






Frog tendon structure and its relationship with locomotor modes

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Abstract

Tendon collagen fibrils are the basic force-transmitting units of the tendon. Yet, surprisingly little is known about the diversity in tendon anatomy and ultrastructure, and the possible relationships between this diversity and locomotor modes utilized. Our main objectives were to investigate: (a) the ultra-structural anatomy of the tendons in the digits of frogs; (b) the diversity of collagen fibril diameters across frogs with different locomotor modes; (c) the relationship between morphology, as expressed by the morphology of collagen fibrils and tendons, and locomotor modes. To assess the relationship between morphology and the locomotor modes of the sampled taxa we performed a principal component analysis considering body length, fibrillar cross sectional area (CSA) and tendon CSA. A MANOVA showed that differences between species with different locomotor modes were significant with collagen fibril diameter being the discriminating factor. Overall, our data related the greatest collagen fibril diameter to the most demanding locomotor modes, conversely, the smallest collagen fibril CSA and the highest tendon CSA were observed in animals showing a hopping locomotion requiring likely little absorption of landing forces given the short jump distances.

KEYWORDS

anuran locomotion

1 | INTRODUCTION

Collagen fibrils are the basic structural elements that withstand tensile forces in the connective tissues across all animal taxa (Ottani, Raspanti, & Ruggeri, 2001). Although it has previously been reported that collagen fibrils exhibit a wide range of diameters (Parry, Barnes & Craig, 1978; Vogel & Koobs, 1989; Ottani et al., 2001), the functional implications of this structural diversity remain poorly understood. It has been suggested that tendon collagen fibrils determine the mechanical properties and function of the tendon as a whole (Parry et al., 1978; Ottani et al., 2001; Rigozzi, Muller, & Snedeker, 2010; Rigozzi, Stemmer, Müller, & Snedeker, 2011). Differences in the material properties of the tendon in hind limb muscles of turkeys were related to structural differences (Matson, Konow, Miller, Konow, & Roberts, 2012). It has also been suggested that tendons with a larger proportion of larger collagen fibril diameters withstand greater tensile forces because of a higher density of intermolecular cross-links (Parry et al., 1978; Parry,

1988; Franchi, Triré, Quaranta, Orsini, & Ottani, 2007; Galloway, Lalley, & Shearn, 2013) and a positive relationship between fibril diameter and tendon stiffness has been observed (Vogel & Koobs, 1989; Kongsgaard et al., 2010; Cheema, Chuo, Sarathchandra, Showan, & Brown, 2006; Rigozzi et al., 2010). Three studies (Michna & Hartmann, 1989; Cheema et al., 2006; Kalson et al., 2011) have demonstrated that function is likely to be reflected directly in the diameter and number of the collagen fibrils in the tendons. Thus, the relationship between patterns of collagen fibril diameters and tendon function appears straightforward. As tendon function is conveyed as movement, and tetrapod locomotor modes are also conveying different movements, it could be considered that locomotor modes can be good proxies for differences in movement/function of tendons involved in those locomotor modes.

Although considerable effort has been made to determine the ultrastructure of connective tissues with the aid of electron microscopy (e.g., Parry et al., 1978; Parry & Craig, 1984; Gill et al., 2004; Svensson et al., 2007; Rigozzi et al. 2011), most of these studies were performed

to address issues in the fields of sports or veterinary medicine. Yet, this research avenue seems promising to address questions from an eco-morphological perspective. To the best of our knowledge no studies have compared the ultrastructure of tendons among closely related taxa that differ in ecology and locomotor mode, though there is a study from our group that addressed the organization of the collagen fibril within tendons of frogs and lizards in the context of the network theory (Dos Santos et al., 2014).

Anurans are one of the vertebrate groups that have undergone greatest diversification in locomotor modes (Emerson, 1979; Jorgensen & Reilly, 2013). Different species have evolved to occupy different habitats leading to specialized arboreal, aquatic, terrestrial, and even fossorial forms. Moreover, within each of these categories, generalists and specialists can be observed. For example, arboreal species can be grouped into generalists that are excellent jumpers and climbers and fine-branch specialists that are specialized walkers and climbers but poor jumpers. Similarly, among terrestrial species runners, hoppers, and jumpers can be identified, each faced with different constraints imposed upon the locomotor system. Jumping in frogs and the evolution thereof has been studied in great detail (e.g., Gans & Parsons, 1966; Emerson, 1978; Zug, 1978; Shubin & Jenkins, 1995; Nauwelaerts & Aerts, 2003; Azizi & Roberts, 2010; Reilly & Jorgensen, 2011; Herrel, Vasilopoulou-Kampitsi, & Bonneaud, 2014; Herrel et al., 2016; Lires, Soto, & Gómez, 2016; Astley, 2016), with interesting studies devoted to landing (Nauwelaerts & Aerts, 2006; Essner, Suffian, Bishop, & Reilly, 2010; Azizi, Larson, Abbott, & Danos, 2014; Ekstrom & Gillis, 2015; Bijma, Gorb, & Kleinteich, 2016; Reilly et al., 2015, 2016). During landing the forelimbs and body need to absorb forces that may be up to three times larger than the propulsive forces (Nauwelaerts & Aerts, 2006). During landing the forelimbs are extended and the hand placed on the substrate fingers first. Although detailed kinematics of the hand during landing are scarce (Nauwelaerts & Aerts, 2006; Reilly et al., 2016), it is likely that this movement involves significant extension of the finger and hand flexors that need to resist and absorb these landing forces. It has already been shown that tendons act as power attenuators that protect muscles and modulate the energy dissipated by them (Konow, Azizi, & Roberts, 2011; Konow & Roberts, 2015). If so, then differences can be expected between species with different modes of locomotion; for example, we expect that species with longer distance jumps have higher landing forces having greater collagen fibril diameters and overall tendon diameters.

Here, we present an analysis of the flexor tendon ultrastructural anatomy, that is, tendon collagen fibril cross sectional area (CSA), and tendon CSA, in frogs with different modes of locomotion. We selected a digital flexor tendon because of the likely role of the flexor tendons in absorbing landing forces, and also because the proximal or distal (digital) sections of the m. flexor digitorum longus are among the most studied tendons in a comparative context (e.g., Vogel & Koobs, 1989; Michna & Hartmann, 1989; Patterson-Kane, Wilson, Firth, Parry, & Goodship, 1997; Watanabe et al., 2005; Dos Santos et al., 2014). We predict that jumpers will possess a larger number of collagen fibrils and a greater collagen fibril and tendon CSA as the flexor tendons of the fingers are used to absorb landing forces (Nauwelaerts & Aerts, 2006;

Essner et al., 2010). Conversely, we predict that hoppers will show a larger number of the smallest collagen fibrils and a reduced tendon CSA given that they show a bounding gait with very short distance jumps where landing takes place with the forelimb extended (Reilly et al., 2015), likely associated with low landing forces (but see Reilly et al., 2016). Climbers are likely intermediate despite their long-distance jumps as landing in these animals involves the belly in addition to an attachment with the toes of either front or hind limb (Bijma et al., 2016).

2 | MATERIALS AND METHODS

Electron microscopy analysis was conducted with samples of the flexor tendon of the Digit IV obtained from adults of the jumpers *Leptodactylus chaquensis*, *L. latinasus*, the hoppers *Rhinella arenarum* and *Physalasmus biligonigerus*, and the climbers *Scinax nasicus* and *Phyllomedusa sauvagii* (Appendix 1 in supporting information). This study was approved by the Ethics Committee of Universidad Nacional de Tucumán, Argentina) (Exp. No. 1206 2010).

2.1 | Ultrastructural analyses

Samples were placed overnight in 0.1 mol L⁻¹ phosphate buffer with 2.5% glutaraldehyde and 4% paraformaldehyde. The tissue was then immersed in 1% osmium tetroxide, dehydrated in graded acetones, and flat embedded in Epon plastic 812 (Ernest F Fullam, Inc, Latham, NY) in a cross-sectional orientation. Sections (85 nm) were obtained and stained with 0.25% lead citrate and 5% uranyl acetate in 50% acetone and then observed and photographed in a JEOL100CX transmission electron microscope (CIME, Universidad Nacional de Tucumán, Argentina). Collagen fibril diameters of each species were measured on each micro-graph using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, <http://rsbweb.nih.gov/ij/>). Fibrils included in a randomly selected area of 1.5 × 2 μm were counted in each specimen (N = 42) and the diameter of each fibril present in the selected area was measured. To characterize diameter distribution, all fibril diameters measured were plotted per species, within class intervals of 30 nm of the collagen fibril diameter. Based on the density and diameters of the collagen fibrils, the total area of collagen in a surface of comparable size was calculated using the Image Tool 3.0 software. In addition, the CSA of each collagen fibril was obtained by transforming the diameter data into radius, which was squared and multiplied by pi (Appendix 2 in supporting information).

To characterize tendon ultrastructural morphology, the following statistic descriptors were obtained: abundance, estimated as the number of fibrils belonging to a size range per species. Diversity (Table 1), which shows the relative abundance of each group of fibrils, and was calculated using the Shannon–Weaver index ($H' = -\sum p_i \ln p_i$); evenness (Table 1), which expresses how evenly the total of fibrils is distributed in each range, calculated with Pielou's evenness index: $J' = H' / H'_{\max}$; $H'_{\max} = \ln(s)$, where s is number of types of collagen fibrils. J' is constrained between 0 and 1.

TABLE 1 Diversity (Shanon–Wiever index: H') and Equitativity (Pielou's evenness index: J') of the collagen fibril ranges for each species

Species	H'	J'
<i>Leptodactylus chaquensis</i>	2.42	0.78
<i>Leptodactylus latinasus</i>	1.77	0.57
<i>Phyllomedusa sauvagii</i>	2.06	0.66
<i>Physalaemus biligonigerus</i>	1.78	0.57
<i>Scinax nasicus</i>	1.65	0.53
<i>Rhinella arenarum</i>	1.73	0.56

2.2 | Histology

To obtain the CSA of the sampled tendons (supplementary online material, Appendix 2), portions of the flexor tendon of the Digit IV (Figure 1) were dehydrated through a graded ethanol series, cleared in xylenes and embedded in Paraplast. Embedded specimens were sectioned in serial and semi-serial sections of between 5 and 10 μm . Sections were deparaffinated, hydrated, and stained with Hematoxylin–Eosin (H–E) and Mallory (Azán) Trichromic (modified from Totty, 2002). Tendon CSA was calculated by averaging four histological sections that were traced using Corel Draw (R) Graphics Suite X5 (Corel Corp., Ottawa, Canada) and saved as JPG. The captured images were measured and analyzed using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, <http://rsbweb.nih.gov/ij/>); histological sections correspond to the conventional CSA of the tendons. All these procedures were followed because the small size of the tendons prevented us from obtaining a direct measure of their CSA.

2.3 | Statistical analyses

The variables were Log_{10} -transformed to meet requirements of normality (Zar, 1999). First, we calculated a correlation matrix (Pearson correlations) to see whether variables were correlated. Next, we tested for differences in snout-vent length between locomotor modes using an ANOVA. Given that no differences in snout-vent length were detected we ran a MANOVA on the fibrillar and tendon CSA data to test for

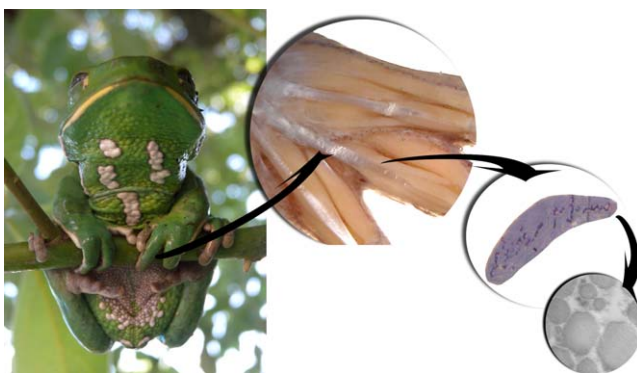


FIGURE 1 *Phyllomedusa sauvagii*, details of the flexor tendon of the Digit IV, of the CSA of this tendon, and of the CSA of collagen fibril [Color figure can be viewed at wileyonlinelibrary.com]

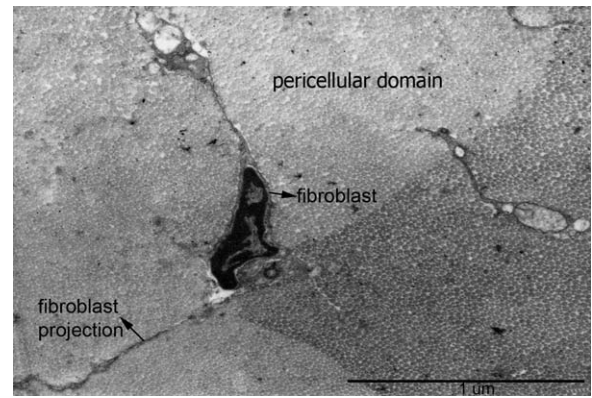


FIGURE 2 *Scinax nasicus*, transmission electron micrograph of the mature flexor tendon of the Digit IV, showing the fibroblast and the collagen fibers delimited by the projections of the fibroblast

differences between species with different locomotor modes. To explore whether and how phylogeny may impact our data we ran a factor analysis with varimax rotation on the Log_{10} -transformed data of snout-vent length, fibrillar CSA, and tendon CSA and plotted the phylogeny in the morphospace using the 'phylo-morphospace' function in R (R Core Team, 2016) implemented in the 'phytools' library (Revell, 2012). Our cladistic hypothesis of the relationships between species was the phylogeny of Pyron and Wiens (2011). We used the phylogenetic position of the sister taxon *L. gracilis* to indicate the phylogenetic position of *L. latinasus* which was not included in the phylogeny.

3 | RESULTS

3.1 | Comparative ultrastructural anatomy

In all samples, the typical tendon configuration of collagen fibers and fibrils is observed. The tendons are composed of collagen fibers delimited by the projections of the fibroblasts, which exhibit a highly retracted cytoplasm characteristic of mature tissue (Figure 2). In all samples, the diameter distribution of collagen fibrils is characteristic of a mature tendon (Figure 2). An extracellular compartmentalization of the collagenous matrix assembly or pericellular domain (Carvalho & Felisbino, 1999) is observed outside the boundaries of the fibroblast (Figure 2). The fibroblasts and their nuclei are elongated (Figure 3). The nuclei are eccentric, with more heterochromatin than euchromatin. The heterochromatin is located peripherally to the nucleus, surrounded by the euchromatin. The nuclear membrane is perforated by the nuclear pores. In the cytoplasm, large mitochondria, many secretory vacuoles, the Golgi apparatus, and the rough and smooth endoplasmic reticulum are visible (Figure 4). Elongated secretory vacuoles containing procollagen fused in a tandem fashion can be seen, surrounded by a thick layer of mature fibrils. The union between muscle and tendon follows the classic pattern of myotendinous junction (Figure 5), consisting of many variable-sized interdigitations of tendon and muscle (Mair & Tomé, 1972). The muscle at the myotendinous junction consists of tongues of muscle fibers that surround single or multiple collagen fibrils, which are immersed among the muscular fibers.

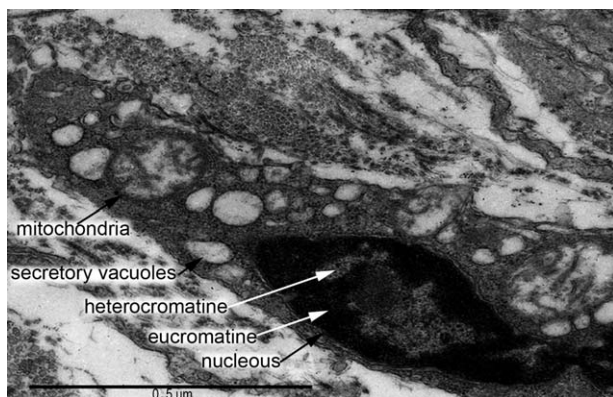


FIGURE 3 *Scinax nasicus*, transmission electron micrograph of the mature flexor tendon of the Digit IV, showing a fibroblast and its nucleus elongated. The heterochromatin is peripheral to the nucleus, surrounded by the euchromatin. In the cytoplasm, big mitochondria and many secretory vacuoles are visible

The frequency histograms show tendons composed of collagen fibrils of different diameters and in different abundance (Figures 6 and 7). In all taxa, most of the fibrils range from 0 to 100 nm, showing that the smaller fibril diameters occur in highest abundance. The only taxa exhibiting larger collagen fibrillar diameters are *Phyllomedusa sauvagii* and *L. chaquensis*. These taxa show the maximum values of evenness (Pielou's evenness index) and diversity (Table 1; Figures 6 and 7). The rest of the sampled amphibians exhibit a collagen fibril range smaller than 350 nm. The distribution of the collagen fibril diameters differs among species. For example, in *L. chaquensis* it is clearly right-skewed (Figure 6).

3.2 | Statistical analyses

Our Pearson correlations showed no relationships between snout-vent length and either of the CSA measures. Moreover, the fibrillar CSA was

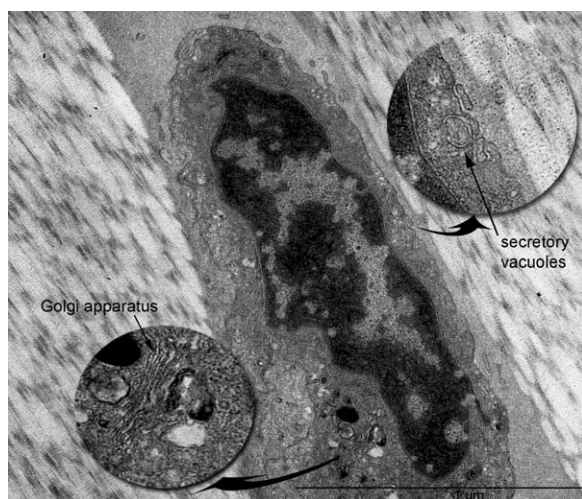


FIGURE 4 *Phyllomedusa sauvagii*, transmission electron micrograph of the mature flexor tendon of the Digit IV. In the cytoplasm, details of the Golgi apparatus and secretory vacuole. The secretory vacuole fuse in a tandem fashion, similar to compound exocytosis

not correlated to the tendon CSA. The factor analysis retained two factors jointly explaining 90% of the variance in the data set (Figure 8). Whereas the first axis was highly correlated with snout-vent length and tendon CSA, the second axis was strongly correlated with fibrillar CSA. On the plot of the two axes, jumping species clearly segregate along the second axis by having larger scores in contrast to hoppers which have the lowest scores (Figure 8). The phylogeny plotted into the morphospace shows that these data are also somewhat structured by phylogeny (Figure 8).

A univariate analysis of variance showed no differences in snout-vent length between locomotor groups ($F_{2,3} = 0.41$; $p = .96$). A MANOVA performed on the \log_{10} -transformed CSA data showed significant differences between locomotor groups (Wilks' lambda = 0.009; $F_{4,4} = 9.62$; $p = .025$). Subsequent univariate ANOVAs showed that differences were significant only for fibrillar CSA ($F_{2,3} = 60.89$; $p = .004$). Bonferroni post-hoc tests showed that all locomotor groups differed from one another with jumpers having the highest and hoppers the lowest fibrillar CSA. Arboreal species are intermediate.

4 | DISCUSSION

Tendons are important sources for storing and recovering elastic energy, minimizing the work that the limb muscles have to do to restore an animal's potential and kinetic energy while running, hopping, or galloping. Tendons are viscoelastic tissues whose elastic properties dominate its overall behavior (Biewener, 2003; Hessel & Nishikawa, 2017). Roberts, Azizi, & Abbott, (2011) have shown that muscle properties of frogs are not enough to determine their locomotor performance (see also Roberts & Azizi, 2011). They found a lack of correlation between available muscle power and jump power, suggesting that non-muscular mechanisms can obscure the link between muscle mechanical performance and locomotor performance. They proposed that the lack of consistency in their data could be attributed to tendon properties. One very interesting outcome of our work is the correlation analysis

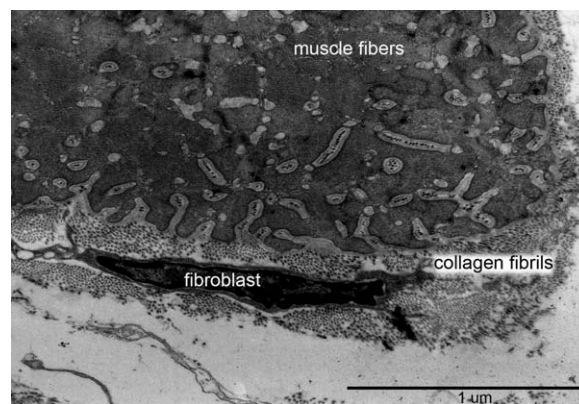


FIGURE 5 *Scinax nasicus*, transmission electron micrograph of the mature flexor tendon of the Digit IV. Myotendinous junction consisting of many varying sized interdigitations of tendon and muscle. The muscles at the myotendinous junction are visible as tongues of muscle fibers the surround single or multiple collagen fibrils, which are immersed among the muscular fibers

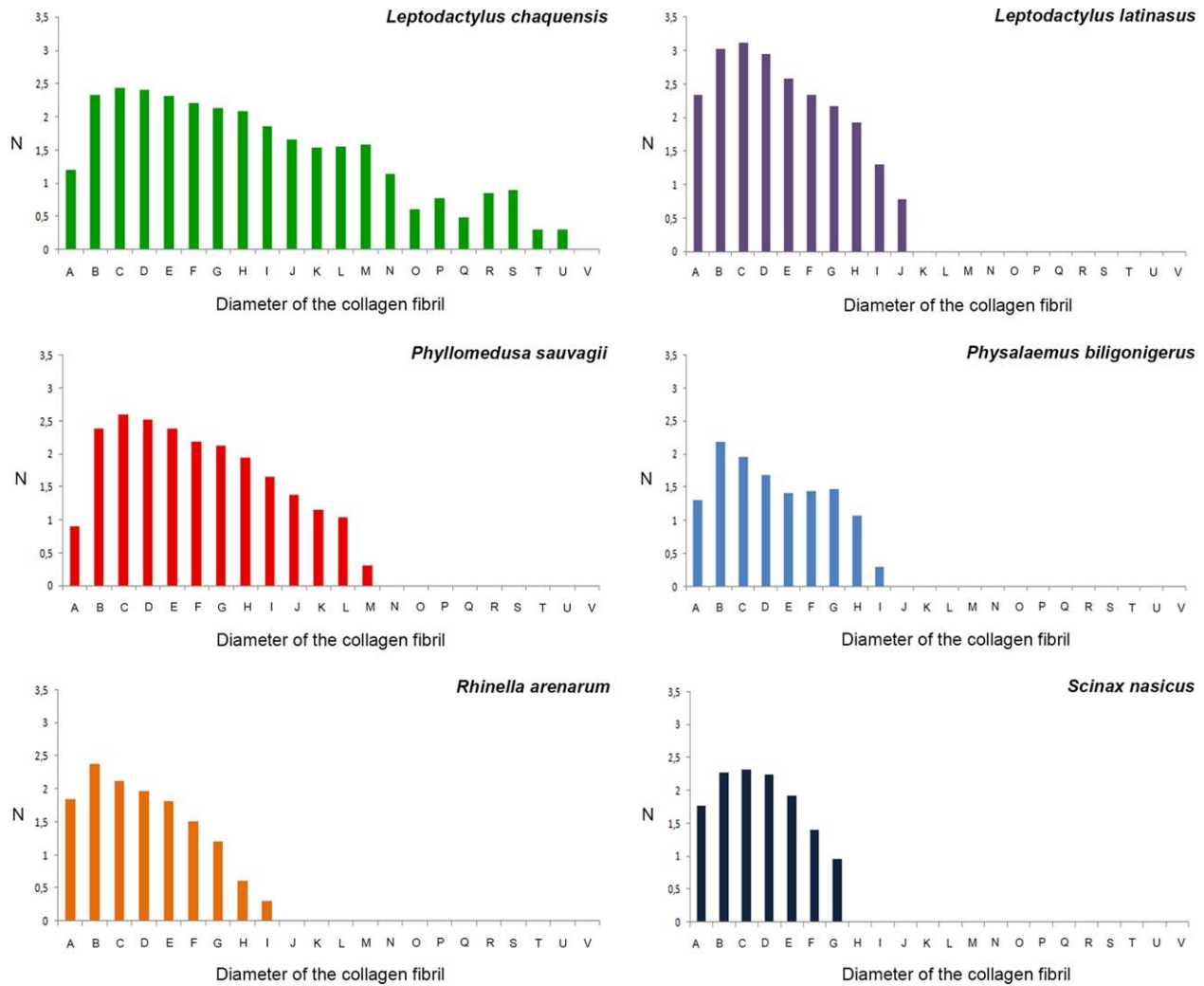


FIGURE 6 Size distribution of collagen fibril diameters of the collagen fibrills from the flexor tendon of the Digit IV. X axis: fibril diameters, each letter (A–V) represents one class interval increasing of 30 nm—that is, A, 10–40 nm; B, 41–70 nm; C, 71–100 nm; D, 101–130 nm; E, 131–160 nm; F, 161–190 nm; G, 191–220 nm; H, 221–250 nm; I, 251–280 nm; J, 281–310 nm; K, 311–340 nm; L, 341–370 nm; M, 371–400 nm; N, 401–431 nm; O, 431–460 nm; P, 461–490 nm; Q, 491–520 nm; R, 521–550 nm; S, 551–580 nm; T, 581–610 nm; U, 611–640 nm; V, 641–670 nm. Y axis: number of fibrils log₁₀ transformed for the sake of clarity [Color figure can be viewed at wileyonlinelibrary.com]

showing that collagen fibrils and tendon diameter are independent of body size. This is an unexpected result because, for example, *L. chaquensis*, a small sized jumper frog (SVL 78 mm) exhibits some of the largest collagen fibril diameters (~550 nm). Reports exist of collagen fibril diameters of horse (~400 nm; Parry, 1988), rabbit (~280 nm; Gill et al., 2004), and humans (121 nm; Svensson et al., 2007; Heinemeier & Kjaer, 2011) all being smaller than those of a small frog. Thus, if body size does not explain the collagen fibril diameter in a tendon, the rationale behind our ecomorphological study is reinforced. The creep-inhibition property of a tendon, that is, its ability to resist plastic deformation, has previously been related to the higher percentage of small-diameter fibrils present (Parry & Craig, 1984; Parry, 1988; Ottani et al., 2001; Franchi et al., 2007; Galloway et al., 2013). Our results show that almost all analyzed species present a unimodal distribution of collagen diameter. By contrast, two of the species, *L. chaquensis* and *Phyllomedusa sauvagii*, exhibiting the broadest ranges of diameters and are those with a unimodal skewed collagen diameter distribution (Parry,

1988; Amey et al., 2002; Gill et al., 2004). Because the specimens sampled in our work were adults, collagen diameter distribution seems not to be related to ontogenetic aspects of tendon growth. It should be also considered that the observed variation in collagen fibril diameters could be an epiphenomenon of their tapered ends (Trotter & Koob, 1989; Canty & Kadler, 2002; Kalson et al., 2011), yet if this were to be the case then a similar broad distribution should be observed in all species if similar sites are examined in the different species.

The taxa showing a skewed-unimodal distribution pattern or the collagen fibrils also exhibit particularities in their movement related behaviors. *P. sauvagii* uses its forelimbs for manipulating prey and during wiping behavior (Gray et al., 1997; Manzano et al., 2008), abilities that require very sophisticated digit movements; *L. chaquensis* is an exceptional jumper. These are highly demanding movements from a mechanical point of view (Nauwelaerts, Ramsay, & Aerts, 2007; Manzano et al., 2008). Overall, our data show that frogs tend to exhibit higher values of collagen fibril diameters compared to mammals (Parry,

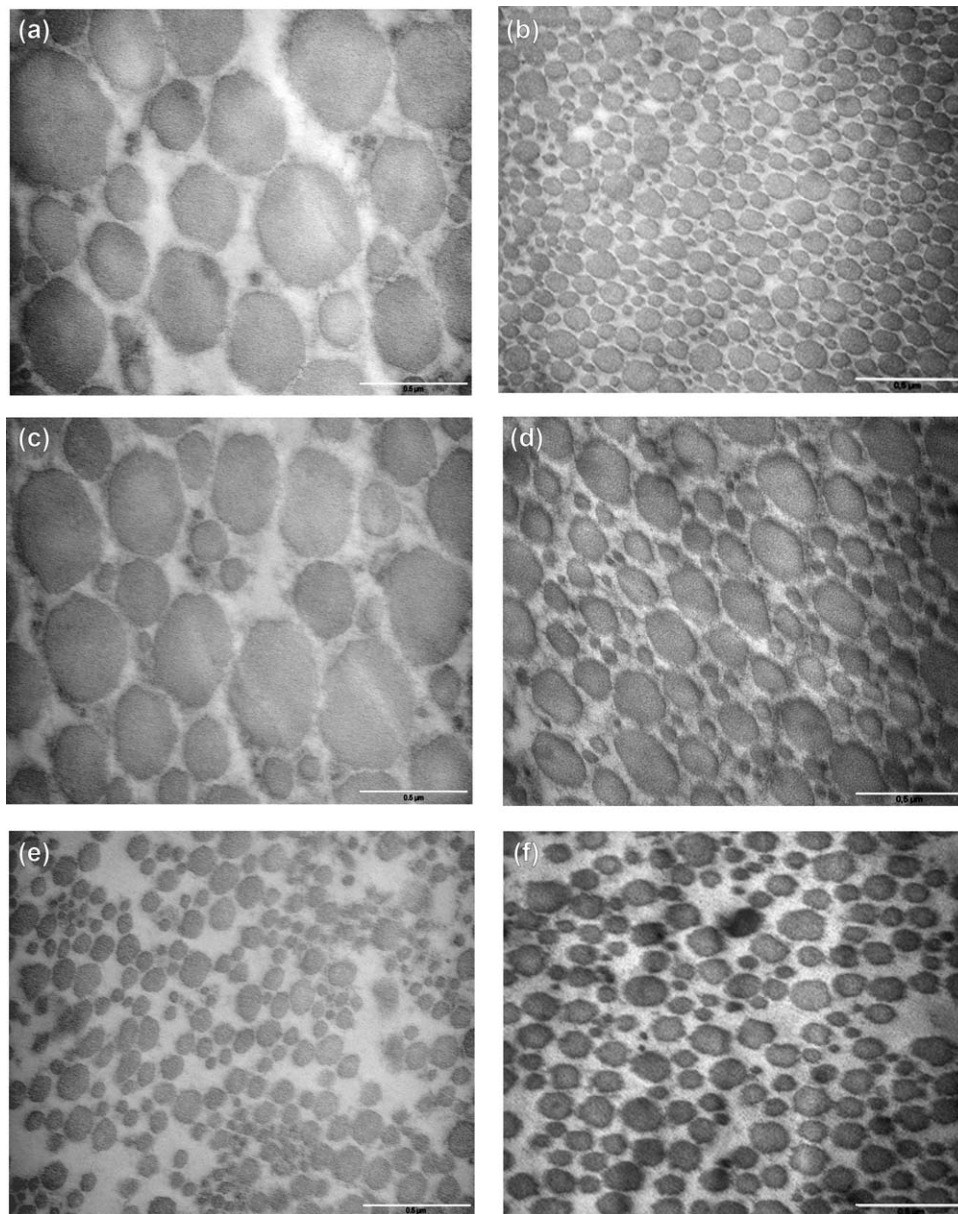


FIGURE 7 Transmission electron microscopic pictures showing transverse sections of the collagen fibrills from the flexor tendon of the Digit IV. (a) *Leptodactylus chaquensis* (b) *Leptodactylus latinasus* (c) *Phyllomedusa sauvagii* (d) *Physalaemus biligonigerus* (e) *Rhinella arenarum* (f) *Scinax nasicus*. Scale bar 0.5 μm

1988; Ameye et al., 2002; Gill et al., 2004). These data could indicate that terrestrial jumping could have imposed an ancestrally high threshold for the CSA of the tendon collagen fibril.

However, despite the large fibrillar diameter in comparison with other vertebrates, our data also show differences between species with different locomotor modes. Specifically, jumpers had a greater fibrillar CSA than arboreal climbers and jumpers, which in turn had a greater fibrillar CSA than hoppers in line with our predictions. Unexpectedly, however, no differences in tendon CSA were detected between species with different locomotor modes. Moreover, we found no correlation between collagen fibril CSA and tendon CSA confirming that measurements of the tendinous CSA do not per se reflect the collagen content and consequently the functional

properties of a tendon (independence of tendon CSA and its functional properties was also highlighted by Magnusson et al., 2003). Our data suggest that variation in collagen fibril dimensions can constitute another important means for tuning mechanical properties of tendons (Matson et al., 2012). The fact that jumpers showed a greater fibrillar CSA suggest that, in line with our prediction, the flexor tendons of the fingers play an important role in absorbing the forces during landing in frogs. Unexpectedly, a study by Reilly et al. (2016) showed higher average forelimb impact forces in a hopper (*Rhinella marina*) compared to a jumper (*Lithobates catesbeiana*). However, total landing forces (including body landing forces) for the specialized jumper were nearly higher than those of the hopper (Reilly et al., 2016). Despite the fact that no detailed kinematics of

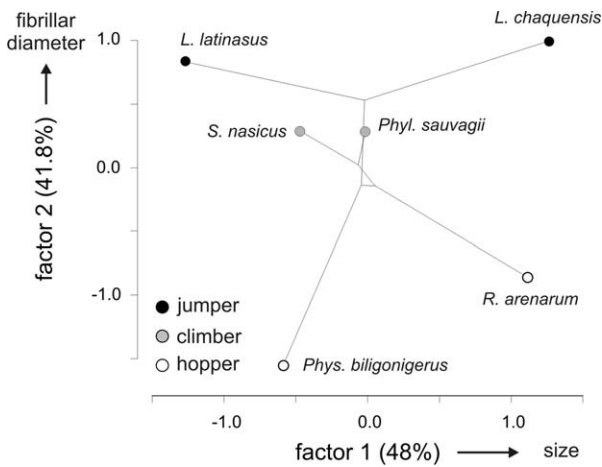


FIGURE 8 Scatterplot illustrating the results of a principal components analysis performed on the tendon measurements and body size. The first axis represents overall frog size and tendon diameter. The second axis explains variation in fibrillar diameter. Frogs with different locomotor modes segregate along the second component with jumpers having the highest fibrillar diameter and hoppers the lowest

the hand and fingers were presented in the paper it appears, however, that the position of the hand is not the same in both species. Future studies would thus benefit from detailed kinematic studies of the hands in different frogs species to better understand the relationships between tendon ultrastructure and function. Moreover, measurements of impact forces for the species studies here would be important in order to validate our prediction suggesting a link between collagen fibril CSA and landing force. In addition, future studies should ground-truth the hypotheses about fibril CSA governing tendon mechanical properties using direct tensile testing as performed in Shadwick (1990), and Matson et al. (2012), among others.

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SUPPORTING INFORMATION

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