SYMPOSIUM

Evolution of the Unique Anuran Pelvic and Hind limb Skeleton in Relation to Microhabitat, Locomotor Mode, and Jump Performance

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Synopsis   Anurans (frogs and toads) have a unique pelvic and hind limb skeleton among tetrapods. Although their distinct body plan is primarily associated with saltation, anuran species vary in their primary locomotor mode (e.g., walkers, hoppers, jumpers, and swimmers) and are found in a wide array of microhabitats (e.g., burrowing, terrestrial, arboreal, and aquatic) with varying functional demands. Given their largely conserved body plan, morphological adaptation to these diverse niches likely results from more fine-scale morphological change. Our study determines how shape differences in Anura's unique pelvic and hind limb skeletal structures vary with microhabitat, locomotor mode, and jumping ability. Using microCT scans of preserved specimens from museum collections, we added 3D landmarks to the pelvic and hind limb skeleton of 230 anuran species. In addition, we compiled microhabitat and locomotor data from the literature for these species that span 52 of the 55 families of frogs and ∼210 million years of anuran evolution. Using this robust dataset, we examine the relationship between pelvic and hind limb morphology and phylogenetic history, allometry, microhabitat, and locomotor mode. We find pelvic and hind limb changes associated with shifts in microhabitat ("ecomorphs") and locomotor mode ("locomorphs") and directly relate those morphological changes to the jumping ability of individual species. We also reveal how individual bones vary in evolutionary rate and their association with phylogeny, body size, microhabitat, and locomotor mode. Our findings uncover previously undocumented morphological variation related to anuran ecological and locomotor diversification and link that variation to differences in jumping ability among species.

Introduction

The emergence of unique postcranial morphotypes has enabled distinct shifts in locomotor modes, such as the pygostyle in birds (Benson and Choiniere 2013), the flight-enabling pectoral girdle of bats (Rayner 1988), and the reduction of the pelvic girdle associated with limbless locomotion in squamates (Gans 1962). In many cases, these synapomorphies also facilitated expansion into previously unoccupied niches, resulting in evolutionary radiations and within-clade niche diversification (e.g., birds, Benson and Choiniere 2013 and pygopodid lizards, Shine 1986). Among tetrapods, frogs and toads (order Anura) have a unique body morph (Handrigan and Wassersug 2007) and exhibit a range of locomotor modes and ecologies, making them an excellent clade for testing how variation in their specialized features affects locomotion and covaries with ecological diversity.

The unique anuran morph consists of distinct hind limb and pelvic features that are critical for their characteristic jump and are conserved among all 7000+ species (Jenkins and Shubin 1998; AmphibiaWeb 2020). First, within the hind limbs, two tarsal bones (tibiale and fibulare) have elongated to form a third leg segment that adds approximately two body-lengths to frogs' maximum jumping distance (Kargo et al. 2002). Second, anurans have a long bony element called the urostyle attached to their sacral vertebra, or sacrum, which provides axial rigidity (Přikryl et al. 2009; Reilly and Jorgensen 2011). Third, anurans have a specialized pelvic morphology that articulates with the sacral vertebra. These morphological changes are thought to have evolved when the clade first emerged, a hypothesis that is supported by fossil evidence (Báez and Basso 1996; Jenkins and Shubin 1998; Báez and Nicoli 2008).

Although the ancestral anuran form was optimal for hopping and eventually jumping (Reilly and Jorgensen 2011), it is unclear how variation in the anuran pelvic girdle and hind limb relates to the subsequent widespread diversification in ecology and locomotion. Extant anurans exhibit a broad range of locomotor modes, such as walking, jumping, burrowing, and swimming, and have colonized diverse microhabitats, such as high grade-streams (torrent), water bodies (aquatic), trees (arboreal), land (terrestrial), underground (burrowing), and intergrades between each (Wells 2013). Locomotor mode and microhabitat are somewhat related (e.g., most aquatic anuran species are primarily swimmers), but species within any given microhabitat exhibit diversity in locomotor modes (e.g., terrestrial species can be walker, hoppers, or jumpers; Fabrezi et al. 2014).

Pelvic and hind limb structures have been shown to vary among microhabitat and locomotor mode, but studies differ in which skeletal features best explain these relationships.

Numerous studies have shown relationships between hind limb morphology and microhabitat (Gomes et al. 2009; Moen et al. 2013; Vidal-García and Keogh 2015; Citadini et al. 2018; Moen 2019) as well as locomotor mode (Enriquez-Urzelai et al. 2015). Namely, shorter, stouter limbs are associated with burrowers and walker-hoppers, and long limbs are associated with arboreal, torrent, and aquatic frogs and jumpers. However, other studies found pelvic variation to be more related to locomotor mode than hind limb variation (Jorgensen and Reilly 2013). Work examining pelvic morphology

has found relationships between expanded sacral vertebral diapophyses (Emerson 1979, 1982; Reilly and Jorgensen 2011; Jorgensen and Reilly 2013, Petrović et al. 2017), narrower sacrum width (Simons 2008; Jorgensen and Reilly 2013), and ilial length (Emerson 1982, Soliz et al. 2017) in burrowing and walking species versus arboreal and jumping species. Studies conflict about whether sacral diapophyseal expansion is part of a suite of changes associated with locomotor mode differences (Emerson 1982) or whether sacral variation is less related to locomotor mode than other pelvic features and better explained by a Brownian Motion model of evolution (Soliz et al. 2017). It is clear that pelvic and hind limb variation is related to microhabitat and locomotor mode, but differences in sampling, measurements, and categorization of locomotor modes and microhabitats among studies leave overall conclusions about related morphological adaptations and their functional consequences unclear.

Pelvic and hind limb features that vary with locomotor mode and microhabitat are hypothesized to influence jumping ability; however, few studies directly link microhabitat-specific and locomotor mode-specific morphologies to jump performance data. Examining 44 species from eight families, Moen (2019) found that anuran species that used the same microhabitat converged on morphology and jump performance. Moen (2019) directly related changes in morphology to jump performance, showing leg muscle mass positively correlated with acceleration and power, while both leg length and muscle mass positively correlated with jump length. Other studies have documented greater jump distances in arboreal, semi-aquatic, and torrent species compared to terrestrial and fossorial species (Zug 1978; Gomes et al. 2009; Citadini et al. 2018). Most studies that have related jump performance and microhabitat-specific morphology have focused on the hind limbs (Gomes et al. 2009; Moen et al. 2013; Citadini et al. 2018; Moen 2019), while ignoring variation in pelvic structures. Jorgensen and Reilly (2013) used performance data from Zug (1972) to relate hind limb length and pelvic type to jump distance, concluding that limb length was the primary predictor of relative jump length, while pelvic type had no effect. With more extensive phylogenetic and morphological sampling, we aim to examine the relationship between a species' jump distance and changes in both pelvic and hind limb morphology related to microhabitat and locomotor mode.

We take advantage of anuran diversity, natural history collections, and 3D geometric morphometrics

to examine how the "classic" anuran pelvic and hind limb morphotype differs among microhabitats and locomotor modes when accounting for both phylogeny and allometry. We then determine how variation in pelvic and hind limb morphology that differentiates among microhabitats and locomotor modes relates to a species' jump distance. Finally, we compare evolutionary rates among individual pelvic and hind limb bones with respect to phylogeny, body size, microhabitat, and locomotor mode to identify potential drivers of bone-specific evolution. Our study more comprehensively samples anuran diversity than previous studies, with 230 species from 52 of the 55 extant families, representing many independent evolutionary transitions in microhabitat, locomotor mode, and body size (Supplementary Fig. S1). This allows us to investigate how pelvic and hind limb morphology reflects microhabitat and locomotor ability at deep evolutionary timescales.

Methods

MicroCT scanning and morphological data collection from museum specimens

We microCT scanned 220 specimens with a Phoenix vjtomejx M (GE Measurement & Control Solutions, Boston, MA) at the University of Florida's Nanoscale Research Facility as detailed in Stepanova and Womack (2020). For 10 additional specimens, we downloaded one microCT scan each from Morphosource.org (specimen and project details are in Supplementary Datatable S1). All specimens belonged to a unique species and were vouchered museum specimens with 121 specimens from the National Museum of Natural History in Washington, DC, 99 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 and Natural History Museum in Lawrence, KS. The specimen's sex was not considered in this study. To account for body size, we measured snout-vent length (SVL; distance from the tip of the rostrum to the caudal end of the ischium) of loaned specimens to the nearest 10th millimeter using a digital caliper (31-415-3, Swiss Precision Instruments Inc., Garden Grove, CA) and measured SVL of all downloaded skeletons in Meshlab (Cignoni et al. 2008). All scans are freely available on Morphosource.org (Project

Number P967) and specimen details can be found in Supplementary Datatable S1.

Microhabitat, locomotor mode, and performance data

We collated species microhabitat data from primary references (Andreone and Luiselli 2003; McCranie and Castañeda 2005; Brito et al. 2012; Matojo 2015) and secondary references (Moen et al. 2016; Moen and Wiens 2017; AmphibiaWeb 2020; IUCN Red List of Threatened Species 2020). We used eight microhabitat categories defined by Moen and Wiens (2017): (1) aquatic—usually in water, (2) arboreal—typically on aboveground vegetation, (3) burrowing—nonbreeding season spent underground or in burrows they dug, (4) semi-aquatic—partially aquatic and partially terrestrial, (5) semi-arboreal—partially arboreal and partially terrestrial, (6) semi-burrowing—partially burrowing and partially terrestrial, (7) terrestrial—found on the ground, under rocks, or in leaf litter, and (8) torrential—found in high-gradient, fast-flowing streams.

Jump performance data were collated from primary references (Zug 1978, 1985; Rogowitz et al. 1999; Smith et al. 2006; Royan et al. 2010; Reilly et al. 2015, 2016; Rebelo and Measey 2019) and one secondary reference (Gomes et al. 2009). For one species (*Hypsiboas geographicus*), we averaged jump distances from two studies (Reilly et al. 2015, 2016). All associated data and references including the sizes of animals used in the performance studies (for comparison with our scanned specimens) are in table S1.

We categorized as many species as possible into one of six locomotor modes (swimmer, jumper/swimmer, jumper, hopper, walker/hopper, and walker) using existing locomotor mode assignments from the literature (Emerson 1978; Simons 2008; Jorgensen and Reilly 2013; Fabrezi et al. 2014; Reilly et al. 2015, 2016; Lires et al. 2016; Soliz et al. 2017; Fratani et al. 2019; Frýdlová et al. 2019; Moreno-Rueda et al. 2019). Walkers rarely hop or jump, hoppers jump less than nine times their body length per jump, and jumpers jump more than nine times their body length (Emerson 1978). Swimmers mainly locomote through water bodies. Mixed locomotor mode categories from previous studies—walker/hopper and jumper/swimmer—were conservatively retained because we could not determine the primary locomotor mode from natural history data. In addition to locomotor mode assignments from the literature, we used jump performance data from the literature (detailed above) to categorize eight species as jumpers based
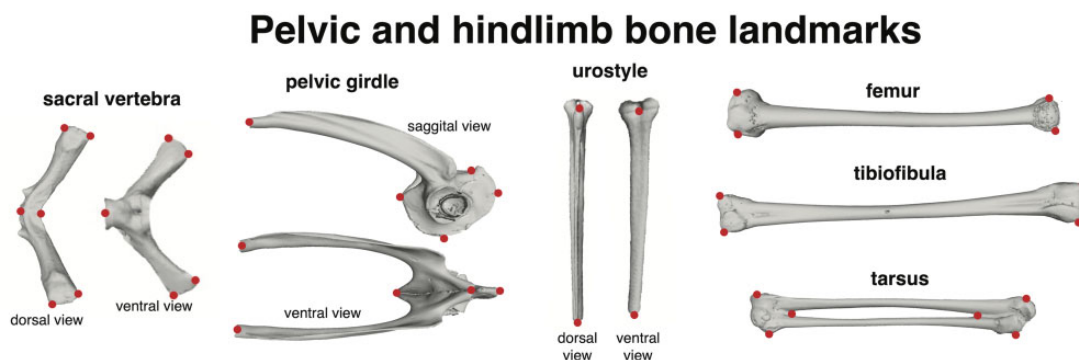
Pelvic and hindlimb bone landmarks

Fig. 1 Diagram of 3D landmarks used in this study. Bone structures not to scale.

on those species having an average jump length greater than nine times their body length (SVL). We also used these jump performance data to categorize six species as hopper/walkers based on average jump lengths less than nine times their body length (SVL). We conservatively categorized these six species as walkers/hoppers instead of hoppers because we could not verify the relative amount of these species hop versus walk. One species (*Tomopterna cryptotis*) had been previously classified as a walker/hopper by Jorgensen and Reilly (2013); however, Rebelo and Measey (2019) report this species' average jump distance as 9.09 times its SVL. We did not change the walker/hopper categorization by Jorgensen and Reilly (2013) because the average jump distance relative to SVL was barely over the jumper threshold of nine times its SVL. We also categorized nine fully aquatic species as swimmers.

Landmark placement

We added 3D landmarks to the pelvic and the right hind limb bones using the R v3.5.1 (R Core Team 2020) package geomorph 3.0.7 (Adams et al. 2018). Each bone had its own set of landmarks that correspond to homologous and repeatable points that define the outer borders of the bony structure (Fig. 1A). For the hind limb, landmark data were the same as those used in Stepanova and Womack (2020): six on the tarsus, four on the tibiofibula, and four on the femur. For the three main bony structures in the pelvic region, seven landmarks defined the sacral vertebra, six the pelvic girdle (ilia, ischium, and pubis), and three the urostyle. Our landmark sampling of the pelvic regions provides three-dimensional shape data as opposed to scoring character states or measuring linear distances or diapophyseal (transverse process of a sacral vertebra) angle (Zug 1972; Emerson 1979, 1982; Simons 2008; Reilly and Jorgensen 2011).

Conversion of pelvic and hind limb landmarks to shape datasets for downstream analyses

We converted pelvic and hind limb bone landmarks to three shape datasets for downstream analysis (pelvic, hind limb, and combined pelvic and hind limb) in geomorph v3.1.2 (Adams et al. 2018). We rotated the pelvic bones into comparable positions across species with the R tool ShapeRotator (Vidal-García et al. 2018) because we wanted to include information about where the sacrum and pelvic girdle meet. Although the position and connection between the ilium and sacrum can be influenced by specimen fixation position, the vast majority of specimens were fixed in a standard, flattened position, and our goal was to standardize the relative angles and positions of the pelvic base, ilia, sacral vertebra, and urostyle so that we could capture clear interspecific differences in where the sacrum and pelvic girdle met. We visually inspected our rotated landmarks and found them to be consistent and comparable across specimens. We performed a Generalized Procrustes analysis (GPA) on the rotated pelvic landmarks 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.

For the hind limb, we focused on changes to individual bone shape and their relative sizes (not their relationship in space), so we did not rotate these bones and instead GPA aligned each bone's landmarks before using the combine.subsets function to combine the three limb bones. We performed a second GPA analysis (using option GPA = TRUE) within combine.subsets to correctly scale bones relative to one another. This allowed us to analyze the entire hind limb while ignoring limb angle differences among preserved specimens.

For our analyses on combined pelvic and hind limb shape, we used combine.subsets to combine

our rotated and GPA aligned pelvic dataset with our three, individually GPA aligned hind limb bones. We performed a second GPA analysis (using option GPA=TRUE) within combine.subsets to correctly scale bones relative to one another.

Analysis of pelvic and hind limb shape in relation to phylogeny, body size, microhabitat, and locomotor mode

We ran a series of analyses using the R package geomorph v3.0.7 (Adams et al. 2018) to determine how pelvic shape, hind limb shape, and combined pelvic and hind limb shape varied with phylogeny, SVL, and microhabitat. For all analyses, we used the three shape datasets (pelvic shape, hind limb shape, and combined pelvic and hind limb shape) and an existing anuran phylogeny inferred from molecular data via maximum likelihood methods (Pyron 2014), which we trimmed to the species in our analyses using phytools v0.6.6 (Revell 2012) and geiger v2.0.6.1 (Harmon et al. 2008). We first used the physignal function to estimate the phylogenetic signal of each of our three shape datasets. We then ran three phylogenetic Multivariate analysis of variances (MANOVAs) using the procD.pgls function with our shape data (pelvic only, hind limb only, or combined pelvic and hind limbs) as the dependent variable, specimen SVL as the first independent variable, and microhabitat as the second independent variable. Each MANOVA was performed with Type I sum of squares and 10,000 permutations. These MANOVAs tested two associations: (1) the association between our three shape datasets and SVL while accounting for phylogeny and (2) the association between our three shape datasets and microhabitat while accounting for both phylogeny and SVL.

For 77 species with known locomotor mode, we repeated the previous analyses to determine how pelvic shape, hind limb shape, and combined pelvic and hind limb shape varied with phylogeny, SVL, and locomotor mode. We ran three phylogenetic MANOVAs using the procD.pgls function with our shape data (pelvic only, hind limb only, or combined pelvic and hind limbs) as the dependent variable, specimen SVL as the first independent variable, and locomotor mode as the second independent variable. Each MANOVA was performed with Type I sum of squares and 10,000 permutations. These MANOVAs tested the association between our three shape datasets and locomotor mode while accounting for both phylogeny and SVL.

Using canonical variate analyses of pelvic and hind limb shape to differentiate among microhabitats and locomotor modes and relating those morphological changes to jump performance

We performed canonical variate analyses (CVAs) in the R package Morpho v2.7 (Schlager 2017) to determine the largest differences in pelvic and hind limb shape among microhabitats and locomotor modes. We first generated size-corrected shape data for our three shape datasets (pelvic, hind limb, and combined pelvic and hind limb) by taking the residuals from a procD.pgls analysis with shape data as the dependent variable and log(SVL) as the independent variable. We used these three size-corrected shape datasets to differentiate among our a priori microhabitat and locomotor mode groups with CVA analyses.

We tested for an association between species' jump performance and the first and second canonical variate axes, which differentiated among our microhabitats and locomotor modes. We performed phylogenetic least-squares (PGLS) analyses in caper v1.0.1 (Orme et al. 2018) to test for an association between each species' jump performance and their position along the first and second canonical axes while accounting for phylogeny and estimating phylogenetic signal (lambda) in the residual error simultaneously with the regression parameters (see Revell 2010). Eight total PGLS analyses tested the association between a species' jump performance (either average jump distance or average jump distance relative to SVL) and their position along either the first or second canonical axes (of either the microhabitat or locomotor CVA).

Analyzing how individual pelvic and hind limb bone shapes evolve in relation to phylogeny, body size, microhabitat, and locomotor mode

We compared evolutionary rates among individual pelvic and hind limb bones using the R package geomorph v3.1.2 (Adams et al. 2018). We first GPA aligned an individual bone's landmarks and then combined the six bones via combine.subsets. We did not perform a second GPA alignment during the combine.subsets function (we used GPA = FALSE) so that bones would be equally sized. This equal sizing avoided biases in evolutionary rates related to minor changes in larger bones having a larger overall effect. We analyzed evolutionary rate differences among pelvic and hind limb bones using the compare.multi.evol.rates function.

We compared how individual pelvic and hind limb bone shapes varied in association with phylogeny, body size (SVL), and microhabitat and ran a series of analyses within the R package geomorph v3.1.2 (Adams et al. 2018). We first GPA aligned each bone's landmarks to

Table 1 Estimates of phylogenetic signal and results from phylogenetic MANOVAs that test for associations between pelvic and hind limb shape and body size, microhabitat, and locomotor mode

	Phylogenetic signal ($N = 230$)	Body size log(SVL) ($N = 230$)	Microhabitat ($N = 230$)	Locomotor mode ($N = 77$)
Pelvic bones	$K = 0.389$ $p < 0.001$***	$F_{1,221} = 12.42$ $Z = 5.36$ $R^2 = 0.05$ $p < 0.001$***	$F_{7,221} = 2.37$ $Z = 2.37$ $R^2 = 0.07$ $p < 0.001$***	$F_{5,70} = 7.21$ $Z = 6.24$ $R^2 = 0.27$ $p < 0.001$***
Hind limb bones	$K = 0.506$ $p < 0.001$***	$F_{1,221} = 18.49$ $Z = 5.05$ $R^2 = 0.07$ $p < 0.001$***	$F_{7,221} = 4.49$ $Z = 5.64$ $R^2 = 0.12$ $p < 0.001$***	$F_{5,70} = 3.68$ $Z = 4.30$ $R^2 = 0.17$ $p < 0.001$***
Pelvic and hind limb complex	$K = 0.468$ $p < 0.001$***	$F_{1,221} = 12.93$ $Z = 5.02$ $R^2 = 0.05$ $p < 0.001$***	$F_{7,221} = 4.47$ $Z = 5.95$ $R^2 = 0.12$ $p < 0.001$***	$F_{5,70} = 5.20$ $Z = 5.47$ $R^2 = 0.23$ $p < 0.001$***

*$p < 0.5$; **$p < 0.01$; ***$p < 0.001$.

obtain shape data for each bone. Next, we used the physignal function to estimate the phylogenetic signal in each of our six bone shapes. We ran six separate phylogenetic MANOVAs using the procD.pgls function with our shape data (a single bone's shape) as the dependent variable, specimen log(SVL) as the first independent variable, and microhabitat as the second independent variable. Each MANOVA was performed with Type I sum of squares and 10,000 permutations. These MANOVAs tested two associations: (1) the association between our three shape datasets and SVL while accounting for phylogeny and (2) the association between our three shape datasets and microhabitat while accounting for both phylogeny and SVL. We then further trimmed the phylogeny to the 77 species with known locomotor mode and ran six additional phylogenetic MANOVAs with each bone's shape as the dependent variable, specimen log(SVL) as the first independent variable, and locomotor mode as the second independent variable. With this reduced locomotor mode dataset, we again assessed the relationship between each bone's shape and microhabitat using six MANOVAs identical to the microhabitat MANOVAs described above. We ran this second set of microhabitat MANOVAs so that we could compare each bone's shape association with locomotor mode to each bone's shape association with microhabitat using the same dataset.

Results

How has pelvic and hind limb shape evolved in relation to phylogeny, body size, microhabitat, and locomotor mode?

Pelvic shape, hind limb shape, and combined pelvic and hind limb shape all showed significant phylogenetic signal (Table 1). When accounting for phylogeny, we found relationships between body size and pelvic shape, hind limb shape, and combined pelvic and hind limb shape; however, body size explained very little of the variation in all cases (Table 1). When accounting for phylogeny and body size, we found microhabitat-associated changes in pelvic morphology, hind limb morphology, and combined pelvic and hind limb morphology (Table 1). Most of the pelvic and hind limb differences among microhabitats are described by the first principal component, where (1) burrowing and semi-burrowing species tended to have relatively large pelvic girdles with shorter urostyles, expanded sacral vertebrae, and relatively short and wide hind limb bones with especially short tibiofibulae and wide tarsi, (2) torrent dwelling, arboreal, semi-arboreal, and semi-aquatic species showed opposite patterns with smaller pelvic girdles, relatively long urostyles, compressed sacral vertebrae, and longer, thinner hind limb bones, and (3) terrestrial and aquatic species possessed pelvic and hind limb morphologies intermediate between the two extremes (Fig. 2A). We found a strong relationship between locomotor mode and pelvic shape, hind limb shape, and combined pelvic and hind limb shape (Table 1; Fig. 2B). In the 77 species for which locomotor mode was known, we saw numerous microhabitats represented within each locomotor mode, except for swimmers, which are almost all aquatic (Fig. 2B and C). Despite this variable relationship between microhabitat and locomotor mode, walkers, walker/hoppers, and hoppers tended to have more burrowing-like pelvic and hind limb morphologies, while jumpers and jumper/swimmers tended to have more arboreal- or torrential-like pelvic and hind limb morphologies. Walkers deviated from the other locomotor modes along the second principal component axis, where walkers had dorsoventrally compressed pelvic bones, a more caudal connection between the ilium and sacrum, and longer, thinner tarsi on average (Fig. 2B).
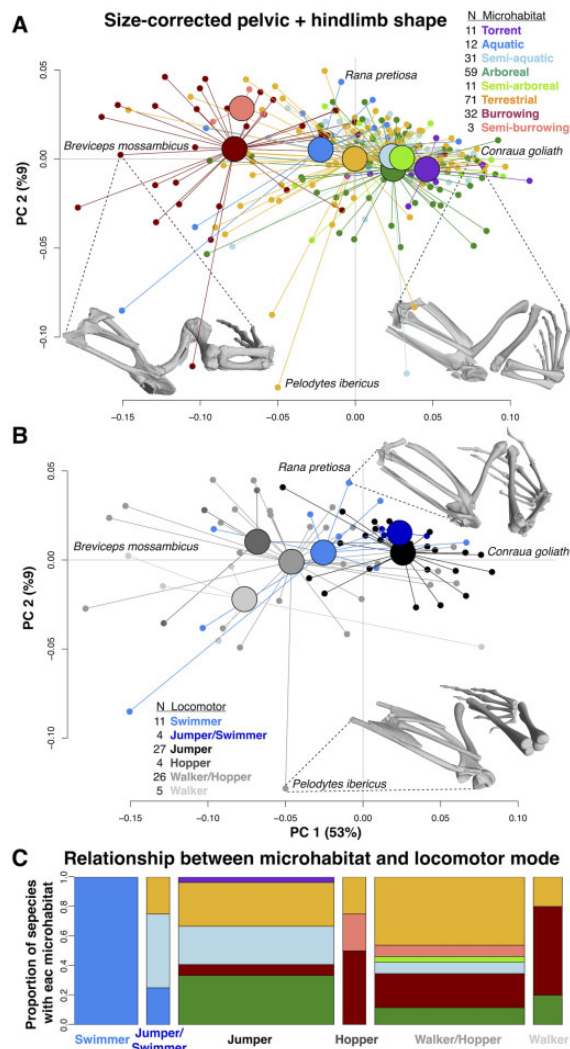
Fig. 2 PCA of major shape differences in pelvic and hind limb bones among species and their relationship with microhabitat and locomotor mode. (A) PCA plot with smaller dots representing the pelvic and hind limb shape of individual species and larger dots showing the centroids of pelvic and hind limb shape for each microhabitat. (B) PCA plot with smaller dots representing the pelvic and hind limb shape of individual species and larger dots showing the centroids of pelvic and hind limb shape for each locomotor mode. For easy comparison between microhabitat and locomotor mode shape differences, (C) was generated with all pelvic and hind limb shape data and not limited to just the 77 species for which locomotor mode is known. Skeleton meshes not to scale. (C) The proportion of species within each locomotor mode that belong to each microhabitat.

Do differences in pelvic and hind limb shape associated with microhabitat predict locomotor ability?

CVAs show that pelvic morphology alone and hind limb morphology alone could predict microhabitat to a similar degree (67% and 64%, respectively). However, combined pelvic and hind limb shapes could predict microhabitat in 86% of cases

(Fig. 3A). Burrowing species were separated from species of other microhabitats along the first canonical variate axis. The average burrowing and semi-burrowing species had relatively large pelvic girdles with expanded sacral diapophyses and slightly shorter urostyles relative to pelvic girdle length. Burrowing and semi-burrowing species also tended to have relatively short and wide hind limb bones with equi-length femurs and tibiofibulae and especially wide tarsi. The average arboreal species (which is similar to an average semi-arboreal and torrential species) had longer, thinner hind limbs with relatively longer tibiofibulae than femurs, smaller pelvic girdles, more rostrocaudally compressed sacral diapophyses, and slightly longer urostyles relative to pelvic girdle length. Aquatic species differ on average from torrential, arboreal, and semi-arboreal species along the second canonical axis by having slightly expanded sacral vertebrae and wider and longer bases of their pelvic girdles (larger ischium, pubis, and caudal/ventral portion of ilium; Fig. 3A). Using jump performance data for 21 species, we highlight a trade-off between average jump distance and more burrower-like pelvic and hind limb morphologies. We found a positive relationship between the first canonical variate axis of our microhabitat CVA and average jump distance (Fig. 3B) but no relationship between the first canonical variate axis of our microhabitat CVA and jump distance relative to SVL (Fig. 3B). We found no relationship between the second canonical variate axis of our microhabitat CVA and average jump distance ($F_{1,19} = 0.00$, $R^2 = 0.00$, $p = 0.991$) or average jump distance relative to SVL ($F_{1,19} = 0.04$, $R^2 = 0.00$, $p = 0.846$).

CVA showed that pelvic morphology alone could predict locomotor mode in 93% of cases and hind limb morphology alone could predict species locomotor mode in 82% of cases. However, pelvic and hind limb shape together could predict locomotor mode in 100% of cases (Fig. 4A). Hoppers had relatively large pelvic regions with longer pelvic girdles and rostrocaudally compressed sacral vertebrae compared to jumpers. Hoppers also had wider, shorter hind limb bones with relatively short tibiofibulae and especially wide tarsi compared to jumpers. Walkers and hoppers had similar changes in pelvic and hind limb morphology compared to jumpers; however, the average walker was differentiated from the average hopper in that it had rostrocaudally extended sacral diapophyses and relatively larger tarsi. Using jump performance data for 21 species, we saw a positive relationship between the first canonical variate axis of our locomotor mode CVA and average jump distance relative to SVL but not average jump
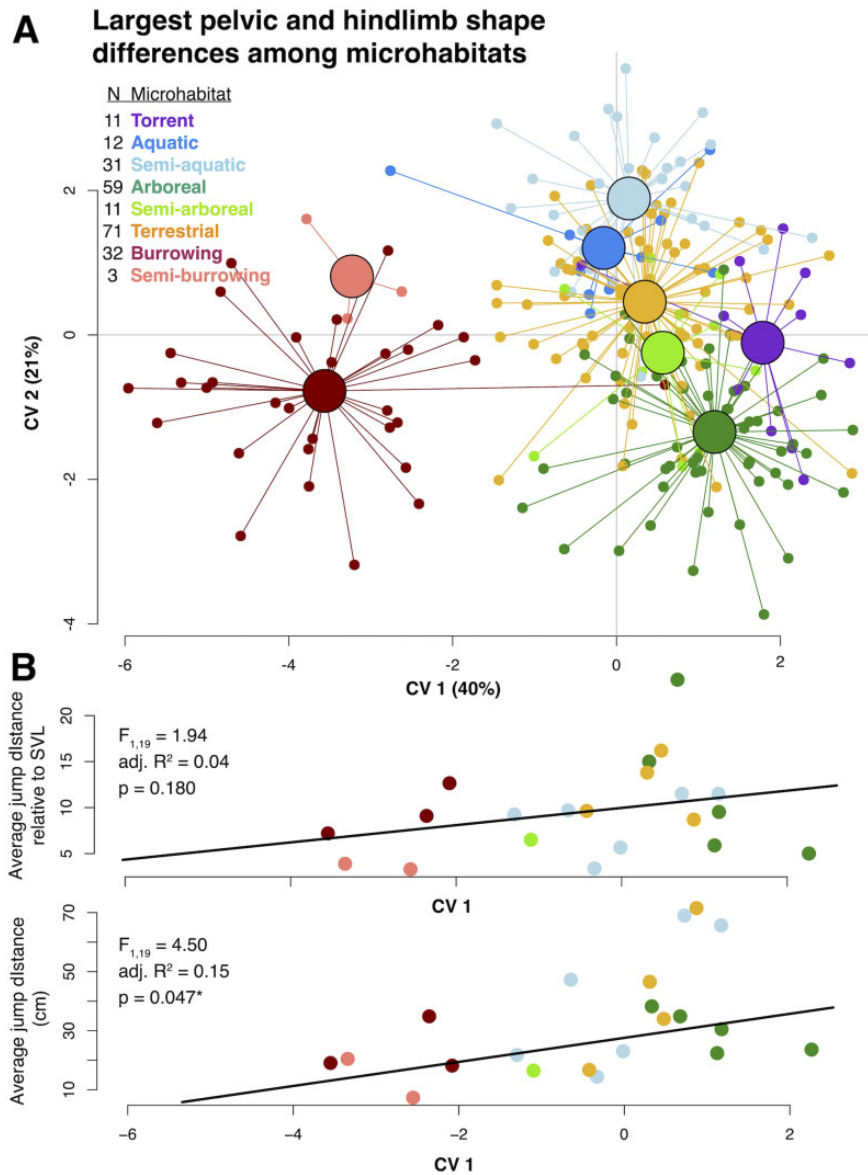
Fig. 3 CVA of pelvic and hind limb morphology differentiating among microhabitats and its relationship with jump performance. (**A**) CVA plot of the first two canonical variates differentiating microhabitats with smaller dots representing the pelvic and hind limb shape of individual species and larger dots showing the centroids of pelvic and hind limb shape for each microhabitat. (**B**) Relationship between jump distance (average and average relative to SVL) and the first canonical axis differentiating pelvic and hind limb morphology by microhabitat. Test statistics and regression lines are from PGLS analyses.

distance (Fig. 4B). We also saw a positive relationship between the second canonical variate axis of our locomotor mode CVA and average jump distance relative to SVL but not average jump distance ($F_{1,19} = 0.16$, $R^2 = 0.01$, $p = 0.692$; Fig. 4C).

Do pelvic and hind limb bones evolve at different rates and are their evolution similarly associated with phylogeny, body size, microhabitat, and locomotor mode?

Individual pelvic and hind limb bones varied in their evolutionary rates of morphological evolution

($p < 0.001$; Fig. 5A). Although all bone shapes showed a significant relationship with phylogeny (Table 2), the degree of that relationship varied among bones (Table 2; Fig. 5B). Notably, the sacrum varied strongly with phylogeny, and the urostyle, femur, and tibiofibula varied little with phylogeny. When accounting for phylogeny, body size (SVL) showed a universally minor association with sacral vertebra, pelvic girdle, and tarsus shape, and no significant association with urostyle, femur, and tibiofibula shape (Table 2; Fig. 5C). Variation in all bones, except the sacral vertebra, showed an
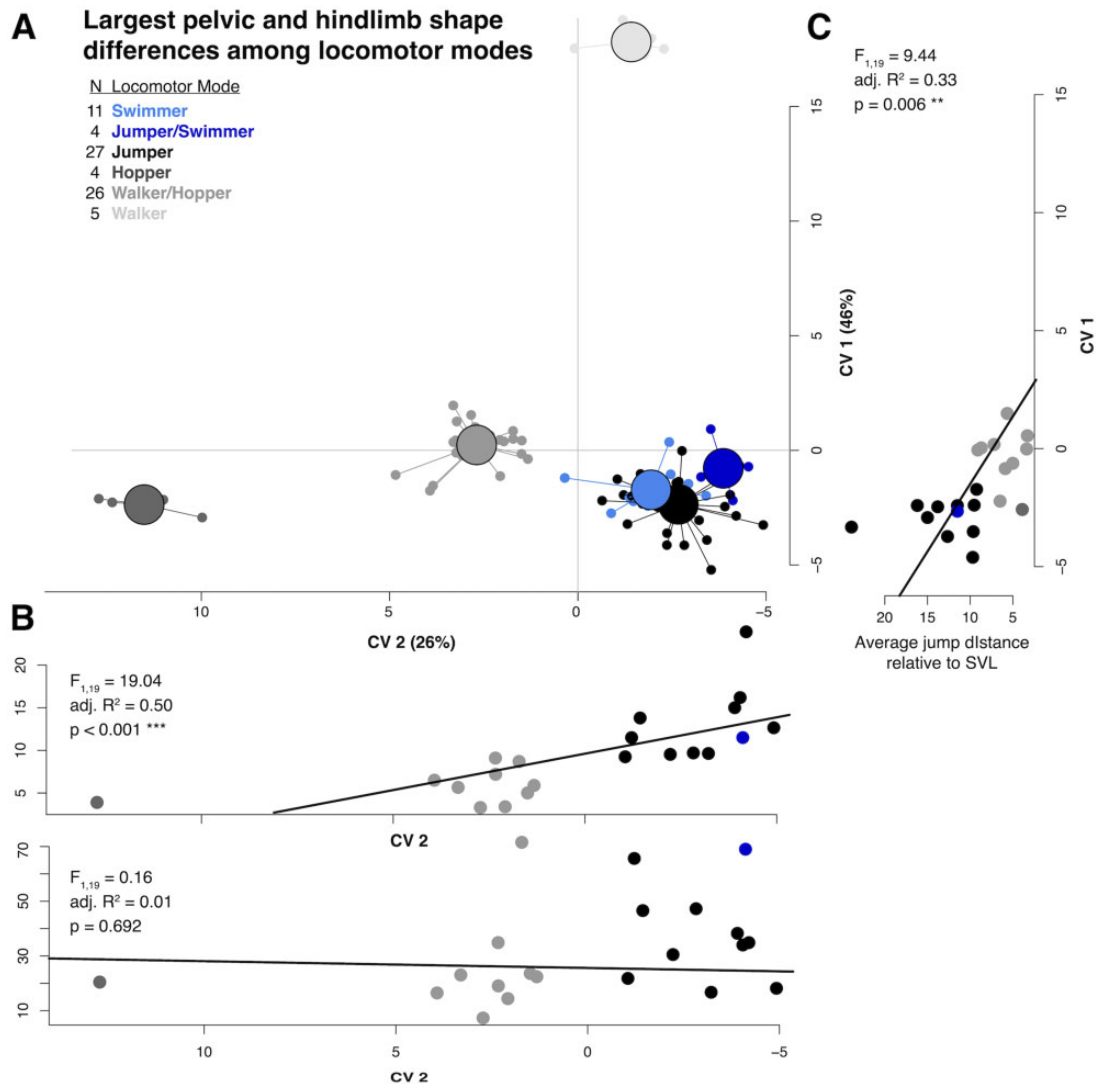
Fig. 4 CVA of pelvic and hind limb morphology differentiating among locomotor modes and its relationship with jump performance. (A) CVA plot of the first two canonical variates differentiating locomotor modes with smaller dots representing the pelvic and hind limb shape of individual species and larger dots showing the centroids of pelvic and hind limb shape for each locomotor mode. (B) Relationship between average jump distance (average and average relative to SVL) and the second canonical axis differentiating pelvic and hind limb morphology by locomotor mode. (C) Relationship between jump distance (average and average relative to SVL) and the first canonical axis differentiating pelvic and hind limb morphology by locomotor mode. In both (B) and (C), test statistics and regression lines are from PGLS analyses.

association with microhabitat, although microhabitat explained more hind limb bone variation in general (Table 2; Fig. 5D). In the 77 species with known locomotor mode, we observed that locomotor mode explains anywhere from 15% to 35% of the variation in individual pelvic and hind limb bone shapes, with locomotor mode showing strong association with pelvic bones in general (Table 2; Fig. 5E). To compare how locomotor mode and microhabitat related to each bone's shape, we assessed the relationships between bone shape and microhabitat in our trimmed locomotor mode dataset. Overall, our locomotor mode dataset ($N = 77$) and

our full dataset ($N = 230$) showed similar relationships between each bone's shape and microhabitat, except the pelvic girdle, which had a relatively weaker relationship with microhabitat in the trimmed locomotor mode dataset (Fig. 5F).

Discussion

We found clear variation in quintessential anuran pelvic and hind limb features related to transitions in ecology and locomotion when looking across 200 million years of anuran evolutionary history. Both pelvic and hind limb bones showed relationships
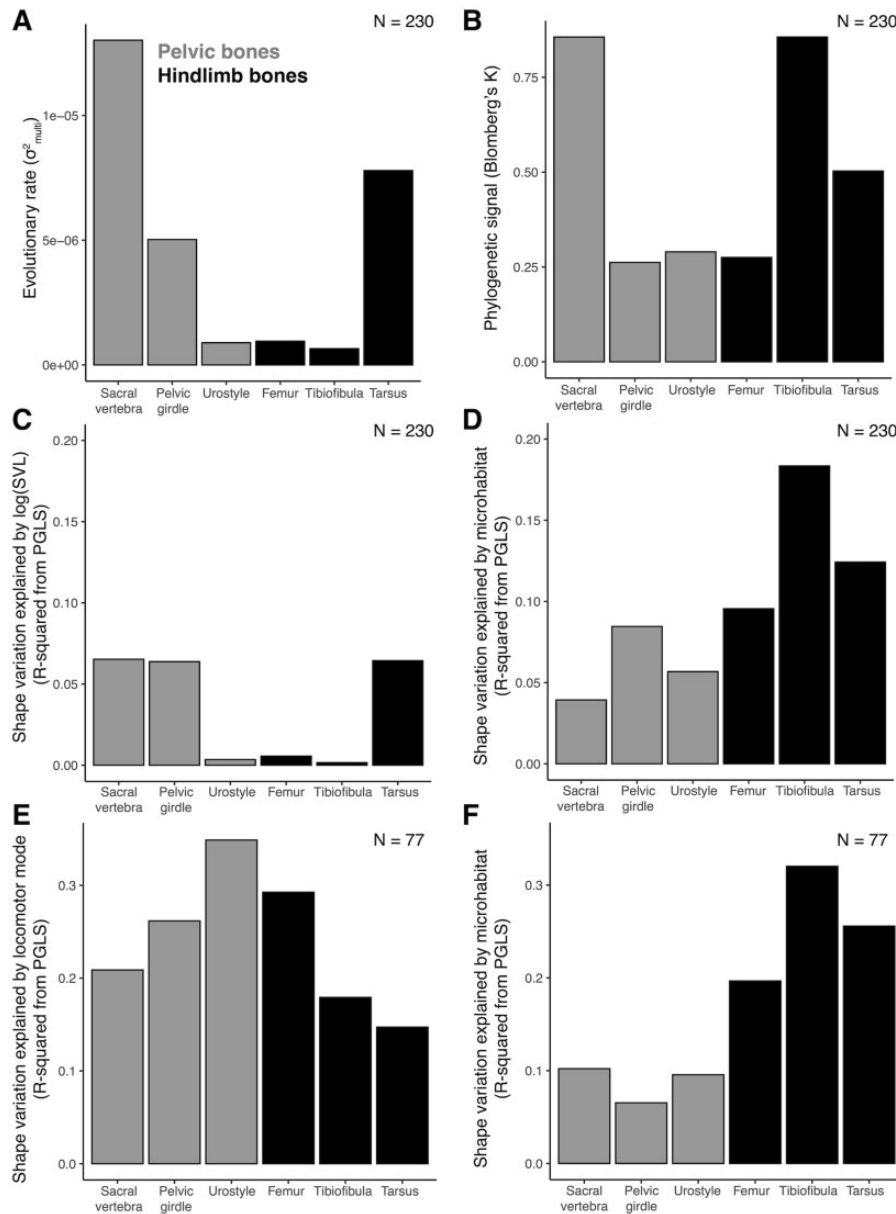
Fig. 5 Evolutionary rates among pelvic (gray) and hind limb (black) bones and the amount of individual bone shape variation explained by phylogenetic signal, body size, microhabitat, and locomotor mode. Sample size is listed for each analysis to indicate whether all species were analyzed ($N = 230$) or only the species with known locomotor mode ($N = 77$). (**A**) Comparison of evolutionary rates among pelvic and hind limb bones. (**B**) Comparison of phylogenetic signal among pelvic and hind limb bone shapes. (**D**) Comparison of pelvic and hind limb bone shape variation explained by microhabitat. (**E**) Comparison of pelvic and hind limb bone shape variation explained by locomotor mode. (**F**) Comparison of pelvic and hind limb bone shape variation explained by microhabitat when analyzed with the subset of species for which locomotor mode is known.

with microhabitat and locomotor mode individually but were better able to differentiate among microhabitat and locomotor modes in combination. This indicates pelvic and hind limb morphology each contribute complementary information related to potential microhabitat and locomotor mode

adaptation. We also found that jumping performance negatively correlated with differences in pelvic and hind limb morphology associated with burrowing. However, the evolution of individual bones varied in their relationship with phylogeny, body size, and microhabitat, and locomotor mode.

Table 2 Estimates of phylogenetic signal and results from phylogenetic MANOVAs that test for associations between individual bone shape and body size, microhabitat, and locomotor mode

	Phylogenetic signal (N = 230)	Body size log(SVL) (N = 230)	Microhabitat (N = 230)	Locomotor mode (N = 77)
Sacral vertebra	$K = 0.86$ $p < 0.001$***	$F_{1,221} = 16.12$ $Z = 4.48$ $R^2 = 0.07$ $p < 0.001$***	$F_{7,221} = 1.39$ $Z = 1.23$ $R^2 = 0.04$ $p = 0.111$	$F_{5,70} = 4.11$ $Z = 3.85$ $R^2 = 0.21$ $p < 0.001$***
Pelvic girdle	$K = 0.26$ $p = 0.002$**	$F_{1,221} = 16.55$ $Z = 4.42$ $R^2 = 0.06$ $p < 0.001$***	$F_{7,221} = 3.14$ $Z = 3.78$ $R^2 = 0.08$ $p < 0.001$***	$F_{5,70} = 6.89$ $Z = 4.91$ $R^2 = 0.26$ $p < 0.001$***
Urostyle	$K = 0.29$ $p < 0.001$***	$F_{1,221} = 0.82$ $Z = 0.31$ $R^2 = 0.00$ $p = 0.432$	$F_{7,221} = 1.91$ $Z = 1.70$ $R^2 = 0.06$ $p = 0.036$*	$F_{5,70} = 7.97$ $Z = 4.17$ $R^2 = 0.35$ $p < 0.001$***
Femur	$K = 0.28$ $p = 0.004$**	$F_{1,221} = 1.38$ $Z = 0.75$ $R^2 = 0.01$ $p < 0.233$	$F_{7,221} = 3.36$ $Z = 3.80$ $R^2 = 0.10$ $p < 0.001$***	$F_{5,70} = 6.91$ $Z = 4.86$ $R^2 = 0.29$ $p < 0.001$***
Tibiofibula	$K = 0.86$ $p < 0.001$***	$F_{1,221} = 0.43$ $Z = -0.41$ $R^2 = 0.00$ $p < 0.671$	$F_{7,221} = 7.11$ $Z = 4.86$ $R^2 = 0.18$ $p < 0.001$***	$F_{5,70} = 3.16$ $Z = 2.38$ $R^2 = 0.18$ $p = 0.007$**
Tarsus	$K = 0.50$ $p < 0.001$***	$F_{1,221} = 17.54$ $Z = 4.22$ $R^2 = 0.06$ $p < 0.001$***	$F_{7,221} = 4.84$ $Z = 4.91$ $R^2 = 0.12$ $p < 0.001$***	$F_{5,70} = 2.90$ $Z = 2.84$ $R^2 = 0.15$ $p = 0.001$**

*$p < 0.5$; **$p < 0.01$; ***$p < 0.001$.

Pelvic and hind limb variation associated with microhabitat and locomotor mode

An organism's "ecomorphology" describes the relationship between its morphology and environment (Wainwright 1991; Melville et al. 2006; Losos 2011). For example, *Anolis* ecomorphs differ in their leg lengths depending on microhabitat (e.g., trunk or grass-bush), suggesting adaptation to best exploit their niche (Losos 1992), though the relationship between different ecomorphs and jump performance is complicated (Toro et al. 2004). We see evidence for skeletal ecomorphs in frogs, with the most obvious changes found in burrowing species, which have shorter hind limbs, relatively large pelvic girdles, and expanded sacral vertebra, as seen in other studies (Emerson 1976; Zug 1978; Moen 2019; Fig. 2). Short hind limbs are known to increase the force generated while scooping substrate (Emerson 1976; Vidal-García et al. 2014), and the wide tarsus would allow burrowing species to more efficiently dig in densely packed substrates. Although less obviously distinct in our principal component analysis, our CVA shows that aquatic and semi-aquatic species have an expanded pelvic base, which may provide more attachment area for larger average leg muscles that are associated with aquatic species (Moen 2019).

Species that are semi- and fully arboreal or torrent-dwelling have thinner and longer hind limb and pelvic bones, which may partially explain the lower relative body mass of arboreal species compared to others (Santini et al. 2018). Long hind limbs may also aid arboreal and torrent frogs in surface adhesion since they are known to press their bodies onto a surface and spread their limbs out when adhering at shallow angles (Endlein et al. 2013). Terrestriality appears to be a less specialized microhabitat, having intermediate phenotypes on average with a wide range in species' morphology (Fig. 2). Citadini et al. (2018) also found higher morphological variance in terrestrial species. However, the broad range in terrestrial phenotypes could also be due to their large range in locomotor modes (jumper/swimmer, jumper, hopper, walker/hopper, and walker; Fig. 2).

Following the same logic as ecomorphology, "locomorphology" refers to morphological variation with locomotor mode. Our results support the existence of pelvic and hind limb locomorphs within Anura. However, we find little pelvic and hind limb distinction between jumpers, swimmers, and jumper/swimmers (Fig. 4). The similar pelvic and hind limb morphology between jumpers and swimmers may explain the lack of performance trade-offs found in intraspecific (Nauwelaerts et al.

2007) and interspecific (Moen 2019) jumping and swimming ability. We find the largest pelvic and hind limb differences between swimmers/jumpers and hoppers and walkers (Fig. 4). Both walkers and hoppers have larger pelvic girdles than jumpers and shorter, wider leg bones, which is expected given that shorter legs decrease jumping distance (Gomes et al. 2009) and we define hoppers by shorter jumps relative to SVL. This pattern of shorter hind limbs in walking species matches patterns in mammals, where walkers have more similarly sized fore- and hind limbs (Astley 2016; Reynaga et al. 2018). The slightly longer urostyle relative to pelvic girdle length in jumping anurans is consistent with a study on hylid frogs (Soliz et al. 2017), wherein the authors postulated that longer urostyles correlate with longer muscles, which may improve jumping and swimming performance. We find that walkers are distinguished from hoppers by having even wider tarsi and a more pronounced rostrocaudal sacrum expansion, which correlates with a looser connection between the sacrum and the ilia and allows for even more lateral rotation and anteroposterior movement when walking (Emerson 1979; Emerson and De Jongh 1980; Jorgensen and Reilly 2013; Reilly et al. 2016).

Comparing pelvic and hind limb morphology of "ecomorphs" and "locomorphs"

Microhabitat and locomotor mode do not have a straightforward relationship. We found that microhabitat is more strongly associated with variation in hind limb bones while locomotor mode is more related to variation in pelvic structures (Fig. 5). This suggests that pelvic structures are more shaped by locomotor mode than microhabitat, which may be related to locomotor mode associations with previously defined pelvic "types" (Emerson 1982; Reilly and Jorgensen 2011; Jorgensen and Reilly 2013). In contrast, individual hind limb bones appear more important for differing demands among microhabitats, which aligns with previous findings and hypotheses (Gomes et al. 2009; Moen et al. 2013; Vidal-García and Keogh 2015; Citadini et al. 2018; Moen 2019).

The most morphologically similar ecomorphs and locomorphs are the burrowers/semi-burrowers and the walkers, walker/hoppers, and hoppers (Fig. 2B and C), which tended to have relatively large pelvic girdles and expanded sacral vertebrae. These characters have been associated with burrowing, walking, and hopping previously (Jorgensen and Reilly 2013) and are thought to be ideal for lateral bending and anteroposterior movement (Emerson 1979, 1982).

Thus, walking, hopping, and burrowing can all be achieved with similar pelvic morphologies and short hind limbs. However, using a walker/hopper classification may have concealed pelvic and hind limb distinctions between species that predominantly hop and species that predominantly walk. The typical morphology of jumper/swimmers and jumpers is clustered with semi-aquatic, semi-arboreal, arboreal, and torrent morphologies (Fig. 4). This portion of morphospace encompasses skeletal characteristics needed to jump well, such as longer hind limbs and compressed sacral diapophyses, which increase their ability to rotate the pelvic girdle dorsoventrally (Zug 1972, 1978; Emerson 1979; Choi et al. 2003; Gomes et al. 2009; Enriquez-Urzelai et al. 2015; Citadini et al. 2018; Moen 2019).

Differences in pelvic and hind limb bones related to microhabitat and locomotor mode somewhat correlate with jumping performance

Here we show that differences in pelvic and hind limb morphology that best predict microhabitat and locomotor mode correlate with species' jumping ability to varying degrees (Fig. 3, 4). However, it is important to note that, within a species, jump distance increases with body size (Gomes et al. 2009; Enriquez-Urzelai et al. 2015; Citadini et al. 2018; Rebelo and Measey 2019) and thus the average jump distances we analyzed may be higher or lower than a true species' average jump distance if the animals tested were particularly large or small. This potential bias in species' jump distance along with our limited sampling of species' performance data means our results should be interpreted with caution. Nevertheless, these results lay out hypotheses for how pelvic and hind limb morphologies associated with particular microhabitats may affect jumping ability.

Despite microhabitat data being categorical and often difficult to assess, differences in pelvic and hind limb morphology that best predict microhabitat also correlated with species' jumping ability. The major axis of variation that distinguished among microhabitats and indicates long, thin leg bones, small pelvic girdles, and rostrocaudally compressed sacral vertebrae showed a significant positive relationship with absolute average jump distance (Fig. 3B). The association between long hind limbs and greater jump length, as well as take-off speed, is supported by several studies (Zug 1972, 1978; Choi et al. 2003; Gomes et al. 2009; Enriquez-Urzelai et al. 2015; Citadini et al. 2018; Moen 2019). Our results indicate a trade-off between jumping performance

and burrowing, which has been seen and hypothesized in other studies and seems due to biomechanical differences needed for jumping versus digging movements and the shorter hind limbs of burrowers (Emerson 1976; Duellman and Trueb 1994; Gomes et al. 2009; Wells 2013; Vidal-García and Keogh 2015).

The correlation between relative jump distance and pelvic and hind limb morphologies associated with locomotor modes is expected and unremarkable, given we define jumpers and hoppers based on relative jump distance and the majority of our jump distance data are from species with those two locomotor modes. However, the correlation between longer, thinner hind limb morphologies, and relative jump distance further indicates that these morphologies are important for a species' jumping ability (Fig. 4B and C).

Pelvic and hind limb bones differ in how quickly they evolve and what factors affect their evolution

We found individual pelvic and hind limb bones varied greatly in their evolutionary rate (Fig. 5A). Differences in the number of landmarks could be contributing to differences in evolutionary rate among bones because more landmarks may inherently capture more shape variation. However, a previous study found similar evolutionary rate differences among hind limb bones when controlling for the number of landmarks on each bone (Stepanova and Womack 2020). It is also important to remember that the results discussed in this section refer to an individual bone's shape analyzed in isolation. Thus, any changes in the size of each bone relative to other structures were not considered.

Among pelvic bones, the sacral vertebra had the highest evolutionary rate and a high phylogenetic signal (Fig. 5A and B). Jorgensen and Reilly (2013) found that the sacrum exhibits the most structural variation and varies with locomotor mode. However, similar to Soliz et al. (2017), we found sacral variation to be less related to locomotor mode and microhabitat, and more driven by clade-specific shifts in morphology (Supplementary Fig. S2) as indicated by its high phylogenetic signal. Meanwhile, the pelvic girdle's relatively high evolutionary rate seems less influenced by phylogeny and more influenced by microhabitat and to some extent, locomotor mode (Fig. 5). Previous studies found variation in ilial length was not related to locomotor mode (Jorgensen and Reilly 2013) or was part of a suite of pelvic features associated with locomotor mode (Emerson 1982). The stronger relationship shown

here may be due to our increased species sampling and examination of ilial length in relation to the rest of the pelvic girdle's 3D shape. We did not capture dorsoventral variation in the ilia, which varies among species and may allow further distinction among microhabitat and locomotor mode using pelvic girdle shape. The urostyle showed a relatively low evolutionary rate and little association with phylogeny and microhabitat but a relatively high association with locomotor mode (Fig. 5). Other studies either measured the urostyle (Simons 2008; Soliz et al. 2017) or focused on whether it was fused to the sacrum or free and whether it had a dorsal ridge (Emerson 1979; Reilly and Jorgensen 2011; Jorgensen and Reilly 2013; Soliz et al. 2017). While our landmarks mainly provide information about the length of this bone, the dorsocaudal landmark grants some information on urostyle height, which could explain why, unlike Simons (2008), urostyle shape was related to locomotor mode in our dataset. We find body size only minorly influenced pelvic bone shapes, where the sacrum and pelvic girdle shape showed minor relationships with SVL and urostyle shape exhibited no allometric relationship.

The differences among hind limb bone evolutionary rates shown in this study were first described and discussed in Stepanova and Womack (2020). This study expands on what was previously found by comparing hind limb bone evolutionary rates to pelvic bone evolutionary rates and examining their relationship with locomotor mode. The tarsus has the second-highest evolutionary rate of all pelvic and hind limb bones after the sacral vertebra, and shows a relationship with many factors, including phylogeny, microhabitat, and locomotor mode, highlighting its importance in ecological and locomotor adaptation (Fig. 5). Despite the tibiofibula's low evolutionary rate, its variation showed associations with phylogeny and microhabitat (Fig. 5B, D, and F). Interestingly, of the hind limb bones, the femur was least associated with phylogeny and microhabitat but most associated with locomotor mode, indicating that femur morphology may importantly differentiate among locomotor modes but lacks importance in microhabitat adaptation. Similar to pelvic bones, the hind limb bones showed little allometric influence, with only tarsus shape varying with SVL (Fig. 5C).

Concluding remarks

Here we show how unique anuran morphologies are evolving in association with ecological and locomotor diversification while accounting for phylogeny

and allometry. Pelvic and hind limb shapes provide complementary information about a species' microhabitat and locomotor mode. However, individual pelvic and hind limb bones evolve at varying rates and appear driven by different selective and drift forces. Our study informs anuran jumping and swimming biomechanics by highlighting key skeletal variation among species that vary in locomotor performance. We also find pelvic skeletal variation in tendon, ligament, and muscle attachment sites that indicate soft-tissue adaptations—such as an expansion of the pelvis at leg muscle attachment sites in aquatic species. However, more jumping and swimming performance data are needed to test how the evolution of pelvic and hind limb structures shown here affects how species move in their environment.

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Authors' contributions

S.M.B. contributed to the data collection, writing, and editing of this manuscript. N.S. contributed to the data collection and editing of this manuscript. M.C.W contributed to the conception of the study, data collection, data analysis, writing, and editing of the manuscript.

Supplementary data

Supplementary data available at *ICB* online.

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