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Shape, variance and integration during craniogenesis: contrasting marsupial and placental mammals

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Abstract

Studies of morphological integration can provide insight into developmental patterns, even in extinct taxa known only from skeletal remains, thus making them an important tool for studies of evolutionary development. However, interpreting patterns of integration and assessing their significance for organismal evolution requires detailed understanding of the developmental interactions that shape integration and how those interactions change through ontogeny. Thus far, relatively little comparative data have been produced for this important topic, and the data that do exist are overwhelmingly from humans and their close relatives or from laboratory models such as mice. Here, we compare data on shape, variance and integration through postnatal ontogeny for a placental mammal, the least shrew, *Cryptotis parva*, and a marsupial mammal, the gray short-tailed opossum, *Monodelphis domestica*. Cranial variance decreased dramatically from early to late ontogeny in *Cryptotis*, but remained stable through ontogeny in *Monodelphis*, potentially reflecting functional constraints related to the short gestation and early ossification of oral bones in marsupials. Both *Cryptotis* and *Monodelphis* showed significant changes in cranial integration through ontogeny, with a mixture of increased, decreased and stable levels of integration in different cranial regions. Of particular note is that *Monodelphis* showed an unambiguous decrease in integration of the oral region through ontogeny, potentially relating to their early ossification. Selection at different stages of development may have markedly different effects if patterns of integration change substantially through ontogeny. Our results suggest that high integration of the oral region combined with functional constraints for suckling during early postnatal ontogeny may drive the stagnant variance observed in *Monodelphis* and potentially other marsupials.

Introduction

Delineating the relationships among different parts of an organism can provide novel and unpredicted information about the genetic, developmental and functional influences on an organism's morphology. Patterns of phenotypic trait relationships are termed morphological integration, and the relevance of morphological integration to the evolution of shape and diversity has been a topic of considerable interest since the publication of Olson & Miller's groundbreaking book (Olson & Miller, 1958). The bulk of studies on this topic have focused on identifying patterns of integration in model systems, such as the mandible and cranium of laboratory-reared mammalian species, but the diversity of studies has increased markedly in the last decade. Recent comparative analyses in large clades, such as Primates (Ackermann & Cheverud,

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2004; Marroig & Cheverud, 2004; Marroig *et al.*, 2004; de Oliveira *et al.*, 2009) and Carnivora (Goswami, 2006b), and across Mammalia (Hallgrimsson *et al.*, 2004; Goswami, 2006a; Porto *et al.*, 2009) have bridged the microevolutionary and macroevolutionary scales and provided data supporting the relative conservation of patterns of cranial integration, although important differences among taxa remain.

Another aspect of keen interest is the relationship between development and morphological integration (Schoch, 2006; e.g. Hallgrimsson *et al.*, 2009). Patterns of morphological integration can reflect genetic, developmental and functional interactions among traits, but these are ultimately expressed through development (Klingenberg, 2010). For this reason, understanding how integration changes through ontogeny is central to identifying the influences on and evolutionary implications of morphological integration. A comparison between placentals and marsupials will illuminate the ways in which morphological integration is related to evolutionary changes in reproduction and development independent of adult function, because the marsupial mode of reproduction produces highly altricial newborns compared with highly precocial placentals, despite evolutionary convergences in the form and function of adults (Lillegraven, 1975). Many hypotheses exist on the potential effects of birth and weaning on cranial integration, but little work has been conducted outside of humans, other primates and rodents, and not a single marsupial–placental comparison has ever been made. Establishing whether there is a common developmental trajectory to cranial integration or identifying the causes underlying the disparate patterns observed in different groups is an important step in understanding the influence of development on adult morphology and morphological diversity.

Here, we extend the data on cranial development and integration in mammals by describing patterns of cranial shape, variance and integration through postnatal ontogeny for a marsupial opossum, *Monodelphis domestica*, and a placental shrew, *Cryptotis parva*. These data are the first of their kind from a marsupial, thus greatly expanding the breadth of comparative data on the ontogeny of integration, and are also the first of their kind from a placental taxon other than rodent or primate. The selection of these two species presents a contrast in terms of reproductive strategy and rate. *Monodelphis domestica* neonates are born ~15 days after conception and wean at ~50 days (Nowak, 1999). By contrast, *Cryptotis parva* neonates are born after 21 days and wean 18 days later (Nowak, 1999). At birth, only the dentary, maxilla and premaxilla of *Monodelphis* are well ossified, whereas the majority of skull bones are ossified by birth in *Cryptotis*. *Monodelphis* has been established as an important model for biomedical research and belongs to the basal-most clade of living marsupials (Keyte & Smith, 2008). Previous studies presenting craniogenetic data for *Cryptotis parva* did not identify any highly derived or aberrant pattern of ossification sequence (Koyabu *et al.*, 2011) in this group of shrews. There are specializations in the reproductive biology of soricine shrews, to which *Cryptotis* belongs, but this altricial and very small mammal provides a relatively conservative representation of a placental mammal and is an important laboratory model (Mock, 1982).

The data from *Monodelphis* are especially relevant to understanding how cranial integration has evolved because marsupials are born in an extremely altricial state, only a few weeks after conception, and their crania have an immediate function in attaching to the teat for suckling. At birth, most of the skeleton is unossified, except the forelimbs and masticatory apparatus (Smith, 2001). The embryonic newborn crawls or otherwise locates their mother's teat, usually enclosed in a protective pouch, and spends the rest of their development suckling. In contrast, placental mammals spend much longer in the womb, being born at a relatively advanced state with the central nervous system and most of the skeletal system well developed. These differences have been hypothesized to constrain forelimb evolution in marsupials (Sears, 2004) and are reflected in different patterns of phenotypic integration across limbs in the three clades of living mammals (Young & Hallgrimsson, 2005; Bennett & Goswami, 2011a; Kelly & Sears, 2011).

This early functional role of the facial region of the skull in marsupials is a factor that is expected to influence cranial integration, as has been suggested in studies of heterochrony in therian mammals (Smith, 1996), as well as cranial disparity (Bennett & Goswami, 2011b; Goswami *et al.*, 2011). Studies testing for coordination of heterochronic shifts that relate to cranial modules have found no significant relationship (Goswami, 2007b; Goswami *et al.*, 2009), except for the apparent integration of face (Goswami *et al.*, 2009), or more generally the neural crest-derived bones (Koyabu *et al.*, 2011) in the skeletogenesis of eulipotyphlans, the group including shrews, moles and hedgehogs. Concerning the marsupial–placental dichotomy, proposed phenotypic modules and patterns of skeletal heterochrony are strongly correlated in the post-cranium (Goswami *et al.*, 2009). Heterochronic differences in therians, as well as the observed differences in post-cranial developmental modularity, are most clearly expressed when comparing marsupials and placentals. A long series of work has identified a number of heterochronies that characterize marsupials and placentals, as well as subclades within those groups (Smith, 1996, 1997, 2001, 2002, 2006; Sánchez-Villagra, 2002; Sánchez-Villagra *et al.*, 2008; Weisbecker *et al.*, 2008), and these differences are well known to relate to the divergent reproductive strategies of these two clades.

For each of the two species studied here, we analyse morphometric data derived from three early postnatal

stages, chosen based on the presence of multiple ossified cranial bones and representing an approximate doubling in size from the earliest to latest stage. Given the differences in timing of skull bone ossification, weaning time, and developmental rate, *Monodelphis* specimens span a longer range of raw time than *Cryptotis* specimens, but both are similar in terms of change in size along sampled stages and the presence of multiple ossified cranial elements in the first stage sampled. This dataset was used to quantify patterns of shape change, variance and integration through early craniogenesis and to assess the relationship among these aspects of developmental morphology. The comparison between two species with strikingly different reproductive patterns provides a unique system for understanding cranial ontogeny and broader questions on developmental influences on morphological evolution.

Methods

Specimens

Specimens were obtained from breeding colonies of *Cryptotis parva* at Kirksville College of Osteopathic Medi cine and of *Monodelphis domestica* at Duke University. Specimens were cleared and double-stained with alcian blue for cartilage and alizarin red for bone, following standard protocols (Prochel, 2006). After initial study of cleared and double-stained specimens of each species, three stages were selected for further analysis of cranial integration. In both species, the first stage was chosen based on the appearance of ossification centres for several cranial bones. In both species, as has been previously reported (Sánchez-Villagra *et al.*, 2008), the dentary, maxilla and premaxilla were the first bones to ossify and do so quite early, particularly in *Monodelphis*. However, to increase the ability to compare across stages, the first specimens were chosen based on appearance of at least some vault and basicranial bones. The second and third stages were chosen based primarily on body size, with the third stage representing an approximate doubling in size from the first stage, and the second stage representing the temporal midpoint between the other two stages. Because of the difficulty in obtaining and preparing early postnatal specimens while minimizing the age range within any individual stage, specimen numbers were necessarily limited. After discarding outliers (identified after Procrustes superimposition), the following stages and specimen numbers were used in analyses: *Monodelphis domestica* 15-day postnatal (n = 8, mean skull length = 10.8 mm), 30-day postnatal (n = 10, mean skull length = 16.3 mm) and 45-day postnatal (n = 9, mean skull length = 24.9 mm); and *Cryptotis parva* 2-day postnatal (n = 8, mean skull length = 8.3 mm), 5-day postnatal (n = 11, mean skull length = 10.8 mm) and 9-day postnatal (n = 11, mean skull length = 13.3 mm) [Fig. 1].

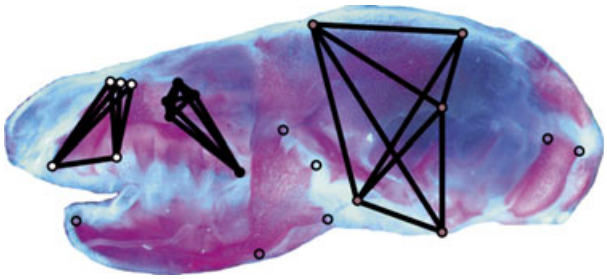

Fig. 1 Landmarks and module associations used in analyses, shown on 5-day-old *Cryptotis parva*. White circles represent landmarks of the oral module, black circles represent the orbit module, and grey circles represent the vault module. Open circles from the mandible and basicranium are used in analyses of shape and variance but are not included in module-specific analyses because of inability to capture enough landmarks in a module due to late ossification of some bones or difficulty in accurately identifying relevant landmarks at early stages of ossification.

Landmarks

Landmarks used in analyses were limited to bones that were present in the first stage for each species. Twenty-two landmarks were selected that were accurately identified in both species and all stages (Table 1, Fig. 1). Because specimens had not yet formed most sutures,

Table 1 Landmarks used in analyses. Numbers refer to Fig. 1. All landmarks were taken from elements on the left side of the cranium. Region refers to cranial regions used in analyses of integration. Because bones are not sutured in most specimens, landmark descriptions refer to extremal points on individual bones, rather than sutures, as used in studies of older specimens.

Number	Landmark description	Region
1	Premaxilla – anterior medial (ventral)	Oral
2	Premaxilla – anterior dorsal	Oral
3	Premaxilla – posterior dorsal	Oral
4	Maxilla – anterior lateral (ventral)	Oral
5	Maxilla – anterior dorsal	Oral
6	Maxilla – posterior dorsal	Orbit
7	Maxilla – posterior lateral (on zygomatic arch)	Orbit
8	Lacrimal – anterior dorsal	Orbit
9	Lacrimal – anterior ventral	Orbit
10	Lacrimal – posterior dorsal	Orbit
11	Squamosal – anterior medial (along vault)	Vault
12	Squamosal – posterior ventral	Vault
13	Parietal – anterior medial	Vault
14	Parietal – posterior medial (dorsal)	Vault
15	Parietal – posterior ventral	Vault
16	Exoccipital – medial dorsal	
17	Exoccipital – lateral dorsal	
18	Dentary – anterior medial	
19	Dentary – posterior dorsal coronoid process	
20	Dentary – posterior lateral articular process	
21	Dentary – posterior angular process	
22	Dentary – ventral intersection of horizontal and vertical rami	

landmarks were placed on the extremities of the growing bones, which likely represent future suture boundaries. 3D morphometric data were collected directly from specimens with a Reflex microscope, which has a reported accuracy of 3 μm in the *x* and *y* directions and 5 μm in the *z* direction. All landmarks were digitized three times and then averaged, with landmarks showing high variance across repetitions removed from further analysis. Specimens were oriented in lateral and dorsal views and fixed into position with insect pins during data collection. These views were then unified with a least squares algorithm in Mathematica 7.0.1 (Wolfram Research Inc., Champaign, IL, USA) using a minimum of seven overlapping landmarks.

All 22 landmarks were used in comparisons of ontogenetic shape and variance. However, because of sample size limitations, landmarks were subdivided into three cranial regions for analyses of integration: oral, orbit and vault, with five landmarks in each of the three regions (Table 1, Fig. 1). Because of the late timing of ossification for some bones or structures, the mandible and basicranial regions were not included in analyses of integration. Similarly, the late development of teeth precluded the gathering of landmarks based on the positions of dentition.

Analyses

Ontogenetic shape and variance

Landmarks were first subjected to generalized Procrustes analysis (Rohlf, 1990) to remove nonshape variation resulting from rotation, translation and size. For analyses in which the three cranial regions were analysed separately, each region was also subjected to separate Procrustes analysis. The resulting Procrustes coordinates were then used to generate covariance–variance matrices.

To first assess the reliability of the datasets, matrix repeatability analyses were conducted in Mathematica 7.0.1 (Wolfram Research), as an assessment of the statistical robustness of the estimated trait correlation patterns (Goswami & Polly, 2010b). Repeatabilities were assessed with a self-correlation approach. Each dataset was resampled 100 times, with sample sizes held constant. Then, a correlation matrix was calculated for each resampled matrix and compared back to the original matrix with matrix correlation analysis. The matrix repeatability measure was the average matrix correlation from 1000 repetitions for the whole skull dataset (22 landmarks), as well as for each of the three cranial regions that were analysed separately (oral, orbit and vault, five landmarks each).

Following the matrix repeatability analyses, the effects of allometry were quantified across different stages, as well as within individual stages, separately for *Monodelphis* and *Cryptotis*. Allometric regressions were conducted on Procrustes coordinates using log centroid size in MorphoJ (Klingenberg, 2008a). Pooled within-group regressions were used for comparisons across stages. A permutation test (10 000 rounds) was used to assess the significance of allometric effects.

Principal components analysis was then conducted on Procrustes coordinates in MorphoJ (Klingenberg, 2008a) to examine changes in ontogenetic morphology for *Cryptotis* and *Monodelphis* together and separately. Variance within each ontogenetic stage for each species was quantified in Mathematica 7.0.1. The significance of differences in variance between different stages within each species was determined with a delta variance permutation test (Boughner *et al.*, 2008). This test generates a null distribution for the difference in variance between two datasets by randomly sampling residuals from the mean shapes across both datasets (with replacement), in this case 5000 times, which can then be compared to the observed value.

Ontogenetic integration

To assess whether there are changes in cranial integration through ontogeny, stage-specific correlation matrices were generated for each cranial region. For these analyses, two different measures of trait correlation were used: the canonical correlation coefficient (Hotelling, 1936) and the congruence coefficient (Burt, 1948) (see review in Goswami & Polly, 2010b). Whereas the canonical correlation coefficient is more widely used, the congruence coefficient, which treats a landmark as a single unit rather than allowing its three coordinates to vary independently, may better represent biological information. As discussed in detail elsewhere (Goswami & Polly, 2010b), this coefficient has been criticized as underestimating covariance in opposite directions (Klingenberg, 2008b), but this only has a significant effect when two landmarks have highly linear patterns of variation at near 90° to each other.

Following generation of both types of correlation matrices for each stage and cranial region, eigenvalue dispersion was quantified. Eigenvalue dispersion provides a useful metric for summarizing the level of integration or modularity in a dataset. Because principal components reflect the covariances among variables, a relatively integrated system will have most of its variance explained by one or a few principal components. In such a case, eigenvalue dispersion will be high, with a few axes having very high eigenvalues and most having very small ones. In a more modular system, more axes will have moderate eigenvalues, and eigenvalue dispersion will be relatively lower (Pavlicev *et al.*, 2009; Goswami & Polly, 2010b). Here, we use the relative standard deviation of the eigenvalues, which is more robust to differences in trait numbers than is eigenvalue variance (Pavlicev *et al.*, 2009), to assess changes in integration within each cranial region across ontogeny.

We further analysed ontogenetic changes in integration using matrix correlation analysis. Correlation matrices for each cranial region, again using the two

different correlation metrics described above, were compared between different stages for each species. The significance of the matrix correlation between each pair of stages was assessed using Mantel's test, a commonly used test that generates a null distribution of matrix correlations by randomly permuting the rows and columns of one of the matrices being compared and then recalculating the matrix correlation several times (in this case, 10 000 times). This distribution is then compared to the observed value to determine whether it is > 95% or 99% of the randomly generated matrix correlations (Mantel, 1967).

Results

Matrix repeatability

Matrix repeatability was assessed across the entire cranium and for each of the three cranial regions (Table 2). For the whole skull dataset (22 landmarks), repeatabilities were quite high and similar in both species and across all stages, ranging from 0.841 (2-day *Cryptotis*) to 0.871 (45-day *Monodelphis*). Within each of the three modules, repeatabilities were unsurprisingly higher due to the fewer numbers of landmarks and ranged from 0.911 (2-day *Cryptotis* vault) to 0.977 (30-day *Monodelphis* orbit). Repeatability is an important measure to consider in analyses as this one, where sample size is necessarily limited by the difficulty in obtaining specimens of a specific early postnatal stage for nonmodel organisms. The high repeatabilities observed, particularly within the individual region datasets, suggest that these sample sizes are sufficient for analysing shape and covariance structure when relatively few landmarks are being assessed.

Allometric effects

As this study focused on ontogenetic patterns in shape and integration, allometric change was an important factor. The ontogenetic series for both datasets represented an approximate doubling in size and thus, unsurprisingly, allometric effects were large and highly significant when comparing across ontogenetic stages. In *Cryptotis*, allometry accounted for 39.68% ($P < 0.0001$) of the total variation across all three stages, whereas for *Monodelphis* it accounted for 58.80% ($P < 0.0001$) of the total variation across all stages. In contrast, allometric effects were not significant in most individual ontogenetic stages, within which there was relatively little size variation. The only stage in which allometry explained a significant proportion of the variation was 30-day *Monodelphis* (25.3%, $P = 0.0018$).

Ontogenetic morphology

Geometric morphometric analyses of Procrustes coordinates for 22 landmarks were conducted separately for *Cryptotis* (Fig. 2) and *Monodelphis* (Fig. 3) to identify major changes in morphology that occur during early postnatal ontogeny in each species.

In *Cryptotis*, the first principal component explained 47.2% of the total variance in the dataset, with PC2 and PC3 explaining a further 12.6% and 7.8%, respectively. Ontogenetic stages were well separated on PC1, but did not form distinct clusters on PC2 (Fig. 2). The two older stages were better separated on PC3 than on PC2. PCs4-8 each explained between 5.4% and 2.5% of the variance, but all stages overlapped extensively on these axes. It is clear from Fig. 2 that the 2-day-old specimens, the youngest stage, showed much greater variance than the later stages, and this was tested explicitly below.

On PC1, ontogenetic changes in *Cryptotis* were arranged from the positive end, dominated by 2-day specimens, to the negative end, dominated by 9-day specimens, and involved the ventral and postero-dorsal expansion of the maxilla, the postero-ventral expansion of the parietal and the posterior expansion of the squamosal. Within the dentary, the major change involved the dorsal expansion of the coronoid and the development of a pronounced articular process.

On PC2, the ontogenetic stages overlapped, but 2-day specimens defined the negative end, whereas 5-day specimens defined the positive end. Nine-day specimens

Table 2 Matrix repeatability (1000 permutations) for entire skull (22 landmarks) and each cranial region (five landmarks each) in *Cryptotis* and *Monodelphis*.

	Whole	Oral	Orbit	Vault
Cryptotis				
2-day	0.841	0.915	0.936	0.911
5-day	0.862	0.959	0.959	0.935
9-day	0.870	0.958	0.926	0.960
Monodelphis				
15-day	0.848	0.967	0.931	0.938
30-day	0.850	0.968	0.977	0.945
45-day	0.871	0.946	0.950	0.952

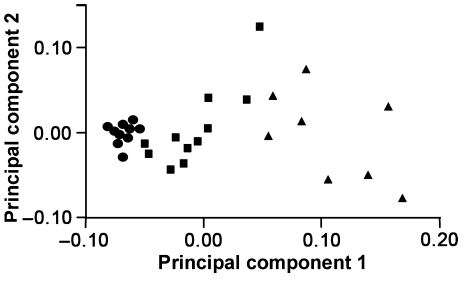

Fig. 2 Principal components analysis of ontogenetic stages for *Cryptotis*, showing distribution of specimens on PC axes 1 and 2. Triangles represent 2-day specimens, squares are 5-day specimens, and circles are 9-day specimens.

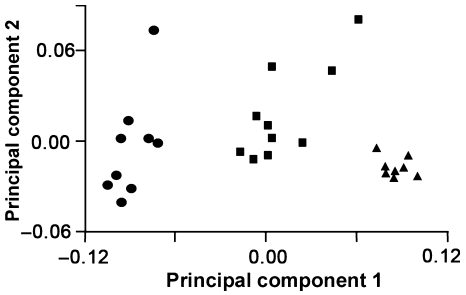

Fig. 3 Principal components analysis of ontogenetic stages for *Monodelphis*, showing distribution of specimens on PC axes 1 and 2. Triangles represent 15-day-old specimens, squares are 30-day-old specimens, and circles are 45-day-old specimens.

were tightly clustered in the middle. Specimens near the negative end displayed a more dorsally expanded parietal and antero-posteriorly shorter squamosal compared with the positive end. On PC3, all stages overlapped extensively. Changes along this axis related to a general flattening of the skull, with posterior expansion of the dorsal part of the parietal, and more distinct and dorsoventrally expanded lacrimal, from the negative end to positive end.

In *Monodelphis*, PC1 explains 60.4% of the total variance and was the only axis to completely separate each stage (Fig. 3). PCs 2 and 3 explained 10.4% and 7.3% of the total variance, respectively. No other axes explained more than 4% of the total variance. PC3 separated the two older stages (30- and 45-day) better than PC2, although there was some overlap between the youngest and oldest stages on PC2.

In moving from the positive end, dominated by 15-day specimens, to the negative end, dominated by 45-day specimens, of PC1, the major shape changes in *Monodelphis* involved an antero-posterior expansion of the maxilla and premaxilla, and general flattening of the skull. PC2 was dominated by the expansion of the angular process of the dentary, and a ventral rotation of the squamosal. PC3 reflected a shift in the lateral inflection of the angular process and lateral rotation of the exoccipital, although, as noted above, changes along with PCs 2 and 3 do not correspond well to ontogenetic stages.

Variance through ontogeny

Variance in *Cryptotis* specimens, measured across the 22 landmark dataset, dropped significantly between the earliest and latest ontogenetic stages (Table 3). Whereas the difference between the 2- and 5-day samples was not significantly different ($P = 0.063$), the differences between the 2- and 9-day samples and the 5- and 9-day samples were both highly significant ($P \ll 0.01$). In contrast, none of the ontogenetic stages of *Monodelphis* differed significantly in variance with each other ($P = 0.38$–0.66, Table 3), suggesting that early cranial ossification in *Monodelphis* may be more constrained than that of *Cryptotis*. Moreover, variance within stages was higher in 2- and 5-day *Cryptotis* samples than in any *Monodelphis* stages. Only the oldest *Cryptotis* stage (9-day), which showed the lowest variance within *Cryptotis*, overlapped in amount of variance with any stage of *Monodelphis*.

Table 3 Variance in cranial shape (diagonal elements) and significance of differences in variance, as determined by a delta variance permutation test (5000 permutations) between pairwise comparisons of ontogenetic stages (off-diagonal elements).

Cryptotis	2-day	5-day	9-day
2-day	0.0122		
5-day	0.052	0.0082	
9-day	< 0.001	< 0.001	0.0031
Monodelphis	15-day	30-day	45-day
15-day	0.0031		
30-day	0.133	0.0044	
45-day	0.954	0.160	0.0032

Ontogenetic integration

Eigenvalue dispersion analysis produced mixed results, both across cranial regions as well as between the two correlation metrics. Within *Cryptotis*, eigenvalue relative standard deviation, calculated using the congruence coefficient, dropped from 2- to 9-day specimens in the oral and orbital regions, suggesting a decrease in integration through ontogeny that has been found in previous studies (Table 4, Fig. 4). The vault region produced an unexpected result of first increasing from 2- to 5-day specimens and then falling back to its original value in the 9-day specimens. The canonical correlation produced markedly different results, with oral and vault integration increasing from 2- to 9-day specimens, whereas orbit integration remained relatively stable.

Table 4 Relative standard deviations of eigenvalues for each stage and cranial region of *Cryptotis*. Results are presented for both the congruence coefficient and the canonical correlation coefficient, as shown in Fig. 4.

	Oral	Orbit	Vault
Congruence			
2-day	0.462	0.493	0.381
5-day	0.366	0.374	0.512
9-day	0.417	0.397	0.400
Canonical			
2-day	0.413	0.517	0.496
5-day	0.417	0.437	0.411
9-day	0.482	0.507	0.565

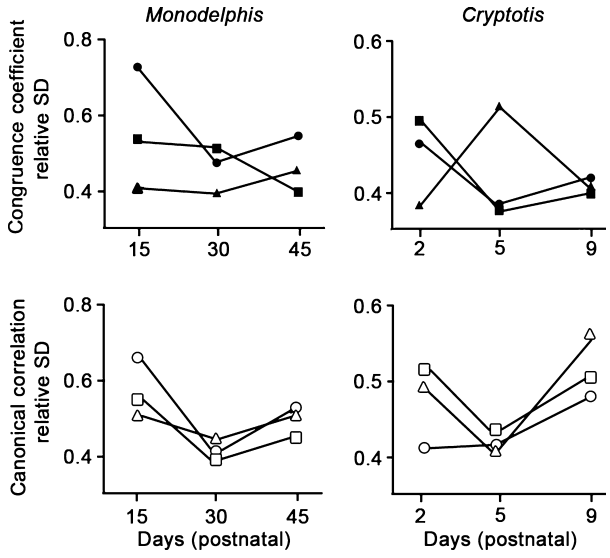

Fig. 4 Relative eigenvalue standard deviations for each stage and cranial region of *Monodelphis* and *Cryptotis*. Results are shown for both the congruence coefficient (closed symbols) and canonical correlation (open symbols). Circles indicate the oral region; squares, the orbit; and triangles, the vault.

Within *Monodelphis*, eigenvalue dispersion calculated using the congruence coefficient again showed a decrease in integration within the oral and orbital regions from the 15-day-old specimens to the 45-day-old specimens (Table 5, Fig. 4). The vault region showed slightly increased integration during the same period. When canonical correlations are used, the oral and orbital regions again show a decrease in integration from 15- to 45-day specimens, whereas the vault region remains stable. In all of the regions, the 30-day specimens displayed the lowest eigenvalue dispersions among the three stages.

As with eigenvalue dispersion, matrix correlation analysis was conducted separately for each of the three cranial regions (oral, orbit, vault). In analyses using the congruence coefficient as the measure of correlation, only a single result, between the oral regions in the 5- and 9-day stages of *Cryptotis*, was significantly correlated at the $P = 0.05$ level (Table 6). None were significant at the $P = 0.01$ level. Analyses conducted using the more traditional canonical correlation metric were generally similar in significance, though not in magnitude. Again only a single correlation was significant among *Cryptotis* stages, again in the oral region, but this time between the 2- and 5-day *Cryptotis* samples ($P = 0.017$).

Among *Monodelphis* stages, no matrix correlations were significant when the congruence coefficient was used (Table 7). When the canonical correlation was used, the oral region showed a significant matrix correlation between the 30- and 45-day stages ($P = 0.008$). Matrix correlations were also significant in the orbital regions between both the 15- and 30-day stages and between the 30- and 45-day stages ($P = 0.008$ and $P = 0.018$, respectively).

Table 5 Relative standard deviations of eigenvalues for each stage and cranial region of *Monodelphis*. Results are presented for both the congruence coefficient and the canonical correlation coefficient, as shown in Fig. 4.

	Oral	Orbit	Vault
Congruence			
15-day	0.729	0.537	0.409
30-day	0.470	0.515	0.394
45-day	0.545	0.400	0.454
Canonical			
15-day	0.666	0.543	0.517
30-day	0.402	0.398	0.436
45-day	0.527	0.449	0.506

Discussion

A series of studies on the ontogenetic dynamics of integration in rats and mice returned the surprising result that cranial integration is repeatedly repatterned during ontogeny (Zelditch, 1988; Zelditch & Carmichael, 1989a,b; Zelditch *et al.*, 2006). More specifically, some data suggest that developmental sources of integration dominate in early ontogeny, whereas later integration more closely reflects functional influences (Zelditch *et al.*, 1992). Studies on humans and gorillas have also found that repatterning is prevalent during ontogeny (Ackermann, 2005; Mitteroecker & Bookstein, 2009), but further studies of *Mus musculus* have found more stability during ontogeny than previously reported (Willmore *et al.*, 2006). The earliest stage sampled in this last study is 35 days older than the rodent samples in other studies, which may partially explain the discrepancy, if repatterning of

Table 6 Results of matrix correlation analyses within each cranial region across ontogenetic stages for *Cryptotis*. The upper triangle reports results using canonical correlations, whereas the lower triangle reports results using the congruence coefficient. Entries in bold are significant at the $P < 0.05$ level.

	2-day	5-day	9-day
Oral			
2-day	1	**0.647**	0.537
5-day	0.793	1	0.647
9-day	0.725	**0.913**	1
Orbit			
2-day	1	0.359	0.198
5-day	0.891	1	0.399
9-day	0.718	0.800	1
Vault			
2-day	1	0.121	0.227
5-day	0.721	1	0.485
9-day	0.699	0.748	1

Table 7 Results of matrix correlation analyses within each cranial region across ontogenetic stages for *Monodelphis*. The upper triangle reports results using canonical correlations, whereas the lower triangle reports results using the congruence coefficient. Entries in bold are significant at the $P < 0.05$ level.

	15-day	30-day	45-day
Oral			
15-day	1	0.307	0.210
30-day	0.747	1	**0.885**
45-day	0.727	0.767	1
Orbit			
15-day	1	**0.659**	0.424
30-day	0.695	1	**0.623**
45-day	0.817	0.693	1
Vault			
15-day	1	0.249	0.208
30-day	0.768	1	0.590
45-day	0.867	0.627	1

integration stabilizes later in ontogeny. The repatterning that occurs through ontogeny complicates attempts to understand these effects purely through studies of adult morphology, as multiple layers of effects can obscure any single pattern (termed the 'palimpsest' problem by Hallgrimsson *et al.*, 2009).

The study presented here focused exclusively on early craniogenesis, before skull ossification is complete. As detailed above, marsupials are born after extremely short gestation periods and have prolonged lactation periods relative to placentals. Thus, unlike previous studies, landmarks were limited to extremal points of bones, rather than suture junctions, which had not yet formed in most of the specimens examined here. Despite this difference in the analytical approach, the results of this study are generally similar to previous studies of placental mammals.

Firstly, within *Cryptotis*, integration changed significantly from stage to stage in most cranial regions. Only the oral region showed any significant similarity in integration between stages. This result suggests a possible correspondence with developmental timing, as a previous analysis shows that the bony elements of the oral region are significantly integrated in timing of ossification within Eulipotyphla, the clade that includes *Cryptotis* (Goswami *et al.*, 2009; Koyabu *et al.*, 2011). This result also corresponds to a degree with previous studies of the cotton rat which showed stronger integration corresponding to developmental origin in the orofacial region during early ontogeny, with later repatterning along functional boundaries (Zelditch & Carmichael, 1989b). That study also suggested that the neurocranial region showed greater variation in integration along ontogeny, which is also supported by the data presented here for the cranial vault module in both *Cryptotis* and *Monodelphis*. Whereas the two facial modules, oral and orbit, are both composed entirely of bones of neural crest origin, the cranial vault module as defined in this study includes bones of both neural crest (squamosal) and paraxial mesodermal (parietal) origin (Noden & Schneider, 2006). This developmental complexity of the cranial vault thus perhaps drives the greater variation in integration for this region reported here.

Whereas no significant similarities were observed between stages of *Monodelphis* when the congruence coefficient was used, both the oral and orbital regions showed significant matrix correlations between some stages with the canonical correlation. Given the functional requirements of the oral region at an early stage of maturity in marsupials, some consistency across stages might be expected. Nonetheless, the majority of matrix correlations between stages of *Monodelphis* were not significant, and so it appears that repatterning of cranial integration though ontogeny is ubiquitous in therian mammals.

Another relevant comparison with previous studies is offered by the results of the eigenvalue dispersion analyses. Some studies have noted that integration increases during the course of ontogeny (Zelditch *et al.*, 1992), whereas other studies have suggested that level of integration is stable or even decreases during ontogeny (Zelditch & Carmichael, 1989b; Willmore *et al.*, 2006). In a recent study of ontogenetic integration in a well-sampled series of macaques, we found that cranial integration decreases, and modularity increases, from infancy to adulthood (Goswami & Polly, 2010b).

As with the matrix correlation analysis, some results differed with use of the congruence coefficient and the canonical coefficient. In most of the analyses, eigenvalue dispersion, and thus integration, decreased from the earliest to latest stages in the oral and orbital regions, whereas the level of integration, though not its pattern, was usually stable across stages in the vault region. However, the canonical correlation results for *Cryptotis* showed instead that integration was increasing in the oral and orbital regions. The cause of the discrepancy between the two measures of correlation is unclear, but, given that the majority of results agree, it is likely that the general pattern within the oral and orbital regions is a decrease in integration through ontogeny, with the vault region remaining relatively stable. Increases, decreases and stability in the level of cranial integration through ontogeny have all been reported previously, as discussed above. It appears from the results observed here that a mixture of these patterns, or at least a mixture of decreasing and stable integration, characterize cranial ontogeny in *Cryptotis* and *Monodelphis*.

As Zelditch & Carmichael (1989b) suggested, if character correlations, as described by patterns of integration, change through ontogeny, selection that acts at different points in ontogeny may have different effects on cranial evolution. As necessitated by their reproductive strategy, marsupial neonates are under strong functional constraints at a much earlier stage of maturity than

their placental relatives. In the case of *Monodelphis*, suckling begins just over 2 weeks after conception and lasts more than three times longer than gestation. Results from analysis of eigenvalue dispersion using both the congruence coefficient and the canonical correlation show that integration of the oral region is strongest in 15-day-old specimens. Analyses of variance also show that there is no change in cranial variance from the youngest to oldest stages of *Monodelphis*. This consistency is in striking contrast to *Cryptotis*, which showed much higher variance than *Monodelphis* in early stages and also showed a significant decrease in variance from the earliest to latest ontogenetic stages, as is also evident in the PCA plots. Previous studies of rodents have similarly shown a strong decline in variance from early ontogeny to later stages (Zelditch *et al.*, 2004), suggesting that divergent trajectories of ontogenetic variance observed for *Cryptotis* and *Monodelphis* may reflect broader differences between placentals and marsupials.

Combined, these results suggest that strong functional constraints early in ontogeny when cranial integration is at its highest may be responsible for the relatively low variance observed during early ontogeny in *Monodelphis*. Unfortunately, the mixed results of the eigenvalue dispersion analyses for *Cryptotis* complicate a straightforward comparison with *Monodelphis*. However, placental mammals undergo most of their cranial ontogenetic changes prior to birth (Wilson, 2011) and place much less emphasis on lactation for development than marsupials do. Thus, it is likely that there are fewer functional constraints, and lower selection pressure, on cranial morphology of placentals during early postnatal ontogeny, when cranial integration is strongest, relative to the case in marsupials. The decrease in variance from 2- to 9-day *Cryptotis* specimens suggests that functional requirements later in development may impose similar constraints on the placental cranium, and in particular on the oral apparatus, to that in marsupials.

At present, there is much theory but little consensus on the evolutionary implications of morphological integration, with different authors having argued on the one hand that integration is expected to constrain trait evolution and, on the other hand, that integration facilitates trait change (Wagner, 1996; Wagner & Altenberg, 1996; Schlosser & Wagner, 2004; Marroig *et al.*, 2009). One study testing this relationship in a few clades of placental mammals found that for the most part, integration and disparity are not strongly linked. However, where significant results occurred, they suggest that strong integration mainly constrains morphological disparity in the skull (Goswami & Polly, 2010a). Further studies of the evolutionary analysis between patterns of integration and trait evolution are sorely needed in a broader range of taxa.

Nearly all of studies of integration focus on placentals, usually primates or rodents, with only a few considering marsupials at all (Goswami, 2006a, 2007a; Bennett & Goswami, 2011a; Kelly & Sears, 2011). Moreover, to date, no comprehensive comparison of cranial morphological diversity between marsupials and placentals has been conducted, although work along these lines is currently in progress (Bennett & Goswami, 2011b). Much more data are needed to address this question, but the results presented here suggest that the collusion of high levels of integration and strong selection pressures during early craniogenesis may have significant effects on morphological evolution.

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