Evolution of Sexual Dimorphism in Mustelids

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Abstract

The existence of striking sexual dimorphism in morphology, such as extreme differences in body size and mass between males and females across various animal taxa, has inspired a wealth of research. However, the narrow focus on specific aspects of dimorphic variation in adults and lack of integration between various sources of information leaves many unresolved questions in sexual dimorphism evolution. I have studied dimorphism at different levels of organization in model mammalian species (mustelid carnivores and laboratory rat), with a focus on the least understood aspects of morphological evolution: 1) phylogenetic relationship between sexual size dimorphism and body mass in mustelids, 2) ontogenetic origins of adult sexual size and shape dimorphism in the growing pelvic skeleton, 3) evolution of sex differences in shape and phenotypic covariation in the adult pelvic skeleton, 4) developmental origin and intraspecific and interspecific divergence and maintenance of patterns of trait relationships in the skull. Collectively, results emphasize the evolution of sexual dimorphism as a product of evolutionary changes in both males and females. Greater rates of male body size evolution relative to females, coupled with a more rapid body size evolution in some taxa, produced correlated evolution of body size and sexual dimorphism. Sexual divergence in ontogenetic trajectories of bone remodeling of the pelvis was tied to hormonal events occurring at puberty and was related to reproductive function of female pelvis, ultimately leading to specific expression of shape dimorphism in adults. Evolutionary divergence of shape of the pelvis in adult males and females was also found to be the result of reproduction-related variation (females had more spacious birth canal reflecting demands of increasing neonatal size in more dimorphic species), as well as sexual differences in bone size (bones were more robust in males having larger muscle site

attachments). Both the pelvis and the skull showed sexual dimorphism in details of trait relationships at both intra- and interspecific levels, possibly related to integration differences in early and late development. The research of mechanisms producing developmental variation leading to differential expression of adult morphology in males and females may hold a key to understanding sexual dimorphism evolution.

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Chapter 1

Introduction and Overview

"…the whole organization is so tied together during its growth and development, that when slight variations in any one part occur, and are accumulated through natural selection, other parts become modified. This is a very important subject, most imperfectly understood."

Charles Darwin, *The Origin of Species*

Darwin (1871) was only the first to acknowledge that biological organization is complex and highly regular. Even today the problem of how complex morphologies are organized and evolve remains one of the central themes in morphological evolution research. In 1958, Olson and Miller introduced the term "morphological integration", to refer to the lack of independence among morphological characters. Any two measurements taken on the same bone are likely to be correlated to some extent, beyond the correlation due to effects of size. In reality, we take multiple bone measurements to quantify and represent complex morphologies such as the skull or the pelvis. Thus, we work with sets of relationships within the whole form that may represent patterns of morphological integration. Morphological integration research addresses questions of what causes such groupings of relationships, whether there are differences among groups of related traits, and to what magnitude these differences exist.

Studies of morphological integration received critical attention and have proliferated since the publications by Cheverud (1984, 1989, 1995). In particular, Wagner (1996) placed morphological integration into a rigorous theoretical framework of development and evolution. Development is a highly dynamic process that translates genotypic information into phenotypic variation, thus introducing new variants upon which natural selection acts. Consequently, sorting of phenotypic variation by selection leads to morphological evolution. However, the introduction of new variants is also biased because of the integration among

morphological parts. This integration is due to the high regulatory control during development, which allows only a limited number of viable developmental outcomes. As a result phenotypic variation and morphological evolution are constrained due to morphological integration, which in turn limits aspects of variation available to natural selection. Cheverud (1996) recognized three different levels of morphological integration. First, morphological characters are related through their relationships and common regulatory control in development or due to performance of a common function. Second, such characters frequently assume common genetic control due to gene pleiotropy or linkage disequilibrium, which is reflected in integration at the genetic level. Third, genetically integrated traits evolve together, which leads to morphological integration at the evolutionary level.

Morphological integration in mammalian skeleton is extensively documented by Cheverud et al. (1984, 1995, 2001, 2004) in primates, and Hallgrímsson et al. (2002) in primates and laboratory mice, both with almost exclusive focus on the integration of the skull. One of the major findings of these authors is that morphological organization of the skull is modular, meaning that correlation between traits sharing developmental origin and function (e.g., belonging to the same module) is higher than between unrelated traits. Further, morphological integration is evolutionary stable, with different primate taxa sharing similar patterns of integration. Despite being novel and influential, limited focus of these studies leaves many open questions, which I address in my thesis:

1) Are the causes of integration consistent across mammalian orders?
2) Does modularity exist in skeletal regions other than the skull?
3) Is integration sexually dimorphic?
4) Does evolution of morphological integration correspond to evolutionary divergence in skeletal morphology?

I chose family Mustelidae as an excellent model to study morphological integration and the evolution of morphology and sexual dimorphism. The evolution of this family was rapid, resulting in remarkable ecomorphological diversity. The family includes four major clades: terrestrial/subterranean weasels, arboreal martens, aquatic otters, and terrestrial badgers and skunks. Such diversity in natural history comes with significant variation in body size (spanning four orders of magnitude), in size and shape of the skeletal regions, and in sexual dimorphism, from species with no dimorphism to extremely dimorphic species, where males are twice as large as females (such as weasels).

Specifically, I addressed hypotheses of morphological integration and evolution in two skeletal regions of mustelids: the skull and the pelvis. I asked following questions concerning the evolution of mustelid skull. Do mustelids show patterns of craniofacial modularity similar to primates and mice? Is sexual dimorphism in integration, if it exists, correlated with sexual dimorphism in skull shape? Does integration evolve in a group of closely related but morphologically divergent species such as mustelids? Similarly to my study of the skull, I tested *a priori* hypotheses of the existence of several distinct modules within the whole pelvic form, based on independent evidence of developmental and functional complexity in this skeletal region. I examined sexual dimorphism in pelvis integration relative to pelvic shape dimorphism due to parturition and sexual dimorphism in size. I hypothesized that the evolution of morphological integration in mustelid pelvis was related to the evolutionary divergence in pelvic shape resulting from differential locomotory function in different mustelid clades.

The resulting thesis is a collection of four manuscripts presented here as four chapters that follow this introduction; each of them deals with different aspects of evolution of complex morphologies and evolution of sexual dimorphism. The details of each chapter are briefly summarized below.

Chapter 2. Phylogenetic history of the evolution of sexual size dimorphism in mustelids

One of the most striking differences between sexes is in body mass. Sexual dimorphism in body mass is correlated with overall body size, pattern consistent across a variety of animal groups (Rensch 1959; Webster 1992; Fairbairn and Preziosi 1994; Colwell 2000; Kratochvil and Frynta 2002). This phenomenon remains a topic of much discussion, with many mechanisms proposed to explain this correlation (Reiss 1986; Fairbairn 1997). Most comparative studies of the pattern focus on present-day relationships at the "tips" of phylogeny, contrasting expression of sexual dimorphism in body size with differences in natural history between sexes. At the same time, phylogenetic history of any clade presents an opportunity to explain present-day phenomena from historical perspective, by direct reconstruction of evolutionary events (Harvey and Pagel 1991). Since sexual dimorphism is the result of evolutionary changes and constraints posed on each of the sexes independently, this work was an attempt to understand the origin of the pattern by tracing the evolutionary divergence in body sizes of males and females individually. I found sexual dimorphism in body mass within mustelids to be a consequence of unequal rates of male and female body size evolution, with male body mass being less constrained in its evolution than female mass. Greater rates of male size divergence, coupled with a more rapid body size evolution in some taxa, produced correlated evolution of body size and sexual dimorphism. These results suggest an existing constraint on female body mass in mustelids and/or selection for increased male size, which can generate further hypotheses of the relationship between body size and sexual dimorphism. As one example, since adult body size is the direct result of growth, comparative analysis of the events responsible for differences in growth patterns between sexes may increase resolution of the pattern. Next chapter gives one example of significance of studying growth to understanding sexual dimorphism.

Chapter 3. Pelvic growth: Ontogeny of size and shape sexual dimorphism in rat pelves

Neonate males and females of mammalian species are nearly identical in size and shape. Therefore, any expression of adult sexual dimorphism is necessarily a product of differences in patterns of growth between the sexes. This fact is ignored in current studies of sexual size dimorphism, and especially in studies of shape dimorphism. Whereas the evolution of genetically based sexual size dimorphism in adults is extremely slow, there is a rapid evolution of differences between males and females in growth patterns, and these differences evolve not just among related species, but also among populations and even among different traits within an organism (Maunz and German 1996; Badyaev et al. 2001; Badyaev 2002). Developmental studies demonstrate a variety of ontogenetic pathways that can lead to adult dimorphism, mostly through differences in rates and duration of growth (McNamara 1995). Largely, sex-specific growth patterns are due to temporal differences between males and females in the secretion of growth hormones and spatial differences in regulation of their activity in growing tissue (Agrawal and Shapiro 2001), resulting both in adult size and shape dimorphism. This chapter focuses on testing hypotheses of sexual dimorphism in postnatal remodeling (size and shape alteration) of pelvic bones against well-understood developmental and endocrinology background of the laboratory rat (*Rattus norvegicus*), with the goal to understand proximate mechanisms that generate adult dimorphism in shape of the adult pelvis. Landmark-based geometric morphometrics methodology tested for differences in the rates, magnitudes and directional patterns of shape change during growth. Males achieved larger pelvic sizes by growing faster throughout ontogeny. However, the rates of shape change in the pelvis were greater in females. Both sexes underwent similar bone remodeling until puberty. After puberty, but before reproductive maturity, shapes of the sexes diverged due to specific changes in the female pelvis, possibly due to the influence of gonadal estrogens. Pattern of male pelvic bone

remodeling remained the same throughout ontogeny, suggesting that androgen effects on male pelvic morphology were constant and did not contribute to specific shape changes at puberty. Collectively, the results indicate that sexual dimorphism in growth patterns (and thus adult dimorphism) of the pelvis is tied to major hormonal events occurring at puberty and is further related to reproductive function of female pelvis.

Chapter 4. The evolution of sexual shape dimorphism and morphological integration in the mustelid pelvis

The popularity of studies of sexual dimorphism in body size and craniofacial features detracted attention from the postcranial skeleton, including the pelvic bones, which is surprising given their central location in the mammalian skeleton and functional significance in parturition, skeletal support and locomotion. This chapter focuses on the relative contributions of allometry, phylogeny, reproduction and function to the evolution of shape dimorphism in the pelvic bones of mustelids. Landmark-based geometric morphometrics methodology was used to address the long-standing issue of quantification of shape independently of size, which was never applied in studies of the pelvic form before. In addition, hypotheses of morphological integration within the pelvis were tested with resampling-based matrix correlation approaches. Morphological integration is a term used to describe patterns of trait covariation within a form. Morphology organizes itself in evolution by forming complexes of traits related through commonality in genetic control, development or function (Olson and Miller 1999). This non-independence of traits determines the variation exposed to selection and thus is essential for understanding morphological evolution (Cheverud 1995). Unlike skull, the pattern of morphological integration in the mammalian pelvis remains to be unknown. Results showed that species differed in pelvic shape based on the degree of locomotor specialization. Sexual shape dimorphism was the result of both sexual differences in bone size (bones were more robust in males having larger muscle site attachments) and parturition-related variation (females had more spacious birth

canal reflecting demands of increasing neonatal size in more dimorphic species). Tests of alternative hypotheses of morphological integration suggest that growth and adult function are significant determinants of pelvic trait relationships. Besides sexual dimorphism in pelvic shape, sexual dimorphism also exists in the overall magnitude of integration at both intra- and interspecific levels. This suggests that removing the effects of sex in studies of morphological integration may conceal significant biological information on the evolution of sexual differences in complex anatomical structures.

Chapter 5. The evolution of phenotypic integration structure in the mustelid skull

This chapter addresses the question of the evolution of phenotypic patterns of trait relationships in the mammalian skull. While many studies of the evolution of craniofacial morphology exist, evolutionary dynamics of integration (and sexual dimorphism of integration) across mammals largely remain unresolved. The hypotheses of origin, sexual dimorphism and evolution of morphological integration were tested in a model clade of mustelids. Following Cheverud's (Cheverud 1995; Marroig and Cheverud 2001) study of phenotypic integration in primate skulls, it was hypothesized that both size and developmental/functional relationships structure trait relationships in phenotype. Another set of hypotheses addressed the question whether skull morphological integration is sexually dimorphic as a result of dimorphism in morphology, and evolves differently in males and females. Results indicate that, similarly to what we known from primates and laboratory mice, developmental/functional relationships are important determinants of mustelid skull phenotypic covariance structure, which suggests that basic observed pattern of trait relationships is conserved across mammalian orders. Sexual dimorphism was not found in basic modular composition of the skull, but in levels of integration within modules. Observed sexual dimorphism in patterns of trait relationships was not related to sexual dimorphism in shape, size or early development. This suggests that differences in growth patterns (and

craniofacial bone remodeling) may govern the variation in trait relationships between sexes. The effects of postnatal growth patterns on the skull covariance structure require further investigation, since this would help to understand how differences in trait relationships in ontogeny map onto differences in adult phenotypic integration patterns, consistent or not consistent with differences in adult skull morphology. The evolution of integration proceeded differently in two sexes. Interspecific variation in phenotypic covariance was detected, in males it corresponded to phylogenetic patterns in the clade, and in females to morphological divergence among species.

Future research

Despite the large volume of research and many sources of information, there is a remarkable lack of integration between the different approaches and levels to the study of sexual dimorphism. A growing understanding of the evolution of developmental processes is one key to answering the questions left unresolved. In future I intend to study the molecular and genetic mechanisms of sexual dimorphism ontogeny to understand the variation in the origin and maintenance of sexual dimorphism across various animal groups, which holds the great potential to advance our understanding of sexual dimorphism evolution.

Chapter 2

Phylogenetic History of the Evolution of Sexual Size Dimorphism in the Family Mustelidae (Carnivora, Mammalia)

ABSTRACT

Comparative studies of the evolution of sexual size dimorphism often reveal allometric tendencies, where existing proportional size differences between sexes depend on changes in overall body size, a trend known as a Rensch's rule. Most studies focus exclusively on non-directional comparisons in extant taxa. However, because sexual dimorphism is a result of historical changes in individual sexes, studying differences in phylogenetic history of the sexes can explain its evolution more directly. In this paper, I (1) tested the relationship between sexual dimorphism and body size (consistency with Rensch's rule) within family Mustelidae and its monophyletic subclades; (2) determined exactly what differences in phylogenetic history of the sexes lead to extant patterns of sexual size dimorphism and Rensch's rule allometry within this family. Male-biased dimorphism within mustelids was a consequence of unequal rates of male and female body size evolution, with male body size being less constrained in its evolution than female size. Greater rates of male size divergence, coupled with a more rapid body size evolution in some taxa, produced an allometric pattern consistent with Rensch's rule.

Key words: Rensch's rule, body size, sexual dimorphism, rates of evolution, phylogenetic constraints, mustelids

Selection acting differently on the two sexes, together with non-selective factors such as allometry and phylogenetic history, result in sexual dimorphism in morphological, physiological and behavioral traits (e.g., Ralls 1976; Nylin and Wedell 1994; Fairbairn 1997; Plavcan 2001). The occurrence of the most striking difference between sexes in animals, sexual size dimorphism (SSD), has motivated a great deal of research (e.g., Ralls 1976; Cheverud et al. 1985; Hoglund 1989; Shine 1989; Bjorklund 1991; Fairbairn and Preziosi 1994; Fairbairn 1997; Kraushaar and Blanckenhorn 2002).

Quantitative studies of SSD in various groups of organisms often show allometric trends in SSD, where proportional size differences between sexes either increase or decrease with changes in overall body size (e.g., Webster 1992; Fairbairn and Preziosi 1994; Colwell 2000; Kratochvil and Frynta 2002). Rensch (1959) was first to document this allometric trend in various arthropod and avian taxa, where sexual dimorphism increases with body size in species where males are larger than females (in female-biased taxa this relationship is inverse), a pattern now referred to as a Rensch's rule. The validity and generality of Rensch's rule (which applies only to subspecies of a species, to related species of a genus, or to related genera of a family) has been established by comparative studies of Fairbairn (1997) and Abouheif and Fairbairn (1997).

Webster (1992) summarized several hypotheses for the evolutionary mechanism generating this size/dimorphism relationship. The general pattern of evolutionary divergence leading to allometry for SSD among contemporary species consists primarily of correlated changes in size in males and females, allometry being produced because one sex (usually males) shows a greater magnitude of change than the other (Abouheif and Fairbarn 1997). Both SSD and body size often show strong phylogenetic effects. For example, as a species

evolves a larger or smaller body size, the sexual size dimorphism present in the ancestral species is carried along, and magnified or reduced with the evolution of body size, as a consequence of phylogenetic history (Leutenegger and Cheverud 1982; Cheverud et al. 1985; Abouheif and Fairbairn 1997).

Most comparative investigations of allometric patterns in sexual dimorphism and evolution of SSD in general (e.g., Fairbairn and Preziosi 1994; Fairbairn 1997; Abouheif and Fairbairn 1997) focus on relationships between present day species employing non-directional phylogenetic techniques for phylogenetic correction. At the same time, phylogenetic history of a clade presents an opportunity to work with comparative data at yet another level, and may reveal broad patterns of dimorphic character evolution, explaining present-day phenomena from historical perspective (Harvey and Pagel 1991). In particular, constraints imposed on character evolution by phylogenetic history play an important role in the evolution of morphological character states. The nature of these constraints, loosely grouped together under name of "phylogenetic inertia", remains debatable (e.g., Derrickson and Ricklefs 1988; McKitrick 1993; Griffiths 1996; Blomberg and Garland 2002). Quite different processes, such as developmental constraints, niche conservatism or stabilizing selection, may explain same patterns of channeled character evolution (slow rates of evolutionary change and tendency of related species to resemble each other) within a single clade (Morales 2000; Blomberg and Garland 2002; Blomberg et al. 2003).

In this paper, I show that independent of the cause, specific characters may have different constraints and different phylogenetic history in different sexes. This may lead to the expression of dimorphism in extant species. Because sexual dimorphism is a result of changes in individual sexes, large-scale evolutionary patterns of sexual dimorphism observed

in body size and other morphological characters can be explained by difference in the rates of character evolution between males and females, i.e. strength of phylogenetic inertia.

Specifically, I (1) tested the relationship between sexual dimorphism and body size (consistency with Rensch's rule) within family Mustelidae and its monophyletic subclades; (2) determined exactly what differences in phylogenetic history of sexes lead to extant patterns of sexual size dimorphism and Rensch's rule allometry within this family.

The family Mustelidae contains sixty-five species with significant variation in sexual dimorphism of body size (from no dimorphism to extreme dimorphism). This group of animals has long been an object of controversy in studies of Rensch's rule, being used both as an example of taxa supporting and contradicting this allometric trend (Moors 1980; Ralls and Harvey 1985; Reiss 1986; Fairbairn 1997; Abouheif and Fairbairn 1997). Based on my results, a significant positive relationship between sexual dimorphism and body size, an allometric pattern consistent with Rensch's rule, was found only in *Lutrinae*. Furthermore, phylogenetic history proceeded differently in both sexes and different clades, producing different patterns of sexual dimorphism in present-day species.

MATERIALS AND METHODS

All measurements used here were taken as species averages from the literature sources (Ognev 1962; Ternovski 1977; Mason and Macdonald 1986; Silva and Downing 1995; Nowak 1999; Johnson et al. 2000) (see Table 1). The unit of analysis for this study is a sex within a species or a species itself. Because sample size corresponds to the number of species, using more species of Mustelidae compared to previous studies of mustelid SSD increased degrees of freedom of the phylogenetic analysis. The phylogenetic tree used in the

analysis is part of a composite supertree phylogeny of order Carnivora by Bininda-Emonds et al. (1999).

The analyses included forty-nine species of the family Mustelidae divided into four monophyletic clades of *Mustela* (11 species), *Martes* and related genera (15 species), *Lutrinae* (11 species) and *Mephitinae* (six species) (Fig. 1). The analyses were performed at the family level, and for each monophyletic subclade individually. Data for each species included measurements of male and female body mass. For the analysis, mean species size was also calculated as a mean of male and female body masses.

I calculated sexual dimorphism index as logarithm of male size to female size ratio. This index is commonly used in various sexual dimorphism studies, and takes care of statistical problems with ratios transforming the multiplicative relationship between the numerator and denominator to an additive relationship: $\log(X/Y) = \log X - \log Y$ (LaBarbera 1989; Ranta et al. 1994). This operation removes some distributional and spurious correlation problems (e.g., Albrecht 1978; Atchley and Anderson 1978; Ranta et al. 1994).

I tested relationships between male and female body sizes, as well as sexual dimorphism and species size using different methods based on both tips data and information on phylogenetic relationships between species. I chose these methods because they involve historical analysis of events leading to extant patterns, and are built on different assumptions, estimating the same relationships from slightly different perspectives, as it is described below.

Tips analysis. —I performed tips analysis on data, assuming a star phylogeny (ignoring branching pattern of character evolution), using statistical package SYSTAT 9.0 (Wilkinson 1999). All body mass measurements were logarithmically transformed prior to

analyses. Pearson correlations and ordinary least square regressions were used to test relationships between male and female body sizes, as well as sexual dimorphism and species sizes. Positive allometric relationships indicated support for Rensch's rule.

Felsenstein's independent contrasts. —Independent contrasts estimated the same relationships taking phylogenetic relationships into account, removing statistical problems associated with non-independence of tips as data points (Felsenstein 1985). Sets of independent contrasts were generated using PDAP phylogenetic programs (Garland et al. 1999; Garland and Ives 2000). Tips were logarithmically transformed prior to analysis. I performed the tests based on two different models of character evolution. Under the Brownian motion model, or gradual model of evolution, the branch lengths of phylogeny are taken into account, which standardizes contrasts at each node based on degree of accumulation of variance of change. All polytomies were treated as soft, inserting branches of zero length as recommended by Felsenstein (1985) and Purvis and Garland (1993). In each case, I plotted values of standardized contrasts versus their standard deviations, following the diagnostic of Garland et al. (1992). If any pattern was noted, appropriate transformations of branch lengths were performed in order to give contrasts equal weightings in subsequent analyses. Under the punctuational model of evolution, all branch lengths were set to unit length, therefore ignoring times of divergence. Relationships between male and female sizes, and sexual dimorphism and species size, were tested with standardized contrasts of each variable using correlation and regression analyses.

Rate tests for character evolution. — This method was used to estimate evolutionary rates of divergence (β) of characters of interest, based on the idea that each independent contrast from Felsenstein's analysis represents an index of the minimum amount of

evolutionary change that has occurred since divergence from the most recent common ancestor of two tips or nodes (Garland 1992; Webster and Purvis 2002). β estimates of each character divergence rates were calculated using PDAP (Garland et al. 1999; Garland and Ives 2000) program. To test for the significance of differences in beta estimates of male and female size evolution rates, as well as differences in sexual dimorphism and species size evolution rates, I performed ANOVA and Kruskal-Wallis tests on independent contrasts generated by PDAP.

Randomization tests for phylogenetic signal. —The basic idea of the randomization tests is to ask whether a given phylogeny better fits a set of tip data as compared with the fit when the data have been randomly placed across the tips of the tree by permutation, in this way destroying any phylogenetic signal that may have existed. The detection of phylogenetic signal in a set of comparative data implies a tendency for related species to resemble each other, indirectly indicating slow rates of character change across phylogeny (Blomberg et al. 2003). All randomization tests (1000 permutations) were performed in MatLab module PHYSIG (Blomberg et al. 2003) for each character individually. Due to concerns about the statistical power of the randomization analysis (which performs poorly with fewer than 20 species), the Mephitinae (six species) clade was ignored in this analysis. Because of the same concerns, and to support findings of subclade analyses in different parts of phylogeny, Mustela (11 species) and Martes with related genera (15 species) were combined together in a larger monophyletic clade of Mustelinae (27 species), as well as Lutrinae (11 species) and Mephitinae (six species) were grouped together as a monophyletic clade of 22 species.

RESULTS

Tips analysis. —At the family level, n = 49 (Fig. 1), male and female sizes were highly correlated ($r = 0.99$, $p < 0.05$), species size and sexual dimorphism showed significant negative relationship ($r = -0.382$, $p = 0.007$). Within *Mustela*, species size and sexual dimorphism showed no significant correlation ($r = 0.19$, $p = 0.57$, $n = 11$). *Martes* with closely related genera, like *Mustela*, had no significant relationship between size and sexual dimorphism ($r = 0.20$, $p = 0.47$, $n = 15$). The *Lutrinae* and *Mephitinae* clades showed no significant relationship between sexual dimorphism and species size as well ($r = 0.372$, $p = 0.260$, $n = 11$; $r = -0.49$, $p = 0.328$, $n = 6$ respectively). The sizes of both sexes were highly correlated in all subclades ($r = 0.99$, $p < 0.05$).

Felsenstein's independent contrasts. —Mustelidae-wide, there was no significant relationship between species size and sexual dimorphism ($r = 0.10$, $p = 0.5$). The *Mustela* clade showed no apparent relationship between dimorphism and size ($r = 0.11$, $p = 0.77$). *Martes* with related genera exhibited no relationship between size and dimorphism ($r = 0.49$, $p = 0.08$). *Lutrinae* showed significant positive allometry for sexual size dimorphism ($r = 0.67$, $p = 0.049$). Size and dimorphism were not significantly correlated in *Mephitinae* ($r = -0.16$, $p = 0.85$). Male and female body sizes were significantly correlated in all clades ($p < 0.05$). Tests based on two different models of evolution (gradual vs. punctuational) gave similar results.

Rate tests for character evolution. —The ANOVAs and non-parametric Kruskal-Wallis tests found no significant differences in rates between sexual dimorphism and body size within clades ($p > 0.05$). The Kruskal-Wallis tests for differences in character evolution rates among clades found no significant differences in rates of species size or dimorphism

evolution. However, among-clade comparisons of sex sizes revealed that *Lutrinae* are different from *Mustela* and *Martes* with related genera in rates of male body size evolution ($p = 0.034, 0.026$ respectively), corresponding differences between female body size rates were not significant ($p = 0.064, 0.074$).

Randomization tests for phylogenetic signal. —At the family level, female size showed more phylogenetic signal than male size ($k = 1.20, 1.02$ respectively). The size of species had stronger phylogenetic signal than sexual dimorphism ($k = 1.10, 0.63$ respectively). In general, species size showed strong phylogenetic signal in all clades except for *Lutrinae*. Within subclades, no phylogenetic signal in sexual dimorphism was detected ($p > 0.05$). Male size within subclades exhibited a strong signal, except for *Lutrinae*, where it was not significant. Female size within clades was constrained more than male size, it was not significantly constrained in *Lutrinae*. Combining *Mustela* and *Martes* with related genera into one clade *Mustelinae* produced results that were consistent with results from individual clades. Combining *Lutrinae* and *Mephitinae* into one clade gave the same results as *Mustelinae*.

Discussion

Only the *Lutrinae* clade had a significant pattern of allometry consistent with Rensch's rule. Thus, Rensch's rule cannot be applied to the whole family Mustelidae (therefore supporting results of Abouheif and Fairbairn (1997)) or any other subclade within the family.

Based on randomization tests, sexual dimorphism did not show strong phylogenetic signal (phylogenetic inertia or constraint) within clades, although at the family level species

tended to resemble each other in the degree of sexual dimorphism. Species size showed a strong phylogenetic signal in all clades except for *Lutrinae*, suggesting that body size in otters has rapid rates of evolution compare to other clades. Species size seemed to be more constrained than sexual dimorphism in all cases.

The observed phylogenetic constraint (e.g., measured as a strength of phylogenetic signal) was always a variable property of a clade, which could be changed by evolutionary events in particular parts of phylogeny. The phylogenetic signal observed on a large scale (e.g., Mustelidae-wide) clearly depended on smaller evolutionary events within individual subclades. This suggests that phylogenetic inertia should not be viewed as a direct constraint by phylogeny in sense of Cheverud (Cheverud et al. 1985). It merely indicates the presence of phylogenetic signal, tendency of related species to resemble each other, possibly due to slow rates of evolutionary change in particular groups of species.

Male size was less constrained in its evolution than female body size. Comparisons among clades revealed that in all clades female body size evolves at the same rates. Any differences in sexual dimorphism among clades could be attributed solely to different rates of male body size evolution. *Lutrinae*, in particular, exhibited greater rates of male size divergence.

Collectively, these results suggest that Rensch's rule pattern observed in *Lutrinae* originated historically as a result of differential rates of size evolution in males and females, where male otters tended to have higher rates of body size evolution than females, in contrast to more equal sex size evolution in other mustelid clades.

No current model satisfactorily explains the causal link between size and dimorphism (Plavcan 2001). A combination of factors may explain differential evolution of body sizes in

males and females (Fairbairn 1997). Intrasexual selection is one of them, driving the evolution of sexual size dimorphism due to increase in male size, where increase in male size is decisive in gaining access to females (Darwin 1871; Moors 1980; King 1990). In addition, there could be fewer competing species and more resources at larger sizes, freeing males to increase size without competition for food (Plavcan 2001). Body size of females may be constrained for reproductive reasons. Specifically, mustelid females are known to be under selection for smaller size in order to achieve early maturation so that they can breed at a younger age (Erlinge 1979; Ralls and Harvey 1985). These explanations are not mutually exclusive, and selection may act simultaneously on body sizes of males and females in one clade. As the results indicate, at least female explanation may be plausible for mustelids, since rates of female size evolution were equally slow in clades of varying body sizes.

These results revealed historical patterns of size evolution, only as an outline of differential evolutionary mechanisms in males and females responsible for the dimorphic pattern observed in present-day species. Further clarification of the mechanisms causing enhanced rates of male size evolution in otters and relatively constrained female size evolution in mustelids in general requires causal hypothesis testing at the population level (Lauder 1996). Since size dimorphism is the direct result of different growth patterns in males and females, comparative studies of mechanisms producing different growth patterns in the sexes may also hold a great promise for resolving the pattern behind Rensch's rule.

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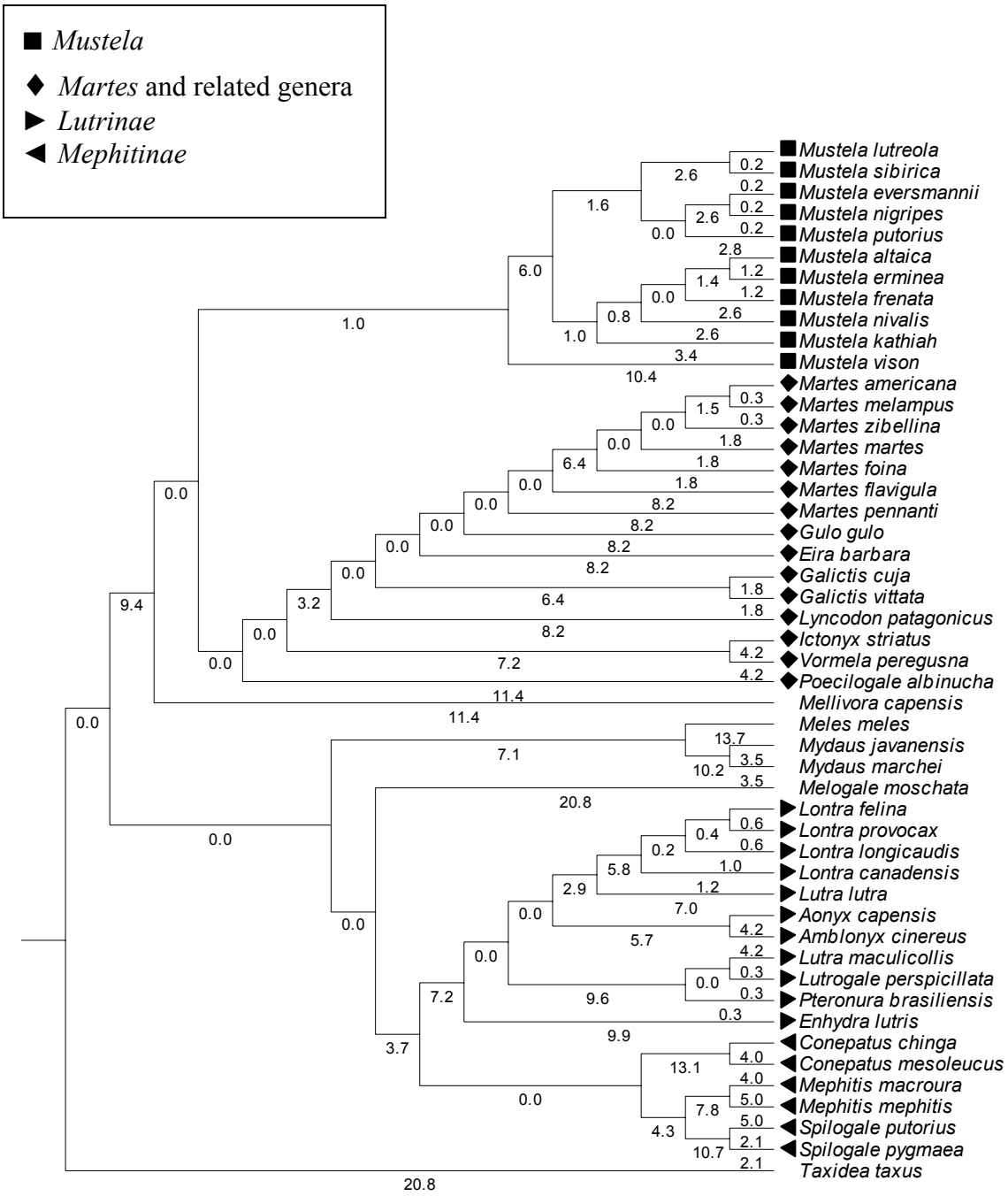
TABLE 1. Taxa, data on male and female body sizes, as well as calculated mean species sizes and sexual size dimorphism index used in this study.

Species	Male mass (kg)	Female mass (kg)	Species mean mass (kg)	Sexual size dimorphism
Mustela lutreola	1.03	0.69	0.86	0.17
Mustela sibirica	0.52	0.31	0.42	0.23
Mustela eversmannii	1.33	0.65	0.99	0.31
Mustela nigripes	1.02	0.81	0.92	0.10
Mustela putorius	1.38	0.65	1.02	0.33
Mustela altaica	0.3	0.22	0.26	0.13
Mustela erminea	0.21	0.12	0.17	0.24
Mustela frenata	0.31	0.17	0.24	0.26
Mustela nivalis	0.07	0.047	0.06	0.17
Mustela kathiah	0.36	0.2	0.28	0.26
Mustela vison	1.19	0.71	0.95	0.23
Martes americana	0.97	0.77	0.87	0.10
Martes melampus	1.56	1.01	1.29	0.19
Martes zibellina	1.35	1.03	1.19	0.12
Martes martes	1.31	0.98	1.15	0.13
Martes foina	1.83	1.45	1.64	0.10
Martes flavigula	2.5	2.5	2.50	0.00
Martes pennanti	4.05	2.25	3.15	0.26
Gulo gulo	14.8	10.6	12.70	0.15
Eira barbara	4.85	3.4	4.13	0.16
Galictis cuja	1.85	1.85	1.85	0.00
Galictis vittata	2.35	2.35	2.35	0.00
Lyncodon patagonicus	0.23	0.23	0.23	0.00
Ictonyx striatus	0.97	0.71	0.84	0.14

Vormela peregusna	0.67	0.53	0.60	0.10
Poecilogale albinucha	0.5	0.34	0.42	0.17
Mellivora capensis	8.58	7.59	8.09	0.05
Meles meles	12.32	10.9	11.61	0.05
Mydaus javanensis	2.44	2.44	2.44	0.00
Mydaus marchei	2.5	2.5	2.50	0.00
Melogale moschata	1.2	1.08	1.14	0.05
Lontra felina	4	4	4.00	0.00
Lontra provocax	7.5	7.5	7.50	0.00
Lontra longicaudis	10	7.5	8.75	0.12
Lontra canadensis	8.1	6.7	7.40	0.08
Lutra lutra	9.6	6.75	8.18	0.15
Aonyx capensis	13.7	11.9	12.80	0.06
Amblonyx cinereus	3.5	3.5	3.50	0.00
Lutra maculicollis	4.38	3.5	3.94	0.10
Lutrogale perspicillata	10.29	7.3	8.80	0.15
Pteronura brasiliensis	26	24	25.00	0.03
Enhydra lutris	33.5	23.5	28.50	0.16
Conepatus chinga	2.37	1.47	1.92	0.21
Conepatus mesoleucus	2.13	1.89	2.01	0.05
Mephitis macroura	0.89	0.72	0.81	0.09
Mephitis mephitis	2.8	2	2.40	0.15
Spilogale putorius	0.4	0.28	0.34	0.16
Spilogale pygmaea	0.5	0.23	0.37	0.34
Taxidea taxus	8.6	5.62	7.11	0.18

FIGURE 1. Composite phylogeny of family Mustelidae (by Bininda-Emonds et al., 1999), with four monophyletic subclades used in the analyses. All branch lengths are in millions of years before present. Branches of length zero indicate soft polytomies.



Chapter 3

Pelvic Growth: Ontogeny of Size and Shape Sexual Dimorphism in Rat Pelves

ABSTRACT

The mammalian pelvis is sexually dimorphic with respect to both size and shape. Yet little is known about the differences in postnatal growth and bone remodeling that generate adult sexual dimorphism in pelvic bones. We used Sprague-Dawley laboratory rats (*Rattus norvegicus*), a species that exhibits gross pelvic size and shape dimorphism, as a model to quantify pelvic morphology throughout ontogeny. We employed landmark-based geometric morphometrics methodology on digitized landmarks from radiographs to test for sexual dimorphism in size and shape, and to examine differences in the rates, magnitudes and directional patterns of shape change during growth. Based on statistical significance testing, the sexes became different with respect to pelvic shape by 36 days of age, earlier than the onset of size dimorphism (45 days), although visible shape differences were observed as early as at 22 days. Males achieved larger pelvic sizes by growing faster throughout ontogeny. However, the rates of shape change in the pelvis were greater in females for nearly all time intervals scrutinized. We found that trajectories of shape change were parallel in the two sexes until age of 45 days, suggesting that both sexes underwent similar bone remodeling until puberty. After 45 days, but before reproductive maturity, shape change trajectories diverged due to specific changes in the female pelvic shape, possibly due to the influence of estrogens. Pattern of male pelvic bone remodeling remained the same throughout ontogeny, suggesting that androgen effects on male pelvic morphology were constant and did not contribute to specific shape changes at puberty. These results could be used to direct additional research on the mechanisms that generate skeletal dimorphisms at different levels of biological organization.

KEYWORDS: pelvis, geometric morphometrics, growth, sexual dimorphism, size, shape

INTRODUCTION

Sexual dimorphism exists between the sexes of any species as differences in either shape or size of a structure. While dimorphism in some characters in mammals exists at birth, the difference between the sexes more typically develops over the course of ontogeny. Because many sexually dimorphic characters are relevant to mate choice, reproduction, and other adult functions, the ontogenetic history of dimorphic characters is often overlooked. Yet such a study of ontogeny can provide insight into the evolutionary origin of sexual dimorphism, as well as into the physiological basis, at the tissue, cellular or genetic level, for the generation of such dimorphism.

The mammalian pelvis is formed from the fusion of three endochondral bones (the ilium, ischium, and pubis) into the bilaterally paired innominate bones. This complex structure differs in both shape and size between the sexes in nearly all mammalian species, including those that do not have gross body size or shape sexual dimorphism (Arsuaga and Carretero, 1994; Chapman et al., 1994; Iguchi et al., 1995; Krystufek, 1998; Tyler, 1987). Sexual dimorphism of the pelvis is commonly held to be a consequence of its differential role in male and female reproduction. Since neonates pass through the female birth canal, formed by pelvic bones, selection for fitness at parturition drives shape differences between the sexes (Hausler and Schmidt, 1995; LaVelle, 1995; Leutenegger and Larson, 1985; Leuttenegger, 1974; Ridley, 1995; Wood and Chamberlain, 1986).

Although comparative studies support this parturition explanation for adult pelvic shape dimorphism, little is known about differences between the sexes in the postnatal growth of the pelvis, or the relationship of the timing of sexual dimorphism during growth to other endocrinological events that are critical for reproduction. The best data exist for

humans, where some sexual differences in size exist prenatally (Merrot et al., 2001), and persist through childhood (LaVelle, 1995). Yet, during adolescence the pelvis undergoes significant remodeling in both shape and size, presumably, caused by the changing hormonal milieu (Tague, 1995). Studies of mice and rats suggest both pre- and post-nataly significant roles for sex steroids in the generation of sex-specific pelvic shapes (Bernstein and Crelin, 1967; Iguchi et al., 1989; Tague, 1995; Uesugi et al., 1992).

A simplistic understanding of the current theory of the ontogeny of pelvic shape dimorphism, from an endocrinological perspective, is that the female shape is the default and all or some aspects of male differences are androgen dependent. Hormone manipulation studies have repeatedly shown that certain dimorphic features are affected by removal of sex hormones, while others are not. Of eleven sexually dimorphic features of the adult rat pelvis described by Bernstein and Crelin (1967), six develop normally (i.e., as in controls) in males castrated at birth and five depend on presence of gonadal androgens. Ovariectomy at birth results only in one change in the pelvis of females: the length of the pubic symphysis is significantly increased, a feature thought to be dependent on estrogens. Similarly, Uesugi et al. (1992) show that gonadectomy of males at either 10, 15, 20, 30, or 60 days of age results in no difference from controls in elongation and broadening of the ischium, and elongation of the pubis, again suggesting that not all aspects of male pelvic growth are dependent on androgens. Removing androgens in mice prior to 60 days of age inhibits morphogenesis of the male form, but later gonadectomy has no effect: the overall male form persists.

Androgen receptors are known to be present in osteoblasts, osteoclasts, and the growth plate chondrocytes of periosteal and trabecular bone tissue. In males, androgen levels are low and relatively unchanging from birth until puberty, at which time they increase

(Selmanoff et al., 1977). Androgens have been shown to have a global role in promoting the accrual of bone mass at puberty, and the maintenance of bone in adulthood. The role of androgens in pelvic growth, at specific developmental stages and tissues, is largely unknown. However, there is now wide spread agreement among researchers of human bone growth that the role of androgens in promoting the pubertal growth spurt and skeletal maturation is minimal, and less critical than previously thought (Grumbach, 2000).

Existing evidence of estrogen receptor (ER) distribution in the pelvis is contradictory. As noted, Uesugi et al. (1992) report that by 60 days of age all pelvic bones in the mouse express estrogen receptors, although at that time there was no distinction between ER-alpha and ER-beta, two forms suggested to have different actions in bone growth and maintenance (Nilsson et al., 2001; Rickart et al., 1999). Karsenty's (1999) review of the literature, however, suggests that that there are relatively few estrogen receptors in bone tissue. Prior to puberty, there appears to be no role for estrogen in bone growth in either sex as shown by the result that bone phenotype in the estrogen receptor knock-out mouse is unaffected prior to puberty (Nilsson et al., 2001). At puberty, estrogen promotes bone growth and the accrual of bone mass. Later in puberty, estrogen stops further growth by stimulating the fusion of the epiphyses. During adulthood, estrogen is critical in bone maintenance. It is now well established that both male and female skeletal growth is affected by estrogen, though the timing of these effects during puberty differs between the sexes. In fact, in humans "the evidence currently available supports a critical role for estrogen, and not testosterone, in the pubertal growth spurt of the male and in the development of normal skeletal proportions" (Grumbach, 2000).

Testing specific hypotheses about the developmental timing of morphological

differences between the sexes is an important step in understanding the mechanisms producing skeletal sexual dimorphism. While providing valuable clues about the timing of pelvic dimorphism, the previously mentioned endocrinological studies did not intend to address questions about the onset of shape and size sexual dimorphism, or reach a complete understanding of the trajectories of ontogenetic shape change in the sexes. There are additional drawbacks in studies that attempt to describe the sexual dimorphism in growth of skeletal elements such as the pelvis. The pelvis is an anatomically complex structure. Studies employing conventional morphometric metrics, based on such shape approximations as angles, linear distances, and their ratios, capture shape incompletely, inevitably lose information, and are hard to interpret and compare (Iguchi et al., 1995; Walranth and Glantz, 1996; Wood and Chamberlain, 1986). An equally significant problem is the conflation of dimorphism in shape and size. Attempts to scale for body size, e.g., taking ratios to whole body length, present statistical problems that compromise the analytical results (Albrecht, 1978; LaBarbera, 1989). The consequence of these methodological drawbacks is that pelvic shape is not well described, either at a single point in time, or over the course of an animal's growth.

Our objective was to quantify pelvic morphology over ontogeny and to test hypotheses about the ontogenetic origins of pelvic dimorphism, specifically examining size and shape dimorphism independently. We hypothesized that the rates and patterns of ontogenetic shape change would not be the same in males and females, that both size and shape dimorphism would be evident only after the onset of puberty, and that pelvic size and shape dimorphisms would be firmly established at reproductive maturity. To avoid the methodological problems of previous studies, we applied landmark-based geometric

morphometrics to longitudinal radiographic data collected from Sprague-Dawley rats (*Rattus norvegicus*), a species that exhibits gross size sexual dimorphism. Results from this study will serve as a baseline against which additional hypotheses about the effects of genetic and environmental factors that perturb growth and alter sexual dimorphism may be tested.

MATERIALS AND METHODS

Animals and Husbandry

Data used in this study were collected from a sample of Sprague-Dawley laboratory rats (*Rattus norvegicus*) used in a larger, longitudinal study on gross somatic growth (Miller and German, 1999; Reichling and German, 2000) conducted in the Department of Biological Sciences, University of Cincinnati. University of Cincinnati IACUC approved all husbandry and procedures (protocol #91-05-27-01). Rats were maintained on a 12:12h light:dark cycle, had *ad lib.* access to food and water, and were housed individually in conventional shoebox cages with standard rodent bedding. We used a specially formulated diet (#111147 *Dyets*, Bethlehem, PA) based on AIN-93G standard. This diet had 3.4kCal/kg, with 27.6% calories by weight from protein (casein), and supported maximum growth rates associated with increased protein uptake (Edozier and Switzer, 1978). All rats were weighed daily (Ohaus Lume-O-Gram Lo-Pro, Ohaus Scale, Florham, NJ) to ensure good health and to provide data for other analyses.

Radiography

Beginning when rats were weaned at age 21days (21d), we radiographed each in the dorsoventral and lateral planes three times per week. Briefly, animals were lightly anaesthetized in a small induction chamber using an Ohio Compact Anesthesia Machine (Anaquest, Liberty Corner, NJ) delivering 2-3.5% isoflurane per liter of O_2. Depending on

the size of the rat, we set kV equal to 44-47 and mA at 75 for ¼ sec. using a Bennett Mammography Machine (Bennett X-Ray, Copiague, NY). Once anesthetized, rats were placed on a cassette loaded with Kodak MRM-film, aligned based on the skull bi-lateral axis of symmetry and the vertebral column, and radiographed. Rats recovered from the anesthesia within minutes. This procedure results in no harmful effects to the animals and does not affect skeletal growth (Fiorello and German, 1997). As growth slowed, the frequency of radiography sessions decreased, ending when an accurate estimate of final size was determined and fusion of femur epiphyses was evident in radiographs. For this study only radiographs in the dorsoventral view were used.

Data Collection

From each animal, greater than 50 radiographs were taken between ages 21-150d. Because it was difficult to position each animal in an absolutely bi-laterally symmetrical fashion with respect to pelvis orientation (left-right *ossa coxae*), many radiographs were deemed unacceptable for use in this study. We therefore used a cross sectional subset of 100 radiographs (44 from males, 56 from females) taken during five discrete age ranges. Table 1 provides age ranges and number of radiographs used for each sex. These age ranges corresponded to developmental time points along animals' growth trajectories, and were chosen based on body mass differences between males and females over time (Miller and German, 1999; Reichling and German, 2000), and known maturation events (Reichling, 1999). At **age group 1,** (22-24d) there are no differences in body mass between the sexes. The onset of puberty occurs just prior to age 40d, and thus in **age group 2** (31-36d) we predict that the hormonal milieu to be differentially changing, possibly impacting the rate of pelvic elements' growth. Gross weight dimorphism occurs at **age group 3** (45-50d). Both

males and females are fully reproductive by **age group 4** (60-65d), and have achieved overall adult skeletal size by **age group 5** (86-93d).

INSERT TABLE 1 ABOUT HERE

Twenty-four homologous and repeatable anatomical landmarks (Fig. 1, Table 2), consistently visible on all radiographs, were converted to Cartesian co-ordinates by digitizing radiographs in the *DIGIT* program (written by David Hertweck). These landmarks provided comprehensive and even coverage of the entire pelvic area.

INSERT FIGURE 1 ABOUT HERE

INSERT TABLE 2 ABOUT HERE

Data Analysis

A combination of landmark-based geometric morphometrics analyses was used to address questions concerning the ontogeny of pelvic *shape* dimorphism: testing for shape differences at discrete age groups, testing for differences in the direction of shape change over time, and testing for differences in rates (magnitudes) of shape change. To summarize these analyses, PCA was used as an exploratory step, two-way MANOVA and Goodall's F-test were used to test for differences between sexes and among age groups, MANCOVA compared directions of shape change, and linear regressions with Procrustes distances compared rates of shape change. Details of data analyses follow.

First, we carried out Generalized Least-Squares Procrustes superimposition (GLS) of digitized landmark coordinates in the program *CoordGen6f* of the IMP series software (Sheets, 2004). This procedure quantifies shape by removing such "nuisance" parameters as initial differences in centroid size, position and orientation of the specimens (Rohlf and Slice, 1990; Rohlf, 1996), and defines the shape of each specimen in terms of Procrustes residuals,

which serve as the starting point of the statistical analysis of shape. The superimposed coordinates represent the locations of shapes in the multidimensional shape space defined by the number of landmark coordinates in a single shape (Rohlf, 1996). This shape space is non-Euclidean and has fewer dimensions than there are landmark coordinates. To permit the use of conventional multivariate analyses for the landmark coordinates, specimens located in the non-Euclidean Kendall's shape space were projected onto a Euclidean space that is tangent to shape space and has the same number of dimensions. Following Bookstein (1996), the location of a sample's average shape was used as the point of tangency (reference shape) for all analyses of the sample. Before applying multivariate methods, we used the *tpsSmall* 1.20 program (Rohlf, 2003) to determine whether the amount of variation in shape contained within our data set was small enough to permit statistical analyses to be performed in the linear tangent space, approximating non-linear Kendall's shape space. The correlation between Procrustes distances in shape space and the corresponding distances in tangent space was 1.0 and the regression slope through the origin was 0.999574. This indicates that there were only small amounts of variation in specimen shape space locations and negligible distortion was introduced by projection of shape space distances into Euclidian space.

As an exploratory tool, we used a principal component analysis on Procrustes residuals to visualize and describe the ontogenetic shape trajectories of each sex in multivariate shape space (Berge and Penin, 2004; Cobb and O'Higgins, 2004; Dryden and Mardia, 1998; Mitteroecker et al. 2004). Projection of Procrustes residuals onto principal components gave principal component scores that could be plotted to examine patterns of shape similarity and difference between superimposed landmark configurations. Variation along the first principal axis, which was closely correlated with temporal scale, represented

ontogenetic variation in shape for data quantifying growth. Means of each group (each sex at a given age group in this case) in the multivariate shape space were connected by vectors to provide better visualization of the direction and magnitude of shape change. Principal components analysis was carried out in the *PCAGen6l* program of the IMP software (Sheets, 2004).

To proceed with multivariate statistical analyses testing for significant differences in shape variation between groups, a thin-plate spline interpolation function was used to describe the shape of each specimen in the sample in relation to the GLS consensus configuration (i.e., the reference point, which is the tangency point between shape space and the linear tangent space) by a set of forty-two partial warps representing non-uniform components and two uniform components (Bookstein, 1996). These calculations were performed using the *tpsRegr* 1.28 program (Rohlf, 2003). The non-uniform components represent regionally differentiated, and localized aspects of shape variation; the uniform components, however, are descriptors of shape change of the whole organism, measuring shape effects that apply anywhere in the form (Zelditch et al., 2004). To test if the shapes were different between the sexes and age groups, we used a two-way multivariate analysis of variance (MANOVA) with complete sets of the non-uniform and uniform components of shape change as dependent variables. Age group and sex were the independent variables, with five age levels and two sex levels. We also looked at the interaction terms between sex and age. An interaction in this case meant that the shape change in males through time was different from the shape change in females through time, implying that the direction of shape change was not concordant. Following Adams and Funk (1997), in addition to performing a MANOVA on all components of shape at the same time, we repeated the analysis on the

non-uniform and uniform components separately to assess to what extent localized or global changes influenced ontogenetic shape variation in the two sexes. Based on initial exploration of ontogenetic shape trajectories, a visible divergence between sex trajectories occurred at age group 3. Therefore, we divided the single trajectory of each sex into two half-trajectories, one spanning age groups 1, 2 and 3, and another spanning age groups 3, 4 and 5. Two-way MANOVAs were performed in SYSTAT 10 (Wilkinson, 2003), on matrices of partial warp scores and uniform components computed and saved from the *tpsRegr* 1.28 program (Rohlf, 2003).

Using the same matrices of shape components, we further compared the regression slopes of shape on age for each sex to understand shape dimorphism. We used a MANCOVA model with sex as categorical variable and age as covariate in *tpsRegr* 1.28 (Rohlf, 2003). These regressions were tested for differences in the slope of the shape regression on age and differences in the intercept of these regressions, whenever they proved to have homogeneous slopes. MANCOVAs were performed for both single vector trajectories and half-trajectories of ontogenetic shape change.

Following these analyses, we performed pair-wise tests of shape differences between the sexes (Adams and Funk, 1997; Douglas et al., 2001; Zelditch et al., 2003) at a range of age groups, to determine which groups specifically exhibited differences in shape. The statistical significance, using Bonferroni adjustment ($p<0.002$), of pair-wise shape differences was tested by bootstrap version of Goodall's F-test (1600 bootstraps). We performed all pair-wise tests with *TwoGroup6e* (Sheets, 2004). In addition, Procrustes distances were calculated between mean forms of the sexes at particular age groups to assess the magnitude of sexual dimorphism. Procrustes distances are the sums of squared distances

between corresponding homologous landmarks of GLS-superimposed configurations (Rohlf and Slice, 1990). Ninety-five percent confidence intervals were placed to test for significant differences in Procrustes distances between age groups.

The analyses above dealt with both magnitude and direction of shape change. To dissociate the magnitude from direction and examine shorter time intervals (spanning only two age groups at a time), we performed univariate magnitude tests with Procrustes distances. To estimate the magnitude of shape change, for each sex, we calculated Procrustes distances between the shape of the smallest individual and each individual shape in the ontogenetic data set. Each set of Procrustes distances was then regressed against the animal's age to yield estimates of the slopes representing the rate of change of shape over time (Zelditch et al., 2000; Zelditch et al., 2003). Rates of shape change were estimated within time intervals spanning just two age groups at a time (i.e., between age groups 1 and 2, 2 and 3, 3 and 4, 4 and 5). This design, based on short time intervals, was developed to satisfy the assumption of a linear relationship between shape change and age. Constructing bivariate plots of Procrustes distance versus age also tested the assumption of linear increase of Procrustes distance with age. This was an important consideration because our rate comparisons were based on linear regression. The resulting short magnitude trajectories were compared in a pair-wise manner between sexes at corresponding time intervals. As we did to calculate sexual dimorphism at particular age groups, we also calculated Procrustes distances between mean shapes of the sexes of adjacent age groups to estimate magnitudes of change within these same time intervals (Zelditch et al., 2004). Ninety-five percent confidence intervals on each slope and distance were estimated by bootstrapping with 2500 repetitions. Between- and within-sex comparisons of regression slopes and distances representing

different growth trajectory intervals were based on comparing 95% confidence intervals for statistical significance. Procrustes distances from the reference were calculated in *Regress6f* (Sheets, 2004), their bootstrap slopes were compared in SYSTAT 10 (Wilkinson, 2000), and Procrustes distances between group means and their confidence intervals were calculated using *TwoGroup6e* (Sheets, 2004).

Two analyses addressed questions concerning pelvic *size* dimorphism: testing for sex differences in size within each of the five age groups, and testing for sex differences in the rates of pelvic size increase through incremental age groups. Pelvic size was represented by centroid size, the sum of squared distances of each landmark from the centroid (the average position of all landmarks in the configuration). Centroid size is the only measure of size not correlated with shape and therefore appropriate in geometric morphometric analyses, where shape is the property of a figure invariant to the scale, position and rotation (Bookstein, 1991; Bookstein, 1996).

RESULTS

Ontogenetic Shape Trajectories

Based on a principal component analysis summarizing variation in multivariate shape space, the ontogenetic trajectories of shape change were not the same for males and females (Fig. 2). Trajectories spanning age groups 1, 2 and 3 appeared to be parallel, however not coincidental. At age group 3 (45-50 days) the female trajectory departed from relative linearity, apparently following a sex-specific pattern of shape change, at the same time the male trajectory remained the same. Clearly, complete shape trajectories of sexes should not be described as single vectors because the patterns of shape change are complex. Trajectories appeared to have the same start point with overlapping groupings of males and females,

although females started with higher scores on the first principal component and appeared to have longer trajectory.

INSERT FIGURE 2 ABOUT HERE

Shape Differences Due to Sex and Age

Two-way MANOVAs of shape variables, with sex and age as factors, revealed statistically significant differences ($p < 0.01$) between the sexes and among age groups (Table 3). Working with all groups together, we found significant differences between sexes and among age groups. The interaction between sex and age was also significant, implying that directions of male and female shape change differ significantly. The interaction term for the uniform component was not significant, but interaction for the non-uniform component was, implying that directions of shape change were different due to localized, sex-specific changes only. Based on our principal component exploration of shape trajectories we also examined half-trajectories (spanning groups 1,2,3 and groups 3,4,5). The trajectories spanning groups 1,2,3 showed significant differences between sexes and among age groups, but the interaction term was not significant for any component of shape change, implying that each sex had same pattern of shape change through age group 3. Trajectories for each sex spanning groups 3,4,5 (after reproductive age) also exhibited significant differences in size and age. As with the younger age groups, the interaction term for non-uniform shape component was significant, but the interaction term for uniform component was not significant, implying that from this age on sexes underwent directionally different regional shape modifications. These results were consistent with our interpretation of principal component plot visualizing shape trajectories.

INSERT TABLE 3 ABOUT HERE

Pair-wise tests performed with Goodall's F-tests ($p < 0.002$ following Bonferroni adjustment), with bootstrapping used for significance testing, indicated a lack of sexual dimorphism in shape at age group 1 (Table 4). However, all consecutive ages had significant shape differences between sexes.

INSERT TABLE 4 ABOUT HERE

Procrustes distances between mean male and female shapes at particular age groups indicated that the amount of difference between the sexes increased over time to generate adult shape dimorphism (Table 4). The correlation between sexual dimorphism in shape and age was 0.9948. We acknowledge that this test was not an explanation of how the dimorphism arises (multivariate analyses in this paper do), but it showed that the sexual dimorphism, estimated as a Procrustes distance, was a function of age.

INSERT FIGURE 3 ABOUT HERE

We visualized the sexual dimorphism implied by the principal component axes as wireframe displacements of the mean pelvic shape (Fig. 3A). There was general increase in iliac crest - ischial tuberosity distance in males, narrowing of pelvic bones in females, as well as all following changes in females relative to males: strong increase in interischial distance in the female, shortening of the ischium, shortening of the pubic symphysis, caudo-medial displacement of acetabular area and lateral displacement of iliac blades.

Directionality of Shape Change

Applying MANCOVA design for all age groups, slopes of regressions of shape on age were different between the sexes (Wilks $\Lambda = 0.2622$, $p = 2 \times 10^{-5}$). For the uniform component of shape change, the slopes of the sexes did not differ (Wilks $\Lambda = 0.9890$, $p = 0.5903$), although the intercepts were different (Wilks $\Lambda = 0.8715$, $p = 0.0014$). For the non-

uniform component representing regional pattern of shape change, the slopes of regressions of shape on age were different between sexes (Wilks $\Lambda = 0.2706$, $p = 8 \times 10^{-6}$).

For the portion of the data set spanning age groups 1, 2 and 3, no differences were found between the slopes of shape regression on age (Wilks $\Lambda = 0.1228$, $p = 0.2083$). However, shape was significantly sexually dimorphic when age was held constant (Wilks $\Lambda = 0.0455$, $p = 0.0026$) meaning that the intercept for males and females differed. This suggested that until reproductive age, males and females varied along separate, although parallel, ontogenetic trajectories. Results on the uniform and non-uniform shape components were consistent: slopes of shape regression on age were not different between the sexes (uniform: Wilks $\Lambda = 0.8894$, $p = 0.4742$; non-uniform: Wilks $\Lambda = 0.1293$, $p = 0.1101$), although the intercepts differed (uniform: Wilks $\Lambda = 0.7549$, $p = 0.0006$; non-uniform: Wilks $\Lambda = 0.4754$, $p = 0.0005$).

Regressions of shape on age for the portion of the data set covering age groups 3, 4 and 5, showed that the slopes were significantly different between males and females (Wilks $\Lambda = 0.0683$, $p = 0.0006$), meaning that the sexes had different ontogenetic trajectories of shape change. However, the uniform component of shape changed in the same way in males and females (Wilks $\Lambda = 0.8929$, $p = 0.0374$), and intercepts were not different as well (Wilks $\Lambda = 0.8701$, $p = 0.0165$). The non-uniform component of shape changed differently in males and females (Wilks $\Lambda = 0.0842$, $p = 0.0004$). Apparently, localized changes in pelvic shape caused ontogenetic shape divergence between sexes at age group 3.

INSERT TABLE 5 ABOUT HERE

Rates of Shape Change

Comparisons of rates of shape change revealed that shape change between age groups

1 and 2 was greater in females than in males, rates between age groups 2 and 3 were the same in both sexes, shape change in females was greater between age groups 3 and 4, and it was less in females than in males between age groups 4 and 5 (Table 5). The same comparisons based on calculations of Procrustes distances traveled between adjacent age groups, instead of slopes representing rates of shape change, gave somewhat different results. Females appeared to have greater magnitudes of shape change than males for the first three time intervals (between age groups 1-2, 2-3, 3-4), and less magnitude than males between age groups 4 and 5, which was roughly consistent with rate estimates. However, none of these differences were statistically significant based on comparisons of confidence intervals. Differences between results of both approaches may be explained by the different sources of uncertainty that enter into the estimates of the rates (Zelditch et al., 2004), and due to the large confidence intervals surrounding the estimates of Procrustes distances. Both regressing Procrustes distance on age and computing Procrustes distances traveled between age groups within each sex revealed that the magnitude of pelvic shape change consistently decreased over time in both males and females (Table 5). In general, the patterns of shape and size change were opposite: when males were increasing in size (growing) faster than females, they were changing shape at a slower rate than females.

INSERT FIGURE 4 ABOUT HERE

Interpretation of Shape Changes

Based on the results of multivariate analyses and the exploration of shape space, ontogenetic shape changes in the two sexes between age groups 1 and 3 (parallel trajectories of shape change), and age groups 3 and 5 (divergent trajectories) were visualized as wireframe displacements and compared, revealing specific shape changes that generated

adult sexual shape dimorphism (Fig. 4).

For males, there was an elongation and widening of the iliac bones, caudal shortening and narrowing of the pubic bones, and elongation and narrowing of the ischial bones between age groups 1 and 3. The acetabular area underwent shortening in the caudal direction, allowing the iliac bones to elongate. The interischial distance (subpubic angle) decreased, therefore reducing the pelvic outlet area. The pubic symphysis elongated in cranial direction. This pattern of shape change remained the same between age groups 3 and 5, but was of a lesser magnitude (Fig. 4A).

In females, the pelvic shape changes between age groups 1 and 3 were essentially same as in males, although somewhat accelerated. However, between age groups 3 and 5, the pattern of shape change was altered: the interischial distance increased rather than decreased, the pubic symphysis moved caudally, and the ischial bones shortened rather than elongated (Fig. 4B).

Age-related shape changes common to both sexes, as implied by principal component axes, were also visualized as wireframe displacements of the mean pelvic shape (Fig. 3B). There was general narrowing of the ischial and pubic bones, elongation and widening of iliac bones, and elongation of the pubic symphysis in the pelvic shape of both sexes as animals aged.

Sexual Size Dimorphism

Pair-wise comparisons of centroid size between the sexes at each age group indicated that the sexes became significantly different with respect to pelvic size at age group 3 ($p < 0.01$) (Table 4). There were also significant differences in the rates of size increase between and within the sexes across age groups ($p < 0.05$) (Table 5). Regressing centroid size on age,

and comparing the regression slopes of incremental age intervals, showed that both sexes grew at a slower rate as they aged (Table 5). For example, in males the slope of size increase from age groups 1-2 (0.278) was significantly greater than the slope from age groups 2-3 (0.224). Males had consistently higher rates of growth than females throughout all time intervals. Both sexes continued to increase in size through age group 5.

DISCUSSION

Our analyses treated the ontogeny of size dimorphism and shape dimorphism independently, and our results suggested that the development of shape differences was, to some extent, decoupled from the development of size differences through ontogeny. Whereas shape dimorphism in the pelvis was evident as early as at the onset of puberty (31-36 days), size dimorphism appeared only at a later age (45-50 days). Although comparisons of our results with results of earlier work on sexual dimorphism in the rat pelvis are confounded by differences in methodology, we interpret this result on size sexual dimorphism as being consistent with that of Bernstein and Crelin (1967), who report that at 46 days of age (analogous to our age group 3, 45-50 days) the male pelvis is larger than that of the female. We attributed size dimorphism to males' greater rates of pelvic growth, a result similar to sex differences in the rates of craniofacial and appendicular skeletal growth (Miller and German, 1999; Reichling and German, 2000). Thus *size* dimorphism in the pelvis may be a phenomenon reflective of global differences between the sexes in skeletal growth trajectories that are likely accentuated by hormonal differences that occur at puberty.

The circumstances for the ontogeny of *shape* dimorphism are more complex. The current theory of the ontogeny of sex shape differences in the pelvis, developed on the basis of conventional morphometrics studies by Bernstein and Crelin (1967), Iguchi et al. (1989),

and Uesugi et al. (1992), holds that testicular androgens are necessary to redirect the *default* female pelvic shape change trajectory into the *derived* trajectory of the male by modifying some or all aspects of shape change in the male. This theory is supported by results from endocrine manipulation studies in rodents which show that hormonal changes occurring in the male at puberty are largely responsible for differences between the sexes (Bernstein and Crelin, 1967; Iguchi et al., 1995; Iguchi et al., 1989; Uesugi et al., 1992). In part, our results do not contradict earlier results. With respect to shape dimorphism, we found that males and females statistically differed at age group 2 (onset of puberty), but it is important to remember that there were differences in shape as early as age group 1, notably the subpubic angle (interischial distance) was smaller in males than in females. Thus, the "starting point" in shape space was not the same for the sexes. In the adult rat pelvis, we found most of the eleven dimorphic shape features that Bernstein and Crelin report (Bernstein and Crelin, 1967), such as the greater iliac crest – ischial tuberosity distance, narrower subpubic angle, shorter and wider pubis, longer and wider ischium, longer pubic symphysis and thicker iliac bones in males (Fig. 3B). We also found the temporal pattern of changes in dimorphism to be similar: significant differences appeared at 32 days of age (age group 2) (narrower subpubic angle in males, slight shifts of iliac and pubic bones in caudal directions in females, resulting in a shortening of the ischium and pubic symphysis in females), at 46 days (our age group 3) the dimorphic features were consistent with the previous age group and all features of adult dimorphism were in place at 60 days (age group 4), at an earlier age than reported by Bernstein and Crelin (at 76 days). However, we found the appearance of dimorphism to be a reflection of the complex combination of initial sex differences followed by dissimilarities in rates and directions of shape change between sexes. We also found that the eleven dimorphic

shape features of the pelvis that Bernstein and Crelin identify are oversimplified by linear measurements and, in fact, represent complex patterns of localized modifications and shifts in growing pelvic bone tissue.

The trajectories of shape change were parallel until age group 3 (45-50d), with no interaction between sex and age group. This meant that bones were growing and being remodeled in very similar ways in both sexes (Fig. 4). During this time interval, sexually dimorphic shape changes involved alteration of both non-uniform and uniform components of shape. We interpreted this result as consistent with earlier studies. In rats, removing androgens prior to 60 days of age affects only some features of male pelvic growth; other features are androgen independent (Bernstein and Crelin, 1967). We further suggest that in females these changes were not estrogen dependent either. Instead, they may be reflective of global sex differences in the mechanisms that regulate early skeletal growth, especially those involved with the greater initial rates of shape change in females than males. In other specific pelvic loci, removing androgens and their general growth stimulating effects prior to sexual maturation, may change the mode and pace of skeletal remodeling such that the pelvis of a gonadectomized male looks like that of a female. These effects can be reversed in males by treatment with exogenous male hormones (Iguchi et al., 1989, 1995). Thus, until approximately age 45 days the female could be considered the default configuration.

At age group 3 (45-50d), the trajectories of male and female shape change diverged in shape space. The presence of an interaction between sex and age group beginning at age group 3, and the difference in the direction of female shape change at this time compared to earlier ages, suggested to us that it is the female pelvis, undoubtedly under the sex-specific influence of estrogens, that becomes radically different from the male. The male pelvis

continued to change shape, but the remodeling generating those changes followed the same pattern as in earlier ages. There were no longer significant sex differences in the uniform component of shape change—those effects that appeared everywhere in the form. The observed sexual dimorphism in shape change can be attributed solely to the non-uniform components — those effects that were localized. These specific localized changes in the female, notably the shortening of the pubic symphysis and increase in the subpubic angle relative to males, obviously prepared the female pelvis for its role in reproduction. As we know from previous studies of rat ontogeny (Miller and German, 1999), by 45-50 days (age group 3) female rats can successfully become pregnant and deliver litters 21 days later, or shortly after the end of age group 4 (60-65 days). The onset of reproductive senescence begins at ~90 days, the last age used in group 5 (86-90 days). Although the rates of female pelvic shape change slowed across all post-weaning age intervals, female pelves continued to change throughout the most fecund portion of their reproductive lives. This is in contrast to Hodsman et al. (1998) who report no open epiphyseal growth cartilage in the 84 day old female rat pelvis, an age earlier than age group 5. Hormonal changes in estrogen levels in females at this time period may be essential for successful reproduction. A temporal examination of the distribution of estrogen receptors in the pelvic bone of males and females would begin to explain how estrogen differentially affects the trajectories of pelvic shape change in each sex. To our knowledge, no such study has been conducted. Within this context, and given the effects of estrogen on bone remodeling, it seems to us that the female shape cannot be called the default, at least not for the entire span of the shape change trajectory. Even with removal of gonadal androgens, the male pelvis would not be able to reach adult female shape without effect of gonadal estrogens.

In keeping with the claims of Grumbach (2000) concerning the limited role of androgens in human bone growth, we suggest an alternative interpretation to the "female default" theory based on the following results. The mechanisms that generated pelvic shape dimorphism were in place as early as at weaning (21days) and therefore not tied to the effects of pubertal sex hormones. Neither size nor shape was statistically different between the sexes at weaning. However, there were differences: the probability of wrongly rejecting the null hypothesis that the shapes were the same at age group 1 was 0.047. The pattern of sexual dimorphism in rates of shape change before puberty was complex. The effects of these rate differences, acting on existing size and shape differences (already in place at weaning), accrued over early development, such that shape differed even more at the onset of puberty, and size before reproductive maturation. Differences in rates simply accumulated and accentuated change along a common trajectory; the patterns of changes in shape were not themselves different between the sexes until age group 3. Based on these findings, we cannot agree that androgens redirect the female trajectory of shape change to male trajectory, and we propose the following model. The effects of androgens are more or less constant throughout ontogeny and do not contribute to the pubertal growth spurt in male shape change. Androgens may play role in accentuating preexisting sex differences by controlling rates of bone remodeling. Thus, all differences before age group 3 (puberty in general) are attributable to more global differences in growth between males and females. At puberty we assume some fundamental difference in the distribution of estrogen receptors between males and females such that specific regions of the female pelvis now come under the stimulating effects of estrogen (whether directly or indirectly). We believe that the significance of estrogens was likely underestimated in previous studies. Differences in estrogen

concentrations, in the distribution of estrogen receptors, or in downstream target of estrogens' effects are what must underlie the abrupt change in direction of the female shape trajectory because regions of the female pelvis are being remodeled in a different way than the corresponding regions of the male pelvis (which continues to change as it had prior to sexual maturation). The regions that are affected are those that will become critical in the female as her anatomy "prepares for" pregnancy and parturition. Thus, our model is not one of a female default form after the age of 45-50 days. Rather, the uniform component of shape change is related to those aspects of the pelvis that are less affected by sex hormones generally. After the onset of puberty the uniform component is not significantly different between the sexes, and localized differences in shape remodeling are due to estrogens.

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TABLE 1. Sample size by age range and group.

Age range (days)	Age group	Life history marker	Sample size	
			Male	Female
22-24	1	No differences in body weight	8	9
31-36	2	Onset of puberty	8	12
45-50	3	Sexual dimorphism in body weight	8	12
60-65	4	Fully reproductive	12	11
86-90	5	Adult stature	8	12

TABLE 2. Twenty-four homologous landmarks and their anatomical description. Landmark numbers correspond to those in Fig. 1.

Landmark number	Landmark description
1	Left caudal dorsal iliac spine at the posteriolateral part of the first sacral vertebra
2	Left cranial dorsal iliac spine
3	Left cranial ventral iliac spine
4	Left caudal ventral iliac spine
5	Anteriomost point on margin of left acetabulum
6	Posteriomost point on margin of left acetabulum
7	Left ischiadic tuber
8	Left ischiadic spine
9	Right ischiadic spine
10	Right ischiadic tuber
11	Posteriomost point on margin of right acetabulum
12	Anteriomost point on margin of right acetabulum
13	Right caudal ventral iliac spine
14	Right cranial ventral iliac spine
15	Right cranial dorsal iliac spine
16	Right caudal dorsal iliac spine at the posteriolateral part of the first sacral vertebra
17	Right iliopubic eminence
18	Anteriomost point of right obturator foramen
19	Posteriomost point of right obturator foramen
20	Pubic symphysis at ischiadic arch
21	Pubic symphysis at ventral pubic tubercle
22	Posteriomost point of left obturator foramen
23	Anteriomost point of left obturator foramen
24	Left iliopubic eminence

TABLE 3. Two-way MANOVA comparing uniform and non-uniform components of shape, concurrently and individually. Three different analyses were performed with data spanning different combinations of age groups based on initial exploration. ** indicates statistically significant differences ($p < 0.01$).

Source	Wilks Λ	F-Statistic	df	P
Groups 1,2,3,4,5				
All components of shape				
sex	0.0410	24.99	44, 47	0.0005**
age	0.0001	9.38	176, 190	0.0005**
sex x age	0.0238	1.68	176, 190	0.0002**
Uniform				
sex	0.8693	6.69	2, 89	0.0020**
age	0.6995	4.35	8, 178	0.0001**
sex x age	0.8818	1.44	8, 178	0.1810
Non-uniform				
sex	0.0458	24.33	42, 49	0.0005**
age	0.0001	9.89	168, 198	0.0005**
sex x age	0.0263	1.76	168, 198	0.0001**
Groups 1,2,3				
All components of shape				
sex	0.0228	7.80	44, 8	0.0023**
age	0.0003	11.25	88, 16	0.0005**
sex x age	0.0328	0.82	88, 16	0.7286
Uniform				
sex	0.7499	8.34	2, 50	0.0008**
age	0.6887	5.13	4, 100	0.0008**
sex x age	0.8815	1.63	4, 100	0.1732
Non-uniform				
sex	0.0327	7.03	42, 10	0.0012**
age	0.0003	13.03	84, 20	0.0005**
sex x age	0.0454	0.88	84, 20	0.6709
Groups 3,4,5				
All components of shape				
sex	0.0072	43.86	44, 14	0.0005**
age	0.0022	6.40	88, 28	0.0005**
sex x age	0.0125	2.53	88, 28	0.0034**
Uniform				
sex	0.8694	4.21	2, 56	0.0199
age	0.9584	0.60	4, 112	0.6622
sex x age	0.8754	1.93	4, 112	0.1110
Non-uniform				
sex	0.0117	32.05	42, 16	0.0005**
age	0.0028	6.87	84, 32	0.0005**
sex x age	0.0187	2.40	84, 32	0.0033**

TABLE 4. Pair-wise size and shape comparisons between sexes at each age group. MANOVA revealed significant differences in size and shape between the sexes and among the age groups. Post-hoc comparisons showed that males were larger than females at age of reproduction. Pair-wise Goodall's F-test showed significance differences in shape from age group 2 through the end of the study. ** $p < 0.01$

Age Group	Size	Shape		
	Pair-wise comparisons	Goodall's F-test		Sexual Dimorphism
	p	p	F	Procrustes Distance
1	0.1967	0.0468	2.22	0.022 (0.0188-0.0342)
2	0.1055	0.0004**	7.37	0.0306 (0.0264-0.0371)
3	0.0001**	0.0004**	7.65	0.0366 (0.0284-0.0469)
4	0.0000**	0.0004**	10.78	0.0427 (0.0379-0.0529)
5	0.0000**	0.0004**	11.65	0.0476 (0.0362-0.0634)

TABLE 5. Differences in the rates of pelvic size increase and shape change. Shape change was estimated both as slope of regression of Procrustes distance on age and as Procrustes distance traveled between means of groups of interest. For both sexes, all regression slopes were significant for size and shape ($p<0.05$). Ninety-five percent confidence intervals are provided in parentheses for all measures of size and shape change.

Time Interval	Size (slope)	Shape (slope)	Shape (distance traveled)
male			
1-2	0.2778 (0.2772-0.2784)	1.225 (1.216-1.234)	0.0391 (0.0364-0.0469)
2-3	0.2243 (0.2238-0.2248)	1.178 (1.174-1.182)	0.0237 (0.0216-0.0310)
3-4	0.1174 (0.1169-0.1179)	0.688 (0.685-0.692)	0.0207 (0.0198-0.0300)
4-5	0.0568 (0.0565-0.0571)	0.493 (0.491-0.495)	0.0236 (0.0202-0.0372)
female			
1-2	0.2481 (0.2477-0.2484)	1.686 (1.677-1.695)	0.0429 (0.0398-0.0490)
2-3	0.1898 (0.1895-0.1901)	1.173 (1.168-1.178)	0.0253 (0.0215-0.0334)
3-4	0.0947 (0.0945-0.0949)	1.039 (1.035-1.043)	0.0228 (0.0212-0.0331)
4-5	0.0433 (0.0431-0.0434)	0.375 (0.372- 0.378)	0.0181 (0.0161-0.0296)

FIGURE CAPTIONS

Figure 1. A schematic of the rat pelvis with 24 digitized landmarks. Numbered anatomical landmarks correspond to those in Table 2. These landmarks provided comprehensive coverage of the shape of the entire pelvic region. Pairs of landmarks were linked in a wireframe to facilitate visualization of pelvic anatomy. Landmarks 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-20, 20-9, 9-10, 10-11, 11-12, 12-13, 13-14, 14-15, 15-16, 16-17, 17-21, 21-24 and 24-1: pelvic outline. Landmarks 20-21: pubic symphysis. Landmarks 18-19 and 22-23: obturator foramina.

Figure 2. Principal components analysis on Procrustes residuals, following Procrustes superimposition representing multivariate shape space, within which shape trajectories are located. Means of groups are connected by vectors for clearer visualization of ontogenetic shape trajectories for each sex (solid lines). The dashed lines represent shape ontogenetic trajectory approximated by a single vector. Principal component axis 1 and axis 2 explain 41% and 17% of the variation in the data, respectively. Age groups are labeled on the graph.

Figure 3. Changes in the mean pelvic shape in shape space as implied by principal component axes. A) Sexual dimorphism. Solid line represents male, dashed line is female. Note the general iliac crest - ischial tuberosity elongation in males, narrowing of pelvic bones in females, as well as all following changes in females relative to males: strong increase in interischial distance in the female, shortening of the ischium, shortening of the pubic symphysis, caudo-medial displacement of acetabular area and lateral displacement of iliac blades. B) Changes due to age. Solid line represents young, dashed line represents old animals. Note general narrowing of the ischial and pubic bones, elongation and widening of iliac bones, and elongation of the pubic symphysis as animals age.

Figure 4. Visualizing the ontogeny of shape change based on exploration of shape space. Shape changes in the two sexes between age groups 1 and 3 (parallel trajectories of shape change), and age groups 3 and 5 (divergent trajectories) were visualized as wireframe displacements and compared. In all diagrams the younger age group is represented by solid line and the older by dashed line. A) Males. Between age groups 1 and 3, there was an elongation and widening of the iliac bones, caudal shortening and narrowing of the pubic bones, elongation and narrowing of the ischial bones. The acetabular area underwent shortening in caudal direction, allowing iliac bones to elongate. The interischial distance (subpubic angle) decreased, therefore reducing pelvic outlet. The pubic symphysis elongated in the cranial direction. Shape changed in the same way between age groups 3 and 5, although to a lesser degree. B) Females. Essentially, pelvic shape changes between age groups 1 and 3 were similar to males. However, between age groups 3 and 5, the pattern of shape change was altered: the interischial distance increased rather than decreased, the pubic symphysis moved caudally, and the ischial bones shortened rather than elongated.

Figure 1

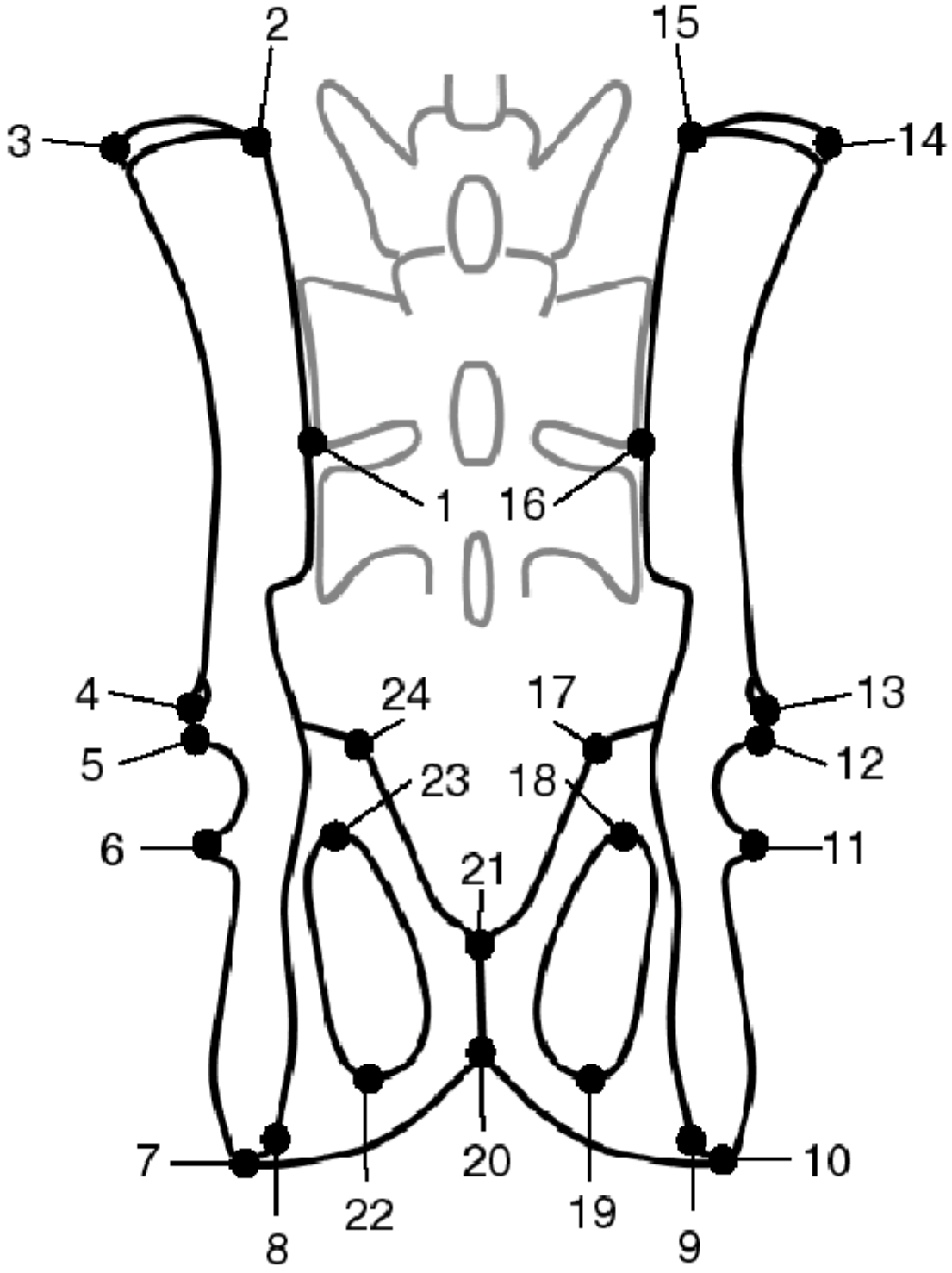


Figure 2

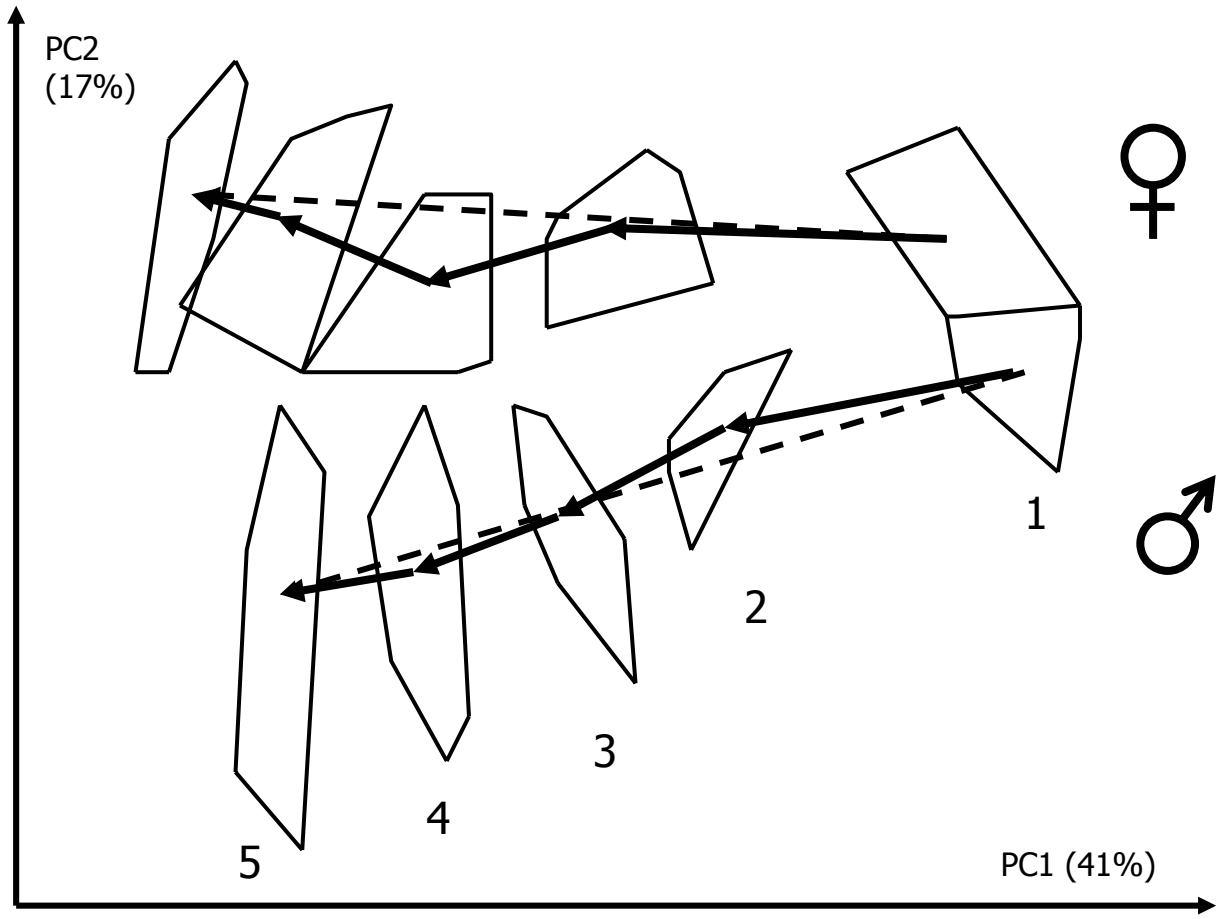
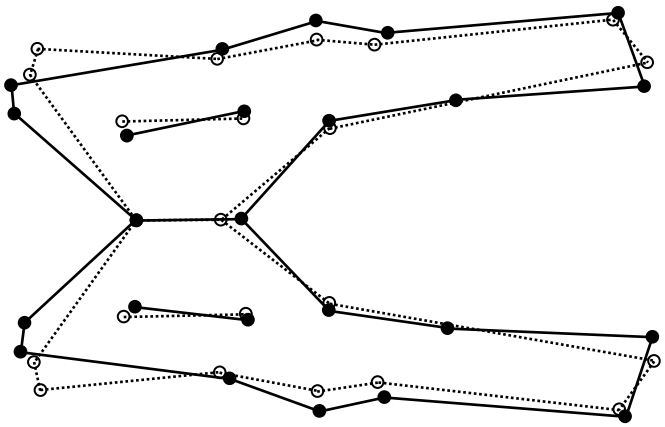


Figure 3

A: Sex



B: Age

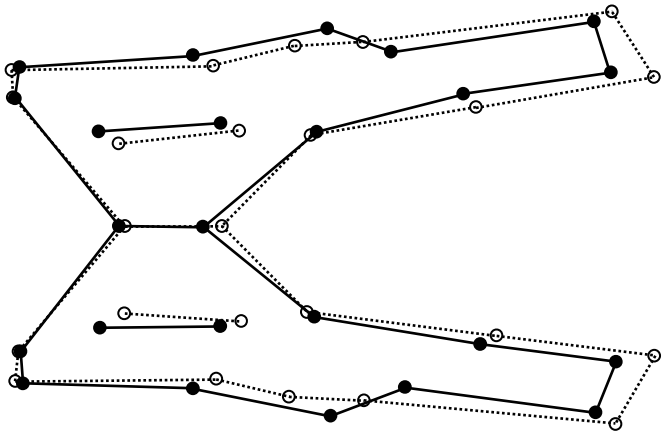
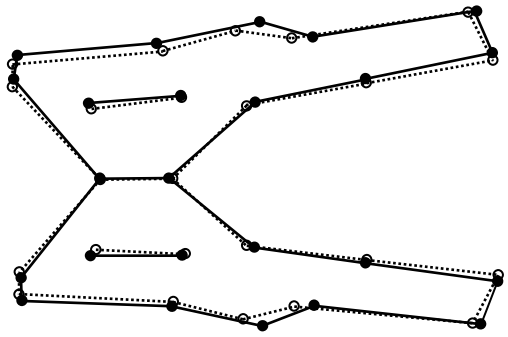
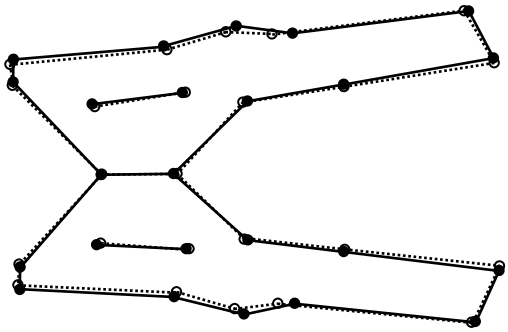


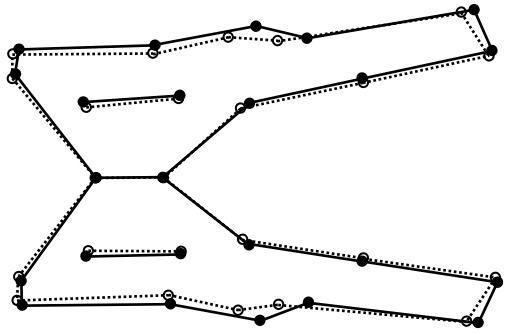
Figure 4

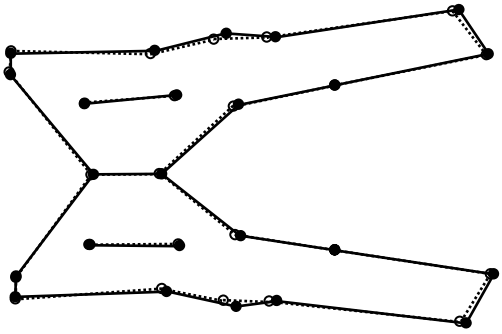
A: Males





B: Females





Age groups 1 to 3

Age groups 3 to 5

Chapter 4

The Evolution of Sexual Shape Dimorphism and Morphological Integration in the Mustelid Pelvis

Abstract

The pelvis in mammals is usually sexually dimorphic, and has multiple functional roles, in both locomotion and parturition. We tested the relative contributions of allometry, phylogeny, reproduction and locomotion to the evolution of sexual dimorphism in shape of this feature in a representative clade of mustelid species. We used geometric morphometrics methodology to visualize and analyze variation in shape. Resampling-based Mantel's tests were used to test hypotheses of morphological integration within the pelvis. Sexual shape dimorphism was the result of both sexual differences in bone size (bones were more robust in males having larger muscle site attachments) and parturition-related variation (females had more spacious birth canal reflecting demands of increasing neonatal size in more dimorphic species). Species differed in pelvic shape based on the degree of locomotor specialization, falling into three major categories: terrestrial walkers, with pelvic shape reflecting greater limb abduction, terrestrial runners with the pelvis suited more for flexion and extension, and locomotor specialists (aquatic and arboreal species) with pelvic specializations for increased column flexion and limb adduction. Tests of alternative hypotheses of morphological integration suggest that postnatal growth and adult function are more important determinants of pelvis covariance structure than shared early development. Besides sexual dimorphism in pelvic shape, sexual dimorphism also exists in the overall magnitude of integration at both intra- and interspecific levels. Removing the effects of sex in studies of morphological integration may conceal significant biological information on the evolution of sexual differences in complex anatomical structures.

Key words allometry; parturition; geometric morphometrics; modularity; mustelids

Introduction

The pelvis is a complex structure, critical for two significant functions in mammals: locomotion and parturition. Evolution in mammalian species has produced significant variation in the pelvis, both between sexes and among species (e.g., Edwards & Marchinton, 1981; Lochmiller et al. 1984; Tyler, 1987; Uesugi et al. 1992; Tague, 1995, 2000, 2003; Krystufek, 1998; Sargis, 2002). This variation has been documented most extensively in primates, specifically humans and hominids (e.g., Arsuaga & Carretero, 1994; Hausler & Schmid, 1995; LaVelle, 1995; Walrath & Glantz, 1996; Tague, 2000). However, little attention has been paid to the relationships among variation in pelvic form, sexual dimorphism and the links between form and function for other mammals.

The pelvis differs in both shape and size between the sexes for most mammalian species, including those that do not show dimorphism in overall body size or shape (e.g., Tyler, 1987; Arsuaga & Carretero, 1994; Chapman et al. 1994; Uesugi et al. 1992; Krystufek, 1998; Tague, 2003). The differential function of the pelvis in male and female reproduction is responsible for the differences in shape between sexes. Since neonates pass through the female birth canal, formed by the pelvic bones, selection for fitness at parturition is one factor that drives shape dimorphism (Leutenegger, 1974; Leutenegger & Larson, 1985; Wood & Chamberlain, 1986; Hausler & Schmidt, 1995; LaVelle, 1995; Ridley, 1995). In support of this parturition theory, Leutenegger (1974) and Ridley (1995) show that species with newborns that are large relative to the female pelvic inlet are more dimorphic in shape. Another factor producing dimorphism in shape is differences in size between male and female pelves (e.g., Wood, 1976; Steudel, 1981; Leutenegger & Cheverud, 1985). The relative significance of parturition and sexual size dimorphism in producing sexual shape

dimorphism is debated, as well as which specific morphological aspects of the shape dimorphism in the pelvis can be attributed to either of these two factors.

There are many sources of variation in pelvic shape among species. Interspecific allometry, differences in shape among species as a function of size, stems from the support for body mass that this structure provides (Calder, 1984; Jungers, 1985; LaBarbera, 1989; Swartz & Biewener, 1992). Further, because the pelvis is the origin or insertion for many muscles of the trunk, hip, and leg, differences in locomotion among species are likely to correlate with shape differences in sites of muscle attachment.

Evolutionary history is rarely considered in studies of the pelvic form. However when it has been for other anatomical regions, phylogeny is a significant factor in the constraint of the evolution of morphology in a group of related species (Cheverud et al. 1985; Harvey & Pagel, 1991; Blomberg & Garland, 2002). The relative contribution of these factors, functional, allometric and phylogenetic, to variation in pelvic morphology has not been examined, particularly in an evolutionary comparative context.

Previous studies of pelvic shape relied on conventional morphometric methodology, which is deficient in several ways. Metrics of conventional morphometrics studies, based on such shape approximations as angles, linear distances, and their ratios, capture shape incompletely, inevitably lose information, and are hard to interpret and compare (e.g., Wood & Chamberlain, 1986; Iguchi et al. 1989; Walrath & Glantz, 1996). Another significant methodological drawback is the conflation of shape and size in allometric studies. Attempts to scale shape to body size, by taking ratios of a specific measurement to body length or mass, present statistical problems that compromise the analytical results (Albrecht, 1978; LaBarbera, 1989). To avoid these methodological problems, we applied landmark-based

geometric morphometrics methodology (Bookstein, 1991; Dryden & Mardia, 1998) to quantify shape independently of size, and ask questions about the evolution of pelvic shape in a model taxonomic group, mustelids.

Mustelidae is a functionally and morphologically diverse family of carnivores that includes such species as weasels, ferrets, mink, tayra, martens, badgers, otters and skunks. The phylogenetic relationships of the clade are well resolved, based on molecular data (Bininda-Emonds et al. 1999; Koepfli & Wayne, 2003; Sato et al. 2003; Marmi et al. 2004; Flynn et al. 2005). Mustelid lineages underwent rapid evolutionary diversification in six different radiation episodes since the Early Eocene (Marmi et al. 2004). At the same time, evolutionary diversification was accompanied with the striking degree of ecomorphological divergence, evident even among closely related species within a genus (Koepfli & Wayne, 2003). Mustelids range from taxa that are fossorial (badgers *Taxidea*) to terrestrial (skunks *Mephitis* and weasels *Mustela*) and semi-arboreal (martens *Martes* and tayras *Eira*) forms, to others that are semi-aquatic (otters *Lutra* and sea otters *Enhydra*). This variation in ecology is reflected in the diversity of locomotory behaviors of these species (Taylor, 1989). In addition, this group has a significant variation in both body size (spanning four orders of magnitude) and sexual size dimorphism (from species with no dimorphism to extremely dimorphic species where males are roughly twice as large as females).

Our objectives were to quantify and visualize the evolutionary changes in pelvic morphology of this diverse clade, treating shape variation independently of variation in size. We hypothesized that the evolution of sexual shape dimorphism in the pelvis is explained by two major factors: sexual size dimorphism and parturition. We hypothesized that the

evolution of interspecific differences in mustelid pelvic shape is explained by three factors: species size, phylogeny and ecology (reflected in locomotory function of the pelvis).

Erroneously, the evolution of characters is often assumed to be independent, despite much evidence to the contrary, and the fact that the total phenotype is selected (Cheverud, 1982). The genetically, developmentally and functionally related characters within a form vary in a coordinated fashion in the phenotype, meaning that morphological variation of organisms is integrated (e.g., Olson & Miller, 1999; Cheverud, 1982; Cheverud, 1996). Integration can be expressed as the coordination of morphological change in ontogeny or phylogeny, in space, time, magnitude or direction (Roth, 1996). As a result of this integration each structure forms a functioning whole, and the characters that tend to be correlated within a form evolve as a unit or module. The anatomical organization of an organism is modular, for example the skull consists of a neurocranium and a viscerocranium, each in turn composed of individual bones. Therefore, integration appears to be modular in nature, showing higher levels of covariation within modules than between them (Klingenberg et al. 2001, 2003; Hallgrimsson et al. 2004). If organisms, and the structures within organisms, are integrated, analyses of covariation between traits may help to determine how morphology structures itself (by understanding hierarchical interrelationships between traits) and responds to selection (Cheverud, 1982, 1996; Wagner, 1996; Hallgrimsson et al. 2002; Pilbeam, 2004). Despite its modular anatomical organization and complex developmental history, no studies of integration of the pelvis exist.

One of our goals was to understand the structural integration underlying pelvic shape complexity. The bony pelvis is a complex structure consisting of three separate bones (the ilium, ischium and pubis) that fuse together early in postnatal development. We tested three

hypotheses of morphological integration in these bones, based on evidence of developmental and functional relationships in mammalian pelvis.

The morphogenesis of the pelvis begins by the migration of mesenchymal cells derived from the paraxial mesoderm to their locations at the basis of the lower limb bud. Further, mesenchymal cells condense into three separate bone morphogenetic fields, becoming precursors of pelvic cartilages and bones (Olsen et al. 2000). The pelvic bones develop through process of endochondral ossification, replacement of cartilage by bone. Each bone has a distinct ossification center, defined by its morphogenetic origin, and is independent from the others in its ossification timing. The human pelvis demonstrates a particular chronological sequence of ossification, starting with the ilium at 9 weeks and the ischium at 16 weeks, with the pubis following a few weeks later (Delaere & Dhem, 1999; Stec et al. 2003). In fetal pigs, ossification in the pelvis starts first with the ilium as well (at 34-35 days), followed by ischium and pubis at 44-45 days of age (Ichikawa et al. 1993). Lacertidae lizards also exhibit a strict order of pelvic element maturation: ilium first, followed by pubis and then by ischium, all three developing bones being separated by rows of less differentiated cells (Malashichev, 2001). The similar pattern is observed in all species of the Anura, the pelvis developing from iliac and puboischiadic centers, with the ilium being the first bone to undergo ossification (Pugener & Maglia, 1997; Rockova & Rocek, 2005). Thus, patterns of pelvic development are conserved across animal classes as diverse as amphibians, reptiles and mammals.

Much less is known about the exact genes and molecular mechanisms involved in the pelvic patterning. The three distinct cell populations that give rise to the pelvis are patterned by at least four genes: *Hoxc10*, *Hoxc11*, *Emx2*, and *Ptx1*. The regulatory effects of these

genes are now known, based upon knockout experiments in chicks and mice. Genes of the *Hoxc* complex are not expressed in the bones directly, but play a role in the correct positioning of the pelvic elements, specifically appropriate positioning of the ilium relative to the sacrum (Nelson et al. 1996; Pilbeam, 2004). The homeobox containing gene *Emx2* is expressed exclusively in the ilium, mutants lacking this gene fail to develop the major part of this bone, except for the small portion forming the acetabulum (Pellegrini et al. 2001). The knockout of *Ptx1*, a bicoid-related gene, shows the absence of ilium and a slight reduction in the size of the ischium and pubic bones in mice embryos (Lanctot et al. 1999). Less is known about the genetic regulation of development of the ischium and pubis, which potentially could be under the same genetic control. However, a study of mycotoxin exposure that disrupted normal rat development demonstrated complete agenesis of only the ischium, pointing to the independence of its origin (Wangikar et al. 2004). Based on the evidence of relative independence of developmental regulation in the three pelvic bones, we hypothesized that the pelvis is an integrated structure containing three distinct modules, corresponding to anatomical positions of the ilium, ischium and pubis.

The other alternative source of integration could be the functional nature of the pelvis. Some studies suggest that function is the primary factor shaping phenotypic covariation and diversity, revealing the transitory nature of developmental constraints (Beldade et al. 2002). Badyaev & Foresman (2004) pointed to the primary significance of function in patterning phenotypic covariance structure in the skull, demonstrating that there is strong integration of traits involved in the attachment of the same muscle. In the case of the pelvis, the functional explanation may be equally applicable. Both iliac blades and ischial bones provide attachment area for major groups of pelvic muscles (adductors/abductors and

flexors/extensors of the thigh), while pubic bones and lower ilium are less involved in muscle attachment and are the exact portions of the pelvis that form the bony portion of the birth canal. Therefore, the second integration hypothesis we tested predicted the pelvis to consist of two functional modules, one uniting ischial tuberosities and iliac blades as sites of major pelvic muscle attachments, another spanning the rest of the pelvis involved in forming the birth canal.

Finally, a third possibility is that variation in postnatal growth patterns of the pelvis, as opposed to the embryologic development, could be the source of the evolution of differences in covariance patterns (Riska, 1989). Different parts of the bony pelvis are known to differ in rates and duration of their growth. Pubic bones and lower ilium undergo complete fusion before sexual maturity is attained and cease growing earlier than ischium and iliac blades, which continue to grow through adulthood (LaVelle, 1995; Tague, 2003). Based on this, we tested a third alternative hypothesis of integration via postnatal growth. In this case, we predicted the presence of two modules in the pelvis, one corresponding to common growth pattern of the pubis and lower ilium, and another uniting the ischium and iliac blades on the basis of their growth.

In general, the evolution of observed patterns of covariation and what factors govern that evolution, is also debated. Several authors have argued for evolutionary stability of integration patterns. Marroig & Cheverud (2001) and Young (2004) found little correspondence of covariation patterns with phylogeny in monkey crania and the hominoid scapula, but did find a correspondence to functional similarity and morphological distance. On the contrary, Ackermann & Cheverud (2000) found that interspecific similarity in hominoid cranial covariation patterns corresponded to their phylogenetic relationships,

suggesting that covariance structure evolves. For mustelids, we tested the hypothesis that covariance structure in the pelvis remains evolutionary stable, based on rapid divergence of mustelid species and overall stability of developmental patterns in different groups. We also tested whether pelvic function and morphological diversification are significant factors in the evolution of pelvic covariance structure.

Hypotheses of sexual dimorphism in integration patterns are seldom addressed. Instead, sex is often treated as a confounding factor (e.g., Cheverud, 1996; Marroig & Cheverud, 2001; Young, 2004). Based on the expectations of differential evolution in the pelvic morphology of males and females, we hypothesized that patterns and/or levels of integration are different in males and females of the same species. We also addressed a related hypothesis that patterns of covariance evolve differently in males and females.

Materials and methods

Sample

A total of 498 museum specimens of pelvic bones of extant mustelids were examined in this study. The material is housed in the collections of the American Museum of Natural History, Field Museum of Natural History, Smithsonian Museum of Natural History and Harvard Museum of Comparative Zoology. The sample included nine species, belonging to six genera: *Mustela erminea*, *M. frenata*, *M. vison*, *Martes americana*, *M. pennanti*, *Eira barbara*, *Lutra canadensis*, *Mephitis mephitis* and *Taxidea taxus*. Only complete, adult specimens of known sex were used in the analysis. Pelvic specimens were judged as belonging to adults by presence of the complete fusion (i.e., no visible fusion traces) of the ilium, ischium and pubis. Only intact pelvic specimens, fused at the pubic symphysis, were

used to preserve the structure of the birth canal and the bilateral nature of the pelvic bones. The total sample included 275 males and 209 females over the nine species (Table 1).

Data collection

Each pelvic specimen was digitally photographed in ventral view using Canon G2 PowerShot digital camera with 1600x1200 pixel resolution. To eliminate distortion due to parallax effects, all images were taken with the camera positioned at a sufficient distance to ensure that the specimen occupied only the part of the field free of the distortion. A ruler was included in all images, in the same plane as the specimen, to allow computation of pelvic size. Each pelvic specimen was oriented so that landmarks 1 and 2 (pubic symphysis), 4 and 23 (dorsolateral ends of ischial tuberosities), and 8 and 19 (dorsal cranial portions of iliac blades) were parallel to the plane of focus (Figure 1). This view and orientation capture variation in the ilium, ischium and pubis simultaneously, as well as variation in the birth canal space formed by bilateral fusion of these bones at the pubic symphysis. Thirty homologous and repeatable anatomical landmarks (Fig. 1, Table 2), consistently visible on all photographs, were converted to two-dimensional (2D) Cartesian coordinate data by digitizing photographs using the tpsDig software (Rohlf, 2003). These landmarks were chosen for their capacity to provide comprehensive and even coverage of the entire pelvic area. Pelvic size was computed as a centroid size, which is the square root of summed squared distances of landmarks from their centroid (Slice et al. 1996), calculated for all landmark configurations. Centroid size is the only measure of size not correlated with shape and therefore appropriate in geometric morphometrics analyses, where shape is the property of a figure invariant to the scale, position and rotation (Bookstein, 1991, 1996). One randomly chosen specimen from each species was placed, photographed, and digitized 10

times. These 10 sets of landmark coordinates were superimposed and plotted. Digitizing errors at each landmark were small, circular and similar in size, indicating there were no consistent errors in positioning the specimen or in locating individual landmarks. All photography and digitizing was performed by a single individual (S.B.).

Data analysis

We used a variety of landmark-based geometric morphometrics methods to address questions concerning the evolution of pelvic shape dimorphism and interspecific differences in pelvic shape. Multivariate analysis of variance and Goodall's F-tests tested for shape differences between sexes and among species and multivariate analysis of covariance tested for the relationship between shape and size across sexes and species. The contribution of phylogeny, lifestyle and parturition to the pelvic shape were tested by multiple regressions and Mantel's matrix correlation tests. Details of shape quantification and data analysis of shape variables follow.

Shape quantification

First, digitized landmark coordinates were subjected to a Generalized Least-Squares Procrustes superimposition (GLS) in the program *CoordGen6f* of the IMP series software (Sheets, 2004). This procedure quantifies shape by removing such "nuisance" parameters as initial differences in centroid size, position and orientation of specimens (Rohlf & Slice, 1990; Rohlf, 1996), and defines the shape of each specimen in terms of Procrustes residuals, which serve as the starting point of the statistical analysis of shape. The superimposed coordinates represent the locations of shapes in the multidimensional shape space, which is defined by the number of landmark coordinates in a single shape (Rohlf, 1996). This shape space is non-Euclidean and has fewer dimensions than there are landmark coordinates,

reducing statistical degrees of freedom needed to perform conventional multivariate analyses. To correct for this, specimens located in the non-Euclidean Kendall's shape space were projected onto a Euclidean space that is tangent to shape space and has the same number of dimensions. Following Bookstein (1996), the location of a sample's average shape was used as the point of tangency (reference shape) for all analyses of the sample.

Before applying multivariate methods, we used the *tpsSmall* 1.20 program (Rohlf, 2003) to determine whether the amount of variation in shape contained within our data set was small enough to permit statistical analyses to be performed in the linear tangent space, approximating non-linear Kendall's shape space. The correlation between shape distances in shape space and the corresponding distances in tangent space was calculated. A high correlation ($r^2 = 0.9999$) was found and this indicated that only a small amount of variation in specimen shape space locations exists and negligible distortion was introduced by projection of shape space distances into Euclidian space.

To produce the shape variables for various multivariate statistical analyses of shape in this study, a thin-plate spline interpolation function was used to describe the shape of each specimen in the sample in relation to the GLS consensus configuration (i.e., the reference point, which is the tangency point between shape space and the linear tangent space) by a set of forty-two partial warps representing non-uniform components and two uniform components (Bookstein, 1996). These calculations were performed using the *tpsRegr* 1.28 program (Rohlf, 2003). The non-uniform components represent regionally differentiated, and localized aspects of shape variation; the uniform components, however, describe shape change across the whole organism, measuring shape effects that apply anywhere in the form (Zelditch et al. 2004).

Procrustes analysis

Procrustes distances were calculated between mean forms of the sexes and species to assess the magnitude of sexual dimorphism and interspecific shape differences. Procrustes distances are the sums of squared distances between corresponding homologous landmarks of GLS-superimposed configurations (Rohlf & Slice, 1990). Procrustes distance calculation provides a single number calculation of the amount of shape difference between two groups of interest. This metric was used to assess magnitude of shape differences between groups and to construct interspecific morphological shape distance matrices for Mantel's tests (described in the following sections). Ninety-five percent bootstrap confidence intervals were used to test for significant differences in Procrustes distances between sexes and among species. Procrustes distances between group means and their confidence intervals for 2D data were calculated using *TwoGroup6e* program of the IMP series software (Sheets, 2004).

While differences in geometric scale were removed during the GLS, aspects of shape variation correlated with size (allometric components of shape) were not. Previous geometric morphometrics analyses demonstrated that there is a large component of shape variation that is correlated with size in mammalian skulls (e.g., Singleton, 2002; Berge & Penin, 2004). Therefore, Procrustes tests were repeated on size-adjusted shape data as well, which permitted a test for shape differences not confounded by size differences between groups. Shape adjustments for size were carried out by shape regressions on size using *Standard6* program of the IMP software (Sheets, 2004).

Principal component analysis (PCA)

As an exploratory tool, we used a principal component analysis on Procrustes residuals to visualize and describe the pelvic shape of each sex and species in multivariate shape space

(Dryden & Mardia, 1998; Berge & Penin, 2004; Cobb & O'Higgins, 2004; Mitteroecker et al. 2004). Projection of Procrustes residuals onto principal components gave principal component scores that could be plotted to examine patterns of shape similarity and difference between superimposed landmark configurations. Principal components analysis was carried out in the *Morphologika* program (O'Higgins & Jones, 2004).

Two-way multivariate analysis of variance (MANOVA)

To test if the shapes were significantly different between the sexes and species, we used a two-way multivariate analysis of variance (MANOVA) with complete sets of the non-uniform and uniform components of shape change as dependent variables. Species and sex were the independent variables, with nine species levels and two sex levels. Interaction terms were calculated between sex and species. A statistically significant interaction in this case meant that the shape sexual dimorphism is expressed differently in different species. Following Adams & Funk (1997), in addition to performing a MANOVA on all components of shape at the same time, we repeated the analysis on the non-uniform and uniform components separately to assess to what extent localized or global changes influenced shape variation in the two sexes. Two-way MANOVAs were performed in SYSTAT 10 (Wilkinson, 2003), on matrices of partial warp scores and uniform components computed and saved from the *tpsRegr* 1.28 program (Rohlf, 2003).

Following these analyses, we performed pair-wise tests of shape differences between sexes (Adams & Funk, 1997; Douglas et al. 2001; Zelditch et al. 2003) for all species examined, to determine which species specifically exhibited significant shape dimorphism. Interspecific shape differences were examined in a pairwise manner as well. The statistical significance, using Bonferroni adjustment ($p<0.0011$), of pair-wise shape differences was

tested by a bootstrap version of Goodall's F-test (data resampled 100 times). We performed all pair-wise tests with *TwoGroup6e* (Sheets, 2004). Both MANOVA and pairwise comparisons were done on the original shape variables and on size-adjusted shapes.

Multivariate analysis of covariance (MANCOVA)

Using the same matrices of shape components, we further examined the relationship between shape and size across various species of mustelids with the help of MANCOVA model in *tpsRegr* 1.28 (Rohlf, 2003). Sex and species were categorical variables. Centroid size was transformed into natural logarithms and was used as the covariate. First, separate MANCOVAs were run within each species to determine whether scaling patterns were similar between sexes within species. We tested for differences in the slope of the shape regression on size and differences in the intercept of these regressions, whenever they proved to have homogeneous slopes.

Multivariate regression

To test the relationship between female pelvic shape and relative neonatal size, multivariate multiple least-squares linear regression was performed on the matrices of size-adjusted partial warps and uniform components, together and individually, with average relative neonatal size (ratio of individual newborn mass to maternal mass) of a species as the independent variable.

Matrix correlations

Mantel's test for statistical significance of matrix correlations was used to test the contribution of phylogeny and lifestyle to shape differences among species. Previously computed Procrustes distances were used to construct similarity matrices of shape distances among species. Divergence times between species (in millions of years) were used to

construct matrices of phylogenetic distances. Information on times of species divergence in mustelid phylogeny came from the Order Carnivora supertree phylogenetic hypothesis of Bininda-Emonds et al. (1999). Lifestyle matrices were produced coding species belonging to the same lifestyle group as 1 and coding species belonging to different lifestyle groups as 0. The lifestyle groups used in this study were defined as fossorial (*Taxidea taxus*), terrestrial (*Mephitis mephitis, Mustela erminea, M. frenata, M. vison*), semiarboreal (*Martes americana*, *M. pennanti*, *Eira barbara*) and aquatic (*Lutra canadensis*).

Mantel's test of matrix correlation was also used to examine similarity in observed integration structure between sexes and among species. Integration structure in pelvic bones of sexes and species was assessed by constructing variance/covariance matrices of the landmark configurations in SYSTAT 10 (Wilkinson, 2003). Matrix correlation is a measure of the strength of association among matrices varying from –1.0 to +1.0. A matrix correlation of zero indicates no structural similarity among the matrices. A matrix correlation equal to one indicates identity of covariation pattern in the two matrices, although overall magnitude of integration may still differ (Cheverud et al., 1989). Statistical significance of matrix correlations was evaluated using randomization tests, where the observed correlation between two matrices was compared to an empirically derived distribution of 1000 matrix correlations produced by randomly permuting the rows and columns of one matrix and correlating the randomized matrix with the second matrix. If the observed correlation exceeded 95% of the randomized correlations, the integration patterns in the two matrices were considered significantly similar. Mantel's tests were performed with the help of PopTools 2.6 program (Hood, 2005). The magnitude of overall integration in each matrix was measured as the variance of the covariance matrix's eigenvalues, following Cheverud

(1989) and Hallgrimsson et al. (2004). High variances of eigenvalues are indicative of high overall levels of integration (Wagner, 1984; Cheverud et al. 1989; Hallgrimsson et al. 2004).

Visualization

For visualization purposes, and to better elucidate the shape changes associated with the different PCA axes, hypothetical pelvic shapes were reconstructed along the principal component axes. This was achieved by adding the coordinates of the consensus landmark configuration to the product of the principal component scores of a specimen and the eigenvectors for the principal component of interest, which was carried out in *Morphologika* program (O'Higgins & Jones, 2004). Shape differences implied by extreme values along the principal component axes were visualized as landmark wireframe displacements. A similar operation was performed with the vector coefficients from the multivariate regressions.

Results

The wireframe figures suggest differences both between sexes and among species in the overall shape of the pelvis (Fig. 2).

Procrustes analysis

Interspecific Procrustes distances, which measure magnitude of differences among species in the shape of the pelvis, calculated for males, before (Table 2, upper half) and after removing the effects of size (Table 2, lower half), showed that all interspecific differences were significant, both before and after removing the allometric component of shape variation. Procrustes distances between sexes within species, reflecting magnitude of sexual dimorphism, indicated significant sexual shape dimorphism in all species, except *Lutra canadensis* (Table 3). The decrease in intraspecific Procrustes distances after adjusting for the effects of size, thus representing sexual dimorphism in shape independent of sexual

dimorphism in size, pointed to the significance of contribution of allometry to sexual shape dimorphism (Table 3).

Principal component analysis (PCA)

Principal component analysis on Procrustes residuals for both sexes in all species effectively partitioned shape variation due to size and sex (Fig. 3). The first four principal components explained 79.9% of shape variation in the data set. Scores on the first principal component axis (41.8% of variation) were highly correlated with log(centroid size) ($r = 0.83$, $p < 0.05$) and therefore represented the allometric component of shape variation. The second principal component explained 24.9% of shape variation and showed separation of sexes. Patterns of shape variation represented by the first two principal component axes, visualized as wireframe displacement diagrams (Fig. 4) demonstrated the changes in pelvic shape resulting from increase in pelvic size and presence of sexual dimorphism. There was a general roughening of pelvic outline, in particular longer and laterally flexed iliac blades (thus increasing sacral space), wider acetabula, more prominent ischial tuberosities, reduced pubic symphysis (Fig. 4A) as size increased. Shape variation due to sex included a more spacious pelvic inlet, longer arcuate lines, reduced pubic symphysis and wider placed ischial bones in females, all of which produced increasing space for the birth canal. Other aspects of the pelvic shape, such as iliac blades and acetabula, were not affected by sex (Fig. 4B).

The interspecific differences in shape not related to size or female-specific function such as parturition, were different in the principal component analyses of the male-only Procrustes residuals (excluding female residuals) from those analyses including both sexes (Figs. 5, 6). The first four principal components of the male-only interspecific residuals explained 72.7% of shape variation in size-standardized data. The first principal component

(39.63% of interspecific pelvic shape variation) separated strictly terrestrial species with relatively short, wide and robust pelves (skunk *Mephitis mephitis* and badger *Taxidea taxus*) from species with long, narrow and slender pelvis, such as aquatic Canadian river otter (*Lutra canadensis*) and arboreal marten (*Martes americana*), fisher (*Martes pennanti*) and tayra (*Eira barbara*) (Fig. 5). The second principal component (12.9% of interspecific size-independent shape variation) separated species like *Mephitis mephitis* with narrowly positioned iliac bones and extremely well developed and widely positioned ischial bones from species such as *Lutra canadensis* with wide flared ilia and underdeveloped narrow ischia (Fig. 5). The third principal component (11.3% of interspecific variation) summarized variation in ischial morphology (Fig. 6), whereas the fourth principal component (8.9% of the variation) separated species with short pubic symphysis and closely positioned pelvic bones versus species with long pubic symphysis and widely positioned pelvic bones (Fig. 6).

Two-way multivariate analysis of variance (MANOVA)

Differences in shape between sexes and among species, tested with two-way MANOVA, were significant for both the shape effects that apply anywhere in the form, such as general elongation or widening of the whole pelvis (uniform component of variation), and the regionally differentiated, localized aspects of shape variation, such as specific, small-scale differences in shape of the iliac blades (non-uniform component).

Looking at localized aspects of shape variation expressed only in specific parts of the pelvis, there were significant shape differences among species (Wilks $\Lambda = 0.0000024$, $F = 24.92$, $df = 432, 2616$, $p = 0.000$) and between sexes (Wilks $\Lambda = 0.4401$, $F = 7.73$, $df = 54, 328$, $p = 0.000$). The interaction term between sex and species was also significant (Wilks $\Lambda = 0.0466$, $F = 2.8641$, $df = 432, 2616$, $p = 0.000$), meaning that the shape sexual dimorphism

was expressed differently in different species. For the shape effects spanning the whole pelvic structure, thus representing global shape differences such as widening or elongation of the pelvic form, there were significant shape differences among species (Wilks $\Lambda = 0.2285$, $F = 51.87$, $df = 16,760$, $p = 0.000$) and between sexes (Wilks $\Lambda = 0.9543$, $F = 9.09$, $df = 2,380$, $p = 0.00014$) as well. The interaction term was also significant (Wilks $\Lambda = 0.8759$, $F = 3.25$, $df = 16, 760$, $p = 0.000017$).

Following the MANOVA, pair-wise tests of shape differences between the sexes were performed for the nine species to determine specifically which species exhibited significant shape dimorphism. All species exhibited significant shape sexual dimorphism, except for *Lutra canadensis* (Table 3). When the same tests were performed after removing the effects of size differences on shape dimorphism, only five species were sexually dimorphic for shape (*Mustela erminea*, *M. vison*, *Martes americana*, *Mephitis mephitis*, *Taxidea taxus*) and four were not (*Mustela frenata*, *Martes pennanti*, *Eira barbara*, *Lutra canadensis*) (Table 3). Size-independent shape differences in pelvic morphology between sexes were expressed as shorter pubic symphysis, longer pubic bones, longer and more curved arcuate lines and more laterally positioned ischium in females.

Multivariate analysis of covariance (MANCOVA)

The allometric responses of sexes within each species were the same, with no species having a significant interaction between sex and centroid size in the MANCOVA analysis that used centroid size as a covariate. The only exception was *Lutra canadensis*, which did not have an allometric relationship for either sex.

MANCOVA on the regionally localized aspects of shape variation at the interspecific level revealed significant differences in allometric slopes of the sexes (Wilks $\Lambda = 0.6927$, $F =$

2.81, df = 54, 342, p = 0.000), indicating that the expression of particular shape sexual dimorphism depends on a size dimorphism of a particular species. The MANCOVA analysis of the global shape effects, spanning the whole pelvic structure, at the interspecific level revealed no significant differences in slopes (Wilks Λ = 0.9989, F = 0.2209, df = 2, 394, p = 0.8019) and intercepts (Wilks Λ = 0.9883, F = 2.34, df = 2, 395, p = 0.0981) of allometric responses of the sexes. That indicates that sexes do not differ in their global allometric shape response (i.e., elongation of the pelvis) to an evolutionary change in species size, but do differ in smaller scale, localized shape allometric responses.

Sexual shape dimorphism and parturition

The effects of parturition on female pelvic shape, tested by multivariate regression of shape on relative neonatal size were significant. A significant relationship between female pelvic shape and relative neonatal size was found for the regionally localized shape variation only (Wilks Λ = 0.2014, F = 8.29, df = 54, 113, p = 0.000). This relationship for the large scale, uniform, component of shape variation was not significant (Wilks Λ = 0.9911, F = 0.74, df = 2,165, p = 0.4774), indicating that parturition influences only small-scale localized aspects of female pelvic shape. We also found a positive relationship between relative neonatal size and the overall magnitude of shape dimorphism in the pelvis, expressed as Procrustes distance between mean shape of the sexes) (r = 0.69, p = 0.0401).

Larger mustelids tended to have less offspring than small species, but of larger size. There was a significant relationship between female centroid size and relative neonatal size (r = 0.88, p = 0.0017). The relationship between female centroid size and average number of newborns in a litter was negative (r = -0.90, p = 0.0009). The relationship between average number of newborns in litter and their size was negative (r = -0.85, p = 0.0039). Larger

species also had a longer gestation, there was a significant relationship between female size and gestation length ($r = 0.69$, $p = 0.0419$). We also found a positive relationship between relative neonatal size and length of gestation ($r = 0.68$, $p = 0.0448$).

Matrix correlations

The contribution of phylogeny and ecology to shape differences between species was significant, as tested by Mantel's tests. The interspecific Procrustes distance matrices (Table 2), when correlated with constructed matrices of phylogenetic distances, taxonomic affinity and similarity in ecology showed high correlation with both phylogeny ($r = 0.907$, $t = 5.33$, $p = 0.001$) and taxonomic rank ($r = 0.916$, $t = 5.39$, $p = 0.001$). The correlation of shape differences with ecology was lower, but still significant ($r = 0.609$, $t = 3.62$, $p = 0.001$). The same correlations after adjusting for the effects of size were also significant, but lower: with phylogeny ($r = 0.803$, $t = 4.72$, $p = 0.001$), with taxonomic rank ($r = 0.840$, $t = 4.94$, $p = 0.001$), and with ecology ($r = 0.584$, $t = 3.48$, $p = 0.003$).

The significant and high correlations between variance/covariance matrices of sexes and species indicated high levels of similarity in integration structure of the pelvis intra and interspecifically (Table 4). Variance/covariance matrices were more similar between sexes within species than among species (Table 4). For females, interspecific variance/covariance matrix correlations were lower than corresponding correlations for males (Table 4). All correlations in Table 4 were statistically significant. Integration similarity matrices of the sexes (Table 4) were correlated with matrices of phylogenetic distance, taxonomic affinity and ecology, factors hypothesized to influence integration structure of the pelvis. For males, covariance structure of the pelvis was not correlated with phylogeny ($r = 0.027$, $t = 0.06$, $p = 0.562$) or taxonomic rank ($r = 0.027$, $t = 0.06$, $p = 0.585$), but was correlated with ecology (r

= 0.811, t = 1.81, p = 0.001). For females, on the contrary, pelvic covariance structure was significantly influenced by phylogeny (r = 0.856, t = 1.71, p = 0.001) and taxonomic rank (r = 0.856, t = 1.71, p = 0.001) and was not correlated with ecology (r = 0.042, t = 0.09, p = 0.647).

The observed patterns of covariance in the pelvis were weakly, although significantly, correlated with predicted covariance structure derived from the information on pelvic prenatal development and function (Table 5). Covariance structure of the pelvis predicted on the basis of postnatal growth had a stronger support, correlations between observed and predicted covariance structure were higher (Table 5). Based on postnatal integration hypothesis, there were two modules in the pelvis. One of them consisted of the iliac blades and ischial bones; another spanned the rest of the ilium and the pubis (Fig. 7). The overall magnitude of pelvic integration was higher in females of all species (Table 5), meaning that even if modular organization of the pelvis was largely the same in both sexes, the correlations between different parts of the pelvis were higher in females.

Discussion

The relationship between shape and size dimorphism

Differences in shape between sexes, such as we observed, may simply reflect the differences in size. An evolutionary increase in pelvic size dimorphism, as seen in species of primates, would necessarily produce changes in shape, particularly to accommodate and support that larger mass (Steudel, 1981; Leutenegger & Larson, 1985; Arsuaga & Carretero, 1994). As is true of primates, differences in mustelid pelvic shape resulting from differences in size reflected an increased robustness in the male pelvis. Males had longer, wider and more laterally positioned iliac blades, thus accommodating larger sacrum, wider iliac shafts, longer

ischial bones (with enlarged ischial tuberosities) and pubic symphysis, as well as larger acetabula. These areas are the attachment sites for the major muscle groups of the hind limb, and the site of femur-acetabulum articulation. One proximal mechanism for producing some of these morphological differences is the differences in rates and duration of adult growth that exist between males and females. The ischium and acetabulum generally continue growth throughout life, unlike ilium and pubis that cease growth in adulthood (LaVelle, 1995; Tague, 2003).

Shape Dimorphism and Parturition

The pelvic shape differences independent of size dimorphism were found in the portions of the pelvis relevant to parturition. The elongation of the iliac shafts and pubic bones, shortening of the pubic symphysis, and more lateral position of the ischial bones relative to pubic symphysis resulted in a wider pelvic inlet and more spacious birth canal in the female pelvis. Other mammals, including primates and rodents, show similar morphological specializations of the female. Differences in pelvic inlet transverse diameter, pubic length and lower iliac length are features of crucial significance to parturition in primate species (Steudel, 1981; Leutenegger & Larson, 1985; Wood & Chamberlain, 1986; Ridley, 1995; Tague, 1995). Distance between ischial bones, while less emphasized as a dimorphic feature, exists in rodents (Bernstein & Crelin, 1992) and humans (Tague, 2000). Sexual dimorphism in the length of pubic symphysis also facilitates parturition in rodents (Bernstein & Crelin, 1992; Uesugi et al. 1992). These sexual dimorphic features are usually exaggerated in species with precocious offspring. Because all mustelid species are altricial, it is interesting that parturition-related sexual dimorphism in the pelvis varies in a group of related altricial species with different offspring and adult sizes. Some of the shape differences dependent on

size were similar to shape differences related to parturition. The pubic symphysis is shorter and iliac shafts are thinner in females, thus enlarging space for the birth canal as well and adding to specific shape differences related to reproductive function.

One explanation for the evolution of the distinctive female pelvic morphology could lie in interspecific differences in relative neonatal size. Larger mustelids tended to have larger offspring relative to their body mass and pelvis size**,** whereas the smaller mustelids, which were less dimorphic for pelvic shape, had relatively small offspring. This is consistent with findings in rodents. The larger cavy species, one of the most extreme examples of this, have few precocial offspring, which weigh almost 20% of their mother's weight at birth (Farmer & German, 2004; Kraus et al. 2005). Smaller species of mice and rats have much larger litters of small altricial infants. The negative relationship we found between the number of newborns in the litter, and the size of the female in mustelids was offset by the positive correlation among relative size of the newborns, maternal size and length of gestation. These relationships reflect the differences in reproductive strategies of mammals of different sizes. Larger species, in general, tend to have smaller litter sizes and longer gestation, but invest in larger offspring as a result (Martin & MacLarnon, 1985; Promislow & Harvey, 1990; Kraus et al. 2005). Such larger offspring could produce a selective pressure for a more pronounced shape dimorphism in the pelvis, as is seen in primates (Ridley, 1995). In the case of caviomorphs, with extremely large offspring, this sexual dimorphism is exaggerated due to partial absorption of the pubic bones and relaxation of the pubic symphysis during parturition (Todd, 1925). Large size species of mustelids, such as skunks, badgers and otters, also have prolonged and more difficult labor relative to small species, most likely due to the large size of the neonates. For instance, the duration of parturition in

small-size long-tailed weasel *Mustela frenata* is around 30 minutes (DeVan, 1982), while in larger sized Canadian river otter *Lutra canadensis*, parturition lasts several hours (Liers, 1951). These behavioral differences could result in evolutionary pressures for differential female pelvic shape across different sized species.

Interspecific shape differences

The evolution of differences in pelvic shape among species that we found was related to locomotor specializations. Our results supported our hypothesis that a positive relationship existed between phylogeny, changes in pelvic shape and locomotion. On the basis of our PCA of pelvic morphology, we found three groups of species: the terrestrial skunks and badgers preferring a slow walk; the terrestrial cursorial weasels and minks; and the species specialized for locomotion in particular environments, the aquatic otters and the arboreal martens and tayras. In particular, the first axis distinguished the species based on the degree of locomotor specialization, from the most generalist terrestrial walkers to the most specialized aquatic and arboreal species.

The striped skunk *Mephitis mephitis*, exhibits a primitive, ancestral mode of mustelid locomotion, which is terrestrial walking (Van De Graaff et al. 1982). The American badger *Taxidea taxus* is similar to the skunk in its locomotor habits, routinely preferring a slow walk, with no reports of its using fast gaits as the run or the gallop (Long, 1973). Cursorial weasels and minks of the genus *Mustela* are more specialized in their locomotion, capable to go from a walk to the half-bound (a form of galloping) and known to climb on occasions (Dagg, 1973; Gambaryan, 1974; Williams, 1983). The arboreal (martens of the genus *Martes* and tayra *Eira barbara*) and the aquatic (Canadian river otter *Lutra canadensis*) mustelid

species are most specialized, capable of moving in different environments (Tarasoff, 1972; Tarasoff et al. 1972; Hildebrand, 1982).

The locomotion of more primitive mustelid species, primarily walking, involves more abduction of the lower limb than for cursorial species (Jenkins & Camazine, 1977; Goslow & Van De Graaf, 1982; Van De Graaf et al. 1982). The pelvic morphology of these species suggests several adaptations for walking. The shape and position of the pelvis ensured that the femur projects laterally rather than being in the sagittal plane. The more laterally positioned pelvic bones in skunks and badgers generate a more laterally positioned limb. The sacrum and iliac blades of these species were broad and well developed relative to other species, reflecting increased mass of gluteals, the major limb abductors. The pubic symphysis was extremely short and pubic bones were thin, which suggested a reduction in mass of adductors, gracilis and rectus abdominis muscles, the major adductors of the limb. The sites of attachment of gemelli and quadratus lumborum muscles on the ischial bones, as well as ischial tuberosities were well developed, also pointing to an increase in abduction, retraction and rotation of the hind limb.

Cursorial, aquatic and arboreal species have different kinematic requirements for their locomotion (Taylor, 1989). Cursorial species have adductor and abductor muscles that are reduced or positioned in a way that they have a flexion extension function (Hildebrand, 1982). Strong limb adduction is nearly always found in arboreal species, as it is essential for successful and rapid vertical climbing (Sokolov & Sokolov, 1971; Taylor, 1989). Aquatic species in general show a greater flexion of the vertebral column. All these types have a lesser need for limb abduction (Tarasoff et al. 1972; Taylor, 1989). The otters and martens, mustelids specialized for aquatic and arboreal locomotion, had narrow pelvis, with reduced

sites of attachments for abductor muscles and increased sites for attachments of adductor muscles. In particular, the pubic symphysis was long and well developed, with thick pubic bones and a prominent pubic tubercle, indicative of the increased function of muscles adducting the limb, specifically gracilis, pectineus and adductors, and of the muscle flexing the vertebral column, rectus abdominis. Correspondingly, cursorial weasels exhibited pelvic morphology intermediate between terrestrial walkers and specialized arboreal and aquatic mustelids.

Other principal components separated the pelves of species inhabiting two very different environments, the aquatic river otter from the terrestrial mustelid species. The otters had slender and streamlined pelves, with the effect of reducing the proportion of the hind limb protruding from the otter body, which in turn reduces the drag in water and improves swimming performance (Tarasoff et al. 1972). We found the attachments for the flexor and extensor muscles of the limb to be underdeveloped in otters, which was reflected in narrow and light ischial bones with weak ischial tuberosities and almost absent tubercles for the rectus femoris. This could be related to reduced mobility of otters on land compared to most terrestrial mustelids because of their short legs, knowing that well developed flexor and extensor muscles are usually good indicators of long limbs and cursoriality in terrestrial species (Tarasoff 1972; Van Valkenburgh, 1986). In turn, psoas minor and pubic tubercles, attachment sites for the psoas minor and rectus abdominis muscles (flexors of the back), were well developed, reflecting increased flexibility of the back, which is necessary for grooming and feeding in the aquatic environment (Estes, 1989).

Morphological integration

Integration has been commonly studied in the craniofacial skeleton (e.g., Cheverud, 1982; Roth, 1996; Smith, 1996; Marroig et al. 2004; Hallgrimsson et al. 2004)**.** The pattern of phenotypic integration in craniofacial structures usually matches the pattern of integration in early organogenesis, the result of both common embryologic history and shared mechanisms of genetic control (e.g., Cheverud, 1995; Marroig et al. 2004). Besides the cranium, integration through development also exists in primate limbs (Hallgrimsson et al. 2002) and the hominoid scapula (Young, 2004). For the pelvis, however, we observed that the structure of phenotypic covariance was weakly correlated with integration, as predicted by embryologic development, despite the fact that it is highly conserved across different animal classes (Malashichev, 2001; Stec et al. 2003; Rockova & Rocek, 2005). Because integration among mustelid species was independent of shared early development, more flexible aspects of development such as variation in rates and timing of growth are likely to influence integration structure in the pelvis. This provides stronger support for the postnatal growth hypothesis. However, as our knowledge of the molecular basis of early morphogenesis in the pelvis grows, it is likely that this conclusion may not hold.

In structures such as the skull, where integration through prenatal development is strongly supported, each developing unit usually matches its future function (Cheverud, 1995), and functional integration accompanies developmental integration. In case of the pelvis, there is no apparent match between embryologic development of individual bones and their function. Each bone with its independent development has multiple functions that are shared across bones. The iliac bone is a good example of this, attaching abductor/adductor and flexor/extensor musculature (function shared with the ischium) and forming part of the

birth canal (function shared with the pubis). However, there was a match with patterns of postnatal growth in these bones. It is possible that early development, postnatal growth and function jointly influence integration patterns in the pelvis, with greater contribution of events occurring later in animal's life.

In general, our results indicated that interspecific similarity in covariance structure was related to morphological distance between species, suggesting the significance of function in the evolution of pelvic integration. Within species, covariance structure was found to be more similar between sexes than among species, with no significant differences between sexes, although females exhibited overall higher levels of integration. Significant sexual dimorphism in the patterns of integration was detected at the interspecific level. For females, interspecific correlations of covariance matrices were lower than for males, meaning that females resemble each other less in covariance structure, which could be related to interspecific variation in female reproductive output. For females, interspecific integration similarity was correlated with phylogeny and not correlated with ecology. On the contrary, in males, interspecific similarity in integration patterns was correlated with ecology and not correlated with phylogeny, which is consistent with results for the primate skull (e.g., Cheverud, 1995; Marroig et al. 2004), and for the hominid scapula (Young, 2004). Because sexual dimorphism in integration existed at both intra- and interspecific levels, removing the effects of sex in studies of morphological integration may conceal significant biological information on the evolution of sexual differences in complex anatomical structures.

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Figure legends

Table 1. Sample sizes by species and sex. The ecology and natural history data of mustelid species are listed, as used in the hypotheses testing.

Table 2. Interspecific shape differences calculated as Procrustes distances between mean forms of males of different species. Values before shape standardization for size are given above the diagonal, values after size standardization are given below the diagonal.

Table 3. Sexual shape dimorphism in the pelvis of mustelid species represented as a Procrustes distance between mean shapes of males and females. Ninety-five bootstrap confidence intervals estimated for all Procrustes distances are given in parentheses. ** indicates statistically significant shape dimorphism, as tested by Goodall's F-test ($p < 0.01$). ns indicates no sexual shape dimorphism.

Table 4. The matrix of intra- and interspecific similarity in pelvic form integration patterns. Correlations between covariance matrices of males and females of the same species are given on the diagonal, interspecific correlations of male covariation matrices are above the diagonal and corresponding correlations for females are below the diagonal.

Table 5. Correlations of species variance/covariance matrices with the three integration hypotheses. Postnatal growth hypothesis of integration had the strongest support. Overall magnitude of integration in the pelvis is given as variance of eigenvalues.

Figure 1. A schematic of the mustelid pelvis with 30 digitized landmarks. These landmarks provided comprehensive coverage of the shape of the entire pelvic region. Pairs of landmarks were linked in a wireframe to facilitate visualization of pelvic anatomy. Pubic bones are represented by landmarks 1, 2 (pubic symphysis), 14, 19 (iliopubic eminences), and 15, 18 (cranial rims of the obturator foramina). The ischium is described by landmarks 3, 4, 29, 30

(ischial tuberosities), 16, 17 (caudal rims of the obturator foramina) and 5, 28 (caudalmost points on the acetabula). The ilium is represented by landmarks 8, 9, 10, 23, 24, 25 (iliac blades), 11, 12, 13, 20, 21, 22 (arcuate lines), 7, 26 (tubercles for the rectus femoris), and 6, 27 (cranialmost points on the acetabula).

Figure 2. Wireframe diagrams showing differences between sexes and among species in the overall shape of the pelvis.

Figure 3. Principal component analysis of GLS Procrustes superimposition aligned shape configurations of males and females of all nine mustelid species. Male clusters are represented by solid lines, females are given as dash lines. The first principal component axis summarizes allometric shape variation in the pelvis and describes 41.8 % of the variation. The second principal component describes 24.9 % of shape variation and represents sex.

Figure 4. A. A schematic showing allometric shape variation in the mustelid pelvis. Solid line represents small size species, dashed line represents large size species. B. Variation in the pelvic shape due to sex. Male form is given by solid line, females are visualized as dashed line.

Figure 5. Principal components analysis of shapes of males of all nine mustelid species. The first principal component axis explains 39.63% and the second axis explains 12.9% of interspecific shape variation. Shape changes as implied by the two principal component axes are visualized as wireframe displacements and are given in the boxes.

Figure 6. Principal components analysis of shapes of males of all nine mustelid species. The third principal component axis explains 11.3% and the fourth axis explains 8.9% of the interspecific variation in mustelid pelvic shape. Shape changes as implied by the two principal component axes are visualized as wireframe displacements.

Figure 7. A schematic showing an integration pattern in the mustelid pelvis common to all species. Based on the covariation patterns in the pelvic form, the three pelvic bones form an integrated structure consisting of two distinct modules. One of the modules consists of the iliac blades and ischial bones (solid circles connected by solid lines), another spans the rest of the ilium and the pubis (empty squares connected by dashed lines). Areas of the pelvis that hypothetically could belong to either of these two modules are shown as dotted lines.

Table 1.

Species	Males	Females	Ecology	Log(newborn size)	Average litter size	Gestation length
Mustela erminea	44	48	terrestrial	0.93	6.0	28
Mustela frenata	35	16	terrestrial	0.82	4.5	27
Mustela vison	54	34	terrestrial	0.90	4.0	31
Martes americana	46	28	arboreal	1.52	2.9	27
Martes pennanti	31	31	arboreal	1.28	3.0	30
Eira barbara	8	13	arboreal	1.82	2.0	65
Lutra canadensis	17	11	aquatic	1.83	2.0	62
Mephitis mephitis	17	21	terrestrial	1.47	4.2	66
Taxidea taxus	23	7	fossorial	2.03	2.0	45

Table 2.

Species	*M. erminea*	*M. frenata*	*M. vison*	*M. americana*	*M. pennanti*	*E. barbara*	*L. canadensis*	*M. mephitis*	*T. taxus*
M. erminea	**0**	0.0521	0.0973	0.1094	0.1238	0.1188	0.1906	0.1896	0.2449
M. frenata	0.0337	**0**	0.0650	0.0790	0.0913	0.0863	0.1663	0.1601	0.2066
M. vison	0.0500	0.0487	**0**	0.0772	0.0868	0.0659	0.1309	0.1470	0.1722
M. americana	0.0553	0.0577	0.0764	**0**	0.0318	0.0707	0.1335	0.1773	0.1790
M. pennanti	0.0683	0.0736	0.0922	0.0483	**0**	0.0801	0.1343	0.1789	0.1719
E. barbara	0.0916	0.0933	0.0904	0.0940	0.0820	**0**	0.1495	0.1700	0.1794
L. canadensis	0.1008	0.1149	0.1059	0.1161	0.1254	0.1411	**0**	0.2030	0.1639
M. mephitis	0.1348	0.1301	0.1382	0.1729	0.1825	0.1783	0.2021	**0**	0.1484
T. taxus	0.0973	0.0922	0.1025	0.1273	0.1542	0.1667	0.1659	0.1227	**0**

Table 3.

Species	Procrustes Distance	Size-adjusted Procrustes Distance
M. erminea	0.0380 (0.0350-0.0439)**	0.0154 (0.0103-0.0260)**
M. frenata	0.0416 (0.0351-0.0500)**	0.0149 (0.0137-0.0297)ns
M. vison	0.0380 (0.0288-0.0513)**	0.0180 (0.0146-0.0319)**
M. americana	0.0280 (0.0252-0.0361)**	0.0146 (0.0123-0.0225)**
M. pennanti	0.0289 (0.0257-0.0339)**	0.0064 (0.0073-0.0182)ns
E. barbara	0.0389 (0.0362-0.0548)**	0.0250 (0.0196-0.0475)ns
L. canadensis	0.0278 (0.0270-0.0523)ns	0.0227 (0.0218-0.0421)ns
M. mephitis	0.0356 (0.0286-0.0473)**	0.0218 (0.0187-0.0360)**
T. taxus	0.0587 (0.0464-0.0729)**	0.0407 (0.0256-0.0630)**

Table 4.

Species	*M. erminea*	*M. vison*	*M. americana*	*M. pennanti*
M. erminea	**0.876**	0.683	0.729	0.629
M. vison	0.652	**0.669**	0.705	0.676
M. americana	0.599	0.600	**0.733**	0.730
M. pennanti	0.624	0.629	0.655	**0.772**

Table 5.

Species	Prenatal	Postnatal	Function	Overall integration
Males				
Mustela erminea	0.214	0.614	0.409	8.44E-09
Mustela vison	0.245	0.357	0.333	6.78E-09
Martes americana	0.245	0.501	0.293	4.41E-09
Martes pennanti	0.388	0.333	0.140	4.41E-09
Females				
Mustela erminea	0.214	0.65	0.497	8.95E-09
Mustela vison	0.426	0.216	0.096	1.79E-08
Martes americana	0.265	0.345	0.154	4.93E-09
Martes pennanti	0.382	0.316	0.237	5.36E-09

Figure 1.

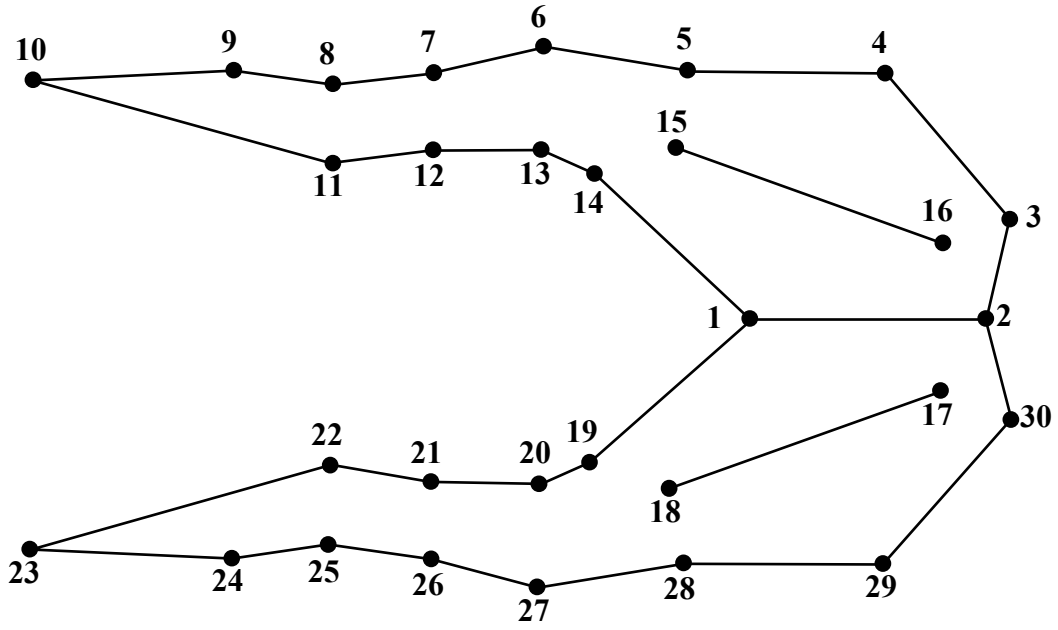
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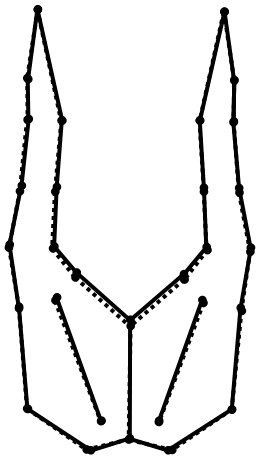
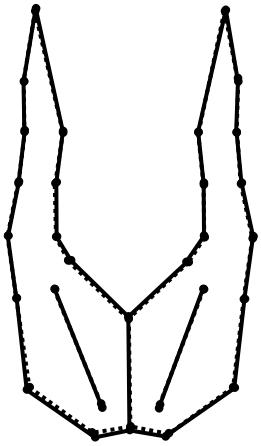
Figure 2.



Mustela erminea



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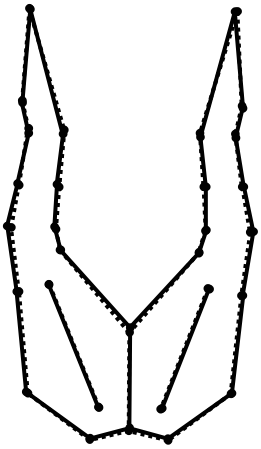

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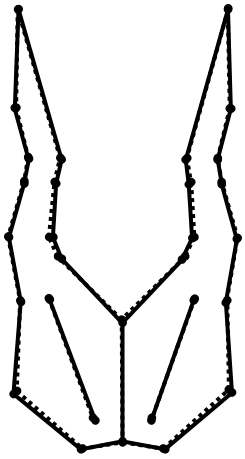

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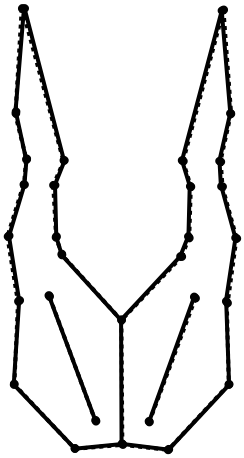

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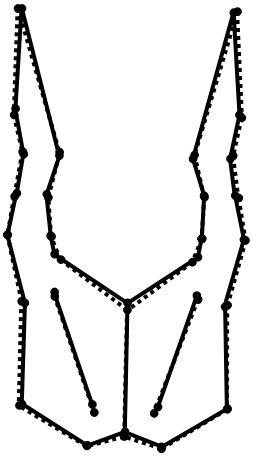
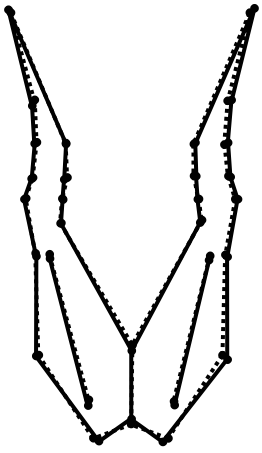
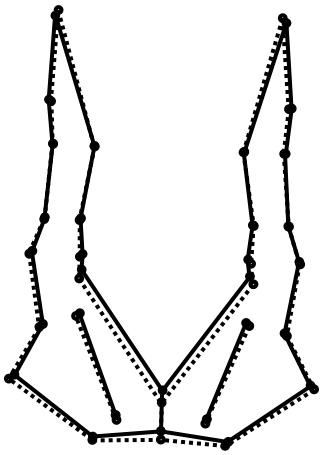


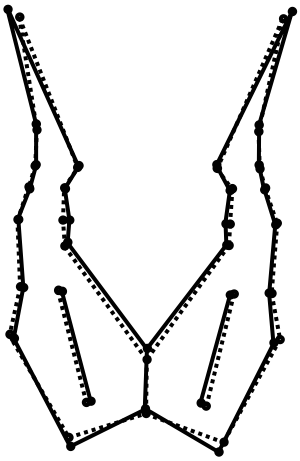
Eira barbara



Lutra canadensis

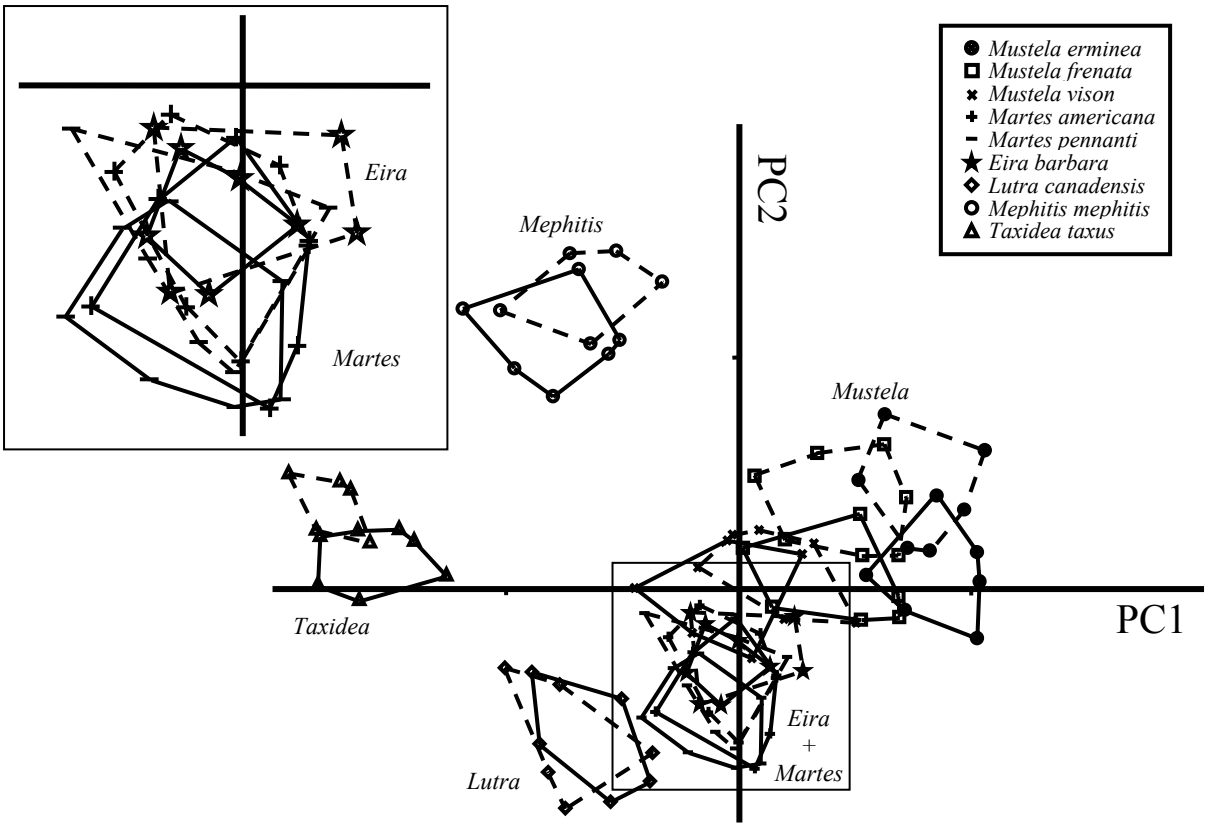


Mephitis mephitis



Taxidea taxus

Figure 3.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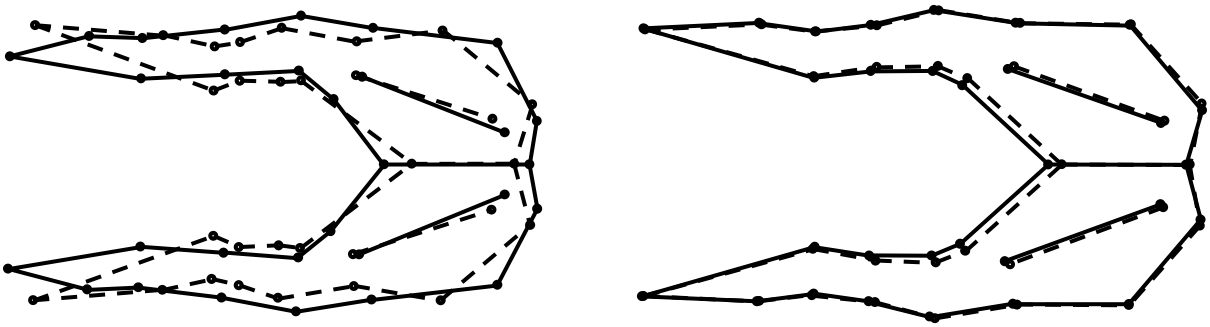
Mustela frenata
Mustela vison
Martes americana
Martes pennanti
Eira barbara
Lutra canadensis
Mephitis mephitis
Taxidea taxus
PC2
PC1
Eira
Martes
Mephitis
Mustela
Taxidea
Lutra
Eira
+
Martes

Figure 4.



A. Size B. Sex

Figure 5.

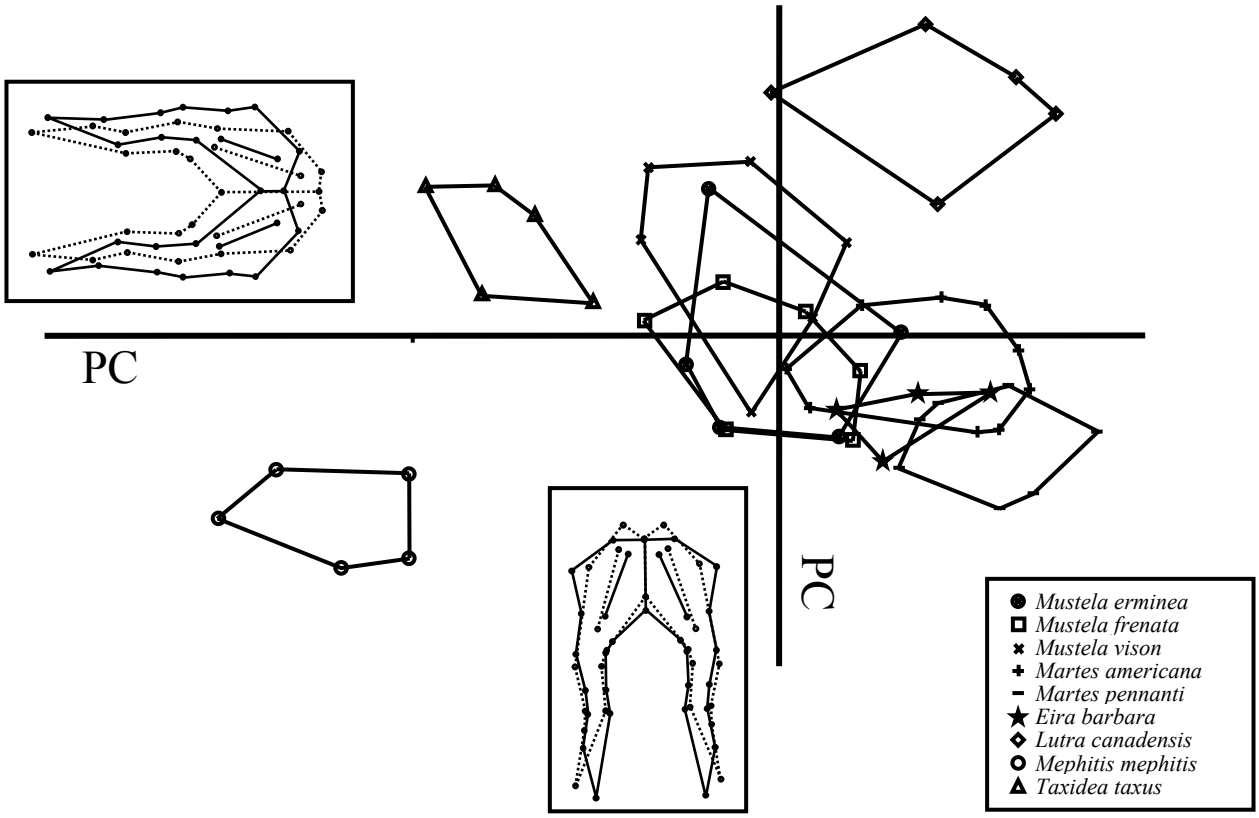

PC
PC
Mustela erminea
Mustela frenata
Mustela vison
Martes americana
Martes pennanti
Eira barbara
Lutra canadensis
Mephitis mephitis
Taxidea taxus

Figure 6.

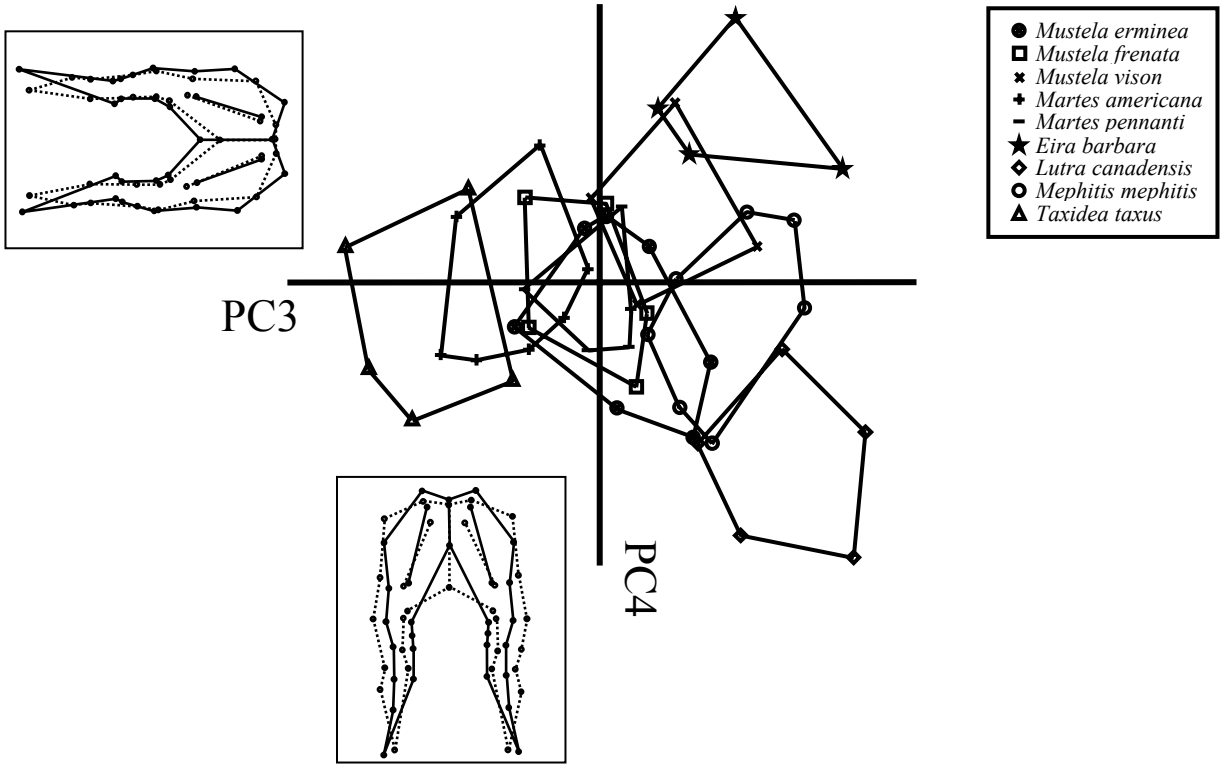
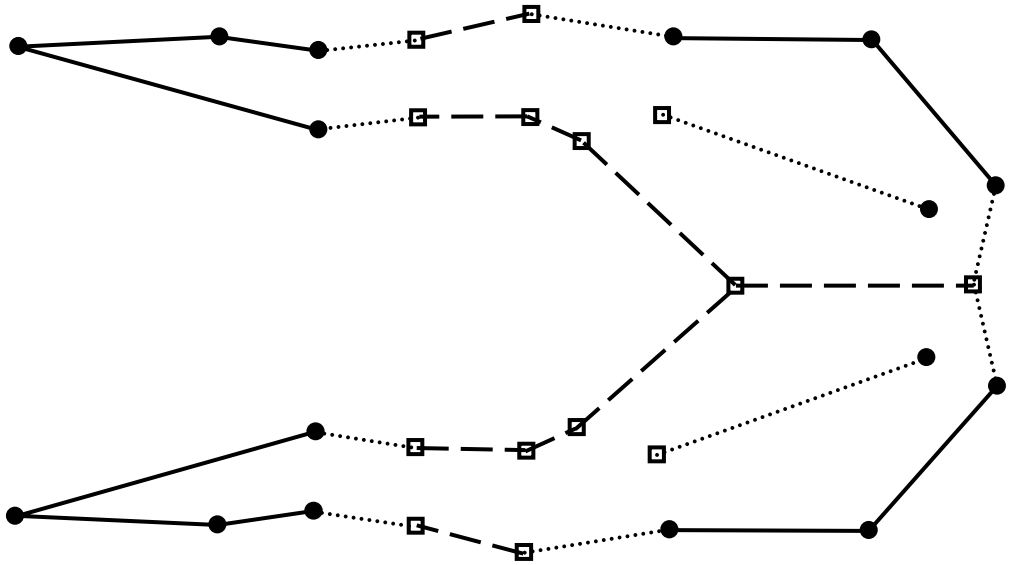

PC3
PC4
Mustela erminea
Mustela frenata
Mustela vison
Martes americana
Martes pennanti
Eira barbara
Lutra canadensis
Mephitis mephitis
Taxidea taxus

Figure 7.



Chapter 5

The Evolution of Phenotypic Integration Structure in the Mustelid Skull

ABSTRACT

The mammalian skull is a composite, sexually dimorphic structure showing complex patterns of phenotypic relationships among craniofacial structures. We tested the hypotheses of origin, sexual dimorphism and evolution of morphological integration in a model clade of mustelid species, using resampling-based Mantel's matrix correlation tests. We hypothesized that both size and developmental/functional relationships influence trait relationships in phenotype. Based on developmental/functional relationships, we hypothetically divided the cranial bones into four semi-independent modules: the cranial base, cranial vault, oral and upper face. Another set of hypotheses addressed whether skull integration is sexually dimorphic and if it evolves differently in males and females. Results indicate that, similarly to primates, developmental/functional relationships are important determinants of mustelid skull phenotypic covariances, suggesting that the observed pattern of integration is common across mammals. Sexual dimorphism was not found in the basic modular composition of the skull, but was in levels of integration within modules. The evolution of integration has proceeded differently in the two sexes: in males it corresponded to phylogenetic diversification patterns in the clade, and in females to morphological divergence among species.

Keywords: allometry, sexual dimorphism, modularity, mustelids.

The mammalian skull is a complex structure shaped partly by the functional requirements of the various organs of the head. As studies of primate and mouse models show, significant morphological integration exists among the bones of the skull (e.g., Cheverud 1982; Roth 1996; Smith 1996; Marroig et al. 2004; Hallgrímsson et al. 2004). Regions of the skull exhibit a specific covariance structure, forming complexes of strongly correlated and developmentally related traits, with lesser correlations among the regions (Waddington 1957; Cheverud 1982; Olson and Miller 1999). Several underlying factors are capable of producing sets of highly correlated traits within an anatomical region. Previous studies of skull morphological integration of primates and rodents suggest that patterns of function or development common to all mammals explain the covariance structure of the cranium (Cheverud 1982; Smith 1996; Hallgrímsson et al. 2004). The most detailed work, in primate clades, suggests that phenotypic covariance is stable across species because of high interspecific similarity in developmental mechanisms (Marroig and Cheverud 2001; Marroig et al. 2004; Young 2004). Both the more general developmental hypothesis, and this specific finding for primates, remain to be tested for other mammalian groups.

Several factors are potentially responsible for evolutionary changes in covariance structure in the skull. Development, allometry, phylogeny and morphological divergence influence patterns of variation in primates (Cheverud 1996; Badyaev and Hill 2000; Hallgrímsson et al. 2002; Marroig et al. 2004). Despite the documented significance of these factors in the evolution of integration patterns, the questions of how much divergence in covariance structure is observed during cranial evolution, and what is the nature of changes in trait relationships during morphological diversification, remain to be tested across a wider group of mammals. This study focuses on the origin and potential evolution of the

relationships among cranial traits by testing hypotheses of the evolution of sexual dimorphism and interspecific variation in skull phenotypic correlation and covariance patterns in seven species belonging to four genera of mustelids.

Mustelidae is a functionally and morphologically diverse family of carnivores with evolutionary relationships well resolved based both on morphological and molecular data (Bininda-Emonds et al. 1999; Koepfli and Wayne 2003; Sato et al. 2003; Marmi et al. 2004; Flynn et al. 2005). Mustelid lineages underwent rapid evolutionary diversification in six different radiation episodes since the Early Eocene (Marmi et al. 2004). At the same time, evolutionary diversification was accompanied with the striking degree of ecomorphological divergence, evident even among closely related species within a genus (Koepfli and Wayne 2003). This group has a significant variation in both size (spanning four orders of magnitude) and sexual dimorphism in body size (from species with no dimorphism to extremely dimorphic species where males are roughly twice as large as females) and craniofacial features (Ewer 1973; Holmes 1988; Gittleman and Van Valkenburgh 1997; Lee and Mill 2004). Mustelids are an excellent model to study the evolution of integration patterns, because close genetic relationships among species are accompanied with significant evolutionary divergence in morphology, and possibly with divergence in correlation patterns among craniofacial traits. We ask the following questions about morphological integration in the mustelid skull:

1) Do mustelids show patterns of functionally/developmentally based craniofacial modularity similar to primates and mice?

2) What is the nature of sexual dimorphic pattern of covariance in the skull, if it exists?

3) Does covariance structure evolve in a group of closely related but morphologically divergent species?

The first question addresses the hypothesis that the modular structure of skull phenotypic covariation is a reflection of the underlying modularity in developmental organization (Wagner 1996; Magwene 2001; Marroig and Cheverud 2001). Because some anatomical regions or structures grow as units (i.e. modules), morphological correlation within such modules will be high, but *among* such semiautonomous units correlation will be much lower (Hall 1995; Wagner 1996; Magwene 2001; Schlosser 2002). No study comparable to Cheverud's (1995) analyses of integration modularity in primates exists for any other mammalian clade. Given that the processes and tissue level patterns of cranial development are common across most mammals, we predict that patterns of modularity in mustelids will conform to this model.

Cheverud (1995) outlines the roles of hormonal influence, embryonic origin, tissue interactions and modes of ossification in the general developmental patterns in Eutherian skulls in terms of predicting the modular structure of phenotypic integration. Following this approach, we compared hypothetical modular patterns of correlation among skull traits to observed ones, and determined whether functional/developmental integration is a source of morphological integration. We hypothesized that cranial bones were divided into the four semi-independent modules defined by Cheverud (1995), which also correlate with development and embryology: the cranial base, cranial vault, upper face and oral modules.

The two major cranial regions, the neurocranium, including the cranial vault and the cranial base, and the viscerocranium, which can be divided into the upper face module and the oral module, are distinguished on the basis of their growth patterns. The growth of the

neurocranium depends almost entirely on the growth of the brain, occurs prenatally and neonatally, and completes its growth soon after birth. The neurocranial region is subdivided into the cranial base and cranial vault modules by the mode of ossification. Much of the cranial base derives from paraxial mesoderm (Sperber 2001), and is formed by endochondral ossification. The cranial base functions as a link between other craniofacial structures and plays a significant role in determining craniofacial shape (Lieberman et al. 2000a). The cranial vault elements are derived from neural crest and are formed by intramembranous ossification, closely matching growth patterns of the brain (Sperber 2001). In contrast, a majority of facial growth occurs after the brain has stopped growing (Smith 1996). The face consists of an oral and a facial module. The oral module includes the maxillary and premaxillary structures surrounding the dentition and is largely affected by teeth development and stresses generated by mastication. The remaining facial structures are combined in a face module, including distances linking orbital, zygomatic and nasal landmarks, which are influenced by the combined effects of brain growth (facial width), muscle activity and masticatory stress (Hallgrímsson et al. 2004).

Hallgrímsson et al. (2004) and Young (2004) propose and test an alternative hypothesis of overall skull integration due to effects of size. Size is the single most important determinant of cranial shape in most mammalian species and a strong correlation between size and shape is thus a significant component of integrated phenotypic variation in shape (Frost et al. 2003). Therefore, size is expected to play a large effect on the integration of the skull as a single unit. We therefore tested whether size played a significant role in overall mustelid skull integration.

Our second question dealt with the significance of sexual dimorphism in covariance structure of the skull. Most studies of morphological integration treat sex as an extraneous factor, removing effects of sex from the observed covariance structure (e.g., Cheverud 1996; Marroig and Cheverud 2001; Young 2004). Therefore, sexual dimorphism in morphological integration is not well documented. Sexes of the same mustelid species are expected to be highly similar in specifics of their developmental and functional organization, possibly differing only slightly in rates and timings of developmental events (Ternovsky 1977; King 1989; Gittleman 1993). Therefore, we predict intra-specific sexual dimorphism in covariance structure to be less than interspecific covariance structure differences. Sexual dimorphism in covariance structure could be expressed in various ways, but we predicted the basic pattern of trait relationships to be the same in males and females, with possible sexual variation of integration levels within modules.

Our third question asked whether the observed covariance structure in the skull evolves, and in response to what factors. Several authors argue for evolutionary stability of integration patterns. Marroig and Cheverud (2001) and Young (2004) find little correlation of covariation patterns with phylogeny in monkey crania and the hominoid scapula. They do document a correlation with functional similarity and morphological distance. On the contrary, Ackermann and Cheverud (2000) show that interspecific similarity in hominoid cranial covariation patterns corresponds to phylogenetic relationships, suggesting evolution in covariance structure. For mustelids, we tested the hypothesis that covariance structure in the skull remains evolutionary stable, based on rapid divergence of mustelid species and overall stability of skull developmental mechanisms. We also tested whether divergence in the skull morphology among mustelid species was accompanied by changes in

morphological covariance structure, which could be either in overall pattern of craniofacial trait relationships or in levels of integration within the four skull modules.

Materials and Methods

Sample

A total of 1051 mustelid skulls were examined and 632 skulls were measured in this study. The material is housed in the collections of the American Museum of Natural History, Field Museum of Natural History, Smithsonian Museum of Natural History and Harvard Museum of Comparative Zoology. A complete list of examined specimens may be obtained from the authors upon request. The sample included seven species, belonging to four genera: *Mustela sibirica* (40 males, 24 females), *M. erminea* (34 males, 55 females), *M. frenata* (56 males, 58 females), *M. vison* (55 males, 57 females), *Martes americana* (47 males, 36 females), *Eira barbara* (40 males, 47 females) and *Lutra canadensis* (41 males, 42 females). Only complete, adult specimens of known sex were used in the analysis. Specimens were judged as belonging to adults by presence of the totally erupted and functional dentition, and complete fusion of skull sutures. Whenever possible, only specimens from the same geographical area were used in analyses to reduce sample heterogeneity due to geographical variation. The total sample included 313 males and 319 females, representing seven species.

Data Collection

Each skull was digitally photographed in dorsal and ventral views using Canon G2 PowerShot digital camera with 1600x1200 pixel resolution. To eliminate distortion due to parallax effects, all images were taken with the camera positioned at a sufficient distance to

ensure that the specimen occupied only the part of the field free of the distortion. A ruler was included in all images, in the same plane as the specimen, to allow computation of skull size. A total of 63 (28 in dorsal view and 35 in ventral view) homologous and repeatable anatomical landmarks, consistently visible on all photographs, were converted to the form of two-dimensional (2D) Cartesian coordinate data by digitizing photographs using the *tpsDig* software (Rohlf 2003). These landmarks were chosen for their capacity to provide comprehensive and even coverage of the entire skull (fig. 1, table 1). Skull size was characterized as centroid size, square root of summed squared distances of landmarks from the skull's centroid (Slice et al. 1996), calculated for all landmark configurations. One randomly chosen specimen from each species was placed, photographed, and digitized 10 times. These 10 sets of landmark coordinates were superimposed and plotted. Digitizing errors at each landmark were small, circular and similar in size, indicating there were no systematic errors in positioning the specimen or in recognizing individual landmarks. Data used in this study were photographed and digitized by a single individual (S.B.).

Covariance and Correlation Structure

Digitized landmark coordinates were subjected to a Generalized Least-Squares Procrustes superimposition (GLS) in the program *CoordGen6f* of the IMP series software (Sheets 2004). This procedure superimposes a set of skull landmarks, mathematically removing such "nuisance" parameters as initial differences in size, position and orientation of specimens (Rohlf and Slice 1990; Rohlf 1996). The resulting sets of superimposed landmarks represent the size-invariant landmark variation around landmark means of the sample. This

procedure is common in geometric morphometrics analyses and details of it are presented elsewhere (e.g., Bookstein 1996; Rohlf 1996).

Following Procrustes superimposition, two different approaches were used to quantify and represent phenotypic variance/covariance and correlational structure in the mustelid skull. First, phenotypic variance/covariance matrices (further referred to as covariance matrices) were computed for the dorsal and ventral sets of Procrustes superimposed landmark configurations in *SYSTAT 10* (Wilkinson 2003). These matrices estimated phenotypic covariance patterns of the entire skull for all sexes and species in the sample. Secondly, sets of Euclidean distances (in millimeters) were calculated in the IMP *Tmorphgen6* program (Sheets 2004) among landmarks of both ventral and dorsal sides to assess correlational structure and levels of integration in the skull. Two sets of 48 linear distances in dorsal view and 47 distances in ventral view describing cranial morphology were calculated from the coordinate values. These sets were reduced to a 31 measurement set in dorsal view and 30 measurements in ventral view, averaging the measurements present on both left and right sides of the skull. These 61 measurements were classified in functional/developmental groups or modules following Cheverud (1995) (table 2). A total of four sets of measurements were created to represent four skull modules: the cranial vault (11 measurements) and face (20 measurements) in dorsal view, and the cranial base (14 measurements) and oral modules (16 measurements) in ventral view (table 2). Collectively, they represented two major developmental/functional modalities in the skull: the neurocranium (the cranial vault and cranial base modules) and viscerocranium (the face and oral modules). Pearson correlation matrices were computed from interlandmark distances in

SYSTAT 10 (Wilkinson 2003), to estimate phenotypic correlational structure in the skull, for the entire skull in dorsal and ventral views, and for each of the four modules separately.

Integration Hypotheses and Levels

The a priori hypotheses of integration pattern were tested in two ways. First, we performed a principal component analysis (PCA) on Procrustes residuals to calculate the pattern of variation in landmarks. PCA scores for the first principal component were plotted against centroid size to test if size accounts for a significant proportion of total variance. PCA tests whether the skull is integrated as one overall module due to the underlying effects of overall size on variation in landmarks (Hallgrímsson et al. 2004; Young 2004). Principal components analysis was carried out using the *Morphologika* program (O'Higgins and Jones 2004). Secondly, the hypothesis of integration through developmental/functional relationships in the skull was tested following the matrix correlation methodology of Cheverud (Cheverud 1982; Cheverud et al. 1989). Matrices of observed patterns of covariance and correlation of species, by sex, were compared to connectivity matrices representing hypothetical developmental/functional integration pattern. Hypothetical integration matrices were constructed by placing a one where two traits were hypothesized to be related in development, and a zero where no relationship was predicted (Cheverud et al. 1989). Correlation of the observed phenotypic covariance or correlation matrix to hypothetical connectivity matrix was tested by Mantel's test of matrix correlation, for all sexes and species. The matrix correlation is a measure of the strength of association among matrices varying from –1.0 to +1.0. A matrix correlation of +1.0 indicates identity of integration pattern in the two matrices. A matrix correlation of zero indicates no structural

similarity between the matrices. Statistical significance of matrix correlation representing support for integration hypotheses was assessed with the help of randomization tests. The rows and columns of the observed covariance or correlation matrix were randomly permuted and the randomized matrix was then correlated with the hypothetical connectivity matrix, repeating this procedure 1000 times. The observed correlation between covariance/correlation and connectivity matrices was compared to an empirically derived distribution of 1000 random matrix correlations. The integration patterns were considered significantly similar, and integration hypothesis supported, if the observed correlation exceeded 95% of the random correlations. All Mantel's tests and randomizations were performed in the *PopTools 2.6* program (Hood 2005).

The magnitude of overall integration in the whole skull, as well as within each of the four developmental modules, was measured as the mean Pearson correlation for a correlation matrix (Cheverud et al. 1989; Marroig and Cheverud 2001). Alternatively, the levels of integration were measured as the variance of the covariance matrix's eigenvalues. High eigenvalue variances are indicative of the high overall level of integration (Wagner 1984; Cheverud et al. 1989; Hallgrímsson et al. 2004).

Sexual Dimorphism and Evolution of Integration Patterns

Statistical significance of sexual dimorphism and evolution of integration patterns were assessed using randomization-based Mantel's tests. High correlations between covariance or correlation matrices of males and females indicate low sexual dimorphism. Measures of species sexual dimorphism in integration were correlated with measures of sexual dimorphism in shape to test the hypothesis that sexual dimorphism in integration

results from the evolution of sexual differences in phenotype. Procrustes distances provided a single number calculation of the amount of shape difference between the sexes (table 3), calculated as the sums of squared distances between corresponding homologous landmarks of Procrustes-superimposed configurations (Rohlf and Slice 1990). Procrustes distances were calculated in the *TwoGroup6e* program of the IMP series software (Sheets 2004).

The hypotheses of evolution in integration were tested by Mantel's matrix correlations of interspecific integration similarity matrices and constructed matrices of phylogenetic, taxonomic and morphological distances among species (Marroig and Cheverud 2001). Interspecific integration similarity matrices were computed as matrices of correlations among covariance or correlation matrices of seven mustelid species. Matrices of interspecific morphological distances were represented as Procrustes distances calculated among mean forms of the seven species (table 3). Taxonomic matrices were constructed assigning one to congeners, two to species of the same subfamily and three to species of the same family (table 3). Divergence times between species (in millions of years) were used to construct matrices of phylogenetic distances (table 3). Information on times of evolutionary divergence in mustelid species was used from the Order Carnivora supertree phylogenetic hypothesis of Bininda-Emonds et al. (1999). All covariance and correlation matrices of observed phenotypic integration were computed separately for males and females of each species. In case of correlational matrices, tests were done for the four developmental modules individually as well, in order to assess modular pattern of the evolution in integration.

Results

The wireframe diagrams suggest differences both between sexes and among species in the overall shape of the skull (fig. 2).

Hypotheses of Integration

The hypothesis that overall integration in skull landmarks was due to variation in skull size was not supported. The scores for the first principal component axis were not significantly correlated with centroid size in any of the mustelid species. Average percent variance explained by the first principal component was only 33.2% for Procrustes superimposed landmarks on dorsal side and 23.3% for ventral landmarks. Corresponding percentages for the second principal component were 14% for dorsal side and 13.5% for ventral.

The matrix correlations between observed correlation structure in the skull (measured as matrix of interlandmark distance correlations) and hypothetical modular structure predicted on the basis of development or function (using our connectivity matrix assigning 1 to developmentally related and 0 to unrelated traits) were significant for all species-sex groups. High correlations existed among interlandmark Euclidean distances within each of the four modules defined by function or development (cranial vault, face, cranial base, oral) for each species-sex group (table 4). There was no obvious difference between the modules from the dorsal view relative to those from the ventral view. The two sets of between module correlations, cranial vault/face in the dorsal view and cranial base/oral in the ventral view, were lower (table 4).

Overall Magnitude of Integration

The overall magnitude of integration in the skull, showed different patterns for dorsal and ventral measurements based on the two metrics we used, the average correlation value of interlandmark distance correlations and the variance of eigenvalues for a set of landmarks. For dorsal modules, these two metrics agreed as to level of integration (fig. 3). There was a discrepancy for ventral modules; there was no relationship between average correlation and eigenvalues variance in this view (fig. 3). Variances of eigenvalues were significantly higher in the dorsal landmarks than in the ventral landmarks (fig. 3). Some interspecific variation existed in the values of correlations and variances of eigenvalues, but there was small, if any, difference between the sexes in these measures. Species of the genus *Mustela* (weasels) had the highest average correlations and variances of eigenvalues for dorsal traits, followed by related *Martes* (martens) and *Eira* (tayra) species. The Canadian river otter (*Lutra canadensis*) exhibited the lowest correlations and eigenvalue variances.

The interlandmark distances were correlated to a different degree within each of the four skull modules (fig. 4). The oral module showed the highest interlandmark distance correlations compared to other modules. The cranial vault was second in the degree of correlation to the oral module, followed by the face. Cranial base had the lowest correlations of all (fig. 4). Within modules, correlations were similar between the sexes, although higher in females than in males, with the face as the only exception (fig. 4).

Integration and Sexual Dimorphism

There were no differences between the sexes in the integration structure of the skull, as measured by the correlations between correlation or covariation matrices of the sexes

(table 5). All correlations were significant at the 0.001 level.

Sexual dimorphism in integration was significantly correlated with sexual dimorphism in shape only in case of ventral landmark covariation matrices ($r = -0.905$, $p = 0.005$). For ventral correlation matrices, and for dorsal covariation and correlation matrices, there was no correlation with sexual dimorphism in morphology (dorsal correlation matrix: $r = -0.245$, $p = 0.596$; ventral correlation matrix: $r = -0.183$, $p = 0.695$; dorsal covariance matrix: $r = 0.270$, $p = 0.559$).

Correlations of both covariation and correlation matrices indicated that both sexes exhibited more sexual dimorphism for integration in the ventral relative to the dorsal side. In particular, as Mantel's correlation analysis of modules demonstrated, sexes were most dimorphic in measurement correlation sets belonging to the cranial base and oral modules of the skull, and less dimorphic in the correlation structure of the upper face and cranial vault modules (fig. 5). Sexual dimorphism in the correlation structure of the cranial vault was significantly correlated with sexual dimorphism in the correlation structure of the face ($r = 0.93$) and oral ($r = 0.75$) modules, and not correlated with sexual dimorphism in the structure of the cranial base ($r = 0.42$) (fig. 5).

Evolution of Integration Patterns

All interspecific correlation or covariation matrix correlations were significant at the 0.001 level (table 5). Species exhibited higher correlations in interspecific matrix correlations for dorsal traits than for ventral traits. Interspecific correlations of the correlation matrices by sex demonstrated that males of mustelid species are more similar to each other than females are.

We examined interspecific similarity in integration by estimating relationships among correlation or covariance matrices of all species. There was a significant relationship between phylogenetic distance and interspecific similarity in correlation structure. This general pattern was maintained with the exception of the female ventral similarity matrix (table 6). Phylogenetic distance and taxonomy exhibited very similar pattern of correlation with the observed interspecific integration similarity matrices; phylogeny showing higher correlations than taxonomic rank. Significant correlations with morphological distance were found only in females (table 6). In the case of male dorsal interspecific similarity, significant matrix correlations with phylogeny and taxonomy were largely due to high interspecific correlations in the face module (table 6). All other significant correlations of similarity matrices with phylogeny, taxonomy or morphological distance were module-independent.

Discussion

Patterns and Levels of Integration

Integration in the two large-scale modules of the mammalian skull (neurocranium and viscerocranium) results from developmental and functional factors that are synapomorphic for mammals (Smith 1996). The overall pattern of phenotypic covariation that we found in mustelid skulls largely corresponded to these craniofacial integration patterns, best described in primates (Cheverud 1995) and in laboratory mice (Hallgrímsson et al. 2004). These results provide strong support for Cheverud's (1982) hypothesis that the common modularity pattern shared by most mammalian orders reflects the evolutionarily conserved patterns of mammalian skull development.

The basic pattern of integration in the mustelid skull was similar to primates and

mice, although we found some significant differences. One important difference was the role of size in overall skull integration, as our results did not support Hallgrímsson et al.'s (2004) hypothesis that overall integration in skull landmarks was due to variation in size. Other differences were found in more minor areas, such as in the levels of integration within finer-grained skull modules, e.g., cranial base or oral module. The highest magnitude of integration existed in the oral module, similar to results reported for primate species (Cheverud 1995; Marroig and Cheverud 2001; Marroig et al. 2004). Such high levels of oral integration are likely explained by highly coordinated development of the maxilla, mandible and dentition, which in turn reflect the functional demands of feeding, and in particular, mastication (Stock 2001).

Cheverud (1996) suggests that the significant integration in the cranial vault and the well-defined separation of braincase and face modules are only characteristic of primate groups due to the prolonged early growth period leading to larger brains in primates, relative to other mammals. Our results, however, show that this pattern of integration is not unique to primates. Cranial vault and face modules exhibited higher integration within than among them, and integration in the mustelid cranial vault was second highest in the skull. The magnitude of cranial vault integration was as high as it has been shown for the saddle-back tamarins (Cheverud 1995) and even higher than it was reported for most New World monkeys (Marroig and Cheverud 2001, Marroig et al. 2004). High integration of the cranial vault of mammals in general may reflect a unique developmental origin and growth of the mammalian brain, independent of duration of early growth or size of the brain. On the other hand, it may be that carnivores, with brains relatively larger than other mammals, have the same patterns of cranial vault growth and integration as primates (Iwaniuk et al. 2001;

Kruska 2005). Data on additional taxa would clarify the evolutionary extent of the pattern of cranial vault integration across Eutherian mammals.

The lower integration levels in the face, relative the cranial vault or oral modules, could stem from the varied and complex functional and development factors influencing this module, consisting of the zygomatic arches, orbits, and nasal cavity. The factors relevant to the face include diverse muscle activity, growth of sense organs, mastication stresses, and indirect influences of brain growth. The basicranium was the least integrated skull module, consistent with integration levels reported in primate crania (Cheverud 1995; Marroig and Cheverud 2001; Marroig et al. 2004). One suggestion is that low integration levels may result from correlations among functionally and developmentally independent traits (Cheverud 1995), which is possible due to the basicranium interactions with components of both brain and face in development (Lieberman et al. 2000b).

Examination of results from different modules suggests that the magnitude of integration depends on the complexity of underlying developmental and functional factors defining each module. Highly integrated modules such as oral dentition and cranial vault have unique developmental origins and a basic set of functions to perform. On the contrary, less integrated modules, such as face and cranial base, are complex in their development and function. This conclusion, in turn, suggests a modification of the long standing hypothesis that integration is much higher in the viscerocranium than in the neurocranium (e.g., Marroig and Cheverud 2001), as finer scale modules with distinct and variable levels of integration clearly exist within each of these broadly defined units. Lower integration in the neurocranium may be only a reflection of the very low integration of the basicranium, ignoring strong integration of the cranial vault. Similarly, higher integration in the

viscerocranium is probably just a reflection of high oral integration. The use of neurocranium and viscerocranium as distinct entities may not be useful, and may mask important biological patterns (Maunz and German 1996).

Integration and Sexual Dimorphism

Males and females were not different in the basic modular structure of the skull. The similar patterns of modularity in both sexes are not surprising considering the fact that general patterns of skull development are highly conserved across sexes and species of mammals. However, males and females of the same species did differ in magnitudes of integration within and across modules. Differences in levels of integration between sexes were as high as differences in integration levels among species. This is contrary to the prediction of intraspecific integration differences being less than interspecific differences, stemming from the fact that sexes of the same species are more functionally and genetically similar than different species. Sexual dimorphism in integration was greater than interspecific differences for some species, but the opposite was true for other species. For example, in *Mustela sibirica* and *Martes americana* differences between sexes were greater than most differences among species. Only *Mustela erminea* and *Eira barbara* consistently showed less difference between sexes than among species.

Sexual dimorphism in skull size or morphology did not predict sexual dimorphism in covariance structure, or integration. In particular, sexes were most different in integration of craniofacial measurements belonging to the cranial base and oral modules, which were least dimorphic for shape. The sexes were least dimorphic in the correlation structure of the cranial vault, although a significant portion of sexual dimorphism in carnivoran skull morphology is found in the relative size of the braincase (Gittleman 1994). The lack of a

relationship, or at best a weak relationship, between sexual dimorphism in covariance and development patterns suggests that a significant portion of sexual dimorphism in integration could be attributed to ontogenetic growth mechanisms (Badyaev 2002). Variation in relative rates and timings of growth in neurocranial and viscerocranial structures produces similar patterns of craniofacial trait relationships (Zelditch et al. 1992). Longitudinal growth studies of the cranium in both metatherian and eutherian mammals show that sexes of the same species frequently differ in relative timings of growth, rates of growth and relationships among growing craniofacial bones (integration), which may lead both to similar and divergent adult morphologies (Maunz and German 1996; Lightfoot and German 1998). Mustelid species are known to be sexually dimorphic in growth and timing of maturation events (King 1989; Gittleman 1993), which could include differences in relative growth of the craniofacial structures as well. The effects of postnatal growth patterns on the skull covariance structure require further investigation, since this would help to understand how differences in trait relationships in ontogeny map onto differences in adult phenotypic integration patterns not consistent with differences in adult skull morphology.

Evolution of Integration Patterns

The significant relationship between integration structure and phylogeny shows that the integration of the skull in mustelid clade did not remain constant over time. The significant association between skull integration structure and phylogeny was strongest in the face and cranial base modules, i.e., those modules which were also the least integrated. The only significant correlation with morphological distance, in the female ventral similarity matrix, was largely due to high correlations in the cranial base as well.

The modules, such as the cranial base, with weaker integration and a more complex developmental/ functional framework, may have been more susceptible to evolutionary change. Cheverud (1982) and Wagner (1996) predict this pattern, demonstrating that correlations among traits constrain the rates of evolutionary change. If the cranial base is an agent of evolutionary change, as shown in primates, the potential for cascading effects on other regions of the skull exists (Lieberman et al. 2000a,b).

Morphological distance among species was significantly, but not strongly correlated with phylogeny, which indicates morphological divergence in closely related mustelid species, consistent with rapid mustelid evolution. However, for the most part, evolution of integration in mustelids followed the phylogenetic pattern, meaning that related species tended to resemble each other in covariance structure. This result suggests that interspecific similarity in genetics of developmental control left basic relationships among craniofacial features relatively constant, even though the skull phenotype was simultaneously evolving.

The evolution of the skull covariance structure differed in the two sexes. In general, females showed greater correlations of interspecific integration similarity with morphological distance. Females also resembled each other less in relationships among craniofacial traits, interspecifically. This divergence in integration structure, related to interspecific changes in morphology, was largely due to discrepancies in the cranial vault and the cranial base modules. One of the explanations may be that the neurocranium of females underwent more rapid evolutionary change relative to males. The evolution of the relative size of the female brain is known to be independent of the evolution of relative male brain size in terrestrial Carnivora (Gittleman 1994). The basis of that change may be due to the evolutionary flexibility conferred by lower levels of integration.

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Table and Figure Captions

Table 1. Anatomical descriptions of the 63 homologous landmarks digitized on mustelid skulls. The label D (dorsal) or V (ventral) after the landmark description indicates which view the landmark was digitized in. Landmarks descriptions are also given in Figure 1.

Table 2. Forty-eight dorsal and forty-seven ventral skull measurements calculated as Euclidean interlandmark distances. Measurements (in millimeters) are grouped on the basis of their belonging to the four developmental/functional modules and two major cranial regions of the skull. Table 1 defines each landmark anatomically and Figure 1 shows their locations in a consensus mustelid skull.

Table 3. Phylogeny, taxonomy and morphological distance matrices used in integration hypotheses testing. Phylogenetic distances (divergence times in millions of years) are presented below the diagonal, taxonomic affinity above the diagonal. Morphological distance was calculated as a Procrustes distance between consensus skull shapes of Procrustes superimposed landmark configurations. Male Procrustes distances are given below the diagonal, female distances are above the diagonal, distances representing sexual dimorphism are on the diagonal.

Table 4. Integration levels within skull modules for males and females of seven mustelid species. Integration magnitude was assessed as a mean Pearson correlation calculated for interlandmark distance correlations within the four skull modules. “Vault/face” and “oral/base” are “between-modules” corresponding to sets of non-integrated skull traits.

Table 5. Matrices of integration similarity between sexes and among species of mustelids, given as values of correlations between correlation or covariance matrices of sexes and species. Values below the diagonal are for interspecific integration similarity of males, the

diagonal is intraspecific integration similarity or sexual dimorphism, and above diagonal correlations are for females. All comparisons, as tested by Mantel's test, were significant at the 0.001 level.

Table 6. Correlations between interspecific correlation similarity matrices and matrices representing morphological distance, phylogenetic distance and taxonomic affinity among species. "All" are complete interspecific matrices, not divided into modules, that were calculated using correlation matrices of species computed from all landmark distances on dorsal or ventral sides. Asterisk denotes statistically significant correlations at the 0.05 level.

Figure 1. The 63 landmarks, 28 in dorsal view and 35 in ventral view, are plotted on the consensus skull shape of the 632 specimens. Left: dorsal view. Right: ventral view. For anatomical definition of landmarks, see Table 1. Links are drawn to make anatomical structures clearer. Landmarks 15-11-9-7-6-8-10-12-16-5 outline the cranial vault. Landmarks 13-17 and 14-18 are zygomatic arches. Landmarks 19-21-23-20-22-24: orbital cavities. Landmarks 1-27-2-28 outline the external nasal opening. Landmarks 3 and 4 belong to the nasal bones, and landmarks 25 and 26 are on the facial outline of the maxillary bones. Occipital condyles and foramen magnum are located between landmarks 31-34-32-33-35. Landmarks 36-38-40 and 37-39-41 belong to the medial outline of tympanic bullae along basioccipital and basisphenoid bones. Landmarks 42-44 and 43-45: mandibular fossae. Landmarks 46-48 and 47-49: molars. Landmarks 48-52-50 and 49-53-51: carnassials. Landmarks 52-54 and 53-55: 2nd premolars. Landmarks 58-60 and 59-61: canines. Landmarks 62-29-63: incisors.

Figure 2. Wireframe diagrams showing differences between sexes and among species in the overall shape of the skull.

Figure 3. Overall integration levels in the skull of mustelid species measured as the average correlation value and the variance of eigenvalues. For dorsal side, the two measures correspond to each other. There is a discrepancy for ventral side; low variances of eigenvalues in ventral landmarks correspond to high average interlandmark distance correlations.

Figure 4. Integration levels among skull modules measured as mean Pearson correlation values of interlandmark distance correlations within modules. “Vault/face” and “oral/base” are “between-modules” corresponding to sets of non-integrated skull traits.

Figure 5. Sexual dimorphism in integration measured as a correlation between male and female correlation matrices, calculated for sets of traits representing four skull modules. Low correlations imply high sexual dimorphism. Correlations values for the cranial vault, which has the least amount of dimorphism, are plotted against sex correlations for other modules. Dotted line represents 1:1 relationship with the cranial vault. Sexual dimorphism in face integration is closely related to the cranial vault, both have low sexual dimorphism (A). The oral and cranial base modules show higher sexual dimorphism in integration, not related to the cranial vault or face (B and C).

Table 1.

Landmark(s)	Description	Position(s)
1	intradentale superior, D	midline
2	nasale, D	midline
3	nasion, D	midline
4	nasal midline at zygomaxillare, D	midline
5	frontal midline at constriction, D	midline
6	external occipital protuberance, D	midline
7, 8	cranial vault at maximum parietal breadth, D	left, right
9, 10	posterior of zygomatic process of temporal, D	left, right
11, 12	anterior of zygomatic process of temporal, D	left, right
13, 14	zygo-temporal suture, D	left, right
15, 16	postorbital constriction, D	left, right
17, 18	frontal process of zygomatic bone, D	left, right
19, 20	zygomatic process of frontal bone, D	left, right
21, 22	zygomaxillare superior, D	left, right
23, 24	zygomaxillare inferior, D	left, right
25, 26	maxillar outline at the level of nasion, D	left, right
27, 28	external naris, D	left, right
29	intradentale superior, V	midline
30	posterior nasal spine, V	midline
31	basion, V	midline
32, 33	occipital condyle, V	left, right
34, 35	paroccipital process, V	left, right
36, 37	jugular foramen, V	left, right
38, 39	posterior carotid foramen, V	left, right
40, 41	foramen for auditory tube, V	left, right
42, 43	zygo-temporal suture, V	left, right
44, 45	postarticular process, V	left, right
46, 47	maxillary tuberosity, V	left, right
48, 49	molar-premolar juncture, V	left, right
50, 51	carnassials, V	left, right
52, 53	2nd premolar-carnassial juncture, V	left, right
54, 55	anterior of 2nd premolar, V	left, right
56, 57	palatine fissure, V	left, right
58, 59	upper canines, V	left, right
60, 61	premaxillary suture at the alveolus, V	left, right
62, 63	upper incisors, V	left, right

Table 2.

Measurement	Module	Region	View
1-27, 1-28	face	viscerocranium	dorsal
2-27, 2-28	face	viscerocranium	dorsal
25-27, 26-28	face	viscerocranium	dorsal
23-25, 24-26	face	viscerocranium	dorsal
21-23, 22-24	face	viscerocranium	dorsal
19-21, 20-22	face	viscerocranium	dorsal
19-23, 20-24	face	viscerocranium	dorsal
17-23, 18-24	face	viscerocranium	dorsal
17-19, 18-20	face	viscerocranium	dorsal
13-17, 14-18	face	viscerocranium	dorsal
4-19, 4-20	face	viscerocranium	dorsal
19-20	face	viscerocranium	dorsal
21-22	face	viscerocranium	dorsal
23-24	face	viscerocranium	dorsal
25-26	face	viscerocranium	dorsal
27-28	face	viscerocranium	dorsal
3-4	face	viscerocranium	dorsal
2-3	face	viscerocranium	dorsal
2-4	face	viscerocranium	dorsal
1-2	face	viscerocranium	dorsal
6-7, 6-8	cranial vault	neurocranium	dorsal
7-9, 8-10	cranial vault	neurocranium	dorsal
9-11, 10-12	cranial vault	neurocranium	dorsal
11-15, 12-16	cranial vault	neurocranium	dorsal
6-9, 6-10	cranial vault	neurocranium	dorsal
6-15, 6-16	cranial vault	neurocranium	dorsal
7-8	cranial vault	neurocranium	dorsal
9-10	cranial vault	neurocranium	dorsal
11-12	cranial vault	neurocranium	dorsal
15-16	cranial vault	neurocranium	dorsal
5-6	cranial vault	neurocranium	dorsal
29-62, 29-63	oral	viscerocranium	ventral
58-60, 59-61	oral	viscerocranium	ventral
29-56, 29-57	oral	viscerocranium	ventral
52-54, 53-55	oral	viscerocranium	ventral
48-52, 49-53	oral	viscerocranium	ventral

Table 2 (continued).

Measurement	Module	Region	View
50-52, 51-53	oral	viscerocranium	ventral
46-48, 47-49	oral	viscerocranium	ventral
46-60, 47-61	oral	viscerocranium	ventral
30-46, 30-47	oral	viscerocranium	ventral
29-30	oral	viscerocranium	ventral
46-47	oral	viscerocranium	ventral
48-49	oral	viscerocranium	ventral
58-59	oral	viscerocranium	ventral
56-57	oral	viscerocranium	ventral
62-63	oral	viscerocranium	ventral
42-44, 43-45	oral	viscerocranium	ventral
32-34, 33-35	cranial base	neurocranium	ventral
31-32, 31-33	cranial base	neurocranium	ventral
34-36, 35-37	cranial base	neurocranium	ventral
36-38, 37-39	cranial base	neurocranium	ventral
38-40, 39-41	cranial base	neurocranium	ventral
40-44, 41-45	cranial base	neurocranium	ventral
30-40, 30-41	cranial base	neurocranium	ventral
32-33	cranial base	neurocranium	ventral
34-35	cranial base	neurocranium	ventral
36-37	cranial base	neurocranium	ventral
38-39	cranial base	neurocranium	ventral
40-41	cranial base	neurocranium	ventral
44-45	cranial base	neurocranium	ventral
30-31	cranial base	neurocranium	ventral

Table 3.

Phylogeny and Taxonomy							
	sibirica	*erminea*	*frenata*	*vison*	*americana*	*barbara*	*canadensis*
sibirica	**0**	1	1	1	2	2	3
erminea	5.5	**0**	1	1	2	2	3
frenata	5.5	2.5	**0**	1	2	2	3
vison	10.7	10.7	10.7	**0**	2	2	3
americana	13.6	13.6	13.6	13.6	**0**	1	3
barbara	13.6	13.6	13.6	13.6	2.6	**0**	3
canadensis	20.8	20.8	20.8	20.8	20.8	20.8	**0**
Morphological Distance							
dorsal							
	sibirica	*erminea*	*frenata*	*vison*	*americana*	*barbara*	*canadensis*
sibirica	**0.055**	0.070	0.064	0.085	0.085	0.073	0.112
erminea	0.085	**0.029**	0.064	0.108	0.110	0.110	0.150
frenata	0.069	0.071	**0.037**	0.058	0.090	0.083	0.132
vison	0.049	0.094	0.064	**0.029**	0.087	0.074	0.119
americana	0.091	0.106	0.092	0.088	**0.031**	0.081	0.148
barbara	0.051	0.101	0.085	0.063	0.086	**0.023**	0.104
canadensis	0.098	0.138	0.137	0.109	0.157	0.101	**0.015**
ventral							
	sibirica	*erminea*	*frenata*	*vison*	*americana*	*barbara*	*canadensis*
sibirica	**0.023**	0.051	0.022	0.047	0.088	0.095	0.097
erminea	0.049	**0.018**	0.045	0.089	0.130	0.137	0.131
frenata	0.026	0.044	**0.018**	0.047	0.089	0.100	0.099
vison	0.049	0.086	0.047	**0.017**	0.056	0.068	0.069
americana	0.098	0.131	0.093	0.063	**0.023**	0.064	0.088
barbara	0.093	0.129	0.094	0.065	0.070	**0.010**	0.081
canadensis	0.094	0.124	0.095	0.069	0.099	0.086	**0.008**

Table 4.

Species	Sex	Dorsal Modules			Ventral Modules		
		cranial vault	face	vault/face	cranial base	oral	oral/base
Mustela sibirica	male	0.592	0.631	0.523	0.351	0.518	0.327
Mustela erminea	male	0.563	0.684	0.512	0.369	0.700	0.412
Mustela frenata	male	0.435	0.351	0.250	0.310	0.505	0.255
Mustela vison	male	0.417	0.426	0.284	0.436	0.563	0.374
Martes americana	male	0.551	0.528	0.438	0.515	0.708	0.497
Eira barbara	male	0.569	0.426	0.319	0.366	0.579	0.336
Lutra canadensis	male	0.461	0.338	0.268	0.330	0.412	0.253
Mustela sibirica	female	0.482	0.512	0.227	0.520	0.743	0.530
Mustela erminea	female	0.694	0.710	0.582	0.572	0.832	0.625
Mustela frenata	female	0.600	0.522	0.419	0.521	0.698	0.476
Mustela vison	female	0.449	0.397	0.263	0.364	0.546	0.330
Eira barbara	female	0.534	0.403	0.333	0.290	0.488	0.226
Martes americana	female	0.421	0.267	0.015	0.213	0.313	0.064
Lutra canadensis	female	0.538	0.259	0.215	0.345	0.427	0.248

Table 5.

correlation dorsal							
	sibirica	*erminea*	*frenata*	*vison*	*americana*	*barbara*	*canadensis*
sibirica	**0.649**	0.72	0.54	0.674	0.573	0.523	0.413
erminea	0.828	**0.901**	0.733	0.758	0.524	0.544	0.5
frenata	0.657	0.65	**0.693**	0.676	0.429	0.503	0.519
vison	0.729	0.707	0.713	**0.857**	0.552	0.566	0.538
americana	0.683	0.727	0.654	0.651	**0.404**	0.587	0.481
barbara	0.464	0.472	0.548	0.543	0.705	**0.755**	0.592
canadensis	0.392	0.432	0.625	0.559	0.479	0.552	**0.732**
correlation ventral							
	sibirica	*erminea*	*frenata*	*vison*	*americana*	*barbara*	*canadensis*
sibirica	**0.513**	0.539	0.666	0.644	0.245	0.532	0.566
erminea	0.659	**0.706**	0.739	0.566	0.234	0.453	0.352
frenata	0.588	0.668	**0.651**	0.695	0.374	0.519	0.549
vison	0.672	0.607	0.552	**0.655**	0.513	0.601	0.509
americana	0.67	0.682	0.552	0.587	**0.415**	0.495	0.271
barbara	0.603	0.569	0.392	0.525	0.628	**0.644**	0.449
canadensis	0.561	0.491	0.397	0.466	0.501	0.495	**0.461**
covariance dorsal							
	sibirica	*erminea*	*frenata*	*vison*	*americana*	*barbara*	*canadensis*
sibirica	**0.691**	0.690	0.506	0.482	0.773	0.385	0.427
erminea	0.504	**0.756**	0.614	0.473	0.700	0.463	0.428
frenata	0.333	0.518	**0.543**	0.358	0.481	0.370	0.454
vison	0.800	0.548	0.528	**0.507**	0.410	0.288	0.356
americana	0.338	0.606	0.343	0.436	**0.627**	0.451	0.419
barbara	0.586	0.620	0.468	0.594	0.575	**0.620**	0.438
canadensis	0.263	0.499	0.516	0.476	0.376	0.465	**0.590**
covariance ventral							
	sibirica	*erminea*	*frenata*	*vison*	*americana*	*barbara*	*canadensis*
sibirica	**0.432**	0.468	0.479	0.557	0.344	0.45	0.356
erminea	0.516	**0.597**	0.725	0.458	0.357	0.515	0.45
frenata	0.515	0.519	**0.542**	0.457	0.352	0.54	0.438
vison	0.495	0.546	0.553	**0.585**	0.59	0.577	0.564
americana	0.456	0.379	0.488	0.551	**0.527**	0.484	0.565
barbara	0.432	0.435	0.481	0.612	0.497	**0.652**	0.461
canadensis	0.464	0.391	0.491	0.493	0.446	0.352	**0.66**

Table 6.

Hypothesis	Sex	Dorsal			Ventral		
		all	face	cranial vault	all	oral	cranial base
morphology	male	0.346	0.417	0.416	0.234	0.280	0.412
phylogeny	male	0.654*	0.764*	0.328	0.646*	0.076	0.291
taxonomy	male	0.720*	0.742*	0.409	0.648*	0.034	0.344
morphology	female	0.450*	0.066	0.189	0.660*	0.226	0.648*
phylogeny	female	0.607*	0.264	0.024	0.453	0.137	0.414*
taxonomy	female	0.707*	0.353	0.111	0.505	0.093	0.483*

Figure 1.

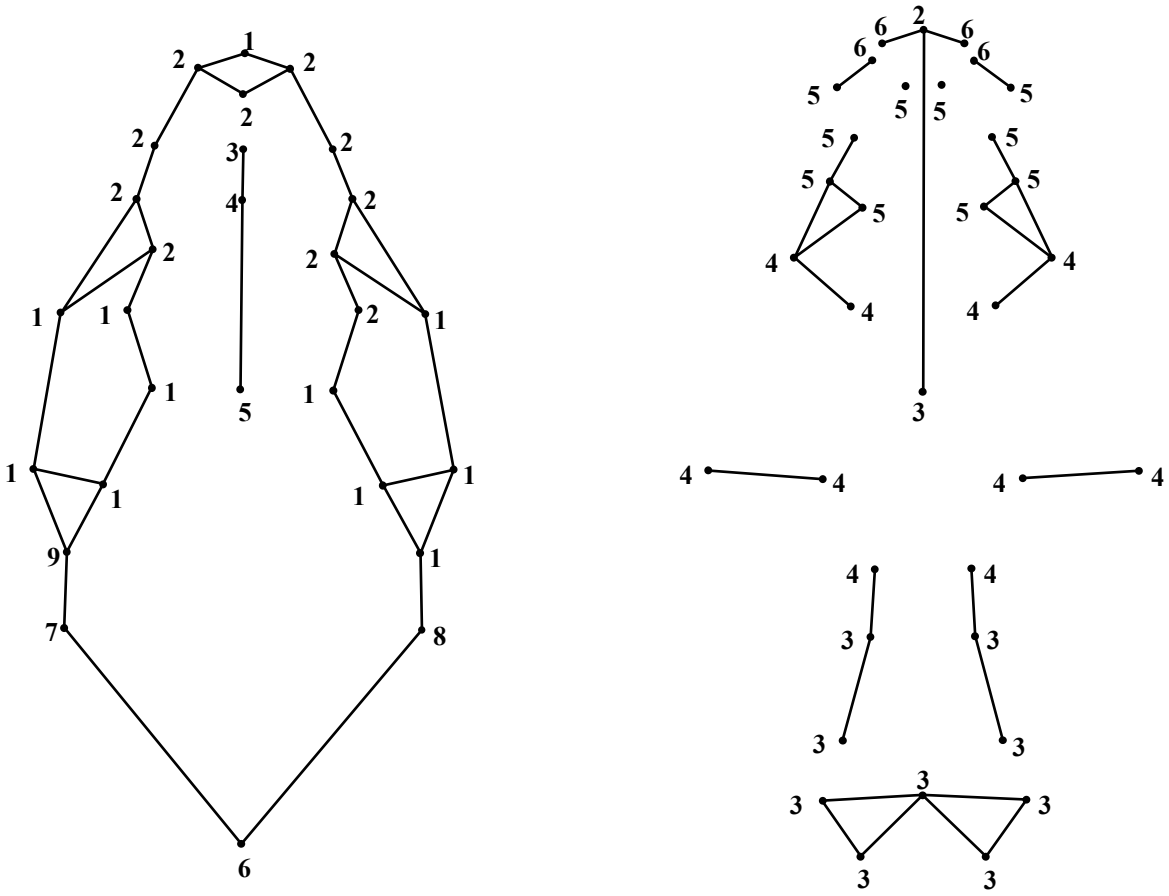
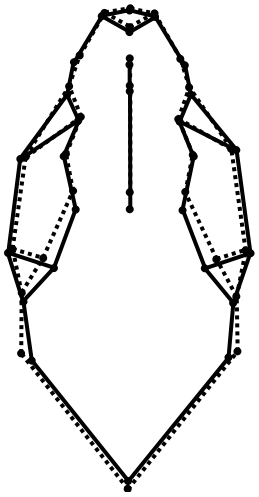
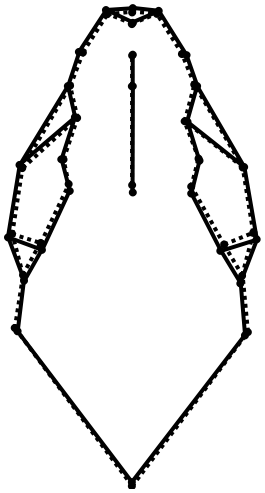


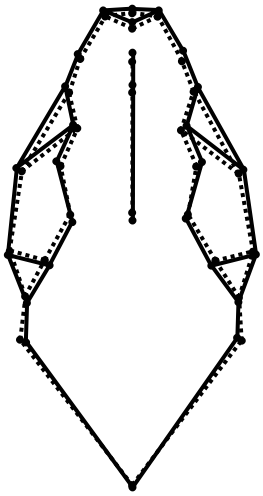
Figure 2.



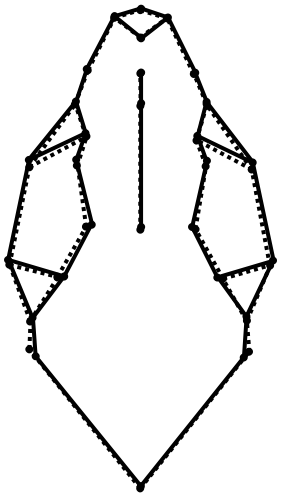
Mustela sibirica



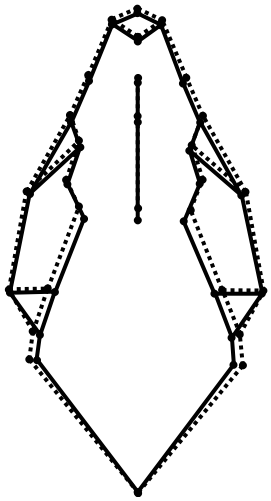
Mustela erminea



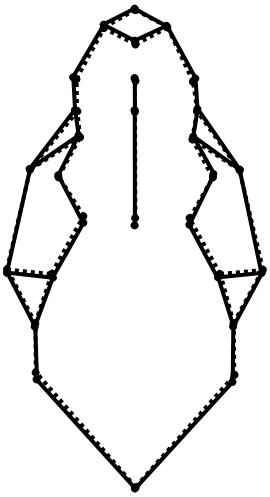
Mustela frenata



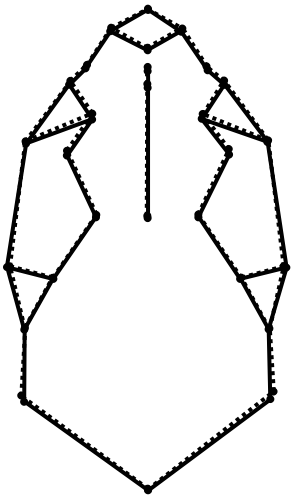
Mustela vison



Martes americana



Eira barbara



Lutra canadensis

Figure 3.

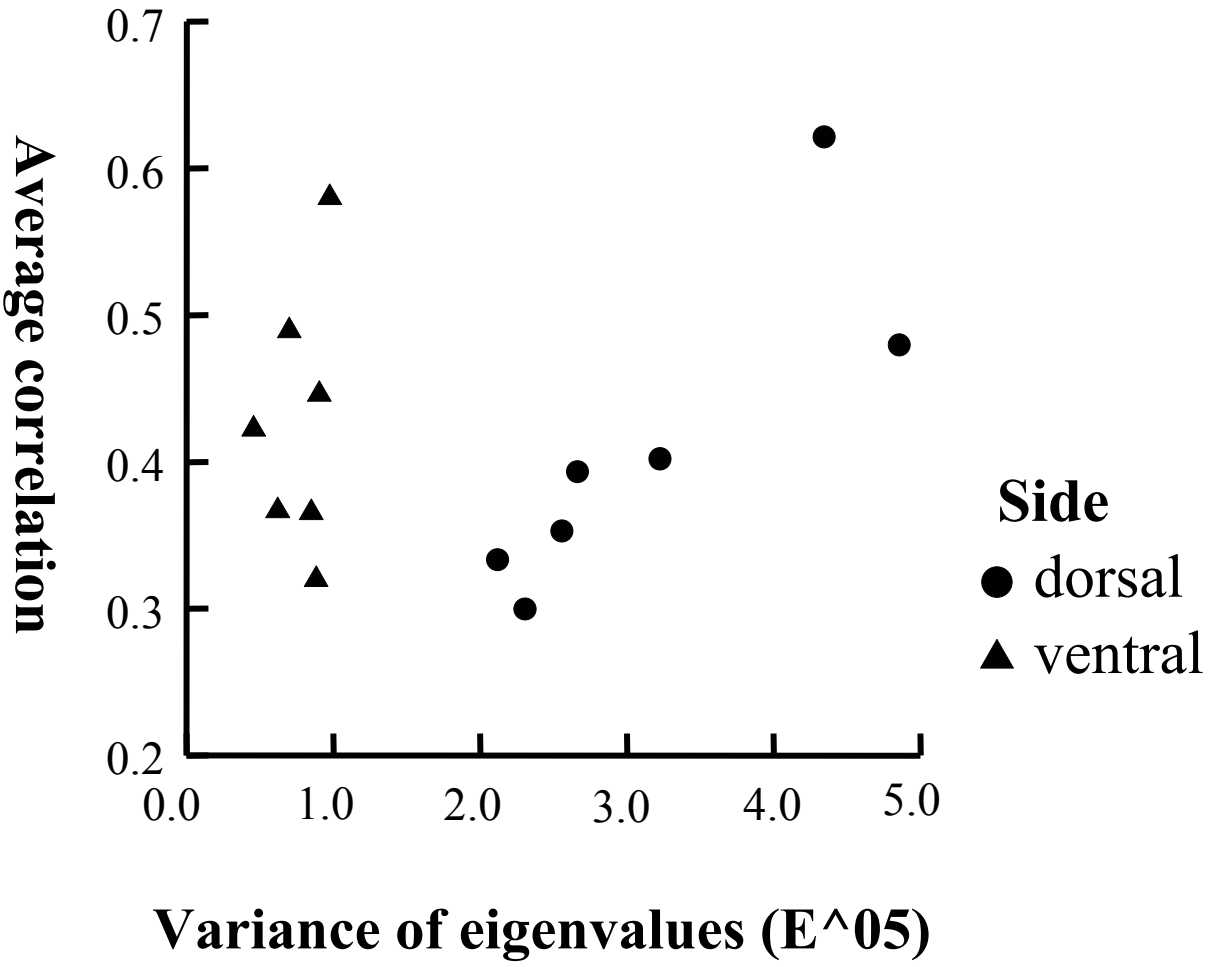

0.7
0.6
0.5
0.4
0.3
0.2
Average correlation
0.0
1.0
2.0
3.0
4.0
5.0
Variance of eigenvalues (E^05)
Side
dorsal
ventral

Figure 4.

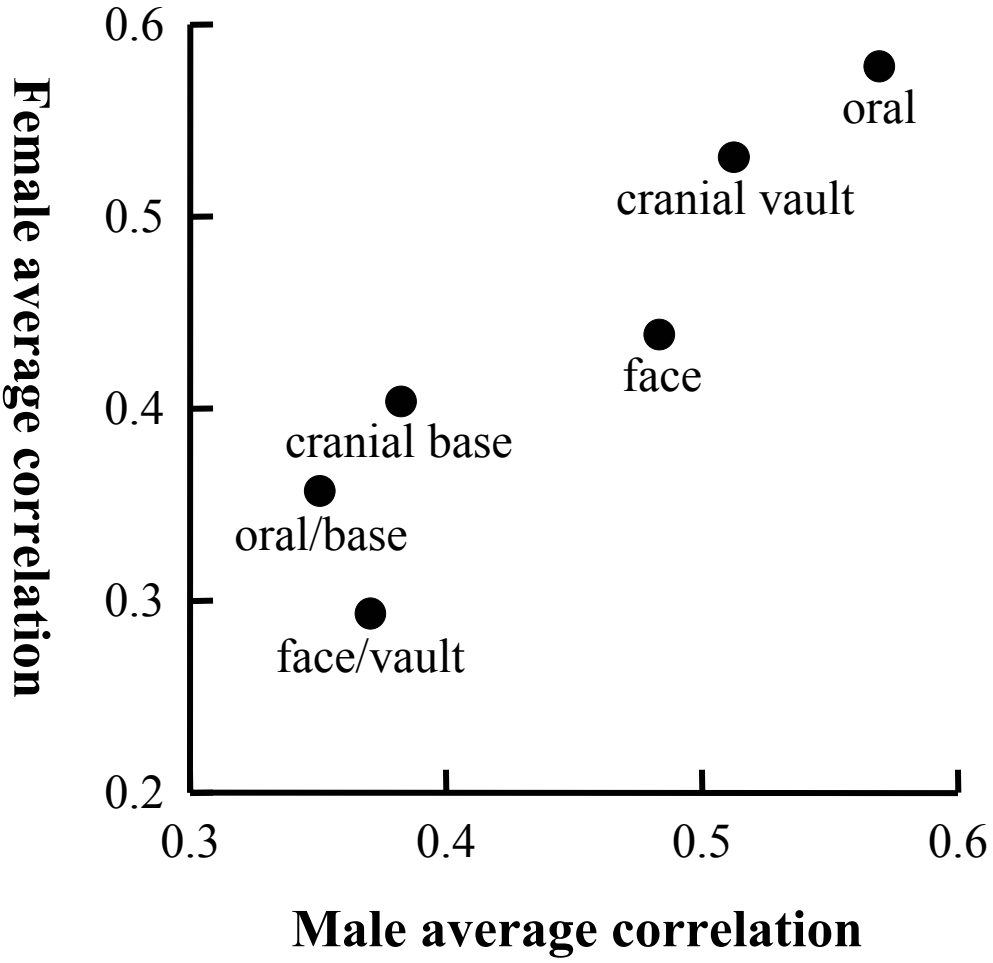
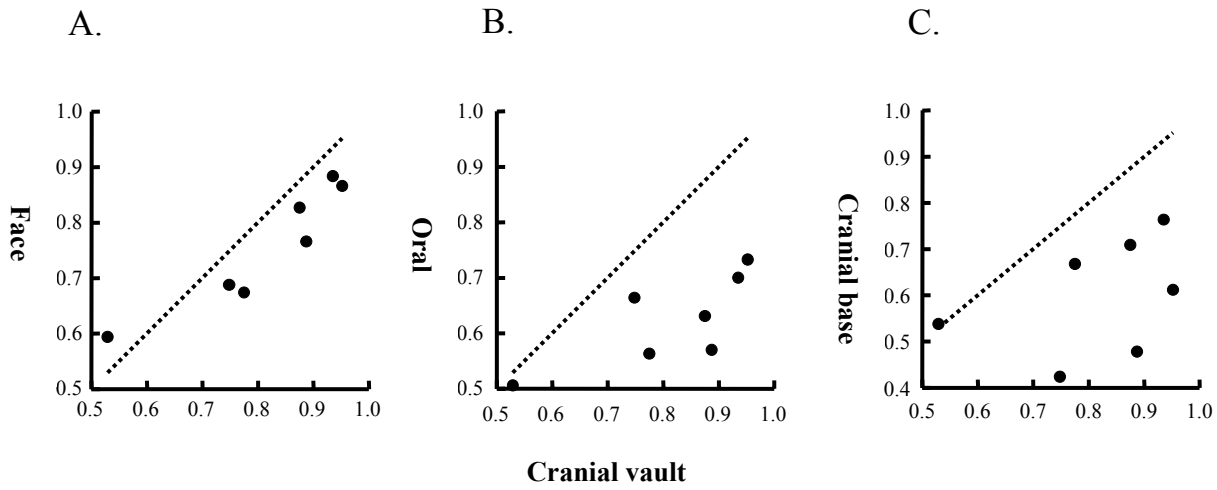

0.6
0.5
0.4
0.3
0.2
0.3
0.4
0.5
0.6
Female average correlation
Male average correlation
oral
cranial vault
face
cranial base
oral/base
face/vault

Figure 5.


A.
B.
C.
Face
Oral
Cranial base
Cranial vault
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.5
0.6
0.7
0.8
0.9
1.0

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