Comparative limb bone loading in the humerus and femur of the tiger salamander *Ambystoma tigrinum*: testing the “mixed-chain” hypothesis for skeletal safety factors

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Summary statement (15-30 words): Safety factors against skeletal failure differ between the forelimbs and hind limbs of salamanders; this may relate to differing locomotor roles, potentially bearing on the invasion of land by vertebrates
Locomotion imposes some of the highest loads upon the skeleton, and diverse bone designs have evolved to withstand these demands. Excessive loads can fatally injure organisms; however, bones have a margin of extra protection, called a “safety factor” (SF), to accommodate loads that are higher than normal. The extent to which SFs might vary amongst an animal’s limb bones is unclear. If the limbs are likened to a chain composed of bones as “links”, then similar SFs might be expected for all limb bones because failure of the system would be determined by the weakest link, and extra protection in other links could waste energetic resources. However, Alexander proposed that a “mixed-chain” of SFs might be found amongst bones if: 1) their energetic costs differ, 2) some elements face variable demands, or 3) SFs are generally high. To test if such conditions contribute to diversity in limb bone SFs, we compared the biomechanical properties and locomotor loading of the humerus and femur in the tiger salamander (*Ambystoma tigrinum*). Despite high SFs in salamanders and similar sizes of the humerus and femur that would suggest similar energetic costs, the humerus had lower yield stresses, higher mechanical hardness, and larger SFs. SFs were greatest in the anatomical regions where yield stresses were highest in the humerus and lowest in the femur. Such intraspecific variation between and within bones may relate to their different biomechanical functions, providing insight into the emergence of novel locomotor capabilities during the invasion of land by tetrapods.
INTRODUCTION
Bones must regularly withstand applied forces, or loads, imposed internally by the contraction of muscles and externally by interactions with the environment. When bones are unable to withstand loads, injury to the skeleton could lead to inferior predator evasion, inability to acquire food, or other detriments including death (Biewener, 1993). Terrestrial locomotion is particularly noteworthy in this context, because limb bones must accommodate the forces imposed by body support and propulsion, generating some of the highest demands upon the skeleton (Biewener, 1993). Despite these demands, bones can normally withstand loads greater than those they typically experience. This ratio between the typical load sustained and the maximum load the structure can withstand is called a “safety factor” (SF), and provides a margin of protection to structures for performing functions with variable demands (Alexander, 1981; Alexander, 1997; Alexander, 1998; Diamond, 2002).

SFs for bones commonly allow protection against loads ranging from two to ten times greater than ordinary, with variation across taxa and among the limb bones within a species (Alexander, 1981; Biewener, 1993; Blob et al., 2014; Currey, 2002; Diamond, 2002; Sheffield and Blob, 2011). Several factors contribute to interspecific variation in SFs (Blob and Biewener, 1999; Blob et al., 2014), but explanations for intraspecific variation are less intuitive. For a single structure, the SF is expected to be sufficiently high to prevent it from being compromised by applied loads, but low enough to minimize the energetic costs to produce such a structure (Alexander, 1997).

Yet, the performance of one structure may influence the performance of another within a skeleton. Structures are organized into interconnected systems based on shared biological functions, and the interdependency of structures within a system can limit the performance of individual structures. Alexander (1997) described the integrated nature of structures using a metaphor of chains in which a biological system represents a “chain” composed of interconnected “links”, such as the bones within the leg. Given that a chain’s overall strength depends upon the strength of its weakest link, it might be assumed that all components within a system should have comparable biological performance, thus avoiding wasted energy in the production of higher-quality components that would be superseded by the inferior performance of weaker ones (Alexander, 1997). However, Alexander (1997) proposed several scenarios under which variation in SFs, or a “mixed-chain”, might be expected within an organism. First, structures that are energetically costly to move or maintain could have lower SFs. Second, structures that experience more variable loading regimes than the rest of the skeleton might have higher SFs, protecting against occasionally higher loads. Third, for species in which all...
structures of the skeleton exhibit high SFs, there might be greater opportunity for variation in SFs across elements. Diamond (2002) further suggested higher SFs in structures with higher penalties for failure. For instance, a broken nasal bone might impair olfaction, but a broken cranium could be fatal, so greater SFs would be expected for the cranium.

Limited empirical evidence has supported the presence of “mixed-chains” of SFs in the locomotor skeleton. Currey (2002) found a higher incidence of fracture (implying lower SFs) in the distal limb bones of racehorses, compared to proximal bones. Similarly, Blob and Biewener (1999) found lower SFs in the tibia (distal bone) versus the femur (proximal bone) in the hind limbs of iguanas and alligators. Comparisons between bones of the forelimb and hind limb are also appropriate to consider in the context of “mixed-chains” because, although the girdles and vertebrae intervene between these limbs, both limbs function to support the body in quadrupeds, and a break in any leg would impair locomotion. However, data for such comparisons are more limited, with a single study finding higher SFs in the humerus versus the femur of alligators (Blob et al., 2014). With respect to proposed factors contributing to “mixed-chains” (Alexander, 1997; Diamond, 2002), the higher humeral SFs of alligators were attributed to the generally high SFs in the limbs of reptiles and the smaller size of the humerus that might make high SFs less costly than for the femur (Blob et al., 2014). However, with such patterns evaluated for only a single species, their generality is unclear.

Understanding the prevalence of “mixed-chains” of limb bone SFs could inform how the different functions of forelimbs and hind limbs contributed to the invasion of land. Fossil evidence suggests that terrestrial capabilities occurred in the forelimb before the hind limb, and while the forelimbs could have powered propulsion on land in some of the earliest amphibious stem tetrapods (Nyakatura et al., 2014; Pierce et al., 2012), hind limbs were the primary propulsor on land thereafter for many tetrapods, and may have contributed to hind limb-driven aquatic locomotion in sarcopterygian fishes (King et al., 2011) and some early stem tetrapods (Pierce et al., 2013). Salamanders are often used as modern locomotor analogs to early stem tetrapods given their morphological and ecological similarities (Gao and Shubin, 2001). Thus, salamanders are an intriguing system to test the “mixed-chain” hypothesis and explore how locomotor function can leave biomechanical signatures in bones, providing a foundation for inferring locomotor capabilities of fossil taxa. Femoral stresses have been evaluated for the tiger salamander Ambystoma tigrinum during terrestrial locomotion (Sheffield and Blob, 2011), but corresponding analyses for the humerus have not been performed. Combined with work on Alligator mississippiensis (Blob et al., 2014), comparisons of locomotor loading between the humerus and femur of A. tigrinum would help identify factors that drive structural and
functional diversity within the locomotor system. Additionally, information regarding form-function relationships in the locomotor system of a modern analog to early stem tetrapods can facilitate modeling early stages in the invasion of land.

To more broadly test the prevalence of “mixed-chains” of SFs within the appendicular system, biomechanical properties and loading mechanics during terrestrial locomotion were compared between the humeri and femora of Ambystoma tigrinum. Given that its humerus and femur are subequal in size and might require similar energy to move, similar SFs might be expected for these bones (Blob et al., 2014). Alternatively, a “mixed-chain” of SFs might emerge between the limbs in tiger salamanders for several reasons. First, different muscle configurations between salamander limbs (Walthall and Ashley-Ross, 2006) result in fewer muscles that are active during stance spanning the midshaft (and contributing to stress) in the humerus than in the femur (Fig. 1), potentially resulting in different sustained loads (the denominator of SF calculation) between the limbs. Alexander’s second condition predicted higher SFs with increased load variation. In another sprawling quadruped, Tiliqua scincoides intermedia, long axis rotation was greater in the humerus (78°) than the femur (53°) (Nyakatura et al., 2014), potentially increasing variation in humeral loads. Increased load variation could also result from the non-locomotor roles of the humerus, such as burrowing (Semlitsch, 1983). In addition, relatively high SFs for tiger salamander femora (~10: Sheffield and Blob, 2011) suggest the potential for variation in SF across salamander limb bones (Alexander, 1997; Blob et al., 2014).

We used a biomechanical model to estimate locomotor stresses (as proxies for loads) and SFs (specifically, ratio of yield stress to mean peak locomotor stress) for the humeri and femora of tiger salamanders by integrating measurements of bone geometry, Vickers hardness (HV), muscle moment arms, and anatomy as well as calculations of ground reaction forces (GRFs) and kinematics. Through our tests of the “mixed-chain” hypothesis of SFs between salamander limb bones, we evaluated (1) whether the femur bore greater stresses due to its greater contribution to acceleration (Kawano and Blob, 2013) or muscular configuration (Walthall and Ashley-Ross, 2006), and (2) whether variation in hardness across a limb bone corresponded with regional differences in locomotor stresses. Moreover, these data establish a foundation for considering “mixed-chains” of limb bone SFs in generalized quadrupeds, in context to transitions in limb function amongst stem tetrapods.
MATERIALS AND METHODS

Animals

Bone loading mechanics were analyzed for adult, male tiger salamanders (*Ambystoma tigrinum* Green 1825) used in a previous study on GRF production (Kawano and Blob, 2013). Two trials from this earlier study were excluded herein because they generated unrealistic estimates of bone stress (e.g., calculations suggested that limb retractor muscles did not activate during stance). Following completion of experiments, animals were humanely euthanized with buffered tricane methanesulfonate (MS-222; 2 g/L), and frozen for subsequent anatomical measurements (Tables 1-3). Experimental and animal care procedures were approved by the Clemson University Institutional Animal Care and Use Committee (Protocols 2009-071, 2010-066).

Collection of synchronized three-dimensional (3D) kinematics and kinetics

Methods for collecting synchronized 3D kinematic and kinetic data for salamanders have been documented (Kawano and Blob, 2013; Sheffield and Blob, 2011), but are summarized with additional details herein. Dorsal and lateral views of animals walking across a custom-built, multi-axis force platform (K&N Scientific, Guilford, VT, USA) were recorded at 100 Hz with digitally synchronized high-speed digital video cameras (Phantom v. 4.1, Vision Research Inc., Wayne, NJ, USA). Data on the force production of individual limbs were recorded at 5000 Hz with LabVIEW (v. 6.1, National Instruments, Austin, TX, USA), and calibrated daily. A 4 × 9 cm aluminum insert reduced the contact area of the platform, facilitating data collection from isolated limbs (see Fig. 1 of Kawano and Blob, 2013). The platform was covered with shelf liner to prevent damage to salamander skin. Data from the force platform and high-speed videos were synchronized with a 1.5 V pulse on the force traces that matched the onset of a light pulse on the lateral video of each trial.

Stance phase kinetics were processed in R (v. 3.1.2) to generate mediolateral, anteroposterior, and vertical components of the GRF, and angles of orientation in the mediolateral and anteroposterior directions. Force magnitudes were normalized to units of body weight (BW) for each animal to standardize for minor differences in body size. Data on GRFs were padded at the beginning and end to avoid edge effects (Smith, 1989), and then filtered with a second order, zero phase, low-pass Butterworth filter using the `signal` package in R. Filter parameters were determined using custom specifications, with normalization to Nyquist frequency to prevent aliasing of data (Smith, 1997). Filtered data were then interpolated to 101 points with a cubic spline using `signal::interp1(method='spline')`. 
Standardization to 101 points allowed data to be analyzed throughout stance at 1% increments (0% = beginning of stance, 100% = penultimate to swing), facilitating direct comparison between kinematics and kinetics. 50 and 48 trials were evaluated for the forelimb and hind limb, respectively, with about ten trials from each of five individuals for each limb. Comparisons were performed throughout stance, when GRFs were greatest (“peak net GRF”; Table 4), and during peak tensile stresses (Table 5).

Kinematics were quantified by digitizing coordinate data from the dorsal and lateral (right) views of each trial with DLTdv3 in MATLAB (Hedrick, 2008). High-speed videos were cropped to encompass stance. Joint and anatomical landmarks digitized in each video included: 1) the tip of the longest digit of the manus/pes, 2) metacarpophalangeal/metatarsophalangeal joint, 3) wrist/ankle, 4) elbow/knee, 5) shoulder/hip, and 6) two points along the midline of body that were dorsal to the pectoral/pelvic girdles (Fig. S1). Every other frame was digitized for videos longer than 40 frames. Otherwise, every frame was digitized. Digitized coordinates were then smoothed with a quintic spline using pspline::smooth.Pspline. Since generalized cross-validation is unreliable for high-speed videos (Walker, 1998), custom smoothing parameters were quantified as the variation of each variable obtained from a single person (S.M.K.) digitizing the first ten frames of a trial for each limb three times. The variance amongst the three digitizing attempts was used as a separate smoothing parameter for each anatomical landmark in each perspective (dorsal and lateral).

Several criteria were used for quality control of data. Trials were excluded if the animal: (1) turned, stopped, or fell on the platform; (2) moved diagonally across the platform; (3) did not have its manus/pes completely on the platform; or (4) had other parts of its body (e.g., head, throat, belly) contact the platform during stance. If the peak of the net GRF occurred within ≈ 5% of the beginning or end of stance, that trial was excluded because the animal’s body likely contacted the platform while shifting between its limbs. Acceptable trials had negligible differences in speed between the limbs (Table 6). For trials selected for analysis, data were excluded when the limb overlapped with another body part (e.g., hind limb during a forelimb trial) to ensure that calculations of GRF, moments, and bone stresses resulted from isolated limbs.

**Calculation of bone stresses**

Bone stresses were evaluated using conventions for the anatomical planes of the limbs for sprawling animals, accounting for their rotation during stance (Blob and Biewener, 2001; Butcher and Blob, 2008; Sheffield and Blob, 2011). Bone stresses were analyzed at the mid-
shaft, where the most complete records of the biomechanical loading regime are stored (Sanchez et al., 2010) and loads are predicted to be greatest (Biewener and Taylor, 1986). A biomechanical model for calculating locomotor stresses in *A. tigrinum* femora was applied to the femur data and modified for the humerus. Although data on the loading of *A. tigrinum* femora during terrestrial locomotion are available (Sheffield and Blob, 2011), new data were collected to directly compare fore- and hind limb function within individuals.

In addition to accounting for bone stresses imposed by the GRF, mathematical models were used to evaluate the contributions of limb muscles to bone stress due to moments imposed by the GRF (Fig. S2). These models incorporated only muscles that are likely active during stance and capable of countering the GRF. Joints were considered to be in static rotational equilibrium (Biewener, 1983), allowing contributions of muscle forces ($F_m$) to bone stresses to be calculated as:

$$F_m = \frac{R_{GRF} \times GRF}{r_m}$$

(1)

where $R_{GRF}$ is the moment arm of the GRF relative to the joint (obtained from force platform analyses), GRF is the ground reaction force, and $r_m$ is the moment arm of the muscle needed to counter the GRF moment about the joint. Muscles that did not span the mid-shaft could contribute to joint moments countering the GRF, but not to mid-shaft bending stresses (Blob and Biewener, 2001; Sheffield and Blob, 2011). If more than one muscle counteracted the GRF to maintain equilibrium at a joint, a mean moment arm was calculated for the group weighted by the Physiological Cross-sectional Areas (PCSAs) of the contributing muscles (Alexander, 1974; Biewener, 1983; Sheffield and Blob, 2011). Muscular moment arms were measured during post-mortem dissections, while stabilizing the limb in a mid-stance orientation. Detailed descriptions of salamander myology, including origins and insertions of muscles, are described in Walthall and Ashley-Ross (2006).

Muscles assumed to contribute to humeral joint moments and stresses included retractors and adductors, and elbow and wrist extensors (Fig. 1A,B; Table 2). Forelimb muscle activity were inferred from electromyography (Delvolvé et al., 1997; Székely et al., 1969), anatomical descriptions of *Taricha torosa* (Walthall and Ashley-Ross, 2006), and direct observations of *A. tigrinum* *Latissimus dorsi* (LD) and *coracobrachialis longus* (CBL) were considered to retract the humerus (Fig. 1A,B; red). The four bundles of the *anconaeus* complex were inferred to act as elbow extensors, and subdivided into two functional units due to their anatomical positions: *anconaeus scapularis medialis* and *anconaeus coracoideus* (ASMAC; purple), and *anconaeus humeralis lateralis* and *anconaeus humeralis medialis* (AHLAHM;
blue). ASMAC was inferred to exert an additional retractor moment due to its moment arm at the shoulder. Wrist extensors included the *flexor digitorum communis* (FDC), *flexor antebrachii et carpi radialis* (FACR), *flexor antebrachii et carpi ulnaris* (FACU), and a deep complex of carpal plantiflexors (DCF). These muscles were assumed to be active to oppose the moment of the GRF tending to dorsiflex/extend the wrist, with FDC, FACU, and FACR also spanning the extensor aspect of the elbow joint (Fig. 1A,B: yellow). *Pectoralis* (PEC) and *supracoracoideus* (SC) insert on the crista ventralis of the humerus (proximal end), and adduct the humerus (Fig. 1A,B: orange). Of these muscles that exert moments about the joints, only three (ASMAC, AHLAHM, and CBL) spanned the mid-shaft of the humerus and contributed directly to bone stresses.

The bone loading model for the femur incorporated ankle extensors, and femoral retractors and adductors (Fig. 1C,D; Table 3), with these actions inferred from electromyographic (Ashley-Ross, 1995) and anatomical (Ashley-Ross, 1992) data. The model was detailed in Sheffield and Blob (2011), but a brief summary follows. *Caudalipuboischiotibalis* (CPIT), *caudofemoralis* (CDF), and *iliofemoralis* (ILFM) retract the femur (Fig. 1C,D: red). *Ischioflexorius* (ISF) is a multi-articular muscle that contributes to femoral retraction, and spans distally to extend the ankle (Fig. 1C,D: magenta). *Flexor primordialis communis* (FPC) is situated to extend the ankle and knee (Fig. 1C,D: yellow). Three muscles [*puboischiotibialis* (PIT), *pubotibialis* (PTB), and *puboischiofemoralis externus* (PIFE)] contribute to femoral adduction and countering the abductor moment of the GRF (Fig. 1C,D: orange). Muscles that span the mid-shaft and, thus, could contribute to femoral stress include the ISF, PIT, PTB, and PIFE. Knee extensors were not incorporated into the biomechanical model because the muscles acting to extend the knee in salamanders (i.e., *iliotibialis anterior* and *posterior*) do not have a consistent phase of activity during stance (Ashley-Ross, 1995).

Thus, differences in muscle configuration and PCSA between the limbs could contribute to differences in loading between the humerus and femur. Consequently, these wrist extensors reduce the force that primary elbow extensor muscles (e.g., *anconaeus* complex) must generate to counter the elbow flexor moments typically imposed by the GRF, without increasing humeral stresses. In contrast, ankle extensors spanning the knee add to its flexor, rather than its extensor moment, often requiring elevated (rather than reduced) forces from knee extensors (Sheffield and Blob 2011). Also, a lower proportion of forelimb muscles contribute to bone stresses. Only 30% of the forelimb muscles considered in the biomechanical model were likely to contribute to humeral stresses (Table 2), with a cumulative PCSA of about
13 mm² (25% of the total PCSA for the forelimb). In contrast, 50% of the hind limb muscles contributed to femoral stresses (Table 1), with almost 20 mm² constituting about 54% of the total hind limb PCSA.

Forces acting on the bones were resolved into axial and transverse components. These were combined with bone length, cross-sectional area, second and polar moments of area, and the bending moment arms imposed by shaft curvature (rc: Biewener, 1983b) (Table 1) to calculate axial compressive stress and bending stresses in the anteroposterior plane (σb:AP, influenced by humeral retractors) and dorsoventral plane (σb:DV, influenced by elbow extensors) (Blob and Biewener, 2001). The second moment of area (reflecting resistance to bending) and polar moment of area (reflecting resistance to torsion) (Lieberman et al., 2004) were measured with BoneJ (Doube et al., 2010) in ImageJ64 (v. 1.47t; Bestheda, MD, USA).

The magnitude of the net bending stress at the mid-shaft was calculated as the vector sum of stresses in two planes, allowing the orientation of peak bending stress (relative to the AP axis) to be calculated as:

\[ \alpha_{b:net} = \tan^{-1} \left( \frac{\sigma_{b:DV}}{\sigma_{b:AP}} \right). \]  (2)

The net neutral axis of bending was determined as perpendicular to this axis of peak stress (Sheffield et al., 2011).

Torsional stresses (τ) produced by the GRF were calculated as:

\[ \tau = T \left( \frac{y_t}{J} \right) \]  (3)

where T is calculated as the orthogonal distance of the GRF vector relative to the long axis of the bone, yt is the distance of the centroid from the bone cortex, and J is the polar moment of area (Table 1) that is calculated as the sum of the second moments of area in the DV and AP directions (Lieberman et al., 2004).

**Mechanical testing of salamander humeri and femora**

Microindentation was used to compare hardness between and within bones. Right humeri and femora were air-dried, mounted in Caroplastic (Carolina Biological, Burlington, NC, USA), a non-infiltrating resin, and sectioned transversely at the mid-shaft. Cut surfaces from the distal half were polished to visualize cross-sectional geometries and prepare for microindentation. Mounted specimens were affixed to a 100 x 61 x 2 mm Plexiglas slide with cyanoacrylate glue, and loaded onto an automated polisher (EXAKT Technologies, D-4000, Oklahoma City, OK, USA). Samples were ground with moistened silicon carbide paper of decreasing grit sizes (P800, P1200, P2500, P4000), for 5 minutes each. Agglomerate-free alumina suspensions were
used to polish the specimens to 3.0 μm (Baikalox Type 3.0 CR Alpha), 0.3 μm (Baikalox Type 0.3 CR Alpha), and finally to 0.05 μm (Buehler Micropolish II) using a polishing pad (Buehler, Lake Bluff, IL, USA) for 3 minutes each. Grinding and oscillation speeds were set at 30 rpm, with a 99.3 g weight applied. Samples were rinsed with deionized water after each step to remove abrasive particulates, air dried, and then stored at -20°C for less than 72 hours. Prior to indentation, samples were equilibrated to room temperature and cleaned with methanol. These procedures allowed mechanical testing of hydrated bones. \( H_V \) was measured with a Digital Display Microhardness Tester (Model HVS-1000B, Beijing, China) configured with a Vickers indenter tip, 0.49 N load, and 15 sec dwell time, following procedures for microindentation of salamander femora (Sheffield and Blob, 2011). About five indents were performed in the dorsal, ventral, anterior, and posterior regions to test for regional heterogeneity in hardness. Data were collected away from cavities and edges of the bone to avoid potential edge effects. No cracks or pile-up were observed.

Sample preparation and testing conditions can influence hardness measurements, but were likely minimal in this study. \( H_V \) (1) is consistent for dwell times up to 30 secs (Johnson and Rapoff, 2007), (2) does not differ between bones that were fresh versus frozen at -20°C for three months, and (3) is only 4% lower in bones that are embedded in infiltrating media rather than non-embedded (Evans et al., 1990). We used a non-infiltrating plastic to stabilize the bones and, therefore, expect the difference between mounted and unmounted bones to be minimal. Hardness values have been up to \( \approx 50\% \) higher for bones tested dry rather than wet with a nanoindenterator (Hoffler et al., 2005), but only about 9% greater for bones that were dried for two days or longer and tested with a microindenter (Johnson and Rapoff, 2007). Our use of a non-infiltrating resin kept the bones hydrated. Although the humerus of Individual #1 underwent slightly different testing conditions (0.981 N load, and no data from the posterior region), available data still followed general patterns observed between the humeri and femora (Fig. S3). Also, hardness is consistent for applied loads between 15-300 g (Zysset, 2009),
encompassing the 0.49 N and 0.981 N used in this study. Thus, our protocol likely had minimal effect on hardness comparisons.

HV data were entered into a linear regression equation (Wilson et al., 2009), derived using empirical data from various tetrapod bones (Hodgkinson et al., 1989), to estimate tensile yield stress ($\sigma_{y(t)}$; MPa):

$$\sigma_{y(t)} = 32.571 + 2.702 \times H_V$$

(Tensile yield stress has important consequences for organisms because bone failure tends to occur on the tensile side during bending (Currey, 2002). Compressive yield stress was also estimated from HV to evaluate regional heterogeneity of bone biomechanics. Data on compressive yield stress are not available for salamanders, so estimates were based on the assumption that tensile yield stresses are 25% lower than compressive yield stresses (Currey, 1984). SFs were then calculated as:

$$SF = \frac{\sigma_{yield stress}}{mean\ peak\ stress}$$

and “worst-case” scenario estimates (SF$_{WC}$) as:

$$SF_{WC} = \frac{\sigma_{yield\ stress} - 2 \times \text{s.d.}\sigma_{yield\ stress}}{mean\ peak\ stress + 2 \times \text{s.d. mean peak stress}}$$

Hv, yield stress, and SF were reported separately for each of the anatomical regions (Tables 7, S1). Calculations of yield stresses and SFs were based on dorsal and posterior regions being loaded in tension, and anterior and ventral regions loaded in compression.

**Statistical analyses**

Linear mixed-effects models (LMMs), fitted by Restricted Maximum Likelihood via lme4::lmer, were used to evaluate differences within response variables, with individual as a random effect (RE) for a random intercepts model (Bates et al., 2014). REs represented subsamples of a population and an additional source of variation (e.g., individuals within species) whereas fixed effects (FEs) were factors to compare (e.g., forelimb vs. hind limb) (Bolker et al., 2009). Regional heterogeneity of HV within a bone was assessed with anatomical region as a FE. Otherwise, limb bone was the FE. Given that HV within an anatomical region may vary amongst individuals, anatomical region was also added as a RE to create a random intercepts and slopes LMM. Pair-wise comparisons between anatomical regions and bones were fit with a contrast matrix in multcomp::glht. P-values provide limited information regarding the strength of evidence to support conclusions (Anderson et al., 2001), so LMMs were reported in terms of effect sizes and an estimate of precision [e.g., $\Omega^2$: (Xu, 2003)].
emphasizing the magnitude of the differences and the level of uncertainty in supporting those differences, respectively.
RESULTS

Kinematic comparison of forelimbs and hind limbs

Although the limbs have similar kinematic profiles, numerous differences were identified. At the beginning of stance, the shoulder and hip are adducted by ≈ 10-15° (Fig. 2A) with the wrist and ankle showing similar degrees of extension (Fig. 2B). The femur is more protracted than the humerus until about 80% of stance (Fig. 2C), and the elbow more flexed than the knee until about 90% of stance (Fig. 2D). Flexion and extension of the elbow and knee follow similar profiles; however, the ankle becomes flexed almost twice as much as the wrist towards mid-stance. Another major difference between the limbs is that the femur remains adducted (e.g., knee closer to the ground than the hip) throughout stance (Fig. 2A), but the humerus becomes abducted (elbow higher than shoulder) after about 30% of stance. Additionally, although both bones begin in a protracted orientation (i.e., distal joint cranial to the girdle for almost all of stance), the humerus is initially nearly perpendicular to the long axis of the body (≈ 0° in Fig. 2C) and retracts at about 10% of stance, whereas retraction of the femur is more evenly split between protracted and retracted orientations (Fig. 2C).

Moments produced by the GRF about limb joints

GRF production was generally similar between the forelimbs and hind limbs (Table 4, Fig. 3), contributing to similarities in the joint moments imposed by the GRF (Fig. 4). The GRF imposes a dorsiflexion (positive) moment about the wrist and ankle (Fig. 4A) due to the anterior position of the GRF relative to these joints. To maintain equilibrium at these joints, wrist and ankle extensors need to be active to counter the flexor moments imposed by the GRF. The primarily vertical orientation of the GRF throughout stance (Fig. 3) tends to impose an abductor moment on the shoulder and hip, though for the latter this shifts to an adductor moment approximately 75% into stance (Fig. 4B). The GRF also imposes a protractor moment about the shoulder and hip, though protraction at the shoulder is lower in magnitude and occurs later in stance (40%) than at the hip (10% stance) (Fig. 4C). Finally, torsional moments imposed by the GRF are similar between the humerus and femur (Fig. 4D), changing from a tendency to impose anterior axial rotation to posterior rotation at about 60% of stance.

Despite these similarities, different configurations of the forelimb and hind limb influence how the GRF imposes moments on these limbs (Fig. S2). In salamanders (and most quadrupeds), the elbow points posteriorly whereas the knee points anteriorly. However, the GRF is directed essentially vertically for most of stance for both limbs (Fig. 3). Consequently, the flexor/extensor moment of the GRF tends to change in different directions for these joints,
shifting from a flexor to an extensor moment for the knee at \( \approx 50\% \) stance, but vice versa for the elbow at \( \approx 75\% \) stance (Fig. 4E). Also, analogous moments were greater in the hind limb than in the forelimb [e.g., ankle versus wrist in dorsiflexion (Fig. 4A), hip versus shoulder in anteroposterior and dorsoventral rotations (Fig. 4B,C), and knee versus elbow in flexion and extension (Fig. 4E)].

Comparison of bone stresses
Lower stresses were estimated for locomotor loads upon the humerus, though less pronounced for shear (Table 6). Peak tensile and compressive stresses occurred later in stance for the humerus \( (\approx 67\%) \) than the femur \( (\approx 22\%) \) (Table 6, Fig. 5A,B). This pattern corresponds with the peak net GRF, which also occurred later in stance \( (\approx 61\%) \) for the forelimb than the hind limb \( (\approx 33\%) \) (Table 4, Fig. 3). After accounting for variation amongst individuals, total external forces ("net GRF") at the time of peak tensile stresses for each bone were \( 0.061 \pm 0.016 \) BW lower in the humerus, with vertical and anteroposterior components lower by 0.04 and 0.115 BW, respectively (Table 5).

The neutral axis of bending for the humerus was directed such that the posterodorsal region was loaded in tension and the anteroventral region in compression through the time of mid-stance to peak loading (Fig. 5C,D). The neutral axis of bending was aligned closer to the anatomical anteroposterior axis at peak tensile stress for the femur, placing the dorsal portion in tension and the ventral portion in compression. Nonetheless, the anterodorsal cortex of the femur was loaded in tension and the posteroventral cortex in compression at 50\% of stance (Fig. 5C,D).

Biomechanical properties and SFs of the salamander humeri and femora
Comparisons indicated higher Hv for the humerus and regional heterogeneity within each bone (Tables 7, S1). The LMM explained about 68\% of the variation in Hv based on \( \Omega^2 \) (Xu, 2003), an analog of R\(^2\) for LMMs. The greatest magnitude of Hv, and thus estimated yield stresses, at these mid-shafts were generally in the posterodorsal region (Table 7), corresponding with the typical location of tensile loads about the neutral axis of bending (Fig. 5B).

Femoral SFs ranged from \( \approx 9-10 \) (Table 7), corresponding with the published estimate of 10.5 (Sheffield and Blob, 2011). However, humeral SFs were almost twice those of the femur, ranging from \( \approx 20-24 \). The greatest SFs at femoral mid-shafts were in the dorsal cortex, where Hv was greatest, yet SFs at humeral mid-shafts were greater in the anteroventral region, where Hv was lower (Table 7). This difference was largely due to peak bone stresses that were
about two times lower in the humerus (Table 6), although higher yield stresses in the humerus also contributed to SF differences from the femur (Table 7). The “worst-case scenario” \( (\text{SF}_{\text{wc}}) \) was about two times lower than standard SF calculations for both bones, but still indicated ample margins of safety \( (\approx 8-13 \text{ for the humerus, } \approx 3-6 \text{ for the femur: Table 7}) \). Biomechanical differences along the dorsoventral and anteroposterior planes of the bones were also reflected in their structural response to bending, as evidenced by second moments of area that were greater in the dorsoventral plane for the humerus and the anteroposterior plane for the femur (Table 1).
DISCUSSION

Mechanisms underlying elevated SFs in salamander humeri

Humeri have higher SFs (≈ 22) than femora (≈ 10) in salamanders, a disparity greater than that reported in alligators (8.4 for the humerus versus 6.3 for the femur: Blob et al., 2014). The difference between humeral and femoral SFs relates primarily to differences in yield strain for alligators (Blob et al., 2014), but from both lower stresses and structural reinforcement in salamander humeri (Tables 6, 7).

Critical factors that are likely contributing to the relatively lower stresses in the salamander humerus, compared to the femur, include the configuration of joints and disposition of muscles. Because of the range of motion of the arm (Fig. 2) and orientation of the elbow in *A. tigrinum*, the GRF only exerts a flexor moment at the elbow late in stance (Fig. 4E). This reduces the need for elbow extensors (e.g., anconaeus complex) to counter GRF moments at the elbow, reducing the stress they place on the humerus (Table 2, Fig. 1). Humeral stresses are further reduced by contributions of wrist extensors (FDC, FACR, FACU) to the elbow extensor moment, further reducing the force that elbow extensors spanning the humeral mid-shaft must exert. Moreover, adductor muscles contributing to forelimb movement (PEC and SC) insert proximally on the humerus, and do not contribute to stresses experienced at mid-shaft.

Beyond these stress-reducing characteristics of forelimb musculature in salamanders, Hv of the humerus is generally greater than that of the femur, with different patterns of regional heterogeneity (Tables 7, S1) between the bones. The highest SFs corresponded with areas loaded in tension (dorsal and posterior) for the femur, but compression (anterior and ventral) for the humerus. Moreover, the femur has a larger second moment of area in the anteroposterior direction (I<sub>AP</sub>), but the humerus has a greater second moment of area in the dorsoventral direction (I<sub>DV</sub>) (Table 1). These results suggest that these limb bones show differences in structure and mechanical response that reduce bending stress in different directions. Given the extent to which humeral SFs (≥ 20) of salamanders are greater than those of the femur (≈ 10), it is difficult to envision how the entire magnitude of differences in humeral and femoral SFs of salamanders could be adaptive. Nonetheless, elevated SFs supported by greater Hv and structural modifications suggest the possibility that, to some extent, the high
load-bearing capacity of salamander humeri may facilitate the multi-functional role of the forelimbs for locomotor and non-locomotor behaviors (e.g., burrowing).

Relevance of “mixed chains” to tetrapod evolution
Comparisons of SFs for the humerus and femur of salamanders provide an additional empirical example of a “mixed-chain” (Alexander, 1997; Alexander, 1998) within the locomotor skeleton of tetrapods. “Mixed-chains” of SFs were identified between proximal and distal limb bones in horses (Currey, 2002) and iguanas and alligators (Blob and Biewener, 1999). However, data herein reinforce additional patterns observed in alligators (Blob et al., 2014), which demonstrated different SFs between the proximal bones of the forelimb and the hind limb. As in alligators (Blob et al., 2014), the humerus had a higher SF than the femur in salamanders (Table 7).

Some of the factors proposed by Alexander (1997) that might contribute to differences in SFs between these bones in alligators do not apply to salamanders. For example, alligator humeri are smaller than the femora, which might allow for more economical maintenance of a high SF (Blob et al., 2014). However, the humerus and femur are roughly equal in length in salamanders (Table 1), so Alexander’s first condition for “mixed-chains” likely does not apply. Alexander’s second condition for “mixed-chains” of SFs also likely does not apply to salamanders. Similar to alligators, the salamander limb bone that was exposed to greater variation in loads (i.e., femur) did not have higher SFs (Table 6), suggesting that elevation of humeral SFs in salamanders likely was not an adaptive response for protection against unpredictable high loads.

SFs for salamander limb bones, like those of alligators, are generally high (> 7) compared to many taxa, including mammals and birds (Blob et al., 2014). Thus, differences between humeral and femoral SFs for salamanders might simply reflect an increased opportunity for variation in SF across the skeleton (Alexander’s third condition). Though this reason has been invoked as a factor contributing to “mixed-chains” in alligators (Blob et al., 2014), data on the limb configuration, muscle disposition, and regional heterogeneity of Hv in tiger salamanders also suggest mechanistic reasons for high SFs in limb design. Collectively, the elevated structural and material reinforcement to withstand loads in the humerus, and anatomical features of the forelimb promoting low loads, suggest that stochastic variation
associated with large SFs may not completely account for differences in humeral and femoral SFs observed in salamanders.

In addition to the three conditions promoting “mixed-chains” of SFs proposed by Alexander (1997), higher SFs may be found in structures with higher penalties for failure (Diamond, 2002). Forelimbs hind limbs play different roles in legged locomotion (McElroy et al., 2014), which may provide insight into SF variation in salamander limb bones. Although hind limbs provide the primary propulsion in many non-mammalian quadrupeds, forelimbs still make important contributions to locomotion (Kawano and Blob, 2013; Nyakatura et al., 2014), and loss of locomotor function may be more detrimental for the forelimb. Early work on salamander locomotion (Evans, 1946) demonstrated that forward propulsion could be achieved solely by the forelimbs but not the hind limbs, suggesting that the forelimbs play a more important locomotor role than passive body support (at least in terrestrial salamanders such as Taricha and Ambystoma). Moreover, there do not appear to be ready examples (among non-bipedal vertebrates) in which the complete loss of the pectoral appendages occurred while the pelvic appendages remained fully intact: when vertebrates lose an entire appendage, it is typically the pelvic appendages (e.g., siren salamanders, amphisbaenids, cetaceans, sirenian mammals, scincid lizards, and fishes from 100 families; Gans, 1975; Lande, 1978; Yamanoue et al., 2010). Even when limb loss is associated with the evolution of fossorial or aquatic life styles (e.g., amphisbaenians and cetaceans), the forelimbs are typically retained rather than the hind limbs (Caldwell, 2003). Limb reduction, including the loss of digits, can be found in the forelimb rather than the hind limb in some taxa (Lerista lizards: Skinner et al., 2008), but the loss of proximal limb elements or the entire limb is generally less common for forelimbs. Additional studies are required to verify whether there are strong mechanical or selective advantages for forelimb retention in non-bipedal vertebrates, or whether the conservatism of forelimb retention is due to developmental constraint. For instance, hind limbs develop after forelimbs (Tanaka and Tickle, 2007), and structural reduction typically occurs in the reverse order from which structures develop (Lande, 1978), potentially making hind limbs more susceptible to loss via developmental truncation.

Further investigations of how loads vary across limb bones could yield insights into the morphological evolution of limb bones as vertebrates became terrestrial. The vertebrate musculoskeletal system shifted from being essentially weightless due to buoyancy underwater to counteracting the effects of gravity on land, drastically shifting the loading regime imposed upon the locomotor structures. This shift may have made the evolution of long, tubular limb bone shafts advantageous compared to their blocky precursors (Currey,
Microanatomical analyses of a wide range of tetrapods have differentiated aquatic and terrestrial lifestyles from limb bone histology (Laurin et al., 2011), facilitating the inference of the locomotor biomechanics of fossil taxa such as the Devonian fish *Eusthenopteron* (Laurin et al., 2007) and stem stegocephalians (Laurin et al., 2004). Identifying stronger form-function relationships between limb morphology and locomotor movements would facilitate efforts to reconstruct the transition from water to land by tetrapods (Nyakatura et al., 2014; Standen et al., 2014). For instance, mechanical properties of bones from extant taxa were combined with palaeopathology to theorize the loading conditions that could have fractured the radius in the early stem tetrapod *Ossinodus pueri* in the context of walking on land (Bishop et al., 2015), suggesting the utility of bone loading data during terrestrial locomotion to address the mechanisms that influenced how vertebrates became terrestrial. Further application of data on locomotor stresses from extant taxa could help answer questions regarding the functional
consequences of morphological changes observed in extinct tetrapodomorphs spanning the transition from water to land (Hohn-Schulte et al., 2013; Kawano and Blob, 2013).

LIST OF ABBREVIATIONS AND SYMBOLS

\(J\) = polar moment of area (mm\(^4\))

\(\sigma_b\) = bending stress (MPa)

\(\sigma_{y(t)}\) = tensile yield stress (MPa)

\(\Omega_0^2\) = analog for coefficient of determination for LMMs

\(\Theta\) = angle between the muscle and the long axis of the bone

\(\tau\) = torsional stress (MPa)

\(T\) = orthogonal distance of the GRF vector relative to the long axis of the bone

\(y_t\) = deviation of the centroid from the bone cortex (mm)

AHLAHM = anconaeus humeralis lateralis and anconaeus humeralis medialis

AP = anteroposterior

ASMAC = anconaeus scapularis medialis and anconaeus coracoideus

BW = body weight

CBL = coracobrachialis longus

CDF = caudofemoralis

CPIT = caudalipuboischiotibialis

CV = coefficient of variation

DCF = deep complex of plantar flexors of the carpus

DV = dorsoventral

FACR = flexor antebrachii et carpi radialis

FACU = flexor antebrachii et carpi ulnaris

FDC = flexor digitorum communis

\(F_m\) = muscular forces (N)

GRF = ground reaction force

\(H_V\) = Vickers hardness

ILFM = iliofemoralis

ISF = ischioflexorius

LD = latissimus dorsi

LMM = linear mixed-effects model
ML = mediolateral
PEC = pectoralis
PCSA = physiological cross-sectional area (mm$^2$)
PIFE = puboischiofemoralis externus
PIT = puboischiotibialis
PTB = pubotibialis
$R_{GRF}$ = moment arm of the GRF relative to the joint (m)
$r_m$ = moment arm of the muscle forces (mm)
SC = supracoracoideus
SF = safety factor
ACKNOWLEDGMENTS
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COMPETING INTERESTS
The authors declare no competing interests.

AUTHOR CONTRIBUTIONS
S.M.K. collected and analyzed the data, D.R.E., M.S.K., and D.D. provided the mechanical testing equipment, and trained/supervised S.M.K in mechanical testing. R.W.B. developed the biomechanical model, provided equipment for quantifying kinematics and kinetics, and supervised analyses. S.M.K. and R.W.B. led the conception and design of the research. All authors contributed to writing the manuscript.
REFERENCES


Figures

Figure 1. Superficial musculature of the forelimbs (A: dorsal, B: ventral) and hind limbs (C: dorsal, D: ventral) of salamanders that were incorporated into the biomechanical model of limb bone stress production. Illustration is based on myology figures of the California newt, *Taricha torosa* (Walthall and Ashley-Ross 2006), with permission to re-use from John Wiley and Sons, Inc. Details of these anatomical structures and their associated acronyms can be found in Walthall and Ashley-Ross (2006). Muscles active during stance were assumed to contribute to joint moments to counter the GRF, but could only contribute to bone stress if they spanned the mid-shaft of either the humerus or the femur. Two deep muscles (DCF in the forelimb and ISLM in the hind limb) were modeled as wrist and ankle extensors, respectively, but are not illustrated. The top of each figure is oriented in the anterior direction of the animal. Scale bars represent 1 cm.
Figure 2. Comparison of stance phase kinematic profiles between the forelimbs (dashed red) and hind limbs (solid blue) of tiger salamanders (A. tigrinum). Lines and adjacent shading represent means ± s.e.m. pooled across all trials for the forelimbs (N = 50) and hind limbs (N = 48), with all trials normalized as a percentage of stance. Negative values are highlighted in grey, and indicate adduction in A and retraction in C. Kinematic comparisons included: (A) abduction/adduction angle of the limbs, (B) extension/flexion of the wrists and ankles, (C) protraction/retraction angle of the limbs, and (D) extension/flexion of the elbows and knees. See Fig. S1 for illustration of these kinematic angles.
Figure 3. GRF production by the forelimb (dashed red) and hind limb (solid blue) of *A. tigrinum* throughout stance. Lines and adjacent shading represent means ± s.e.m. pooled across all trials for the forelimbs (*N* = 50) and hind limbs (*N* = 48), with all trials normalized as a percentage of stance. Anteroposterior angles were set relative to vertical (0°), so that negative values indicated a GRF directed posteriorly. Mediolateral angles were also relative to vertical (0°), so that negative values indicated a GRF directly medially. Grey rectangles
distinguish negative values within each plot. Although the peak values for the net GRF and the vertical component were similar (≈ 0.45 BW) and the GRF remained medial, positive anterior values throughout stance indicated that the hind limbs had a greater acceleratory role than the forelimbs.
Figure 4. Comparison of moments exerted by the GRF for the forelimb (dashed red) and hind limb (solid blue) of *A. tigrinum*. Lines represent mean values obtained from data pooled across all trials for the forelimbs (*N* = 50) and hind limbs (*N* = 48), with the shading depicting the standard error. Negative values are highlighted in grey, and the directions of rotation indicated by positive and negative values are labeled in the panels. Girdle refers to the shoulder
and hip. See Fig. S2 for illustration of GRF moment arms relative to limb joints.
Figure 5. Bone stresses were lower in the humerus (dashed red) than the femur (solid blue) for *A. tigrinum*, and varied in magnitude across these bones. (A) Maximum tensile and (B) compressive stresses, and (C) the angle of the neutral axis of bending from the anatomical AP axis. (D) Orientation of the neutral axis of bending (solid red line) relative to the AP axis (dashed black line) at peak tensile stress (top) and 50% of stance (bottom), mapped onto cross sections of the humeral and femoral mid-shafts. Dark regions of the bone are in compression, and light regions are in tension. Compared to the humerus, magnitudes of the peak tensile (A) and compressive (B) stresses were about 1.7 and 2.3 times greater, respectively, in the femur. At both 50% of stance and the timing of the peak tensile stress (C,D), the absolute angle of the neutral axis was greater than 30° for the humerus but less than 20° for the femur. Tables
### Table 1. Comparison of anatomical data for the humeri and femora of *A. tigrinum*

<table>
<thead>
<tr>
<th></th>
<th>Humeri</th>
<th>Femora</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>15.244 ± 0.463</td>
<td>14.906 ± 0.478</td>
</tr>
<tr>
<td>Cross-sectional Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>1.007 ± 0.201</td>
<td>0.879 ± 0.343</td>
</tr>
<tr>
<td>Moment arm due to curvature (AP; r&lt;sub&gt;AP&lt;/sub&gt;) (mm)</td>
<td>0.099 ± 0.056</td>
<td>0.040 ± 0.031</td>
</tr>
<tr>
<td>Moment arm due to curvature (DV; r&lt;sub&gt;DV&lt;/sub&gt;) (mm)</td>
<td>-0.349 ± 0.128</td>
<td>-0.138 ± 0.103</td>
</tr>
<tr>
<td>Distance from neutral axis to cortex (AP; y&lt;sub&gt;AP&lt;/sub&gt;) (mm)</td>
<td>1.406 ± 0.089</td>
<td>0.613 ± 0.029</td>
</tr>
<tr>
<td>Distance from neutral axis to cortex (AP; y&lt;sub&gt;DV&lt;/sub&gt;) (mm)</td>
<td>1.368 ± 0.062</td>
<td>1.000 ± 0.077</td>
</tr>
<tr>
<td>Second moment of area (AP; I&lt;sub&gt;AP&lt;/sub&gt;) (mm&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>0.134 ± 0.048</td>
<td>0.201 ± 0.107</td>
</tr>
<tr>
<td>Second moment of area (DV; I&lt;sub&gt;DV&lt;/sub&gt;) (mm&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>0.191 ± 0.072</td>
<td>0.131 ± 0.048</td>
</tr>
<tr>
<td>Polar moment of area (I&lt;sup&gt;J&lt;/sup&gt;) (mm&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>0.325 ± 0.118</td>
<td>0.333 ± 0.154</td>
</tr>
</tbody>
</table>

Values are means ± s.d. (n=5 individuals for each group).
All of the listed variables except “length” were evaluated at the mid-shaft of the bone.
Statistical comparisons were not conducted due to small sample size.

AP = anteroposterior direction; DV = dorsoventral direction.
For r<sub>AP</sub>: positive = concave posterior, negative = concave anterior.
For r<sub>DV</sub>: positive = concave central, negative = concave dorsal.

<sup>J</sup> = I<sub>AP</sub> + I<sub>DV</sub> (Lieberman et al. 2004)
Table 2. Comparison of anatomical data for the forelimb muscles of *A. tigrinum*

<table>
<thead>
<tr>
<th>Muscle Type</th>
<th>Muscle</th>
<th>PCSA (mm(^2))</th>
<th>Θ (degrees)</th>
<th>(r_m) – shoulder (mm)</th>
<th>(r_m) – elbow (mm)</th>
<th>(r_m) – wrist (mm)</th>
<th>Bone Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humeral retractors</td>
<td>LD</td>
<td>4.010 ± 0.632</td>
<td>N/A</td>
<td>2.402 ± 0.239</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>CBL</td>
<td>3.092 ± 0.550</td>
<td>6 ± 2.236</td>
<td>4.060 ± 0.991</td>
<td>N/A</td>
<td>N/A</td>
<td>Yes</td>
</tr>
<tr>
<td>Humeral adductors</td>
<td>PEC</td>
<td>10.105 ± 2.902</td>
<td>N/A</td>
<td>4.342 ± 0.589</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>11.165 ± 2.781</td>
<td>N/A</td>
<td>4.008 ± 1.039</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td>Elbow extensors</td>
<td>ASMAC*</td>
<td>2.460 ± 0.652</td>
<td>6 ± 2.236</td>
<td>1.820 ± 0.253</td>
<td>1.690 ± 0.380</td>
<td>N/A</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>AHLAHM</td>
<td>7.565 ± 1.698</td>
<td>0 ± 0</td>
<td>N/A</td>
<td>1.942 ± 0.735</td>
<td>N/A</td>
<td>Yes</td>
</tr>
<tr>
<td>Wrist extensors</td>
<td>FDC†</td>
<td>6.719 ± 1.603</td>
<td>N/A</td>
<td>N/A</td>
<td>1.546 ± 0.382</td>
<td>1.708 ± 0.726</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>FACR†</td>
<td>3.150 ± 0.836</td>
<td>N/A</td>
<td>N/A</td>
<td>1.356 ± 0.440</td>
<td>1.006 ± 0.326</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>FACU†</td>
<td>1.570 ± 0.594</td>
<td>N/A</td>
<td>N/A</td>
<td>1.500 ± 0.401</td>
<td>0.948 ± 0.334</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>DCF</td>
<td>2.694 ± 0.971</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0.890 ± 0.293</td>
<td>No</td>
</tr>
</tbody>
</table>

Values are means ± s.d. (n = 5).
PCSA = Physiological Cross-Sectional Area, Θ = angle between the muscle and the long axis of the bone, \(r_m\) = moment arm of the muscle forces about the joint. See List of Abbreviations and Symbols for muscle names. Rightmost column indicates whether the muscle was assumed to contribute to bone stress.
Statistical comparisons were not conducted due to small sample size.
* ASMAC also exerts a humeral retractor moment about the shoulder.
† FDC, FACR, and FACU also exert extensor moments at the elbow.
Table 3. Comparison of anatomical data for the hind limb muscles of *A. tigrinum*

<table>
<thead>
<tr>
<th>Muscles</th>
<th>PCSA (mm²)</th>
<th>Θ (degrees)</th>
<th>rₘ – hip (mm)</th>
<th>rₘ – knee (mm)</th>
<th>rₘ – ankle (mm)</th>
<th>Bone stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral retractors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPIT</td>
<td>3.708 ± 0.473</td>
<td>N/A</td>
<td>4.072 ± 0.834</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>CDF</td>
<td>4.895 ± 0.915</td>
<td>N/A</td>
<td>5.942 ± 1.198</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td>ILFM</td>
<td>2.243 ± 0.711</td>
<td>N/A</td>
<td>2.668 ± 0.574</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td>Femoral adductors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIFE</td>
<td>7.357 ± 0.713</td>
<td>11 ± 2.236</td>
<td>2.124 ± 0.185</td>
<td>N/A</td>
<td>N/A</td>
<td>Yes</td>
</tr>
<tr>
<td>PIT</td>
<td>8.475 ± 0.998</td>
<td>9 ± 2.236</td>
<td>2.946 ± 0.530</td>
<td>3.946 ± 0.730</td>
<td>N/A</td>
<td>Yes</td>
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<tr>
<td>PTB</td>
<td>2.219 ± 0.406</td>
<td>10 ± 0</td>
<td>2.198 ± 0.380</td>
<td>2.308 ± 0.603</td>
<td>N/A</td>
<td>Yes</td>
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<tr>
<td>Ankle extensors</td>
<td></td>
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<tr>
<td>ISF*</td>
<td>1.595 ± 0.447</td>
<td>8 ± 2.739</td>
<td>5.944 ± 0.635</td>
<td>3.234 ± 0.408</td>
<td>2.668 ± 0.466</td>
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<tr>
<td>FPC</td>
<td>6.152 ± 1.321</td>
<td>N/A</td>
<td>N/A</td>
<td>1.064 ± 0.188</td>
<td>1.482 ± 0.619</td>
<td>No</td>
</tr>
</tbody>
</table>

Values are means ± s.d. (n = 5).

PCSA = Physiological Cross-Sectional Area, Θ = angle between the muscle and the long axis of the bone, rₘ = moment arm of the muscle forces about the joint. See List of Abbreviations and Symbols for the muscle names.

Rightmost column indicates whether the muscle was assumed to contribute to bone stress.

Statistical comparisons were not conducted due to small sample size.

*ISF also exerts a hip retractor moment
Table 4. Comparison of GRF parameters at the time of peak net GRF for *A. tigrinum*

<table>
<thead>
<tr>
<th></th>
<th>Forelimb</th>
<th>Hind limb</th>
<th>FE ± s.e.m.</th>
<th>FE ± s.e.m.</th>
<th>t-value</th>
<th>Ω₀²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of peak net GRF (%)</td>
<td>61.080 ± 1.008</td>
<td>32.667 ± 1.628</td>
<td>28.294 ± 1.824</td>
<td>32.786 ± 1.854</td>
<td>15.51</td>
<td>17.69</td>
</tr>
<tr>
<td>Net GRF (BW)</td>
<td>0.457 ± 0.009</td>
<td>0.479 ± 0.010</td>
<td>-0.022 ± 0.012</td>
<td>0.478 ± 0.017</td>
<td>-1.798</td>
<td>28.266</td>
</tr>
<tr>
<td>Vertical GRF (BW)</td>
<td>0.447 ± 0.009</td>
<td>0.439 ± 0.013</td>
<td>0.007 ± 0.014</td>
<td>0.440 ± 0.020</td>
<td>0.475</td>
<td>21.609</td>
</tr>
<tr>
<td>Mediolateral GRF (BW)</td>
<td>-0.068 ± 0.004</td>
<td>-0.071 ± 0.007</td>
<td>0.002 ± 0.007</td>
<td>-0.069 ± 0.009</td>
<td>0.206</td>
<td>-7.377</td>
</tr>
<tr>
<td>Anteroposterior GRF (BW)</td>
<td>-0.028 ± 0.008</td>
<td>0.151 ± 0.009</td>
<td>-0.179 ± 0.012</td>
<td>0.151 ± 0.008</td>
<td>-15.39</td>
<td>18.14</td>
</tr>
<tr>
<td>Mediolateral angle (degrees)</td>
<td>-8.671 ± 0.531</td>
<td>-9.271 ± 0.897</td>
<td>0.360 ± 0.964</td>
<td>0.374</td>
<td>0.374</td>
<td>-9.031 ± 1.171</td>
</tr>
<tr>
<td>Anteroposterior angle (degrees)</td>
<td>-3.206 ± 0.996</td>
<td>-9.271 ± 0.897</td>
<td>-23.225 ± 1.782</td>
<td>0.374</td>
<td>-13.04</td>
<td>-9.031 ± 1.171</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. (*n* = 50 and *n* = 48 trials averaged across five individuals for the forelimb and hind limb, respectively). Timing of the peak net GRF is represented as a percentage into the stance phase of the limb cycle. Peak net GRF values were determined for each individual trial, and then averaged across all trials to produce the mean and s.e.m. Mean values assume all trials were independent.

FE = fixed effect. Fixed effect estimates account for non-independence due to sub-sampling of individuals, and values for the humerus indicate differences from the femur (e.g., net GRF was about 0.02 BW lower in the humerus than in the femur).

Statistical analyses based on the model: $\text{lm}r(y \sim \text{Limb} + (1 | \text{Individual}), \text{REML} = \text{True})$. The hind limb was treated as the intercept, by default.

$Ω₀²$ represents a coefficient of determination for LMMs, whereby values closer to 1.0 indicate stronger concordance between the data and the LMM.

The t-value represents the test statistic based on a t-distribution, and can be used to estimate how likely the differences between the forelimb and hind limb could have been derived by chance through a null hypothesis testing framework (Note: t-values are not used in inference herein, but are presented for convenience).
Table 5. GRF parameters at the timing of peak tensile stress for *A. tigrinum* forelimbs and hind limbs

<table>
<thead>
<tr>
<th></th>
<th>Forelimb</th>
<th>Hind limb</th>
<th>Ω²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.e.m.</td>
<td>FE ± s.e.m.</td>
<td>t-value</td>
</tr>
<tr>
<td><strong>Net GRF (BW)</strong></td>
<td>0.376 ± 0.011</td>
<td>-0.061 ± 0.016</td>
<td>-3.927</td>
</tr>
<tr>
<td><strong>Vertical (BW)</strong></td>
<td>0.369 ± 0.011</td>
<td>-0.039 ± 0.015</td>
<td>-2.508</td>
</tr>
<tr>
<td><strong>Mediolateral (BW)</strong></td>
<td>-0.052 ± 0.004</td>
<td>0.028 ± 0.010</td>
<td>2.854</td>
</tr>
<tr>
<td><strong>Anteroposterior (BW)</strong></td>
<td>0.001 ± 0.007</td>
<td>-0.115 ± 0.010</td>
<td>-12.08</td>
</tr>
<tr>
<td><strong>Mediolateral angle (degrees)</strong></td>
<td>-8.337 ± 0.628</td>
<td>2.940 ± 1.349</td>
<td>2.180</td>
</tr>
<tr>
<td><strong>Anteroposterior angle (degrees)</strong></td>
<td>0.355 ± 1.029</td>
<td>-16.21 ± 1.495</td>
<td>-10.98</td>
</tr>
</tbody>
</table>

Analyses were based on n = 42 and n = 29 trials averaged across five individuals for the forelimb and hind limb, respectively, due to data that were removed during times of overlap with other body structures. FE = fixed effect.

GRF parameters were evaluated at the timing of peak tensile stress, and then averaged across all trials to produce the mean and s.e.m. for each limb.

Statistical analyses based on the model: lmer(y ~ Limb + (1 | Individual), REML = True). The hind limb was treated as the intercept, by default.

Format of statistical analyses follows that described for Table 4. Timings of the peak tensile stress are reported in Table 6.
Table 6. Timings and magnitudes of peak bone stresses for *A. tigrinum*, with average speed during stance

<table>
<thead>
<tr>
<th></th>
<th>Humerus</th>
<th>Femur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.e.m.</td>
<td>CV</td>
</tr>
<tr>
<td>Peak tensile stress (MPa)</td>
<td>7.006 ± 0.282</td>
<td>0.285</td>
</tr>
<tr>
<td>Peak compressive stress (MPa)</td>
<td>-7.376 ± 0.292</td>
<td>-0.280</td>
</tr>
<tr>
<td>Peak axial stress (MPa)</td>
<td>-0.930 ± 0.063</td>
<td>-0.476</td>
</tr>
<tr>
<td>Peak shear stress (MPa)</td>
<td>-3.261 ± 0.171</td>
<td>-0.371</td>
</tr>
<tr>
<td>Time of peak tensile stress (%)</td>
<td>66.540 ± 1.508</td>
<td>0.160</td>
</tr>
<tr>
<td>Time of peak axial stress (%)</td>
<td>36.540 ± 1.589</td>
<td>0.308</td>
</tr>
<tr>
<td>Time of peak compressive stress (%)</td>
<td>65.640 ± 1.446</td>
<td>0.156</td>
</tr>
<tr>
<td>Time of peak shear stress (%)</td>
<td>24.560 ± 1.484</td>
<td>0.427</td>
</tr>
<tr>
<td>Average speed during stance (cm s⁻¹)</td>
<td>10.347 ± 0.311</td>
<td>0.213</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. (n = 50 and n = 48 trials averaged across five individuals for the humerus and femur, respectively). FE = fixed effect. CV = s.d./mean
Timings of peak stresses are represented as a percentage into the stance phase of the limb cycle.
Peak stress values were determined for each individual trial, and then averaged across all trials to produce the mean and s.e.m.
Statistical analyses based on the model: lmer(y ~ Bone + (1 | Individual), REML = True). The femur was treated as the intercept, by default.
Format of statistical analyses follows that described for Table 4.
Table 7. Regional heterogeneity of hardness values and safety factors across the limb bone mid-shafts of *A. tigrinum*

<table>
<thead>
<tr>
<th></th>
<th>Humerus</th>
<th>Femur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
<td>Dorsal</td>
</tr>
<tr>
<td>Hardness ($H_v$)</td>
<td>36.3 ± 0.9</td>
<td>41.7 ± 1.5</td>
</tr>
<tr>
<td>$H_v$ sample sizes</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Mean yield stress (MPa)$^1$</td>
<td>174.1 ± 3.3</td>
<td>145.2 ± 4.0</td>
</tr>
<tr>
<td>Standard SF</td>
<td>23.6 ± 0.5</td>
<td>20.7 ± 0.6</td>
</tr>
<tr>
<td>CV of SF</td>
<td>0.113</td>
<td>0.188</td>
</tr>
<tr>
<td>Worst case SF</td>
<td>12.3 ± 0.3</td>
<td>8.1 ± 0.3</td>
</tr>
</tbody>
</table>

$^1$For dorsal and posterior regions (under tension), calculated using the equation: $32.571 + 2.702*H_v$ (Wilson et al., 2009). For anterior and ventral regions (under compression), calculated as (tensile yield stress)/0.75.

CV = coefficient of variation. Values represent means ± s.e.m.

Statistical analyses based on the model: lmer(y ~ Bone/AnatomicalLocation + (1 + AnatomicalLocation | Individual), REML = True)

Statistical comparisons can be found in the Supplementary Material – Table S1.