Diversity of Limb-Bone Safety Factors for Locomotion in Terrestrial Vertebrates: Evolution and Mixed Chains


Synopsis During locomotion over land, vertebrates’ limb bones are exposed to loads. Like most biological structures, limb bones have a capacity to withstand greater loads than they usually experience, termed a safety factor (SF). How diverse are limb-bone SFs, and what factors correlate with such variation? We have examined these questions from two perspectives. First, we evaluated locomotor SF for the femur in diverse lineages, including salamanders, frogs, turtles, lizards, crocodilians, and marsupials (opossums). Comparisons with values for hind-limb elements in running birds and eutherian mammals indicate phylogenetic diversity in limb-bone SF. A high SF is primitive for tetrapods, but low magnitudes of load and elevated strength of bones contribute to different degrees across lineages; moreover, birds and eutherians appear to have evolved lower SFs independently. Second, we tested the hypothesis that SFs would be similar across limb bones within a taxon by comparing data from the humerus and femur of alligators. Both in bending and in torsion, we found a higher SF for the humerus than for the femur. Such a “mixed chain” of different SFs across elements has been predicted if bones have differing variabilities in load, different costs to maintain, or high SF values in general. Although variability in load is similar for the humerus and femur, a high SF may be less costly for the humerus because it is smaller than the femur. The high SFs of alligators also might facilitate differences in SF among their limb bones. Beyond these specific findings, however, a more general implication of our results is that evaluations of the diversity of limb-bone SFs can provide important perspective to direct future research. In particular, more complete understanding of variation in SF could provide insight into factors that promoted the evolutionary radiation of terrestrial locomotor function in vertebrates.

Introduction The invasion of land presented animals with numerous functional challenges related to the different physical properties of aquatic and terrestrial habitats (Clack 2002; Coates et al. 2008; Ashley-Ross et al. 2013). Among the foremost of these were issues of body-support and locomotion (Kawano and Blob 2013; Pierce et al. 2013). Once out of water, animals require a structural framework that can resist the newly significant effects of gravity, as well as allow effective transmission of force to the substrate to enable propulsion. In most terrestrial vertebrates, this framework is provided by the bones of the appendicular skeleton. A variety of actinopterygian fish species have evolved the ability to traverse over land using combinations of fins that are supported primarily by flexible bony rays (Pace and Gibb 2009, 2014; Gibb et al. 2013; Kawano and Blob 2013). However, a much more diverse terrestrial radiation emerged among the tetrapods, in which the appendages were constructed around stiff limb bones connected by mobile joints. Although limb bones initially evolved among tetrapod ancestors in an aquatic environment (Shubin et al. 2006; Coates...
et al. 2008; Clack 2009), the combination of stiffness and flexibility that these elements convey to appendages likely facilitated the proliferation of tetrapods across terrestrial habitats (Kawano and Blob 2013).

Since their initial evolution, the limb bones of terrestrial tetrapods have diversified considerably, exhibiting a wide variety of shapes, proportions, and mechanical properties (Alexander 1983; Currey 1984, 2002; Blob 2000; Erickson et al. 2002; Garcia and da Silva 2006; Wilson et al. 2009). Phenotypic diversification of structures commonly results from variation in natural selection on their functional performance (Wainwright and Reilly 1994; Herrel et al. 2006); thus, a key to understanding how such structural and functional diversity emerged in limb bones is to identify how it relates to differences in the functional demands to which limb bones are exposed. Because the main demands placed on bones are to resist and transfer mechanical loads (Currey 1984, 2002), and the activity typically viewed as imposing the most severe and frequent loads on the limb bones of terrestrial vertebrates is locomotion (Biewener 1990, 1993), comparisons of terrestrial locomotor loading across taxa should provide critical insight for understanding the diversity of limb bones. Such comparisons are challenging, however, because the significance of a given load depends on its context. Loads of the same magnitude or regime could carry very different consequences for bones of different sizes, or for bones having different material resilience.

One way of accounting for such differences in comparisons of limb-bone loading across species (or their skeletal elements) is to consider how closely bones approach their maximum load capacity. In other words, do limb bones differ in the amount of extra protection they have against failure? In engineered structures, such margins of extra protection are commonly called safety factors (SFs). The consideration of SF in a biological context was pioneered by Alexander (1981, 1997, 1998), and has since been advanced for a wide range of organismal structures and measurements of performance (e.g., Rubin and Lanyon 1982; Biewener 1983, 1993; Currey 1984, 2002; Diamond and Hammond 1992; Corning and Biewener 1998; Diamond 1998). In its most basic expression, SF can be regarded as the ratio of the load that causes a structure to fail, compared with the usual load that the structure experiences (Alexander 1981). How such loads are evaluated may depend on several factors (Blob and Biewener 1999; Skedros et al. 2003; Kokshenev 2007; Butcher and Blob 2008; Vogel 2013), but even with such potential challenges, the concept of SF can provide a useful context for broad comparisons of functional diversity and capacity of biological structures.

For vertebrates’ limb bones, analyses by Biewener (1982, 1983, 1990, 1991, 1993) provided the earliest broad comparative framework for considering how loading and SF might vary across species. These comparisons focused primarily on eutherian mammals (particularly ungulates) and on ground-dwelling birds, and identified two general patterns: (1) bending was the primary loading regime for most limb bones in these taxa and (2) SF for limb bones in these species generally ranged between 2 and 4. The dramatic differences in body-plan between species like horses and chickens suggested the possibility that these patterns might be similar across all vertebrates. However, despite many differences, eutherians and ground birds also share important characteristics that are not typical of other lineages, including an endothermic metabolism with high rates of remodeling and repair of bone (de Ricqlès 1975; Lanyon et al. 1982; Burr et al. 1985; de Ricqlès et al. 1991), and generally parasagittal limb kinematics (Biewener et al. 1983, 1988; Gatesy 1999; Reilly 2000) that could predispose limb bones of these taxa to bending loads (Bertram and Biewener 1988). Other vertebrate lineages, such as amphibians, turtles, crocodilians, and squamates, are ectothermic and might exhibit lower capacities for remodeling and repair of bone (Owerkowicz and Crompton 1997); moreover, they use postures in which the limbs move outside of parasagittal planes (Walker 1971; Brinkman 1981; Gatesy 1991; Ashley-Ross 1994; Marsh 1994; Reilly and DeLancey 1997; Blob et al. 2008), placing the limbs at a different orientation to locomotor forces and impacting skeletal loading regimes (Blob and Biewener 1999, 2001). With such differences across vertebrate lineages, it might be expected that the patterns of limb-bone loading and SF found in eutherians and birds are not typical of all vertebrates, and that these lineages instead independently converged on such patterns through evolution (Blob and Biewener 1999, 2001; Sheffield et al. 2011). Recent studies have considerably expanded the diversity of lineages from which bone loading and SF data have been collected for terrestrial locomotion (Butcher and Blob 2008; Butcher et al. 2008, 2011; Sheffield and Blob 2011; Gosnell et al. 2011), but comparisons of these data in an explicitly phylogenetic context remain limited (Sheffield et al. 2011).

Because the limbs are composed of multiple skeletal elements, and many vertebrates use quadrupedal locomotion that imposes loads both on forelimbs and hind limbs, there is a potential for intraspecific variation in limb-bone SF as well as for interspecific
variation. Drawing an analogy between the bones that make up the limbs and the links of a chain, intraspecific variation in limb-bone SF might be expected to be limited (Alexander 1997, 1998). If the “chain” of bones from which the limbs are constructed would always fail at its weakest link, and such failure might put the survival of the whole animal at risk, then little selective advantage would be expected for some limb bones to have greater SFs than others, because the energy to make and maintain that extra protection would not prevent overall failure of the individual. Such expectations provide an intuitive null hypothesis, but Alexander (1997, 1998) proposed three exceptions for which different SFs might be advantageous across the elements of limbs (or, at least, not disadvantageous), forming what he described as a “mixed chain”. These conditions included: (1) elements that are more costly to move or maintain might have lower SFs; (2) elements that have more variable loads than the rest of the skeleton might have higher SFs that could protect against occasional high loading peaks; and (3) when SFs are high for all the elements of the skeleton, there might be more opportunity for variation in SF across different elements. Computational models supported the potential of these conditions to facilitate a “mixed chain” of SFs across limb bones (Alexander 1997, 1998). In addition, limited empirical data comparing frequencies of fractures in the limb bones of racehorses (Currey 2002), as well as differences in SF between the femur and tibia for iguanas and alligators (Blob and Biewener 1999), indicate the presence of lower SFs in distal elements of limbs for these taxa. However, systematic comparisons of intraskeletal variation in SF have not formally evaluated correlations with all three conditions outlined by Alexander (1997, 1998).

In this article, we evaluate the functional diversity of limb bones used for terrestrial locomotion across and within tetrapod species, using SF as a framework for our perspective. First, we perform a phylogenetic comparison of limb bone SF across vertebrate lineages, providing insight into the evolutionary diversification of this trait and evaluating how factors such as variations in loading and differences in the mechanical properties of bone, contribute to that diversity. Second, we compare new SF data for the humerus of alligators to previous data for the femur (Blob and Biewener 1999) to test for a “mixed chain” of SFs in this species, and to ascertain which of Alexander’s (1997, 1998) proposed conditions might facilitate its presence. Through these analyses, we can consider how diversity in features such as limb-bone SF may have contributed to the radiation of the structure and function of limbs that evolved after vertebrates moved onto land.

Materials and methods

Calculations of SF

To standardize our comparisons, we sought a common format for evaluating SFs across different taxa and limb bones. Recalling Alexander’s (1981) conception of SF as the ratio of “failure load” to “ordinary load”, we established the following standards for these terms, with the goal of maximizing our ability to draw on published data that appropriately reflected significant parameters for the survival of species in our comparisons. First, “failure load” was considered to be the point of yield (i.e., the point at which the bone has been damaged by plastic deformation), rather than fracture (Currey 1990; Biewener 1993). Second, to the extent possible, we standardized comparisons of failure and ordinary loads in terms of strains, rather than stresses. Although strain-based comparisons complicated our incorporation of data from salamanders (see below), it allowed us to include new data from anurans, and provided a complementary perspective to previous stress-based comparisons of diversity of SF offered by Sheffield et al. (2011). Third, we considered values reported as “mean peak loads” to represent “ordinary” loads, where “mean peak loads” reflect the average load under standard locomotor activities requiring high levels of exertion. Such values indicate different behaviors in different taxa (e.g., turtles versus horses), but represent an effort to make comparisons that are meaningful within the context of functional capacities for limb bones. Finally, for interspecific comparisons, our analyses focused both on “failure” and “ordinary” loads in bending, again maximizing our ability to compare across diverse taxa. For intraspecific comparisons of elements in alligators, however, we compared data both for bending and for shear (i.e., torsional loading).

Assembly of data on bone loading, mechanical properties, and SF

Phylogenetic comparisons

To obtain a broad sample of tetrapod lineages for phylogenetic comparisons, we compiled published data on loading during terrestrial locomotion, mechanical properties (typically yield strains, see above), and SF for skeletal elements from the hind limb. Data for eutherian mammals and birds were extracted from the analysis by Biewener (1993), which provided 10 values of peak strains from elements of the hind limbs (from four eutherian and
two avian species), and an estimate of $-7000$ microstrain ($\mu e$) as the average yield strain of bone (Reilly and Burstein 1975; Currey 1984). Data for other lineages were primarily from our own published studies, and focused on values for the femur in particular because other elements were typically too small for attaching strain gauges. Data included mean in vivo peak strains, mechanical properties of bones, and calculations of SF for American alligators (Alligator mississippiensis) (Blob and Biewener 1999), green iguana lizards (Iguana iguana) (Blob and Biewener 1999), Argentine black and white tegu lizards (Tupinambis merianae) (Sheffield et al. 2011), river cooter turtles (Pseudemys concinna) (Butcher et al. 2008), and marsupial Virginia opossums (Didelphis virginiana) (Butcher et al. 2011).

To include information from amphibians in our analysis, we added a stress-based estimate of femoral SF from tiger salamanders (Ambystoma tigrinum) (Sheffield and Blob 2011) to our dataset, along with a value of yield strain for this species derived from a different study (Erickson et al. 2002). We also compiled previously unpublished data from in vivo recordings of femoral strains for two anuran species: the bullfrog (Lithobates catesbeiana; $N = 9$, body mass 215–444 g) and cane toad (Chaunus marinus; $N = 3$, body mass 147–174 g). Animals were purchased from commercial suppliers, housed in large plastic enclosures, and daily provided with clean, aged water, and crickets for food.

Procedures for collection and analysis of in vivo strains for frogs followed those previously published for the femur for other taxa (Butcher et al. 2008, 2011; Sheffield et al. 2011), with the following additional clarifications. First, anesthesia of frogs was induced via immersion in a buffered solution of tricaine methanesulfonate (MS-222, 2 g/l), and was maintained during surgical implantation of gauges by covering the skin (except the surgical limb) with gauze soaked in MS-222 (0.5 g/l). Second, effort was made to implant three gauges on the midshaft of each femur, with single-element gauges at all locations except the dorsal surface of some of the largest bullfrogs, to which a rosette gauge was attached. Lead wires from these gauges emerged through a small incision at the shoulder, and were soldered into a microconnector plug. The wires were protected by wrapping them into a cable using self-adhesive bandage, and the cable was stabilized by wrapping it in a belt of self-adhesive bandage around the belly of the frog. Third, after 24 h of recovery, strains were recorded at 2500 Hz from single, forward jumps in trackway arenas, with several minutes of rest between jumps. Finally, at the conclusion of recordings, frogs were euthanized in buffered MS-222 (4 g/l), and the limb bones with implanted gauges were extracted to verify the position of gauges and allow sectioning for analyses of planar strains to evaluate how much peak strains may have exceeded recordings of strains (Biewener and Dial 1995; Lieberman et al. 2004; Butcher et al. 2008, 2011). Mean values of peak strain for each species were compared with published data on the mechanical properties of their femora (Wilson et al. 2009) to evaluate SF.

Intraspecific comparisons

To assess potential differences in SF across elements of limb bones within a species, previously published data from the alligator femur (Blob and Biewener 1999) were compared with new recordings of strains collected from the humerus, along with new mechanical property data both for the humerus and femur. To conduct new strain recordings, four animals were obtained from the Rockefeller Wildlife Refuge that were similar in size (body mass 1.7–2.0 kg, total length 0.81–0.89 m) to animals from which previous femoral strain recordings had been collected. Animals were fed chicken hearts, gizzards, and frankfurters, and were housed in large artificial ponds maintained in a greenhouse under conditions of natural summer light and temperature.

Procedures for the collection and analysis of strains from the alligator humerus followed previously published methods (Butcher et al. 2008, 2011; Sheffield et al. 2011), with the following additional clarifications. First, initial sedation and analgesia were provided by intramuscular injections of butorphanol (1 mg/kg), ketamine (30 mg/kg), carprofen (2 mg/kg), and detomidine (0.2 mg/kg). Alligators were then intubated and maintained at a surgical plane of anesthesia by administration of isoflurane (initially 3–4% at $O_2$ supply of 11/min, reduced to 0.5–1% after 20–30 min and further reduced to 0% after attachment of gauges). Second, gauges were implanted onto the midshaft surface of the humerus, between reflections of the brachialis and humeroradialis muscles (Meers 2003), via a ventral incision on the arm. Lead wires from implanted gauges emerged through a small incision at the shoulder, and were soldered into a microconnector plug. The wires were protected by wrapping them in a cable using self-adhesive bandage, and the cable stabilized by wrapping it in a belt of self-adhesive bandage wrapped around the chest of the alligator. Third, after 48 h of recovery, strains (recorded at 2500 Hz) were collected over the next 1–2 days during bouts of locomotion on a treadmill. Bouts of ~30 s duration were conducted at 0.3–0.4 m/s (comparable to previous
recordings from the femur) (Blob and Biewener 1999), with several minutes of rest between bouts. Finally, at the conclusion of recordings, alligators were euthanized by an overdose of sodium pentobarbital solution (Beuthanasia, 120 mg/kg intraperitoneal injection), and animals were frozen for later extraction of limb bones to conduct analyses of planar strains and tests of mechanical properties.

Although a value of yield strain in bending (6495 µε) had been published previously for the alligator femur (Blob and Biewener 1999), to calculate SF for the humerus and femur during both bending and torsion, additional data on mechanical properties were required. Available specimens of humerus and femur were extracted from animals frozen after use both in current and earlier studies of femoral strains (Blob and Biewener 1999). Bending and torsional tests were conducted on thawed specimens, using the methods and the Instron materials-testing machines used for frogs (Wilson et al. 2009).

Reconstruction of the evolution of SF for tetrapod femora

To evaluate how SFs for bones of the tetrapod hind limb have changed through evolution, and which groups show significant changes from their ancestors, we mapped values of SF onto a cladogram of tetrapod relationships, and used comparative methods to reconstruct ancestral states of SF at internal nodes of the phylogeny. Our analysis used the generalized least squares (GLSs) approach for continuous characters presented by Martins and Hansen (1997), as implemented in the COMPARE 4.6 software package (Martins 2004). In this approach, values and standard errors for the character states of ancestral taxa are estimated from character-state values for the taxa under study, a phylogeny of taxonomic relationships, data on branch-lengths for that phylogeny, and a model of character-change. This approach can also incorporate information about the standard errors of the values of traits for each terminal taxon (Martins and Lamont 1998). Based on available data, it is difficult to represent, in terms of typical standard errors, what is known about variation in SF among members of the taxa we analyzed. However, to acknowledge the presence of uncertainty in those SF values, for each terminal taxon we applied an estimated standard error of 10% of the SF, basing this estimate on common magnitudes of standard errors for measurements of mechanical properties of bones (Blob and Snelgrove 2006).

Estimates of tetrapod phylogeny and branch-lengths for the taxa we considered were taken from the literature (Laurin 2004; Donoghue and Benton 2007; Hugall et al. 2007; Kumazawa 2007; Vidal and Hedges 2009; Pyron 2011; dos Reis et al. 2012). GLS analyses were run in COMPARE using the “Linear” option, which uses Brownian motion as a model of character-change and assumes that divergence between taxa increases linearly with phylogenetic distance. Although objections have been raised to this model (e.g., Butler and King 2004), our objective was to represent the unpredictability of the evolution of characters under natural selection, rather than a genuinely random process (Schluter et al. 1997; Blob and Snelgrove 2006). Our specific goal in these analyses was to evaluate whether eutherian mammals, birds, or any other lineage showed SF values that deviated significantly from those of their ancestors. Toward this goal, we compared whether the magnitude of evolutionary change in SF along any branch of the phylogeny exceeded a factor calculated as 1.96× (average standard error of the two taxa marking the branch). Such lineages could be considered to show a magnitude of change greater than a rough 95% confidence interval of expected change for that lineage (Martins and Lamont 1998; Martins 2004).

Our analysis of ancestral states for the SF for limb bones should be approached with a measure of caution. First, critical evaluations of methods for reconstructing ancestral character states have noted a variety of limitations to such analyses (Cunningham et al. 1998; Cunningham 1999; Losos 1999; Omland 1999; Polly 2001; Webster and Purvis 2002). These include the potential for the standard errors of reconstructed states to become quite large, limiting the potential for identifying significant change through evolution (Schluter et al. 1997; Martins 1999; Moran 2004). This particular limitation actually helps to make our consideration of evolutionary change in SF conservative, as only the most robust changes should be identified as significant. Another potential issue is that SF is itself a ratio of two variables, each of which might evolve independently. To address this issue, we report values for both variables that contribute to calculations of SF for each taxon, allowing qualitative appraisal of potentially distinct evolutionary paths for the loading and mechanical properties of limb bones. However, because these values are reported as a mix of both tensile and compressive values in the original studies from which they were derived (and actually as stresses, rather than strains, for salamanders) (Sheffield and Blob 2011), it is difficult to formally reconstruct ancestral values for these traits without a variety of further assumptions. In addition to these general
issues, the details of our reconstruction of phylogenetic relationships, as well as its branch-lengths, might also be called into question (e.g., the historically contentious placement of turtles) (Chiari et al. 2012; Shaffer et al. 2013). Although a variety of such considerations could be adjusted and explored further, for our present analysis we have focused on identifying at least one strongly supported phylogenetic context, and examine the perspective it can give for evaluating the evolution of SF for limb bones during terrestrial locomotion.

**Results**

**Phylogenetic comparisons**

*In vivo* strains from the femora of frogs

Recorded femoral strains from both species of frogs indicate predominantly tensile axial loads on the dorsal and anterior surfaces that grade into compressive loads toward the anteroventrail aspect of the cortex (Table 1). The presence of both tensile and compressive strains around the cortex of these elements indicates that bending is a significant loading regime in the femur of both species (e.g., Biewener et al. 1983; Blob and Biewener 1999). Rosette recordings from the dorsal surface of bullfrogs show principal strains that approach $1.7 \times$ greater magnitudes than axial strains; moreover, the angle of principal tensile strains to the long axis of the femur ($\phi_t$) averages $44.6 \pm 20.2^\circ$, approaching the $45^\circ$ expected for pure torsion (Table 1). The large standard deviation for this variable indicates that individual jumps probably do not ever actually experience pure torsion, a result corroborated by the values of axial strain also recorded from the rosette gauges (Table 1). However, a predominance of high $\phi_t$ values above and below $45^\circ$, along with values of shear strains that closely match those of principal strains (Table 1), indicates that torsion may be at least similar in importance to bending as a loading regime for the limb bones of jumping frogs.

Entry of the average axial strains recorded from each location into analyses of planar strains that account for the positions of gauges (Biewener and Dial 1995; Blob and Biewener 1999; Lieberman et al. 2004) indicate values of peak tensile and compressive axial strains of $+488 \mu \varepsilon$ and $-1260 \mu \varepsilon$ for bullfrogs, and $+342 \mu \varepsilon$ and $-1401 \mu \varepsilon$ for cane toads. The presence of higher magnitudes of compression versus tension on the cortex indicates that axial compression (likely from the support of body weight) is superimposed on bending and torsion in both species. Peak loads for toads are comparable (possibly even slightly higher) than those for bullfrogs, a potential surprise given the explosive jumps typical of bullfrogs, relative to the seemingly less dramatic hopping behaviors typical of toads (Marsh 1994; Wilson et al. 2009). Because estimates of peak compressive loads in bending were much higher than those for peak tensile loads in both species, these estimates of peak compressive loads were compared with measurements of mechanical properties for the femora of both species (Wilson et al. 2009) to calculate SF values for phylogenetic analyses. After correcting originally reported tensile mechanical properties to account for bone typically being 25% weaker in tension than in compression (Currey 1984; Biewener 1993), femoral SF in bending was calculated as $7.7$ for *L. catesbeiana* and $8.5$ for *C. marinus* (Fig. 1).

**Reconstruction of ancestral values and patterns of evolutionary change in limb-bone SF for terrestrial locomotion in tetrapods**

The phylogenetic distribution of SFs for bones of the hind limb in bending during terrestrial locomotion, along with magnitudes of peak load and mechanical properties, is illustrated in Fig. 1. All three of these parameters show considerable variation across tetrapod lineages. Eutherian mammals and birds have the

<table>
<thead>
<tr>
<th>Species</th>
<th>Gauge location</th>
<th>$\varepsilon_{\text{axial}}$ ($\mu \varepsilon$)</th>
<th>$\varepsilon_t$ ($\mu \varepsilon$)</th>
<th>$\varepsilon_c$ ($\mu \varepsilon$)</th>
<th>$\phi_t$ (°)</th>
<th>Shear ($\mu \varepsilon$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. catesbeiana</em></td>
<td>Dorsal</td>
<td>282 ± 264 (144, 9)</td>
<td>470 ± 316 (64, 5)</td>
<td>$-393 \pm 783$ (64, 5)</td>
<td>$44.6 \pm 20.2$ (58, 5)</td>
<td>$475 \pm 6112$ (64, 5)</td>
</tr>
<tr>
<td></td>
<td>Anterior</td>
<td>229 ± 142 (98, 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Anteroventral</td>
<td>$-371 \pm 234$ (145, 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. marinus</em></td>
<td>Dorsal</td>
<td>198 ± 184 (175, 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anterior</td>
<td>208 ± 100 (111, 3)</td>
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<tr>
<td></td>
<td>Anteroventral</td>
<td>$-181 \pm 184$ (121, 2)</td>
<td></td>
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Notes: Values are means ± SD across all individuals; the numbers of jumps analyzed and individuals tested, respectively, are listed in parentheses after each mean. Angles of principal tensile strains to the long axis of the bone ($\phi_t$) are also reported (positive angles indicate inward/medial rotation).
lowest SFs, whereas salamanders and iguanas have the highest. However, taxa that might be regarded as having higher values of SF can achieve them in different ways. For example, in alligators, SF is high primarily because of low loads; in opossums, SF is high primarily because yield strains are high; and in iguanas, SF is high both because loads are low and yield strains are high (Fig. 1).

Reconstructions of ancestral state lead to an estimate of $6.8 \pm 2.4$ for the ancestral value of SF for tetrapods (Fig. 1). Across tetrapod phylogeny, only four branches show an absolute change in SF of $>2$, in each case between a node and a branch-tip represented by a terminal taxon. These include decreases in SF of $>2$ for the branches leading to eutherian mammals and to birds, and increases in SF of $>2$
for the branches leading to salamanders and iguanas. Based on Martins’ (2004) criterion for significance, that the amount of change along a branch must exceed a rough 95% confidence interval for the amount of change expected for that branch, only the evolutionary changes in SF for birds and salamanders are significant (Table 2). However, the decrease in SF for eutherian mammals is nearly significant: actual change differs from expected change by only 0.21, <10% of mean eutherian SF (compared with a shortfall of 0.82 between actual and expected change for the branch leading to iguanas). The significant increase in SF on the branch leading to salamanders should also be regarded with caution, as the estimate of SF for this taxon was derived from force-platform analyses (Sheffield and Blob 2011), which can lead to higher estimates of SF than obtained by in vivo strain data (Biewener et al. 1983; Butcher and Blob 2008; Butcher et al. 2008; Schoenfuss et al. 2010). As a result, the primary evolutionary changes in SF for limb bones, in bending, during terrestrial locomotion, appear to be evolutionary decreases in birds and, potentially, eutherian mammals.

Intraspecific comparisons

In vivo strains from the humerus of alligators

Recorded strains from the humerus of alligators indicate predominantly tensile axial loads on the anterior and anteroventral surfaces that grade into compressive loads toward the ventral aspect of the cortex

Table 2 Comparisons of magnitude of evolutionary change in limb bone SF for bending during terrestrial locomotion along branches of tetrapod phylogeny, with rough 95% confidence intervals for each branch

<table>
<thead>
<tr>
<th>Branch</th>
<th>Branch length (millions of years)</th>
<th>Rough 95% confidence interval for branch</th>
<th>Evolutionary change in SF along branch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root to node 2 (Amphibians)</td>
<td>57.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>4.19</td>
<td>0.71</td>
</tr>
<tr>
<td>Node 2 to Salamanders</td>
<td>292.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.88</td>
<td>2.96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Node 2 to node 3 (Frogs)</td>
<td>163.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.02</td>
<td>0.40</td>
</tr>
<tr>
<td>Node 3 to Lithobates catesbeiana</td>
<td>129.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.88</td>
<td>-0.24</td>
</tr>
<tr>
<td>Node 3 to Chaunus marinus</td>
<td>129.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.95</td>
<td>0.56</td>
</tr>
<tr>
<td>Root to node 4 (Amniotes)</td>
<td>12.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.43</td>
<td>-0.16</td>
</tr>
<tr>
<td>Node 4 to node 5 (Mammals)</td>
<td>164.5&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>3.23</td>
<td>-1.88</td>
</tr>
<tr>
<td>Node 5 to Didelphis virginiana</td>
<td>172.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.75</td>
<td>0.31</td>
</tr>
<tr>
<td>Node 5 to Eutherians</td>
<td>172.8&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.50</td>
<td>-2.29&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Node 4 to node 6 (Reptiles)</td>
<td>52.8&lt;sup&gt;f,g,h&lt;/sup&gt;</td>
<td>1.08</td>
<td>-0.06</td>
</tr>
<tr>
<td>Node 6 to node 7 (Lepidosaurs)</td>
<td>105.1&lt;sup&gt;f,g,h&lt;/sup&gt;</td>
<td>3.95</td>
<td>1.71</td>
</tr>
<tr>
<td>Node 7 to Iguana iguana</td>
<td>179.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.29</td>
<td>2.47&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Node 7 to Tupinambis merianae</td>
<td>179.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.09</td>
<td>0.47</td>
</tr>
<tr>
<td>Node 6 to node 8 (Turtles + Archosaurs)</td>
<td>20.6&lt;sup&gt;f,g,h&lt;/sup&gt;</td>
<td>1.83</td>
<td>-0.36</td>
</tr>
<tr>
<td>Node 8 to Turtles</td>
<td>263.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.29</td>
<td>-1.86</td>
</tr>
<tr>
<td>Node 8 to node 9 (Archosaurs)</td>
<td>18.9&lt;sup&gt;g&lt;/sup&gt;</td>
<td>1.97</td>
<td>-0.19</td>
</tr>
<tr>
<td>Node 9 to Crocodilians</td>
<td>245.0&lt;sup&gt;f,h&lt;/sup&gt;</td>
<td>2.62</td>
<td>0.24</td>
</tr>
<tr>
<td>Node 9 to Birds</td>
<td>245.0&lt;sup&gt;f,h&lt;/sup&gt;</td>
<td>2.33</td>
<td>-2.76&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: Branches are named based on the numbering of nodes in Fig. 1. Rough 95% confidence intervals are calculated as 1.96 × (average standard error of the two taxa marking the branch) (Martins 2004). Magnitudes of evolutionary change are calculated as (descendent node SF—ancestral node SF), so that positive values indicate an evolutionary increase in SF through evolution, and negative values indicate an evolutionary decrease.

<sup>a</sup>Laurin (2004).

<sup>b</sup>Pyron (2011).

<sup>c</sup>Changes with absolute values greater than the corresponding rough confidence interval.

<sup>d</sup>Dos Reis et al. (2012).

<sup>e</sup>Other changes with an absolute magnitude >>2 considered in the text.

<sup>f</sup>Hugall et al. (2007).

<sup>g</sup>Kumazawa (2007).

<sup>h</sup>Donoghue and Benton (2007).

<sup>i</sup>Vidal and Hedges (2009).
Comparisons of SF from the humerus and femur of alligators: testing for the presence of a mixed chain and proposed correlated conditions

New measurements of yield strains in bending and torsion for the alligator humerus were compared with corrected values of tensile bending strain and shear strain to calculate SF for this bone in both loading regimes (Table 4). New measurements of yield strain in torsion for the alligator femur were also collected, allowing refined comparison of humeral SF values to previously published (Blob and Biewener 1999) estimates for the femur (Table 4). These comparisons show the humerus to exceed the SF of the femur by $>2$ in both loading regimes: 8.4 versus 6.3 in bending and 7.8 versus 5.0 in torsion (Table 4). These differences are primarily due to differences in yield strain between these elements, as the magnitudes of load are nearly identical (Table 4).

After establishing the presence of a “mixed chain” of SF between the alligator humerus and femur, we considered the three conditions that Alexander proposed might be correlated with such a pattern. The presence of a lower SF in the femur than in the humerus is consistent with Alexander’s (1997, 1998) first predicted condition, because the femur is larger than the humerus in alligators (Dodson 1975; Livingston et al. 2009). Thus, the femur likely requires greater energetic resources than the humerus in order to be moved and maintained, potentially making a lower femoral SF advantageous. In contrast, our results do not support Alexander’s (1997, 1998) second condition, which proposed that elements with more variable loads might have higher SF. For both the humerus and femur of alligators, coefficients of variation for the magnitudes of loads (25–52%) are much greater than typical values for the limb bones of mammals (~8%) (Biewener 1991); however, between the limb bones of alligators, the humerus has a higher SF than the femur, but comparable or lower coefficients of variation for load-magnitudes in both bending and torsion (Table 5). Finally, our results do appear to support Alexander’s (1997, 1998) third condition, which proposed that when SFs are high for all the elements of the skeleton, there might be more opportunity for variation in SF across different elements. Both the humerus and the femur have SF $>5$ for all loading regimes (Table 4). This benchmark SF value may be lower than the ancestral value for all tetrapods, but is higher than the values found in taxa for which a significant evolutionary decrease in SF has been identified, such as birds and, potentially, eutherian mammals (Fig. 1).

**Discussion**

Whether SFs for any structure are a target of natural selection has been a topic of considerable debate (Lowell 1985; Lanyon 1991; Diamond 1998; Garland 1998; Currey 2002; Vogel 2013). Nonetheless, differences in SF across taxa or structures carry functional consequences, whether SF was considered the three conditions that Alexander proposed might be correlated with such a pattern. The presence of a lower SF in the femur than in the humerus is consistent with Alexander’s (1997, 1998) first predicted condition, because the femur

**Table 3** Axial ($\varepsilon_{\text{axial}}$), principal tensile ($\varepsilon_t$), principal compressive ($\varepsilon_c$), and shear strains recorded from the humerus of American alligators (*Alligator mississippiensis*) during terrestrial locomotion

<table>
<thead>
<tr>
<th>Gauge location</th>
<th>$\varepsilon_{\text{axial}}$ ($\mu$)</th>
<th>$\varepsilon_t$ ($\mu$)</th>
<th>$\varepsilon_c$ ($\mu$)</th>
<th>$\phi$ ($)</th>
<th>Shear ($\mu$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral</td>
<td>$-243 \pm 152$ (247, 3)</td>
<td>$355 \pm 90$ (149, 2)</td>
<td>$-416 \pm 159$ (149, 2)</td>
<td>$23 \pm 16$ (149, 2)</td>
<td>$520 \pm 261$ (149, 2)</td>
</tr>
<tr>
<td>Anteroventral</td>
<td>$357 \pm 85$ (98, 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>$255 \pm 77$ (247, 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: Values are means $\pm$ SD across all individuals; the numbers of steps analyzed and individuals tested, respectively, are listed in parentheses after each mean. Angles of principal tensile strains to the long axis of the bone ($\phi$) are also reported (positive angles indicate inward/medial rotation).
and intraspecific diversity in SF can provide insight into factors that have shaped the diversity of locomotor function in tetrapods since this lineage invaded terrestrial habitats.

Interspecific diversity in the SFs of limb bones for terrestrial locomotion: what evolutionary changes are we trying to understand?

Birds and eutherian mammals have lower limb-bone SFs than do other tetrapod lineages. However, an important perspective that emerges from our phylogenetic comparisons is that these lineages (allowing slightly generous criteria for eutherians) also show the best evidence for significant changes in SF from their closest ancestors, with both exhibiting decreases (Fig. 1 and Table 2). This insight helps to focus consideration of the kinds of explanations that should be sought. Rather than asking why higher SFs have emerged in amphibians and non-avian reptiles through evolution, it is more appropriate to ask why lower SFs have emerged in eutherians and birds.

A variety of factors might have facilitated evolutionary decreases in SF for the limb bones of eutherians and birds (Blob and Biewener 1999, 2001). First, the high levels of sustained or peak activity commonly shown by species from both of these lineages might make maintaining or carrying the bone material needed for high SFs energetically costly (Lanyon 1991), so that lower SFs were advantageous. Second, the generally greater capacity for remodeling and for microdamage repair of bone in endotherms like mammals and birds, compared with amphibians and non-avian reptiles (Enlow 1969; de Ricqlès 1975; Lanyon et al. 1982; Burr et al. 1985; de Ricqlès et al. 1991; Owerkowicz and Crompton 1997), might facilitate the capacity of mammals and birds to operate successfully with lower margins of safety for limb bones. In a similar context, a third factor might relate to the greater predictability of the loads encountered by mammalian and avian limb bones (Bertram and Biewener 1988; Biewener 1991) compared with other taxa (e.g., Table 5). Predictable loads might also facilitate the capacity of mammals and birds to operate successfully with lower SFs (e.g., Lowell 1985; Alexander 1997), making it advantageous for potential energetic costs of high SFs to be reduced.

Such arguments, although appealing, encounter complications. For example, data from opossums (Butcher et al. 2011; Gosnell et al. 2011) may not fit the above scenarios as neatly as data from other mammals. Although opossums are endotherms and should have predictable loading and strong capacities for repair of microdamage, their femora had some of the highest yield strains of any of the taxa we evaluated (Fig. 1). In addition, although our interspecific analyses focused on bending loads during terrestrial locomotion, other loading regimes (e.g., torsion) and behaviors (e.g., digging) could be significant for the design of limb bones in amphibians or non-avian reptiles (Blob and Biewener 1999, 2001; Butcher and Blob 2008; Sheffield and Blob 2011). Considerations of diversity in SF that accounted for such factors might provide different comparisons to values for mammals and birds.

Nonetheless, beyond such complications and the specific factors potentially contributing to the evolutionary changes we identified, our phylogenetic analysis of the SF for limb bones leads to a further broad question. Reconstructions of ancestral states indicate that limb-bone SF for the earliest tetrapods approached values of nearly 7 (Fig. 1). Since this lineage evolved in an aquatic environment where the forces imposed by body weight were likely negligible (Shubin et al. 2014), why do their limb bones have SFs of this magnitude? It is possible that this ancestral margin of safety evolved incidental to factors related to support of the body, and may have emerged as a consequence of factors such as the

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**Table 4** Comparison of yield strains, calculated peak strains, and SFs between the humerus and femur of American alligators (*Alligator mississippiensis*) during terrestrial locomotion

<table>
<thead>
<tr>
<th>Loading regime</th>
<th>Bone</th>
<th>Yield strain (με)</th>
<th>Calculated peak strain (με)</th>
<th>SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bending</td>
<td>Humerus</td>
<td>8423 ± 3090 (5)</td>
<td>1005</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>Femur</td>
<td>6495 (1)*</td>
<td>1027*</td>
<td>6.3*</td>
</tr>
<tr>
<td>Torsion</td>
<td>Humerus</td>
<td>11,455 ± 450 (3)</td>
<td>1472</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>Femur</td>
<td>7509 ± 1386 (4)</td>
<td>1489*</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Notes: Yield strain values are means ± SD, with the number of bones tested in parentheses and tensile values reported for bending. Calculated peak strains are derived from recorded strains and correct for results of planar strain analyses (see text). Note that SF is at least 2.1 higher for the humerus in both loading regimes.

*Values derived from Blob and Biewener (1999).

**Table 5** Coefficients of variation (CV) for mean recorded bending and shear strains from the humerus and femur of American alligators (*Alligator mississippiensis*) during terrestrial locomotion

<table>
<thead>
<tr>
<th>Bone</th>
<th>Bending</th>
<th>Shear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humerus</td>
<td>25.4%</td>
<td>50.2%</td>
</tr>
<tr>
<td>Femur</td>
<td>48.9%</td>
<td>51.6%</td>
</tr>
</tbody>
</table>

Note that, counter to predictions from the Alexander (1997, 1998) "mixed chain" model, CV is either essentially equal between the bones (shear), or lower in the humerus, the bone with the higher SF (bending).
need for a bone that was sufficiently large to provide attachment for locomotor muscles (Butcher et al. 2008). However, the presence of such a margin of safety may have facilitated the invasion of land, and potentially contributed to the success of tetrapods over other lineages with different appendicular constructions (Kawano and Blob 2013).

**Perspectives and directions for future research**

Our analyses examined the diversity of SFs for limb bones for tetrapods at both interspecific and intraspecific scales. Beyond the phylogenetic patterns just discussed, our comparisons also emphasized that the diversity of SFs seen across tetrapods (and their skeletal elements) emerges from independent variation both in the magnitudes of loads and in the mechanical properties of bones (Fig. 1). Although the mechanical properties of limb bones have been described as conservative (Erickson et al. 2002), variation, both across taxa (Fig. 1) (Wilson et al. 2009) and across skeletal elements (Table 4), contribute to differences in SF that may have significant functional implications. Moreover, the presence of multiple combinations of load-magnitudes and mechanical properties that lead to similar SFs in limb bones reflects the “many-to-one” mapping of structure to function that promotes the diversification of organismal design (Alfaro et al. 2005; Wainwright et al. 2005).

The presence of a “mixed chain” of SFs across limb bones in alligators, with the femur showing lower values than the humerus (Table 4), supports Alexander’s (1997, 1998) hypothesis and two of his three proposed sets of contributing conditions. Of the two proposed contributing conditions found to apply for alligators, the advantages of lower SFs in the energetically costlier femur suggest a selective explanation for the “mixed chain”. The presence of lower SFs in the tibia of alligators, compared with the femur, may further support a selective explanation for “mixed chains”, as the more distal position of the tibia likely makes it more energetically costly to move and might favor a lower SF for this bone (Blob and Biewener 1999). However, the possibility that differences in SF among the humerus, femur, and tibia in alligators could simply reflect a greater opportunity for variation among elements with high SFs (Alexander 1997, 1998) might, instead, indicate a stochastic explanation. To further test the potential role of selection in promoting intraspecific variation in SF, additional comparisons are needed from other taxa with high SF values, including amphibians and non-avian reptiles (Fig. 1). Identifying instances where “mixed chains” were present, but more costly elements did not have lower SFs, would implicate stochastic explanations for differences in SF across elements. Currently, however, the lack of such instances favors selective explanations.

Efforts to evaluate the SFs of limb bones for additional systems could provide important perspective for phylogenetic comparisons, as well as for evaluations of the “mixed chain” hypothesis. The phylogenetic diversity of estimates of SF for limb bones is still quite limited, with most lineages characterized by values for only one or two species (Fig. 1). Expansion of this phylogenetic diversity would provide a foundation for further creative testing of hypotheses with regard to variation in SF. For example, could comparisons of a broader range of taxa test the potential for an optimal level of excess capacity in limb bones? How do such values compare to the demands placed on other components of the locomotor system, such as muscles and tendons? How might such demands combine to proscribe the limits of locomotor function, and, how might skeletal SFs promote or constrain the evolution of major functional transitions, such as the invasion of land (Kawano and Blob 2013), the evolution of upright posture (Blob 2001), or the origin of flight (Dial 2003)? Although the evidence for diversity in the SFs of limb bones is convincing, we are still in the early stages of understanding its functional and evolutionary significance.

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References
Evolution of limb-bone safety factors


