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RESEARCH ARTICLE

The diversity and evolution of locomotor muscle properties in anurans

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ABSTRACT

Anuran jumping is a model system for linking muscle physiology to organismal performance. However, anuran species display substantial diversity in their locomotion, with some species performing powerful leaps from riverbanks or tree branches, while other species move predominantly via swimming, short hops or even diagonal-sequence gaits. Furthermore, many anurans with similar locomotion and morphology are actually convergent (e.g. multiple independent evolutions of 'tree frogs'), while closely related species may differ drastically, as with the walking toad (*Melanophryniscus stelzneri*) and bullfrog-like river toad (*Phrynoides aspera*) compared with other Bufonid toads. These multiple independent evolutionary changes in locomotion allow us to test the hypothesis that evolutionary increases in locomotor performance will be linked to the evolution of faster, high-power muscles. I tested the jumping, swimming and walking (when applicable) performance of 14 species of anurans and one salamander, followed by measurement of the contractile properties of the semimembranosus and plantaris longus muscles and anatomical measurements, using phylogenetic comparative methods. I found that increased jumping performance correlated to muscle contractile properties associated with muscle speed (e.g. time to peak tetanus, maximum shortening speed, peak isotonic power), and was tightly linked to relevant anatomical traits (e.g. leg length, muscle mass). Swimming performance was not correlated to jumping, and was correlated with fewer anatomical and muscular variables. Thus, muscle properties evolve along with changes in anatomy to produce differences in overall locomotor performance.

KEY WORDS: Amphibian, Contractile, Kinetics, Muscular, Evolution, Frog

INTRODUCTION

Animals have evolved a wide array of locomotor modes (e.g. walking, swimming, flying, slithering) to traverse their habitats, find food and mates, and avoid predators. Performance within these modes is also tremendously variable, from the slow trudge of a giant tortoise to the rapid dash of a cheetah. These differences across species must be ultimately due to corresponding differences in the integrative biomechanical systems that produce these movements, and disentangling the roles of various components of these systems in determining organismal performance is a key challenge in biomechanics. While comparative skeletal and gross muscle morphology have been correlated to performance in a wide variety of taxa, fewer studies (Tobalske, 1996; Bonine et al., 2001, 2005; Chadwell et al., 2002; Scales et al., 2009; Roberts et al., 2011; Vanhooydonck et al., 2014) have focused on corresponding intraspecific differences in the fundamental motor of much of animal movement: muscle.

To what extent are interspecific differences in performance linked to corresponding differences in muscle power, contractile speed and activation? While the mechanical output of muscle can be affected by size or muscular anatomy, changes to the force–velocity (*F/V*) curve can change muscle power output and shortening speed independently of overall muscle dimensions and anatomy, and activation/deactivation kinetics determine how rapidly a muscle can respond to neural activation or the cessation of activation. Some prior studies have attempted to address interspecific differences in muscle physiology via two- and three-species comparisons (Abu-Ghalyun et al., 1988; Chadwell et al., 2002), which may be confounded by other, untested differences between species (Garland and Adolph, 1994; Garland et al., 2005). Others have indirectly assessed muscle properties via histological fiber type (Tobalske, 1996; Bonine et al., 2001, 2005; Scales et al., 2009), which, while it can yield insights, does not always directly correspond to muscle contractile properties (Wilson et al., 2004). Finally, meta-analysis using literature values (Medler, 2002) suffers problems of comparing across non-homologous muscles under highly variable testing protocols, especially without phylogenetic comparative methods (Felsenstein, 1985; Garland et al., 2005). More recently, studies using phylogenetic comparative methods have shown changes in muscle contractile properties with animal size (James et al., 2015) and a tradeoff between *in vitro* work-loop power and fatigue resistance (Vanhooydonck et al., 2014), though the link between animal performance and muscle properties was inconsistent (Vanhooydonck et al., 2014). Consequently, it remains unknown whether muscle contractile properties are broadly conserved, evolve rapidly with changes in organismal performance, or show no correlation to locomotor evolution. However, intra-specific studies showing correlations between muscle properties and whole-organism performance (Wilson et al., 2002; Wilson and James, 2004; James et al., 2005; Navas et al., 2005) suggest that selection may indeed act upon organismal performance (Arnold, 1983), and, through this, lead to changes in muscle physiology.

Anuran locomotion represents an excellent system for understanding the evolution of muscle physiology. Anuran locomotor performance in tasks such as jumping and swimming is readily tested, varies widely across species (Zug, 1978; Richards, 2010), strongly correlates with habitat and morphology (Zug, 1972, 1978; Emerson, 1988; Gomes et al., 2009), and can be linked to fundamental aspects of muscle physiology (Lutz and Rome, 1994; Richards and Biewener, 2007). Furthermore, anurans show numerous convergences and divergences in locomotor performance and associated morphological traits across their

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phylogeny (Fig. 1) (Emerson, 1988). Arboreal jumpers have evolved independently several times (*Hylidae*, *Rhacophoridae*, *Hyperolidae*, etc.), while the true toad family (*Bufonidae*) contains not only the well-known terrestrial hoppers but also riparian bullfrog-like leapers (*Phrynoidis aspera*), terrestrial walkers (*Melanophryniscus stelzneri*) and even arboreal toads (*Pedostibes hosii*) (Fig. 1). Consequently, this model system provides a large number of high-contrast nodes for phylogenetic comparative methods, which should allow us to detect correlations between locomotor performance and muscle physiology.

In order to examine the evolutionary relationships between muscle and locomotor performance, I quantified jumping, walking and swimming performance, musculoskeletal gross anatomy and the contractile properties for two major locomotor muscles across a diverse sample of amphibians (14 frogs and one salamander). Using phylogenetic comparative methods, I tested for relationships between locomotor performance, muscle properties and anatomy, as well as the effect of a transition to primarily walking rather than hopping/leaping. I hypothesize that muscle physiology will correlate to observed performance across the frog evolutionary tree, such that frogs with greater jumping performance will have faster muscle kinetics than lower-performing relatives, though muscle relaxation time will be faster in species with shorter jumps (such as toads, which often move in a series of short hops) or that display walking behavior.

MATERIALS AND METHODS
Animals
I purchased adult amphibians of 15 species (Fig. 1) from commercial suppliers, selected based on reported locomotor performance of the taxon or close relatives (Zug, 1978) and relationships in order to maximize evolutionary contrasts between species and avoid redundancy. Size could not be controlled for because of different adult sizes of crucial taxa, and thus two measures of size [snout–vent length (SVL) and mass] were included as variables in the analysis. Animals were kept in housing appropriate to their natural habitat with an air temperature of 22°C (with an additional heat gradient up to 28°C for tropical species), fed

Fig. 1. Ultrametric phylogeny of the species in this study. The tree was pruned from Isaac et al. (2012), with habitat and presence of walking labeled at the tips. *N* denotes the approximate number of individuals used per species, though because of incomplete data, some species may have a greater or lesser *N* for a given variable.

crickets, given continuous access to water, and kept on a 12 h:12 h light:dark schedule, with all experiments conducted during daylight hours. Semi-aquatic species (*Lithobates pipiens*, *Litoria aurea*, *Bombina orientalis*) were housed in plastic tubs of 46×91 cm, tilted at a shallow angle and partially filled with dechlorinated water to create a water and land area. Large terrestrial species (*Phrynoides aspera*, *Ambystoma tigrinum*) were housed in the same tubs, but placed horizontally with a layer of cypress mulch bedding and a bowl of dechlorinated water. Smaller species were housed in 20×30 cm plastic tubs with wet paper towel substrate for tree frogs and cypress mulch for all others. Cypress mulch and paper towels were moistened daily. Animals were housed at no more than three per container for between 1 and 4 weeks prior to use, and all fed willingly when offered food.

Locomotor trials
I attempted to induce a minimum of five trials of high-performance jumping, walking and swimming from all animals via hand movements and light touching, with failure defined as refusal to perform the behavior after 5 min of continuous attempts and a minimum rest period of 5 min between trials. During all locomotor trials, animals were maintained at a temperature range of 28–30°C, as this is at or near the known thermal optimum for many species (Hirano and Rome, 1984; John-Alder et al., 1988; Londos and Brooks, 1988; Knowles and Weigl, 1990; Marsh, 1994), and likely close for those species with unknown thermal optima. Although these limited sample sizes are unlikely to include true physiologically maximal performance trials (Astley et al., 2013), the large differences in performance between species suggest that these observations are at least reflective of true interspecific differences in maximal performance.

Jump forces were measured using a custom-built force plate (previously used by Roberts et al., 2011, and calibrated as described in that paper) for most species. Strain-gauge outputs were amplified (Model 2120A, Vishay Precision Group, Raleigh, NC, USA), collected and converted to digital form (NI BNC 2110 and USB 6251, National Instruments, Austin, TX, USA), and recorded at 10 kHz using Igor v6 (Wavemetrics, Lake Oswego, OR, USA), then smoothed via a low-pass filter at 15 Hz. A custom-written script was used to calculate jump takeoff velocity (normalized by SVL), peak jump force (normalized by body weight), and jump work and peak jump power (both normalized per kg muscle mass). For species either too small (body mass <10 g) or that refused to jump from the center, sensing region of the plate (*P. aspera*), I used two synchronized high-speed video cameras at 125 frames s^{-1} (Photron 1024, Photron Inc., San Diego, CA, USA). High-speed video recordings were calibrated and digitized with a MATLAB script package (Hedrick, 2008), and a custom-written script was used to calculate the same jump metrics from the splined displacement of a point on the tip of the animal's snout. Only *A. tigrinum* and a single individual of *Phrynomantis bifasciatus* failed to jump despite repeated stimulation. For all animals, the peak jumping performance based on takeoff velocity was selected for analysis.

Swimming trials were conducted in an 84×40 cm clear plastic tub filled with between 5 and 8 cm of water and maintained at a temperature range of 27–29°C for a minimum of 5 cycles. A single high-speed camera recorded swimming trials from directly overhead at 60 frames s^{-1}, while a ruler beneath the tub provided scale. I selected the fastest cycle based on the average velocity of the tip of the animal's snout, and in each frame of this cycle, the snout tip and toe tip were digitized and used to determine average swimming
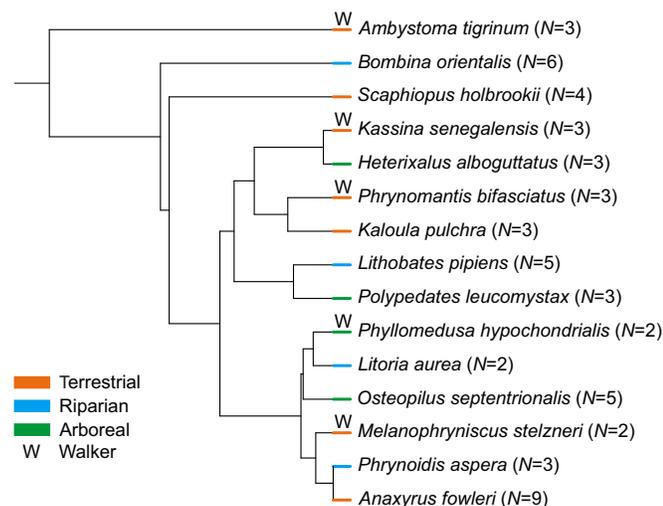
velocity across a cycle and peak swimming velocity within a cycle (normalized by SVL), swim frequency (in Hz) and swim duty factor (kick duration/cycle duration). As *Scaphiopus holbrookii* swam using alternate-leg kicking (Wyman, 1856), I calculated these values based on the complete cycle of one limb. For *A. tigrinum*, which swam via lateral undulation of the body, duty factor was given as 0.5 and peak swimming velocity was set equal to average swimming velocity. No species refused to swim. For all animals, the best swimming performance based on average swimming velocity was selected for analysis.

Walking tests were recorded with two calibrated, synchronized high-speed video cameras in the same configuration as when used for jumping. As with swimming, a point was digitized at footfall of the most visible hindlimb and used to select the fastest cycle and calculate stride frequency (in Hz) and walking speed (averaged across the cycle, normalized by SVL). Only five species performed coordinated walking movements characterized by alternating movements of the forelimbs and hindlimbs and multiple successive cycles without coming to rest: *A. tigrinum*, *Kassina senegalensis*, *M. stelzneri*, *P. bifasciatus* and *Phyllomedusa hypochondrialis*. A categorical variable was assigned to all species to indicate whether they walked or did not walk during trials. For all walking animals, the best walking performance based on walking speed was selected for analysis.

In vitro muscle tests and anatomical measurements

Following locomotor trials and a minimum 24 h rest period, animals were chilled and killed via double-pithing. Frogs were immediately immersed in oxygenated Ringer's solution (prepared as in Peplowski and Marsh, 1997), and the semimembranosus (SM) and contralateral plantaris (PL) were dissected free along with small segments of proximal and distal attachments for clamping. The SM and PL were selected because both are large muscles known to be active and shortening during jumping (Lutz and Rome, 1994; Olson and Marsh, 1998; Astley and Roberts, 2012), but with different roles during the jump. The SM is a hip extensor with a minimal tendinous component (Dunlap, 1960) which contracts at a constant velocity corresponding to the speed of peak power output in the *F/V* curve (Lutz and Rome, 1994). The PL is an ankle extensor with a prominent tendon (Dunlap, 1960), and is known to be involved in elastic energy storage (Astley and Roberts, 2012). During dissection to expose the muscles, I recorded total body mass, SVL, the length of each leg segment as well as total leg length, width of the proximal and distal sections of the PL tendon, mass of the non-tested SM and PL, mass of the viscera (heart, lungs, digestive system, kidneys and gonads, including fat bodies; no specimens had eggs), combined mass of all other proximal limb muscles, and combined mass of all other shank muscles. Mass was recorded on a digital scale to the nearest mg, length was measured via calipers to the nearest mm. In order to measure PL fiber length, muscle length and pennation angle, muscles tested *in vitro* were subsequently fixed in 10% buffered formalin and sectioned in a plane perpendicular to the aponeurosis and parallel to the long axis of the muscle. Large muscles were sectioned using a scalpel and examined under a dissecting scope, while small muscles were cryosectioned (100 µm thickness) and examined under polarized light in a backlit dissecting microscope, with a transparent ruler in the same field of view of both (0.1 mm gradations). These measurements were used to calculate the anatomical variables used in subsequent analysis: total body mass, SVL, relative muscle mass (combined bilateral leg muscle mass/total body mass), relative viscera mass (viscera mass/total body mass), relative leg length (leg length/SVL), relative tarsal

length (tarsal length/tibia length), distal PL tendon expansion (distal/proximal tendon width), PL fiber relative length (PL fiber length/whole muscle length), PL pennation angle and PL relative mass (PL mass/total bilateral leg muscle mass). For *A. tigrinum*, the ischioflexoris and flexor primordialis communis (FPC) were chosen as the homologous muscles to the SM and PL, respectively (Duellman and Trueb, 1994; Ashley-Ross and Lauder, 1997). Certain variables could not be meaningfully calculated for *A. tigrinum* (tarsal/tibia length, distal/proximal tendon width, PL fiber length/whole muscle length, and PL pennation angle) because the tarsals are not elongated as in frogs and the FPC is not pennate and the distal tendon does not have the discrete proximal and distal segments seen in frogs, and these values were set to either zero or one as appropriate. Size was retained as a variable rather than attempting to size-correct via regressions, as the latter method can be statistically problematic (Garcia-Berthou, 2001; Freckleton, 2009).

Muscles for *in vitro* testing were clamped securely at their proximal bony attachment, and connected to the servomotor via either a lightweight chain or a Kevlar thread tied at their distal attachment with a surgical silk suture. Because the PL lacks a discrete and strong distal bony attachment, it was tied at the distal muscle–tendon junction immediately proximal to the thickened 'bulb' present in the tendon of all frog species tested. For most muscles, a servomotor and controller with a 10 N maximum load was used, but for *M. stelzneri* and *K. senegalensis*, I used a smaller motor with a 0.5 N maximum load (models 6650LR and 6350, respectively, Aurora Scientific Inc., Aurora, ON, Canada). Muscles were immersed in an oxygenated Ringer's solution bath maintained at a temperature of 28°C via a temperature-controlled water bath and pump. Muscles were stimulated using an S48 stimulator (Grass Products, Warwick, RI, USA) via an amplifier (Crown DC300A Series 2, Crown Audio Inc., Elkhart, IN, USA) connected to parallel platinum plate electrodes running the full length of the muscle. Force and displacement outputs were converted to digital form (NI BNC 2110 and USB 6251, National Instruments), and recorded at 10 kHz using Igor v6, then smoothed via a low-pass filter at 15–25 Hz, with the cutoff frequency selected to minimize noise while not distorting the force traces. The SM was typically tested first, while the PL was maintained in room-temperature Ringer's solution with 100% oxygen bubbled through, though both tests were completed within a 5 h period. All tetanic contractions were followed by a 5 min rest period to minimize fatigue.

Voltage for supramaximal stimulation was determined by progressive increases until maximal twitch force with the muscle held at the length that showed the smallest detectable passive tension, followed by tetanic contractions with further voltage increases if needed. Preliminary experiments with three species (*Anaxyrus fowleri*, *Osteopilus septentrionalis* and *L. pipiens*) showed that once fused tetanus was achieved, further increases in stimulation frequency did not increase force, and thus a uniform frequency of 100 pulses (0.2 ms pulse duration) per second was chosen, as it was substantially higher than the minimum frequency needed in any example species; all species tested showed fully fused tetani. After determining pulse train duration needed to achieve peak tetanic force by progressive increases until a clear plateau was seen, a series of isometric contractions were used to determine the peak of the length–tension relationship (optimum length, L_0) in order to take subsequent data (e.g. peak twitch, *F/V* measurements) at this length. Full length–tension curves were not reconstructed because of concerns about muscle fatigue from more contractions combined with the limited utility of this information without corresponding

sonometric verification of lengths and excursions used *in vivo*. Because of the compliance of the PL tendon, twitches were used rather than tetanic contractions for the length–tension determination, but subsequent contractions accounted for the longer muscle length at peak twitch versus tetanic isometric force (Holt and Azizi, 2014).

A maximal isometric twitch (Fig. 2A,C) and tetanic contraction at L_0 were recorded, followed by a series of at least seven isotonic tetanic contractions at decreasing fractions of maximal force, in which isotonic velocity was determined at L_0; all tetanic contractions (isometric and isotonic) were separated by at least 5 min rest. A subsequent isometric tetanic contraction at L_0 was used to adjust for fatigue during the isotonic contraction series (always <10% decline). Finally, a 1 Hz series of isometric tetanic contractions of 300 ms stimulus duration for 100 s was used to assess fatigue. Isotonic contractions were normalized for peak tetanic force, fiber length, and tension loss due to fatigue, and a F/V curve was fitted to the combined data for all individuals of a species using a Hill curve (Hill, 1938) via a custom-written MATLAB script (Fig. 2B,D) using the fit() function with non-linear least-squares and the default Levenberg–Marquardt algorithm. The choice to collect seven isotonic contractions from each individual, then pool individuals, was due to concerns for fatigue effect and the viability of the second muscle (stored in oxygenated Ringer's solution until the completion of the first test), as well as the scarcity of specimens of many of these species; excessive fatigue during the isotonic series would render the entire dataset dubious, without assurances of additional specimens. From these data, I quantified the following variables for each muscle: peak twitch time (time from stimulation to peak of twitch force), twitch half-relaxation time (time from peak twitch until 50% force decline), twitch/tetanus ratio (peak twitch force/peak tetanic force), peak tetanus time (time from start of stimulation until peak tetanic force), half-tetanus time (time from start of stimulation to 50% of peak tetanic force), tetanic half-relaxation time (time from end of stimulation until 50% force decline), fatigue resistance (time to 50% loss of force in fatigue protocol), V_{max} (maximum unloaded tetanic isotonic shortening velocity, normalized by fiber length), $\dot{W}_{max}$ (maximum tetanic isotonic power, normalized per kg muscle mass) and velocity at $\dot{W}_{max}$ [relative shortening velocity (V/V_{max}) at peak power ($\dot{W}_{max}$)].

Statistics

I used a single mean value of each variable per species, except for locomotor trials, in which I selected the values of the trial with a maximum jump takeoff velocity and peak swimming speed across individuals. As noted above, variables were normalized by the appropriate scaling metric (length, cross-sectional area, mass) whenever possible in order to minimize the effects of size. Given the reliance of multivariate methods, I retained the traditional threshold for statistical significance as $P=0.05$.

In order to thoroughly capture the variation between species, and avoid unfounded assumptions of which variables would be significant, I quantified a large number of variables (see above). I then used phylogenetic principal component analysis (phyl.PCA in R, based on Revell, 2009) to both account for likely correlations between associated variables and reduce the number of variables while still capturing the bulk of observed variation. I selected an ultrametric tree with dated nodes (Isaac et al., 2012) containing all taxa in the sample to calculate independent contrasts. Alternative trees in the recent literature (Frost et al., 2006; Roelants et al., 2007; Pyron and Wiens, 2011) differ only at one or two nodes for the taxa
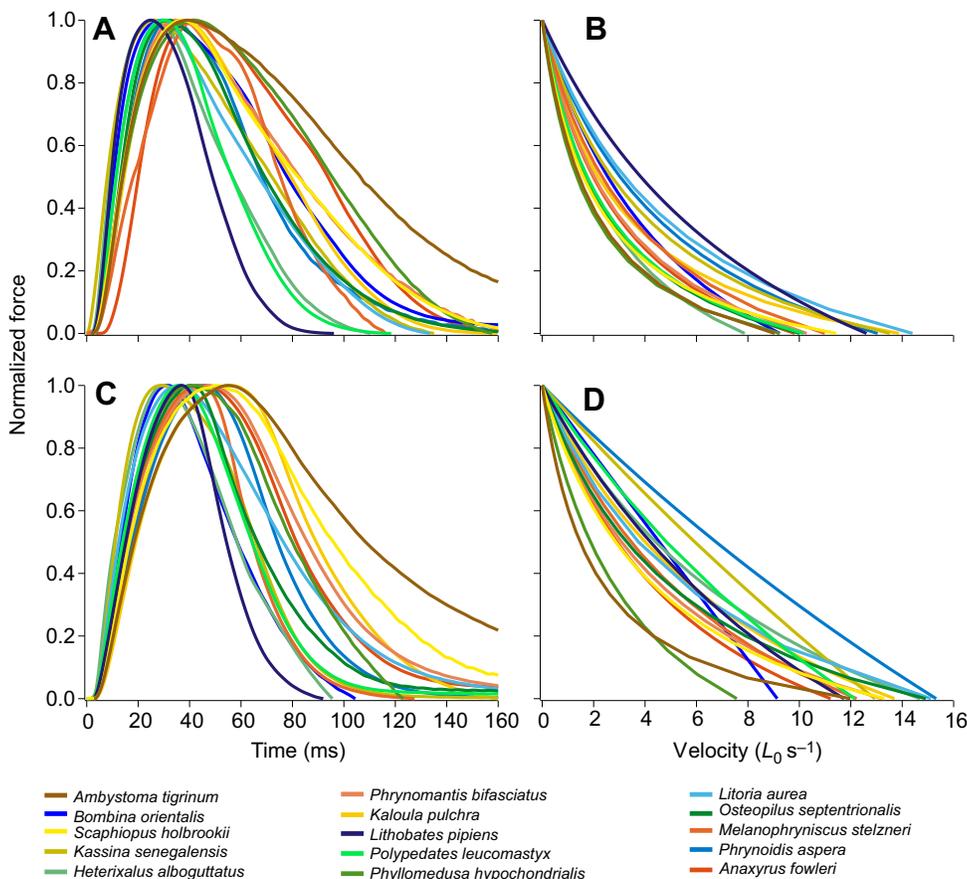

Fig. 2. Twitches and force–velocity (F/V) curves. All twitches were normalized to peak twitch force at L_0 (optimum length); all F/V curves were normalized to P_0 (isometric force at optimum L_0) and L_0. Twitches were selected based on similarity to the average peak twitch values for the species. Species ID is given in the key, with colors broadly corresponding to the habitat: terrestrial species are yellow–orange–red, riparian species are shades of blue, arboreal species are shades of green. (A) Semimembranosus (SM) twitches. (B) SM F/V curves. (C) Plantaris (PL) twitches. (D) PL F/V curves.

- *Ambystoma tigrinum*
- *Bombina orientalis*
- *Scaphiopus holbrookii*
- *Kassina senegalensis*
- *Heterixalus alboguttatus*
- *Phrynomantis bifasciatus*
- *Kaloula pulchra*
- *Lithobates pipiens*
- *Polypedates leucomastyx*
- *Phyllomedusa hypochondrialis*
- *Litoria aurea*
- *Osteopilus septentrionalis*
- *Melanophryniscus stelzneri*
- *Phrynoidis aspera*
- *Anaxyrus fowleri*

Table 1. Phylogenetic principal component analysis (PCA) loadings of jumping and swimming variables

	Jump Performance PC1 (60.5%)	Swim Performance PC2 (25.8%)
Peak jump force (BW)	−0.9534	−0.2309
Takeoff velocity (BL s^{-1})	−0.8354	0.4029
Jump work (J kg^{-1})	−0.9330	−0.1272
Peak jump power (W kg^{-1})	−0.9318	−0.0925
Mean swim velocity (BL s^{-1})	−0.1707	0.9513
Swim frequency (Hz)	0.8064	0.3543
Swim duty factor	0.7852	0.2174
Peak swim velocity (BL s^{-1})	−0.4418	0.8654

BW, body weight; BL, body length.
Percentages in parentheses indicate the proportion of variation accounted for by the axis.

in the sample. I performed phylogenetic PCA (pPCA) on groups of related variables: locomotor, anatomical, SM and PL. For each pPCA, I retained axes that would account for approximately 85–90% of the variation. Locomotor pPCA variables were then compared via linear models (phylolm in R) to three sub-groups of variables (anatomical variables, SM variables and PL variables) in order to avoid having more variables than data points in a given comparison (Dunn et al., 2013). Finally, I performed phylogenetic MANOVA to determine whether walking influenced performance, anatomy or muscle properties. Because walking taxa were all sister to non-walking taxa, I performed a non-phylogenetic orthogonal linear regression between speed and stride frequency to determine whether sufficiently strong correlation was present to use only one, and coded walking as a presence/absence variable.

RESULTS
Phylogenetic PCAs
Strong correlations appeared throughout locomotor performance variables, collapsing performance into two PC axes, termed '**Jump Performance**' and '**Swim Performance**' that accounted for 86.3% of variation (Table 1; all PCA axes are denoted henceforth using initial capitalization and bold font to avoid confusion). All jump

Table 2. Phylogenetic PCA loadings of anatomical variables

	Leg Size PC1 (42.4%)	Distal Element Elongation PC2 (20.7%)	PL Size PC3 (14.4%)	Distal Tendon Expansion PC4 (10.5%)
Body mass (g)	−0.8236	−0.4112	0.3306	0.1563
Snout–vent length (mm)	−0.7480	−0.5802	0.2574	0.0152
Relative muscle mass (%)	−0.8670	−0.2143	−0.3220	−0.1487
Relative viscera mass (%)	0.6676	−0.4833	−0.2349	−0.2709
Relative leg length (%)	−0.7667	0.1684	−0.4578	−0.3145
Tarsal/tibia ratio	−0.1545	0.9336	0.1276	−0.1138
PL aponeurosis expansion ratio	−0.3377	0.4352	−0.4180	0.6653
PL relative fiber length (%)	0.7243	−0.2752	0.1602	0.4923
PL pennation angle (deg)	−0.7322	−0.0452	0.1886	0.3066
PL proportion of leg muscle mass	0.1342	−0.3670	−0.8046	0.1928

PL, plantaris. Percentages in parentheses indicate the proportion of variation accounted for by the axis.

Table 3. Phylogenetic PCA loadings of semimembranosus (SM) contractile property variables

	SM PC1 (46.5%)	SM PC2 (22.3%)	SM PC3 (12.8%)	SM PC4 (7.5%)
SM twitch/tetanus ratio	0.4165	0.8492	−0.1153	0.1865
SM time to peak twitch force (ms)	−0.8563	0.2164	0.1016	0.0302
SM time to twitch half-relaxation (ms)	−0.6476	0.6671	−0.0064	0.0484
SM time to peak tetanic force (ms)	−0.8786	0.2077	−0.2496	−0.0999
SM time to half-peak tetanic force (ms)	−0.8354	−0.4322	−0.1378	−0.1831
SM time to tetanic half-relaxation (ms)	−0.2266	0.0021	−0.9081	0.2548
SM fatigue ratio (%)	−0.5355	0.7746	0.2514	−0.0773
SM V_{max} (L s^{-1})	0.5836	0.3227	−0.1393	−0.6889
SM $\dot{W}_{max}$ (W kg^{-1})	0.7680	0.2397	−0.4625	−0.1180
SM V at $\dot{W}_{max}$ (V/V_{max})	0.7628	0.1675	0.2120	0.3247

Percentages in parentheses indicate the proportion of variation accounted for by the axis.

variables loaded together and very highly (albeit negatively) on the **Jump Performance** axis, which also showed correlations with swim frequency and duty factor; powerful jumpers had low-frequency swimming movements and low duty factor, consisting of short propulsive kicks interspersed with long glide phases (Table 1). The second axis, **Swim Performance**, was primarily loaded by both peak instantaneous and mean swim speed, with minimal loading of jump variables (Table 1).

Anatomical variables collapsed to four PC axes, with the first displaying high loading of many variables, while the other three primarily loaded a single variable each, accounting for a total of 88.2% of variation (Table 2). The first axis, broadly renamed '**Leg Size**', showed strongly correlated changes in relative muscle mass, relative leg length, PL pennation angle and fiber length, and relative viscera mass (Table 2). Although all variables had been size-normalized by the appropriate measurement, absolute size still loaded on the **Leg Size** axis, suggesting that these correlated morphological changes also correlate with changes in animal size (Table 2). The remaining axes were renamed based on the most strongly loaded variable, albeit slightly re-phrased to avoid confusion (Table 2).

Table 4. Phylogenetic PCA loadings of PL contractile property variables

	PL PC1 (39.1%)	PL PC2 (21.8%)	PL PC3 (15.9%)	PL PC4 (9.3%)
PL twitch/tetanus ratio	0.4832	−0.3604	0.7863	0.0568
PL time to peak twitch force (ms)	−0.7818	0.0428	0.2482	−0.2874
PL time to twitch half-relaxation (ms)	−0.3203	0.8024	0.0585	0.3352
PL time to peak tetanic force (ms)	−0.9644	−0.0049	−0.0030	−0.1266
PL time to half-peak tetanic force (ms)	−0.8104	0.5109	−0.0614	−0.2573
PL time to tetanic half-relaxation (ms)	−0.3679	−0.0649	0.8384	−0.1968
PL fatigue ratio (%)	−0.1451	−0.6351	−0.3276	−0.0071
PL V_{max} (L s^{-1})	0.5694	0.5902	0.2710	0.2478
PL $\dot{W}_{max}$ (W kg^{-1})	0.6202	0.6194	−0.0836	−0.3801
PL V at $\dot{W}_{max}$ (V/V_{max})	0.7147	0.0527	−0.0859	−0.6354

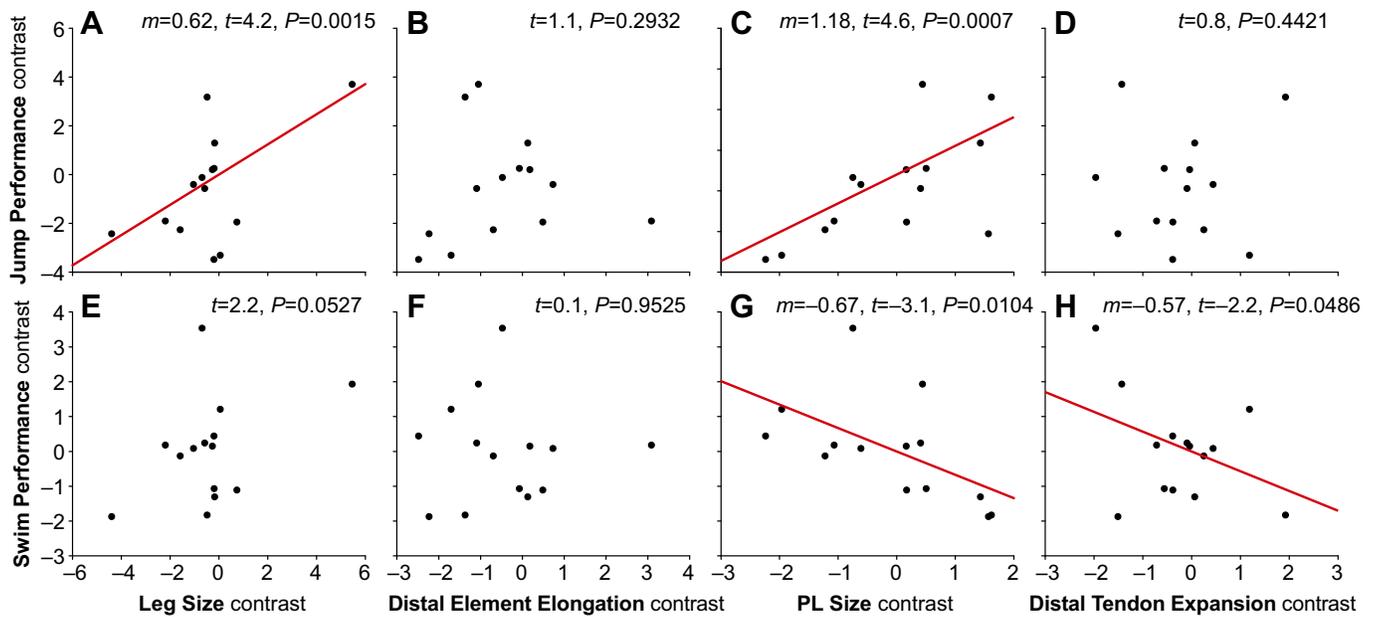Percentages in parentheses indicate the proportion of variation accounted for by the axis.

Fig. 3. Locomotor performance contrasts versus anatomical contrasts. Jump Performance (A–D) and **Swim Performance** (E–H) versus **Leg Size** (A,E), **Distal Element Elongation** (B,F), **PL Size** (C,G) and **Distal Tendon Expansion** (D,H). The text in the top right of each graph gives the *t* statistic and *P*-value of each relationship, and the slope (*m*) if the relationship is significant. Red lines are the linear regressions of significant relationships.

SM and PL muscle property variables each collapsed to four axes accounting for 89.0% and 86.0% of variability, respectively. In both muscles, the first axis showed heavy loadings of variables associated with the rate of force rise and the *F/V* relationship (Tables 3 and 4). Variables characterizing relaxation time and fatigue loaded on the remaining three axes, with some influence of PC1 variables in the PL (Tables 3 and 4). Axes were simply named based on the two-letter abbreviation for the muscle and axis number (e.g. 'SM PC3').

Phylogenetic linear models

Locomotor performance was significantly correlated with several anatomical and muscle property variables, as revealed by phylogenetic linear models (Figs 3–5). **Jump Performance** (primarily loaded by peak jump force) was strongly linked to **Leg Size** (with strong loadings of both relative leg length and relative muscle mass) and to 'PL Size' (Fig. 3A,C), while **Swim Performance** (primarily loaded by average swim speed) was linked to **PL Size** and 'Distal Tendon Expansion' (Fig. 3G,H).
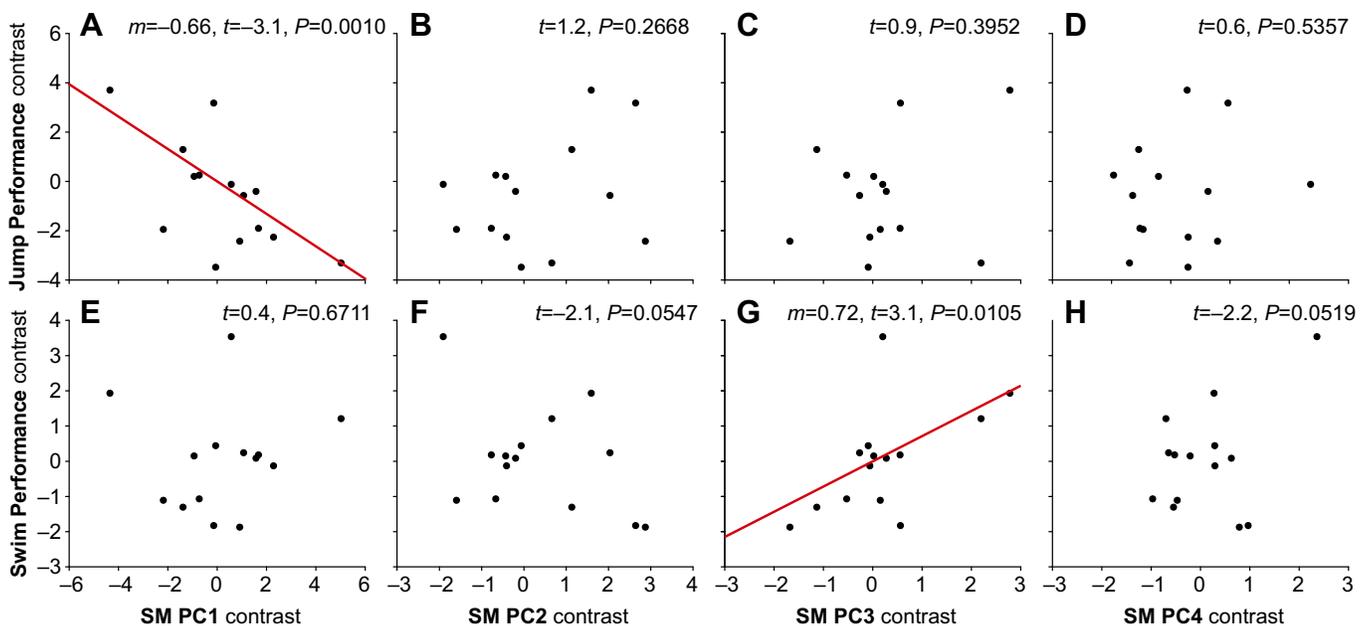

Fig. 4. Locomotor performance contrasts versus semimembranosus (SM) contrasts. Jump Performance (A–D) and **Swim Performance** (E–H) versus **SM PC1–4**. The text in the top right of each graph gives the *t* statistic and *P*-value of each relationship, and the slope (*m*) if the relationship is significant. Red lines are the linear regressions of significant relationships.
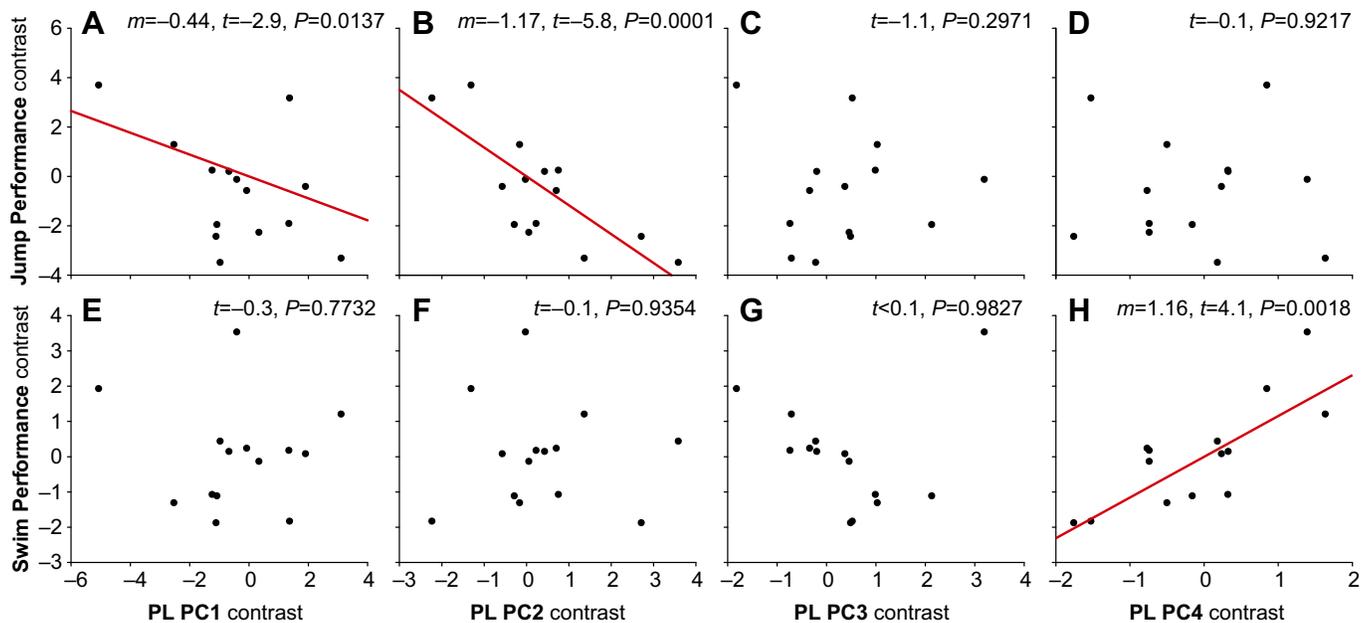
Fig. 5. Locomotor performance contrasts versus plantaris (PL) contrasts. Jump Performance (A–D) and Swim Performance (E–H) versus **PL PC1–4**. The text in the top right of each graph gives the *t* statistic and *P*-value of each relationship, and the slope (*m*) if the relationship is significant. Red lines are the linear regressions of significant relationships.

Jump Performance was also linked to changes in properties of the SM (**SM PC1** – primarily loading times to peak twitch and tetanic force, as well as *F/V*-linked properties; Fig. 4A) and PL (**PL PC1** – primarily loading times to peak twitch and tetanic force, as well as *F/V*-linked properties; and **PL PC2** – primarily loading relaxation time; Fig. 5A,B), while **Swim Performance** was linked to changes in **SM PC3** (primarily loading relaxation time) and **PL PC4** (primarily loading *V* at $\dot{W}_{max}$) (Figs 4G and 5H). Because these correlations deal with contrasts, not tip data, their interpretation will be left to the Discussion, in which the nature of these relationships is inferred based in part on comparable tip data analysis.

Walking effects

A series of phylogenetic ANOVA of walking showed a significant effect of walking on anatomical ($F_{4,10}=4.0$, $P=0.01399$) and locomotor ($F_{2,11}=4.6$, $P=0.01299$) variables, with no effect on SM ($F_{4,10}=1.4$, $P=0.1658$) or PL ($F_{4,10}=2.3$, $P=0.05894$) properties. Within anatomical and locomotion variables, *post hoc* effects tests showed walkers are have elevated values of **Leg Size** ($P=0.03922$) and **Jump Performance** ($P=0.008139$) compared with non-walkers. However, many salient variables (e.g. relative leg length, relative muscle mass, peak jump power) load negatively on these axes; thus, when translated into raw variables, walkers are shown to have shorter and less muscular legs and consequently reduced jump performance compared with non-walkers (see raw variable graphs in Fig. 6).

DISCUSSION

Raw results

Initial data collection yielded a substantial raw dataset, with potential use beyond this study, and which has therefore been included in the supplementary information as both means±s.d. (Table S1) and individual data (Table S2). While the locomotor and anatomical diversity of anurans is well known (Zug, 1972, 1978; Emerson, 1988), inspection of the raw muscle data shows a range of muscle contractile properties (Fig. 2; Table S1). For example, V_{max} of the PL varies by a range of over twofold among species (Fig. 2; Table S1). Comparison with similar variables in the only other comparable dataset of properties of homologous muscles collected under standard conditions across a clade (lacertid lizards) (James et al., 2015) reveals a slightly greater variability in anuran muscle properties. However, it remains unknown whether this difference is due to the range of locomotor performance in each group, relative ages of each clade, or other factors.

Another notable aspect of the raw data is the number of species that exhibit high peak mechanical power per kg muscle mass during jumping (Table S1). Several species of frog are known to use power amplification, storing muscular energy early in the jump by stretching elastic tendons then rapidly releasing this energy to achieve power outputs beyond the limits of muscular isotonic contraction (Peplowski and Marsh, 1997; Astley and Roberts, 2012, 2014; Astley et al., 2013). To test for power amplification, I compared peak jump power to maximum isotonic muscular power using the following equation:

$$\text{Power amplification} = \frac{\dot{W}_{\text{jump}}}{((\dot{W}_{\text{max,PL}} \times R_{\text{PL}}) + (\dot{W}_{\text{max,SM}} \times (1 - R_{\text{PL}}))),} \quad (1)$$

where $\dot{W}_{\text{jump}}$ is peak instantaneous power during the jump divided by total frog muscle mass, R_{PL} is the mass of the PL muscle (known to be used in frog power amplification; Astley and Roberts, 2012) divided by the total leg muscle mass (equal to the variable 'PL proportion of leg muscle mass' in Table 2), and $\dot{W}_{\text{max,SM}}$ and $\dot{W}_{\text{max,PL}}$ are peak isotonic muscle power output per kg muscle mass of the SM and PL, respectively. Power amplification values over one require elastic energy storage, though power amplification may still occur during submaximal jumps (Astley and Roberts, 2012). These data show that seven species produce jump power substantially above peak muscular power output of representative muscles, and therefore likely use power amplification (Fig. 7A). Furthermore, several other species show values near one, including a known power-amplifier (*Lithobates pipiens*; Astley and Roberts, 2012),
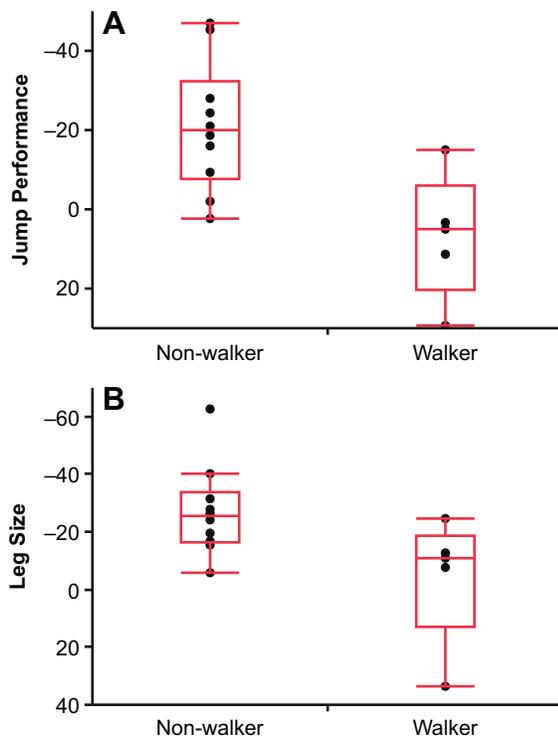
Fig. 6. Effects of walking in frogs (raw data). **Jump Performance** and **Leg Size** axes have been vertically flipped to reflect the negative loadings of key variables in the phylogenetic principal component analyses (pPCA), such that now higher vertical positions in each graph indicate more powerful jumps and longer, more muscular legs. (A) **Jump Performance** of walkers versus non-walkers. The zero value is from the tiger salamander (*Ambystoma tigrinum*). (B) **Leg Size** of walkers versus non-walkers.
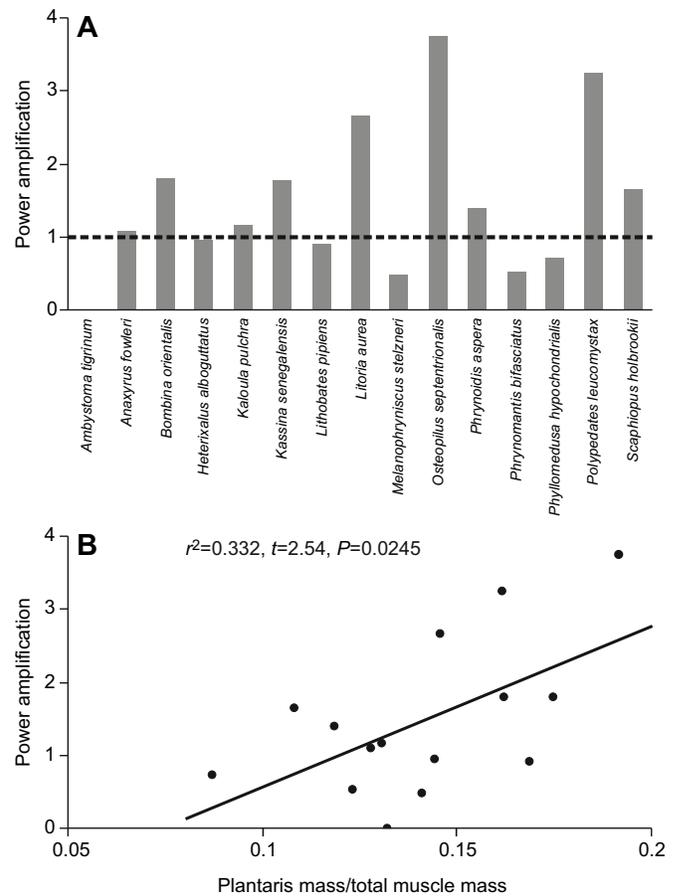

Fig. 7. Power amplification in frogs. (A) Power amplification values of the maximum jump recorded for each species, calculated according to Eqn 1. Values greater than one indicate power amplification must be occurring, though it may also occur in sub-maximal jumps. (B) Power amplification versus relative PL muscle mass (tip values, not contrasts). Increased relative PL muscle mass was significantly associated with increased power amplification.

which could be due to error in either estimated muscle power output or jumping power, though the latter is likely to be an underestimate without extremely high numbers of trials (Astley et al., 2013). However, these species may warrant further scrutiny, and, if confirmed, power amplification may be widespread within anurans, possibly even representing the ancestral state (Fig. 7A).

Evolutionary correlations among related variables

Initial use of pPCAs to reduce variables while retaining data showed a variety of interesting correlations between variables in related groups (i.e. anatomy, performance, SM properties, PL properties). Some of these correlations were expected or even necessary, such as the strong loading of peak jump force, takeoff velocity, jump energy and peak jump power (Table 1). These variables are tightly linked by the physics of jumping (Marsh, 1994), and thus the successful recovery of this association provides some level of confidence in these analyses. Similarly, the first principal component of both muscle pPCAs showed heavy loadings of all three variables associated with the *F/V* curve as well as rates of force development during both twitch and tetanic activation (Tables 3 and 4), all properties that are influenced strongly by acto-myosin crossbridge kinetic rates (Bárány, 1967; Stevens and Renaud, 1985; Rome et al., 1999). Additionally, the first axis of the anatomical pPCA shows strong correlation in a number of morphological traits across anurans (Table 1), supporting the repeated convergence of anurans onto a specialized morphology for jumping characterized by larger absolute size, reduced viscera mass, PL muscles with shorter,

more pennate fibers, and longer, more muscular legs, mirroring and expanding prior findings in an explicitly phylogenetic context (Zug, 1972; Emerson, 1988).

In some cases, the lack of expected correlations was notable and informative. While frogs with high jump performance use different swimming kinematics, the minimal loading of average swim velocity on the **Jump Performance** axis (and the inverse situation on the **Swim Performance** axis) indicates that there is no tradeoff between swimming and jumping among frog species (Table 1), as seen within species (Nauwelaerts et al., 2007). Similarly, the low loading of tetanic relaxation time on the first principal component of both muscles suggests that it is influenced by different molecular kinetics, such as calcium sequestration and reuptake by the sarcoplasmic reticulum (Tables 3 and 4). It also suggests that differences between muscular properties among frog species are not simply a matter of differing ratios of fiber types, or all of the variables would load highly on a single axis; the weak or modest relationships between fatigue and rates of force rise in both muscles particularly underscore this (Tables 3 and 4).

Walking

Walking has evolved numerous times independently within anurans as a common method of terrestrial locomotion (Emerson, 1979, 1988), though this study shows that these frogs can indeed still jump

and swim effectively (Fig. 6A; Table S1). The reduced **Leg Size** may be a solution to walking effectively with the highly unequal forelimb and himblimb lengths seen in anurans, consequently imposing a tradeoff on **Jump Performance**. However, the reduced **Jump Performance** may also reflect a reduced behavioral reliance on jumping and consequent reduction in the body mass invested in long, muscular legs. These two hypotheses, though broadly similar, offer differing predictions about the consequences of longer legs on walking: if long legs are a direct impediment to walking, species with longer legs should suffer reduced performance (e.g. speed, metabolic cost, maneuverability, etc.), but if reduced leg size is merely a case of economizing body mass, no such penalties will be apparent. Testing these hypotheses requires a greater diversity of walking frog morphologies than present in this dataset and a greater understanding of the mechanics of anuran walking; thus, I must leave these questions to future studies.

Locomotor performance versus anatomy and muscle physiology

Jump Performance was strongly affected by **Leg Size** (Fig. 3A), as expected based on both prior studies (Zug, 1972; Emerson, 1988) and the physics of jumping (Marsh, 1994). In addition, **Jump Performance** was linked to the relative mass of the PL (Fig. 3C), a muscle with a prominent tendon which is involved in elastic energy storage and release (Roberts and Marsh, 2003; Astley and Roberts, 2012, 2014). *Post hoc* testing revealed a significant increase of observed power amplification with increased relative mass of the PL (Fig. 7B), consistent with power-amplifying frogs dedicating a larger portion of their muscle mass to the elastic system. Interestingly, **Swim Performance** also displayed a positive relationship with relative mass of the PL [Fig. 3G – appears negative as a result of negative loading on the **PL Size** PCA axis (Table 2)], in spite of the minimal benefits to elastic energy storage during frog swimming (Richards and Sawicki, 2012). This may be due instead to the need for a strong ankle extensor moment, either to generate propulsive foot rotations (as in *Xenopus*) or to balance hydrodynamic forces during foot translation (as in *Lithobates* and *Anaxyrus*) (Richards, 2010).

As with anatomical variables, **Jump Performance** seemed to have the strongest association with muscular variables, particularly the first principal components, which showed high loadings of raw variables associated with muscle speed (e.g. V_{max}, time to peak tetanus; Figs 4A and 5A, Tables 3 and 4). Powerful jumpers had significantly faster contractile properties of both the SM and PL muscles, as well as reduced fatigue resistance and increased relaxation time in the PL (Figs 4A and 5AB, Tables 3 and 4). In contrast, increased **Swim Performance** was only associated with decreased SM tetanic relaxation time and a steeper PL *F/V* curve (Figs 4G and 5H, Tables 3 and 4); relaxation kinetics have been implicated as a limiting factor in high-speed cyclical motion previously (Marsh, 1988; Johnson et al., 1993; Swoap et al., 1993). These results suggest that muscle contractile properties not only are labile on evolutionary timescales but also do indeed evolve in concert with morphology and performance.

This pattern of results suggests that jumping is the primary driver of changes in both anatomy and muscle physiology in frogs, with swim velocity showing correlation to considerably fewer changes. Perhaps the species studied suffer greater predation pressure while in terrestrial habitats, or are more reliant on escape behavior than crypsis on land versus water, respectively. Alternatively, perhaps the benefits of improved swimming velocity are limited by hydrodynamic drag, which rises with the square of velocity and

thereby imposes escalating costs for increased performance. However, I would be remiss to omit the possibility that this difference is an artefact of the dataset, due to the lack of data from highly aquatic genera such as *Xenopus*, *Lepidobatrachus* and *Calyptocephalella*. Additionally, swimming in frogs may either be almost jump-like, consisting of a powerful thrust phase followed by a long glide (low duty factor, Table 1) or highly cyclic, with retraction starting almost immediately after the completion of thrust phase, and in the latter, work loops may be necessary to truly capture the muscle properties in an appropriately integrative fashion.

More generally, the significant and strong association between jumping and both SM and PL properties shows that muscle properties can evolve to match locomotor demands, rather than being broadly conserved within a clade. While this dataset does not have a sufficient sample size to recover an accurate measure of phylogenetic signal (Münkemüller et al., 2012), the detection of functional signal at least shows that frogs are not entirely jumping with their ancestor's muscles. Examination of raw values (Table S1) shows the tremendous range of variation in muscle properties within clades such as Bufonidae and Hylidae, further suggesting that phylogenetic signal may be limited. Evolutionary lability of muscle contractile properties opens new avenues for animal adaptation, allowing organisms to increase muscle power output without increasing muscle mass, and conversely serves as a cautionary note about inferring performance based on sister taxa.

While many of the same muscular variables in both the SM and PL correlate with **Jump Performance** (Tables 3 and 4, Figs 4A and 5A), it remains unknown whether this reflects a simple commonality of function or a more global change in muscle properties across the organism. If the latter is the case, the evolution of muscle properties may be influenced by other aspects and stages of the frog's life history. All of the anurans in this study call to attract mates, a mechanically demanding muscular task strongly influenced by muscle contractile properties (Marsh, 1999). Furthermore, all of the species in this study have an aquatic larval stage, and larval amphibians often experience strong predation and high mortality (Brockelman, 1969; Morin, 1983; Werner, 1986), which may result in strong selection for locomotor performance at this stage. Selection for fast muscle properties in the tadpole could potentially result in commensurate changes in the adult, though prior studies across individuals have shown no consistency in performance ranking across metamorphosis (Watkins, 1997; Johansson et al., 2010). Future contractile property testing of diverse body muscles and accompanying performance tests across life history stages would be needed to explore this possibility further.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.142315.supplemental

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