

# Anuran limbs reflect microhabitat and distal, later-developing bones are more evolutionarily labile

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Tetrapod limbs have been used as a model system to investigate how selective pressures and constraints shape morphological evolution. Anurans have had many independent transitions to various microhabitats, allowing us to dissect how these factors influence limb morphology. Furthermore, anurans provide a unique system to test the generality of developmental constraints proposed in mammals, namely that later-developing limb bones are under less constraint and show more variation. We used microcomputed tomography scans of 236 species from 52 of 55 families, geometric morphometrics, and modern phylogenetic comparative methods to examine how limb bones are related to microhabitat, phylogeny, allometry, and developmental timing. Although there was significant phylogenetic signal, anuran limb shape showed a relationship with microhabitat and to a lesser extent, body size. We found that distal bones had higher evolutionary rates than proximal bones, providing evidence that developmental constraints are reduced in later-developing bones. Distal bones also showed increased selection related to allometry and microhabitat, providing an additional explanation for higher evolutionary rates. By looking at the evolution of limb shape across a diverse clade, we demonstrated that multiple factors have shaped anuran limbs and that greater evolutionary lability in later-developing limb bones is likely a general trend among tetrapods.

**KEY WORDS:** Burrowing, convergent evolution, developmental constraint, frogs, geometric morphometrics, morphology.

Determining how extrinsic selective pressures and intrinsic constraints interact to form observed morphological diversity is a long-standing goal of evolutionary research. Morphology is often tightly correlated with ecology or function, leading to convergence across species and variation within clades that reflects the ecological diversity among species (Losos 1990; Wainwright and Reilly 1994; Kaboli et al. 2007; Grizante et al. 2010; Muschick et al. 2012; Openshaw and Keogh 2014). However, phylogeny and development can bias morphological evolution along certain axes (Gould and Lewontin 1979; Foote 1997; Cooper and Stepan 2010; Goswami et al. 2014; Kazi and Hipsley 2018;

Watanabe 2018). This can lead to constraints on morphology (Lilje et al. 2003; Stoessel et al. 2013) or imperfect convergence, where independent lineages exhibit similar lifestyles but have evolved disparate morphologies, often because of differences in ancestral phenotype (Samuels et al. 2013; Collar et al. 2014). Limbs provide an excellent system for addressing how extrinsic and intrinsic factors interact to shape morphological diversity, as they have a shared genetic architecture, a well-studied developmental pathway, and are functionally related to a species' ecology (Young 2017). Furthermore, evolutionary patterns found in mammals have led to testable predictions for how differing

developmental constraints among limb bones may influence limb evolution (Young and Hallgrímsson 2005; Weisbecker 2011; Martín-Serra et al. 2015). Here we use frogs and toads (anurans) to test the generality of proposed developmental constraints on tetrapod limbs and study the interactions of allometry, phylogeny, and ecological selection pressures in shaping anuran limb evolution.

Due to their shared general morphology, range of body sizes, and repeated evolutionary transitions to different microhabitats, anurans provide a particularly diverse and tractable system for dissecting how extrinsic and intrinsic factors affect limb evolution at macroevolutionary scales. Anurans form the largest group of amphibians, with over 7000 species distributed in 55 families found on every continent except Antarctica (AmphibiaWeb 2019). They have a unique derived morphology specialized for jumping that has been present in its basic form since at least the early Jurassic (Shubin and Jenkins 1995). Key limb changes include fusion of the ulna and radius, fusion of the tibia and fibula, and lengthening of proximal tarsal elements to form an additional long bone. The ancestral microhabitat remains unclear, with different hypotheses placing the origin of frogs in terrestrial, riparian, and aquatic microhabitats (Gans and Parsons 1966; Přikryl et al. 2009; Herrel et al. 2016). Despite a conserved general morphology, which allows for comparison between distant relatives, frogs have repeatedly diversified into a variety of microhabitats (e.g., terrestrial, aquatic, arboreal, torrent, and burrowing; Duellman and Trueb 1986; AmphibiaWeb 2019). This repeated convergence across the phylogeny allows us to untangle the effects of phylogeny, ecology, and body size on phenotypic evolution in a diverse clade.

Limb morphology is often under strong extrinsic selective pressures related to a species' ecology because of their role in traversing and interacting with the environment. Some of the most defining studies of convergent evolution have focused on the relationships between limbs and ecology (Losos 1990; Losos et al. 1998), with many other examples found in mammals (Samuels et al. 2013; Curran 2015; Fabre et al. 2015), birds (Zeffer et al. 2003; Kaboli et al. 2007; Hinić-Frlog and Motani 2010), and lizards (Melville and Swain 2000; Herrel et al. 2002; Grizante et al. 2010; Ord and Klomp 2014; Foster et al. 2018). Studies within anurans show less clear relationships between limb morphology and ecology. Anurans occupy a variety of microhabitats (e.g., aquatic, arboreal, burrowing) that likely select for different limb morphologies. Although microhabitat and locomotor mode are not synonyms and frogs of different microhabitats exhibit similar jumping performances (Moen 2019), overall limb morphology has been linked to both locomotor mode (Emerson 1988; Enriquez-Urzelai et al. 2015) and microhabitat in a subset of anuran families (Gomes et al. 2009; Moen et al. 2013; Vidal-García and Keogh 2015; Vidal-García and Keogh

2017; Citadini et al. 2018; Moen 2019). Other studies have found no correlation between anuran limbs and microhabitat except in burrowing frogs (Jorgensen and Reilly 2013; Vidal-García et al. 2014) or have found differences between fore- and hindlimbs in the relationship of morphology and microhabitat (Jorgensen and Reilly 2013; Citadini et al. 2018). Intriguingly, the two studies with the largest sampling of families disagree on whether limb morphology shows a relationship with microhabitat (37 families, Jorgensen and Reilly 2013; 31 families, Moen 2019). We seek to reach a consensus by sampling the majority of families and maximizing ecological diversity within each clade to determine whether microhabitat relates to limb morphology when integrating over clade-specific morphologies and deep evolutionary timescales.

Body size may impose intrinsic constraints on anuran limbs because changes in overall body size can necessitate changes in the relative size or shape of different elements to retain the same biomechanical performance (Gould 1966; Pounds et al. 1983). In mammalian carnivores, body size was found to have a stronger influence on limb shape than locomotor behavior (Martín-Serra et al. 2014), underscoring the importance of the relationship between body size and limb shape (limb allometry). Many anuran studies are interested in limb proportions and studies frequently correct for size in analyses, but few have focused on the relationship between limb shape and body size. Enriquez-Urzelai et al. (2015) found that larger frogs from the western Mediterranean had lower femur-tibiofibula length ratios relative to body size in some locomotor modes (jumper-terrestrial and burrower/walker/hopper) but not others. In myobatrachid frogs, across microhabitats there was a weak allometric relationship between limbs and body size with only 4.29% of limb shape variation correlated with size (Vidal-García and Keogh 2017). The influence of body size on anuran limb evolution remains unclear beyond these clades.

In addition to contributing to distinct evolutionary patterns among clades, developmental constraints can lead to distinct evolutionary patterns among limb bones. The growth plates of distal limb bones typically close later in development in mammals (Geiger et al. 2014). As a result, the shape and dimensions of distal limb bones are hypothesized to be less limited by developmental constraints and more subject to change from other selection pressures (Young and Hallgrímsson 2005; Weisbecker 2011; Martín-Serra et al. 2015). This observation underlines how changes in developmental timing can impact morphological diversity. For example, because marsupials must have well-developed forelimbs to crawl to the pouch, their forelimbs develop earlier and show less variation than in placentals (Cooper and Stepan 2010). On the other hand, the humerus of monotremes and moles, which experiences delayed development, shows greater specialization than distal bones (Weisbecker

**Table 1.** Evolutionary model comparisons for anuran limb evolution using mvMORPH.

Model	Forelimbs	Hindlimbs	Forelimbs + Hindlimbs
Brownian motion (BM)	$\Delta AICc = 32$ $AICc = -1928$	$\Delta AICc = 65$ $AICc = -2201$	$\Delta AICc = 87$ $AICc = -2144$
BM with differing rates among all 7 microhabitats (BMM)	$\Delta AICc = 39$ $AICc = -1921 (\pm 2)$	$\Delta AICc = 21$ $AICc = -2245 (\pm 2)$	$\Delta AICc = 54$ $AICc = -2177 (\pm 2)$
Ornstein-Uhlenbeck (OU) single peak	$\Delta AICc = 17$ $AICc = -1943$	$\Delta AICc = 50$ $AICc = -2216$	$\Delta AICc = 59$ $AICc = -2172$
OU with 7 microhabitat peaks (OUM)	<b><math>\Delta AICc = 0</math></b> <b><math>AICc = -1960 (\pm 2)</math></b>	<b><math>\Delta AICc = 0</math></b> <b><math>AICc = -2266 (\pm 4)</math></b>	<b><math>\Delta AICc = 0</math></b> <b><math>AICc = -2231 (\pm 3)</math></b>

The best supported model for each limb set is in bold. For BMM and OUM models, we report the average AICc (with standard error) for 20 replicate models, each with a unique stochastic ancestral reconstruction of microhabitat.

2011). Limb development in frogs also follows a proximal-distal direction (Fabrezi et al. 2017), but unlike in amniotes, limb formation occurs later and is decoupled from organogenesis in the phylotypic stage, relaxing constraints against changes to the number of distal elements (Galis et al. 2001; Irie and Sehara-Fujisawa 2007). Changes to the size and shape of elements happen in later development (Galis et al. 2001), but these differences in early constraints may still impact differences in amphibian and amniote limb lability. Alternatively, constraints related to late developmental timing found in mammals may similarly affect frog limb development, in which case, we predict that distal bones show greater evolutionary lability and greater response to ecological selection pressures than proximal bones. No work has been done to determine if these developmental constraints are evident in anurans, which would indicate a more ancient evolutionary constraint on tetrapod limb diversity.

Here we use microcomputed tomography (microCT) scans, three-dimensional geometric morphometrics, and phylogenetic comparative methods to study variation related to phylogeny, allometry, ecology, and developmental timing in anuran limbs. We examined 236 anuran species with representatives from 52 of 55 total anuran families. These 236 species were selected for their range of body sizes and independent evolutionary transitions to various microhabitats, allowing us to tease apart the relative roles that microhabitat, allometry, and phylogenetic constraint play on anuran limb evolution. Specifically, we were interested in these questions: (1) how have limbs evolved in association with microhabitat, body size, and phylogeny, (2) do fore- and hindlimbs show similar evolutionary patterns, and (3) are distal, later-developing limb bones more evolutionarily labile than proximal bones and if so, is there evidence for increased selection on distal bones related to body size and microhabitat?

## Methods

### SPECIES DATA AND MICROCT SCANNING MUSEUM SPECIMENS

Of the 236 specimens used in this study (each belonging to a unique species), 226 specimens were microCT scanned for this study and an additional 10 specimen scans were downloaded from Morphosource.org. All specimens used in this study are vouchered museum specimens with 125 specimens from the National Museum of Natural History in Washington, DC, 101 specimens from the Museum of Vertebrate Zoology in Berkeley, CA, four specimens from the California Academy of Sciences in San Francisco, CA, two specimens from the Centre for Ecological Sciences at the Indian Institute of Science in Bengaluru, India, two specimens from the Florida Museum of Natural History in Gainesville, FL, and two specimens from the University of Kansas Biodiversity Institute in Lawrence, KS.

We measured snout-vent length (SVL) of all loaned specimens to the nearest tenth millimeter using a digital caliper (31-415-3, Swiss Precision Instruments, Inc., Garden Grove, CA) and measured SVL of all skeletons downloaded from Morphosource in Meshlab (Cignoni et al. 2008). Microhabitat information for each species (Supporting Information Datatable 1) was taken from primary (Andreone and Luiselli 2003; McCranie and Casta 2005; Brito et al. 2012; Matojo 2015) and secondary literature (Moen et al. 2015; Moen and Wiens 2017; AmphibiaWeb 2019; IUCN 2019). We used seven of eight microhabitat categories defined by Moen and Wiens (2017): (i) aquatic—almost always in water; (ii) arboreal—typically on aboveground vegetation; (iii) burrowing—nonbreeding season spent underground or in burrows they have dug; (iv) semiaquatic—partially aquatic and partially terrestrial; (v) semiarboreal—partially arboreal and partially terrestrial; (vi) terrestrial—found on the ground, under rocks, or in leaf litter; and (vii) torrential—found in

high-gradient, fast-flowing streams, usually on rocks in the stream or under waterfalls. Three of our species were defined as semiburrowing by Moen and Wiens (2017); however, we define them as burrowing for this study because we were interested in variation in limb morphology related to a species' propensity to burrow at all (not amount of time spent burrowing). All associated data and references are in Supporting Information Datable 1.

We microCT scanned the 226 specimens loaned from the National Museum of Natural History and the Museum of Vertebrate Zoology using a Phoenix vjtoemjx M (GE Measurement & Control Solutions, Boston, MA, USA), at the University of Florida's Nanoscale Research Facility. We performed all scans with a 180 kV X-ray tube containing a diamond-tungsten target, with the voltage, current, and detector capture time adjusted for each scan to maximize absorption range. We reconstructed all scans on GE's datosjx software version 2.3 and segmented all skeletons using VG StudioMax (Volume Graphics, Heidelberg, Germany). All scans are available for download on Morphosource (<http://www.morphosource.org>, project number P967).

#### LANDMARK PLACEMENT

We placed landmarks on each specimen's right fore- and hindlimb using R version 3.5.1 (R Core Team 2019) and the package *geomorph* 3.0.7 (Adams et al. 2018) to characterize variation in overall shape of each limb bone. Each limb bone had its own set of landmarks, corresponding to homologous and repeatable points that defined the outer borders of the limb (six on the tarsus, four on the tibiofibula, four on the femur, five on the humerus, and four on the radioulna (Fig. 1); additional landmarked example in Supporting Information Fig. 1). Each bone had four landmarks that characterized the outer bounds of the limb, the humerus had one additional landmark that designated the fusion of the humeral crest to the shaft of the humerus, and the tarsus had two additional landmarks that designated the fusion points of the fibulare and tibiale. We aimed to choose obvious boundary landmarks that could be easily replicated in future studies within anurans and potentially other tetrapod groups. We chose these landmarks after trialing additional landmarks because they could be reliably placed across all frog families and would provide novel information on limb evolution in most frog families that have previously been studied using only linear measurements. Most previous studies used length measurements of either the entire limb (Gomes et al. 2009; Jorgensen and Reilly 2013; Moen et al. 2013; Moen 2019) or of individual bones analyzed together (Emerson 1988; Vidal-García et al. 2014; Enriquez-Urzelai et al. 2015; Vidal-García and Keogh 2015; Citadini et al. 2018). Some studies also measured the maximum width of limb bones (Vidal-García et al. 2014; Vidal-García and Keogh 2015).

#### CONVERSION OF LANDMARKS TO WHOLE LIMB SHAPE DATA

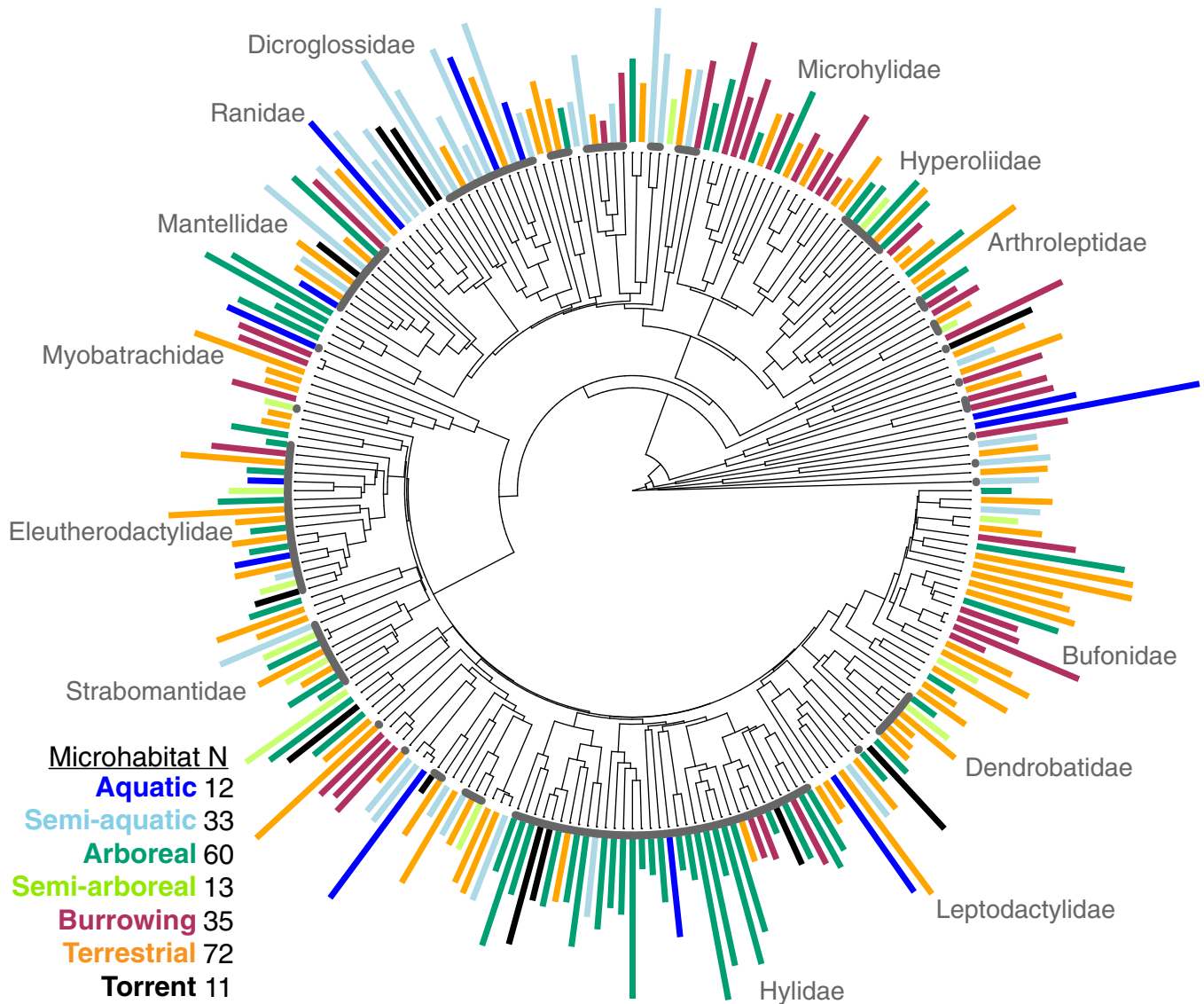
We converted our limb bone landmarks to forelimb, hindlimb, and combined limbs shape datasets in *geomorph* version 3.1.2 (Adams et al. 2018). We first performed a Generalized Procrustes analysis (GPA) on each limb bone to translate all specimens to the origin, scale them to unit-centroid size, and rotate them (using a least-squares criterion) until the landmarks were optimally aligned (Adams et al. 2018). We then used the `combine.subsets` function to combine individual bone landmarks into three shape datasets (forelimbs, hindlimbs, and combined limbs), which allowed us to analyze entire limbs while ignoring limb angle differences among preserved specimens. During implementation of the `combine.subsets` function we performed a second GPA (using option `GPA = TRUE`), which scaled bones to their unit-centroid size and resulted in correct bone proportions.

#### RELATING WHOLE LIMB SHAPE TO PHYLOGENY, SVL, AND MICROHABITAT

We performed all analyses on limb shape evolution within R version 3.6.0 (R Core Team 2019) using our limb shape data (described above) and an existing anuran phylogeny inferred from molecular data via maximum likelihood methods (Pyron 2014), which we trimmed to the 236 species within our study, using *phytools* version 0.6.6 (Revell 2012) and *geiger* version 2.0.6.1 (Harmon et al. 2007). For three species (*Adenomera marmorata*, *Indirana phrynoderma*, *Plectrohyla quechchi*) not on the original phylogeny, we substituted congeneric species names (*Adenomera andreae*, *Indirana leptodactyla*, *Plectrohyla glandulosa*) because they were the only representative samples from these genera in our dataset. Unless specified otherwise, all downstream analyses of limb shape evolution were performed within the R package *geomorph* version 3.1.2 (Adams et al. 2018). We estimated the phylogenetic signal in our forelimb, hindlimb, and combined limbs data using the `physignal` function, which estimates the multivariate version of the *K*-statistic (`Kmult`: Adams 2014).

To determine whether species forelimbs, hindlimbs, or combined limbs showed shape differences in relation to body size and microhabitat, we performed three phylogenetic MANOVAs using the `procD.pgls` function. Specifically, we ran phylogenetic MANOVAs with our trimmed phylogeny (described above), shape data (either forelimb, hindlimb, or combined limbs) as the dependent variable, specimen SVL as the first independent variable, and microhabitat as the second independent variable (shape  $\sim \log(\text{SVL}) + \text{microhabitat}$ ). We tested for pairwise limb shape differences among microhabitats using the `pairwise` function in the R package `RRPP` version 0.4.2 (Collyer and Adams 2018).

In addition to our phylogenetic MANOVA analyses, we compared evolutionary models to determine if a model incorporating individual microhabitat evolutionary optima better



**Figure 1.** Our sampling of 236 anuran species with snout-vent length (bar length) and microhabitat (bar color) indicated by the extending bars. Families are indicated by alternating grey and white bars between the phylogeny tips and the extending bars that indicate body size and microhabitat for each species. Families with more than five species in our sampling are named along the outside of the phylogeny. The molecular phylogeny was trimmed from Pyron (2014).

explained limb shape than other evolutionary scenarios. We first ran three phylogenetic MANOVAs using the `procD.pgls` function with shape data (either forelimb, hindlimb, or combined limbs) as the dependent variable and SVL as the independent variable. We performed principal component analyses (PCA) on the residual shape data from these phylogenetic MANOVAs and used these size-corrected limb shape data to compare evolutionary models within `mvMORPH` version 1.1.1 (Clavel et al. 2015). We only included the first two principal components (PCs) in our evolutionary models because analyzing more PCs resulted in unreliable conclusions from some models. We ran four evolutionary models: a Brownian motion model, a multirate Brownian motion (BMM) model, an Ornstein-Uhlenbeck (OU) model of

evolution with a single evolutionary optimum, and a multi-optima OU (OUM) model that models individual evolutionary optima for each microhabitat. For BMM and OUM models, we mapped microhabitat onto the phylogeny and estimated ancestral states of microhabitat via likelihood using the `make.simmap` function of `phytools` version 0.6.6 (Revell 2012) with one simulation (`nsim = 1`). We ran 20 replicates of each BMM and OUM evolutionary model to verify consistency in model fit over 20, unique `simmap` ancestral reconstructions. For these replicate models, we report the averages and standard errors for model fit statistics. For each size-corrected limb shape dataset, we assessed which evolutionary model was the best fit by AIC comparison.



We further assessed the degree to which limb skeleton shape reflected a species' microhabitat, by performing canonical variate analyses (CVAs) with forelimb, hindlimb, and combined limbs shape data. We ran three separate CVAs in the R package *Morpho* version 2.7 (Schlager 2017), using microhabitat as the grouping variable for each analysis. CVA results did not differ between limb shape data without body size correction and limb shape data with body size correction.

### COMPARING WHOLE LIMB EVOLUTIONARY RATES AND MORPHOLOGICAL DISPARITY

We next compared evolutionary rates and morphological disparity of the forelimbs, hindlimbs, and combined limbs among species that occupy different microhabitats. We again used size-corrected forelimb, hindlimb, and combined limbs shape data that we obtained by running three phylogenetic MANOVAs using the *procD.pgls* function with shape data (either forelimb, hindlimb, or combined limbs) as the dependent variable and SVL as the independent variable. The residual shape data from these phylogenetic MANOVAs were used to compare evolutionary rates of the forelimb, hindlimb, and combined limbs among species that occupy different microhabitats using the *compare.evol.rates* function. To compare the morphological disparity of the forelimbs, hindlimbs, and combined limbs, among species that occupy different microhabitats, we used the *morphol.disparity* function, with SVL as the covariate, which took phylogenetic relationships into account.

### COMPARING EVOLUTIONARY RATES AMONG LIMB BONES

To compare evolutionary rates among individual limb bones, we took GPA aligned landmarks for each individual bone and combined them with *combine.subsets*. To avoid biases associated with unequal landmark numbers among bones, we used only the four boundary landmarks that were applied to all five bones. We performed GPA alignments on each bone after reducing the humerus and tarsus to four landmarks. We did not perform a second GPA alignment during the *combine.subsets* function (GPA = FALSE), so that limb bones would not be scaled to their relative proportions and thus bigger bones would not be biased towards higher evolutionary rates simply due to minor proportional changes having a larger overall effect. We then analyzed evolutionary rate differences among the limb bones using the *compare.multi.evol.rates* function.

### RELATING INDIVIDUAL LIMB BONE SHAPE TO PHYLOGENY, SVL, AND MICROHABITAT

Finally, we analyzed whether differences in individual bone shape were associated with body size and microhabitat. We again analyzed only the four boundary landmarks that could

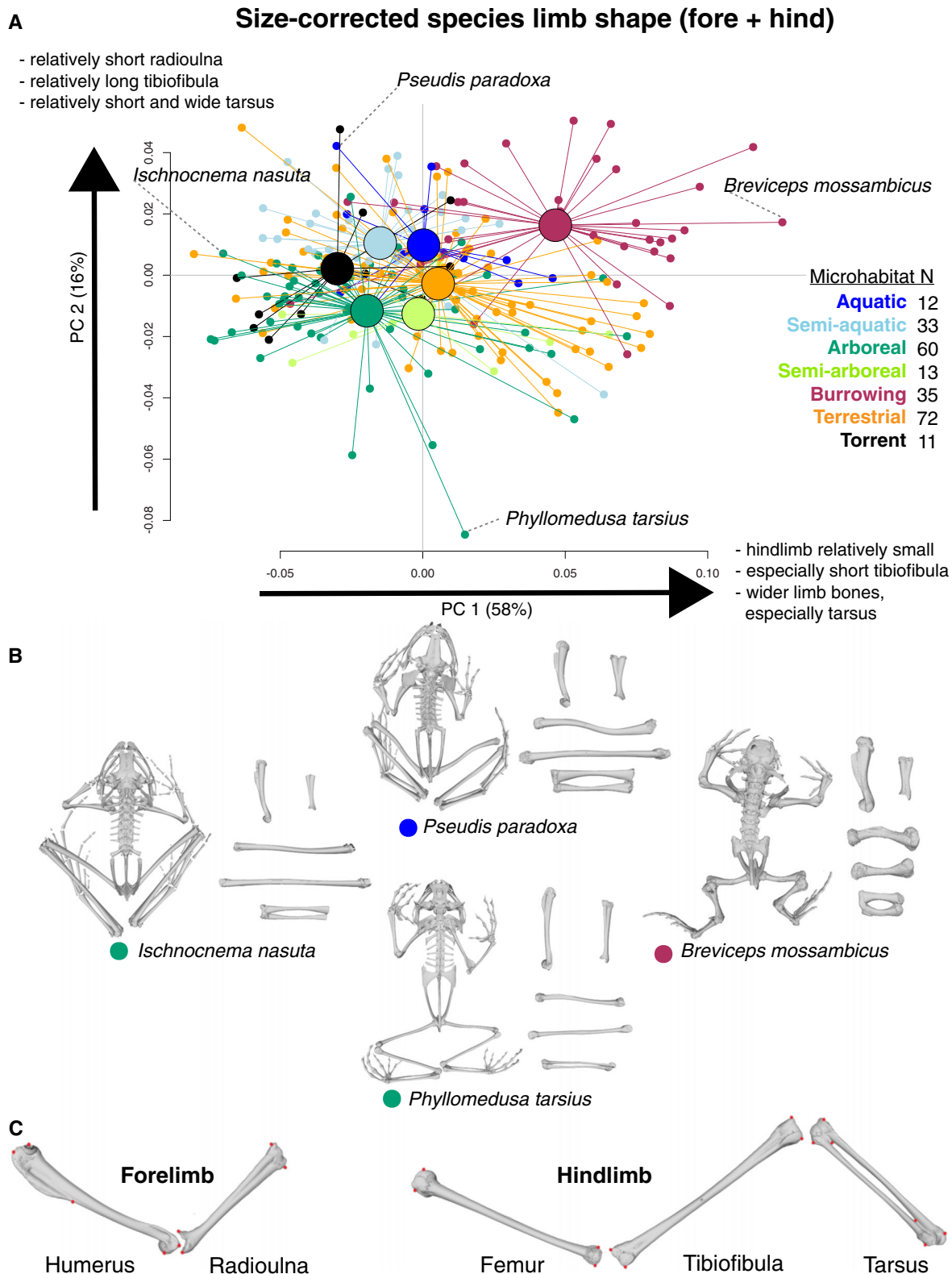
be compared among all five bones, because the main goal of this analysis was to examine factors that might contribute to the evolutionary rate differences seen among individual bones. Next, we estimated the phylogenetic signal of each limb bone's shape using the *physignal* function. We used the individual bone GPA aligned landmarks to run five separate phylogenetic MANOVAs using the *procD.pgls* function with our trimmed phylogeny, shape data (either humerus, radioulna, femur, tibiofibula, or tarsus) as the dependent variable, SVL of the specimen as the first independent variable, and microhabitat as the second independent variable.

## Results

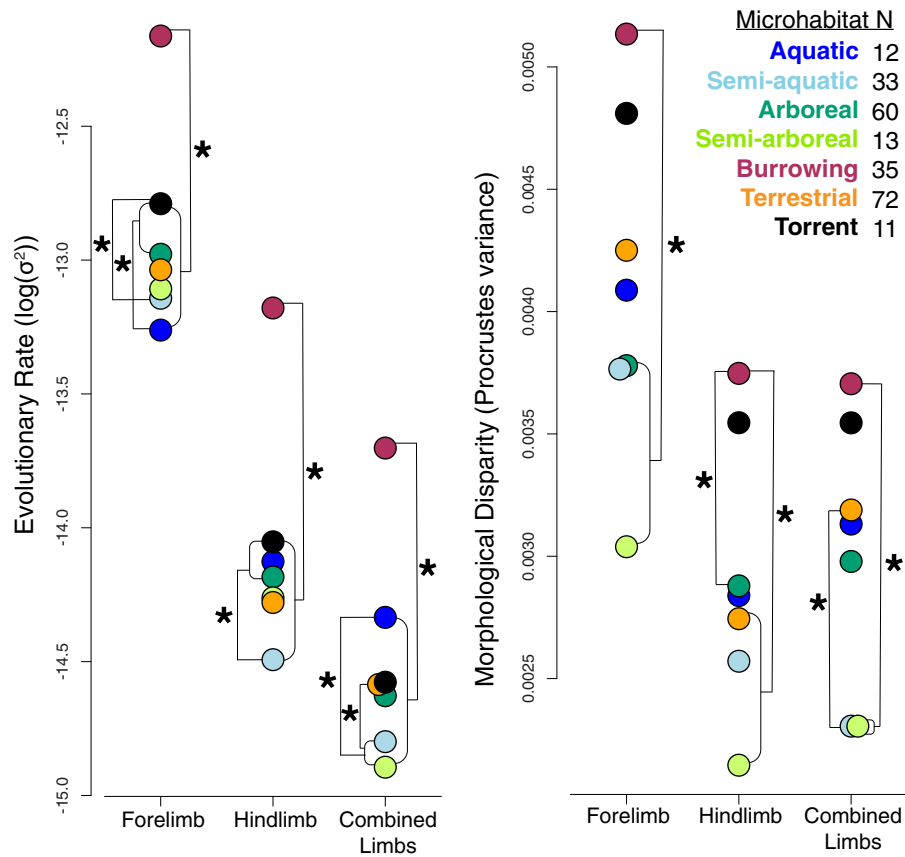
### EVOLUTION OF ANURAN LIMBS IN RELATION TO PHYLOGENY, BODY SIZE, AND MICROHABITAT

When analyzed in combination, fore- and hindlimb shape showed a strong relationship with phylogeny (phylogenetic signal:  $K = 0.494$ ,  $P < 0.001$ ), exemplified by the apparent differences between the two largest families in our study, Hylidae and Bufonidae (Supporting Information Fig. 2A), despite both families containing species ranging in body size and microhabitat. Anuran limbs also showed a weak but significant relationship with body size when accounting for phylogenetic relationships ( $F_{1,235} = 11.14$ ,  $R^2 = 0.04$ ,  $P < 0.001$ ; Supporting Information Fig. 2B), with larger frogs tending to have longer forelimbs relative to hindlimbs and thinner tarsi than smaller frogs. When accounting for body size and phylogenetic relationships, limb shape also showed a relationship with microhabitat ( $F_{6,229} = 5.23$ ,  $R^2 = 0.12$ ,  $P < 0.001$ ; Fig. 2). Evolutionary model comparisons support microhabitat affecting limb evolution with the best fitting model of size-corrected, combined limbs shape being an OU model in which each microhabitat has its own evolutionary optimum (Table 1). Burrowing frogs had the widest limb bones with the shortest tibiofibula relative to other hindlimb bones. Aquatic and semiaquatic species generally had shorter forelimbs relative to hindlimbs and a wider tarsus while arboreal and semiarboreal species showed the opposite pattern. Torrential and terrestrial species exhibited an intermediate limb morphology between arboreal and aquatic species. Furthermore, CVAs accurately classified a species within its microhabitat in 69% of cases when analyzing their fore- and hindlimbs in concert.

In addition to limb bone shape differences, limb evolutionary rates and morphological disparity differed among species that use different microhabitats. When analyzing the limbs in concert, burrowers had higher limb shape evolutionary rates than all other microhabitats but only had higher limb morphological disparity than semiaquatic and semiarboreal species (Fig. 3). Furthermore, semiaquatic and semiarboreal species had lower evolutionary rates compared to all other microhabitats, except arboreal



**Figure 2.** FPrincipal components analysis shows size-corrected combined fore- and hindlimb shape variation among species occupying different microhabitats. Panel A shows the centroids of species occupying each microhabitat as well as individual species combined limbs shape after correcting for the effects of body size. Panel B shows characteristic limb phenotypes from species at the extremes of PC1 and PC2. Panel C depicts all 23 limb landmarks (red dots).



**Figure 3.** Differences in evolutionary rate and morphological disparity of size-corrected limb shape among microhabitats. Pairwise significant differences among microhabitats are indicated by the asterisked lines. Overlapping points are offset for clarity.

and torrential species and lower morphological disparity than terrestrial species in addition to burrowing species (Fig. 3).

#### FORE- AND HINDLIMB SHAPE EVOLUTION MIRROR EACH OTHER WITH RESPECT TO PHYLOGENY, BODY SIZE, AND MICROHABITAT

When analyzed individually, both fore- and hindlimb shape showed significant phylogenetic signal (forelimbs:  $K = 0.343$ ,  $P < 0.001$ ; hindlimbs:  $K = 0.511$ ,  $P < 0.001$ ). Both fore- and hindlimbs also showed a relationship with body size (forelimbs:  $F_{1,235} = 13.16$ ,  $R^2 = 0.05$ ,  $P < 0.001$ ; hindlimbs:  $F_{1,235} = 18.83$ ,  $R^2 = 0.07$ ,  $P < 0.001$ ) when accounting for phylogenetic relationships. Fore- and hindlimbs also showed a relationship with microhabitat when accounting for body size and phylogenetic relationships with similar amounts of variation in fore- and hindlimbs explained by microhabitat (forelimbs:  $F_{6,229} = 4.22$ ,  $R^2 = 0.09$ ,  $P < 0.001$ ; hindlimbs:  $F_{6,229} = 4.88$ ,  $R^2 = 0.11$ ,  $P < 0.001$ ; Fig. 3). Although, both fore- and hindlimb shape showed similar pairwise differentiation among microhabitats (Fig. 4), CVA accurately classified a species within its microhabitat in 63% of cases when using size-corrected hindlimb shape but in only 50% of cases using size-corrected forelimb shape.

Furthermore, an OU model in which each microhabitat has its own evolutionary optimum best fit forelimb and hindlimb shape data (Table 1).

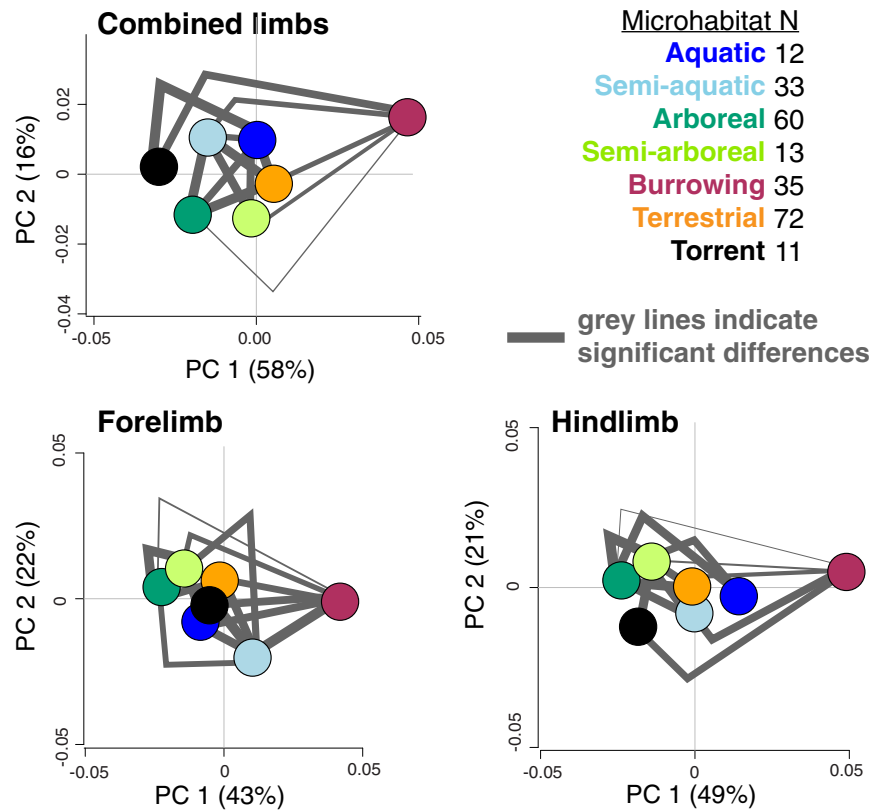
When comparing among species that use different microhabitats, patterns of fore- and hindlimb evolutionary rates and morphological disparity were similar to patterns seen when analyzing the limbs in combination. Burrowers had higher fore- and hindlimb shape evolutionary rates than all other microhabitats and semiaquatic had lower fore- and hindlimb evolutionary rates than many other microhabitats (Fig. 3A). Semiaquatic, semiarboreal, and arboreal species also had lower fore- and hindlimb morphological disparity than burrowing species (Fig. 3B).

#### INDIVIDUAL LIMB BONES VARY IN THEIR EVOLUTIONARY RATES AND THEIR RELATIONSHIPS WITH BODY SIZE, MICROHABITAT, AND PHYLOGENY

Evolutionary rates among the five anuran limb bones differed ( $P < 0.001$ ; Fig. 5A). The distal-most bone had the highest evolutionary rate within both the fore- and hindlimbs (radioulna > humerus,  $P < 0.001$ ; tarsus > tibiofibula and tarsus > femur,  $P < 0.001$ ; Fig. 5A). Similarly, in both the fore- and hindlimb, body size (SVL) explained a greater proportion of shape variation in



## Pairwise differences between microhabitat limb shape



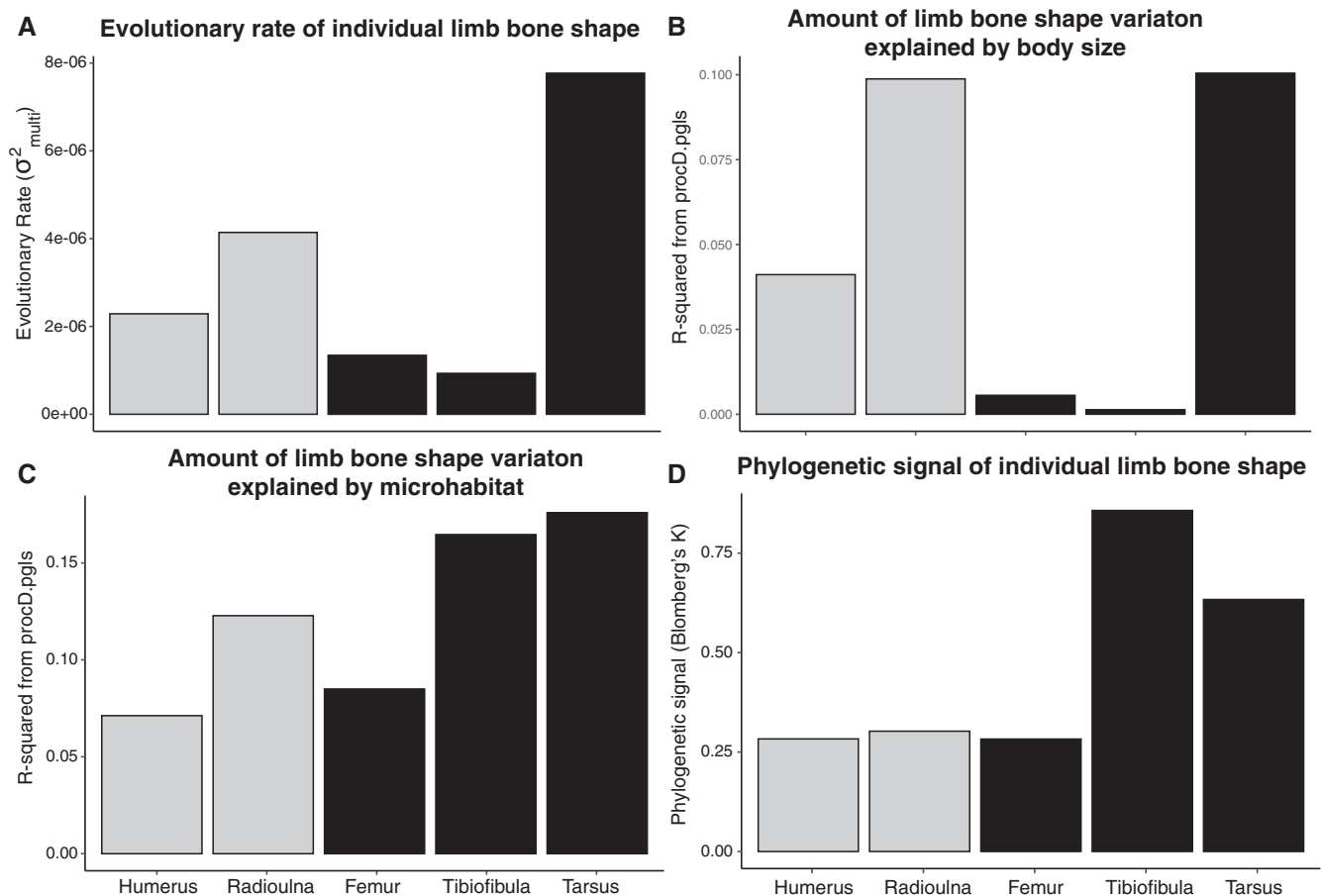
**Figure 4.** Pairwise differences of size-corrected limb shape among species that occupy different microhabitats. Each PCA plot shows the centroids of each microhabitat with gray lines connecting microhabitats with significant pairwise differences. Line thickness scales to the effect size (Z score) of the pairwise comparisons with thinner lines indicating more differentiation between two microhabitats.

the distal-most bone (Fig. 5B; humerus: SVL  $R^2 = 0.04$ ,  $P < 0.001$ ; radioulna: SVL  $R^2 = 0.10$ ,  $P < 0.001$ ; femur: SVL  $R^2 = 0.01$ ,  $P = 0.223$ ; tibiofibula: SVL  $R^2 < 0.001$ ,  $P = 0.733$ ; tarsus: SVL  $R^2 = 0.10$ ,  $P < 0.001$ ), indicating allometry may contribute to higher evolutionary rates in distal limb bones. Limb changes associated with microhabitat may also contribute to higher evolutionary rates in distal limb bones. Within the forelimb, the higher evolutionary rates of the radioulna were accompanied by a higher amount of radioulna shape explained by microhabitat when accounting for body size and phylogenetic relationships (humerus: microhabitat  $R^2 = 0.07$ ,  $P < 0.001$ ; radioulna: microhabitat  $R^2 = 0.12$ ,  $P < 0.001$ ; Fig. 5C). Similarly, within the hindlimb, microhabitat explains over double the amount of shape variation in the distal-most tarsus (microhabitat  $R^2 = 0.18$ ,  $P < 0.001$ ) than in the proximal-most femur (microhabitat  $R^2 = 0.09$ ,  $P < 0.001$ ). However, despite the greater evolutionary rate of the tarsus compared to the medial tibiofibula (Fig. 5A), a similar amount of tibiofibula (microhabitat  $R^2 = 0.17$ ,  $P < 0.001$ ) and tarsus shape variation was explained by microhabitat when accounting for body size and phylogenetic relationships (Fig. 5C). Although all limb bone shapes showed significant phylogenetic

signal (all bones:  $P < 0.001$ ), the tibiofibula had the highest phylogenetic signal, the tarsus had the second highest phylogenetic signal, and the three other limb bone shapes had relatively lower phylogenetic signal (Fig. 5D). Thus, higher phylogenetic signal does not correlate with greater evolutionary rates of distal limb bones.

## Discussion

Our study examined 236 species that ranged in microhabitat, body size, and evolutionary history to determine the role of extrinsic and intrinsic factors shaping anuran limb evolution across deep evolutionary timescales. Microhabitat emerged as a primary driver, although allometry and phylogeny also played a significant role. Forelimbs and hindlimbs showed similar patterns of evolution in relation to these factors, overturning previous notions that anuran forelimbs are more conserved. We also found that distal, later-developing limb bones are more evolutionarily labile than proximal limb bones, agreeing with evidence in mammals and indicating a common developmental constraint on earlier-developing, proximal tetrapod limb bone evolution.



**Figure 5.** Differences in the evolutionary rates of individual bone limb shape (A) and the degree to which each bone's shape variation is explained by body size (B), microhabitat (C), and phylogeny (D). To avoid biases associated with unequal landmark numbers among bones, we used only the four comparable boundary landmarks of each limb bone. Grey bars are forelimb bones and black bars are hindlimb bones.

### THE IMPACT OF MICROHABITAT ON LIMB EVOLUTION

Tetrapod clades show varying relationships between limb morphology and ecology, with phylogeny or allometry playing a more important role in some (Lilje et al. 2003; Stoessel et al. 2013; Martín-Serra et al. 2014). Like many anuran studies (Gomes et al. 2009; Moen et al. 2013; Vidal-García and Keogh 2015; Vidal-García and Keogh 2017; Citadini et al. 2018; Moen 2019), we found a strong relationship between limb morphology and microhabitat, contradicting another large study that found no such relationship (Jorgensen and Reilly 2013). We found that both fore- and hindlimbs had a significant relationship with microhabitat unlike other studies that examined the limbs separately (Jorgensen and Reilly 2013; Vidal-García and Keogh 2017). Our evolutionary model comparisons also support the influence of microhabitat on anuran limb evolution. Combined limbs shape evolution was best explained by an OU model with a unique adaptive peak for each microhabitat. We interpret these model compar-

isons with caution as analyzing seven different ecologies requires a large number of parameters to be accurately estimated and evaluated, increasing potential error (Adams and Collyer 2019). Although we analyzed reduced PC axes, which helped address low sample size to parameter ratios, this inherently biased our dataset (Uyeda et al. 2015). It is at least comforting that the first few PCs of other analyzed datasets seem biased toward supporting Brownian motion models of evolution, yet we find evidence for a more complex model of evolution when analyzing our first two PCs (Uyeda et al. 2015). Furthermore, CVAs were able to predict microhabitat correctly for nearly two-thirds of species based on our limited number of limb landmarks, though prediction accuracy varied among microhabitats. Differences in limb morphology exist between microhabitats, but microhabitats show varying degrees of limb specialization and on its own, limb skeletal morphology cannot be used to predict microhabitat. Adding other aspects of morphology like pelvic morphology and toepad area may increase predictive power.

Differences in average limb morphology between microhabitats fit well with performance studies. Burrowers, which differ from all groups except aquatic frogs, have smaller hindlimbs relative to forelimbs with especially short tibiofibulae and wider bones (especially the tarsus). The distinctiveness of burrowing limb evolution is discussed in the next section. Aquatic and semi-aquatic frogs have a relatively short radioulna, a relatively long tibiofibula, and a relatively short and wide tarsus while arboreal and semiarboreal frogs show the opposite pattern. As the forelimbs are not used in swimming (Abourachid and Green 1999), shortening them may reduce drag in aquatic frogs. For arboreal frogs, long forelimbs can improve reach while thinner bones likely reduce weight, making climbing easier. Torrent frogs seem to have intermediate limb morphologies between aquatic and arboreal frogs, which fit well with their dual climbing-swimming lifestyle. More performance work is required to understand trade-offs between climbing and swimming, and how they influence specialization in microhabitats. Terrestrial frogs show a wide array of limb morphologies, which may be explained by the inclusion of variable locomotor types, including jumpers and walkers, in this microhabitat (Emerson 1988; Enriquez-Urzelai et al. 2015; Petrović et al. 2017).

#### **LIMBS OF BURROWING ANURANS ARE DISTINCT AND HAVE THE HIGHEST EVOLUTIONARY RATES**

We found that burrowers differed in limb shape from species within most other microhabitats, which concords with past studies that found burrowing frogs unique even when other microhabitats were not (Emerson 1988; Gomes et al. 2009; Jorgensen and Reilly 2013; Moen et al. 2013; Vidal-García et al. 2014; Enriquez-Urzelai et al. 2015; Vidal-García and Keogh 2015, 2017; Citadini et al. 2018). Species with the shortest and squat-test limb bones in our study are all burrowers and the limb bones of the three, closely related semiburrowing species that we collapsed with our burrowing species were extremely similar to other burrowing species (Supporting Information Fig. 3). Because the hindlimbs function as out-levers when burrowing (Emerson 1976), shortening the bones increases the generated force and improves burrowing performance. Stouter bones may be better at resisting stress than narrow bones. We also found that burrowing frogs have the highest evolutionary rates compared with frogs from other microhabitats. This may indicate that once frogs start down the path to fossoriality, which allows them to exploit new niches and maintain water balance in arid environments (Bentley 1966; Cartledge et al. 2006), there are high selection pressures to quickly shift their ancestral form to a form better suited for digging.

#### **THE INFLUENCE OF PHYLOGENY AND ALLOMETRY ON IMPERFECT CONVERGENCE OF LIMB MORPHOLOGY**

Despite a clear relationship between limb shape and microhabitat, there is considerable limb shape overlap among species with different microhabitats. This imperfect convergence may be explained by phylogenetic history, as past adaptations, functional trade-offs, and genetic correlations can drive lineages along different evolutionary pathways, even when exposed to similar selection pressures (Alfaro et al. 2005; Samuels et al. 2013; Collier et al. 2014; Morinaga and Bergmann 2017). All limb bones showed strong phylogenetic signal and, despite varying ecologies, families tended to cluster together in morphospace as seen in Hylidae and Bufonidae (Supporting Information Fig. 2A). Such historical contingency has been noted in other anuran families before (Moen et al. 2013; Vidal-García and Keogh 2015) as well as outside of Anura in the limbs of musteloids (Fabre et al. 2015) and lygosomine skinks (Foster et al. 2018). Thus, phylogenetic history plays a vital role in determining how selection shapes morphology in anurans along with other tetrapod groups.

The relationship between limb shape and body size (allometry) can also blur morphological differences between microhabitats. Both forelimbs and hindlimbs together and separately showed small but significant relationships with body size, similar to other studies (Enriquez-Urzelai et al. 2015; Vidal-García and Keogh 2017). Frogs with longer forelimbs relative to hindlimbs and thinner tarsi tend to be smaller while frogs with relatively shorter forelimbs tend to be larger in size. When jumping, shorter forelimbs keep the frog's center of mass backward, increasing propulsion (Emerson 1985; Nauwelaerts et al. 2007); however, longer forelimbs can absorb greater landing forces, which increase with jump distance and affect recovery time between jumps (Nauwelaerts and Aerts 2006), creating a trade-off between two parts of the jump cycle. Our finding may point to larger frogs interacting with this trade-off differently than smaller frogs. Perhaps larger frogs require shorter forelimbs to achieve suitable propulsion due to their greater mass, regardless of the impact on landing. Alternatively, there may be a size at which forelimbs no longer need to be longer to accomplish a good landing. Variation in pectoral girdle morphology between small and large frogs may also impact this trade-off and its influence on forelimb morphology, although more work is required to test these hypotheses.

#### **SHARED EVOLUTIONARY PATTERNS BETWEEN FORELIMBS AND HINDLIMBS**

While many tetrapod clades have shown similar patterns between limbs (Herrel et al. 2002; Martín-Serra et al. 2014; Foster et al. 2018), our finding that fore- and hindlimb shapes show similar associations with microhabitat and body size contradicts previous work in anurans. Unlike previous work (Vidal-García and

Keogh 2017), we did not find a difference in allometric relationships between fore- and hindlimbs. We also find that fore- and hindlimbs both have a relationship with microhabitat, contrary to other anuran studies that found forelimbs to have no relationship to microhabitat (Jorgensen and Reilly 2013; Vidal-García and Keogh 2017; Citadini et al. 2018). With evolutionary model comparisons, we find that both forelimb and hindlimb shape evolution was best explained by an OU model with a unique adaptive peak for each microhabitat. We show that forelimbs generally have greater evolutionary rates and morphological disparity than hindlimbs, further implying that forelimbs are not as conserved as previously believed. At the broad scale studied here, the evolution of fore- and hindlimbs appear to be similarly influenced by these extrinsic and intrinsic factors.

#### **EVIDENCE FOR CONSERVED DEVELOPMENTAL CONSTRAINTS ACROSS TETRAPOD GROUPS RELATED TO DEVELOPMENTAL TIMING OF LIMB BONES**

Of the anuran limb bones, the distal-most bones (radioulna and tarsus) showed the highest evolutionary rates, supporting a hypothesis proposed in mammals that later-developing bones are more evolutionarily labile (Weisbecker 2011; Martín-Serra et al. 2015). It has been proposed that this increased variation is due to a reduction in developmental constraints allowing later-developing, usually distal bones more freedom to change in response to selection pressures. Strengthening this hypothesis are notable heterochronies in monotremes and European moles, where the humerus ossifies after the distal bones and shows more specialization (Weisbecker 2011). Frog limbs develop in a proximal-distal direction (Fabrezi et al. 2017), so the increased evolutionary rates we found may indicate that similar developmental constraints influence anuran limb evolution.

The reduced developmental constraints on the distal radioulna and tarsus may allow them more freedom to respond to selection pressures. We found evidence for multiple different factors contributing to the increased evolutionary rates of distal limb bones. Body size consistently explained more shape variation in distal bones than proximal bones, mirroring the variation in evolutionary rate. Microhabitat explained more shape variation in the distal bones compared to the most proximal fore- and hindlimb bones but a similar amount between the medial tibiofibula and the distal tarsus in the hindlimb, despite the much higher evolutionary rate of the tarsus compared to the tibiofibula. The tibiofibula and tarsus both have much higher phylogenetic signal than other limb bones, which is driven by large clade-specific shifts in morphology. In contrast, phylogenetic signal is fairly intermediate and consistent among forelimb bones. The higher evolutionary rate of the later-developing distal bones may be related to selection pressures related to body size and

microhabitat. In the hindlimb, clade-specific changes in morphology may also contribute to differences in evolutionary rates among hindlimb bones.

During early development, amphibian limbs are under less constraint than amniote limbs due to a decoupling between limb patterning and organogenesis (Galis et al. 2001; Irie and Sehara-Fujisawa 2007). Less work has been done comparing limb evolution in later development, when the shape and size of elements changes, although amniotes do show greater modification of limbs than amphibians (e.g., bat wings, bird feet, etc.; Galis et al. 2001; Young 2013). Our work cannot compare the degree of constraint and evolutionary lability in limbs between amniotes and amphibians, but it does show that, like in most mammals, anuran distal bones are more evolutionarily labile than proximal bones.

## *Conclusion*

By examining limb evolution in a rich taxonomic group with disparate ecologies, sizes, and evolutionary histories, we were able to tease apart and examine the roles of selection and constraint in the evolution of anuran limbs and address more general questions about tetrapod limb evolution. Microhabitat has clearly influenced limb bone shape in frogs. However, convergence remains imperfect, preventing the use of limb bones alone as a predictive tool for microhabitat. Unlike most previous studies, we also looked at individual limb bones and found that the distal bones showed more variation. This supports the hypothesis of reduced developmental constraints on later-developing bones and provides evidence for these general constraints acting across tetrapods. More work will be required to examine how these patterns vary between amniotes and amphibians.

#### **AUTHOR CONTRIBUTIONS**

NS contributed to the data collection, data analysis, writing, and editing of this manuscript. MCW contributed to the conception of the study, data analysis, writing, and editing of the manuscript.

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**DATA ARCHIVING**

Micro CT scans are available for download on Morphosource.org (<http://www.morphosource.org>, project number P967). Landmark data and R scripts used for analysis can be found on Dryad (<https://doi.org/10.5061/dryad.qfttdz0dc>).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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### *Supporting Information*

Additional supporting information may be found online in the Supporting Information section at the end of the article.

#### Supporting Material

**Supplemental Figure 1** - Landmarks used for each bone as shown on CAS125402: a) dorsal view of femur, b) ventral view of femur, c) dorsal view of humerus, d) ventral view of humerus, e) dorsal view of tibiofibula, f) ventral view of tibiofibula, g) radioulna, and h) tarsus.

**Supplemental Figure 2** – Principal components analysis showing how size-corrected shape of the fore- and hindlimbs varies among species that are in different families (Left Panel) and have different body sizes (Right Panel).

**Supplemental Figure 3** – Principal components analysis showing the three semiburrowing species that are collapsed with burrowing species in our analysis are extremely similar to other burrowing species.