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Evaluation of the functional capabilities of fins and limbs for moving on land: insights into the invasion of land by tetrapods

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EVALUATION OF THE FUNCTIONAL CAPABILITIES OF FINS AND LIMBS
FOR MOVING ON LAND:
INSIGHTS INTO THE INVASION OF LAND BY TETRAPODS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

by
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ABSTRACT

Transitions to novel habitats present different adaptive challenges, producing captivating examples of how functional innovations of the musculoskeletal system influence phenotypic divergence and adaptive radiations. One intriguing example is the transition from aquatic fishes to tetrapods. Recent technological advances and discoveries of critical fossils have catapulted our understanding on how fishes gave rise to terrestrial vertebrates. Considerable attention has been paid to legged locomotion on land, but given that the first tetrapods were aquatic, limbs did not evolve primarily for terrestriality. How, then, is the locomotor function of limbs different from fins? Extant amphibious fishes demonstrate that fins can be used on land, and anatomical analyses of the fish relatives of early stem tetrapods indicate that the appendicular bones of fishes could be quite robust. Consequently, there is a need to evaluate the ability of fins to withstand the physical challenges of terrestrial locomotion in order to shed light on how limbs conferred early stem tetrapods with an upper hand for becoming terrestrial.

In the following papers, I have investigated the biomechanical capabilities of different musculoskeletal designs to understand the evolution of terrestrial locomotion in vertebrates. First, I compared the biomechanics of fins and limbs by measuring ground reaction force (GRF) production of mudskipper fishes (*Periophthalmus barbarus*) crutching and tiger salamanders (*Ambystoma tigrinum*) walking on level ground, two strategies for accomplishing terrestrial locomotion. Yet, tiger salamanders are already terrestrial. In order to

understand how limbs function in a more habitually aquatic tetrapod, I conducted similar GRF analyses on a semi-aquatic newt (*Pleurodeles waltl*). Once tetrapods moved onto land, a major question is whether locomotion was primarily driven by the forelimbs or the hind limbs. Thus, I evaluated the ability of the forelimbs and hind limbs of *A. tigrinum* to withstand stresses during terrestrial locomotion. These data provided an opportunity to study whether the bones of different limbs possess different margins of safety against failure. Lastly, I synthesized how extant taxa can be used to model the biology of extinct taxa, advancing our knowledge about how functional innovation of the appendages contributed to one of the greatest revolutions in vertebrate history.

DEDICATION

This dissertation could not have been accomplished without the love, support and encouragement from my father (Itaru), mother (Emilia), and my sister (Sally). My mother and father had to sacrifice their education in order to support their families, and traveled across two continents before they could attain the American dream. Even when we were merely children, our parents engrained upon my sister and me what a privilege it was to obtain an education, and that we could achieve our greatest aspirations with diligence and perseverance. My sister has never ceased to amaze me with her maturity, grace and good nature, and has been there to lend an open heart and a helping hand at my greatest times of need. Thank you for supporting me in all of my endeavors, for being my source of inspiration and encouragement, but most of all, thank you for empowering me to pursue my career aspirations.

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I owe my deepest gratitude to the people who assisted me during the many vicissitudes that I experienced on my journey to becoming a scientist. While this relatively short section does not do them justice, I highlighted many of the influential people who supported me throughout the years.

As an undergraduate at the University of California, Davis, I worked with some remarkable scientists that shaped the researcher that I am today. Dr. Judy Stamps taught me how to apply an integrative approach for understanding phenotypic variation. Dr. Peter Wainwright introduced me to fish functional morphology and biomechanics, and forever changed how I conducted research. Dr. Roi Holzman challenged me to maintain a competitive edge, and develop projects with broad impacts at the forefront of science.

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CHAPTER ONE

INTRODUCTION

The invasion of land by tetrapods marks one of the most seminal events in vertebrate history, and is a classic example of functional innovation and phenotypic divergence driven by new ecological opportunities (Anderson et al. 2013). The morphologies that vertebrates evolved over millions of years in the aquatic realm had to undergo drastic transformations before they could support life on land. Consequently, becoming terrestrial was a slow process, and may have been serendipitous (Clack 2002). For instance, features that facilitated terrestriality appeared first in the pectoral appendage (Clack 2009), but these were later outpaced by changes in the pelvic appendage (Coates et al. 2002). The combination of traits that ultimately allowed early stem tetrapods to move onto land may, therefore, have arisen through evolutionary “trial-and-error”. Given the range of phenotypes possible, what allowed some taxa to become terrestrial, but not others? How did these morphological changes influence how vertebrate animals moved on land?

One of the most intriguing transformations of the vertebrate musculoskeletal system was the evolution of limbs from fins. Yet, contrary to popular belief, the transition to land was not synchronous with a dichotomous change from finned fishes to tetrapods with digit-bearing limbs. Paleontological

examinations indicate that limbs evolved from fins in the aquatic environment (Eaton 1960; Coates 1996), suggesting that limbs did not evolve for the sole purpose of terrestrial excursions. If both fishes and tetrapods were waiting at the water's edge during the Devonian (~400 MYA), why, then, did tetrapods beat fishes in the conquest of land?

One possible explanation could be that there are functional trade-offs associated with using fins and limbs in different environments. Animals are often presumed to swim underwater and walk on land, but fossil evidence suggests that underwater walking was a likely stage during the water-to-land transition in tetrapod evolution (Gunter 1956; Edwards 1989; Lebedev 1997; Boisvert 2005; Ahlberg and Clack 2006; Shubin et al. 2006; Coates et al. 2008; Clack 2009). The functional role of appendages would, thus, change with a switch from axial locomotion (e.g., swimming) to appendicular-based locomotion (e.g., walking) because appendages are used to contact the substrate and potentially prop up the body, thereby loading weight upon the appendages. The mechanics of ambulatory locomotion, such as walking, may be substantially different between aquatic and terrestrial environments due to the drastic physical differences between water and land, and may impose different effects on fins and limbs. Buoyancy likely reduces the magnitudes of forces imposed on appendages by the substrate (i.e., ground reaction forces or GRFs) when they contact the ground during the propulsive phase of locomotion (Martinez et al. 1998). Greater effects of gravity on land may result in greater GRFs on the appendages and

expose appendicular bones to greater stresses than when underwater (Martinez et al. 1998; Gillis and Blob 2001). The shift from short, blocky limb bones in fishes to cylindrical, beam-like limb bones in tetrapods (Kawano, *unpublished data*) could have conferred the bone strength necessary for tetrapods to support movements on land. Yet, no quantitative studies have addressed such predictions to evaluate the magnitudes of skeletal loading differences in the context of tetrapod evolution. Such examinations could help to explain the extent to which changes in skeletal loading were a factor in the morphological changes observed in the appendicular skeleton across the fish-tetrapod transition.

The ability of fins and limbs of extinct tetrapodomorphs (tetrapods and their tetrapod-like fish relatives) to support terrestrial excursions can be difficult to assess from fossil bones alone, but can be facilitated through experimental analyses on extant taxa. When the taxon of interest (e.g., fossil tetrapodomorphs) is difficult to study, extant taxa sharing certain similarities, such as ecology and morphology, can be used as surrogate models (Krebs 1975; Bolker 2009). These “modern analogs” (*sensu lato* Pierce et al. 2013) to fossil tetrapodomorphs offer the benefit of directly testing form-function relationships (Pierce et al. 2013), and allow one to collect anatomical and behavioral data that are unavailable in the fossil record. Also, extant taxa represent evolutionarily successful examples of adaptations that have been used to invade land, and could have been used by fossil tetrapodomorphs (Gordon 1999). Although modern analogs are not exact substitutions for fossil taxa, they represent general

models in which to investigate the basic principles of the question under investigation, such as the functional capabilities of fins and limbs to support movement on land.

Extant amphibious salamanders and fishes are excellent functional models for evaluating the likely locomotor capabilities of fossil tetrapods and tetrapod-like fishes, respectively, due to ecological, morphological, and physiological similarities (Schultze 1999; Long and Gordon 2002). Salamander morphology has remained fairly conserved for at least 150 million years (Gao and Shubin 2001), making them useful models for basal tetrapods. In particular, extant salamanders may be better suited to model early crown tetrapods (Pierce et al. 2013), whereas vertebrate animals with greater aquatic tendencies may better model early stem tetrapods. Mudskipper fishes have robust pectoral fins with functional analogs to elbows (Harris 1960; Pace and Gibb 2009), and use a form of terrestrial locomotion called “crutching” that may resemble how some early stem tetrapods, like *Ichthyostega*, moved on land (Pierce et al. 2012). Although mudskippers are actinopterygians and, thus, are not members of the evolutionary lineage of sarcopterygians that gave rise to the tetrapods, mudskippers still offer valuable information regarding the use of pectoral appendages for “forelimb-driven” locomotion on land. In fact, a number of significant scientific milestones were achieved by studying taxa that were not regarded as standard model systems (Pringle 1966). As with any model, modern analogs simplify more complex biological systems into more pragmatic units for

analysis, contributing crucial insight through the direct testing of biological phenomena (Krebs 1975; Pierce et al. 2013).

For instance, biomechanical analyses of amphibious fishes and salamanders demonstrated specific parameters that could have contributed towards limited terrestrial capabilities in fossil tetrapodomorph fishes. When animals step down on the ground, they experience an equal but opposite force (ground reaction force or GRF) that must be counteracted by the musculoskeletal system to keep the animal supported and balanced on land (Hutchinson and Gatesy 2006). GRF characteristics can determine the types of stresses applied to the appendicular bones and, thus, suggest the physical demands that they must withstand. Recent work on extant mudskipper fishes and salamanders found that fins supported a different distribution of body weight than limbs (Kawano and Blob 2013). These biomechanical differences coincided with the morphological changes between fins and limbs, potentially contributing to the predominance of limbs among terrestrial invaders and the limitation of fins primarily to the aquatic realm.

Such empirical data from modern analogs can be used to test hypotheses regarding the functional morphology of fossil taxa. Observations that numerous lineages of amphibious fishes have independently invaded land demonstrate that finned fishes do exhibit some capabilities to leave the water (Pace and Gibb 2014), with numerous species using their fins for terrestrial locomotion. Yet, there may be physical limitations of fins that have precluded fishes from

becoming as successful as tetrapods in conquering the terrestrial realm. In the late 1800's, Huxley noted that fins and limbs rotate in different directions from the body and these rotations would have created an unrealistic amount of torsion ('twisting') in the humeri of fishes with tetrapod-like appendages, like the crossopterygian *Ceratodus* (Bowler 2007). The increasing robustness of the pectoral girdle/appendage and associated muscles in limbs likely conferred a greater ability to support the weight of the body on land. Direct biomechanical comparisons of the functional role of fins and limbs during terrestrial locomotion could resolve whether the more robust anatomy of limbs actually did confer greater body support, and in what way(s).

Thus, experimental analyses on modern analogs to fossil tetrapodomorphs provide the opportunity to resolve the functional consequences of the morphological changes observed as vertebrates became increasingly terrestrial. For instance, terrestrial adaptations began in the anterior regions of the body in tetrapodomorphs (Lebedev 1997; Boisvert 2005; Clack 2009), suggesting that the pectoral appendages likely had a greater contribution to the initial capacity for ambulatory locomotion on land than the pelvic appendages. This "front-wheel drive" is suggested to have been a basal stage for terrestrial locomotion in early stem tetrapods (Pierce et al. 2012; Nyakatura et al. 2014), with hind limb-driven locomotion or "rear-wheel drive" predominating on land only later in the fossil record when tetrapods had assumed greater terrestrial capabilities. Why did such a shift occur? Rear-wheel drive may have appeared

early, in sarcopterygian fishes, for underwater walking (King et al. 2011), so why was front-wheel drive an early transitional stage in the evolution of terrestrial locomotion? In what ways does the function of forelimbs and hind limbs differ for terrestrial locomotion?

Due to their postural and morphological similarities, salamanders are often used to model the locomotor capabilities of early tetrapods, yet little focus has been placed on the salamander forelimb (but see Evans 1946). Previous work has been conducted on salamander hind limbs during terrestrial locomotion (kinematics: Ashley-Ross 1994, and muscles: Ashley-Ross 1992; Ashley-Ross and Barker 2002), but forelimb data has tended to only be included in analyses of center-of-mass (i.e., whole-body) mechanics (Reilly et al. 2006). Evaluating the loading mechanics of the salamander forelimb during isolated limb cycles could provide vital information for modeling the likely locomotor capabilities of early tetrapods because forelimbs are the first appendicular system to emerge onto land when animals are transitioning between water and land, and because structural transitions of the forelimb skeleton preceded those in the hind limb (Lebedev 1997; Boisvert 2005; Clack 2009).

Previous work on reptiles has shown that forelimbs and hind limbs share many similarities but exhibit different kinematics (Russell and Bels 2001), and the same may be true for salamanders, especially given the similar size proportions of limbs in salamanders. Such a comparison provides the opportunity to evaluate whether the “mixed-chain” hypothesis (Alexander 1997) applies to the

forelimb and hind limb during terrestrial locomotion in salamanders. When animals move on land, their bones experience forces, or loads, created by the contraction of their muscles, and interactions with the environment (e.g., GRFs). Bones must be strong enough to withstand these loads in order to avoid injury, so they have a built-in safety measure, called a 'safety factor', that allows them to accommodate a greater maximum load than what they normally experience. Yet, bones do not operate in isolation. Using a chain of links as an example, Alexander explained that if a chain was only as strong as its weakest link, the links within that chain should be built with the same safety factor since links with a higher safety factor would not elicit a selective advantage and would be more energetically expensive to produce (Alexander 1997). However, Alexander also predicted that uniform safety factors might not be found within a variety of systems. For example, links within the chain that are composed of weaker materials or have unreliable performance are expected to be stronger to compensate for their suboptimal properties. In addition, if the average safety factor of all limb bones were high, greater variation between safety factors might be expected (Blob et al. 2014; Alexander 1997). Bones demanding higher energetic costs for maintenance (e.g., larger elements) or use (e.g., distal elements that swung further from the body) might also have smaller safety factors. Forelimbs and hind limbs can be considered different "links" within the locomotor system (i.e., the "chain" in this analogy), and salamanders provide an intriguing system in which to test Alexander's hypothesis predicting a "mixed-chain" of

safety factors. Because salamander humeri and femora are proximal limb bones with comparable sizes, they should have similar costs associated with growth, maintenance and movement, predicting similar safety factors. However, hind limb safety factors are high for salamanders (Sheffield and Blob 2011; Blob et al. 2014), suggesting the potential for a “mixed-chain” across their limb elements.

In order to investigate how the functional roles of fins and limbs could have contributed to the evolutionary invasion of land by tetrapods, I performed a series of studies that integrated principles from functional morphology, paleontology, engineering, biomechanics, and computer modeling. Chapter 2 compares how the pectoral fins of mudskipper fish (*Periophthalmus barbarus*) and the forelimbs and hind limbs of tiger salamanders (*Ambystoma tigrinum*) are used to move on land. This work was published in *Integrative and Comparative Biology* in 2013 through an invitation to participate in the “Vertebrate Land Invasions – Past, Present, and Future” symposium that was sponsored by the Society for Integrative and Comparative Biology. Chapter 3 builds upon Chapter 2 by investigating the locomotor role of the forelimbs and hind limbs of the semi-aquatic Iberian ribbed newt (*Pleurodeles waltl*). These data establish a framework in which to evaluate the locomotor function of the appendages at key points along the fish-tetrapod transition: fish fin, semi-aquatic limbs, and terrestrial limbs. Multivariate analyses are provided to identify some of the main factors driving the differences amongst these appendages in “biomechanospace”. Chapter 4 describes the ability of tiger salamander

forelimbs and hind limbs to support body weight while moving on land by quantifying the stresses experienced by the appendicular bones (humerus and femur) during terrestrial locomotion, providing crucial information about the functional differences between the two appendicular systems for moving on land in an animal with a general tetrapod *bauplan*. The last chapter serves as a synthesis of how modern analogs have filled major gaps for understanding the conquest of land by tetrapods, and how data on extant taxa can be applied towards inferring the function of extinct taxa. Insight is provided on how the morphological changes observed across the transformation from aquatic tetrapodomorph fishes (e.g., *Sauripterus* and *Eusthenopteron*) to semi-aquatic transitional fish (e.g., *Tiktaalik*) to terrestrial tetrapods (e.g., *Seymouria* and *Captorhinus*) conferred new functional roles to limbs that allowed tetrapods to embark upon one of the most monumental events in the evolutionary history of vertebrates. Collectively, these studies apply an integrative approach to gain a better understanding of how changes to the musculoskeletal system can lead to functional innovation and the exploitation of novel ecological niches.

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CHAPTER TWO
PROPULSIVE FORCES OF MUDSKIPPER FINS AND SALAMANDER LIMBS
DURING TERRESTRIAL LOCOMOTION:
IMPLICATIONS FOR THE INVASION OF LAND

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ABSTRACT

The invasion of land was a pivotal event in vertebrate evolution that was associated with major appendicular modifications. Although fossils indicate that the evolution of fundamentally limb-like appendages likely occurred in aquatic environments, the functional consequences of using early digitated limbs, rather than fins, for terrestrial propulsion have had little empirical investigation.

Paleontological and experimental analyses both have led to the proposal of an early origin of “hind limb-driven” locomotion among tetrapods or their ancestors. However, the retention of a pectoral appendage that had already developed terrestrial adaptations has been proposed for some taxa, and few data are available from extant functional models that can provide a foundation for evaluating the relative contributions of pectoral and pelvic appendages to terrestrial support among early stem tetrapods. To examine these aspects of vertebrate locomotor evolution during the invasion of land, we measured three-dimensional ground reaction forces (GRFs) produced by isolated pectoral fins of mudskipper fishes (*Periophthalmus barbarus*) during terrestrial crutching, and compared these to isolated walking footfalls by the forelimbs and hind limbs of tiger salamanders (*Ambystoma tigrinum*), a species with subequally-sized limbs that facilitate comparisons to early tetrapods. Pectoral appendages of salamanders and mudskippers exhibited numerous differences in GRFs. Compared to salamander forelimbs, isolated fins of mudskippers bear lower vertical magnitudes of GRFs (as a proportion of body weight), and had GRFs that were oriented more medially. Comparing the salamanders’ forelimbs and hind limbs, although the peak net GRF occurs later in stance for the forelimb, both limbs experience nearly identical mediolateral and vertical components of GRF, suggesting comparable contributions to support. Thus, forelimbs could also have played a significant locomotor role among basal tetrapods that had limbs of subequal size. However, the salamander hind limb and mudskipper pectoral fin

had a greater acceleratory role than did the salamander forelimb. Together, data from these extant taxa help clarify how structural change may have influenced locomotor function through the evolutionary invasion of land by vertebrates.

INTRODUCTION

The invasion of land was a pivotal event in vertebrate evolution. The penetration of terrestrial habitats, beginning with shallow shores and marginal habitats before culminating in subaerial substrates, required major changes in the functional demands faced by fishes and tetrapods due to the dramatic physical differences between aquatic and terrestrial environments (Clack 2002; Coates et al. 2008). One of the functions most dramatically affected by these physical differences is locomotion (Martinez 1996; Gillis and Blob 2001), yet there are only limited data on the specific impacts of these differing physical conditions on locomotor performance, and how such performance may have influenced morphological and ecological transitions in early tetrapods.

Although living tetrapods often exhibit a fundamental shift from axial-based swimming in water to appendage-based stepping on land (e.g., Gleeson 1981; Frolich and Biewener 1992; Russell and Bels 2001; Ashley-Ross and Bechtel 2004), the fossil record suggests that underwater walking was a likely stage during the water-to-land transition (Gunter 1956; Edwards 1989; Lebedev 1997; Boisvert 2005; Shubin et al. 2006; Coates et al. 2008; Clack 2009). Thus, one early stage in the evolutionary changes that facilitated the invasion of land

must have been the assumption of a new functional role for the appendages, involving contact with the substrate for propulsion and support. Yet, how did the two appendicular systems, pectoral and pelvic, contribute to this transition?

The earliest structural changes toward a tetrapod-like morphology appear in the pectoral appendage (Lebedev 1997; Clack 2009), with enlargement of the endoskeletal girdle and implied increased musculature present among taxa such as the fossil elpistostegalid *Panderichthyes*, outside of crown group tetrapods (Coates et al. 2002; Boisvert 2005). By the emergence of tetrapods such as *Acanthostega*, which likely was still aquatic (Coates and Clack 1991; Coates 1996), character changes in the pelvic appendage have outpaced those in the pectoral appendage, with the pelvic larger than the pectoral one (Coates et al. 2002; Coates et al. 2008). The appendages also underwent morphological changes including a reduction in the number of axial segments, evolution of digits and distinct wrists and ankles, and the loss of fin rays (Coates 1996; Coates et al. 2008). Behavioral studies of African lungfish (*Protopterus annectens*) suggested an even earlier phylogenetic origin than *Acanthostega* for “hind limb-driven” locomotion (King et al. 2011). When moving along a substrate underwater, *P. annectens* uses gaits that resemble bipedal walking, propelling themselves strictly with the pelvic appendages with the anterior body elevated from the buoyant lungs. However, recent studies modeling the range of motion for each limb joint in the early tetrapod *Ichthyostega* have suggested that the hind limbs could not be used for propulsive substrate contact, and that this taxon

would have propelled itself with simultaneous “crutching” movements of the forelimbs that resembled the patterns in modern seals and mudskippers (Pierce et al. 2012). Though some aspects of hind limb morphology contributing to such a locomotor style might be specialized features of *Ichthyostega*, Pierce et al. (2012) propose that similarities to features in other stem tetrapods, such as *Acanthostega* and *Hynerpeton*, suggest that the range of mobility found in *Ichthyostega* could more broadly reflect appendicular function in ancestral stem tetrapods.

Although knowledge of the fossil taxa spanning the fish-to-tetrapod and water-to-land transitions has grown considerably through recent fossil discoveries and analyses (e.g., Boisvert 2005; Daeschler et al. 2006; Shubin et al. 2006; Boisvert et al. 2008; Pierce et al. 2012), data from extant taxa serving as functional models that provide a foundation for evaluating the relative contributions of pectoral and pelvic appendages to terrestrial support among early tetrapods are much more limited (Fricke and Hissmann 1991; Pridmore 1994; Ashley-Ross and Bechtel 2004; Ijspeert et al. 2007; Macesic and Kajiura 2010; King et al. 2011). Most locomotor studies of terrestrial lineages closest in body plan to early tetrapods, such as amphibians and reptiles, have focused on the hind limb, often with the view that the hind limb is the primary propulsor (Ashley-Ross 1994; Reilly and Delancey 1997; Irschick and Jayne 1999; Blob and Biewener 2001; Gillis and Blob 2001; Sheffield and Blob 2011). Much less is known about forelimb function in such taxa, and empirical data that compare

the locomotor roles of forelimbs and hind limbs within the same animal are uncommon for such species.

One study of a taxon using sprawling posture like that of early tetrapods that did compare the locomotor roles of forelimbs and hind limbs was conducted on the gecko *Hemidactylus garnotti*, a lizard with forelimbs and hind limbs subequal in size, in which ground reaction forces (GRFs) were measured from footfalls of individual feet during trotting over level ground (Chen et al. 2006). In contrast to trotting quadrupeds with upright limb posture like mammals, in which each footfall typically shows deceleration followed by acceleration (Lee et al. 1999; Witte et al. 2002), the forelimbs and hind limbs of *H. garnotti* were found to have different roles. Although vertical forces were comparable between forelimbs and hind limbs, medially directed forces were moderately larger for the hind limbs; moreover, the forelimbs produced only deceleratory forces, whereas the hind limbs produced small deceleratory forces, followed by larger acceleratory forces late in the step (Chen et al. 2006). A comparative study of seven additional lizard species found similar patterns of forelimb deceleration and hind limb acceleration, but also found that as the hind limbs increased in size relative to the forelimbs, medial forces became correspondingly larger for the hind limb relative to the forelimb (McElroy 2009). Data from alligators, in which the hind limbs are considerably larger than the forelimbs, are consistent with these patterns, showing moderately larger medial forces and slight deceleration followed by primarily acceleration for the hind limb; however, the forelimb also

showed slight acceleration at the end of the step after a primarily deceleratory force (Willey et al. 2004).

Despite questions about the ancestry of the use of posterior appendages through the invasion of land, consensus has emerged that limbs evolved from limb-like fins among aquatic animals (Shubin et al. 2006; Boisvert et al. 2008; Coates et al. 2008; Clack 2009). Given that the evolution of digits and the loss of fin rays occurred underwater, what biomechanical factors may have facilitated the use of limbs with digits, or limited the use of fins, during the evolutionary invasion of land? Bowler (2007) suggested potential differences in locomotor performance between fins and limbs, because the fins of the ancestors of stem tetrapods were likely adequate for benthic, underwater locomotion, but a stronger pectoral appendage would have been required for sustained forward propulsion on land. Some structural reinforcement of the pectoral appendage can be observed among amphibious fishes that use their fins to power terrestrial movement. For example, morphological specializations among *Periophthalmus* mudskippers (members of the actinopterygian lineage), such as greater ossification and stiffening of the fin rays (Harris 1960), likely contribute to the capacity of these fish to use simultaneous “crutching” of the pectoral fins to move over terrestrial surfaces (Pace and Gibb 2009). Among basal tetrapodomorphs, the evolution of digits and the loss of fin rays, in addition to enlargement of the endoskeletal bones of the pectoral girdle, probably made the pectoral appendage more robust and efficient at supporting the body off of the ground (Bowler 2007).

However, direct comparisons of appendicular mechanics that could evaluate the relative functional capabilities fish fins (with rays) and tetrapod limbs (with digits) during terrestrial locomotion have not been performed.

Extant amphibious fishes and amphibians could provide informative models for understanding the functional challenges faced by vertebrates through the evolutionary transition from water to land (Graham and Lee 2004; Ashley-Ross et al. 2004). To improve the foundation for understanding the changing roles of pectoral and pelvic appendages, and the contrasting capabilities of fins and limbs, during the evolutionary invasion of land by vertebrates, we compared measurements of three-dimensional GRFs produced during terrestrial locomotion by the pectoral fins of a representative amphibious fish, the African mudskipper (*Periophthalmus barbarus*), and a representative amphibious tetrapod, the tiger salamander (*Ambystoma tigrinum*). There are some limitations to the use of both of these taxa as functional models for the stem tetrapods that spanned the water-to-land transition. For example, because mudskippers are actinopterygians rather than sarcopterygians, they are not on the same evolutionary line that led to tetrapods and do not have homologous limb elements. However, both taxa also have advantages that make them among the best extant models available (Long and Gordon 2004). First, mudskippers and salamanders readily use their appendages for locomotion over ground. Second, the forelimbs and hind limbs of tiger salamanders show limited disparity in size, resembling the limbs of many extinct Paleozoic amphibians. This provides an appropriate comparison for fossil

taxa spanning this evolutionary transition, but in a model that, as an amphibian, might be physiologically more similar to early tetrapods than alternative taxa such as lizards. Third, the projection of the mudskipper's pectoral girdle beyond the body wall provides a functional analogue to the tetrapod elbow (Harris 1960; Pace and Gibb 2009), and recent proposals of crutching as a mode of terrestrial locomotion among some early tetrapods (Clack 1997; Ahlberg et al. 2005; Pierce et al. 2012) make comparisons of force production between stepping and crutching relevant for understanding early stages of terrestrial locomotion. Other amphibious fishes are less appropriate models for appendicular GRF production either because they do not use the pectoral appendages for moving on land (e.g., *Anguilla* eel: Gillis and Blob 2001; climbing perch: Sayer 2005; ropefish: Pace and Gibb 2011), or because they primarily use movements of the axial system to generate thrust while the pectoral fins have less of a locomotor role (e.g., *Claris* catfish: Pace 2009; blennies: Hsieh 2010; stichaeids: Kawano *pers. obs.*).

Our paper thus has the following specific objectives. First, we compare GRFs from the forelimbs and hind limbs of salamanders during terrestrial locomotion to evaluate how their roles in force production might differ in a quadrupedal amphibian. Second, we compare GRFs from salamanders' limbs to data from mudskippers' pectoral fins during terrestrial locomotion, to evaluate potential differences in the functional roles and capacities of fins versus limbs on land. Finally, we consider these data in the evolutionary context of the water-to-

land transition in tetrapods. Our data show a substantial role of the forelimbs in supporting the body of amphibians on land, although they contribute to propulsion differently than do the hind limbs. In addition, our data provide evidence for a significant evolutionary change in GRF orientation between fins and limbs that might contribute insight into the evolutionary success of limbs as propulsive structures on land.

MATERIALS AND METHODS

Animals

Tiger salamanders, *Ambystoma tigrinum* Green 1825, and African mudskippers, *Periophthalmus barbarus* (Linnaeus 1766), were chosen as model taxa for our analyses because they were the largest available species of salamander and fish that regularly use their appendages to move over land. Salamanders were purchased from Charles D. Sullivan Co. (Nashville, TN, USA) and Underground Reptiles (Deerfield Beach, FL, USA), and mudskippers from Fintastic (Charlotte, NC, USA).

Experimental trials were conducted on five adult salamanders (body mass: 61.72 ± 0.07 g; snout-vent length: 0.100 ± 0.001 m; total length: 0.187 ± 0.005 m), and five adult mudskippers (body mass: 25.10 ± 0.53 g, total length: 0.137 ± 0.001 m). All values represent means ± 1 S.E. Animals were housed in individual enclosures, kept on a 12h:12h light:dark cycle, and maintained in

accordance with procedures approved by the Clemson University IACUC (AUP 2009-071 and AUP2010-066).

Collection of data on 3-D ground reaction force (GRF)

Data for GRFs were obtained from isolated ground contacts of appendages from the right side of the body, using a custom-built multi-axis force platform (K&N Scientific; Guilford, VT, USA) connected to bridge amplifiers. Forces were collected at 5000 Hz using a custom LabVIEW (v.6.1; National Instruments, Austin, TX, USA) routine, with amplifier gains adjusted appropriately for the small body masses of the animals so as to maximize the sensitivity of GRF resolution. Force-plate calibrations were performed daily, and the natural frequency of the plate was 190 Hz in all three directions (vertical, anteroposterior, and mediolateral), sufficiently greater than the step frequencies of our animals, thereby avoiding confounding GRF signals. The force platform was inserted into a wooden trackway with a rubberized surface, providing a flush locomotor path with a 4 x 9 cm plate area for isolated foot or fin contacts. Animals were encouraged to traverse the plate by gentle tapping and providing a dark hiding location across the plate from their starting location. Animals were allowed to rest in water treated with water conditioner for several minutes between trials in order to avoid desiccation, and were not tested for more than 30 min per day (with at least one day of rest between testing sessions). Video was collected simultaneously in dorsal and lateral views (Fig. 2.1) using two digitally

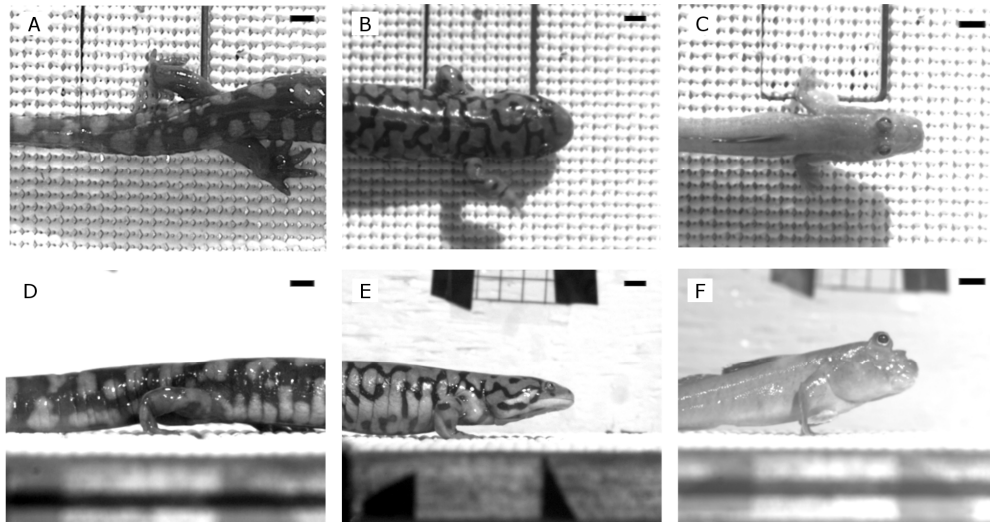


Fig. 2.1. Dorsal (A–C) and lateral (D–F) views from high-speed videos of salamanders' hind limbs (A, D) and forelimbs (B, E) and mudskippers' pectoral fins (C, F) at the time of peak net GRF for each of the appendages. Minor adjustments of contrast and sharpness were made to enhance clarity of the image for reproduction. Black lines in upper right corners represent 1-cm scale bars.

synchronized, high-speed (100 Hz) cameras (Phantom v.4.1, Vision Research Inc.; Wayne, NJ, USA) to evaluate aspects of the appendage cycle, such as durations of stance (propulsive phase) and swing (recovery phase). Video data were synchronized with corresponding data on force by coordinating the onset of an LED light on the video with a 1.5 V pulse on the force traces. Details on the experimental set-up and equipment are described in Sheffield and Blob (2011) and Butcher and Blob (2008).

All traces of force (analyzed only during the propulsive stance phase) were processed and filtered in R (v. 2.15.2; Vienna, Austria). Magnitudes of force were standardized to units of body weight (BW) to facilitate comparisons across individuals of different sizes. Relative magnitudes of the vertical, anteroposterior, and mediolateral components of force were used to calculate the magnitude and

orientation of the net GRF vector. Angular orientations were analyzed with respect to vertical (0 degrees): positive values corresponded to the anterior or lateral directions, whereas negative values corresponded to posterior or medial directions. Prior to filtering, the beginning and end of raw force data were padded to avoid edge effects (Smith 1989). A custom second-order, zero-lag, low-pass Butterworth filter was applied to all raw force using the *signal* package in R (available at <http://www.r-project.org>). Frequency values were normalized to Nyquist frequency to avoid aliasing (Smith 1997). Data filtered during stance were then interpolated to 101 points using a cubic spline to represent 1% increments, from 0% to 100%, of the stance phase.

Several criteria were used to determine whether a trial was valid for inclusion in our analyses. First, the entire right foot/fin was required to contact the force plate. If the pelvic appendage overlapped the pectoral appendage during its contact with the ground (i.e., stance), then those frames of overlap were not included in analyses for either limb. Animals also must have completed a full appendage cycle in a straight line (i.e., no turning). Trials were not used if the peak net GRF was found to occur at 0% or 100% of stance, or if it occurred during a time of overlap with another body part. Although steady speed locomotion can be rare among sprawling taxa (e.g., Farley and Ko 1997), effort was made to select trials with locomotor cycles before and after the cycle of interest that were comparable in speed, with preliminary data for speeds evaluated for each trial by digitizing the movement of a point near the center of

mass of the animal. Linear mixed-effects models fit by restricted maximum likelihood (REML) with individual as a random effect were conducted using the *lme4* package in R (see “Analyzed variables and statistical comparisons” for details). Speeds of trials for the salamander forelimb (9.9 ± 0.3 cm/s) and hind limb (10.4 ± 0.5 cm/s) were not significantly different ($p = 0.811$). Speeds of the trials for the pectoral fin of the mudskipper (7.6 ± 0.3 cm/s) also did not differ from those for the forelimb ($p = 0.391$) and hind limb ($p = 0.444$). All trials represented typical behaviors of the animals.

Analyzed variables and statistical comparisons

Pair-wise comparisons of force between forelimbs and hind limbs, and between fins and limbs, were conducted in R and Microsoft Excel. These comparisons were approached from two perspectives. First, pair-wise linear mixed-effects models fit by REML with appendage type (forelimb, hind limb, or pectoral fin) as a fixed effect, and individual as a random effect (*lme4* package in R), were used to compare response variables. *P*-values were generated using Markov Chain Monte Carlo methods using 10,000 iterations, and were adjusted through sequential Bonferroni correction (Holm 1979) with the *languageR* package. These models were used to compare values of several variables at the time of peak net GRF, providing information about how forces were applied when the weight supported by the appendage was the greatest (Sheffield and Blob 2011). These variables included the timing of the peak net GRF, magnitudes of the

components of GRF, and angles of GRF orientation. Second, vector analysis (Hankison et al. 2006; Cullen et al. 2013; Rivera et al. 2013) was used to qualitatively assess the overall similarity of GRF patterns between pairs of appendicular systems. For each trace of force values through stance, 21 mean values of the variable (calculated for each 5% increment through stance, from 0% to 100%) were used to generate vectors with 21 dimensions. The angle between pairs of these vectors could then be calculated using standard equations (Hamilton 1989). Angles near 0° indicate nearly identical vectors (i.e., two nearly identical force profiles) whereas angles near 90° indicate vectors with perpendicular trajectories, reflecting strong differences between force profiles. In addition to these comparisons of forces, duty factors (i.e., the proportion of an appendicular cycle spent in contact with the ground) were also compared between systems using linear mixed-effects models, as described above. These were evaluated from the videos of each trial, and were viewed as a possible factor contributing to differences in magnitudes of GRFs between systems (e.g., higher duty factors corresponding to lower peak forces) (Biewener 2003).

RESULTS

Comparison of GRFs between salamander forelimbs and hind limbs

Comparisons of GRFs between salamander forelimbs and hind limbs showed several similarities. For both appendicular systems, net GRF magnitudes were slightly less than 0.5 BW with similar magnitudes of the vertical and mediolateral

components when evaluated at peak net GRF (Table 2.1; Fig. 2.2). The GRF also showed a similar medial orientation between both limbs ($p = 0.679$), inclined 8.7° for the forelimb and 11.0° for the hind limb at peak GRF (Table 2.1). Frequency of the locomotor cycle did not differ significantly between the forelimb and hind limb ($p = 0.641$), at 1.45 ± 0.03 and 1.42 ± 0.05 Hz, respectively. Swing duration (FL: 0.19 ± 0.01 s; HL: 0.16 ± 0.02 s) and total appendage cycle duration (FL: 0.71 ± 0.02 s; HL: 0.76 ± 0.03 s) also did not differ (swing duration: $p = 0.424$; cycle duration: $p = 0.544$).

However, salamander forelimbs and hind limbs also showed several significant differences in the values of GRF parameters at the time of peak net GRF. Prominent among these was the time of peak GRF itself, which occurred approximately one-third of the way through the step for the hind limb, but nearly two-thirds of the way through the step for the forelimb (Table 2.1; Fig. 2.2). Also, at the time of peak net GRF, the anteroposterior component was large and positive for the hind limb, but small and negative for the forelimb (Table 2.1, Fig. 2.2). These values corresponded to a substantial anterior (acceleratory) inclination of over 20° for the hind limb, but a slight posterior (deceleratory) inclination averaging just over -3° for the forelimb (Table 2.1). Although duty factor was significantly larger for the hind limb than the forelimb ($p < 0.001$), for both limbs it was very high with only a 6% difference between them (0.80 ± 0.01 for the hind limb and 0.74 ± 0.01 for the forelimb).

Table 2.1. Comparison of mean ground reaction force (GRF) parameters between the forelimb and hind limb of *A. tigrinum* and pectoral fin of *P. barbarus* at the time of peak net GRF

	Hind limb (HL)	Forelimb (FL)	Pectoral fin (PF)	HL vs. FL <i>p</i>-value[†]	FL vs. PF <i>p</i>-value[†]
Time of peak net GRF (%)	32.80 ± 1.60	61.08 ± 1.01	57.16 ± 1.84	< 0.001*	0.297
Net GRF (BW)	0.47 ± 0.01	0.46 ± 0.01	0.42 ± 0.01	0.616	0.118
Vertical GRF (BW)	0.43 ± 0.02	0.45 ± 0.01	0.39 ± 0.01	0.679	0.014*
Mediolateral GRF (BW)	-0.07 ± 0.01	-0.07 ± 0.004	-0.12 ± 0.01	0.679	0.011*
Anteroposterior GRF (BW)	0.15 ± 0.01	-0.03 ± 0.01	0.05 ± 0.01	< 0.001*	< 0.001*
Mediolateral angle (deg)	-11.04 ± 1.73	-8.67 ± 0.53	-17.14 ± 0.90	0.679	0.001*
Anteroposterior angle (deg)	21.69 ± 1.98	-3.21 ± 0.10	7.65 ± 0.83	< 0.001*	0.002*

Values are means ± SE (n=50 steps across five individuals for each group); BW, body weights; **p* < 0.05.

For mediolateral GRF and angle, negative values indicate a medial direction; for anteroposterior GRF and angle, negative values indicate a posterior (deceleratory) direction, whereas positive values indicate an anterior (acceleratory) direction.

[†]*p*-values were generated using Markov Chain Monte Carlo methods (10,000 iterations) and adjusted using sequential Bonferroni corrections

Comparison of GRFs between salamander forelimbs and mudskipper pectoral fins

Some similarities in GRF were also identified between the salamander forelimb and the mudskipper pectoral fin (Table 2.1, Fig. 2.2). The timing of peak net GRF did not differ significantly ($p = 0.297$), occurring at approximately 57% and 60% into stance phase for the pectoral fin and forelimb, respectively. The overall magnitude of the GRF at these points was similar between these pectoral appendages ($p = 0.118$), with values just under 0.5 BW (Table 2.1, Fig. 2.2). In addition, the time spent during the swing phase was not significantly different ($p = 0.706$), at 0.19 ± 0.01 s (forelimb) and 0.20 ± 0.01 s (pectoral fin). Stance duration (FL: 0.53 ± 0.02 s; PF: 0.39 ± 0.01 s; $p = 0.358$), total cycle duration (FL: 0.71 ± 0.02 s; PF: 0.59 ± 0.02 s; $p = 0.422$), duty factor (FL: 0.74 ± 0.01 ; PF: 0.066 ± 0.01 ; $p = 0.303$), and appendage frequency (FL: 1.45 ± 0.03 Hz; PF: 1.78 ± 0.06 Hz; $p = 0.400$) were also not different.

However, salamanders' forelimbs and mudskippers' pectoral fins also showed a number of significant differences in GRF parameters. Differences in all three components of the GRF were observed (Table 2.1). At the time of peak net GRF, the vertical component was greater for the forelimb, but the medial component was greater for the pectoral fin (Table 2.1, Fig. 2.2). As a result, the medial angle of inclination of the GRF for the pectoral fin ($-17.1^\circ \pm 0.9$) was almost twice as large as that for the forelimb ($-8.7^\circ \pm 0.5$). In a further contrast between these appendages, mudskippers' pectoral fins showed a slight anterior

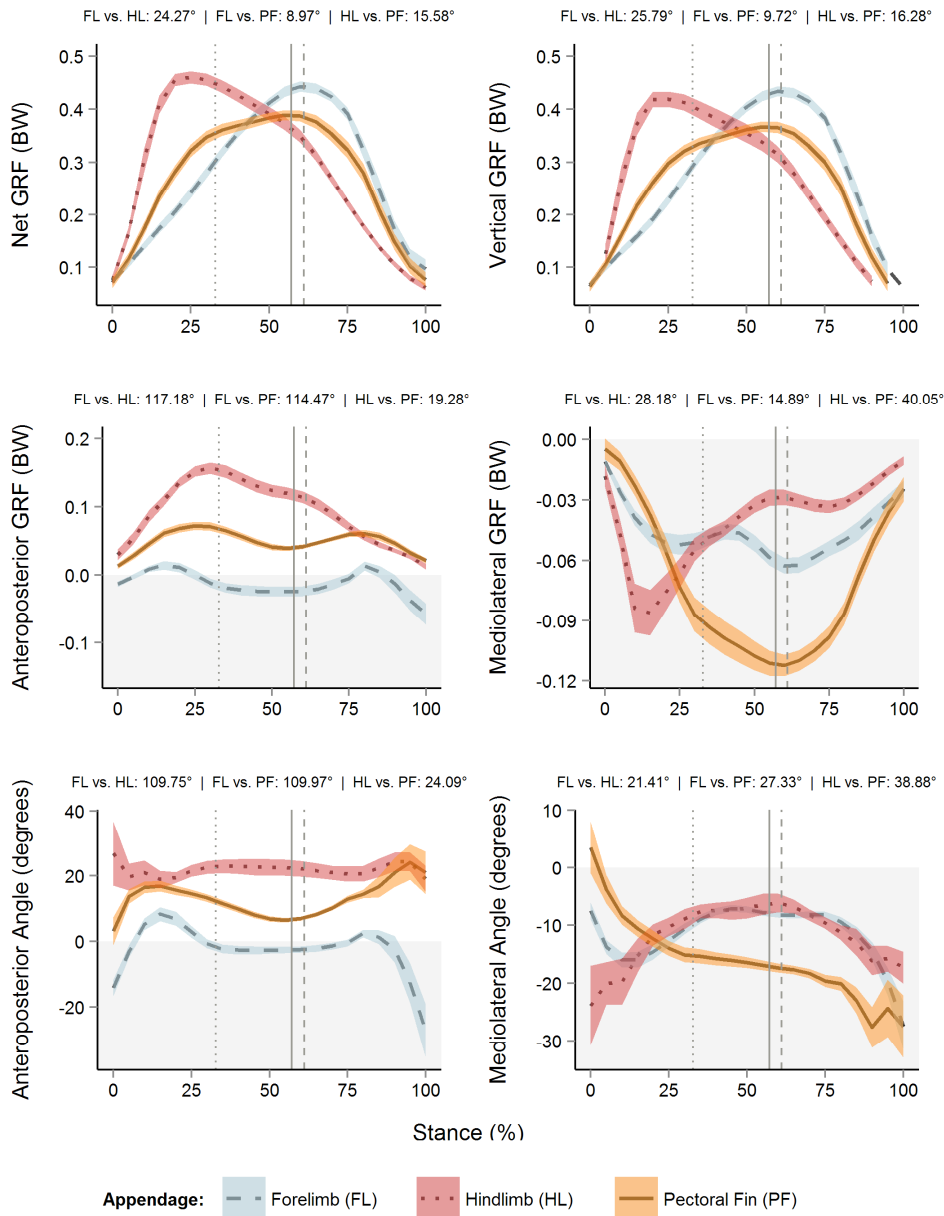


Fig. 2.2. Dynamics of GRF parameters during stance. Lines represent means from pooled trials for each appendage ($N = 50$ averaged across five individuals for each appendage), and shading surrounding each line represents its standard error. Salamanders' forelimb (FL) traces are represented by light blue dashed lines, and hind limb traces (HL) by dark red dotted lines; mudskippers' pectoral fin traces (PF) are in orange solid lines. The gray background in the bottom four plots represents negative values (e.g., medial and posterior in the mediolateral and anteroposterior plots). Vertical lines are coded according to appendage type, identifying the timing of the peak net GRF for each appendicular system. Divergence angles between pairs of appendicular systems are reported above each plot; values close to 0 degrees indicate similarity between pairs of plots whereas values close to 90 degrees indicate strong differences. Darker areas of shading for force traces indicate areas of overlap between standard errors of traces.

(acceleratory) orientation of the GRF, rather than the slight posterior (deceleratory) orientation found in salamanders' forelimbs (Table 2.1, Fig. 2.2).

Comparisons of GRF patterns throughout the duration of stance

Based on comparisons at peak net GRF, salamanders' forelimbs appeared to demonstrate more similarities to salamanders' hind limbs than to mudskippers' pectoral fins. However, comparisons of overall force profiles throughout stance for these appendages complicate this perspective (Fig. 2.2). Vector analyses showed that overall profiles for the medial inclination of the GRF were still most similar (i.e., had the smallest divergence angle) between salamanders' forelimbs and hind limbs. However, the net GRF and the vertical component of the GRF were most similar between the salamanders' forelimbs and mudskippers' pectoral fins, with divergence angles under 10° versus approximately 25° between salamanders' forelimbs and hind limbs. Moreover, with regard to anteroposterior forces and angles, overall profiles were more similar between the mudskippers' pectoral fins and the salamanders' hind limbs, with divergence angles under 25°, than either was to the salamanders' forelimbs, which showed divergence angles of over 100° compared to the other two appendicular systems.

DISCUSSION

The physical properties of the terrestrial environment are drastically different from those of the aquatic realm, in which vertebrates originated and lived for millions

of years. To facilitate the penetration of terrestrial habitats, a wide range of morphological, physiological, and life-history adaptations were ultimately required (e.g., Anderson et al. 2013; Gibb et al. 2013; Jew et al. 2013; Martin et al. 2013; Pierce et al. 2013; Van Wassenbergh and Michel 2013). Among the suites of features that experienced such changes were the appendages; these anatomical structures encountered new demands for supporting body weight to allow locomotion on land. How did the functional differences between fins versus limbs with digits influence the conquest of land by tetrapod vertebrates? To address this broad question we focused on two more specific questions. First, what were the likely contributions of the front and rear appendages to locomotion in early tetrapods? Second, how do the function of fins and limbs differ for locomotion on land? The present study helps to answer these questions using GRFs collected from the forelimbs and hind limbs of salamanders and the pectoral fins of mudskippers, providing a framework for comparing how these structures contribute to locomotion on land.

Functional roles of fore and hind appendages across the fin-to-limb transition

Salamanders present a useful model for gaining insight into the potential capacities for terrestrial locomotion by early tetrapods for several reasons, including their use of a sprawled limb posture with forelimbs and hind limbs of similar size. Our data on GRF patterns from salamanders' hind limbs are largely

concordant with those reported in a previous study (Sheffield and Blob 2011), indicating net magnitudes of GRF just under 0.5 BW with medial inclinations of approximately 10°, and a strong anteriorly directed component. Our new data show how the locomotor role for the forelimb follows these general trends.

Forelimb function shows a number of similarities to hind limb function in salamanders, including having similar total durations and frequencies of limb cycles, similar magnitudes of GRF (e.g., vertical, mediolateral, and net), and similar medial inclinations of GRF (Fig. 2.2, Table 2.1). These results indicate that the forelimbs and hind limbs of salamanders have a similar weight-bearing capacity, much like the gecko *H. garnotti*, which also uses a sprawling posture with similarly sized limbs (Chen et al. 2006). However, the forelimb differed markedly from the hind limb in its anteroposterior GRF, with the hind limb exhibiting a strong acceleratory component at peak net GRF, but the forelimb showing a small deceleratory component. It is possible that drag produced by the tail contributes additional deceleration, which together with the forelimbs would balance the acceleration generated by the hind limbs. In broader comparisons, however, this pattern of deceleration of the forelimb and acceleration of the hind limb also matches that observed in geckos (Chen et al. 2006) and alligators (Willey et al. 2004), suggesting this may be a general pattern for sprawling quadrupeds, with an ancestry deep in the use of stepping locomotion.

These results also call attention to distinct aspects of what has been categorized as “hind limb-driven” locomotion: (1) weight support and (2) the provision of acceleration versus deceleration during an appendage’s contact with the ground. As might be expected, similarly sized limbs bear similar responsibilities for weight support. Thus, even if the hind limbs provided the primary acceleration for early tetrapods, the forelimbs still would have been expected to bear a major responsibility for support of weight, based on the size of these structures (e.g., Coates 1996). Early experiments on salamanders by Evans (1946) suggested that forelimbs played major roles in support of body weight and in forward propulsion. For instance, vertically suspended salamanders could pull themselves back up from the edge of a shelf using only their forelimbs (Evans 1946). However, the extent to which the hind limbs were the primary source of acceleration in a taxon might depend on the size of its tail. In geckos, with rather short tails (~40% snout-vent length based on measurements of published figures) for which dragging was not documented, forelimb GRFs were deceleratory for the entire step (Chen et al. 2006). In contrast, in salamanders with larger tails (87% snout-vent length) that dragged on the ground (dragging of the tail is visible in Fig. 2.1D), forelimb GRFs were initially acceleratory at the beginning of the step (Fig. 2.2), and became only slightly deceleratory by peak GRF (Table 2.1). Such a model may be more appropriate than geckos for comparison with early tetrapods with heavy tails (Coates 1996), and suggests that with a particularly massive tail the forelimb may

have had an even more substantial role in providing acceleration. For instance, *Alligator mississippiensis* has a relatively large tail that accounts for about 8% of its total body weight, and although the forelimb has a net deceleratory role, it plays a slight acceleratory role later in stance when the acceleratory role of the hind limb has decreased (Willey et al. 2004). A similar late acceleratory peak for forelimbs can be seen in our data on salamanders after hind limb acceleration declines sharply near the end of the step (Fig. 2.2). Empirical data on tail dragging are currently unavailable for mudskippers, but Harris (1960) estimated that the tail supported about 10% of the body weight of the mudskipper, which is comparable to values for *A. mississippiensis* (Willey et al. 2004). Thus, some acceleration contributed by the pectoral fins of mudskippers might serve to offset the frictional forces produced by tail drag in addition to contributing towards forward propulsion.

Viewing the GRFs of mudskippers' pectoral fins in this context, a striking point of comparison is that the pectoral fins show an anterior component of GRF that was acceleratory throughout the entire duration of stance (Fig. 2.2). In this way, the role of these fins appears to more closely resemble that of salamanders' hind limbs than of salamanders' forelimbs, a conclusion further suggested by our vector analysis that showed the smallest divergence angle between force trace of the hind limb and the pectoral fin (Fig. 2.2). This comparison underscores the dramatic change in functional role between pectoral appendages that drag the body via crutching versus those that contribute to propulsion via stepping.

Body support on land: consequences of using fins versus limbs

In addition to differing in anteroposterior components of GRF, mudskippers' pectoral fins also differed from both fore and hind appendages of salamanders in vertical and medial components of GRF (Table 2.1). With lower vertical but higher medial forces, mudskippers' pectoral fins experienced a much more medially inclined GRF at peak force (-17.1°) than either the forelimb (-8.7°) or hind limb (-11.0°). Although differences in speed can influence the magnitudes of the components of the GRF (McLaughlin et al. 1996), such an explanation does not seem likely to explain the higher medial force of mudskippers (Table 2.1, Fig. 2.2), given the similar speeds between mudskippers and salamanders (see Materials and Methods). The presence of such a difference in orientation of the GRF across these taxa is striking, because comparisons of GRFs across a broad range of species (amphibians to mammals) and limb postures (sprawling to parasagittal), including turtles (Jayes and Alexander 1980; Butcher and Blob 2008), iguanian (Blob and Biewener 2001) and scleroglossan (Sheffield et al. 2011) lizards, crocodylians (Blob and Biewener 2001; Willey et al. 2004), and a variety of mammals (Biewener 1983; Biewener et al. 1983; Gosnell et al. 2011) have all found remarkably consistent medial inclinations of the GRF, typically about 10° or less. *Hemidactylus* geckos represent an exception to this general pattern, with medial inclination averaging just over 30° (Chen et al. 2006). This difference may be related to locomotor speed, as GRFs were measured in geckos running at an average of 7.8 SVL/s (Chen et al. 2006), but speeds for

other sprawling taxa were typically 1 BL/s or less (Willey et al. 2004; Butcher and Blob 2008; this study). However, iguanas from which GRFs were measured also ran at speeds approaching 8 SVL/s, and still showed medial GRF inclinations of only 8° at the time of peak bone stress (Blob and Biewener 1999; 2001). It is possible that some differences in the orientation of the GRF in mudskippers versus most other sprawling and parasagittal taxa are inherent to their different modes of locomotion (i.e., crutching versus stepping). However, it is also possible that despite the wide range of variation in the shape and proportions of limbs, and in posture among tetrapods, it is the fin-to-limb transition that produces some of the most dramatic consequences for orientation of GRF during terrestrial locomotion (Fig 2.3). This change in orientation might be related to the presence of the elbow joint in limbs, which would cause the distal segment of the limb to be directed more vertically compared to the pectoral fin of the mudskipper. As a result, the mudskipper could provide a better functional model for appendicular function in stem tetrapods, such as elpistostegalids, than limbed tetrapods with digits. The posture of the pectoral appendage reconstructed for the elpistostegalid *Tiktaalik*, in which the entire appendage is held at an angle from the body axis (Shubin et al. 2006), strongly resembles that of the mudskipper pectoral fin, potentially correlating with similarities in force production as well.

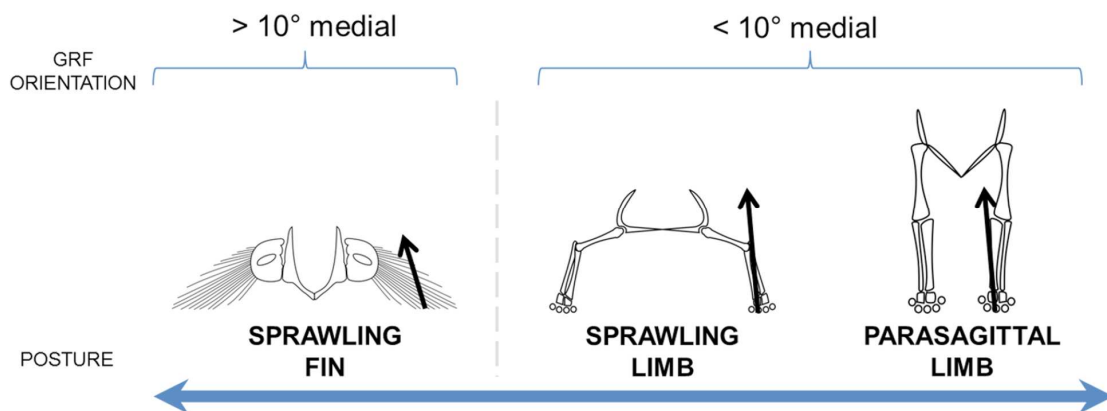


Fig. 2.3. Data on mudskippers' GRF from this study provide insight into the evolution of the orientation of GRF in vertebrates (indicated by black arrows). Although tetrapods exhibit a wide diversity of postures of the limb and foot, the medial inclination of the GRF is relatively similar across taxa at about $\sim 10^\circ$ or less from vertical. The mudskipper has a sprawling fin posture, and has a GRF oriented more medially than all tetrapods. Inclusion of the mudskippers' GRF data demonstrates how the fin-to-limb transition may have marked a major change in the orientation of the GRF, which can impact the weight-bearing capacities of the appendicular system. Images of the mudskipper from Harris (1960) were used as a guide for illustrating this figure.

What functional consequences might such large medial inclinations in GRF have for the use of fins as locomotor structures on land? One potential impact could be on how the skeletal structures of the appendages are loaded. With a nearly vertical GRF at its peak net magnitude, both sprawling (Blob and Biewener 2001; Sheffield and Blob 2011) and more upright tetrapods (Biewener 1989; Biewener 1990) are able to minimize moments of the GRF at the elbow and knee joints, reducing the muscular forces required to maintain joint equilibrium and, thereby, limiting exposure of the limb to bending stresses. Although mudskipper fins do not have a joint homologous to the elbow, the joint between the radials and the fin rays serves a functional analogous role. In this context, the greater medial inclination experienced by fins moving over land

could increase joint moments of the GRF and potentially elevate bending. In addition, such medial inclination could also increase the distance of the GRF vector from the long axis of the radials, increasing its moment arm for axial rotation and potentially elevating the importance of torsion as a loading regime. Consistent with this possibility, in the late 1800's, Huxley wrote that fins and limbs rotated in different directions from the body and that these rotations would have created an unrealistic amount of torsion in the humeri of fishes with tetrapod-like appendages (Bowler 2007). Because bone performs poorly both in bending and torsion compared to axial compression (Wainwright et al. 1976), the orientation of loads placed on fins could require substantial structural reinforcement to avoid an excessive risk of failure. Measurement of stresses and safety factors of fins during terrestrial locomotion could give insight into this question, and could ultimately provide a basis for modeling the stresses experienced by the appendages of early tetrapods (e.g., Blob 2001), using a variety of models of their locomotor patterns (e.g., Pierce et al. 2012). Such models could, in turn, provide insight into the transformation of skeletal morphology between aquatic fins and terrestrial limbs, particularly between the robust morphology of appendicular elements exhibited by early tetrapodomorphs taxa to the long, tubular bones found in early tetrapods that were more terrestrial.

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CHAPTER THREE

PROPULSIVE FORCES OF THE SEMI-AQUATIC NEWT, *PLEURODELES*
WALTL:

INSIGHTS INTO THE FUNCTIONAL EVOLUTION OF TERRESTRIAL
LOCOMOTION IN EARLY STEM TETRAPODS

ABSTRACT

Modern analogs to early stem tetrapods have been used to infer the biology of extinct taxa, providing insight into the evolutionary history of vertebrates. Studies of salamanders have been a particular focus in examinations of locomotor function in stem tetrapods. Investigations of walking biomechanics have typically focused on more terrestrial salamanders and, thus, may best reflect the capabilities of terrestrial, crown tetrapods. However, given that the earliest tetrapods were likely aquatic, a salamander group with greater aquatic tendencies may serve as a more appropriate model for the incipient stages of terrestrial locomotion in early stem tetrapods. In the present study, locomotor biomechanics were assessed from the semi-aquatic *Pleurodeles waltl*, a newt that spends most of its adult life in water, using data on the ground reaction forces imposed upon individual limbs. Our findings indicate that limb kinetics of *P. waltl* are generally intermediate between those of the pectoral fins of mudskipper fish (*Periophthalmus barbarus*) moving over land, and those of more

terrestrial salamanders (*Ambystoma tigrinum*). *Pleurodeles waltl* forelimb forces were most similar to those from fish fins, whereas *P. waltl* hind limb forces were more similar to those from *Ambystoma* hind limbs. These data provide a framework for modeling stem tetrapods using an early stage of rear-wheel drive, with forelimb kinetics still sharing similarities to fins.

INTRODUCTION

The fossil record provides some of the most compelling evidence for the evolutionary steps taken as vertebrates became terrestrial, yet this evidence can be strengthened by the integration of complementary approaches (see reviews in Maidment et al. 2013; Pierce et al. 2013). While fossils of bones that are uncrushed and well preserved can yield important information about the musculoskeletal system of extinct taxa, they are subject to some limitations for interpreting how these structures are moved to accomplish behavioral tasks, such as locomotion. Fossil trackways have helped fill some gaps in our knowledge of the locomotor behaviors of extinct taxa by offering crucial insight about their gait (Maidment et al. 2013), but do not always allow direct measurements of locomotor dynamics for an extinct track maker, particularly factors that might impact more proximal limb elements (e.g., humerus, femur). One complementary approach for addressing these challenges is to use living taxa as analogs for extinct taxa, contributing perspective into evolutionary history through functional models (Pierce et al. 2013). With a similar objective as Extant

Phylogenetic Bracketing (Witmer 1995), one may employ 'functional bracketing' by studying a range of modern analogs to infer the functional capabilities of fossil taxa. Living taxa represent form-function solutions to different selective pressures and often serve as a foundation for estimating biologically realistic reconstructions of the soft tissue and movements of fossils, bracketing the likely function of extinct taxa (Witmer 1995, Pierce et al. 2012).

In 1929, August Krogh (Krogh 1929) advocated that many biological problems that can be difficult to study in a focal taxon could be investigated by using an appropriate animal or small subset of animals as surrogate models (*sensu lato* Bolker 2009; in contrast to exemplary models). Although originally intended to spur alternative approaches for studying human physiology, Krogh's principle can be invoked to gain perspective into the biology of extinct taxa (Krebs 1975). In the same sense that mice serve as valuable vessels in which to model biological processes in humans, despite the fact that there are noticeable differences between mice and humans, extant taxa can serve as informative models to explore in what ways fossil taxa could or could not have functioned. One of the utilities of models is to provide simplified versions of biological phenomena by distilling complex systems into more basic units for analysis (Krebs 1975; Bolker 2009; Anderson et al. 2012). Although the use of models inherently involves some generalization, powerful results can be achieved because the models still represent the fundamental principles under study.

The use of extant taxa as modern analogs (*sensu lato* Pierce et al. 2013), or functional models, allows one to link morphology to locomotor behaviors and functional performance. One benefit of studying extant taxa is that form-function relationships can be experimentally tested, allowing specific measures to be evaluated (Pierce et al. 2013). For instance, Nyakatura and colleagues (Nyakatura et al. 2014) studied the limb mechanics of the blue-tongued skink (*Tiliqua scincoides*) in order to understand how belly-dragging influenced sprawling locomotion, a stage proposed to be intermediate between the forelimb-driven, crutching movements of early stem tetrapods such as *Ichthyostega* on land, (Pierce et al. 2012) and the sprawling diagonal couplet of basal crown tetrapods (Nyakatura et al. 2014). By studying this modern analog walking on land, they were able to quantify the forces that were exerted on the limbs and the long axis rotation of the limb bones, compare the functional role of forelimbs and hind limbs, and propose an important intermediate stage in which the locomotion of tetrapods shifted from being forelimb-driven to hind limb-driven on land (Nyakatura et al. 2014). Thus, modern analogs offer valuable measurements from which to understand how organisms function as well as generate new hypotheses about the events that transpired over the course of evolution.

While it is optimistic to think that a single animal could adequately model the initial pioneer of the terrestrial invaders, it is more pragmatic to pursue a range of carefully selected taxa that represent key aspects along the transition to land since vertebrates underwent a series of gradual changes before becoming

terrestrial (Pierce et al. 2013; Nyakatura et al. 2014). For instance, Pierce and colleagues (Pierce et al. 2012) used five extant tetrapods (*Ambystoma tigrinum* salamander, *Crocodylus niloticus* crocodile, *Ornithorhynchus anatinus* platypus, *Haliobalopterus grypus* seal, and *Lutra vulgaris* otter) to validate their estimates of limb joint mobility in the early tetrapod *Ichthyostega* (Pierce et al. 2012).

Studying these taxa allowed the authors to evaluate the contributions of soft tissue to limb mobility, a factor difficult to estimate from fossil bones alone, and to also investigate fundamental properties of limbs. Similarities amongst these diverse tetrapods could potentially signify basal conditions of digit-bearing limbs whereas differences could set a precedent to generate hypotheses about how the phylogenetic, morphological, and/or ecological differences amongst these taxa could be influencing their limb function.

In the context of studying the evolution of terrestrial locomotion, living amphibious fishes, amphibians and reptiles have been used as functional models to infer the biology of extinct tetrapodomorphs (tetrapods and their sarcopterygian fish relatives) (Pierce et al. 2013; Nyakatura et al. 2014), with extant taxa representing alternative strategies for invading land and potentially simulating different time points along the adaptive steps towards becoming terrestrial. Investigations of extant taxa exhibiting morphological and/or behavioral traits that are consistent with those of fossil tetrapodomorphs offer particularly intriguing potential to gain insight into how tetrapods were able to leave the water's edge (Pierce et al. 2013).

In considerations of locomotor evolution during the invasion of land, salamanders are often used as functional analogues for basal tetrapods since they regularly move between water and land (Karakasiliotis et al. 2012), and exhibit a relatively generalized tetrapod *bauplan* that has not changed substantially for at least 150 million years (Gao and Shubin 2001). Previous studies have used living salamanders to gain perspective into the functional performance of extinct stem tetrapods, including the biomechanics and muscle physiology of walking underwater (Frolich and Biewener 1992; Azizi and Horton 2004; Ashley-Ross et al. 2009; Deban and Schilling 2009) and on land (Frolich and Biewener 1992; Brand 1996; Delvolvé et al. 1997; Ashley-Ross et al. 2009; Deban and Schilling 2009; Sheffield and Blob 2011; Kawano and Blob 2013), transitioning between water and land (Ashley-Ross and Bechtel 2004), and assessing how bone histology relates to ecological habits (Laurin et al. 2004; Canoville and Laurin 2009). Given the greater effect of gravitational loads on the musculoskeletal system on land, one of the most fundamental requirements for moving in terrestrial environments is the ability to support body weight for posture and locomotion. Evaluations of the weight-bearing capabilities of the limbs of stem tetrapods have been approached through measurements of ground reaction forces (GRFs) experienced by the terrestrial tiger salamander, *Ambystoma tigrinum* (Kawano and Blob 2013). In this species, the forelimbs played a weight-bearing role that was similar to the hind limbs, but the hind limbs had a greater role in acceleration than the forelimbs. However, fossil evidence

suggests that the first tetrapods, such as *Acanthostega*, were still aquatic (Coates 1996), and other early tetrapods, such as *Ichthyostega*, may have had only limited terrestrial capabilities (Pierce et al. 2012). In contrast, *A. tigrinum* are one of the largest terrestrial salamanders in North America, and are found in various terrestrial habitats, ranging from conifer forests to deserts; only rarely are they found in water for reasons other than reproduction (Petranka 1998). As such, they may not provide an optimal model for the initial invaders of land, in which terrestrial capacity may not have been fully developed. How might limb function differ for a species that exhibits greater aquatic tendencies?

Because salamander species have a diverse range of habitat preferences and life histories (Wake 2009), they provide an opportunity to model different evolutionary stages in the adoption of terrestrial habits. In particular, examinations of taxa that use their limbs primarily for aquatic locomotion could yield substantial insight into the limb function of earlier stem tetrapods with digit-bearing limbs. Phylogenetic analyses on the microanatomy of vertebrates indicated that all living amphibians (lissamphibians) descended from a lineage consisting of taxa that were either amphibious or terrestrial (Canoville and Laurin 2009), so the acquisition of a primarily aquatic lifestyle in lissamphibians was likely derived from a secondary land-to-water transition from a terrestrial or semi-aquatic ancestor. Consequently, no primitively aquatic extant salamanders are available. Semi-aquatic salamanders can serve as a model for early stem tetrapods that had not yet acquired full terrestrial locomotor capabilities. In this

study, we compared GRF production of individual limbs by semi-aquatic Iberian ribbed newts, *Pleurodeles waltl* Michahelles 1830, to published data from tiger salamanders, *Ambystoma tigrinum* Green 1825, and African mudskippers, *Periophthalmus barbarus* (Linnaeus 1766) (Kawano and Blob 2013). Our objective in these comparisons was to examine extant taxa that model important stages during the transition to land (i.e., fin, semi-aquatic limb, terrestrial limb), in order to gain insight into the functional changes associated with the evolution of terrestrial locomotion. *Pleurodeles waltl* was chosen because it is one of the better available models of a predominantly aquatic vertebrate with a generalized tetrapod *bauplan* that can be readily induced to use its limbs for terrestrial excursions (see Appendix A for detailed justification). Although *Pleurodeles* undergoes a terrestrial eft phase as part of its life cycle, they still exhibit greater aquatic tendencies than more terrestrial groups, such as *Ambystoma* and, thus, can provide insight into limb use in a taxon that is not fully terrestrial.

Propulsion on land in stem tetrapods may have been dominated by the forelimb ('front-wheel drive') and then transitioned to hind limb dominance ('rear-wheel drive') as the hind limbs assumed a more important locomotor role (Boisvert 2005), with 'rear-wheel drive' potentially appearing as early as in sarcopterygian fishes for aquatic locomotion (King et al. 2011). The proposed 'front-wheel drive' of the sarcopterygian fish *Panderichthys* (Boisvert 2005) and the tetrapod *Ichthyostega* on land (Pierce et al. 2012) have been compared to locomotor behaviors in extant fishes that use the pectoral fins to move over land,

such as walking catfishes and mudskippers, respectively (Pace and Gibb 2014). Correspondingly, terrestrial salamanders, like *A. tigrinum*, can provide an appropriate model for ‘rear-wheel drive’ in early crown tetrapods (Pierce et al. 2013). Comparisons between the kinetics (force production) of the pectoral fin of mudskippers and the forelimbs and hind limbs of terrestrial salamanders have demonstrated that the GRF of fins is directed more medially (~17 vs. <11 degrees), potentially exposing fin bones to greater bending stresses than limbs during terrestrial movements (Kawano and Blob 2013). Our new data from the semi-aquatic *P. waltl* have the potential to give insight into the nature of the transition between these conditions. Simply by having limbs, locomotor force production by *P. waltl* may be similar to that of *A. tigrinum*, yet habitual limb use for aquatic locomotion in adult *P. waltl* might lead to kinetic similarities to fish fins. These comparisons carry broader implications for generating hypotheses regarding how functional capacities can evolve, whether through close coupling with major structural changes (i.e., fin to limb), or through gradual steps potentially decoupled from structural changes.

MATERIALS AND METHODS

Animals

Five adult *P. waltl* (body mass: 16.60 ± 0.40 g; snout-vent length: 0.083 ± 0.001 m; total length: 0.186 ± 0.003 m) were obtained from a commercial vendor. All values represent means ± 1 S.E. Animals were individually housed in glass

aquaria aerated with sponge filters, kept on a 12h:12h light:dark cycle, and fed every 1-2 days on a diet of frozen bloodworms and krill. Animal husbandry and experimental procedures complied with procedures approved by the Clemson University IACUC (AUP2010-066).

Collection of data on 3-D ground reaction forces (GRFs)

Experimental procedures from a previous study on the GRFs of tiger salamanders and mudskipper fishes (Kawano and Blob 2013) were replicated in the present study (see Appendix A) to obtain forelimb ($N=50$) and hind limb ($N=49$) GRFs from *P. waltl* (Appendix A - Fig. A1). The focal taxa examined herein represent models for distinct potential stages during the evolution of terrestrial locomotion: front-wheel drive in a terrestrial vertebrate with limited capabilities of the pelvic appendages (terrestrial mudskipper fish), a semi-aquatic early stem tetrapod (semi-aquatic *P. waltl* newt), and rear-wheel drive in a stem tetrapod that is highly terrestrial (terrestrial *A. tigrinum* salamander). Although the mudskipper is not fully terrestrial and the newt undergoes a terrestrial eft phase, they are herein referred to as “terrestrial” and “semi-aquatic”, respectively, for simplicity. GRFs in the vertical, mediolateral, and anteroposterior directions were digitally filtered with a custom low-pass, zero phase second order Butterworth filter, and then interpolated to 101 points (0-100% of stance at 1% increments) using a cubic spline with the *signal* package in R.

Comparisons of GRFs were conducted amongst: 1) the forelimbs and hind limbs of newts versus previously collected data from the pectoral and pelvic appendages of mudskipper fishes and tiger salamanders (Kawano and Blob 2013), to assess whether limb kinetics in semi-aquatic newts are more similar to those of mudskipper fins or the limbs of a primarily terrestrial salamander taxon; and 2) the forelimbs and hind limbs of newts, to understand whether, as a model for early stem tetrapods, a taxon with limbs used primarily in an aquatic environment could be forelimb-driven or hind limb-driven on land. Comparisons were performed when the overall magnitude of the GRFs reached a maximum (“peak net GRF”) using linear mixed effects models (see “Statistics”), and over the entire phase of stance, when the foot is in contact with the ground, to examine overall patterns of GRF production using vector analysis (see Cullen et al. 2013 and Appendix A). Stance duration was used as a basis for comparing speeds since stance is the phase in the locomotor cycle where GRFs are produced.

Statistics

Linear mixed effects models (LMMs) were used to compare GRF parameters at the peak net GRF, when the total forces imposed upon the limb bones are the greatest, while accounting for variation in random effects. LMMs were fitted by Maximum Likelihood with lme4::lmer, in order to calculate Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC), which were used to test

for significance by comparing the full model against a null model. *P*-values are not appropriate for mixed model designs (Bates 2006), and recent investigations have suggested that formerly recommended tests using Markov Chain Monte Carlo are not as reliable as other statistical alternatives (Bates et al. 2014). Individual was treated as a random effect, and group (e.g., *Ambystoma* forelimb, *Pleurodeles* hind limb) was used as a fixed effect. To test pair-wise differences, Tukey's post hoc comparisons can be conducted on the least-squares means fitted from linear models using `lsmeans::lsmeans` (Lenth 2014), which employs the Kenward-Roger method (Kenward and Roger 1997) to calculate the degrees of freedom for the post-hoc comparisons. Discriminant function analyses (DFAs) were used to assess overall differences amongst the groups, and Spearman rank correlations (`stats::cor.test`) tested which variables were contributing towards these differences along each DF axis. Convex hulls were drawn around groups in the DFA plot to facilitate group comparisons. Statistical analyses were conducted in *R* (v. 3.1.0).

Assessing forelimb function without hind limbs in an aquatic salamander

Forelimbs may have been the primary propulsor in early stem tetrapods (Pierce et al. 2013; Nyakatura et al. 2014), but the assessment of how lower vertebrates (e.g. fishes and amphibians) accomplish terrestrial excursions with only their forelimbs has been difficult because the hind limbs tend to be the primary propulsors in reptiles (Russell and Bels 2001) and even in salamanders with

comparable lengths of the forelimbs and hind limbs (Kawano and Blob 2013). Thus, preliminary data ($n = 3$) on the forelimb function of two *Siren lacertina* salamanders were collected (AUP 2014-041) to provide insight into the terrestrial limb mechanics of a forelimb-driven amphibian. Data are available in Appendix B, but are not included in statistical analyses due to small sample size.

RESULTS

Comparison amongst the appendages of fishes and salamanders

Differences amongst individual GRF parameters at the peak net GRF were supported by lower AIC and BIC values for the full models compared to the null models (Table 3.1). Comparisons of stance duration indicated that differences amongst the appendicular groups were not substantial, with the pectoral fin only 0.13-0.15 s shorter in duration than the other appendicular groups; stance duration was similar across the limbs ($p = 0.718$). Thus, GRFs were regarded as having been generated under generally comparable durations of stance. Tukey's post-hoc comparisons indicated that the semi-aquatic newt forelimb shared similarities with the terrestrial fish pectoral fin and the terrestrial salamander forelimb at the peak net GRF, but that the latter two appendages exhibited numerous differences (Table 3.2). Although the mediolateral component of the GRF of the semi-aquatic forelimb was similar to both the terrestrial fin and forelimb, the fin exhibited greater medial magnitudes than the terrestrial forelimb. The semi-aquatic forelimb had a GRF that had a medial orientation that was

Table 3.1. Information criterion for evaluating parameters at the peak net GRF

	AIC_{null}	AIC_{full}	BIC_{null}	BIC_{full}
Time of peak net GRF (%)	2074.845	1871.833	2085.409	1896.483
Net GRF (BW)	-528.197	-574.890	-517.633	-550.240
Vertical GRF (BW)	-501.945	-520.173	-491.381	-495.523
Mediolateral GRF (BW)	-768.219	-785.372	-757.654	-760.722
Anteroposterior GRF (BW)	-381.323	-662.740	-370.759	-638.089
Mediolateral angle (°)	1767.304	1764.372	1777.868	1789.023
Anteroposterior angle (°)	2108.306	1890.263	2118.870	1914.913

BW = body weight. Comparisons are assessed between the null and full models for a given information criterion test, with lower values indicating a better model.

intermediate between the fish fin and terrestrial forelimb, with the GRF of the fish fin and semi-aquatic forelimb directed more than 1.5x medially than the terrestrial forelimb (Fig. 3.1, Table 3.2). Comparisons of anteroposterior GRF components and angles indicated that the semi-aquatic forelimb had a lower acceleratory role than the terrestrial fin or the terrestrial forelimb.

The semi-aquatic newt hind limb shared greater similarities to the terrestrial hind limb than its own forelimb at the peak net GRF (Table 3.2). Both the semi-aquatic and terrestrial hind limbs had a peak net GRF occurring around 30% of stance, vertical and net GRF magnitudes of around 0.50 BW, and a net acceleratory role. The semi-aquatic forelimb supported about 10% less than the

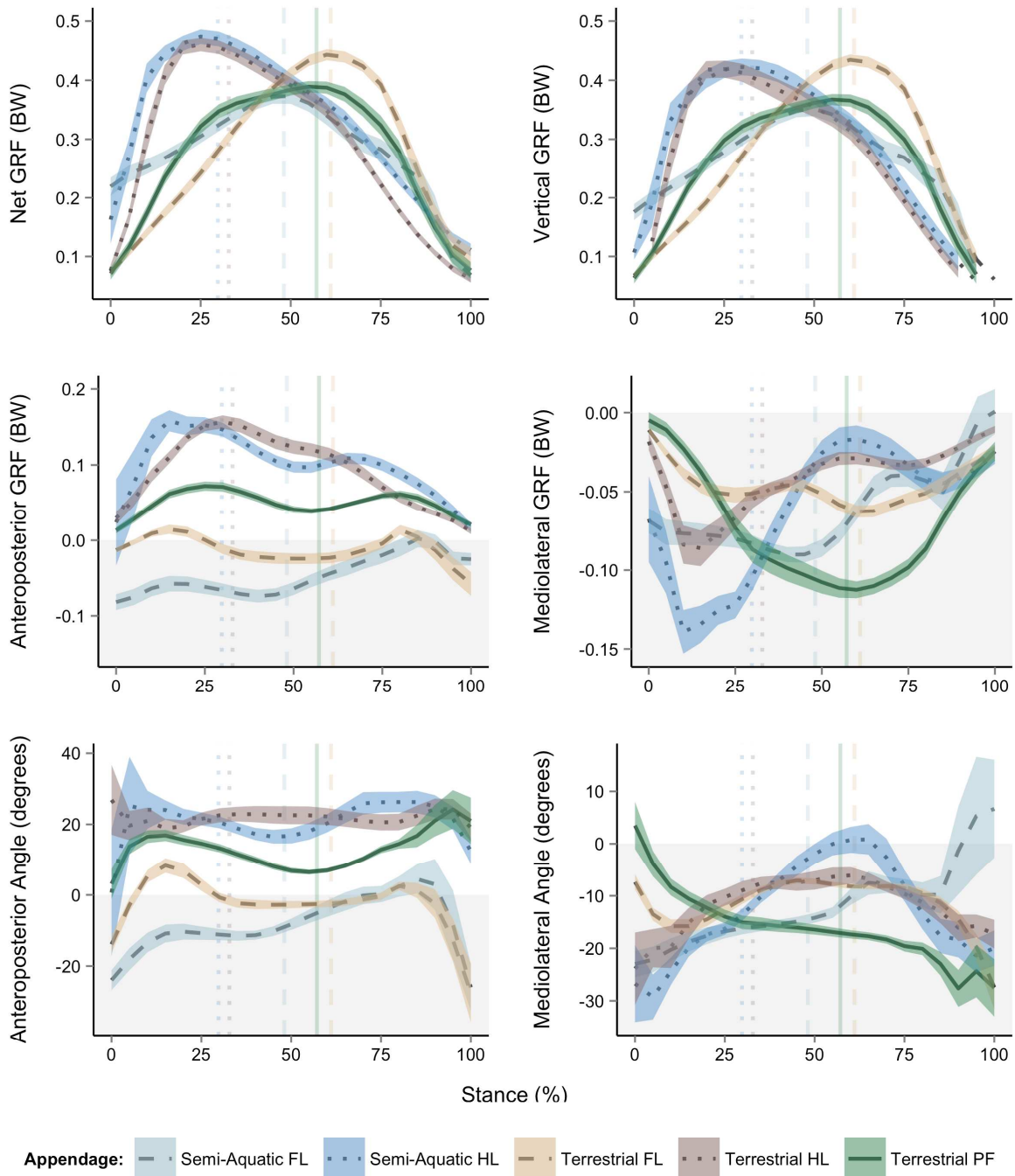


Fig. 3.1. Profiles of GRF parameters throughout stance. Means (curved lines) with standard errors (shading), and the timing of the peak net GRF (vertical lines) for each appendage are color-coded using the conventions indicated at the bottom of the figure.

semi-aquatic hind limb. Despite the disparity amongst these measures, the GRFs were directed medially for all limbs (~11-17°), with the semi-aquatic hind limb having a larger medial orientation than the terrestrial hind limb and forelimb (Table 3.2).

Both tetrapods exhibited a pattern whereby the hind limbs had a greater propulsive role than their respective forelimbs, but relative contributions of the limbs towards bearing weight differed between the semi-aquatic newt and terrestrial salamander. Although the hind limbs of these two taxa both supported about 0.50 BWs at the peak net GRF, the semi-aquatic forelimb supported a lower overall proportion of body weight (0.40) than the semi-aquatic hind limb (0.50), whereas the terrestrial forelimb and hind limb had similar roles in weight-bearing (0.46 and 0.47, respectively; Table 3.2). In addition, the semi-aquatic forelimb decelerated more than the terrestrial forelimb.

Summarizing differences amongst the appendages

Differences amongst the appendages were also observed in “biomechanospace,” where all kinetic data at the peak net GRF were evaluated together in multivariate space. Discriminant function (DF) 1 separated pectoral vs. pelvic appendages, whereas DF 2 differentiated fins vs. limbs (Table S1, Fig. 3.2). Together, DF 1 and DF 2 accounted for ~94% of the between-group variation, with the separation between the pectoral and pelvic appendages accounting for ~85% of this variation. All variables except the mediolateral orientation of the

GRF were significant along DF 1 whereas about half of the variables were significant along DF 2 (Table S1). For these taxa, pectoral appendages were most strongly discriminated from pelvic appendages by possessing a peak net GRF occurring later in stance, with less of an acceleratory role, and a lower magnitude of the GRF in the vertical direction. The amount of overlap was considerably greater between the semi-aquatic and terrestrial hind limbs (almost complete overlap) than the forelimbs. Differences amongst fins and limbs, on DF 2, were most strongly influenced by the GRF being more medial and having less of an acceleratory role in the fin than all of the limbs combined.

The biomechanical distinction between fins and limbs was also supported by the percentage of misclassification from a linear DFA (Table S2). The fin had the highest percentage (86%) of trials that were correctly classified, with the terrestrial forelimb having the highest misclassification (only 12%). Limbs had about 10% fewer correct classifications compared to the fin. Semi-aquatic and terrestrial hind limbs were mistaken for one another in roughly one quarter of the trials, and a similar trend was found between the forelimbs. However, misclassifications of a forelimb for a hind limb, and vice versa, never occurred.

Comparisons of GRF patterns throughout the duration of stance

When evaluating overall GRF profiles during stance (Fig. 3.1), numerous similarities were observed between the amphibian hind limbs as well as between the terrestrial fish fin and the limbs (Table S3 in Appendix A). The fish fin and the

terrestrial hind limb shared some of the greatest similarities for the anteroposterior GRF magnitude and angle, with vector analyses showing angles of differentiation under 25°. In vector analysis, angles close to zero indicate strong similarities whereas angles closer to 90 suggest dissimilarity (Cullen et al. 2013). The semi-aquatic hind limb was also quite similar to the terrestrial hind limb. However, the magnitude and angle of the anteroposterior component of the GRF for the fish fin was intermediate between the hind limbs that had a greater role in acceleration, and the forelimbs that had a greater role in deceleration. The net GRF and vertical component of the GRF for the fish fin and the semi-aquatic forelimb had a broader shape than the other appendages (Fig. 3.1). The GRFs in the mediolateral and vertical directions and net GRF were similar across stance for all of the appendages, but were most similar between the semi-aquatic and terrestrial hind limbs.

Forelimb function in a front-wheel driven salamander

The forelimbs of *S. lacertina* supported a much lower proportion of body weight (~0.2) compared to the other appendicular groups (~0.5), had a lower medial magnitude, and had only a slight role in acceleration (Appendix B). The two tested individuals also demonstrated greater lateral bending than the mudskipper fish, newt, and salamander.

Table 3.2. Comparison of mean GRF parameters at the time of peak net GRF amongst the appendages of the terrestrial fish, semi-aquatic newt, and terrestrial salamander

Variable	Terrestrial PF	Semi-aquatic FL	Semi-aquatic HL	Terrestrial FL	Terrestrial HL
Time of peak net GRF (%)	57.16 ± 1.84 ^{a,e}	48.10 ± 1.39 ^{c,e}	29.78 ± 1.44 ^{d,f}	61.08 ± 1.01 ^a	32.80 ± 1.60 ^{b,f}
Net GRF (BW)	0.42 ± 0.01 ^{a,b}	0.40 ± 0.01 ^b	0.50 ± 0.01 ^c	0.46 ± 0.01 ^{a,b,c}	0.47 ± 0.01 ^{a,c}
Vertical GRF (BW)	0.39 ± 0.01 ^{a,b}	0.38 ± 0.01 ^a	0.45 ± 0.01 ^b	0.45 ± 0.01 ^{a,b}	0.43 ± 0.02 ^{a,b}
Mediolateral GRF (BW)	-0.12 ± 0.01 ^{a,d}	-0.09 ± 0.01 ^{b,d}	-0.13 ± 0.01 ^{c,a}	-0.07 ± 0.004 ^b	-0.07 ± 0.01 ^{b,d}
Anteroposterior GRF (BW)	0.05 ± 0.01 ^a	-0.08 ± 0.01 ^b	0.15 ± 0.01 ^{c,e}	-0.03 ± 0.01 ^d	0.15 ± 0.01 ^e
Mediolateral angle (°)	-17.14 ± 0.90 ^a	-13.62 ± 1.02 ^{a,b}	-16.21 ± 1.37 ^{a,b}	-8.67 ± 0.53 ^b	-11.04 ± 1.73 ^{a,b}
Anteroposterior angle (°)	7.65 ± 0.83 ^a	-11.08 ± 1.15 ^b	19.79 ± 1.80 ^{c,e}	-3.21 ± 1.00 ^d	21.69 ± 1.98 ^e
Number of trials	50	50	49	50	50

Values represent means ± SE for 49-50 steps averaged across five individuals for each group; BW, body weights. PF = pectoral fin, FL = forelimb, and HL = hind limb. For a given variable, dissimilar superscript letters across the appendicular groups indicate pair-wise differences based on Tukey post-hoc comparisons. For mediolateral variables, negative values indicate a medial direction. For anteroposterior variables, negative values indicate a posterior (deceleratory) direction and positive values indicate an anterior (acceleratory) direction. Note that exact values may appear slightly different from the profiles illustrated in Fig. 3.1 because the profiles were generated using the pooled means whereas the values reported in this table were extracted at the peak net GRF for each individual trial, rather than at the average timing of the peak net GRF from the pooled means.

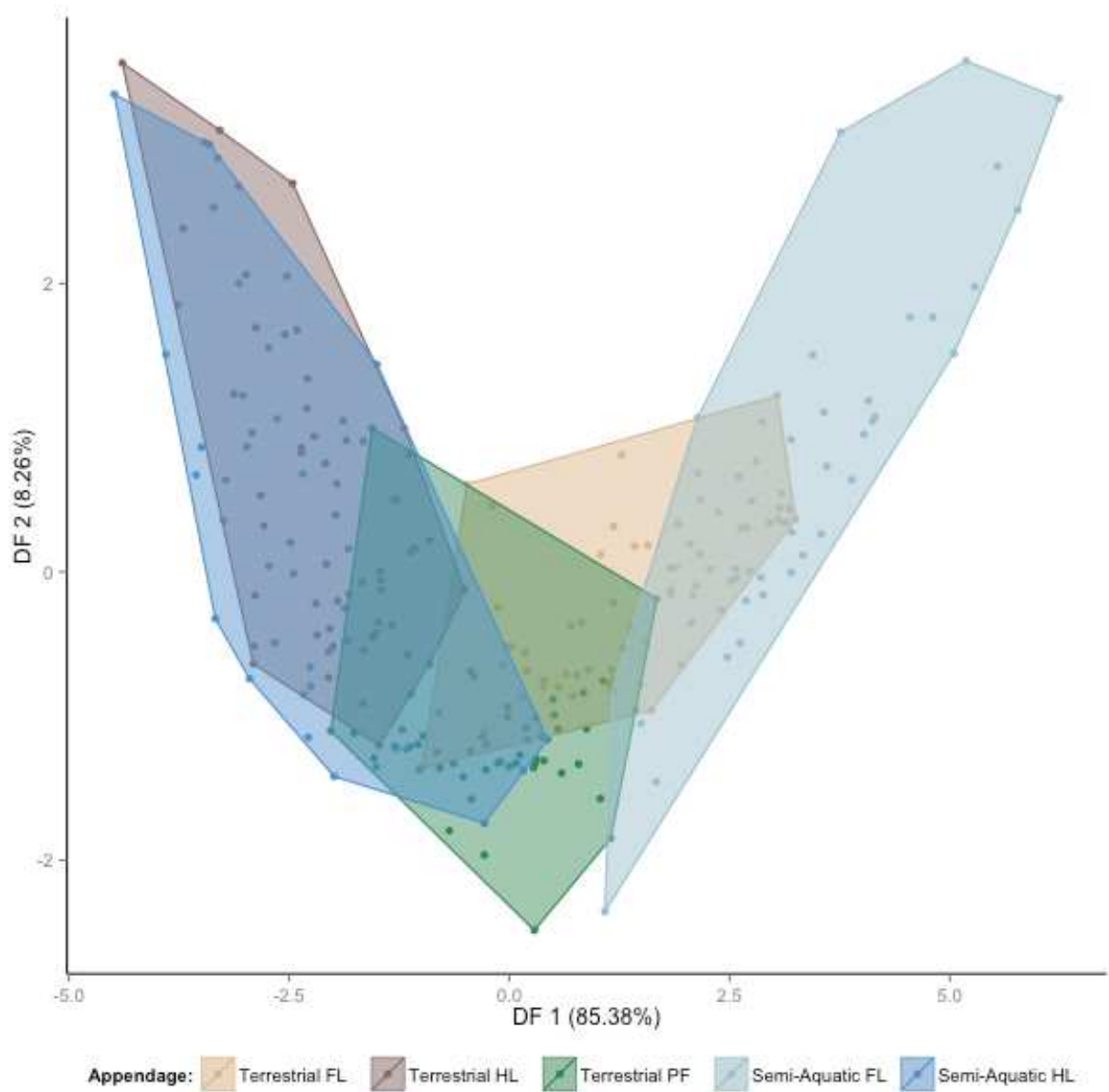


Figure 3.2. A canonical discriminant function analysis illustrates the factors driving the biomechanical differences amongst these groups of appendages. DF 1 separates pectoral vs. pelvic appendages, and DF 2 separates fins from limbs.

DISCUSSION

The propulsive forces of the newt *P. waltl*, a semi-aquatic tetrapod with digit-bearing limbs, exhibited a mosaic of characteristics that resemble aspects of GRF profiles from both fish fins and the limbs of more terrestrial salamanders (Tables 3.1, 3.2 and supplementary tables in Appendix A). Like more terrestrial salamanders (and running lizards: McElroy et al. 2014), the predominant acceleratory forces in this semi-aquatic newt are produced by the hind limb, signifying rear-wheel drive. The numerous similarities between semi-aquatic and terrestrial hind limbs at the time of peak net GRF, and during all of stance (Table 3.2 and S3), may indicate that the use of the hind limbs as a primary propulsor may impose strong selection on limb kinetics. Also, considering all of the parameters we evaluated, forelimb GRFs from semi-aquatic newts were actually more similar to GRFs from terrestrial fins than to profiles for either forelimbs from terrestrial salamanders or hind limbs from semi-aquatic salamanders (Table 3.2). The semi-aquatic newt also had a medial orientation of the GRF upon its limbs that was intermediate between the terrestrial limbs and fins.

Multivariate analyses of these GRF data indicated biomechanical distinctions amongst the locomotor structures studied herein. Hind limbs were distinguished from pectoral appendages (forelimbs and pectoral fins) primarily by a peak net GRF occurring earlier in stance, and having a greater acceleratory role. Differential limb function has been documented across numerous running lizard species, with limb length potentially influencing various biomechanical

parameters of terrestrial locomotion (McElroy et al. 2014). Although the forelimbs and hind limbs are of comparable size in both the semi-aquatic newt and the terrestrial salamander, only in terrestrial salamanders do the two limbs contribute equally to body support (i.e., have equal net and vertical GRF magnitudes); in semi-aquatic newts these GRF components differ by 15-20% (Table 3.2). Overall differences in locomotor function were also greater between the limbs in the semi-aquatic newt, with ~85% of GRF parameters significantly different between forelimbs and hind limbs compared to ~43% in the terrestrial salamander (Table 3.2). In addition, though the terrestrial fin examined in this study is used for front-wheel driven locomotion, the semi-aquatic forelimb (from a rear-wheel drive taxon) shared slightly more GRF similarities with the fin than the terrestrial limb (Table 3.2).

Such disparities in limb function, as well as other differences between biomechanical profiles for semi-aquatic and terrestrial species, could relate to the different demands imposed by the primary environments in which the limbs of these taxa function. For example, the medial orientation of the peak GRF in semi-aquatic newt limbs (14-16°) falls between that of mudskipper fins (17°) and most previously evaluated tetrapod limbs (<11°), including terrestrial salamanders (Fig. 3.3). A shift to a GRF directed less medially could reduce joint moments, and, thus, the stresses experienced by the appendicular bones during terrestrial locomotion (Kawano and Blob 2013). However, the greater medial inclination of the GRF in semi-aquatic newts (Table 3.2) could relate to the

greater lateral spread of their distal limb segments compared to terrestrial taxa, so that the feet are placed lateral to the elbow or knee joint during stance (Fig. A-1A), rather than directly below these joints (as in terrestrial salamanders: Fig. 1A, B in Kawano and Blob 2013). Given that this more pronounced sprawling limb posture is also found in the mudskipper fish, this pattern may be found in taxa that are ancestrally aquatic (fish) and/or use their appendages primarily for aquatic locomotion (semi-aquatic newt). The broadening of the gait that would result from such lateral foot placement might convey additional stability against currents or other flows in aquatic habitats (Martinez et al. 1998) by reducing pitching and rolling (Chen et al. 2006). However, when on land, habitually aquatic species may not be able to adjust to using the more upright orientations of distal limb segments that are seen in terrestrial taxa (Kawano and Blob 2013).

Producing more acute limb angles could facilitate elevating the body off the ground, and shift the bone loading regime to reduce bending and increase compression (Ashley-Ross and Bechtel 2004; Kawano and Blob 2013). Thus, such a limb posture could have major biomechanical consequences that could facilitate terrestrial locomotion. Lateral spread of the distal appendage may also contribute to the high medial orientation of the GRF in mudskippers (Fig. 1C in Kawano and Blob 2013), but may also contribute to stability during terrestrial crutching, given the lack of extended posterior appendages in the mudskipper fish. Although there is the possibility that alternative functions (e.g., amplexus, burrowing, antagonistic interactions) could also be influencing limb function in the

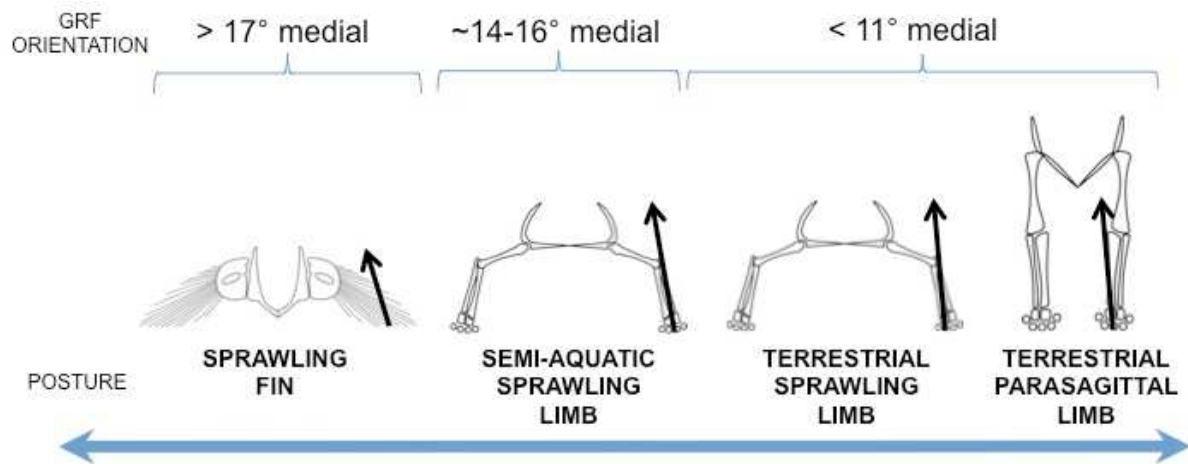


Figure 3.3. Data on the limb kinetics of a semi-aquatic newt add further information towards interpreting the evolution of GRF in vertebrates. The GRF becomes less medial during the shift from fish to terrestrial tetrapods, with the semi-aquatic tetrapod as an intermediate.

salamander and newt, locomotion regularly places some of the highest demands on limb function (Biewener 1990; Biewener 1993) and, thus, is assumed to be the predominant factor driving the differences observed amongst the limbs.

Evidence from the fossil record suggests that terrestrial adaptations first appeared in the anterior regions of the body (Nyakatura et al. 2014), but how rear-wheel drive evolved from stem tetrapods, especially in regards to terrestrial locomotion, is still unresolved. Anatomical evaluations of some of the earliest stem tetrapods, such as the elpistostegalid fish *Panderichthys* (Boisvert 2005) and the Devonian tetrapod *Ichthyostega* (Pierce et al. 2012), indicate that the pelvic appendages were likely not effective propulsors on land. As a result, front-

wheel drive has been proposed to be the basal condition for tetrapod movements on land (Boisvert 2005; Nyakatura et al. 2014). In contrast, rear-wheel drive, in concert with movements by the tail, was likely the primary locomotor mode underwater. Along these lines, empirical work on the African lungfish (*Protopterus annectens*) suggests that rear-wheel drive could have evolved when tetrapods were still aquatic and as early as in sarcopterygian fishes (King et al. 2011), with the acquisition of rear-wheel drive potentially beginning as a modification of a more ancestral swimming mode powered by the posterior region of the body, such as the tail. Further, recent paleontological examinations of the pelvic girdle of the elpistostegolid tetrapodomorph fish *Tiktaalik* (a relative of *Panderichthys*) indicate that this transitional fossil exhibited a mosaic of tetrapod-like and fish-like characteristics, including precursors for achieving rear-wheel drive (Shubin et al. 2014). Our GRF data from *P. waltl* build upon previous work on the kinetics of mudskipper pectoral fins and salamander limbs (Kawano and Blob 2013) to offer additional insight for interpreting evolutionary patterns in the incipient stages of terrestrial locomotion, providing a functional model for semi-aquatic basal tetrapods that exhibit locomotor biomechanics intermediate between those of finned taxa and crownward tetrapods.

Recent work by Nyakatura and colleagues (Nyakatura et al. 2014) suggests that tetrapods may have gone through an intermediate stage during the transition from front-wheel drive to rear-wheel drive. Specifically, their work

evaluated the limb mechanics of a sprawling, belly-dragging lizard, and proposed that belly dragging could have allowed early tetrapods to move on land using less developed appendicular muscles (Nyakatura et al. 2014). The authors propose that early tetrapods were front-wheel driven during this intermediate belly-dragging stage to allow initial capacities for terrestrial locomotion, after which the role of rear-wheel drive gradually increased. Our findings from the semi-aquatic newt, *P. waltl*, may provide a model for a subsequent stage after belly-dragging with front-wheel drive, in which rear-wheel drive has been adopted but the forelimbs have not yet acquired fully terrestrial limb mechanics. Although the extensive lateral bending employed by *S. lacertina* complicates direct comparisons of its forelimb function to those of the other modern analogs that did not exhibit such axial curvature in the trials observed, preliminary data on the forelimb function of *S. lacertina*, salamanders that entirely lack hind limbs, provide a foundation in which to test how lateral bending of the body axis contributes to terrestrial locomotion in tetrapods that are better adapted for aquatic environments, and that have more limited terrestrial adaptations in their limb morphology (Appendix B). Although lateral-sequence walking behaviors were not likely in *Ichthyostega* (Pierce et al. 2012), and possibly other early stem tetrapods, further experimental analyses on the contribution of lateral bending towards limb kinetics in various modern analogs could help resolve to what extent lateral bending could have facilitated the initial forays onto land in the evolution of terrestrial locomotion.

Kinetic data from the semi-aquatic newt may serve as a foundation for building upon two hypotheses regarding how terrestrial locomotion evolved (discussed in Pierce et al. 2013). The first hypothesis suggested a trot with lateral bending of the axial system producing a traveling wave, with the limbs treated as 'struts'. The second hypothesis proposed a lateral-sequence walk involving a standing wave, with the limbs generating propulsion. Given that the semi-aquatic forelimb was deceleratory while the hind limb was acceleratory (Table 3.2, Figure 3.1), *P. waltl* may be using a modified standing wave in which the hind limbs are generating forward propulsion while the forelimbs are being used as 'struts'. Such disparity in the propulsive roles of the limbs is not as pronounced in the terrestrial salamander (Table 1 in Kawano and Blob 2013). A gait similar to one employed by *P. waltl* may have allowed the earliest limbed tetrapods to traverse the terrestrial environment with a musculoskeletal system that still primarily functioned for underwater behaviors, potentially also providing an intermediate stage between sarcopterygian fish that could accomplish rear-wheel drive underwater (King et al. 2011) to crownward tetrapods that used rear-wheel drive on land.

How functional changes evolve has been considered in a variety of systems. Historically, the evolution of locomotor posture had been viewed to exemplify evolutionary change through a sequential series of gradual steps, leading from sprawling to upright (Charig 1972). More recent work highlighted the potential for intermediate taxa to exhibit a highly flexible range of capabilities

between the ends of this functional continuum, rather than a graded series of incremental changes between them (Kemp 1978; Blob 2001). Hind limb function in the tetrapodomorph fish *Tiktaalik* has been described with a wide range of capacities (Shubin et al. 2014), potentially indicating intermediate functional flexibility in an early stage of the fin-to-limb transition. Our data from *P. waltl* suggest that even after such functional flexibility, evolutionary change in some traits, such as the reduction in the medial orientation of the GRF and the acquisition of ‘rear wheel drive’, may still have proceeded gradually. Moreover, these changes may not have been strictly coupled to evolutionary changes in appendicular structure. Synthesis of data from biomechanics and paleontology, therefore, holds promise for developing a more comprehensive understanding of the transformations of the vertebrate musculoskeletal system that led to limbed tetrapods conquering the terrestrial realm, and the nature of functional evolution more broadly.

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CHAPTER FOUR

COMPARATIVE LIMB BONE LOADING IN THE FORELIMBS AND HIND LIMBS OF THE SALAMANDER *AMBYSTOMA TIGRINUM*: TESTING THE “MIXED- CHAIN” HYPOTHESIS FOR SKELETAL SAFETY FACTORS

ABSTRACT

The ability of bones to resist physical demands has important implications for the functional capabilities of vertebrates. However, the capacity of bones to resist loads may be affected by a variety of factors, including the mechanical properties of bone material, the intensity of the loads placed upon the skeleton, and the predictability of such demands. This capacity is typically greater than what is required to accomplish normal tasks. Such excess capacity, or “safety factor,” can serve as biological insurance to reduce the likelihood of failure. Though high safety factors might be advantageous, they might also be selected against because overbuilt structures can be expensive to produce and maintain, and may not actually be advantageous if the structure is linked to another structure that exhibits a lower margin of safety. The “mixed-chain” hypothesis proposes that different safety factors might be found among components within a biological system due to unpredictability in the demands placed upon them, different energetic costs, or overall high safety factors of the components within the system. Studies of skeletal loading during locomotion present opportunities to

test for intraspecific variation in the biomechanical capabilities of components within biological systems because locomotion is a demanding task that requires the coordination of multiple elements that may be subject to different costs or demands. This study compared the mechanical properties and locomotor loading of the humerus and femur of tiger salamanders *Ambystoma tigrinum* in context of the “mixed-chain” hypothesis, in order to evaluate the conditions under which functional diversity in safety factors might emerge. Although the forelimbs and hind limbs appear superficially similar in *A. tigrinum*, bone stresses in the humerus were generally about half those observed in the femur. Safety factors for resisting bending in the humerus were almost twice as large as those for the femur, with regional heterogeneity in bone mechanical properties contributing to larger hardness values in the dorsal and posterior regions of both bones. Such intraspecific variation between and within bones may relate to the different biomechanical functions of these locomotor modules, and provide a refined context for considering the acquisition of novel locomotor capabilities during the evolutionary invasion of land by tetrapods.

INTRODUCTION

Bones must regularly withstand applied forces, or loads, imposed by the contraction of muscles and interactions with the environment. Failure to resist such loads could result in injury to the skeleton, potentially leading to inferior predator evasion performance, inability to acquire food, or other detriments that

could ultimately produce severe consequences such as death (Biewener 1993). Terrestrial locomotion is particularly noteworthy, in this context, because limb bones must accommodate the physical demands associated with generating forward propulsion as well as supporting the body for posture, thus, imposing some of the highest demands upon the skeleton (Biewener 1993). However, limb bones are often capable of resisting loads that are considerably higher than they normally experience. This property is called a “safety factor,” and can be viewed as an extra “reserve” capacity of a structure to perform a biological function with variable demands (Alexander 1981, 1997; Diamond 2002).

Safety factors for limb bones commonly allow protection against loads ranging from 2-10 times greater than ordinary demands, with variation found both across taxa and among the limb bones within a single species (Alexander 1981; Biewener 1993; Currey 2002; Diamond 2002; Vogel 2003; Butcher and Blob 2008; Sheffield and Blob 2011; Blob et al. 2014). Several factors have been proposed to contribute to interspecific variation in safety factors (Blob and Biewener 1999; Blob et al. 2014), but reasons for intraspecific variation are less intuitive. For a single element, the safety factor is expected to be sufficiently high enough to prevent a structure from being compromised by applied loads, but low enough to minimize the energetic costs to produce such a structure (Alexander 1997). However, individual limb bones function as links within an integrated biological system (Alexander 1997). Given that a system or a “chain” is only as strong as its weakest link, it might be expected that all elements within the

system should have comparable safety factors, because it would be disadvantageous for energy to be wasted in the production of elements with higher safety factors when this protection would be undercut by limitations of the weaker components (Alexander 1997). Although this expectation has intuitive appeal, Alexander (1997) proposed multiple scenarios under which intraspecific variation in safety factors, or a “mixed-chain”, might be expected. First, elements that are energetically costly to move or maintain might have lower safety factors. Second, elements that experience more variable loads than the rest of the skeleton might have higher safety factors, thereby protecting against occasionally higher peak loads. Third, for species in which all elements of the skeleton exhibit high safety factors, there might be greater opportunity for variation in safety factors across different elements. Diamond (2002) built upon this framework and suggested that those elements that have higher penalties for failure should possess higher safety factors (Diamond 2002). For instance, a broken nose might only impair an organism’s olfactory capabilities, but a broken skull could have fatal consequences, so greater safety factors would be expected for protecting the skull.

A limited body of empirical evidence has supported the presence of mixed chains of safety factors in the skeletal elements of locomotor systems. For example, Currey (2002) found a higher incidence of fracture (implying lower safety factors) in the distal limb bones of racehorses, compared to their proximal bones. Blob and Biewener (1999) found a similar pattern of lower safety factors

in the tibia (distal element) versus the femur (proximal element) in the hind limbs of iguanas and alligators. Comparisons between forelimb and hind limb elements are more limited, with Blob et al. (2014) finding higher safety factors in the humerus versus the femur of alligators. In the context of Alexander's (1997) proposed factors contributing to mixed chains, the higher humeral safety factors of alligators were attributed to the generally high safety factors found in the limbs of reptiles, as well as the smaller size of the humerus, which might make a high safety factor less costly than the femur (Blob et al. 2014). However, with such patterns evaluated for only a single species, their generality is unclear.

Understanding the generality of “mixed chains” of limb bone safety factors could have implications for understanding a long-standing question in tetrapod evolution, which is how the different functional roles of forelimbs and hind limbs could have contributed to the invasion of land. Fossil evidence suggests that the capacity for terrestrial excursions 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 (Pierce et al. 2012; Nyakatura et al. 2014), hind limbs assumed the role as the primary propulsor not long after forelimbs and may have contributed to aquatic locomotion in sarcopterygian fishes (King et al. 2011). In the context of understanding the incipient stages of terrestrial locomotion, salamanders are often used as modern analogs to early stem tetrapods due to morphological and ecological similarities (Gao and Shubin 2001; Pierce et al. 2013); thus, salamanders provide an intriguing system in which to

test the “mixed-chain” hypothesis. Femoral stresses have been evaluated for the tiger salamander (*Ambystoma tigrinum*; Sheffield and Blob 2011) during terrestrial locomotion, but comparable analyses for the humerus have not been performed. Comparisons of locomotor loading between the humerus and femur of this species could offer specific insights with regard to the “mixed-chain” hypothesis because, in contrast to alligators, the humerus of *A. tigrinum* is slightly larger than its femur (present study), potentially leading to novel differences in the costs and safety factors associated with production/maintenance and movement of these bones.

To more broadly test the generality of “mixed chains” of safety factors between the humerus and femur, bone mechanical properties and loading mechanics during terrestrial locomotion were compared for the forelimb and hind limb of tiger salamanders. The relatively high safety factors previously evaluated for tiger salamander femora (~10; Sheffield and Blob 2011) suggest the potential for variation in this property between limb bones (Alexander 1997; Blob et al. 2014). However, by measuring whether the forelimb and hind limb experience different loads during terrestrial locomotion, it is possible to test whether the femur might bear greater stresses due to its greater contribution to propulsion (Kawano and Blob 2013), or whether the costs associated with the larger size of the humerus in *A. tigrinum* might lead to relatively lower safety factors for this element. Moreover, these data provide a model for inferring a potentially broader presence of a mixed chain of limb bone safety factors in quadrupeds with a

generalized *bauplan*, providing a context for evaluating transitions in the functional roles of the limbs among early tetrapods.

MATERIALS AND METHODS

Animals

Experimental trials were conducted on the same individuals used in a previous study (Kawano and Blob 2013) that evaluated the kinetics of fins and limbs during terrestrial locomotion. Tiger salamanders *Ambystoma tigrinum* Green 1825 were used as functional models for comparing the biomechanical capabilities of limbs to support propulsion on land in comparison with fins. Tiger salamanders had been selected because they are among the largest and most terrestrial salamanders that routinely move on land using their appendages (Kawano and Blob 2013), and have been suggested to share locomotor similarities to basal terrestrial tetrapods (Pierce et al. 2013). Following completion of experimental trials, animals were humanely euthanized with an overdose of buffered tricaine methanesulfonate (MS-222; 2 g/L), and frozen for subsequent measurements of bones and muscles. All experimental and animal care procedures were approved by the Clemson University IACUC (AUP2009-071 and AUP2010-066).

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

Information regarding the collection of synchronized 3D kinematic (movement)

and kinetic (GRF production) data have been documented previously (Sheffield and Blob 2011; Kawano and Blob 2013), but will be summarized with additional details below. Dorsal and lateral views of animals moving 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 appendages were recorded at 5000 Hz using a custom routine in LabVIEW (v. 6.1, National Instruments, Austin, TX, USA), and calibrated daily. An aluminum insert, measuring 4x9 cm, was installed into the force platform in order to constrain the contact area available to record force data, facilitating data collection from isolated appendages. All surfaces along the force platform were covered with shelf liner to provide a homogeneous substrate, a background grid in order to assess video distortion and alignment, and a substrate that would not cause damage to the sensitive skin of salamanders. 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 an LED light on the lateral view video file of each trial.

Quality control procedures were enforced to limit extraneous factors that could influence interpretation of the results. Trials were immediately excluded from consideration if the animal: (1) turned, stopped or fell on the force platform; (2) moved diagonally across the force platform; (3) did not have the distal portion of its appendage completely on the force platform; or (4) had other parts of its body (e.g., head, throat, belly) contact the force plate during stance. A second

round of quality control was performed after GRF data were processed. If the peak of the net GRF (summation of the vertical, mediolateral, and anteroposterior components of the GRF) occurred close (within ~5%) to 0% or 100% of stance, that trial was excluded from analysis because that likely indicated a spike from the animal falling on the plate as the animal shifted between its pectoral and pelvic appendages. Acceptable trials in which the animals moved at comparable speeds were then selected, with no significant differences between the forelimbs and hind limbs of *A. tigrinum*. For the trials selected for analysis, data were excluded during the portions of stance when the appendage of interest overlapped with another body part (e.g., touch-down of the hind limb during a forelimb trial), ensuring that the measurements of GRF, moments, and bone stresses reflected contributions from isolated appendages.

Kinematic variables were quantified by separately digitizing raw coordinate data from the dorsal and lateral (right) views of each trial with DLTdv3 in MATLAB (Hedrick 2008). AVI video files were cropped to contain only the frames observed during stance, the propulsive phase when the appendage is in contact with the ground. The joint and anatomical landmark points that were digitized in each salamander video included the hip/shoulder, knee/elbow, ankle/wrist, metatarsophalangeal/metacarpophalangeal joint, tip of the longest digit of the pes/manus, and two points along the midline of body that were almost immediately dorsal to the pelvic/pectoral girdles.

Data for force production and raw coordinates of the anatomical

landmarks were prepared to evaluate the stance phase of the locomotor cycles. To facilitate collection of coordinate data, every other frame was digitized for video files that were longer than 40 frames, producing a filming rate of 50 Hz. Otherwise, every frame was digitized. Kinetic data were processed in *R* (v. 3.1.0) to generate components of the GRF in the mediolateral, anteroposterior, and vertical directions, and angles of orientation in the mediolateral and anteroposterior directions. All magnitude values were converted to units of body weight (BW) to standardize for size differences across individuals. Data on GRF production were padded at the beginning and end to avoid edge effects (Smith 1989), and then filtered with a custom second order, zero phase, low-pass Butterworth filter using the *signal* package. Filter parameters were determined using custom specifications, with normalization to Nyquist frequency to prevent aliasing of data (Smith 1997). Following smoothing and filtering procedures, all data were then interpolated to 101 points with a cubic spline using the 'spline' option of `signal::interp1`. Standardization to 101 points allowed for the analysis of data throughout stance at 1% increments (0% = beginning of stance, 100% = penultimate frame to the swing phase), and facilitated direct comparison between kinematic and kinetic data. Ultimately, 48-50 trials were included for analysis from each group (salamander hind limb and salamander forelimb, respectively), with about ten trials from each of five individuals within a given group.

Digitized coordinates were then processed for kinematic analysis. Raw coordinate files were smoothed with a quintic spline through

pspline::smooth.Pspline. Generalized cross-validation was not used because it has been found to be unreliable for high-speed videos (Walker 1998). Instead, smoothing parameters were quantified to match the variability of each given variable, in order to create a smoothing algorithm that was appropriate for the specific characteristics of this dataset. Smoothing parameters were determined by having a single person (S.M.K.) digitize the first ten frames of a single trial for each limb group, and then repeat the process three times. Dorsal and lateral views for a given trial were evaluated separately. The variance amongst the three repeated digitizing attempts was then taken as the smoothing parameter for each video file (e.g., dorsal vs. lateral) for a given group, and a separate smoothing parameter was calculated for each anatomical landmark in each perspective (dorsal and lateral views).

Calculation of bone stresses

Bone stresses were evaluated using conventions established to maintain the anatomical planes of the appendicular bones throughout stance for sprawling animals, accounting for the rotation of appendicular bones during stance (Blob and Biewener 2001; Butcher and Blob 2008; Sheffield and Blob 2011). Analyses of bone stresses focused on the mid-shaft of the humeri and femora, 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; Sheffield and Blob 2011).

A biomechanical model for calculating locomotor stresses in the femur of *A. tigrinum* that was developed in a previous study (Sheffield and Blob 2011), was applied to the current data and modified for the forelimb. Although previous data on the loading of *A. tigrinum* hind limbs during terrestrial locomotion are available (Sheffield and Blob 2011), new data were collected for the present study in order to directly compare the functional capabilities of the forelimbs and hind limbs within the same individuals. This was particularly appropriate because the salamanders used by Sheffield and Blob (2011) were slightly larger than those used in this study, potentially complicating comparisons of forelimbs from one group with hind limbs from another.

In addition to accounting for stresses imposed on limb bones by the GRF, models evaluated the contributions of limb muscles to bone stress in response to moments imposed by the GRF. In order to calculate muscular contributions towards bone stresses, joints were measured to be in static rotational equilibrium (Biewener 1983). Consequently, muscle forces (F_m) could be calculated using the following equation:

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

where R_{GRF} is the moment arm of the GRF relative to the joint, GRF is the ground reaction force data obtained from the force platform analyses (Kawano and Blob 2013), and r_m is the moment arm of the muscle needed to counter the GRF moment about the joint. Moment arms of the muscles were determined through direct measurements, obtained with digital calipers while holding the limb in a

mid-stance orientation.

A detailed description of the biomechanical model being used to assess femoral loading has been documented previously (Sheffield and Blob 2011), so focus here is placed on describing modifications for modeling bone loads in salamander humeri. Data on the activity patterns of forelimb muscles in salamanders during terrestrial locomotion are limited, with a single study on the dorsalis scapulae, extensor ulnae (i.e., anconeus), and the latissimus dorsi providing the some of most extensive data currently available (Delvolvé et al. 1997). Consequently, patterns of muscle activity in the forelimb of *A. tigrinum* were based on presumed functions presented by Walthall and Ashley-Ross (2006), as well as direct observations of the anatomy of *A. tigrinum*. Only muscles that are likely active during stance were incorporated into the biomechanical model. In addition, only muscles that spanned the mid-shaft were considered to contribute to bending stresses at this location, where stress analyses were performed in this study (Blob and Biewener 2001; Sheffield and Blob 2011). Thus, although humeral protractors may be active during stance for stabilization, because these muscles (e.g, dorsalis scapulae, procoracohumeralis, humeroantebrachialis) insert at the proximal end of the humerus in salamanders, their contributions to bone stresses were assumed to be negligible. Similarly, since humeral adductors (e.g., pectoralis and supracoracoideus) do not span the mid-shaft, they also likely do not contribute substantially to humeral stresses, even though they contribute towards

generating moments about the shoulder. Although muscles that attach proximally or distally to the mid-shaft of the bone, but do not span it, contribute to moments at the limb joint(s), it is uncertain if these muscles contribute to bending stresses (and to what extent) at the mid-shaft. Rather than making subjective estimates about what proportion of the bone stresses they accounted for, which could introduce error, their contributions to bone stresses were assumed to be negligible, following conventions used in previous studies (Biewener 1983; Blob and Biewener 2001; Sheffield and Blob 2011). Future studies could assess to what extent these additional muscles could contribute towards bone stresses.

Muscles that were expected to contribute to bone stresses at the humerus included wrist extensors, elbow extensors, and humeral retractors. Although other muscles may be considered retractors, coracobrachialis longus (CBL) was the only retractor muscle presumed to contribute to bone stresses since the other muscles (e.g., latissimus dorsi, dorsalis scapulae) did not span the mid-shaft of the humerus. 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 plantarflexors of the carpus (DCF). All four muscles were assumed to be active to oppose the moment of the GRF tending to dorsiflex the wrist. In addition, three of these muscles (FDC, FACU, and FACR) also span the extensor aspect of the elbow joint. Thus, the fraction of total wrist extensor force generated by these muscles, estimated based on their fraction of the total physiological cross-sectional area (PSCA) of the wrist extensors

(Biewener 1983; Sheffield and Blob 2011), also contributes to elbow extension. This is significant for humeral stresses, because it means that these muscles that do not span the humeral mid-shaft reduce the force that primary elbow extensor muscles must generate to counter the elbow flexor moments typically imposed by the GRF (e.g., anconaeus complex, which does span the humeral mid-shaft and contributes to stress). It is also a distinction from models of hind limb muscle function, in which ankle extensors spanning the knee joint add to its flexor, rather than extensor moment, often requiring elevated (rather than reduced) forces from knee extensor muscles (Sheffield and Blob 2011). The elbow extensors also included the four bundles of the anconaeus, which were subdivided into two functional units due to their anatomical positions: anconaeus scapularis medialis and anconaeus coracoideus (ASMAC), and anconaeus humeralis lateralis and anconaeus scapularis medialis (AHLASM). Finally, two muscles were considered to act as humeral retractors: latissimus dorsi (LAT) and coracobrachialis longus (CBL). While both contributed to countering protractor moments imposed by the GRF at the shoulder, only CBL spans the mid-shaft, so only its portion of total retractor force (based on its fraction of retractor PCSA) was considered to impose stress on the humeral mid-shaft. If more than one muscle was determined to counteract the GRF to maintain equilibrium at the joint, a mean moment arm was calculated for the group weighted by the PCSAs of the contributing muscles (Alexander 1974; Biewener 1983; Sheffield and Blob 2011).

Forces acting on the humerus and femur were resolved into axial and transverse components. These were combined with geometric data (bone length, cross-sectional area, second and polar moments of area, and r_c , the bending moment arms imposed by shaft curvature: see Table 4.1) to calculate axial compressive stress and bending stresses in the anteroposterior plane ($\sigma_{b:AP}$, influenced by humeral retractors) and dorsoventral plane ($\sigma_{b:DV}$, influenced by elbow extensors). The magnitude of the net bending stress at the mid-shaft was calculated using the following equation, in both the dorsoventral (DV) and anteroposterior (AP) anatomical planes:

$$\alpha_{b:net} = \tan^{-1}(\sigma_{b:DV}/\sigma_{b:AP})$$

which quantifies the orientation of the peak stress relative to the anteroposterior axis. The neutral axis of a structure is a region where neither compression or tension occur, and is an important measure because the further away a structure is from the neutral axis, the better able it is to withstand bending (Vogel 2003). The net neutral axis of bending can be determined as being perpendicular to the axis of peak stress (Sheffield et al. 2011).

In addition to bending, twisting motions can also impose torsional loading on the bones (Currey 2002). Torsional stresses (τ) produced by the GRF can be calculated as:

$$\tau = T(y_t/J)$$

where T is determined by calculating the orthogonal distance of the GRF vector relative to the long axis of the limb bone, y_t is the deviation of the centroid from

the bone cortex (see Table 4.1), and J is the polar moment of area, 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

Given the relatively small size of the animals, the bones were embedded in a resin to facilitate sample preparation for mechanical testing. Humeri and femora from the right side of the body were sectioned by embedding the bone in Caroplastic (Carolina Biological, Burlington, NC), a non-infiltrating resin, and then cutting transversely at the mid-shaft with a bandsaw. The cut surface was then polished to improve visualization of the cross-sectional geometry, and to prepare the bone for subsequent testing of mechanical properties. Embedded specimens were affixed to a 100x61x2 mm Plexiglas slide with cyanoacrylate glue, and then loaded onto an automated polishing machine (EXAKT Technologies, D-4000, Oklahoma City, OK, USA). Samples were first smoothed with moistened silicon carbide paper of decreasing grit sizes (P800, P1200, P2500, P4000), at 5 mins for each grit size. Agglomerate-free alumina polishing suspensions were then used to polish the specimens further to 3.0 μm (Baikalox Type 3.0 CR Alpha), 0.3 μm (Baikalox Type 0.3CR Alpha), and then finally to 0.05 μm (Buehler Micropolish II) using a polishing pad (Buehler, Lake Bluff, IL, USA) for 3 mins at each step. The 0.05 μm suspension was prepared by mixing 25 g of Micropolish II powder with 100 mL of distilled water, and then mixing for 5-10 mins to produce

a homogenous mixture. All polishing steps were set at grinding and oscillation speeds of 30 rpm, with a 99.3 g weight applied. The sample was rinsed with deionized water after each step of polishing in order to remove particulates that could scratch the surface. Upon completion, samples were air dried, and then stored in a -20° freezer until needed for mechanical testing. Prior to indentation, samples were allowed to equilibrate to room temperature and were cleaned with methanol.

Mechanical properties of the limb bones were obtained through microindentation. Hardness was measured using a Digital Display Microhardness Tester (Model HVS-1000B, Beijing, China) equipped with a Vicker's indenter tip, and configured with a load of 0.49 N and a dwell time of 15 secs. Five indents were performed in the dorsal, ventral, anterior, and posterior regions of the cross-section to test for regional heterogeneity in mechanical properties. Bending of the bone is more dependent on the regional heterogeneity of material properties since bones will fail in bending at the weakest regions of the bone (Currey 2002). Care was taken to perform indents away from cavities and the edges of the bone in order to avoid potential edge effects. Additional indents were also performed in the Caroplastic and the interface between Caroplastic and bone, providing baseline data on the hardness of the bone, surrounding resin matrix, and the transition between the two. Hardness values were based on five femora and four humeri, all originating from the same animals used for measurements of ground reaction forces (Kawano

and Blob 2013) and *in vivo* bone stress (this study).

Mechanical testing and evaluation of bone geometry were performed on the distal halves of the limb bones. To evaluate the strength of the limb bones, Vickers hardness (H_v) data were collected and entered into a published linear regression (Wilson et al. 2009) to calculate tensile yield stress (σ_y):

$$\sigma_y = 32.571 + 2.702 * H_v$$

Although bending is the most common reason for bone failure, focus was placed on tensile strength because bones are usually weaker in tension and failure tends to occur on the side of the bone where tension is producing during bending (Currey 2002). However, in line with tests on regional heterogeneity, assessments of compressive yield stress were also performed. Measures of compressive yield stress are not available for salamanders, but estimates can be calculated based on the evaluation that tensile yield stresses are 25% lower than compressive yield stresses, on average (Currey 1985). Safety factors (SF) were then calculated as:

$$SF = \sigma_{\text{yieldstress}} / \text{mean peak stress},$$

and “worst-case” scenario estimates (SF_{WC}) were produced as:

$$(SF_{WC}) = [\sigma_{\text{yieldstress}} - 2 * SD(\sigma_{\text{yieldstress}})] / \text{mean peak stress} + 2 * SD(\text{mean peak stress}).$$

Hardness values were found to differ in the four anatomical regions tested, so results for hardness, yield stress, and safety factor will be reported separately for each of the anatomical regions. Calculations of yield stresses and safety factors were based on dorsal and posterior regions being loaded in tension, and the

anterior and ventral regions loaded in compression.

Statistical analyses

Linear mixed effects models (LMMs) fitted by Maximum Likelihood (`lme4::lmer`) were used to test for differences amongst groups, with individual treated as a random effect (Bates et al. 2014). Tests for regional heterogeneity of hardness values within a bone were performed using a LMM with anatomical region (dorsal, ventral, posterior, anterior) treated as a fixed effect. All other comparisons were conducted using LMMs with limb bone (humerus or femur) as a fixed effect. Significance of the variables was assessed by determining whether the full LMM was a better fit model than a null model (with individual as a random effect), based on AIC and BIC values. Tukey post-hoc comparisons (`lsmeans::lsmeans`) on the least-square means were then used to perform pairwise comparisons (Lenth 2014).

RESULTS

Kinematic comparison of forelimbs and hind limbs

Although the forelimbs and hind limbs share some general kinematic profiles, numerous differences were found (Fig. 4.1). At the beginning of stance the shoulder and hip are slightly adducted (~10-15°), with the wrist and ankle starting initially flexed to a similar extent. The femur is slightly more protracted than the humerus, and the elbow more flexed than the knee. Flexion and extension of the

knee and elbow follow a similar profile: however, the ankle becomes flexed about twice as much as the wrist towards mid-stance. Another major difference between the two appendicular systems is that the femur remains in an adducted orientation (knee closer to the ground than the hip) through the entire course of stance, but the humerus shifts to an abducted orientation (elbow higher than shoulder) after about 30% of stance. Additionally, although both the femur and humerus begin in a protracted orientation (i.e., distal joint is cranial to the

Table 4.1. Comparison of anatomical data from the forelimbs and hind limbs of *A. tigrinum*

	Humerus	Femur
Length (mm)	15.244 ± 0.463	14.906 ± 0.478
Cross-sectional Area (mm ²)	1.007 ± 0.201	0.879 ± 0.343
Moment arm due to curvature (AP; $r_{c(AP)}$) (mm)	0.099 ± 0.056	0.040 ± 0.031
Moment arm due to curvature (DV; $r_{c(DV)}$) (mm)	0.349 ± 0.128	0.138 ± 0.103
Distance from neutral axis to cortex (AP; y_{AP}) (mm)	0.703 ± 0.044	0.613 ± 0.029
Distance from neutral axis to cortex (DV; y_{DV}) (mm)	0.684 ± 0.031	1.000 ± 0.077
Second moment of area (AP; I_{AP})(mm ⁴)	0.134 ± 0.048	0.201 ± 0.107
Second moment of area (DV; I_{DV})(mm ⁴)	0.191 ± 0.072	0.131 ± 0.048
Polar moment of area (J^1) (mm ⁴)	0.325 ± 0.118	0.333 ± 0.154

Values are means ± SD (N=5 individuals for each group).

AP = anteroposterior direction; DV = dorsoventral direction.

For $r_{c(AP)}$: positive means concave side is posterior; negative means concave side is anterior.

For $r_{c(DV)}$: positive means concave side is ventral; negative means concave side is dorsal.

¹J = $I_{AP} + I_{DV}$ (Lieberman et al. 2004)

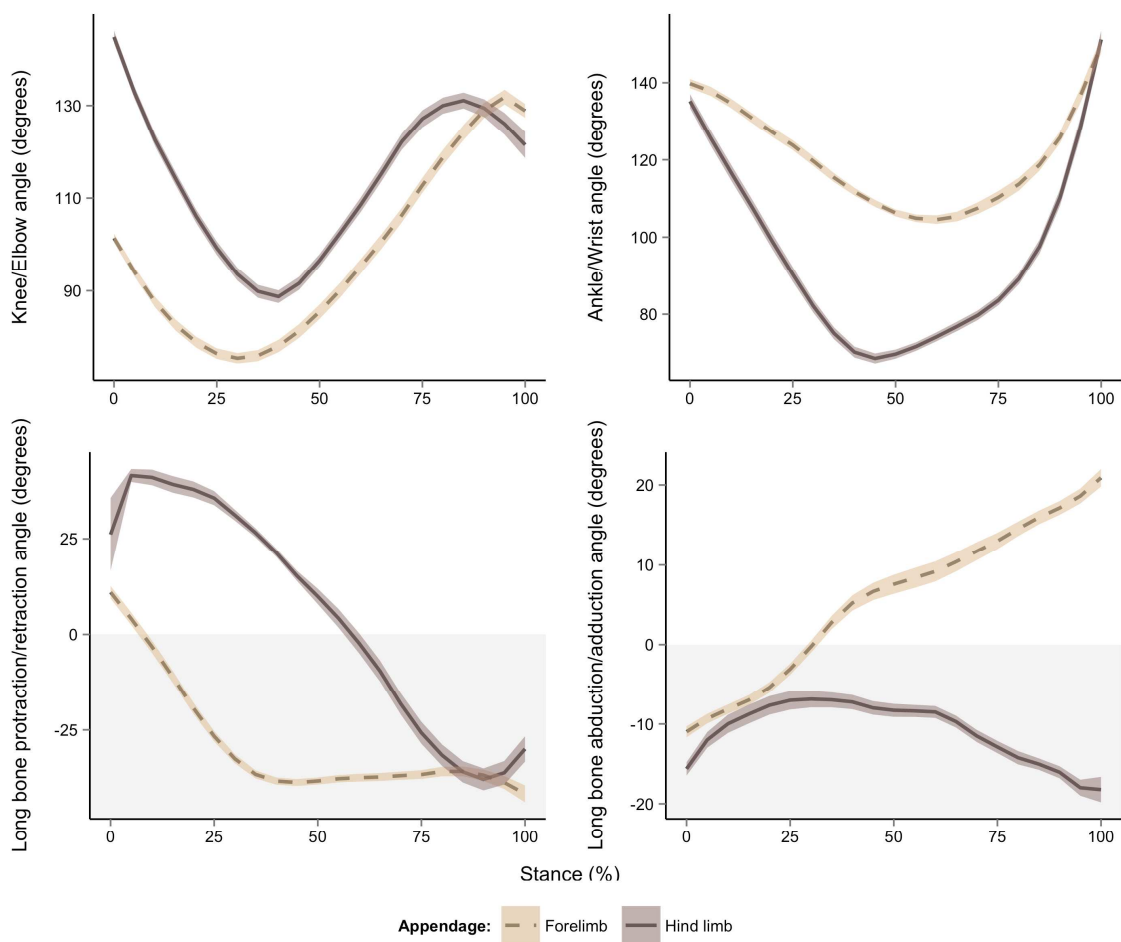


Fig. 4.1. Comparison of the kinematic profiles of the forelimbs and hind limbs during stance. The lines represent the mean pooled across all trials for the hind limbs ($N=48$) and forelimbs ($N=50$), with the shading depicting the standard error. Grey rectangles highlight the negative values, which indicate retraction and abduction in the bottom two plots, respectively.

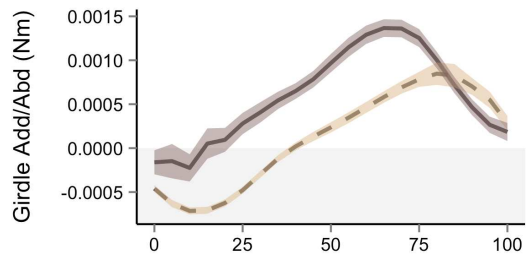
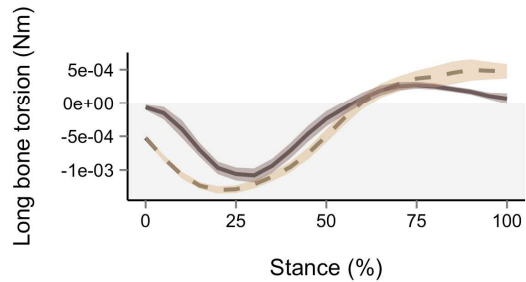
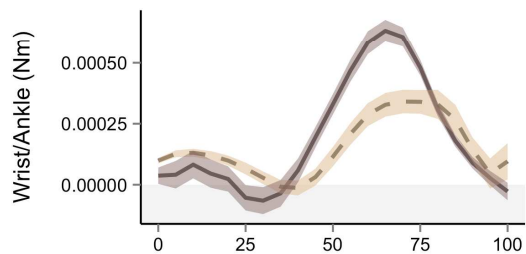
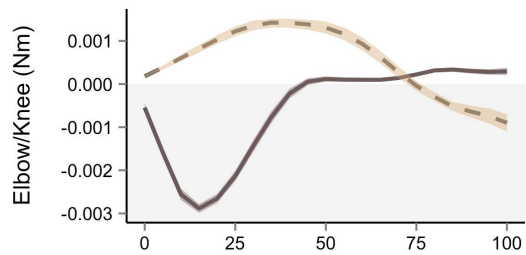
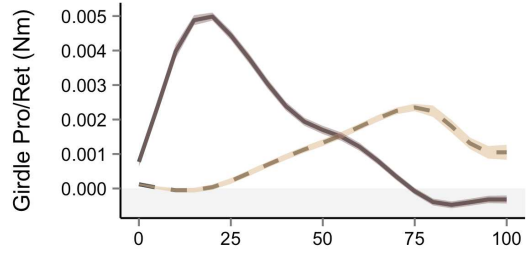


Fig. 4.2. Comparison of the moments exerted by the GRF. Girdle refers to the shoulder and hip. Pro=protraction, Ret=retraction, Add=adduction, Abd=abduction.



Appendage: — Forelimb — Hind limb

hip for almost all of the stance phase, proximal joint), the humerus is initially nearly perpendicular to the long axis of the body (0° in Fig. 4.1) and rotates to a retracted orientation very early in stance, whereas retraction of the femur is much more evenly split between protracted and retracted orientations, with a shift close to half way through stance (Fig. 4.1).

Moments produced by the GRF about the limb joints

In addition to the numerous similarities found between the patterns of GRF production in the forelimbs and hind limbs of these *A. tigrinum* (Kawano and Blob 2013), some similarities in the moments imposed on the bones by the GRF were also observed (Fig. 4.2). For instance, the GRF imposes a dorsiflexion (positive values) moment about the wrist and ankle due to the anterior position of the GRF relative to these joints. In order to maintain equilibrium at these joints, wrist and ankle extensors would need to be active. The primarily vertical orientation of the GRF throughout stance (see Fig. 2 in Kawano and Blob 2013) tends to impose an abductor moment on both the shoulder and though for the hip this moment shifts to become a marginally adductor moment late (>75%) in stance. The GRF also imposes a protractor moment about both the shoulder and hip for almost all of stance, though this is greater for the hip. Finally, torsional moments imposed by the GRF are very similar between the humerus and femur.

Despite these similarities, the different configurations of the forelimb and hind limb also contribute to a strong distinction in how the GRF imposes

moments on these limbs. 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 (Kawano and Blob 2013). As a result, the flexor/extensor moment of the GRF tends to change in different directions for these two joints during stance, shifting from a flexor to an extensor moment at the knee (see also Sheffield and Blob 2011), but from an extensor moment to a flexor moment at the elbow (Fig. 4.2). The shift between flexion and extension, however, occurs at almost the exact same time in stance for these two joints, at almost 75% (Fig. 4.2).

Comparison of the bone stresses

Lower bone stresses were observed for the humerus for all loads, although to a lower extent for shear (Table 4.2). For the forelimb, the timing of the peak tensile stress occurred a little earlier than mid-stance (~40%) while the timing of the peak compressive stress occurred much later in stance (~65%). For the hind limb, the disparity in the timing of these events was much greater: peak tensile stress occurred at about 60% of stance and peak compressive stress at ~18% of stance. Such a pattern may correspond with the patterns of the vertical component of the GRF, which was found to occur later in stance (~61°) for the forelimb than the hind limb (Kawano and Blob 2013). The orientation of the neutral axis of bending (Fig. 4.3) at the time of peak tensile stress for each limb was directed such that the posterodorsal region was loaded in tension and the

anteroventral region was loaded in compression, due to the negative value of the neutral axis angle relative to the anteroposterior plane, except for the femur at 50% of stance (Fig. 4.3). At 50% of stance, the femur shifts so that the anterodorsal region is loaded in tension.

Mechanical properties and safety factors of salamander humeri and femora

Hardness values ranged from 16.200 ± 0.908 (Caroplastic), to 15.500 ± 8.100 (transition between Caroplastic and bone), to 25.105 ± 0.305 (outer edge of bone), and 38.101 ± 0.455 (bone). These values indicate a distinct separation between Caroplastic and bone, with an intermediate value for the transition point between the two materials, providing verification that the hardness values obtained for the salamander humeri and femora are characteristic of bone material and not the surrounding medium. Comparisons of hardness values from the humerus and femur indicated differences between these bones, as well as regional heterogeneity within each bone (Fig. 4.4). The greatest hardness (and thus tensile yield stress) values were generally found in the posterodorsal region of the bone at mid-shaft (Table 4.3, Fig. 4.4), typically corresponding with the location of tensile loads about the neutral axis of bending (Fig. 4.3).

Estimates of femoral safety factor ranged from 9.1-10.4 across the different regions of the bone (Table 4.3), corresponding closely with the previously published estimate of 10.5 (Sheffield and Blob 2011). However, safety factor estimates for the humerus were almost twice those of the femur,

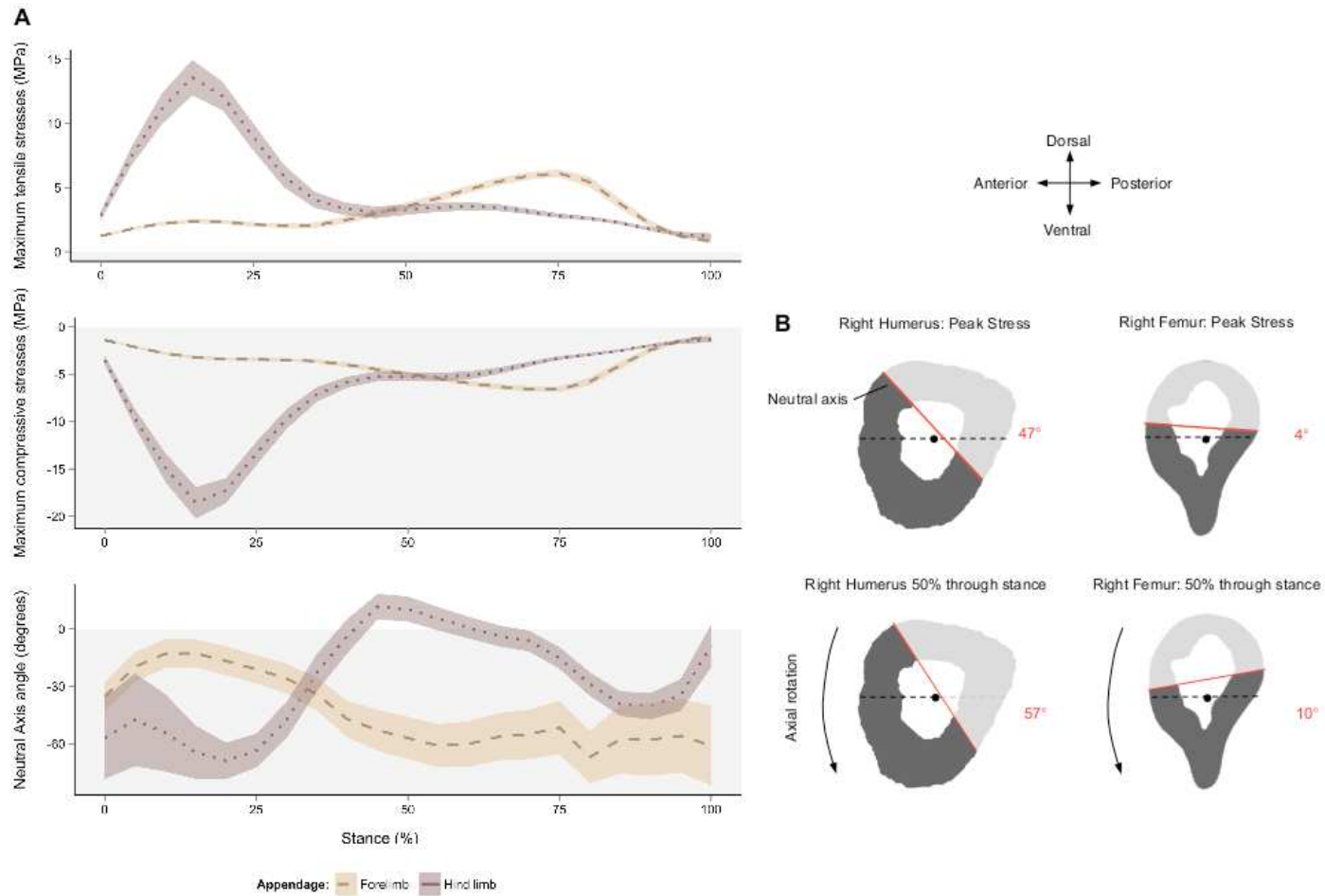


Fig. 4.3. (A) Maximum tensile (top) and compressive (middle) stresses, and the neutral axis angle from the anatomical AP axis (bottom). (B) Illustrations of the neutral axis angle (red line) relative to the AP axis (dashed line) at peak tensile stress (top) and at 50% of stance (bottom). Dark regions of the bone are in compression, and light regions are in tension.

Table 4.2. Timings and magnitudes of peak stresses in *A. tigrinum* limb bones

	Forelimb	Hind limb	AIC_{null}	AIC_{full}
Peak tensile stress (MPa)*	6.970 ± 0.288	12.505 ± 1.051	633.007	610.709
Peak compressive stress (MPa)*	-7.370 ± 0.297	-17.294 ± 1.305	694.719	650.857
Peak axial stress (MPa)*	-0.936 ± 0.062	-2.495 ± 0.161	310.028	250.270
Peak shear stress (MPa)	-3.284 ± 0.167	-3.704 ± 0.360	411.755	412.764
Time of peak tensile stress (%)	40.480 ± 4.616	59.667 ± 4.328	966.412	959.480
Time of peak compressive stress (%)*	64.6 ± 1.956	17.875 ± 0.689	919.653	692.185
Time of peak shear stress (%)*	24.560 ± 1.482	29.938 ± 1.841	766.185	755.645

Values are means ± SE (n=50 trials averaged across five individuals for the forelimb and n=48 for the hind limb). Timings of peak stresses are represented as a percentage into the stance phase of the limb cycle. Asterisks (*) indicate differences between the limbs that were greater than expected by chance.

Table 4.3. Regional heterogeneity of hardness values and safety factor across limb bones in *A. tigrinum*

	Humerus				Femur			
	Anterior	Dorsal	Posterior	Ventral	Anterior	Dorsal	Posterior	Ventral
Hardness (H_v)	36.3 ± 0.9	41.7 ± 1.5	44.4 ± 1.2	36.6 ± 0.9	33.7 ± 1.2	36.0 ± 1.1	34.6 ± 0.9	31.5 ± 1.1
Mean yield stress (MPa) ¹	174.1 ± 3.3	145.2 ± 4.0	152.6 ± 3.2	175.4 ± 3.2	164.8 ± 4.2	129.8 ± 3.0	126.1 ± 2.4	156.7 ± 4.1
Overall SF	23.6 ± 0.4	20.8 ± 0.6	21.9 ± 0.5	23.8 ± 0.4	9.5 ± 0.2	10.4 ± 0.2	10.1 ± 0.2	9.1 ± 0.2
CV of SF	0.092	0.136	0.095	0.085	0.128	0.117	0.095	0.128
Worst case SF	17.8 ± 0.4	9.6 ± 0.4	11.2 ± 0.3	18.3 ± 0.4	6.2 ± 0.2	3.7 ± 0.1	3.8 ± 0.1	5.9 ± 0.2

¹For dorsal and posterior regions (under tension), calculated using the equation: $32.571 + 2.702 \cdot H_v$. For anterior and ventral regions (under compression), calculated as (tensile yield stress)/0.75.

CV = coefficient of variation. Values represent means ± SE.

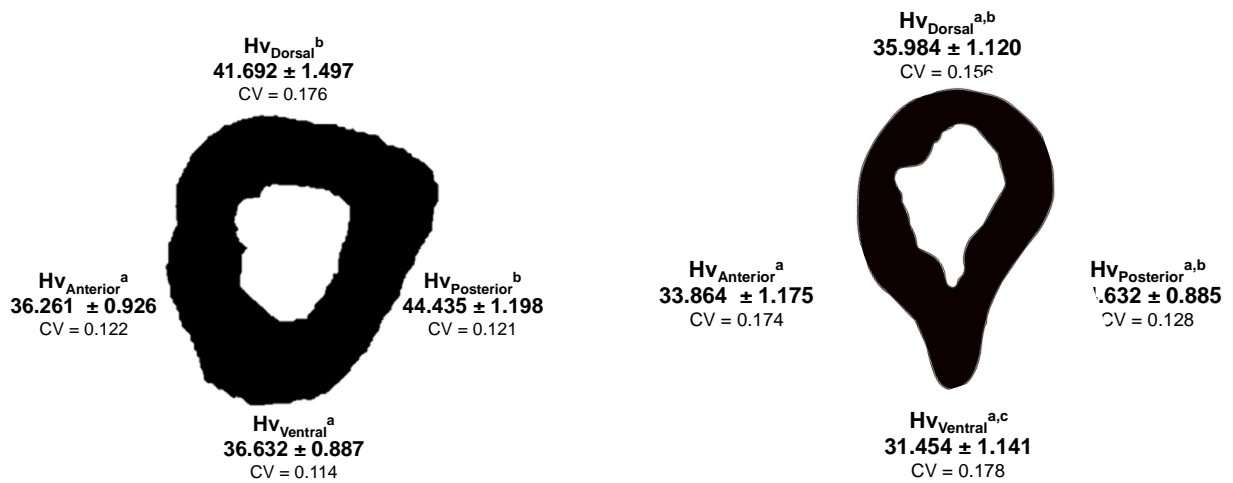


Fig. 4.4. Regional heterogeneity in hardness values was found in both the humerus and femur. CV = coefficient of variation. Hv=Vickers hardness value.

ranging from 20.8-23.8. This difference was largely due to the considerably lower stresses to which the humerus was exposed (Table 4.2), although higher yield stresses in the humerus also contributed to safety factor differences from the femur (Table 4.3). Worst-case scenario estimates of safety factor were considerably lower for both bones, but still indicated ample margins of safety (9.6-18.3 for the humerus, and 3.7-6.2 for the femur: Table 4.3).

DISCUSSION

Comparisons of safety factors for the humerus and femur of tiger salamanders provide an additional empirical example of a “mixed chain” (Alexander 1997) within the locomotor skeleton of tetrapods. Although mixed chains of safety

factors were previously identified between proximal and distal limb bones in horses (Currey 2002) and iguanas and alligators (Blob and Biewener 1999), data from the present study show patterns more like those of alligators (Blob et al. 2014), which characterized different safety factors between the proximal bones of the forelimb versus the hind limb. As described for alligators (Blob et al. 2014), the humerus had a higher safety factor overall than the femur did in salamanders (Table 4.3). However, the difference between these bones was much greater in salamanders (~22 for the humerus versus ~10 for the femur: Table 4.3) than in alligators (8.4 for the humerus versus 6.3 for the femur: Blob et al 2014). In addition, some of the factors proposed by Alexander (1997) that might contribute to differences in safety factor between these bones in alligators do not seem likely to apply to salamanders. For example, unlike alligators, in which the humerus is smaller than the femur and might allow for more economical maintenance of a high safety factor (Blob et al. 2014), in salamanders, the humerus is similar in size or slightly larger in size than the femur (Table 4.1). However, similarly to alligators, load magnitudes do not appear to be substantially more variable for the salamander humerus than for the femur (Table 4.2), suggesting that protection against occasional high peak loads was not a major contributing factor to adaptive elevation of humeral safety factors.

Safety factors for salamander limb bones, like those of alligators, are generally high compared to many taxa (Blob et al. 2014). Thus, differences between humeral and femoral safety factors for salamanders might simply reflect

an increased opportunity for variation in safety factors across the skeleton (Alexander's third condition proposed to lead to mixed chains). Though this reason has been invoked as a factor contributing to mixed chains in alligators (Blob et al. 2014), it may not apply as well to salamanders, which likely have a mechanistic reason for higher safety factors. Not only is the difference between humeral and femoral safety factors much greater for salamanders than for alligators, this difference resulted from a combination of both lower stresses and stronger bone mechanical properties for the salamander humerus compared to the femur. Factors contributing to low humeral stresses in salamanders include the configuration of the forelimb joints and the disposition of forelimb muscle groups. Because of the range of motion of the arm (Fig. 4.1) and orientation of the elbow, the GRF only exerts a flexor moment at the elbow late in stance (Fig. 4.2). This reduces the need for elbow extensors (e.g., anconeus complex) to exert force to counter GRF moments at the elbow, reducing the stress they place on the humerus. Such stresses are further reduced by contributions of wrist extensors that do not span the humeral mid-shaft (e.g. FDC, FACR, FACU, DCF) to elbow extension; in addition, the largest adductor muscles contributing to forelimb movement insert far proximally on the humerus (e.g. pectoralis), further reducing the stresses experienced at the mid-shaft of the bone. Despite these intrinsic stress-reducing characteristics of forelimb design, bone material of the humerus is stronger than that of the femur (Table 4.3), with regional heterogeneity exhibiting different patterns in the bones. The regions with the

highest safety factors corresponded with the areas of the bone that are loaded in tension (dorsal and posterior) for the femur, but compression (anterior and ventral) for the humerus. Moreover, whereas the femur had a larger second moment of area in the anteroposterior direction (I_{AP}) compared to the humerus, the humerus had a greater second moment of area in the dorsoventral direction (I_{DV}) (Table 4.1). These data suggest that these limb bones show structural as well as material modifications to reduce bending stress in different directions. Given that the forelimbs may also be used for antagonistic interactions and burrowing, there is also the possibility that higher safety factors were observed in the humerus because it serves functions in addition to locomotion. Collectively, the incidence of elevated structural and material reinforcement against loads, despite anatomical features of the forelimb promoting low load magnitudes, suggests that stochastic variation associated with large safety factors may not completely account for differences in safety factor observed between the humerus and femur in salamanders.

In addition to the three conditions promoting mixed chains of safety factors proposed by Alexander (1997), higher safety factors may be found in structures that have higher penalties for failure (Diamond 2002). This perspective lends interesting insight into the mixed chain of safety factors in salamander limb bones, and the different role that the forelimbs play in legged locomotion in comparison to the hind limb. Although the hind limbs are the primary propulsors in many non-mammalian quadrupeds, the forelimbs still have an important

locomotor function and forelimb loss may have more detrimental effects than the loss of the hind limbs. Early work on salamander locomotion by Evans (1946) suggested that the forelimbs alone could produce forward propulsion whereas terrestrial locomotion using only the hind limbs was largely ineffective, suggesting that forelimbs play a more important locomotor role than merely passive body support (at least in more terrestrial salamanders such as *Taricha* and *Ambystoma*). It is also interesting to note that there do not appear to be ready examples (among non-bipedal vertebrates) in which loss of the appendages occurred in the pectoral appendages while the pelvic appendages remained fully intact. If a vertebrate animal completely loses an appendicular system, it is typically the hind limbs (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 an iconic stage 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). Additional studies would be required to investigate whether there is a strong mechanical or other selective advantage for forelimb retention in non-bipedal vertebrates, or whether the conservatism of forelimb retention is due to developmental constraint. For instance, the hind limbs develop after the forelimbs (Tanaka and Tickle 2007) and structural reduction is found to occur in the reverse order from which the structures are developed (Lande 1978), potentially making hind limbs more susceptible to loss

via developmental truncation.

Further investigations of how loads vary across regions of limb bones could yield powerful insights into the morphological evolution of limb bones as vertebrates became terrestrial, because functional innovations in the structural integrity of bones may have contributed towards the successful invasion of land. The musculoskeletal system of vertebrates shifted from being essentially weightless due to buoyancy in aqueous environments to having to counteract the effects of gravity on land, resulting in a major shift in 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 2002). A better understanding of additional morphological changes in limb morphology may also be important in reconstructing the transition from water to land by tetrapods. For example, why was the ventral ridge, a process supporting substantial muscle attachment on the humerus, prominent in early stem tetrapods, such as *Sauripterus* and tristichopterids (Kawano *pers. obs.*), but relatively small in more terrestrial crown tetrapods? Further application of data on locomotor stresses from extant taxa could help answer many questions regarding the functional consequences of morphological patterns observed in extinct tetrapodomorphs spanning the transition from water to land.

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CHAPTER FIVE

TAKING THE NEXT STEP FORWARD: MODELING THE LOCOMOTOR CAPABILITIES OF FOSSIL TETRAPODOMORPHS

ABSTRACT

The integration of biomechanics, paleontology, evolutionary biology, engineering, mathematics, and computational science has greatly enhanced our ability to understand the evolutionary patterns observed in the fossil record. The synergism of these seemingly diverse fields has provided the opportunity to explore new avenues that were previously unavailable. Investigations of the morphology of fossil taxa help to generate hypotheses about the ultimate causation for evolutionary changes, which can be tested by applying biophysical principles that have been gleaned from living taxa. Modern analogs to fossil taxa contribute important insight because they represent multiple adaptive strategies for assuming a similar function, and allow direct measurements of a variety of parameters. A brief description is provided to highlight some of the major strides made in interpreting the biology of fossils from early tetrapods, with specific insight into the biomechanical factors that could have contributed towards the evolution of terrestrial locomotion.

INTRODUCTION

In her book, Gaining Ground: the Origin and Evolution of Tetrapods, Professor Jenny Clack aptly described how the journey towards understanding how vertebrates became terrestrial was a rather slow process, much like the evolutionary transition itself (Clack 2002). Only relatively recently has the pace of unraveling this enigma of our evolutionary history begun to pick up through the discovery of new fossil material (Shubin et al. 2006, 2014; Cloutier 2013), and the implementation of new analytical technologies and computational methods designed to glean novel information from existing fossil collections (Pierce et al. 2012; Sanchez et al. 2014). With this continually improving arsenal of tools, scientists are better primed than ever to decipher the clues left as long as 400 million years ago about the obstacles vertebrates faced before they could live on land.

Integrative approaches are a key for examining how the evolution of terrestrial locomotion transpired. The synergism of complementary approaches from diverse fields (e.g., evolutionary biology, paleontology, mathematics, computer science, engineering) can often yield more novel insights than any individual field alone. Advances in computer simulation and animation can reconstruct the movement of extinct taxa by applying biomechanical and form-function relationships derived from modern analogs, allowing us to view paleontological evidence in a new light (Hutchinson and Gatesy 2006). Gould (1989) wrote that if we could replay the tape of life, a different story would unfold

due to historical contingency. Expanding upon this metaphor, in order to better understand evolutionary events, we can also re-enact the “tape of life” using players from today. Extant fishes, amphibians, and non-avian reptiles that walk on land can act as analogues for early tetrapods and tetrapod-like fishes (Ashley-Ross and Bechtel 2004; Graham and Lee 2004; Kawano and Blob 2013; Pierce et al. 2013; Nyakatura et al. 2014; Pace and Gibb 2014), and can demonstrate different evolutionary trajectories for invading the terrestrial realm. Walking fishes, amphibians, and reptiles provide useful modern analogs to encompass the progression from early stem tetrapods that were still somewhat “fish-like” to some of the first tetrapods that achieved full terrestriality. Computational techniques can combine the morphology of fossils with empirical data from living species to estimate the biomechanical limitations of extinct taxa, and how changes in musculoskeletal design paved the way for tetrapods to conquer land.

The combination of paleontological analyses and information gleaned from extant species has served as a powerful “one-two” punch for providing insight into the biology of extinct taxa. Extensive analyses of the microanatomy of a wide range of tetrapods and characterization of limb bone elements by Laurin and colleagues have greatly enhanced our knowledge of how limb bone morphology can serve as an indicator of life history ecology (Laurin et al. 2004, 2007, 2011; Canoville and Laurin 2009; Meunier and Laurin 2012). This impressive database of cross-sectional geometries across hundreds of vertebrates ranging from salamanders to camels has helped to establish the

histological differences between aquatic vs. amphibious/terrestrial tetrapods that can serve as a guide for a better understanding of the ecological niches that fossil taxa may have occupied. The integration of additional cross-sectional geometry measures (e.g., second moment of area) could help to further clarify the biomechanical differences between amphibious and terrestrial taxa. If successful, such data could be useful for evaluating the extent to which early stem tetrapods could support terrestrial excursions. By considering the functional morphology of a broad range of living taxa in concert with extinct taxa, one may gain perspective on the characteristics of the musculoskeletal system that remain relatively conserved over evolutionary time and taxonomic units, thus, establishing a baseline from which to infer how morphological differences could have conferred different functional capabilities. In addition, the estimation of moment arms in the limb bones of various fossil taxa has helped to answer a variety of questions about functional evolution (Maidment et al. 2013), such as the evolutionary shift from sprawling to parasagittal limb postures in non-mammalian therapsids (Blob 2001) and whether *Tyrannosaurus rex* could run fast (Hutchinson and Garcia 2002).

FUTURE DIRECTIONS

Yet, a fundamental question that remains to be answered is: how did changes to the shape of limb bones influence their ability to support the animal's weight on land? According to Wolff's law (Wolff 1986), bones will undergo morphological

changes to adapt to the physical demands being placed upon them.

Gravitational loads on land could impose greater stresses on bones than those found in the aquatic environment, where buoyancy provides weight support, thereby imposing selection on bone morphology that could withstand such loads on land. The microanatomy of limb bones differs between aquatic and terrestrial tetrapods, with the humeri and femora of aquatic taxa generally being denser than terrestrial taxa (Laurin et al. 2011 and references therein). However, how the strength of limb bones correlates with the morphological changes observed during the evolutionary transition to land is unknown. Bone strength can be assessed from cross-sectional geometry and mechanical properties, and may track the terrestrial capabilities of tetrapods, since the time spent counteracting gravitational loads on land should result in a proportional change in bone morphology based on Wolff's law. I am currently using engineering techniques to examine the mechanical capabilities (i.e., bone strength) of the humeri and femora of extant salamanders to model how loads on land could have influenced the evolution of terrestrial locomotion (Kawano and Blob 2013). Salamanders are often used to represent the basal tetrapod *bauplan* (Karakasiliotis et al. 2012), making them excellent tetrapod models. Correlating limb bone geometry and mechanical properties to the locomotor behaviors of diverse salamanders may shed light on the discussion regarding whether the evolution of tetrapod locomotion was powered by the forelimbs ("front-wheel drive;" Pierce et al. 2012), the hind limbs ("rear-wheel drive;" King et al. 2011), or began as front-wheel drive

and then transitioned to rear-wheel drive via an intermediate stage (Nyakatura et al. 2014).

Yet, given that both fishes and tetrapods with digit-bearing limbs were waiting at the water's edge during the Devonian (Shubin et al. 2006), a natural question is how are limbs biomechanically better than fins at withstanding the loads imposed by terrestrial locomotion? Broad surveys of the mechanical properties of bones in various vertebrate animals has suggested that the material properties of bone are relatively conserved (Currey 2002), but these analyses are primarily based on tetrapods. Fish bones are structurally different from tetrapod bones because not all fish bones are cellular (Dean and Shahar 2012). Since stress is a unit of force over a given area, the microanatomical holes resulting from Haversian canals in cellular bones could help to dissipate fractures by exposing microfracture cracks experienced during loading to a greater surface, thereby reducing force transmission (Currey 2002). Unfortunately, the mechanical performance of fish bones is essentially a black box at the moment (Currey 2010; Dean and Shahar 2012), despite the fact that fishes constitute a considerable proportion of the known species of vertebrates. Although the external morphology of the appendicular bones of tetrapodomorph fishes appeared robust, the internal architecture of fish bones could explain the source their biomechanical limitations. A recent study on the tetrapodomorph fish *Eusthenopteron* identified numerous histological differences in the pectoral fin compared to limbs (Sanchez et al. 2014), suggesting that the divergence in fin

and limb bone anatomy has a deep ancestry. Of particular interest was their discovery that the humerus of *Eusthenopteron* lacked capacities for bone remodeling, an important feature for repairing microfractures that can be produced by loads, and trabecular resorption, a process that creates the hollowed cavity that is found in many extant tetrapods (Sanchez et al. 2014) and contributes to the “tubular” bone geometry that is ideal for resisting variable loads (Currey 2002). Preliminary analysis of the mechanical properties of mudskipper pectoral fins suggest that the radials, bones serving a similar functional role as the humerus in the forelimb, exhibit mechanical properties that are remarkably similar to human bones with an elastic modulus of about 22 GPa (Kawano, Singleton, Blob, and Pharr, *unpublished data*). However, these tests were conducted on dry bones, which underestimates viscoelastic properties, potentially exaggerating the stiffness of the bone (Dean and Shahar 2012). The elastic modulus of mudskipper radials were comparable to the metapterygia of the amphibious *Polypterus* fish, which were 17.6 ± 7.8 (Erickson et al. 2002); whereas most values for aquatic fishes were less than 10 GPa (Dean and Shahar 2012), providing an opportunity to test whether life history ecology can influence bone mechanics in fishes. Nanoindentation tests on the viscoelastic properties of the appendicular bones of fishes and salamanders are currently ongoing, and may yield valuable insight into whether the material properties of bones correspond with life history ecology and/or function or remain relatively conserved across diverse taxa.

There are very few studies implementing nanoindentation on fish bones (although see Rho et al. 2001; Roy et al. 2001), with only a few additional studies that have conducted mechanical tests on fish bones using three-point bending (Erickson et al. 2002; Horton and Summers 2009). Given the small sizes of most fish bones, the entire bone is typically loaded to failure in three-point bending studies. Although the knowledge gleaned from such studies is useful for understanding the failure of the entire bone, it is harder to assess regional properties of the bone. Since bones often fail in tension (Currey 2002), three-point bending is expected to fracture a bone at its weakest point. However, bones are composite structures and often do not exhibit homogeneous mechanical properties. Thus, estimates of elastic modulus from three-point bending may not portray the ability of bones to exhibit different strengths in different regions. Nanoindentation offers the ability to conduct alternative investigations on the functional capabilities of bones, including regional heterogeneity in mechanical performance and viscoelastic properties. Assessments of regional heterogeneity in bone strength could yield valuable insight into how bones respond to loads that may be applied non-uniformly across the bone.

However, material properties are only one factor that influences bone strength and mechanics. Information on bone geometry and the loading regime are necessary to take the next step beyond a simple assessment of whether fish fin bones are strong, to asking how strong were they, what types of loads could

they have withstood, and in what ways fin bone geometries could have limited their locomotion. A number of fossil tetrapodomorph fishes have relatively robust elements in their pectoral appendages (Daeschler and Shubin 1997; Shubin et al. 2006; Pierce et al. 2013). While robust, dense bones function as “ballast” to assist with vertical migrations in some secondarily aquatic mammals (e.g., dugongs) (Laurin et al. 2004), terrestrial capabilities, albeit limited, have been hypothesized for tetrapodomorph fishes, such as *Panderichthys* (Boisvert 2005) and *Tiktaalik* (Shubin et al. 2006). Although light, spongy limb bones have been found in some secondarily aquatic mammals, this morphology has been associated with deep divers that collapse their rib cage to reduce buoyancy from the lungs (Laurin et al. 2004). Further, the multiple radiations of amphibious fishes in modern taxa provide compelling evidence that fins are fully capable of supporting terrestrial locomotion (Hsieh 2010; Gibb et al. 2011, 2013; Kawano and Blob 2013; Pace and Gibb 2014). So were tetrapodomorph fishes really restricted to the aquatic realm due to locomotor limitations, or were they constrained by other biological processes such as osmoregulation, desiccation, etc.? If they really could not move on land, why couldn't they occupy regions of morphospace (Raup 1966) that conferred terrestrial capabilities?

Empirical data on the mechanical properties of bones and the stresses experienced during terrestrial locomotion in fins and limbs can ultimately be applied to model plausible locomotor capabilities of extinct tetrapodomorphs spanning the transition from aquatic fishes to terrestrial tetrapods. Observations

of transverse sections at the mid-shaft of humeri and femora from various early stem tetrapods demonstrate that their limb bones shifted from being stout and relatively complex in cross-section in fishes such as *Sauripterus* and tristichopterids (Fig 5.1), to becoming progressively more slender and tubular in more derived tetrapods (e.g., humerus and femur in Fig. 4.4). Many of these geometries do not follow standard beam theory, making calculations of second moment of area with standard conventions unreliable. Instead, finite element analysis (FEA) is necessary for structures that deviate from typical cylindrical

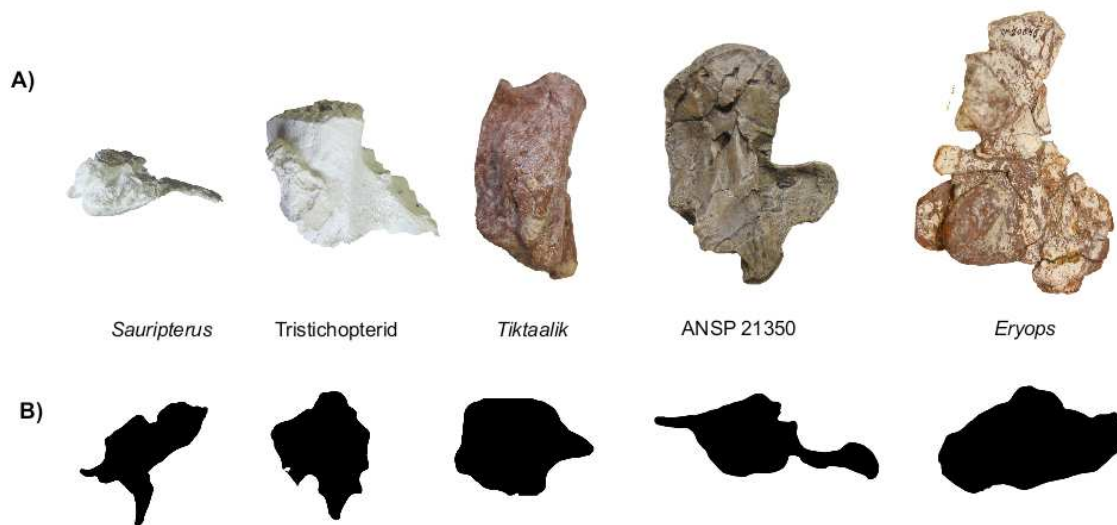


Fig. 5.1. Views of the dorsal (A) and mid-shaft cross-sectional geometries (B) of various tetrapodomorph humeri, ranging from the fish, *Sauripterus*, to the “fish-a-pod”, *Tiktaalik*, to an early stem tetrapod with at least some terrestrial capabilities, *Eryops*, are illustrated to demonstrate the sequence of morphological changes observed as tetrapodomorphs became increasingly terrestrial. Although there is some degree of crushing in ANSP 21350, the other fossil specimens are essentially uncrushed. These important taxa provide a foundation from which to investigate the functional implications of such anatomical transformations. Photos of *Eryops* were provided courtesy of the Carnegie Museum of Natural History (Amy Henrici), and the remaining photos were provided courtesy of the Academy of Natural Sciences, Philadelphia (Ted Daeschler), and the Field Museum (Neil Shubin). These specimens of *Tiktaalik* and *Eryops* are fossils, while the remaining taxa are casts. Note: bones are not to scale.

beam-like shapes, which requires input about the mechanical properties of the structures. Consequently, FEA offers the opportunity to conduct a sensitivity analysis on model parameters to determine how varying aspects of bone strength and loading regime influence the structural integrity and functional capabilities associated with the bone geometries of tetrapodomorphs.

The transformation from short, robust limb bones in the earliest stem tetrapods (e.g., *Sauripterus*, *Eusthenopteron*, *Tiktaalik*) to longer, and more slender limb bones in early limbed stem tetrapods (e.g., *Eryops*) and crownward stem tetrapods (e.g., *Cacops*, *Captorhinus*) may correlate with changing abilities to resist loading regimes. Long bones, such as the humerus and femur, are often modeled as structural beams in order to apply engineering principles that provide an evaluation of a structure's ability to withstand stresses. Although it is intuitive that a perpendicular force will result in the beam being bent, that carries the assumption that the beam is sufficiently long. For example, mechanical studies on sea anemones demonstrates that taller (i.e., longer) sea anemones (*Metridium senile*) responded to a water current (a perpendicular force) by bending, whereas shorter, stout sea anemones (*Anthropleura xanthogrammica*) experienced shearing from the water current, sliding layers of the animal laterally (Koehl 1977).

One hypothesis to explain the morphological transformation of the limb bones in tetrapodomorphs is, that as they became more terrestrial, natural selection favored the hollow, cylindrical structure found in more derived,

terrestrial tetrapods, which conferred greater mechanical performance because such a morphology provides the best structural integrity for withstanding all types of loads – bending, shear, torsion, and compression (Vogel 2003). In addition, the mechanics of tubular bones confer greater abilities to withstand compressive loads and bending moments over relatively long distances (Currey 2002). Such a structural transformation may have facilitated the greater terrestrial habits of amniotes, such as *Captorhinus*, which had hollow limb bone cavities (Kawano *pers. obs.*). Although stem tetrapods, such as *Eryops*, likely had some capacity to move on land, full terrestriality was not achieved until later in geological history, in taxa such as *Pederpes* (Clack 2009). Interestingly, the femur of *Eryops* was still solid (see “EF” in Fig. 3a in Sanchez et al. 2010), so it is plausible that its limb bone morphology limited its ecology. It is also possible that hollowing of the bone was favored under natural selection to make the limbs more lightweight, reducing energetic costs of moving the limbs during locomotion. However, energetic savings due to the reduction in limb bone mass associated with hollowing of the bone cavity has been found to be only 18% (Currey and Alexander 1985). Analyses are currently being pursued that investigate how the sequence of morphological changes observed across the limb bones of tetrapodomorphs influenced their ability to withstand the types of loads imposed by terrestrial locomotion.

Thus, the future of evolutionary biomechanics appears bright. The tools available to investigate the biomechanical capabilities of both extant and extinct

taxa are becoming more readily available and more sophisticated, allowing scientists to explore aspects of the fossil record that were unavailable (or even unimaginable) by their predecessors. The use of photogrammetry techniques and high-resolution synchrotron machines has allowed researchers to even test sub-surface body fossils as well as ichnofossils (e.g., “trackways”), providing crucial information about the locomotor gaits and microanatomy of fossils that were often too delicate to study (or too valuable for destructive analyses) (Tafforeau et al. 2006; Falkingham 2014). Even a decade ago, who would have guessed that it would be possible to 3D-print a replica of a fossil (Schilling et al. 2014)? With such tools at our disposal, we are one step closer towards deciphering how tetrapods left the water to embark upon one of the most monumental events in vertebrate history.

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APPENDICES

Appendix A

SUPPLEMENTARY MATERIAL – CHAPTER 3

Propulsive forces of the semi-aquatic newt, Pleurodeles waltl: insights into the functional evolution of terrestrial locomotion in early stem tetrapods

SELECTION OF EXTANT TAXA AS FUNCTIONAL MODELS

Extant taxa have served as important modern analogs to model the biology of extinct taxa (Pierce et al. 2013). Salamanders, in particular, have often been used as models for basal tetrapods because of their retention of a generalized tetrapod *bauplan* (Kawano and Blob 2013). Nonetheless, evolutionary novelties within the clade have resulted in diverse ecological habits in extant taxa (Wake 2009). In particular, the range of terrestrial capabilities (or lack thereof) in extant salamanders can be used to model locomotor function in fossil species across a range of taxa spanning the invasion of land, from early stem tetrapods to crownward tetrapods. Indeed, Pierce and colleagues (Pierce et al. 2013) suggested that salamanders may better represent models for basal crown tetrapods; however, this may be because many of the taxa in which terrestrial locomotion has been studied have been primarily terrestrial in habitat [e.g., *Ambystoma* (Sheffield and Blob 2011), *Taricha* (Ashley-Ross et al. 2009), *Dicamptodon* (Ashley-Ross 1994)]. Examining a salamander model that is primarily aquatic and employed limb-based locomotion (but with unreduced limbs), could serve as an important functional model for an early tetrapod that

used its digit-bearing limbs during initial excursions of limb-based locomotion on land.

The Iberian ribbed newt, *Pleurodeles waltl* Michahelles 1830, presents such a model, since it is one of the largest species of semi-aquatic salamanders that spends most of its adult life in water (Obst et al. 1988), but can still make terrestrial excursions (Fig. A-1) (Karakasiliotis et al. 2012). Previous studies on the muscle activity (Delvolvé et al. 1997), bone microanatomy (Laurin et al. 2004; Canoville and Laurin 2009), and kinematics and morphology (Karakasiliotis et al. 2012) of *P. waltl* provide a foundation for comparisons with data collected from more commonly used terrestrial ambystomatids (Stokely and Holle 1954; Bennett et al. 1989; Ashley-Ross and Barker 2002; Laurin et al. 2004; Deban and Schilling 2009; Kawano and Blob 2013), which occupy a wide range of habitats (Petranka 1998). Thus, GRF data from the semi-aquatic *P. waltl* and more terrestrial taxa (e.g. *A. tigrinum*) can be used to model two points along the continuum from a semi-aquatic stem tetrapod to a more terrestrial crownward tetrapod.

Other salamander taxa could be considered as models for further investigation. For example, the large, fully aquatic hellbender salamander *Cryptobranchus alleganiensis* (Daudin, 1803) might be considered, though eliciting terrestrial behaviors from larger animals might be more challenging, and their “Threatened” conservation status on the IUCN Red List limits availability for testing. Captive animals in zoo collections might solve this problem. In addition,

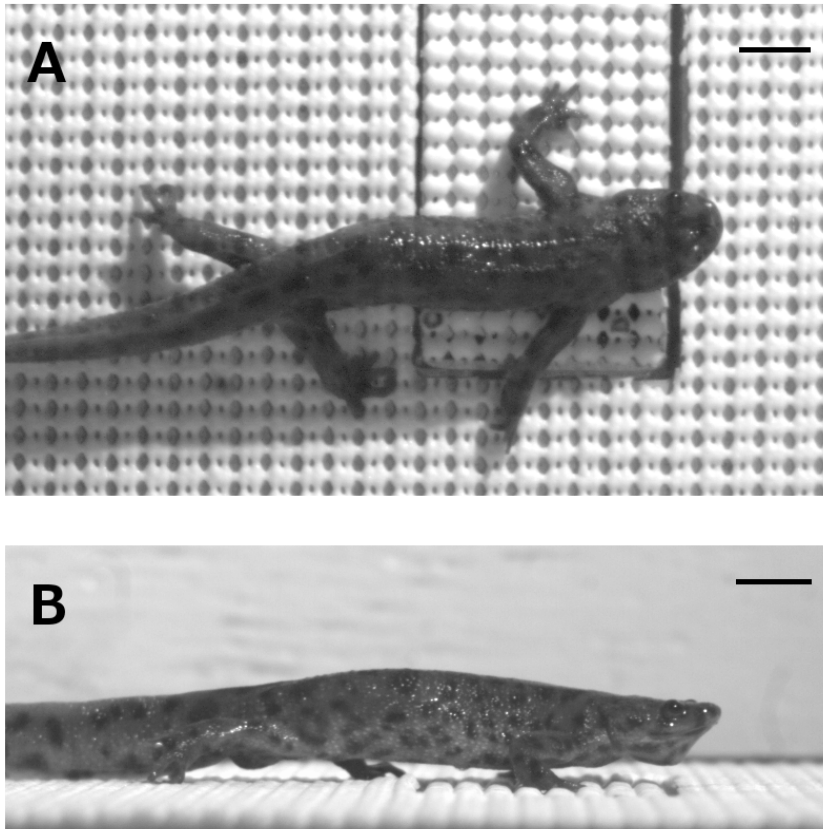


Figure A-1. Dorsal (A) and lateral (B) views of an Iberian ribbed newt walking on a force plate. Scale bar indicates 1 cm.

aquatic *P. waltl* adults have passed through a terrestrial eft phase and, thus, may harbor some ontogenetic influence of terrestriality on their locomotion.

Nonetheless, it seems reasonable to expect that the primary environment in which adult *P. waltl* perform (i.e., water) would have the greatest influence on the capacities they would exhibit as adults, and they still exhibit greater aquatic tendencies than terrestrial salamanders, such as *Ambystoma*. Therefore, although study of other species could provide additional insight, data from *P. waltl* still have better potential to demonstrate how more aquatic limbs can be

used on land than many previously studied species that are more terrestrial (e.g. *A. tigrinum*). The newt, *P. waltl*, thus represents a reasonable functional model for limb function in early stem tetrapods.

CRITERIA FOR TRIAL SELECTION DURING GRF MEASUREMENTS

Several criteria were used to determine whether a trial was valid for inclusion in our analyses. First, the entire right foot (fore or hind) needed to contact the force plate. Second, any frames that included any body parts other than the limb of interest were excluded from analysis. Complete limb cycles performed in a straight line (i.e., no turning or moving diagonally across the plate) were also required. Trials were excluded if the peak net GRF was found to occur at 0% or 100% of stance, or during a time when more than the limb of interest was in contact with the force plate.

EXPERIMENTAL PROCEDURES

Data on three-dimensional GRF production of individual limbs walking over level ground were collected using procedures outlined in published studies from our lab on various fish, amphibians, reptiles, and mammals (Butcher and Blob 2008; Butcher et al. 2011; Sheffield and Blob 2011; Sheffield et al. 2011; Kawano and Blob 2013). Briefly, data on the GRFs imposed on isolated appendages on the right side of the body were recorded (5000 Hz) using a custom-built, multi-axis force plate (K&N Scientific; Guilford, VT, USA) connected to bridge amplifiers, and two digitally synchronized, high-speed cameras (100 Hz; Phantom v.4.1,

Vision Research Inc.; Wayne, NJ, USA) filming the dorsal and lateral views (Fig. A-1). Data from the high-speed cameras and force plate were synchronized by timing the onset of an LED light on the video with the onset of a 1.5 V pulse on the force traces.

Data on GRF production by individual appendages were analyzed during stance, when the foot/fin is in contact with the ground and propulsion is generated. Prior to analysis, raw force traces were padded at the beginning and end, in order to avoid edge effects in the filtering process. Since some of the force traces did not begin at a baseline of zero Newtons, data were padded using the average values calculated at the beginning and end of the trace. Padded force traces were then filtered using a low-pass, zero phase, second order Butterworth filter using the *signal* package in R. The order of the polynomial and the cut-off frequency were determined using `signal::buttord` with the following filter specifications: 2500 Hz frequency, 0.0024 Hz passband frequency, 0.076 Hz stopband frequency, 2 dB passband ripple, and 40 dB stopband attenuation. These frequency values had been normalized to Nyquist frequency to avoid aliasing (Smith 1997). Padding was removed prior to analysis, leaving only data during stance. Data were then interpolated to 101 points to represent 1% increments, from 0% to 100%, of the stance phase using a cubic spline with `signal::interp1`.

Filtered data were then used to calculate the magnitude and direction of the GRFs imposed upon the individual limbs during terrestrial locomotion. All

magnitudes of force were standardized to units of body weight (BW), accounting for size differences. Magnitudes of the vertical, anteroposterior, and mediolateral components of the GRF were used to calculate the magnitude and orientation of the net GRF vector. Angular orientations were analyzed with respect to vertical (0 degrees): positive values indicated a vector directed in the anterior or lateral directions, whereas negative values indicated a vector directed in the posterior or medial directions.

STATISTICAL ANALYSES FOR DIFFERENTIATING APPENDICULAR FUNCTION

Handling of data prior to statistical analyses

Given that all of the variables were continuous, did not include zeros, and did not differ in extreme orders of magnitude (means for variables ranged from about -20 to 60, and standard errors from around 0.004 to 2.0), data were not standardized prior to statistical analyses. All GRF magnitudes were standardized to body mass and, therefore, did not require further standardization. Also, standardization changed the signs of angular measurements, which drastically alters their biological interpretation. For instance, changing angular signs converts the orientation of the GRF from medial to lateral, affecting moment arm calculations and, therefore, estimations of loading regimes upon bones. However, because multivariate statistical tests can sometimes be sensitive to standardization, we compared results from DFAs using standardized and

unstandardized data to evaluate the robustness of our dataset. Conclusions were not altered by standardization, so we considered our data robust, and variables were analyzed without centering and scaling in order to maintain the biological relevance of our interpretations.

Discriminant function analyses

Discriminant function analyses (DFAs) were conducted to identify the major axes that differentiate the five groups of appendages: terrestrial pectoral fin, semi-aquatic forelimb, semi-aquatic hind limb, terrestrial forelimb, and terrestrial hind limb. A canonical DFA, based on Type II error, was conducted with `candisc::candisc` to describe the separation amongst groups. Linear DFA identifies the major axes that describe the separation amongst individuals, and was performed using `MASS::lda` in order to evaluate the percentage of individuals that were correctly classified to their respective appendicular group. All statistical analyses were performed in *R* (v. 3.1.0).

Comparing profiles of GRF production

Vector analysis is a mathematical technique that allows for comparisons of the profiles of a variable between two groups over multiple observations (e.g., throughout the stance phase of the limb cycle) (Cullen et al. 2013; Kawano and Blob 2013). The net GRF and its three components, and the two angles of GRF orientation were evaluated at 5% intervals throughout stance (Fig. 3.1). Data for

the variables of each group (appendage type) are treated as a multidimensional vector, and then angles between these vectors are calculated (Hamilton 1989). Angle values that are close to 0° indicate profiles that are nearly identical, whereas those near 90° indicate profiles that are so different that they have perpendicular trajectories. Calculations for vector analyses were performed in Microsoft Excel.

Table S1. Standardized coefficients and canonical correlations from a canonical DFA on the appendages at peak net GRF

	DF 1	DF 2	DF 3	DF 4
Percent Stance (%)	0.622*	-0.704*	-0.069*	0.085
Anteroposterior angle (degrees)	-1.192*	-1.512*	-0.872	0.108*
Mediolateral angle (degrees)	-0.093	-0.310*	0.199*	0.632
Vertical magnitude (BW)	-1.614*	-3.477	-1.021*	-1.213*
Mediolateral magnitude (BW)	0.081*	1.215*	-0.949*	-0.651*
Anteroposterior magnitude (BW)	-0.173*	0.481	0.637	-0.352*
Net GRF (BW)	0.660*	3.091	0.210*	1.677*
Canonical correlations	0.857	0.514	0.216	0.052
Percentage of total canonical correlation	81.238	14.297	3.718	0.747

BW, body weights. Variables that were correlated with each axis, based on Spearman rank correlation tests, are indicated with an asterisk (*).

Table S2. Misclassification table from a linear DFA on the five appendages at the peak net GRF

		Classified group from LDA				
		Terrestrial PF	Semi-aquatic FL	Semi-aquatic HL	Terrestrial FL	Terrestrial HL
Correct group	Terrestrial PF	84	2	6	6	2
	Semi-aquatic FL	4	78	0	18	0
	Semi-aquatic HL	6	0	58	0	34
	Terrestrial FL	12	14	0	74	0
	Terrestrial HL	0	0	26	0	74

Values are in percentages.

The diagonal indicates the number of trials that were correctly classified whereas the off-diagonals are the misclassified trials.

Table S3. Comparison of GRF profiles using vector analysis

	Terrestrial PF	Semi-aquatic FL	Semi-aquatic HL	Terrestrial FL
Anteroposterior angle (degrees)				
Semi-aquatic FL	131.0			
Semi-aquatic HL	20.4*	124.3		
Terrestrial FL	108.8	55.8	98.0	
Terrestrial HL	24.1	136.1	18.8	109.4
Mediolateral angle (degrees)				
Semi-aquatic FL	55.0			
Semi-aquatic HL	48.2	57.6		
Terrestrial FL	27.1	48.6	27.6	
Terrestrial HL	38.9	38.2	16.3*	21.6
Anteroposterior (BW)				
Semi-aquatic FL	146.1			
Semi-aquatic HL	12.0*	152.0		
Terrestrial FL	113.7	60.3	110.9	
Terrestrial HL	19.3	155.6	13.2	116.5
Mediolateral (BW)				
Semi-aquatic FL	30.2			
Semi-aquatic HL	51.3	29.1		
Terrestrial FL	14.7*	24.6	39.4	
Terrestrial HL	40.0	22.0	14.9*	28.4
Vertical (BW)				
Semi-aquatic FL	9.1			
Semi-aquatic HL	15.4	14.0		
Terrestrial FL	9.7	14.4	24.6	
Terrestrial HL	16.3	17.2	5.3*	25.8
Net (BW)				
Semi-aquatic FL	10.6			
Semi-aquatic HL	15.8	11.7		
Terrestrial FL	8.9	15.6	23.8	
Terrestrial HL	15.6	15.2	6.9*	24.2

All values from the vector analysis are in units of degrees. Bold values indicate pair-wise similarities; values close to 90° indicate dissimilarity. Asterisks (*) indicate pair-wise comparisons with the greatest similarity for the given variable. PF = pectoral fin, FL = forelimb, HL = hind limb.

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PRELIMINARY DATA ON FORELIMB FUNCTION IN *SIREN LACERTINA*

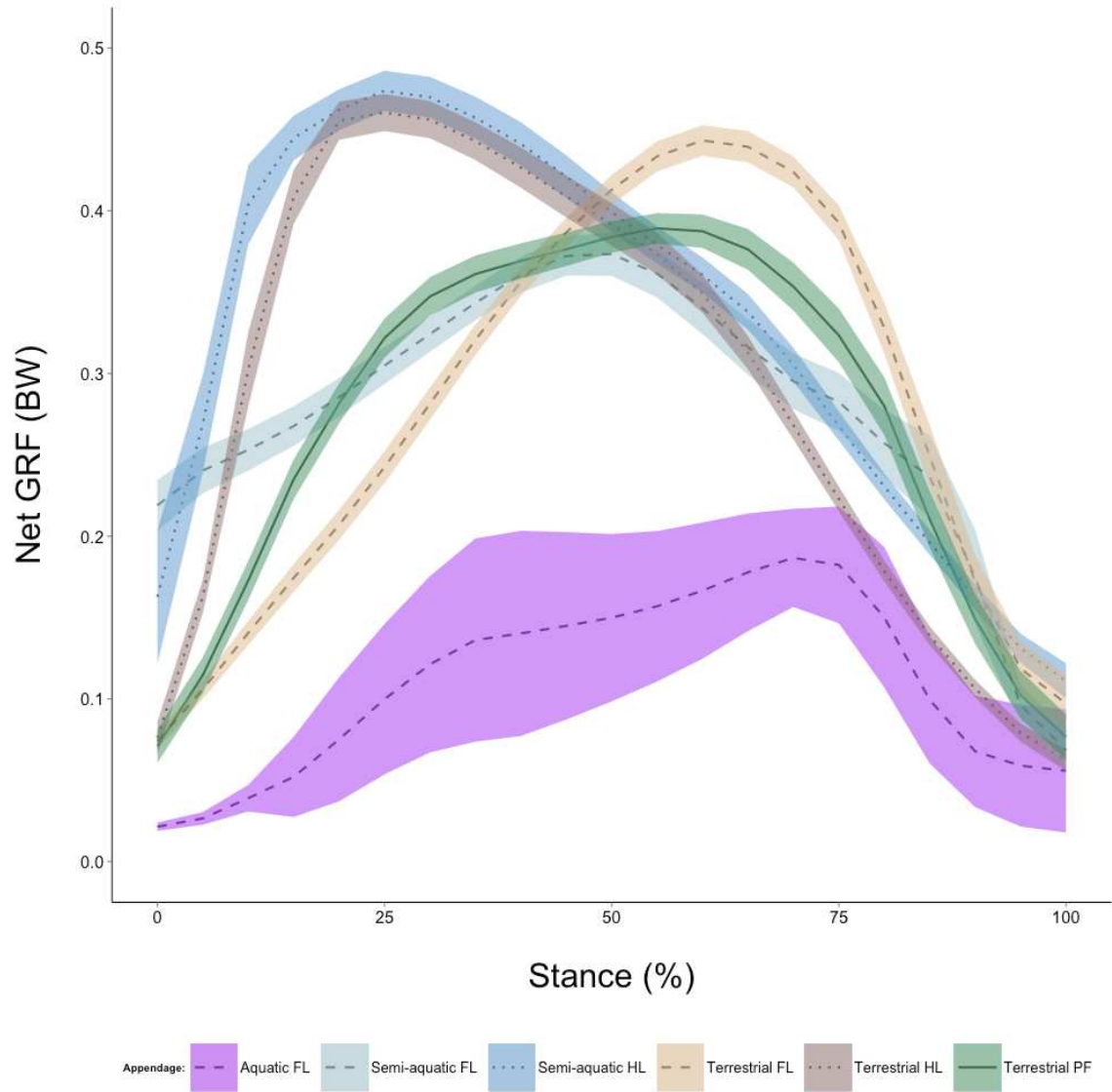


Fig. B-1: Weight-bearing capabilities of the forelimbs in *S. lacertina* were less than half of that observed for the terrestrial *A. tigrinum* limbs, semi-aquatic *P. waltl* limbs, and terrestrial *P. barbarus* pectoral fins. Similar results were observed for the vertical component of the GRF.

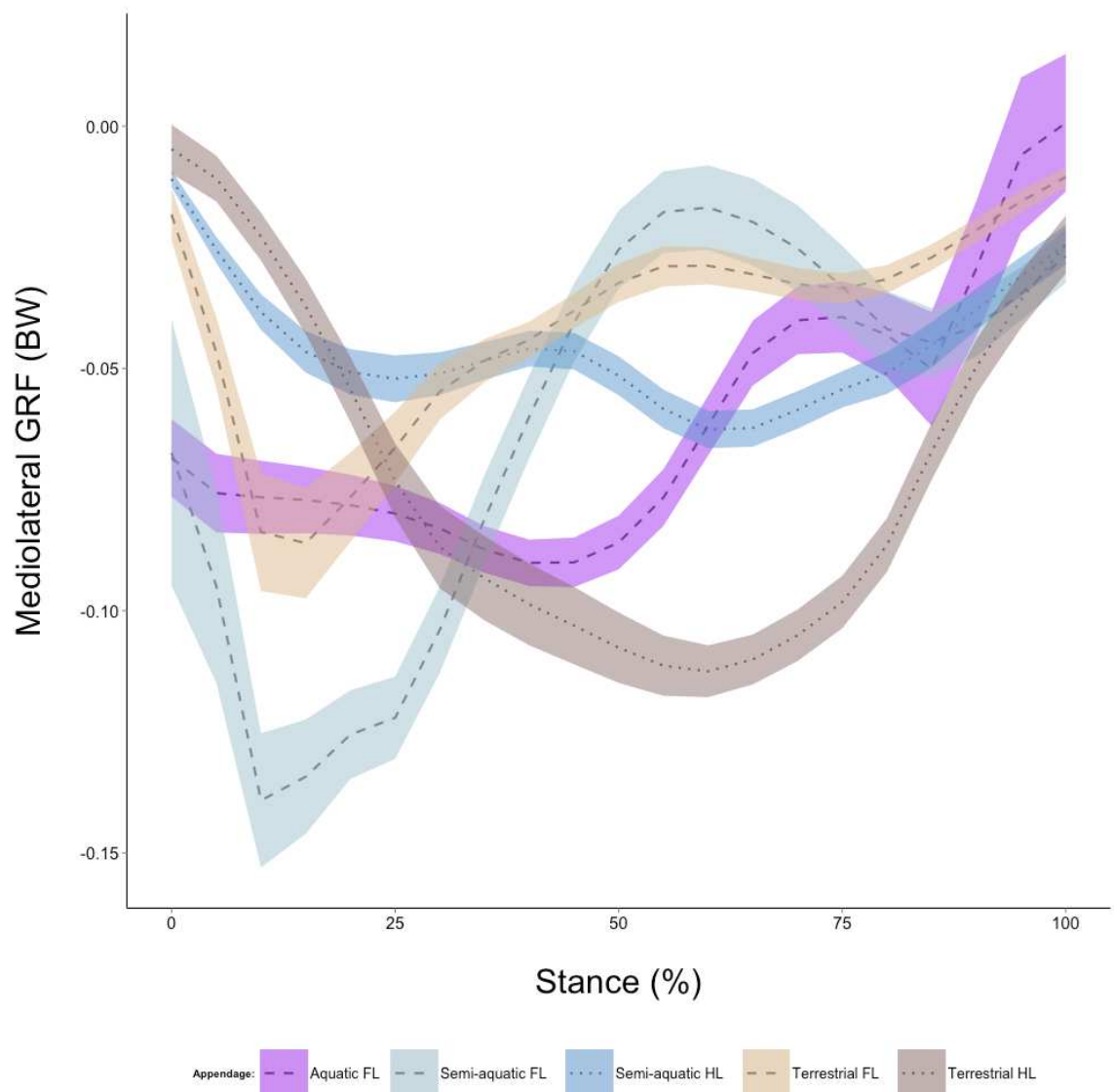


Fig. B-2: The GRF was less medial in *S. lacertina* forelimbs compared to the other appendicular groups. The greater reliance on lateral bending in *S. lacertina* may be influencing how forces are applied to the limb bones.

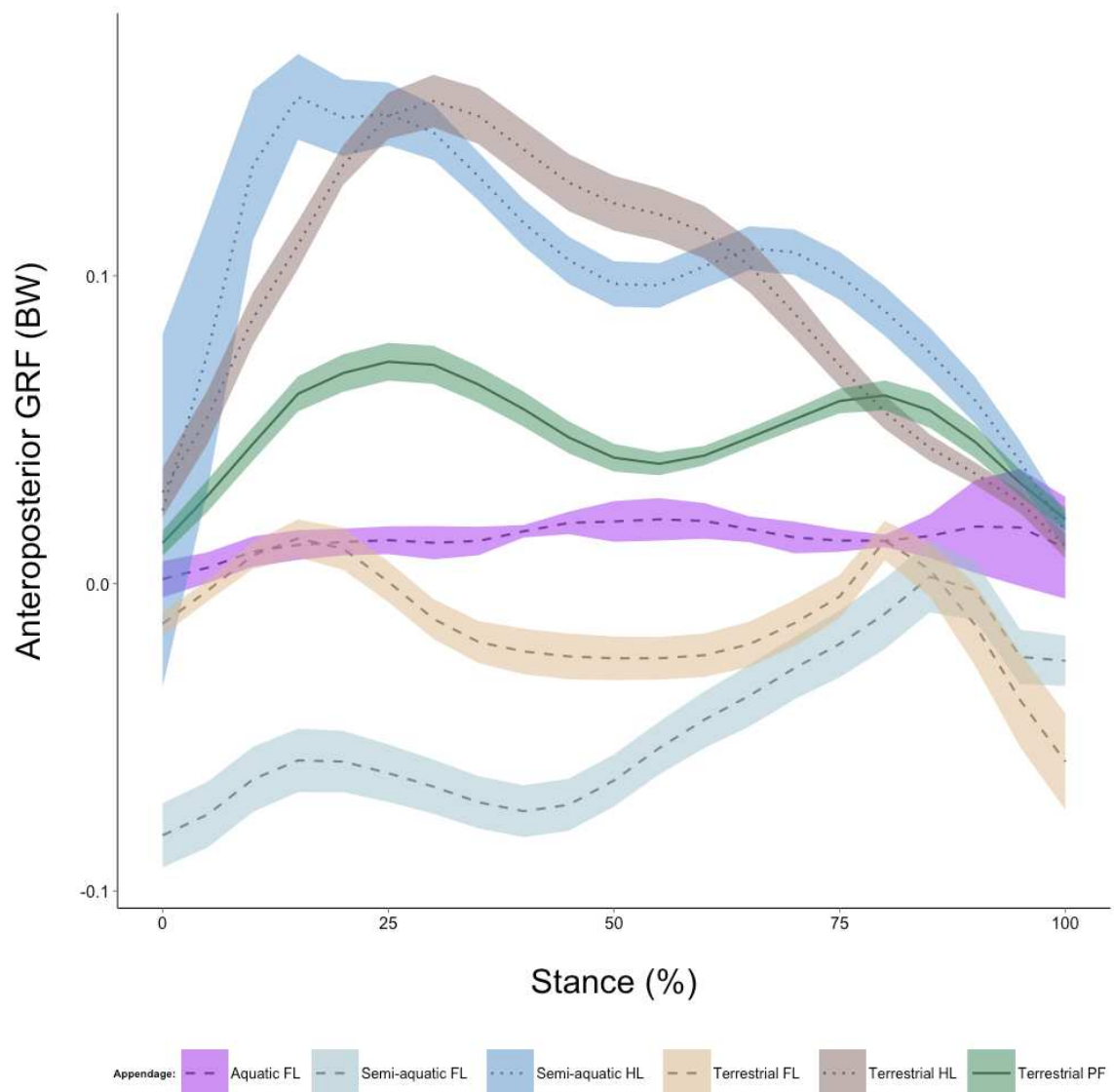


Fig. B-3: The forelimbs of *S. lacertina* had a slight acceleratory role that was intermediate between the deceleratory forelimbs of *A. tigrinum* and *P. waltl* and the acceleratory hind limbs and the terrestrial fish fin.

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