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# Cranial modularity and sequence heterochrony in mammals

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**SUMMARY** Heterochrony, the temporal shifting of developmental events relative to each other, requires a degree of autonomy among those processes or structures. Modularity, the division of larger structures or processes into autonomous sets of internally integrated units, is often discussed in relation to the concept of heterochrony. However, the relationship between the developmental modules derived from studies of heterochrony and evolutionary modules, which should be of adaptive importance and relate to the genotype–phenotype map, has not been explicitly studied. I analyzed a series of sectioned and whole cleared-and-stained embryological and neonatal specimens, supplemented with published ontogenetic data, to test the hypothesis that bones within the same phenotypic modules, as determined by morphometric analysis, are developmentally integrated and will display coordinated heterochronic shifts across taxa. Modularity was analyzed in cranial bone ossification sequences of 12 therian mammals. A dataset of 12--18 developmental events was used to assess if modularity in developmental sequences corresponds to six phenotypic modules, derived from a recent morphometric analysis of cranial modularity in mammals. Kendall's $\tau$ was used to measure rank correlations, with randomization tests for significance. If modularity in developmental sequences corresponds to observed phenotypic modules, bones within a single phenotypic module should show integration of developmental timing, maintaining the same timing of ossification relative to each other, despite differences in overall ossification sequences across taxa. Analyses did not find any significant conservation of developmental timing within the six phenotypic modules, meaning that bones that are highly integrated in adult morphology are not significantly integrated in developmental timing.

## INTRODUCTION

The concept of modules, semi-autonomous sets of highly correlated traits within larger units, has been applied to diverse biological systems, from genes to colonies (Schlosser and Wagner 2004). Modularity is a valuable attribute in that it explains both integration within structures and autonomy among structures. This independence among structures allows unrelated components to vary and evolve separately, but the integration within the units maintains functionally necessary relationships among traits (Wagner 1995, 1996). A number of studies have examined genetic, developmental, and functional modules (Cheverud 1982, 1996, 2004; Cheverud et al. 1983, 1997, 2004; Zelditch 1988; Zelditch and Carmichael 1989a, b; Badyaev and Foresman 2000, 2004; Badyaev and Martin 2000; Klingenberg and Zaklan 2000; Klingenberg and Leamy 2001; Klingenberg et al. 2001, 2004, 2003; Zelditch et al. 2001; Klingenberg 2004; Wagner and Mezey 2004; Zelditch and Moscarella 2004; Goswami 2006), and these studies have provided encouragement that developmental modularity may provide insight into evolutionary processes (Schlosser and Wagner 2004). However, this potential relevance requires a relationship between developmental and evolutionary modules. Whereas developmental modules are derived from ontogenetic associations of structures, evolutionary modules should relate to the genotype–phenotype map and have functional or adaptive importance. This study focuses on one comparison of developmental and evolutionary modularity that is well-suited to comparisons across clades, the relationship between heterochrony (de Beer 1937; Gould 1977, 1982) and phenotypic modularity in the mammalian skull.

Heterochrony is the shift in timing of a developmental event relative to another event (Gilbert 2003) and requires that processes, structures, or sets of structures must be autonomous in developmental timing in order to shift relative to each other (de Beer 1937; Gould 1977). Modularity is defined by the autonomy of groups of events or structures, as well as by the strong associations of events or structures within each of those groups. Therefore, one may hypothesize that heterochronic shifts in developmental events occur among different modules, but not among events within a single module. Modularity is often discussed in studies of developmental

sequences, particularly in terms of ossification sequence heterochrony (Smith 1996; Schoch 2006). A recent study suggested that sets of cranial bones, potentially representing functional or developmental modules, shift in timing of ossification in concert when compared across tetrapod clades (Schoch 2006). Although modularity in the ossification sequences was not explicitly tested, the point was made that if the modularity of ossification sequences is to elucidate evolutionary processes, it needs to be established how developmental modules derived from ossification sequence heterochrony relate to evolutionary modules. There are few comparative studies of modularity and heterochrony that examine this proposed relationship (Poe 2004; Shubin and Davis 2004). Although there are several alternative methods of recognizing evolutionary or developmental modules, both phenotypic modularity, determined by morphometric analysis, and ossification sequence heterochrony are well studied, independently, in the mammalian skull, making it a good system to rigorously test for modularity in heterochrony.

Comparative morphometric studies of cranial modularity focus nearly exclusively on mammals, providing a good foundation for exploring developmental modularity in these groups. Morphometric studies within primates have identified six phenotypic cranial modules (Ackermann and Cheverud 2004), and a study of phenotypic modularity across all mammals (Goswami 2006) demonstrated that six cranial modules are conserved in all therian mammals, but not in monotremes. These six phenotypic modules were defined by quantitative analysis three-dimensional (3D) landmarks in 106 species (Fig. 1), with cluster analyses of trait correlations and significance tests demonstrating that within-module trait correlations were greater than those between modules (Fig. 2). Of the six cranial modules, the anterior oral–nasal group, the molar group, and the basicranial group were the most highly correlated and most conservative. In contrast, the orbit and zygomatic–pterygoid groups usually were weakly integrated, whereas the cranial vault was variable in its integration across taxa. These modules are significantly correlated with hypothesized functional modules for the mammalian skull and can be considered as evolutionary modules.

Heterochrony in mammalian skull development has been extensively researched, with several different methodologies used to identify fundamental differences in sequence heterochrony among higher level taxa (Smith 1996, 1997, 2001a, b, 2002, 2006; Nunn and Smith 1998; Jeffery et al. 2002, 2005; Bininda-Emonds et al. 2003). A series of studies by Smith et al., in particular, has identified that Marsupialia (the crown group of Metatheria; Rougier et al. 1998) and Placentalia (the crown groups of Eutheria; Rougier et al. 1998) differ markedly in the timing of ossification of cranial bones. Because marsupials are born after a very short gestation period, the primary requirement for survival is the development of the masticatory apparatus and forelimb to crawl

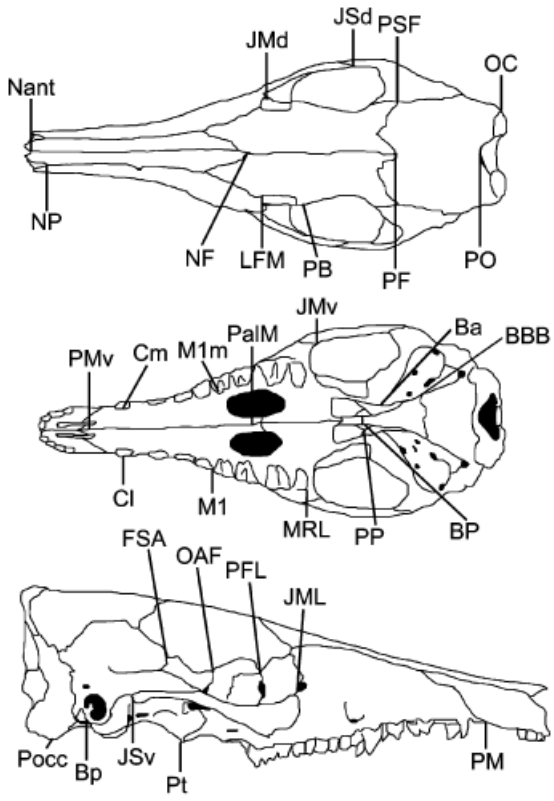


**Fig. 1.** Cranial landmarks, used in analysis of evolutionary modules (Goswami 2006), shown on *Echymipera kalubu*. Symmetrical landmarks shown on one side only.

and attach to a teat and suckle. Therefore, marsupials delay the development of the brain and accelerate the ossification of the masticatory apparatus, relative to placentals (Smith 1996, 1997, 2001a, b, 2002, 2006; Nunn and Smith 1998). Smith (1997) identified event pairs that distinguish marsupials and placentals, and in many cases, bones within the modules identified by Goswami (2006) shifted relative to each other. It is noteworthy that several of these event pair shifts are within the oral–nasal (premaxilla–tooth buds and maxilla–tooth

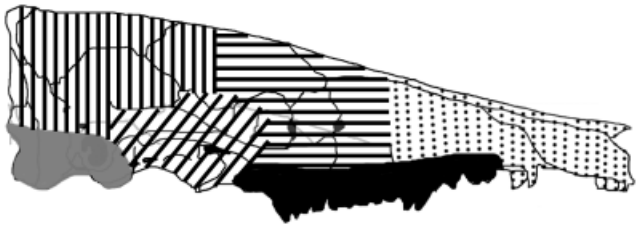

**Fig. 2.** Skull of a peramelid marsupial, *Echymipera kalubu*, showing the six cranial modules tested in this study: anterior oral–nasal (dotted); molar (solid black); orbit (horizontal lines); zygomatic–pterygoid (diagonal lines); vault (vertical lines); and basicranium (solid gray).

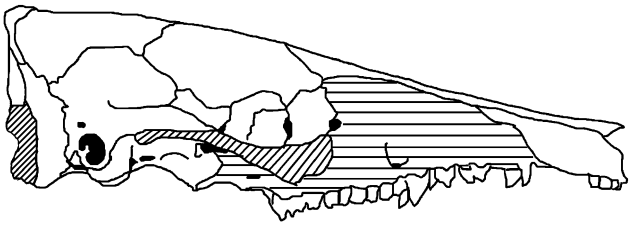

**Fig. 3.** Skull of peramelid marsupial, *Echymipera kalubu*, showing the three bones actively shifting in timing of ossification between placentals (exoccipital and jugal, diagonal lines) and marsupials (maxilla, horizontal lines), according to Jeffery et al. (2005).

buds pairs) and cranial base (exoccipital–basioccipital, exoccipital–squamosal, and exoccipital–parietal pairs) groups, the two most strongly integrated phenotypic modules in adults, suggesting that developmental timing may not be strongly integrated across therians.

Several new methods have been proposed that incorporate an explicitly phylogenetic framework and parsimony analysis to the identification of sequence heterochrony, reducing significant events, and isolating active versus passive events (Jeffery et al. 2002, 2005). Jeffery et al. (2005) identified three major heterochronic changes in skull bone ossification between marsupials and placentals. Marsupials accelerated the ossification of the maxilla, whereas placentals accelerated the jugal and delayed the exoccipital (Fig. 3). These newer methods significantly decreased the number of active heterochronic shifts, while supporting the conclusion that marsupials and placentals display significant heterochronic shifts in cranial bone ossification most likely related to reproductive strategy. Again, these active shifts occurred within observed phenotypic modules, rather than solely between modules.

Three previous studies have explicitly considered modularity in mammalian skull development in a comparative context. Smith (1996) examined three possible types of integrated units in cranial development: evolutionarily integrated units, spatially associated units, and morphologically related units. Her analyses suggested that evolutionary units, such as elements of the first arch, were not developmentally integrated, but that spatially associated units, such as the central nervous system and cranial vault, were integrated. Morphologically related units showed greater variation, with skeletal units showing diversity in ossification patterns, whereas muscular systems showed coordination.

The second study introduced a new method that explicitly tests for the presence of modularity within developmental sequences (Poe 2004). Poe (2004) applied his method to three taxa from Nunn and Smith's (1998) dataset, and rejected the hypothesis that ossification events in the dataset are integrated relative to soft tissue developmental events. Most recently, Schoch (2006) demonstrated that cranial bones can be segregated into five sets (dermal jaw bones, marginal palatal bones, circumorbital bones, braincase bones, and skull roof bones) within which bones shift in concert when compared across tetrapod clades, and suggested that these sets may define functional or developmental modules.

Although the groupings used in the previous studies are only hypothetical modules, a recent study identifying six cranial modules in therians (Goswami 2006) provides an independent measure of evolutionary cranial modularity that can be used to test hypotheses of developmental modularity of ossification sequences in mammals. In this study, Poe's (2004) method will be used to test if the six phenotypic modules observed in the therian cranium correspond to integration in cranial bone ossification sequences. Specifically, if there is a relationship between evolutionary modularity and developmental modularity, as defined by ossification sequence heterochrony, bones within a single evolutionary module should display coordinated heterochronic shifts and should not change in developmental sequence relative to each other.

## METHODS

### Developmental sequences

Ossification sequence data were gathered from original ontogenetic series (Supplementary Table S1) and from published sequences (Nunn and Smith 1998) for six marsupial species, *Mondelphis domestica*, *Caluromys philander*, *Macropus eugenii*, *Trichosurus vulpecula*, *Perameles nasuta*, and *Dasyurus viverrinus*, and six placental species, *Tupaia javanica*, *Tarsius spectrum*, *Mus musculus*, *Felis domestica*, *Manis javanica*, and *Sus scrofa*. Sectioned specimens (Fig. 4) and whole cleared-and-stained specimens (Fig. 5) were used in original data collection. Data derived solely from Nunn and Smith (1998) included the onset of ossification of 11 bones and the first appearance of tooth buds (Table 1 and Supplementary Table S2). The six taxa with original ontogenetic series available included onset of ossification data for 18 bones (Table 1 and Supplementary Table S3). Of these six taxa, five are marsupials

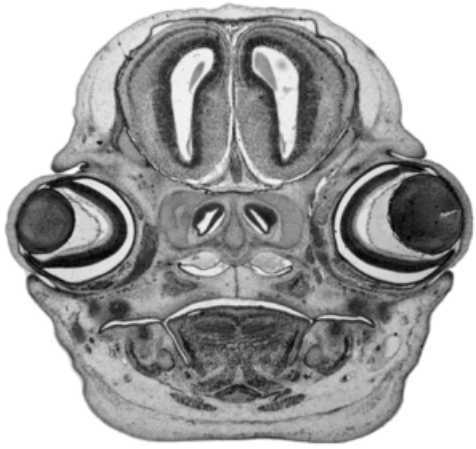

**Fig. 4.** A sectioned histological specimen of *Tupaia javanica*.

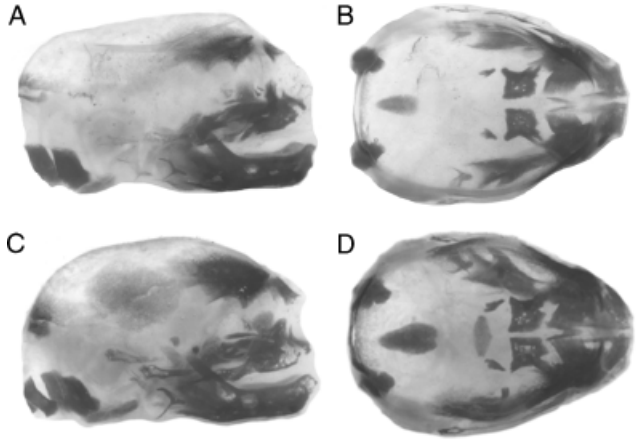


**Fig. 5.** Whole, cleared-and-stained specimens of *Monodelphis domestica*: 2 days postnatal in lateral (A) and dorsal (B) views; and 6 days postnatal in lateral (C) and dorsal (D) views.

and one is a placental (*T. javanica*). Although this sampling prevented the analysis of modularity in developmental sequence among placentals for the expanded dataset, it allowed for a more detailed study of modularity within marsupials.

### Data analysis

Analysis of the integration of developmental events was conducted with the methodology developed by Poe (2004), which builds upon the original rank analysis method of Nunn and Smith (1998). The method tests for the conservation of rank orders within sets of traits across phylogeny, providing a statistical and comparative technique. Poe's method compares only sister taxa, using Kendall's τ, a nonparametric rank correlation measure that is limited to analysis of two sets of variables. Kendall's τ is calculated as

$$\tau = \frac{n_c - n_d}{(n_c + n_d + n_x)(n_c + n_d + n_y)^{1/2}}$$

where $n_c$ is the number of concordant pairs of ranks, $n_d$ is the number of discordant pairs, $n_x$ is the number of tied events in the first taxon, and $n_y$ is the number of tied events in the second taxon.

The method constructed ranks for nodes by averaging the sequences (the temporal ranks of each event) of sister taxa joined at a particular node. The averaged sequence then became the hypothetical ancestral sequence of developmental events for that node and was then compared with the next most closely related taxon or node. This simplistic averaging to reconstruct ancestral sequences is a potential weakness, as it is not necessary that the ancestral condition is an average of the descendents. However, this approach is the logical basis for other widely applied methods, such as independent contrasts, but without the requirement of accurate branch length data. Furthermore, if shifts in developmental sequences are strongly modular, this pattern would be similar in multiple species and should be detectable with this approach to ancestral node reconstruction. The phylogenetic relationships of taxa used in this study are displayed in Fig. 6. Sequences for real terminal taxa, and for pair wise ancestral nodes, are listed in Supplementary Tables S2 and S3.

To test for modularity, Kendall's τ was calculated for subsets of cranial ossification events that correspond to six phenotypic modules identified in adult therian skulls (Goswami 2006). For example, the basicranial module is composed of the exoccipital, basioccipital, basisphenoid, and periotic bones (Table 1). Therefore, Kendall's τ for the onset of ossification ranks for these four bones was calculated for each pair of taxa. The significance of

**Table 1. Developmental events and module associations used in analyses**

| | Event | AON | MOL | ORB | ZYG | VLT | BSE |
|---|---|---|---|---|---|---|---|
| 1 | Premaxilla | X | | | | | |
| 2 | Maxilla | X | X | X | | | |
| 3 | Frontal | | | X | X | X | |
| 4 | Jugal | | X | X | X | | |
| 5 | Parietal | | | | | X | |
| 6 | Squamosal | | | | | X | |
| 7 | Alisphenoid | | | | X | X | |
| 8 | Basioccipital | | | | | | X |
| 9 | Basisphenoid | | | | X | | X |
| 10 | Exoccipital | | | | | X | X |
| 11 | Periotic | | | | X | | X |
| 12 | First appearance of tooth buds | X | | | | | |
| 13 | Nasal | X | | X | | | |
| 14 | Ethmoid | | | | X | | |
| 15 | Palatine | | X | | | | |
| 16 | Orbitosphenoid | | | | X | | |
| 17 | Lacrimal | | | X | | | |
| 18 | Pterygoid | | | | X | | |

1–12 correspond to events used in Nunn and Smith (1998), although the ordering is different.

Modules are anterior oral–nasal (AON), molar (MOL), orbit (ORB), zygomatic-pterygoid (ZYG), cranial vault (VLT), and cranial base (BSE).

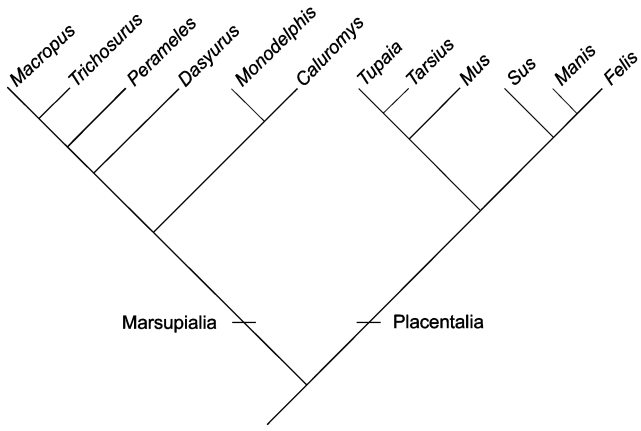


**Fig. 6.** One phylogeny used in analyses (Horovitz and Sánchez-Villagra 2003) with *Dasyurus* basal to other australodelphian marsupials (topology MarsupialiaD as described in Methods). * indicates taxa used in analyses of 18 events.

Kendall's τ was determined by comparison with a null distribution of random sets of developmental events. Because the sets of developmental events were relatively small (four to eight events per module), it was possible to generate a distribution of all possible groupings of events, rather than using random sets. If the set of developmental events is in fact shifting as a module, it should have a higher correlation value than some percentage of all possible similarly sized groupings of events. This study used a 0.05 significance level, necessitating that the correlation among hypothesized modules should be >95% of all possible groupings.

One potential issue was that the cranial morphometric data used to identify phenotypic modules primarily measured sutures (Goswami 2006), which involve more than one bone. Therefore, the boundaries of modules were often defined by the intersection of multiple bones, necessitating that single bones were often involved in more than one module. For example, the anterior portion of the maxilla is part of the anterior oral–nasal module, whereas its ventral part meets the molar module. Its dorsal and posterior portions also extend to the orbital module. To accommodate the contribution of individual bones to multiple modules, each ossification event was included in all modules with which it is involved in the adult skull, resulting in a range of one to three modules per bone (Table 1). This method may increase the number of events included in any one module, and it will tend to mask real modularity, rather than falsely identifying modularity.

As half of the taxa have data only for 12 developmental events, and the other six taxa have data on 18 events, two separate analyses were conducted. The first analysis included all 12 taxa and used the 12 common developmental events. The second analysis was limited to the six taxa with 18 events. Because calculation of the rank correlation coefficient requires at least three events with different ranks, it was not possible to analyze the anterior oral–nasal or molar in the reduced dataset of 12 events. Instead, the anterior oral–nasal and molar groups were combined in that analysis, rather than excluding them entirely, although the combination of the two modules may mask developmental integration of the individual modules. Thus, for analysis of 12 events, five modules were assessed: oral–nasal+molar, orbit, zygomatic-pterygoid, vault, and base, whereas all six modules were included in the analyses of 18 events. In addition, rank correlations among all facial traits and among all neurocranial traits were assessed in the analyses of 18 traits.

The importance of phylogenetic relationships also introduced the need for multiple analyses. Although the relationships among the six placental taxa are well established (Springer et al. 2005), there are two possible phylogenetic relationships among the six marsupial taxa. One recent phylogenetic study found Peramelia (*Perameles*) to be more closely related to Diprotodontia (*Macropus* and *Trichosurus*) than either are to Dasyuromorphia (*Dasyurus*) (Horovitz and Sánchez-Villagra 2003), whereas another study instead placed Peramelia basal to Dasyuromorphia+Diprotodontia (Asher et al. 2004). To account for the uncertainty in these phylogenetic relationships, analyses (12 and six taxa) were conducted twice, with Peramelia basal to the other australodelphian marsupials in this study (MarsupialiaP) and with Dasyuromorphia basal (MarsupialiaD).

## RESULTS

In the analysis of 12 developmental events in 12 taxa, there were no significant rank correlations among bones within a single phenotypic module (Table 2). Rank correlation between taxa ranged from 0.14 to 1.0. The different phylogenetic hypotheses for australodelphian marsupials produced similar results in all analyses. The lowest correlations were observed in the vault group and anterior oral–nasal+molar

**Table 2. Results of analysis for 12 developmental events**

| Taxa A | Taxa B | All | AON/MOL | ORB | ZYG | VLT | BSE |
|---|---|---|---|---|---|---|---|
| *Monodelphis* | *Caluromys* | 0.71 | 0.89 | 0.82 | 0.89 | 0.14 | 0.91 |
| *Macropus* | *Trichosurus* | 0.65 | 0.26 | 0.80 | 0.96 | 0.72 | 0.71 |
| *Diprotodontia* | *Perameles* | 0.89 | 1.00 | 0.82 | 0.89 | 0.59 | 1.00 |
| *Diprotodontia* | *Dasyurus* | 0.86 | 1.00 | 0.82 | 0.80 | 0.50 | 0.91 |
| Diprotodontia+*Perameles* | *Dasyurus* | 0.89 | 1.00 | 0.33 | 0.79 | 0.84 | 0.91 |
| Diprotodontia+*Dasyurus* | *Perameles* | 0.91 | 1.00 | 0.33 | 0.87 | 0.89 | 1.00 |
| Diprotodontia-*Perameles*+*Dasyurus* | *Monodelphis*+*Caluromys* | 0.93 | 1.00 | 1.00 | 1.00 | 0.74 | 1.00 |
| Diprotodontia-*Dasyurus*+*Perameles* | *Monodelphis*+*Caluromys* | 0.90 | 1.00 | 0.33 | 0.87 | 0.74 | 1.00 |
| *Felis* | *Manis* | 0.90 | | | 0.93 | 0.94 | 0.89 |
| *Felis*+*Manis* | *Sus* | 0.71 | 0.77 | 1.00 | 0.60 | 0.38 | 0.91 |
| *Tupaia* | *Tarsius* | 0.94 | 1.00 | 1.00 | 1.00 | 0.82 | 0.80 |
| *Tupaja*+*Tarsius* | *Mus* | 0.57 | 0.77 | 0.50 | 0.67 | 0.14 | 0.91 |
| *Felis-Manis*+*Sus* | *Tupaia-Tarsius*+*Mus* | 0.88 | 0.40 | 1.00 | 0.97 | 0.74 | 1.00 |
| Marsupialia D | Placentalia | 0.74 | 0.18 | 0.82 | 0.82 | 0.60 | 1.00 |
| Marsupialia P | Placentalia | 0.72 | 0.18 | 1.00 | 0.87 | 0.60 | 1.00 |

Kendall's τ is listed for each comparison.
Modules are anterior oral–nasal (AON), molar (MOL), orbit (ORB), zygomatic-pterygoid (ZYG), cranial vault (VLT), and cranial base (BSE).

**Table 3. Results of analysis for 18 developmental events**

| Taxa A | Taxa B | All | AON | MOL | ORB | ZYG | VLT | BSE | NRO | FCL |
|---|---|---|---|---|---|---|---|---|---|---|
| *Monodelphis* | *Caluromys* | 0.70 | 0.60 | 0.82 | 0.67 | 0.88 | 0.14 | 0.91 | 0.77 | 0.64 |
| *Dasyurus* | *Trichosurus* | 0.64 | 0.60 | 0.82 | 0.94 | 0.80 | 0.40 | 0.71 | 0.65 | 0.61 |
| *Perameles* | *Trichosurus* | 0.66 | 0.60 | 1.00 | 0.89 | 0.88 | 0.50 | 0.71 | 0.73 | 0.61 |
| *Dasyurus-Trichosurus* | *Perameles* | 0.86 | 0.89 | 1.00 | 0.74 | 0.89 | 0.82 | 1.00 | 0.94 | 0.89 |
| *Perameles-Trichosurus* | *Dasyurus* | 0.84 | 0.89 | 0.82 | 0.74 | 0.82 | 0.76 | 1.00 | 0.87 | 0.92 |
| *Dasyurus-Trichosurus+Perameles* | *Monodelphis-Caluromys* | 0.80 | 0.89 | 1.00 | 0.74 | 0.62 | 0.74 | 1.00 | 0.77 | 0.94 |
| *Perameles-Trichosurus+Dasyurus* | *Monodelphis-Caluromys* | 0.83 | 0.89 | 1.00 | 0.95 | 0.69 | 0.74 | 1.00 | 0.78 | 0.94 |
| MarsupialiaP | *Tupaia* | 0.79 | 1.00 | 1.00 | 0.95 | 0.85 | 0.45 | 1.00 | 0.81 | 0.79 |
| MarsupialiaD | *Tupaia* | 0.80 | 1.00 | 1.00 | 0.95 | 0.85 | 0.45 | 1.00 | 0.81 | 0.79 |

Kendall's $\tau$ is listed for each comparison.
Modules are anterior oral–nasal (AON), molar (MOL), orbit (ORB), zygomatic-pterygoid (ZYG), cranial vault (VLT), cranial base (BSE), all neurocranial traits (NRO), and all facial traits (FCL).

group. The latter result may be due to the grouping of two modules, as discussed above. There were very low correlations between Placentalia and Marsupialia in the oral–nasal+molar group (0.18), although not in any other module. The cranial base had the highest mean correlations of any module, ranging from 0.80 to 1. Although several analyses returned a rank correlation of 1.0, these results were not significant.

The full set of 12 events also was compared among sister taxa (Table 2). Most sister species had high correlations, although two of the lowest values are for sister species: *Monodelphis* and *Caluromys* (0.71) and *Macropus* and *Trichosurus* (0.65). The lowest correlation overall was from the comparison of *Tupaia+Tarsius* and *Mus* (0.57). The comparison between Marsupialia and Placentalia was 0.72–0.74.

The analyses of the 6 taxa with 18 developmental events also showed no significant rank correlations within modules (Table 3). Use of different phylogenetic relationships among the australodelphian marsupials did not greatly affect results. Among the six modules, the cranial vault again showed the lowest rank correlations, ranging from 0.14 to 0.82. Unlike the previous analysis, rank correlations among oral–nasal traits were high (0.60–1.0). The cranial base again showed the highest correlations across taxa, despite having a broad range of ranks, but the separated molar group showed equally high correlations in this analysis. As before, the lowest rank correlations were often between species, as opposed to between more-inclusive clades or hypothetical ancestral taxa. Rank correlations between marsupials and the placental were similar to comparisons within marsupials.

When all 18 events are included in analyses of rank correlation across species, the three lowest correlations are among species pairs (0.64–0.70), whereas comparisons involving hypothetical ancestors ranged from 0.79 to 0.86. Correlations among neurocranial traits and among facial traits were not significant in any analysis.

## DISCUSSION

None of the analyses in this study showed any significant correlations of ossification events within phenotypic modules. In the study of 12 events across 12 taxa, the oral–nasal+molar group displayed the lowest correlations in comparisons of Placentalia and Marsupialia (0.18), which is congruent with previous work demonstrating that acceleration of the masticatory apparatus was a major difference in cranial development between these clades (Smith 1997, 2001b). Placental taxa displayed a broader range of rank correlations among the oral–nasal+molar traits than marsupials did, and most comparisons between marsupials showed a perfect correlation within this group, possibly indicating a developmental constraint imposed by the accelerated development of the masticatory apparatus in marsupials. One striking exception to this pattern is the low correlation (0.26) between *Macropus* and *Trichosurus*, two diprotodontian marsupials. This result may reflect the unique dental eruption pattern of *Macropus* (Kirkpatrick 1978), but more data from other diprotodontians are needed to explicitly test this hypothesis. Mean rank correlations among oral–nasal traits and among molar traits (treated separately) increased in the analyses of 18 events, which probably reflects the exclusion of most of the placental taxa, but also may be due to the ability to separate the oral–nasal and molar groups.

The cranial vault displayed the lowest rank correlations across taxa in both the 12 and 18 event analyses, and there is a great deal of variation in rank correlations of the cranial vault traits, even within closely related clades, such as Didelphidae (*Monodelphis* and *Caluromys*). The development of the cranial vault is primarily responsive to the growth of the brain (Young 1959; Persson 1983), and marsupials delay the development of the brain, in order to focus early maternal energy on the development of facial structures necessary for suckling (Nunn and Smith 1998). Because there is a large developmental shift in the cranial vault, it would be an ideal test of

whether morphologically integrated bones are also integrated in developmental timing. One caveat, however, is that the cranial vault is one of the weakest phenotypic modules in most taxa, and therefore would not necessarily be expected to be particularly integrated in development either. The results of this study show that the cranial vault is not more integrated, and is instead the least developmentally integrated of the phenotypic modules.

The cranial base group comes closest to displaying developmental modularity across taxa in both analyses. It has the highest mean rank correlations of any of the phenotypic modules, and the cranial base also is one of the strongest phenotypic modules (Goswami 2006). However, these results are not significant. In fact, several comparisons in the oral–nasal group, basicranial group, and zygomatic group returned a correlation of 1, indicating a perfect correspondence among ranks, but were not significantly greater than random groupings of traits.

Grouping bones into the more general neurocranial and facial clusters also did not return significant results, although the correlations within these groups were relatively high. The ranges and means of the neurocranial and facial trait rank correlations are nearly identical, suggesting that neither is more developmentally integrated than the other. Although there are high rank correlations within neurocranial and facial groups, these are not statistically significant, and only a few bones within either group display active heterochronic shifts.

Because significant values were determined by comparison with all of the other possible groupings of the dataset, the lack of statistical significance for any analyses may suggest that most taxa are relatively similar in rankings of cranial bone ossification. Smith (1997) determined that 43% of cranial event pairs, including cartilage, bone, muscle, and central nervous system development, were uniform across all taxa, whereas over half were uniform within placentals or marsupials. Alternatively, the lack of significance for even perfectly correlated sets of events could be a statistical artifact. The number of developmental events encompassed by each phenotypic module is small, and, as discussed above, most events were involved in more than one phenotypic module, which may mask real developmental modularity and make it difficult to statistically reject the null hypothesis of no developmental modularity. Another possible reason for the lack of any significant modularity within the developmental sequences in this study may be the comparability of sequence and morphometric data. Many cranial bones develop from multiple ossification centers. As these are not detectable in adult skulls, morphometric measurements usually rely on reproducible points, such as sutures, which do not correspond well to centers of ossification. A methodology using the onset of ossification for individual ossification centers or quantitative measurements of developing bones, rather than on just the first center for each bone, may be a more accurate reflection of the phenotypic modularity of adult bones. Nonetheless, the differences in ossification sequence rankings among taxa are essentially random with regard to phenotypic modularity of the cranium, and, thus, it is likely that the lack of statistical significance is an accurate reflection of the relationship between phenotypic modularity and developmental timing.

Both analyses produced interesting results with regard to similarity between real sister species. The lowest correlations across all taxa were often between sister species, rather than between hypothetical ancestral reconstructions for more-inclusive clades. This result may reflect developmental apomorphies within individual taxa, which are reduced in the averaged hypothetical ancestral states. The lowest correlation for all events and within most phenotypic modules was for the comparison of *Mus* with *Tupaia+Tarsius*. As Smith (1997) noted, *Mus* differs markedly from other placental mammals in cranial ossification patterns, and these results support that assertion.

As discussed above, previous studies of sequence heterochrony, when considered in combination with independent analyses of cranial modularity, suggest similar results as this study. The bones within the phenotypic modules observed in Goswami (2006) were previously determined by Smith (1996) not to be integrated in developmental timing across therians, but it could not be determined whether they were integrated within marsupials or placentals. This study statistically demonstrates that observed phenotypic modules of the cranium cannot be shown to be integrated in developmental timing across therians or within placentals or marsupials. Furthermore, the conservation of phenotypic modularity across therians (Goswami 2006), despite the disparity in cranial development between marsupials and placentals, strongly suggests that sequence heterochrony is not a significant influence on adult phenotypic modularity.

Statistical analysis of ossification sequences offers a powerful tool for testing hypotheses of developmental integration of event timing based on qualitative observations or on morphometric data. However, developmental integration of cranial bones may be manifested in many ways, from the shape dynamics of developing bones, to interactions among separate ossification centers, to sequence heterochrony in ossification timing. Furthermore, it should be noted that ossification represents a late-stage event in bone differentiation. Earlier markers of bone development may reveal stronger correlations with phenotypic modularity, with decoupling of ossification and modularity in later stages. Examining sequence heterochrony of ossification as a starting point offers the benefit that the ossification of bones is a discrete event and thus is ideal for comparisons across taxa (Kuhn 1987). Future studies should focus on more detailed data on ossification centers and the ontogenetic shape of individual bones, as well

as on earlier markers of bone differentiation, to better compare morphological and developmental integration.

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## SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

**Table S1.** Original ontogenetic material that was examined for the expanded set of 18 developmental events. Unless otherwise noted, lengths refer to body length. P refers to days postnatal. NT refers to Normal Table stages.

**Table S2.** Ranks for real and ancestral taxa for the set of 12 developmental events. Event numbers are as listed in Table 1.

**Table S3.** Ranks for 18 developmental events for six taxa. Event numbers are as listed in Table 1.

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