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Function and position determine relative proportions of different fiber types in limb muscles of the lizard *Tropidurus psammonastes*

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ABSTRACT

Skeletal muscles can be classified as flexors or extensors according to their function, and as dorsal or ventral according to their position. The latter classification evokes their embryological origin from muscle masses initially divided during limb development, and muscles sharing a given position do not necessarily perform the same function. Here, we compare the relative proportions of different fiber types among six limb muscles in the lizard *Tropidurus psammonastes*. Individual fibers were classified as slow oxidative (SO), fast glycolytic (FG) or fast oxidative-glycolytic (FOG) based on mitochondrial content; muscles were classified according to position and function. Mixed linear models considering one or both effects were compared using likelihood ratio tests. Variation in the proportion of FG and FOG fibers is mainly explained by function (flexor muscles have on average lower proportions of FG and higher proportions of FOG fibers), while variation in SO fibers is better explained by position (they are less abundant in ventral muscles than in those developed from a dorsal muscle mass). Our results clarify the roles of position and function in determining the relative proportions of the various muscle fibers and provide evidence that these factors may differentially affect distinct fiber types.

1. Introduction

Skeletal muscles generate movement, and their metabolic properties, such as fatigue resistance and contraction speeds, determine organismal performance during locomotion. Vertebrate limb muscles can be classified as extensors or flexors according to their function, and these functional groups differ in several aspects (Russell and Bauer, 2008). For example, in mammals the hamstring muscles that flex the knee present relatively longer fiber lengths and smaller physiological cross-sectional areas when compared to the extensor quadriceps muscles (Lieber et al., 1990). Limb muscles can also be grouped based on position, being either dorsal or ventral. This evokes their origin from muscle masses initially divided during limb development, and muscles that share a given position do not necessarily perform the same function. Specifically, in tetrapod embryos the limb muscles primarily separate into dorsal and ventral masses along the skeletal core of the appendage (Romer, 1942; Dilkes et al., 2012), assuming a positional dichotomy that is less apparent in adult limbs (Dilkes et al., 2012). The ventral mass in the adult is often involved in the locomotor thrust through plantar flexion of the pes and in the production and control of complex movements of the pes during locomotion (Arnold, 1998; Russell and Bauer, 2008), whereas the dorsal mass usually plays a rather passive role and is essentially active in the swing phase of the limb stroke (Russell and Bauer, 2008). This trend, however, is not a rule (Russell and Bauer, 2008), and the relative position assumed during development (i.e., dorsal or ventral) does not necessarily imply which function the muscle will perform in the adult (i.e., extending or flexing a leg). An emerging question is therefore what determines tissue properties of different limb muscles – position, function, or both.

The properties of a given muscle are defined by its composition. The vertebrate skeletal muscle contains three types of fiber (Peter et al., 1972; Gleeson et al., 1980), and their relative proportions determine the muscle's overall profile. Slow oxidative (SO) fibers resist fatigue and use mainly energy from oxidative reactions, presenting higher levels of the mitochondrial enzyme nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) in comparison with the other two fiber types (Navas et al., 2004). Fast-glycolytic (FG) fibers cannot sustain activity for long periods because they use mainly energy derived from anaerobic pathways (and thus have lower mitochondrial content of NADH-TR), while

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the fibers named FOG (fast oxidative-glycolytic) exhibit intermediate properties (Hochachka and Somero, 1984; Hochachka, 1994). These fiber types also differ in contraction speeds and forces generated. While FG fibers usually contract quickly and produce high forces, oxidative fibers have low contraction speeds and produce low force and power (Rome et al., 1988; James et al., 1995); fibers with medium levels of mitochondrial enzyme are intermediate both in contraction speeds and power (Bonine, 2007; Scales et al., 2009). Due to their functional differences, variation in the relative proportions of different fiber types is likely relevant to the kind of activity performed by different muscles.

Variation in the relative proportions of different fiber types has been reported in several vertebrate lineages, and its association with muscle function and locomotor performance has been particularly well described in lizards (Putnam et al., 1980; Mirwald and Perry, 1991; Reilly, 1995; Bonine et al., 2001, 2005; Higham and Jayne, 2004; Kohlsdorf et al., 2004; Scales et al., 2009; Higham et al., 2011; Kohlsdorf and Navas, 2012). There is a considerable body of evidence supporting that muscle fiber type composition belongs to a coadapted suite of traits, along with relative limb and muscle sizes, that affects animals' locomotor abilities (Bonine et al., 2001; Kohlsdorf and Navas, 2012). Accordingly, oxidative fibers probably play a special role for slow and/or constant locomotion, as, for example, used during foraging (Gleeson, 1983; Abe, 2000), whereas fast-twitch fibers are more likely to be related to sprint running during predator escape (Bonine et al., 2005; Scales et al., 2009). This assumption presumes a clear association between relative proportions of different fiber types and the function performed by a given muscle, an idea supported by empirical data for specific vertebrate groups (Armstrong and Laughlin, 1985; Alvarez et al., 2012). Variation in fiber type relative proportions, therefore, has more often been interpreted in relation to function regardless of muscle position, and integration between the two classifications remains deficient.

The combination of functional and positional effects in the processes determining relative proportions of fiber types in different limb muscles may be expected from a mechanistic perspective. Genetic developmental programs involving activator and repressor molecules determine the future metabolic and contractile properties of different skeletal muscles, whereas postnatal muscle remodeling reactivates early developmental programs and changes the profiles of fiber types (Braun and Gautel, 2011). Given that FG and SO fibers originate from different transcriptional processes (see Hagiwara et al., 2007; Niro et al., 2010; Braun and Gautel, 2011), relative proportions of different fiber types in a given muscle may thus also be influenced by muscle position. While dorsal and ventral muscular masses give rise to the extensor and flexor muscles of the limb, respectively (Francis-West et al., 2003; Schoenwolf et al., 2009), there are some muscles (e.g., the m. ileofibularis in the hind limb) that deviate from this trend. An investigation of the variation in relative proportions of fiber types among limb muscles must therefore include models that not only differ between oxidative and glycolytic fibers (e.g., Scales et al., 2009) but also integrate both positional and functional effects. Here we test which classification, if positional (i.e., dorsal versus ventral), functional (i.e., extensor versus flexor), or the combination of both, better explains the variation in fiber type proportions among limb muscles. We predict that statistical models incorporating both position and function will have a better fit for explaining the variation in fiber types among limb muscles.

For the present study, we used *Tropidurus psammonastes*, a sit-and-wait predator living in the sand dunes of the Caatingas, which relies on bursts of fast running when moving along the sand between refuges that are disposed disjointedly in its natural environment (Kohlsdorf et al., 2004). Our aim was to elucidate which factors mostly affect the proportions of different fiber types in the skeletal musculature. For this purpose, the relative proportions of fibers with high mitochondrial content of NADH-TR (SO fibers), with low levels of this mitochondrial enzyme (FG fibers) and with intermediate properties (medium mitochondrial content; FOG) were determined in six limb muscles, which were classified according to position and function.

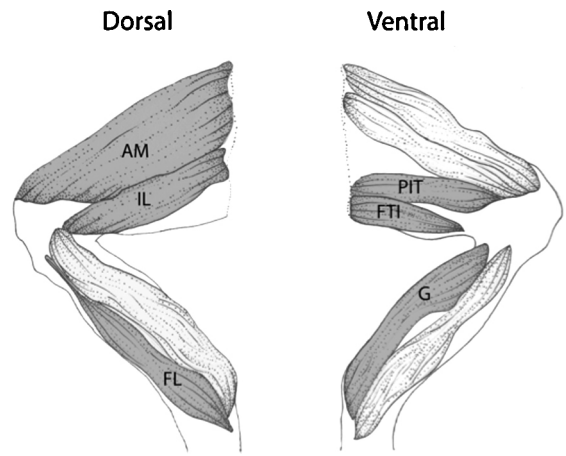

Fig. 1. Illustration of the six limb muscles studied in *Tropidurus psammonastes*. Muscles in dark grey correspond to those analyzed in the present study, muscles in light grey are given only as a spatial reference. *Abbreviations*: AM, ambiens; FL, fibularis longus; FTI, flexor tibialis internus; G, gastrocnemius; IL, ileofibularis; PIT, puboischiotibialis.

2. Materials and methods

Eight specimens of the Brazilian sand lizard *T. psammonastes* (mean snout–vent length [SVL] = 89.0 ± 2.3 mm) were used for the present study. The lizards were collected by noose in the locality of Ibiraba (state of Bahia, Brazil) and transported to the laboratory at Ribeirão Preto (state of São Paulo, Brazil). Permits for capturing and transporting lizards were provided by IBAMA (14109-2/186-2006). All experimental procedures were approved by the Ethics Committee for Animal Research of the University of São Paulo (2006-CEUA/USP).

Proportions of fibers with different mitochondrial contents were determined for different hind limb muscles (illustrated in Fig. 1) using the histochemical techniques detailed in Section 2.1. Data on fiber type proportions were obtained for six muscles: ambiens (AMB), flexor tibialis internus (FTI), fibularis longus (=peroneous longus; FIL), gastrocnemius (GST), ileofibularis (ILF), puboischiotibialis (PIT) (for anatomical location of these muscles see Zaaf et al., 1999).

2.1. Muscle preparations and histochemistry

The lizards were killed with an overdose of anesthetic (xylazine/ketamine 1:2; 400 μl intraperitoneal injection). Right hind limbs were immediately dissected and the entire muscles were removed and dehydrated in commercial talcum, as described in Kohlsdorf et al. (2004). The muscles were then quickly frozen in liquid nitrogen and stored at −80 °C.

Proportions of different fiber types in each muscle were determined using the following histochemical techniques. Serial transverse sections of 10 μm thickness were obtained in a cryostat (Leica CM1850; Leica Biosystems, Nussloch, Germany) at −20 °C and stained with hematoxylin–eosin for morphological examination. Subsequent sections were then stained for nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) (modified protocol by Bancroft and Stevens, 1982) to evaluate oxidative

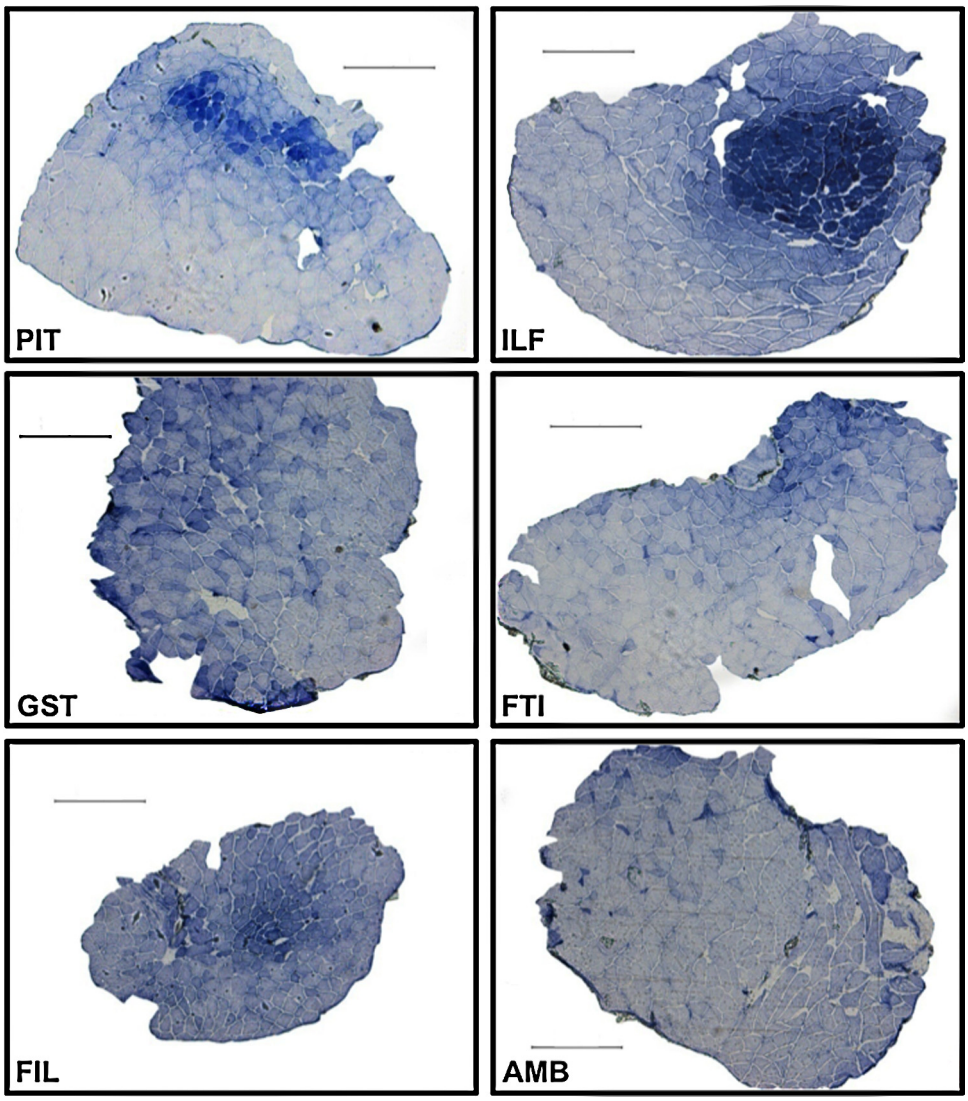

Fig. 2. General profile of the distribution of fiber types in limb muscles from one individual of *Tropidurus psammonastes*. Histological sections of six muscle groups studied (AMB, ambiens; FIL, fibularis longus [peroneous longus]; FTI, flexor tibialis internus; GST, gastrocnemius; ILF, ileofibularis; PIT, puboischiotibialis), stained with the NADH-TR reaction. Dark staining corresponds to fibers with high mitochondrial content (SO), light staining corresponds to fibers with low mitochondrial content (FG), and fibers that stain intermediately are classified as FOG. Scale bars = 500 μm.

activity. NADH-TR is an oxidative enzyme that is present in both the sarcoplasmic reticulum and the mitochondria, and therefore is assumed as indicative of the mitochondrial content of a given muscle fiber. The histochemical reaction used gives a positive (dark) result for oxidative fibers, which are characterized by high levels of mitochondrial enzyme, and a negative (light) result for glycolytic fibers, which have low mitochondrial content of NADH-TR; fibers of mixed metabolic characteristics exhibit intermediate coloration in this staining (Navas et al., 2004), as illustrated in Fig. 2. Based on the staining described, fibers in each section were classified as SO fibers ('slow oxidative', with high mitochondrial content), FG fibers ('fast glycolytic', with low mitochondrial content), and FOG ('fast oxidative-glycolytic', with intermediate metabolic properties). In the literature, NADH-TR staining is often used to classify fibers because it is complementary to the staining obtained from ATPase reactions (e.g., Kohlsdorf et al., 2004; Navas et al., 2004). This complementarity is explained by the characteristic of oxidative fibers to sustain activity aerobically contracting slower than glycolytic fibers, which contract fast but use mainly glycolytic pathways and fatigue quickly.

2.2. Data analysis

Digital images from the histological sections were obtained using a Leica DFC300FX light microscope (Leica Microsystems, Wetzlar, Germany) connected to a computer through a video camera, using the software program Leica QWin version 3.0 for PC (Leica Microsystems, Heerbrugg, Switzerland). One representative section of each muscle for each individual was photographed and all fibers were classified following the Bancroft and Stevens (1982) classification described above. The entire section was quantified and percentages of each fiber type (FG, SO and FOG) were calculated.

Fiber type data have to be treated carefully for statistics, as the three groups (percentages of FG, SO and FOG) are complementary and therefore non-independent. In order to eliminate problems associated with the non-independence of fiber type proportions in statistical analyses, we adopted the transformation proposed by Scales et al. (2009). According to their approach, the proportion of SO fibers was calculated as SO/(SO + FG + FOG), the proportion of FG fibers was calculated as FG/(FG + FOG), and then the

Table 1
Results of tests of mixed linear models, testing for effects of position (dorsal vs. ventral), function (extensor vs. flexor), and the sum of both on the relative proportions of fiber types (FG = fast glycolytic, SO = slow oxidative, FOG = fast oxidative-glycolytic). Individual was considered a random factor. The simple models (M1 and M2) had 39 degrees of freedom; the complex model (M3) had 38 degrees of freedom. Values in bold indicate associations statistically significant in models that had also a significantly better fit in the likelihood ratio tests (results synthetized in Table 2).

Model	*p*	*F*	AIC	logLik
(M1) FG ~ position	0.551	0.363	84.029	−38.015
(M2) FG ~ function	**0.029**	5.118	79.393	−35.697
(M3) FG ~ position + function	0.176/**0.027**	1.901/5.260	79.415	−34.708
(M1) SO ~ position	0.259	1.312	120.475	−56.238
(M2) SO ~ function	0.141	2.263	119.521	−55.760
(M3) SO ~ position + function	**0.001**/0.098	12.560/2.882	109.881	−49.941
(M1) FOG ~ position	0.150	2.153	62.212	−27.106
(M2) FOG ~ function	**0.045**	4.283	60.161	−26.080
(M3) FOG ~ position + function	0.953/**0.048**	0.004/4.190	62.157	−26.079

relative proportions of the three fiber types were logit transformed: $\text{logit}_p = \ln[p/(1-p)]$.

In the present study, we tested whether the variation in the proportion of different fiber types among limb muscles is mainly explained by the function of muscle groups (i.e., flexor vs. extensor), their position (i.e., dorsal vs. ventral), or a combination of both. The six muscles studied were grouped using two classifications (see Fig. 3), one based on their functional roles (extensor = AMB, FIL; flexor = FTI, GST, ILF, PIT) and the other one referring to their position (dorsal = AMB, ILF, FIL; ventral = FTI, PIT and GST). The muscle gastrocnemius (GST) has been ambiguously classified in tetrapods depending on the organism, so we adopted the classification proposed by Russell (Russell, 1993; Russell and Bauer, 2008) for Lepidosauria.

All the statistical analyses were performed in R (version 2.15.2). Using the package 'nlme' in R (implemented in RStudio version 0.97.551), we tested mixed models in which 'fiber type proportion' was a dependent variable, 'function' (extensor or flexor) and 'position' (dorsal or ventral) were fixed factors, and 'individuals' was a random factor. Given the faultiness of evaluating only *p*-values when testing mixed models (see Bolker et al., 2009), we also performed likelihood ratio tests (LRTs) to compare model fits between nested models, specifically to compare the likelihoods of the complex model (M3, function + position) with those of each one of the simple models (M1 and M2).

3. Results

Analysis of *T. psammonastes* limb muscles according to function (i.e. flexor or extensor) or position (i.e. dorsal or ventral) suggests that variation in the proportions of fibers with low or medium mitochondrial content (FG and FOG) is mainly explained by function rather than position (Table 1). The m. ambiens is largely responsible for the high proportions of FG fibers associated to extensor muscles (Fig. 3). In contrast, variation in the proportion of oxidative fibers (SO) is better explained by position, but only in model M3 that also incorporates function (Table 1).

Comparisons of model fit between nested models confirmed these results especially for fibers with low (FG) or high (SO) mitochondrial content. LRTs for FOG fibers suggest that model fit does not significantly differ between the complex model M3 and those with a single factor (Table 2). Variation in the proportion of FG fibers is explained by the function a muscle exerts. Model fit for these fibers is equivalent between M2 (which includes function only) and the complex model M3 with two factors (position and function, although position per se is not significant in this model; see Table 1), but both M2 and M3 fit significantly better than M1 which incorporates position only. In contrast, variation in the relative proportions of oxidative fibers (SO) in limb muscles is explained by position, and the complex model M3 that also incorporates function (although function per se is not significant in this model; see Table 1) has a considerably higher likelihood than the two simple models (Table 2).

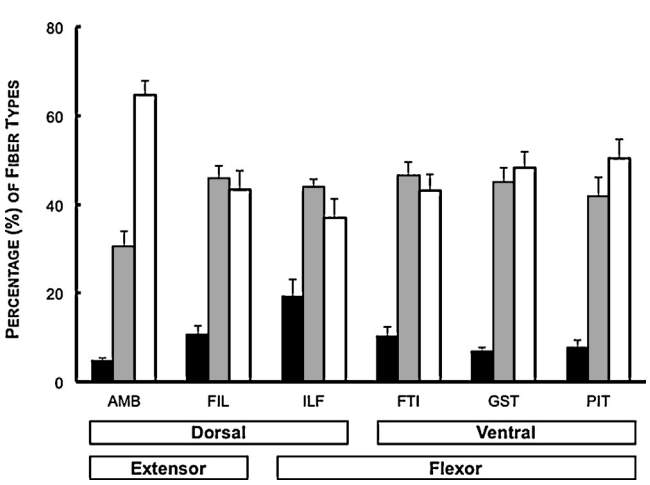

Fig. 3. Percentage of SO (black), FG (white) and FOG (grey) fiber types in the six limb muscles studied in *Tropidurus psammonastes*. Mean values are presented together with the respective standard error.

4. Discussion

Variation in the relative proportions of different muscle fiber types has been widely investigated under specific scenarios involving habitat usage and locomotor performance (e.g., Gleeson and Harrison, 1988; Mutungi, 1989; Young et al., 1990; Mirwald and Perry, 1991; Torella et al., 1998; Bonine et al., 2001, 2005; Kohlsdorf et al., 2004; Cediel et al., 2008; Scales et al., 2009; Alvarez et al., 2012; Kohlsdorf and Navas, 2012). In our present study, we classified muscle fibers according to levels of mitochondrial enzyme

Table 2
Results of likelihood ratio tests (LRT) comparing the fit of nested models (see Table 1 for details) that tested for associations between proportion of fiber types (FG = fast glycolytic, SO = slow oxidative, FOG = fast oxidative-glycolytic) and factor (position, function, or both); both likelihood ratios and the *p*-values of the LRT are presented. Comparisons where p-values were lower than 0.05 are indicated in bold.

Model comparison	Likelihood ratio	*p*-value
FG fibers		
M3 (position + function) × M1 (position)	6.614	**0.010**
M3 (position + function) × M2 (function)	1.978	0.159
SOG fibers		
M3 (position + function) × M1 (position)	12.595	**<0.001**
M3 (position + function) × M2 (function)	11.639	**<0.001**
FOG fibers		
M3 (position + function) × M1 (position)	2.054	0.152
M3 (position + function) × M2 (function)	0.004	0.952

as slow oxidative (SO), fast glycolytic (FG) and fast oxidative-glycolytic (FOG). The complementarity between the reactions staining for metabolic properties (oxidative vs. glycolytic fibers) and those staining for contraction speeds (slow vs. fast contraction; see Kohlsdorf et al., 2004; Navas et al., 2004) supports the prediction that fibers with low mitochondrial content (FG) might contract faster but fatigue sooner than oxidative fibers (SO). The comparison between models for variation in the relative proportions of different fiber types among limb muscles in the Brazilian sand lizard *T. psammonastes* provided evidence that, while the proportions of fast-contraction fibers (FG and FOG) vary according to the function exerted (extending or flexing a limb), variation in the proportion of oxidative fibers (SO) is mainly explained by the muscle position during development (i.e., origin from dorsal or ventral masses; see Romer, 1942; Dilkes et al., 2012). In other words, while the relative proportions of SO fibers seem mostly shaped by developmental processes, the proportions of the other two fibers are mainly determined by functional demands. Our results are novel not only for clarifying the roles of position and function in the specification of relative proportions of different fiber types among limb muscles, but especially for suggesting that these factors may affect in a distinct way the oxidative fibers and those characterized by low or intermediate levels of mitochondrial enzyme.

Fiber type composition of different limb muscles has often been discussed with regard to the use of each muscle during activity, and data comparing different muscles within a single lizard species have been used mainly to test for associations between a muscle's morpho-physiology and its task during locomotion (Mirwald and Perry, 1991; Reilly, 1995; Higham and Jayne, 2004; Higham et al., 2011). This traditional approach has been crucial for setting the conceptual framework necessary for generating hypotheses related to how limb muscles evolved in different organisms and under different selective regimes, as discussed with special attention for the m. ileofibularis (for a broader discussion, see Bonine et al., 2005; Scales et al., 2009). Inter-specific comparisons reveal that the oxidative fibers and those fueled by glycolytic pathways may evolve according to different models, which is consistent with the assumption that selection might act differently on complementary parts of the same functional unit (Scales et al., 2009). Our results suggest, however, that the existing models for the evolution of different fiber types should be expanded so as to include possible effects of muscle position during embryogenesis, which affects in particular the relative proportions of oxidative fibers (see discussion below).

An interesting outcome of our results is the support for differences in the relative proportions of fiber types between extensor and flexor muscles, a pattern which has been described in the literature (e.g., Haines, 1939, 1950; Lieber et al., 1990; Abdala and Moro, 2006; Russell and Bauer, 2008) but has barely been tested using formal statistical approaches. Differences between flexor and extensor muscles reflect architectural correlates but are also physiological (see Asayama, 1916; Liddell and Sherrington, 1925; Fuortes and Hubel, 1956); morphological and physiological variation among muscles likely reflects reaction norms given by interactions between genetic and environmental factors. Muscle functions evolve in the context of a whole-limb performance, and adaptive processes likely favor the overall phenotype instead of the contraction properties of a single muscle in specific ecological settings (Arnold, 1983; Garland and Losos, 1994; Irschick, 2002). However, given that extending a limb is not simply the symmetrical opposite movement of flexing it, function likely plays a special role in determining the differential proportions of FG fibers among muscle groups as reported here. For instance, isolated extensor muscles in mammals usually present both phasic and tonic contraction reflexes to stretch (Liddell and Sherrington, 1925), and their increased proportions of FG fibers likely reflect fast contraction speeds and reduced fatigue resistance.

The present study is one of the first reports sustaining that muscle position (i.e., derivation from a dorsal or a ventral mass) influences the relative proportion of oxidative fibers, whereas the proportions of the fast-contraction fibers (FG and FOG) may be determined only by the function the muscle exerts during limb movement (i.e., extensor or flexor).

Differences in the relative proportions of fiber types among limb muscles seem physiologically relevant for *T. psammonastes*. This sand specialist that rarely jumps exhibits high sprint speeds in comparison with other congenerics (Kohlsdorf et al., 2004; Kohlsdorf and Navas, 2012). Consistently, metabolic scopes are intermediate in this species and the low enzyme activity of citrate synthase recorded in its m. ileofibularis is coupled with a reduced proportion of SO fibers in this muscle when compared with other *Tropidurus* species (Kohlsdorf et al., 2004; Kohlsdorf and Navas, 2012). It is interesting to note that, compared to the other fiber types, SO fibers always represent the lower proportions in limb muscles (Bechtel and Kline, 1987; Bonine et al., 2001; Scales et al., 2009; Alvarez et al., 2012; Kohlsdorf and Navas, 2012). The range of variability in the relative proportions of oxidative fibers seems more restricted than those of the other two fiber types, as relative proportions of SO fibers rarely raise over 15–20% (but see Dearolf et al., 2000); developmental processes seem to play a major role in determining the variation in the proportion of these fibers among the limb muscles of *T. psammonastes*.

Constraints on increasing the amount of oxidative muscle fibers are usually discussed in the context of energetic costs imposed by fibers whose contraction is fueled by aerobic pathways. Metabolic characteristics of animal muscle tissue vary according to the proportions of oxidative and glycolytic fibers and are likely associated with daily locomotor activity patterns (Kernell et al., 1998). Our results suggest that the three fiber types may exhibit different degrees of plasticity, expressed as phenotypic changes triggered by stimuli external to the fiber itself. Our data also corroborate the assumption that oxidative fibers may be more constrained than those with low or intermediate mitochondrial contents. Adult skeletal muscle is plastic in that it responds to environmental and physiological challenges by changing its phenotype in terms of size and composition (Matsakas and Patel, 2009). For example, with chronic immobility, muscles decrease their oxidative capacity through changes in the ratios of oxidative to glycolytic muscle fibers. Muscle plasticity has been discussed in relation to specific parameters, such as training (Gollnick et al., 1973; Howald et al., 1985; Van Damme et al., 2002), injuries (Häggmark et al., 1986), aging (Korhonen et al., 2006), and temperature acclimation (specifically in ectotherms; Johnston and Maitland, 1980; Vieira and Johnston, 1992), but different fiber types are often assumed to have equivalent degrees of plasticity. However, while in adult rodents a fat diet induces an oxidative profile in skeletal muscles, obese humans tend to exhibit higher proportions of FG fibers (Matsakas and Patel, 2009), suggesting that the same environmental signal (in these two examples, diet) may trigger different responses in distinct fiber types. Development of oxidative and glycolytic fibers occurs through genetic processes that are distinct both in embryological and adult muscles (Braun and Gautel, 2011). Such differences may not only explain how muscles can be differentially affected by function and position, but they also shed light on how fiber types might diverge in plasticity, a topic that has to be explicitly incorporated into future discussions about the evolution of fiber type proportions in the skeletal musculature.

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