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Short Communication

Does developmental strategy drive limb integration in marsupials and monotremes?

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Mammal limbs show diverse locomotory specializations, but this range is not evenly distributed throughout the three reproductively distinct extant clades: placentals, marsupials and monotremes. Marsupials, unlike their placental sister group, have not evolved true flight, as observed in placental bats (Chiroptera), or fully aquatic locomotion, as in placental cetaceans, sirenians, and pinnipeds. This lower locomotor diversity in marsupials, relative to placentals, has been hypothesised to result from their unusual reproductive strategy (Sears, 2004).

Marsupials have a much shorter gestation period and lengthened period of lactation relative to that of placental mammals, and they are also the only mammals to complete an obligatory unaided crawl into the pouch (Gemmell et al., 2002) which requires use of the forelimbs at a much earlier stage of development than in monotremes or placentals. However, different marsupial orders reach the pouch by means of distinct birthing methods. Diprotodontians, the most taxonomically diverse marsupial clade, complete an upwards climb, dasyuriformians perform a downwards crawl and peramelids slide down a moistened path into a posteriorly facing pouch (Gemmell et al., 2002). Didelphimorphians have been observed to reduce the distance the young crawls into the pouch by the mother hunching

over (Tyndale-Biscoe and Renfree, 1987). Each method requires a different level of mechanical input from the forelimb of the neonate.

Monotremes form a monophyletic sister clade to marsupials and placentals and are the only extant mammals to retain oviparity. As in marsupials, monotreme gestation is shorter than that of placentals (Griffiths, 1978). In the echidna, eggs are laid directly into the pouch where they hatch and suckle from teats. The platypus, however, does not have a pouch or teats, milk is lactated onto the abdomen and the young suck it from the milk field area on the mother's fur (Griffiths, 1978). Upon hatching, monotremes have large, well-developed forelimbs, hind limb buds and an oral apparatus sufficiently developed for sucking in milk (Hughes and Hall, 1998).

Heterochronic shifts in skeletal development that relate to these differences in reproductive strategy have been explored by a number of authors over the past decade (Smith, 1997, 2001; Sánchez-Villagra et al., 2008; Weisbecker et al., 2008; Goswami et al., 2009). Development of new methods that focus on sequence heterochrony in particular have allowed comparisons across disparate taxa (Smith, 2001; Bininda-Emonds et al., 2003), and as a result, exploration of the developmental component of the marsupial-placental dichotomy has flourished. Sánchez-Villagra (2002) identified marsupial-specific sequence heterochronies to be fundamentally linked to forelimb developmental acceleration, relative to hind limb development. He thus proposed that the acceleration of scapular ossification in relation to the hind limb of marsupials is 'causally correlated' to post-natal

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mechanical demands. Recent work on ossification sequence heterochrony across therians (Weisbecker et al., 2008) has identified specific heterochronies between placentals and marsupials that polarize this heterochrony as a delay in hind limb ossification in marsupials, although prechondrogenic forelimb development is still accelerated in marsupial (Sears, 2009). The same dataset used by Weisbecker et al. (2008) was also used to explore patterns of modularity in skeletal ossification (Goswami et al., 2009), demonstrating that placentals show significant integration in developmental timing of anterior and posterior postcranial development in placental mammals, while marsupials dissociate these regions.

The fore and hind limbs are serial homologues; both limbs share a common developmental programme that has altered in position along the body axis (Hall, 1995). This relationship is also reflected in the comparable patterns of *Hox* gene activity during the development of fore and hind limb elements (Wellik and Capecchi, 2003). *Hox* genes control proximo-distal patterning of segments by transcribing protein switches that control the expression of other genes (turning them 'on' and 'off' over time), and thus driving resultant adult form (Gilbert, 2006). Structures that are derived from the same developmental pathway, such as the one described above, can display integration that relates to their shared developmental history, and these relationships may be conserved over the course of evolution. The grouping of genes, developmental processes, or morphological traits into highly-integrated sets, or modules, is a major feature of biological organisation (West-Eberhard, 2003). Thus an important factor in morphological evolution is variational modularity, the independent variation of sets of covarying traits (Wagner and Altenberg, 1996).

As developmental variation drives phenotypic variation, it follows that changes in development, such as heterochronic shifts or early mechanical demands on subsets of integrated traits, may alter these modules, which should be reflected in the patterns of covariance among phenotypic elements. Functional changes can also influence the integration of elements. Schmidt and Fischer (2009) investigated whether the integration of limb elements across therian mammals reflects the dissociation between serially homologous pairs of bones. Functionally analogous bone pairs have shifted, relative to the ancestral condition, as the scapula has become mobile relative to the rib cage in therians. This freedom to move contributes an additional functional component to the forelimb and thus alters the comparable mechanical relationship between fore and hind limb bones, such that the scapula becomes functionally analogous to the femur (Fischer et al., 2002). Using correlation analysis of limb element length data from 11 marsupial and 178 placental species, they found that hind limb proportions were similar across taxa whilst forelimbs were considerably more varied. From this they tentatively hypothesised that hind limb integration is a result of the common biomechanical requirements for propulsion. In contrast, the lower forelimb integration could reflect a compromise between their more varied functional roles and either persistence of shared developmental factors between fore and hind serial homologues or the incorporation of an additional set of developmental instructions (for the scapula) within the functional module (Schmidt and Fischer, 2009).

Young and Hallgrímsson (2005) conducted a morphometric study of limb covariance within six placental mammals: one bat, two primates, two rodents and a carnivoran. They tested whether functionally divergent fore and hind limbs showed reduced covariation of serial homologues, relative to more generalized species. Their results demonstrated that the fore and hind limbs are generally significantly integrated in adult morphology. Only in the functionally disparate limbs of the bat was the between-limb

covariance markedly weaker, whilst within-limb covariance was high. Interestingly, the two primates use their limbs differently (gibbons spend considerably more time walking bipedally than macaques), and they also have very different limb proportions, yet showed similar levels of covariance between and within limbs.

If the characteristic dissociation of limb developmental timing observed in marsupials affects phenotypic integration, as it appears to influence integration of developmental timing, between limb covariance is expected to be reduced in marsupial species relative to placentals. It is also expected that, as in placentals, functional dissociation of limbs in marsupial species results in lower covariance between limbs than is observed in quadrupedal marsupials.

Here, we present analyses of limb covariance structure in four species of marsupials and two species of monotremes to test for differences among the three mammalian clades. Specifically, we test whether the modularity in developmental timing observed in marsupial limb elements is reflected in adult morphology. As strong associations among elements within a module may constrain variation of individual elements, identification of limb modularity in adult morphology may also relate to the low disparity observed in marsupial forelimbs (Sears, 2004).

We sample some of the phylogenetic and locomotor breadth of marsupials, with one ameridelphian marsupial, *Didelphis marsupialis* (common opossum; $n=14$). Australodelphians, two diprotodontians, *Macropus giganteus* (Eastern grey kangaroo; $n=8$) and *Trichosurus vulpecula* (brushtailed possum; $n=14$), and one dasyuromorphian, *Sarcophilus harrisi* (Tasmanian devil; $n=10$). The two monotreme species sampled were *Tachyglossus aculeatus* (short-beaked echidna; $n=13$) and *Ornithorhynchus anatinus* (duck-billed platypus; $n=13$). Only adult specimens were used in analyses.

Both monotreme species use their limbs for quadrupedal terrestrial locomotion, swimming and digging, and have no mobile elements in their pectoral girdle, unlike therian mammals. The forelimbs of the echidna (as in all marsupials and placentals) are pronated, whereas the platypus retains a more primitive sprawling posture. *M. giganteus* is a large, bipedal, saltatorial mammal with highly specialised hind limbs. *T. vulpecula* and *D. marsupialis* are both scansorial while *S. harrisi* is a fully terrestrial quadruped.

Data were collected from specimens housed at the Natural History Museum in London, Museum für Naturkunde der Humboldt-Universität zu Berlin, Grant Museum of Zoology, University College London, Oxford University Museum of Natural History, University Museum of Zoology Cambridge, American Museum of Natural History New York, Smithsonian Institution National Museum of Natural History, and World Museum Liverpool.

Photographs of one limb bone from each of the stylopodal and zeugopodal segments of both the fore and hind limbs of each species were taken using a Canon EOS 450D digital camera. From the zeugopod, the radius and tibia are used due to reduction of the ulna and fibula in some species (Sears et al., 2007). Photographs were taken of disarticulated bones resting on their ventral surface, as this tends to be flatter and hence will rest at more consistent angle, with the camera positioned directly above mounted on a tripod. Landmarks were digitised in TpsDig2 (Rohlf, 2006).

Limb lengths were calculated as inter-landmark distances for the femur, humerus, tibia and radius of each specimen (Fig. 1) to produce a dataset of four length measurements, comparable to the ELD dataset of Young and Hallgrímsson's (2005) study. Landmark position was kept as comparable as possible across species. However, slightly different landmarks were used between marsupials and monotremes, due to the considerable difference in gross bone morphology and resulting uncertainty in the

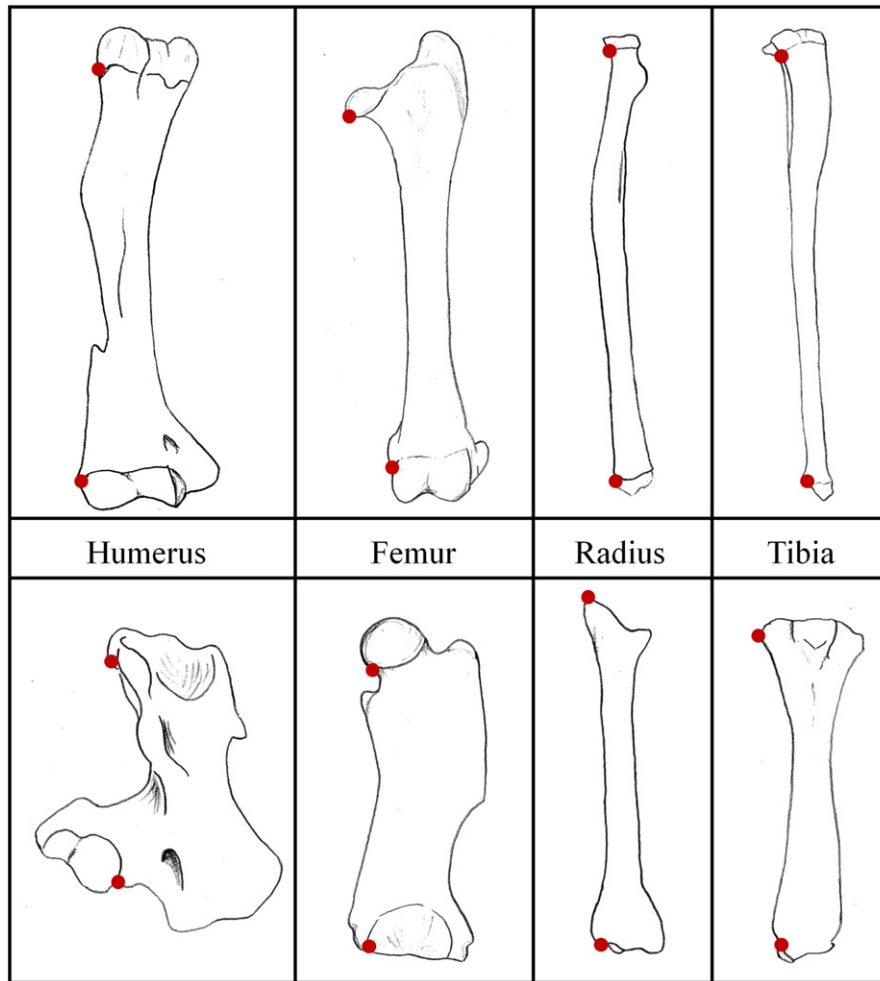


Fig. 1. Positioning of landmarks on bones of a marsupial, *M. giganteus* (top) and a monotreme, *T. aculeatus* (bottom), not to scale.

homologies of processes. These distances were then normalised by the geometric mean within each species. Raw and log lengths were also analysed, but, as all produced similar results, only the results for the geometric-mean normalised lengths are discussed below.

Covariance matrix repeatability was assessed with a Monte Carlo simulation (10,000 replicates) in Poptools (Hood, 2006) for each species to determine if results were robust to sample size (Marroig and Cheverud, 2001). Partial correlation analysis of the four size-adjusted limb lengths was conducted in 'R' (R Development Core Team, 2005), with edge exclusion deviance used to assess significance. In addition, to provide a simple but straightforward comparison between disparate species, mean within-limb and mean between-limb absolute partial correlations were calculated for each species in this study, as well as for placental species from the study of Young and Hallgrímsson (2005).

Covariance matrix repeatability was high in all species, ranging from 0.873 to 0.997 (Table 1). The lowest repeatabilities were for *S. harrisi* and *M. giganteus*, the latter of which had a lower sample size than the other species. *M. giganteus* and *T. vulpecula* showed significant partial correlations only within the forelimb, with no significant correlations among hind limb elements or between limbs (Table 2). *S. harrisi* showed significant correlations between the femur and humerus and between the femur and tibia. *D. marsupialis* showed significant correlation between only the tibia and radius. Partial correlations are markedly higher within the forelimb than within the hind limb in the two diprotodontians

Table 1

Matrix repeatability for taxa analysed in this study.

	Matrix repeatability
<i>M. giganteus</i>	0.910
<i>T. vulpecula</i>	0.978
<i>S. harrisi</i>	0.873
<i>D. marsupialis</i>	0.997
<i>T. aculeatus</i>	0.976
<i>O. anatinus</i>	0.967

(*M. giganteus* and *T. vulpecula*). *S. harrisi*, however, is anomalous among marsupials in displaying higher partial correlations within the hind limb than within the forelimb.

The lack of statistically significant partial correlations within the forelimbs of *S. harrisi* and *D. marsupialis* is perhaps a consequence of the less demanding crawl into the pouch for these species than in diprotodontians (Gemmell et al., 2002). Correspondingly, the high within forelimb correlations observed in both diprotodontians likely relates to the early mechanical requirements of the crawl to the pouch. The relative difficulty or duration of the crawl or locomotory pattern, or both, could be driving the diversity in limb covariance patterns among marsupials observed here. In contrast to the results for marsupials, both monotremes showed statistically significant partial correlations only between limbs, not within limbs (Table 2), and similarly showed higher mean correlations between limbs than

Table 2

Partial correlation matrices with corresponding p values in the upper triangle of each matrix. Numbers in bold indicate significant results ($p < 0.05$).

	Femur	Humerus	Tibia	Radius	Femur	Humerus	Tibia	Radius
	<i>M. giganteus</i>				<i>S. harrisii</i>			
Femur		0.169	0.709	0.305		< 0.001	0.016	0.290
Humerus	0.566		0.411	< 0.001	-0.873		0.080	0.161
Tibia	0.184	-0.380		0.455	0.702	0.581		0.484
Radius	-0.456	0.952	0.350		0.396	0.497	0.275	
	<i>T. vulpecula</i>				<i>D. marsupialis</i>			
Femur		0.354	0.738	0.713		0.513	0.292	0.314
Humerus	0.281		0.381	0.018	0.203		0.573	0.276
Tibia	0.105	0.267		0.076	0.316	0.175		0.029
Radius	-0.115	0.600	0.489		0.303	0.326	0.569	
	<i>T. aculeatus</i>				<i>O. anatinus</i>			
Femur		0.116	0.829	0.664		< 0.001	0.651	0.019
Humerus	0.464		0.036	0.500	0.961		0.457	0.173
Tibia	0.072	0.572		0.334	-0.149	0.241		0.669
Radius	-0.143	0.219	0.307		0.618	-0.413	-0.141	

Table 3

Average partial correlations for the species examined in this study and that of Young and Hallgrímsson (2005). Numbers in bold denote averages for each group.

	Between	Within	Within/ Between
<i>T. aculeatus</i>	0.372	0.145	0.391
<i>O. anatinus</i>	0.490	0.281	0.573
Monotreme	0.431	0.213	0.495
<i>M. giganteus</i>	0.438	0.568	1.297
<i>T. vulpecula</i>	0.288	0.353	1.224
<i>S. harrisii</i>	0.531	0.599	1.128
<i>D. marsupialis</i>	0.313	0.321	1.027
Marsupial	0.392	0.460	1.173
<i>C. brevicaudata</i>	0.172	0.450	2.620
<i>H. lar</i>	0.310	0.362	1.168
<i>M. mulatta</i>	0.259	0.468	1.810
<i>L. flavopunctatus</i>	0.239	0.535	2.236
<i>M. musculus</i>	0.303	0.348	1.149
Placental	0.256	0.434	1.695

Table 4

Within forelimb vs within hind limb partial correlations with averages for marsupials, monotremes and placentals.

	Forelimb	Hind limb	Forelimb/ hind limb
<i>T. aculeatus</i>	0.219	0.072	3.042
<i>O. anatinus</i>	0.413	0.149	2.772
Monotreme	0.316	0.111	2.86
<i>M. giganteus</i>	0.952	0.184	5.174
<i>T. vulpecula</i>	0.6	0.105	5.714
<i>S. harrisii</i>	0.497	0.702	0.708
<i>D. marsupialis</i>	0.326	0.316	1.032
Marsupial	0.594	0.326	1.817
<i>C. brevicaudata</i>	0.454	0.446	1.018
<i>H. lar</i>	0.45	0.273	1.648
<i>M. mulatta</i>	0.444	0.492	0.902
<i>L. flavopunctatus</i>	0.495	0.492	1.006
<i>M. musculus</i>	0.265	0.43	0.616
Placental	0.422	0.427	0.988

within limbs. Both monotremes also showed higher correlations within the forelimb than within the hind limb.

The results of the partial correlation analyses showed that the monotremes differ markedly from the marsupials studied here, and from the results for placental mammals reported by Young and Hallgrímsson (2005). In that study, placentals showed higher within-limb correlations than between limbs overall, similarly to the marsupials and conversely to the monotremes (Table 3). However, placental mammals showed similar within hind limb and forelimb correlations, unlike the condition observed here for monotremes and diprotodontian marsupials (Table 4). When within/between and forelimb/hind limb correlations are plotted against each other, monotremes are clearly separated from the therian mammals (Fig. 2). Marsupial and placental mammals show some overlap between the non-diprotodontian marsupials and the mouse and gibbon, but there is a clear distinction between the diprotodontians and other mammals analysed.

This study examines marsupial and monotreme limb correlation patterns to test for a relationship between developmental strategy and phenotypic integration. That two of the five significant partial correlations within marsupials are between limbs argues against a simple relationship between developmental strategy and integration of adult morphology. However, the absence of significant partial correlation between serially homologous elements in the limbs of *M. giganteus* and *T. vulpecula*, as well as the high within-forelimb correlations, implies an extensive dissociation of the ancestral, serially homologous

developmental pathways of the limbs occurred during diprotodontian evolution that may well relate to their extensive pouch crawl. There is scope for fruitful future research on this topic, particularly in expanding the analyses to include peramelids and *Notoryctes*, the marsupial mole, to further test the relationship between developmental strategy, integration, and morphological diversity. Weisbecker et al. (2008) found that fore and hind limb ossifications were temporally much closer in peramelids than in other marsupials, potentially reflecting the lack of mechanical input from their forelimbs on the journey to the pouch (Weisbecker et al., 2008). In addition, peramelids, such as *Chaeropus*, the pig-footed bandicoot, display more derived adult forelimb morphology than other marsupials and a distinctive pattern of ontogenetic shape change in the scapula (Sears, 2004). Similarly *Notoryctes*, with its reduced and modified manus digits (Kirsch, 1977) would provide an interesting comparison as it has been postulated that young may be directly deposited into the pouch.

The patterns observed in marsupials are in striking contrast to that observed in the two monotremes, with strong correlations between serially homologous bones, which may better represent the ancestral pattern for mammals. However, given that extant monotremes are highly specialised mammals, it is possible that their limb covariance structure is also highly derived from the primitive mammalian pattern. If monotremes do represent the ancestral condition, it may be possible that the incorporation of the scapula into the limb module during the course of therian

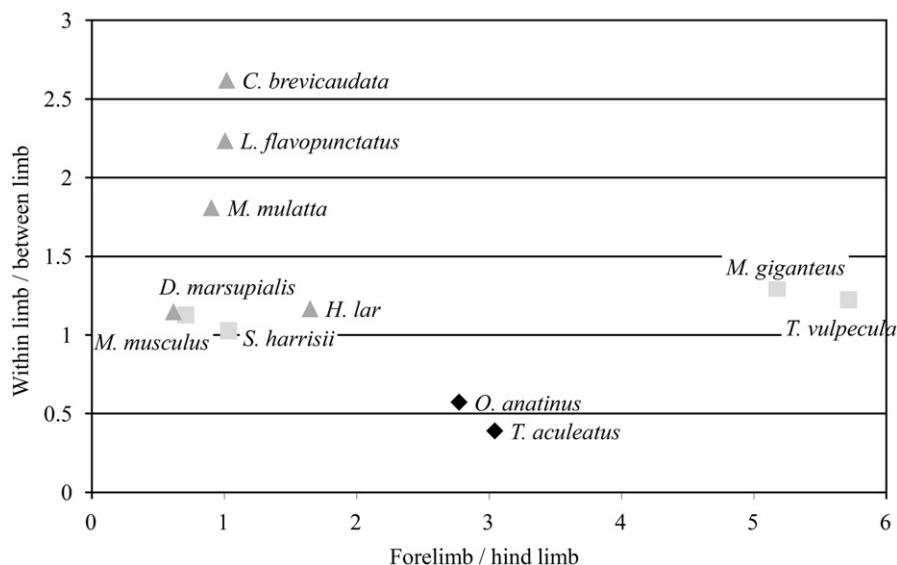


Fig. 2. Scatter plot of within / between limb partial correlations against forelimb / hind limb correlations for monotremes (diamonds), marsupials (squares) and placentals (triangles). Placental data from Young and Hallgrímsson (2005).

evolution (Schmidt and Fischer, 2009) has resulted in a breakdown of the integration between the serial homologues of the fore and hind limb.

The identification of distinct limb covariance patterns for the therian and non-therian mammals, and for diprotodontians versus other marsupials, provides a powerful tool for assessing developmental evolution in extinct taxa. Analysing the limb covariance patterns for fossil mammal species might thus be used to elucidate the timing and sequence of changes involved in the evolution of viviparity in therian mammals and the evolution of the marsupial reproductive strategy.

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