level phylogenetic bias within either group existed. The eutherians include representatives of orders that are presumed to have separated near the basal radiation of eutherians (Novacek, 1990). Likewise, the four metatherian taxa represent major clades within the metatheria and the last branching is thought to have occurred in the Paleocene (Szalay, 1994).

RESULTS

Of the 378 characters examined in the phylogenetic analysis, 163 were uniformly distributed across taxa; 97 were uniform with 1 or two exceptions; 28 absolutely distinguished metatherians and eutherians, and another 27 distinguished the two groups

with a single reversal. The remaining 78 appeared to be distributed with no obvious pattern (*i.e.*, there were three or more reversals across the two groups). The 46 characters that distinguished the two clades with either no or only one reversal may be considered as distinguishing and are summarized below.

The most striking difference is in the timing of development of the central nervous system (CNS) relative to structures of the somatic system. In eutherians, differentiation of the CNS, led by evagination of the telencephalon, precedes all other events in craniofacial differentiation. In metatherians several characters are accelerated relative to the evagination of the telencephalon, including the onset of ossification of the dentary, premaxillary and maxillary bones, the first alignment of the tongue muscle, and the differentiation of the basicranial cartilage (Fig. 1). The maturation of the CNS in placentals follows this first event rapidly, while CNS development is relatively slower in marsupials. As a result the development
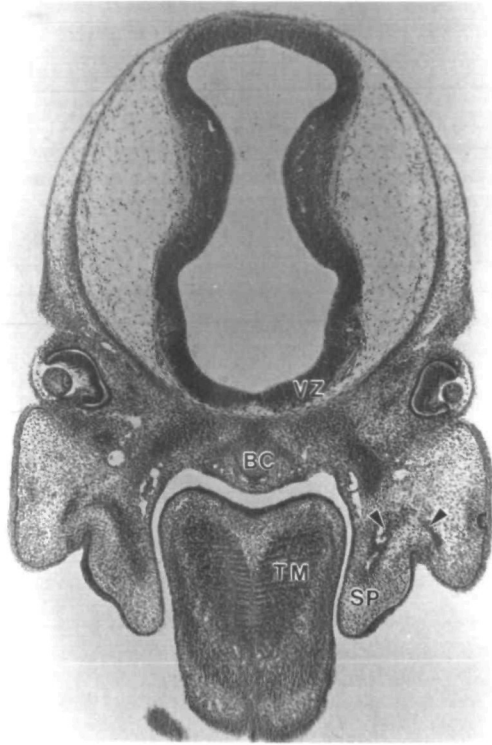
FIG. 1. Photomicrograph of a transverse section through the head of a *Monodelphis domestica* embryo, one half day before birth. Note that the neurons in the central nervous system have just begun to differentiate (the section is through the diencephalon) and as yet there is little differentiation beyond the ventricular zone (VZ). Note also the very early state of differentiation of the eye. In contrast the basicranial cartilage (BC) has begun differentiation, the intrinsic tongue muscles (TM) have started organizing, and the maxillary bone (arrow) has initiated ossification. The secondary palate (SP) has not yet closed.

of many elements of the somatic system in marsupials appears accelerated. For example, in marsupials the ossification of bones such as the frontal and jugal, the differentiation of ear ossicles, the first appearance of striations within muscles, or the closure of the secondary palate, occur early relative to later events in CNS development such as layering in the cortex, the differentiation of the thalamus and hypothalamus or the filling of the lens vesicle by primary lens cells.

Additionally, several patterns of heterochrony appear within the somatic system. For example the following events occur early in marsupials relative to placentals: a)

the ossification of the dentary, maxillary and premaxillary bones relative to the differentiation of the cartilaginous precursors of the ear ossicles or the differentiation of the tooth buds; b) the ossification of the exoccipital relative to most of the bones of the braincase, including the frontal, squamosal, alisphenoid, basioccipital and parietal bones; and c) the closure of the secondary palate relative to the development of striations in, and the full differentiation of, craniofacial muscles.

Although the above patterns are of interest, and will be discussed below, it is important to note that most characters were virtually uniform across these nine taxa. For example, with the exception of the relative timing of ossification of the exoccipital bone, there is little or no variation in the sequence of onset of ossification of bones among the animals studied. However, the rate of ossification of individual bones is different when eutherians are compared to metatherians (see Clark and Smith, 1993). The sequence of differentiation of structures of the nervous system is virtually constant across these taxa, although again, rates differ. In all taxa the sequence of muscle morphogenesis is the same: the tongue is the first muscle to begin differentiation, and all craniofacial muscles follow quickly. Unlike the skeletal or central nervous systems where differences in relative rates differentiate the two groups, in all taxa, the events of muscular development appear to occur rapidly—*i.e.*, to occupy a small and similar proportion of the entire period under consideration (see Smith, 1994 for more detail). The general patterns of the development of these systems in a representative marsupial and placental mammal can be compared in Figure 2.

DISCUSSION

Useful information about developmental integration may be obtained by examining both consistency and change. The characters that are different in eutherians and metatherians may provide insight into which components of craniofacial development are plastic. Characters that distinguish the taxa may reflect specific adaptations of the taxa. In contrast, when characters are uni-
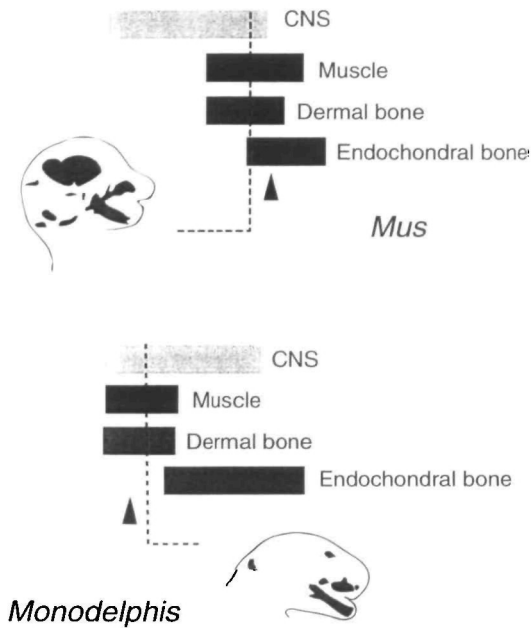
FIG. 2. Overview sketch of cranial development in a eutherian (*Mus musculus*) and a metatherian (*Monodelphis domestica*). The bars represent the relative period during which the events mapped in the phylogenetic study occur (see Table 1). The "other skeletal structures" in Table 1 are not mapped, with the exception of the closure of the secondary palate, which is indicated by an arrow. The drawings of the heads represent tracings of cleared and stained specimens; bone is indicated by shading. The vertical dashed line represents the time at which the sketch of the head is made. Note the fact that CNS development is well in advance of skeletal or muscular development in *Mus*. This is apparent from the time lines as well as from the doming of the head in *Mus* and the flatness of the head in *M. domestica*. Even though both head sketches are made at a time approximately half-way through the period in which bones begin ossification, it is apparent that the dermal bones, in particular of the braincase, have ossified much more extensively in *Mus* than in *Monodelphis*.

form, despite changes in other elements, it is possible that this uniformity is due to developmental integration or constraint.

The adaptations of the marsupial neonate have been discussed elsewhere (*e.g.*, Clark and Smith, 1993; Filan, 1991; Hall and Hughes, 1987; Hill and Hill, 1955; Hughes and Hall, 1988; Maier, 1993; Müller, 1968*a,b*; Nelson, 1992; Smith, 1994; Tyndale-Biscoe and Janssens, 1988 and references therein). However, most previous studies were based on observations of a sin-

gle or relatively few taxa and it has been difficult to place these specializations in a more comprehensive context. The current phylogenetic approach provides detail on the precise patterns of relative acceleration or deceleration of individual characters, across a broad range of taxa.[2]

For example, while previous authors have stated that the tongue develops early in marsupials (Hughes and Hall, 1988), I have found that the tongue is the first cranial muscle to differentiate in both eutherians and metatherians. It is not particularly accelerated in marsupials relative to either other muscles or other facial structures (Smith, 1994). It is reported that the secondary palate closes relatively early in marsupials, but comparative data show that it does not close early relative to the morphogenesis of the cranial skeleton. Instead closure is early relative to certain events of the nervous system and also to some aspects of cranial musculature. The phylogenetic data do show that eutherians and metatherians are consistently distinguished by a heterochronic pattern in the ossification of the exoccipital bone. Finally, the results of the phylogenetic approach confirm the observation that the overriding heterochrony in craniofacial development in the two taxa is the relative timing and rate of CNS differentiation (*e.g.*, Nelson, 1988, 1992; Renfree *et al.*, 1982).

A focus on the differences between one or another taxon provides one interpretive perspective. An alternative perspective may be gained by comparing the patterns of variation and consistency across taxa, and it is this comparison that allows us to understand craniofacial integration. Developmental integration was defined above as a process that associates elements during development by some type of causal mechanism. It was hypothesized that some of

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[2] It is important to note that in this paper acceleration and deceleration have been used in a relative sense (*e.g.*, relative to placentals, character X is accelerated in marsupials, which could as easily be expressed, as character X is decelerated in placentals relative to marsupials). Until the primitive condition is identified through a study of outgroups, the characterization of one or another pattern is arbitrary, and does not indicate polarity or heterochrony in an evolutionary sense.

these mechanisms, particularly epigenetic mechanisms, would be reflected in a spatial or temporal association of events. The comparative data presented here will be used to test specific hypotheses on three types of morphological units that might be expected to be integrated: evolutionarily integrated units, spatially associated units, and units that are all part of a single morphological system.

The first arch complex in mammals is an example of a possible evolutionarily integrated system. During mammalian evolution a suite of characters appear to have been transformed in parallel in several lineages. This suite includes the reduction of post-dentary bones and their transformation into intracranial ear ossicles; the acquisition of a dentary-squamosal joint; the subdivision and re-alignment of masticatory muscles and their attachment points; and the development of complex dental morphology and occlusion as well as diphyodont tooth replacement (e.g., Allin, 1975; Crompton and Parker, 1978; Hopson and Barghusen, 1986; Rowe, 1988). Alberch (1980) proposed that this evolutionary pattern might be indicative of developmental integration or constraint, and that this developmental constraint, rather than simply parallel adaptation, may have been important in the rapid and parallel evolution of first arch elements in the mammal-like reptiles. This view is echoed by Kay (1986) who, in a study of phenotypic integration in the first arch in mice, argued that the first arch elements appear to form a correlated suite of characters, indicative of underlying integration. She also pointed out that several birth defects and many tetragenic factors appear to affect first arch structures as a suite. These data are thought to provide direct evidence for the integration of first arch structures.

Do the elements of the first arch exhibit patterns of temporal or spatial coordination during development across these groups? The sequence data presented here demonstrate that many significant shifts in timing involve first arch/masticatory system elements. For example, in marsupials the maxillary, premaxillary and dentary bones develop early not only relative to elements of

the CNS, but are shifted relative to other first arch elements such as the dentition and the post-dentary elements (ear ossicles). There is a shift of events such as the closure of the secondary palate relative to the development of advanced muscular characters. Muscle development as a whole is somewhat accelerated in marsupials, and first arch muscles in particular develop well in advance of some of their attachment points (Smith, 1994). The differentiation of the jaw joint is perhaps most interesting in this light, as this is one of the most characteristic mammalian features. In eutherians, although a transitory contact between the Meckel's cartilage-malleus-incus and periotic region is formed, it is brief, and is not functionally significant. The dentary-squamosal joint forms relatively early. In marsupials a robust dual articulation exists for an extended period (Fig. 3), and it is not until the rest of the masticatory system is well developed that the dentary-squamosal joint becomes functional. Whether or not the marsupial condition is primitive (e.g., Maier, 1993) or derived (Filan, 1991), the patterns of development are quite different in marsupial and placental mammals. These data indicate that if integration exists, it is not mediated by processes involving temporal or spatial continuity. Therefore according to the criteria developed here, there is little evidence that developmental integration maintains this particular system.

It is of course possible that these data do not reject the hypothesis of integration, but merely the assumption that developmental integration will be reflected by spatial and/ or temporal association. A number of potential mechanisms might produce developmental integration, for example regulatory genes that influence patterns very early in development. There is compelling evidence that the basic patterning of visceral arches is through genetic mechanisms, and that this patterning takes place very early in development (e.g., Hanken and Thorogood, 1993; Noden, 1991). Integration of this system might lie at a deeper level that would not be reflected by later patterns of spatial or temporal association. Two points are important in this context. First, the differences in the rate and pattern of the development
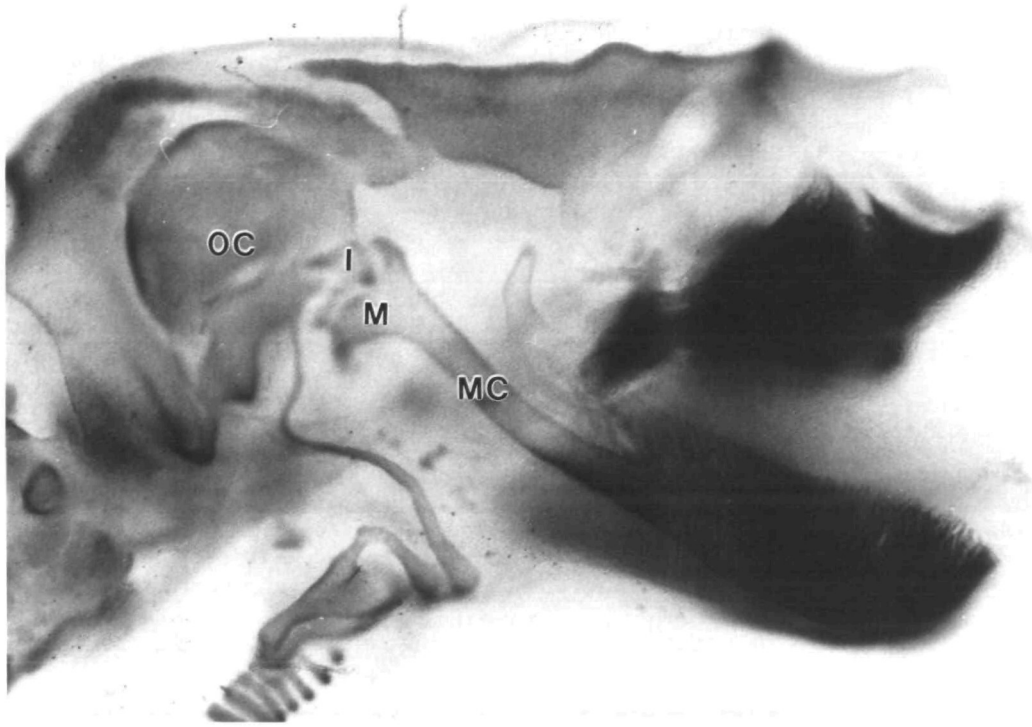
FIG. 3. Photograph of a cleared and stained specimen of a *Macropus eugenii* young, one day after birth. Cartilage appears as light gray; bone is darker. Note the robust jaw articulation formed by Meckel's cartilage (MC), the malleus (M; not yet detached from Meckel's cartilage), the incus (I), and the otic capsule (OC).

of the jaw joint in marsupials and placentals indicates that individual elements within the first arch may be pulled out of a tight developmental pattern and shifted significantly, temporally, spatially and functionally. More importantly, no data exist that suggest that the system of genetic patterning in mammals differs from other tetrapods, or that the first arch differs from other visceral arches. Therefore, it is unlikely that unique patterns of genetic integration exist in the first arch in mammals, as opposed to other arches or other vertebrates that produce particular developmental or evolutionary constraints.

Perhaps the best potential systems for examining developmental integration concern elements that develop in spatial continuity. Such systems are interesting because a wide variety of epigenetic mechanisms—both mechanical and molecular—require spatial and temporal proximity to function (*e.g.*, Hall, 1987; Hanken and Thorogood, 1993;

Herring, 1993*a,b* and references therein). Perhaps the most interesting region in this regard is the braincase. As described above, the most significant heterochrony differentiating marsupial and placental mammals is the timing and rate of CNS growth. The relatively late development of central nervous system tissues in marsupials is accompanied by a relatively slow ossification of the cranial skeleton, especially the dermal bones of the cranial roof. In marsupials these dermal bones are delayed relative to the bones of the face and only begin ossification after there has been significant CNS growth. These results corroborate data from a wide variety of sources suggesting that the bones of the cranium are integrated with CNS differentiation through a number of mechanisms (*e.g.*, Hanken, 1983; Herring, 1993*a,b*; Moss and Salentijin, 1969; Schowing, 1988).

Muscular influences are also well known to have significant impact on cranial ossi-

fication (e.g., Hall and Herring, 1990; Herring, 1993a,b). In eutherians muscular tissue differentiates well after there has been significant growth of central nervous system structures, so that the bones of the braincase in eutherians develop under the influences of both the CNS and the cranial musculature. In metatherians, muscular development occurs well in advance of cranial ossification and CNS differentiation. For example, the temporalis is fully differentiated, striated and presumably functional a few days after birth in *Monodelphis domestica*. However, it is not until 19 days after birth that the major part of the braincase underlying the temporalis and its fascia, the parietal, is ossified in this species. This comparison allows some dissection of the potential dual influences of muscular and CNS growth on braincase ossification. There is consistent association or integration between the CNS and skeleton; integration of skeletal and muscular systems appears to be more plastic.

The exception to the above generalization is the exoccipital bone. This phylogenetic study confirms the hypothesis that the early relative development of this bone is a consistent pattern in marsupials and distinguishes marsupials from placentals (Clark and Smith, 1993). These data demonstrate that the exoccipital bone has achieved developmental independence from the rest of the bones surrounding the cranium. In marsupials its ossification is under the mechanical influence imposed by cervical muscles, which are important in supporting the head during the migration to the teat, and also while the neonate is attached to the teat. These mechanical forces have overridden the dominating effect of the CNS in the ossification of this single bone of the braincase.

The final kind of system that might be integrated is the single morphological system. Do all elements of systems such as the nervous, skeletal or muscular systems, show coordinated change? The data examined here demonstrate that the skeletal system develops as a mosaic, under regional epigenetic influences. On the other hand, comparative data show that the craniofacial muscles develop as a unit, with all muscles

passing rapidly from the first stages (alignment of myoblasts) to the emergence of adult form, rapidly and simultaneously. This pattern of integrated muscle development is discussed in more detail elsewhere (Smith, 1994). The consistent correlation of events within the muscular system, despite changes in the timing and sequence of development of associated structures, suggests that this system is integrated by fundamental regulatory mechanisms.

The major goal of this paper is to explore developmental integration using a historical approach. Historical or phylogenetic analyses of functional integration are now prevalent (e.g., Brooks and McLennon, 1991; Harvey and Pagel, 1991; Lauder, 1981; Liem and Wake, 1985; Wainwright and Reilly, 1994). In the current study, broad hypotheses on developmental integration were presented, and tested with phylogenetic data. Such comparative studies of development are increasingly common (e.g., Alberch and Alberch, 1981; Hanken, 1983, 1992, 1993; Mabee, 1993; Shubin and Alberch, 1986; Velhagen, 1994, 1995). Ultimately an understanding of the relation between development and evolution, or of the networks of developmental integration and possible constraints will require continuing detailed studies of developmental series in a broad comparative context.

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