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Morphological and physiological specialization for digging in amphisbaenians, an ancient lineage of fossorial vertebrates

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Summary

Amphisbaenians are legless reptiles that differ significantly from other vertebrate lineages. Most species dig underground galleries of similar diameter to that of the animal. We studied the muscle physiology and morphological attributes of digging effort in the Brazilian amphisbaenid *Leposternon microcephalum* (Squamata; Amphisbaenia), which burrows by compressing soil against the upper wall of the tunnel by means of upward strokes of the head. The individuals tested (<72 g) exerted forces on the soil of up to 24 N. These forces were possible because the fibres of the longissimus dorsi, the main muscle associated with burrowing, are highly pennated, thus increasing effective muscle cross-sectional area. The muscle is characterized by a metabolic transition along its length: proximal, medial and distal fibres are fast contracting and moderately oxidative, but fibres closer to the head are richer in citrate synthase and more aerobic in nature. Distal fibres, then, might be active mainly at the final step of the compression stroke, which requires more power. For animals greater than a given diameter, the work required to compress soil increases exponentially with body diameter. *Leposternon microcephalum*, and probably some other highly specialized amphisbaenids, are most likely constrained to small diameters and can increase muscle mass and effective muscle cross-sectional area by increasing body length, not body diameter.

Key words: Amphisbaenia, reptile, muscle, digging, *Leposternon microcephalum.*

Introduction

Animals that exploit fossorial microhabitats experience extreme physiological challenges because of the high cost of locomotion associated with burrowing and the paradoxical requirement of sustained activity at presumably low oxygen tensions. These ecophysiological complications, however, have not precluded evolution of a variety of burrowing ectotherm tetrapods. Fossorial ecomorphs are typical of the amphibian order Gymnophiona (Jared et al., 1999) and also characterize several reptilian taxa such as some snakes, dibamids and the whole suborder Amphisbaenia (Lee, 1998). Amphisbaenians stand out among fossorial squamates because of their peculiar habits and early evolutionary origin (Gans, 1977), so that the physiology of extant species might illustrate early steps in the history of vertebrate evolution. The suborder Amphisbaenia exhibits ancient combinations of morphological specializations to a fossorial life that apparently have been conserved since the origin of the suborder, probably before the Cretaceous, about 135 million years ago. Fossil specimens dated 50 million years old are unambiguously identified as amphisbaenians and are extremely similar to extant forms (Gans, 1977).

Amphisbaenians exploit tropical fossorial habitats in America and Africa and are predators of small animals (Gans, 1968). They burrow by compressing the substrate against the walls of the tunnel by means of powerful horizontal, vertical or torsional movements of the head (Gans, 1978). Their ability to sustain burrowing at the low O_2 and high CO_2 concentrations that appear to characterize fossorial microhabitats (McNab, 1966) seems associated with a low rate of O_2 uptake ($\dot{V}_{O_2}$) for reptiles (Abe and Johansen, 1987;

Kamel and Gatten, 1983), significant cutaneous oxygen uptake (Abe and Johansen, 1987), elevated blood oxygen affinity (Johansen et al., 1980), small red blood cells and high haematocrit (Ramirez et al., 1977) and significant amounts of myoglobin, particularly in the muscles associated with digging (Weber et al., 1981). These traits suggest that aerobic metabolism might be relevant to support the efforts associated with production and maintenance of an extensive tunnel matrix and, possibly, with chasing eventual intruders (Gans, 1968). Therefore, amphisbaenian digging muscles must produce forceful, powerful and enduring contractions. Because trade-offs between power output and fatigue resistance have been detected at the whole-muscle (Wilson et al., 2002) and whole-organism (Van Damme et al., 2002) levels, amphisbaenian digging muscles pose intriguing physiological questions. Additionally, as muscle performance is affected by the specific protein isoforms expressed within the fibres (Moss et al., 1995), and the mitochondria and sarcoplasmic reticulum supporting myofibrillar activity (Rome and Lindstedt, 1998), similar trade-offs are also likely to exist at the individual-fibre level.

The purpose of our study is to integrate a diversity of approaches to increase our understanding of the physiology underlying amphisbaenian digging behaviour, to explore the influence of morphology on force production and required effort, and to discuss our findings in terms of animal evolution, ecology, morphology and kinematics. We focus on the Brazilian species *Leposternon microcephalum* [Amphisbaenidae; Fig. 1; see Gans (1971) for a detailed description], a shovel-snouted amphisbaenian that uses only upward strokes of the head to excavate and exhibits a large mass of dorsal musculature associated with upward soil compression strokes. Specifically, we aim to describe the kinematics of head-first digging, measure the maximum digging forces that live animals can produce, describe the metabolic and morphological characteristics of the main muscle associated with digging, analyse the influence of animal diameter on the power output of the digging muscles and evaluate the work requirements of digging for different animal diameters.

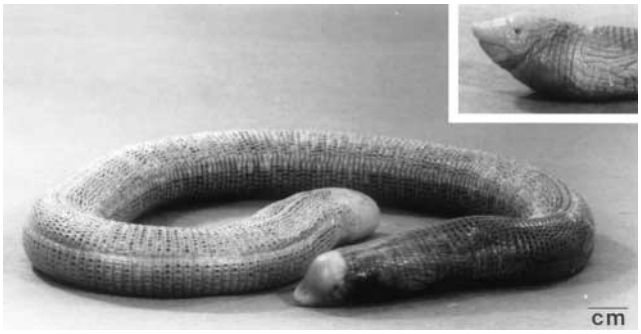

Fig. 1. The amphisbaenian *Leposternon microcephalum*. The inset shows a close-up of the specialized burrowing head. Scale bar, 1 cm.

Table 1. *Morphological parameters of five* Leposternon microcephalum *used in this study*

ID	Body mass (g)	Snout–vent length (mm)	Body length (mm)	Head width (mm)
1	71.0	413	437	10.1
2	49.2	360	383	8.9
3	60.3	387	409	10.0
4	64.4	420	444	11.6
5	41.8	355	372	8.8

The first four individuals are females.

Materials and methods

Study animals

Six individuals of the amphisbaenid *Leposternon microcephalum* Wagler 1824, collected in the state of São Paulo, Brazil, between March and October 2001 (Table 1), were donated by the Laboratory of Herpetology of Instituto Butantan, São Paulo, Brazil to be used in the present study. The small number of animals used in this investigation reflects the enormous difficulty in obtaining live individuals of this species in the field. Animals were kept individually in ventilated plastic boxes containing 10 cm of humus-rich soil that was kept loose and humid. Animals were fed once a week on newborn laboratory mice, minced beef, chicken or fish. The experiments were conducted in April 2002. One animal had to be used for preliminary dissection and pilot muscle physiology tests. The data reported here correspond to the remaining five animals.

Recording of digging activity

Three individuals were filmed in a set-up that consisted of a rectangular glass container partially filled with sifted soil (Fig. 2A), barely wider than the thickest individual. Animals were induced to burrow by tapping the base of their tail, and the digging behaviour was recorded with a Panasonic television camera. The video sequences were later observed and analysed at 60 frames s^{-1} using a time-lapse videocassette recorder system.

Measurement of digging forces

Maximum digging forces for each individual were measured at room temperature (25±1°C) using a custom-built apparatus consisting of a strain gauge (model 1030; UFI, Morro Bay, CA, USA) and a sheet of plastic (Fig. 2B). Animals were induced to push the strain gauge upwards (so as to imitate a soil compression stroke) by gentle squeezing of the posterior end of the animal. The forces produced were calculated from chart recordings of strain gauge voltage output. The strain gauge was calibrated by hanging objects of known weight from it at the same point on the strain gauge that each individual was induced to push against. The maximum diameter of the head of each individual was used to calculate the cross-sectional area of the head region, assuming the head to be a perfect

circle. Each individual performed between five and eight digging movement trials against the strain gauge. The maximum digging force was the peak force imposed on the strain gauge during any one digging movement.

Study muscle

This study focuses on the longissimus dorsi, an extensive dorsal muscle in *L. microcephalum* that starts at the base of the head (proximal part) and runs backwards along the dorsal body of the animal (distal part). This muscle is believed to be homologous to the muscle carrying the same name in snakes, turtles and lizards, although it seems to be less exaggerated in amphisbaenians that use lateral head movements while digging, such as *Amphisbaena alba* (Gans, 1978). The longissimus dorsi is bilateral, with well-defined right and left sides that are separated by a central tendon.

Collection of muscle samples

To collect samples for both muscle fibre and biochemical analyses, animals were placed on a balance to measure body mass to the nearest 0.01 g and then anaesthetized and killed with pentobarbital sodium (50 mg kg^{-1}, intraperitoneally) before dissection. Animals were quickly dissected and the dorsal trunk muscle was photographed. Two samples from each of the proximal, medial and distal parts of the muscle (equal thirds) were quickly removed and frozen immediately in liquid nitrogen and then stored at –85°C until analysis. The time taken between anaesthetic injection and completion of the dissection was always less than 10 min. Samples of the right dorsal muscle were subsequently used for biochemistry, and samples from the left dorsal muscle were used for histochemistry.

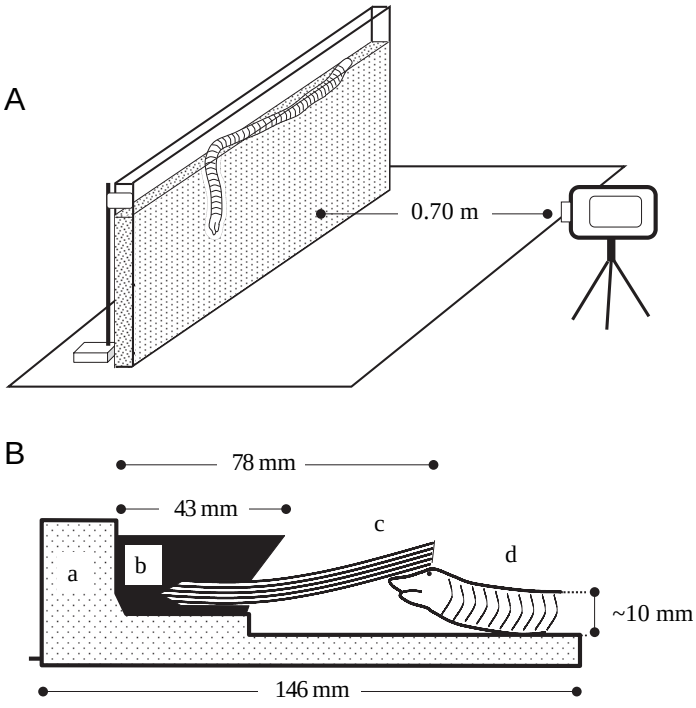

Fig. 2. (A) Diagram of the set-up used to film and photograph digging behaviour in *Leposternon microcephalum*. The glass terrarium used was 0.75×0.60×0.012 m. (B) Diagram of the system used to measure forces produced by *L. microcephalum* during digging: a, purpose-built plastic rig to secure the strain gauge; b, casing for the strain gauge; c, blade assemblage of the strain gauge; d, individual being tested, depicted while 'digging' against the strain gauge.

Muscle histochemistry

Serial transverse sections were obtained by producing 7–9 μm-thick muscle samples using a cryostat at –20°C. Serial sections for each muscle sample were either reacted for haematoxylin-eosin (HE), NADH-TR (nicotinamide adenine dinucleotide tetrazolium reductase) or myofibrillar ATPase. For ATPase reactions, adjacent sections were subjected to either acid (pH 4.5) or alkaline (pH 10.4) pre-incubations, following the procedures detailed in Dubowitz and Brooke (1973). The HE treatment was used to dye muscle tissue to verify section quality and overall fibre morphology. NADH-TR is an oxidative enzyme, present in both the sarcoplasmic reticulum and the mitochondria, that transfers hydrogen to a dark tetrazolium salt. This reaction, therefore, gives a positive result (dark) for oxidative fibres and a negative result (light) for glycolytic fibres but also identifies fibres of mixed metabolic characteristics (intermediate colouration), usually referred to as oxidative-glycolytic. The alkaline myofibrillar ATPase identifies myosin ATPase by the breakdown of ATP and subsequent formation of calcium phosphate and, after treatment with cobalt chlorate and ammonium sulphite, cobalt sulphite (which is dark) is produced. This reaction is considered positive (dark) for fast fibres and negative (light) for slow fibres (opposite results are expected from acid pre-incubation). Thus, it is possible to classify the fibres as oxidative or glycolytic and, regarding general velocity of contraction, as fast or slow. The NADH-TR and myofibrillar ATPase reactions are complementary because slow fibres tend to be highly oxidative and fast fibres tend to be either glycolytic or oxidative-glycolytic.

The results of histochemical reactions were analysed from digital images obtained from laminae using a compound microscope attached to a computerized image analysis system (Stereo Investigator 2000). For each section obtained, all fibres of six non-overlapping and randomly selected fields (at 10× magnification) were typed and quantified, including proportions and area. Fibres were classified as either fast or slow according to the results of the myofibrillar ATPase reaction and as either highly oxidative or moderately oxidative from the NADH-TR reaction (no glycolytic fibres were found). One intact section per reaction was analysed for each muscle region in each of the five individuals.

Muscle biochemistry

Muscle samples were homogenized using a Teflon–glass homogenizer (Marconi Ltd, Piracicaba, SP, Brazil) in ice-cold 20 mmol l^{-1} imidazole (pH 7.4) buffer

with 2 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 20 mmol l^{-1} NaF, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton X-100. The homogenates were then submitted to sonication using a U-200S control unit (IKA-Labor Technik, Staufen, Germany) for three 10 s intervals and directly used in the assays. Measurements were made of the maximal activities of pyruvate kinase (PK) and lactate dehydrogenase (LDH), key enzymes in the glycolytic pathway, and citrate synthase (CS), which plays a major role in the TCA cycle. Measurements were obtained at 25°C with a Beckman DU-70 spectrophotometer (Fullerton, CA, USA), following the changes in the absorbance of nicotinamide adenine dinucleotide reduced form (NADH) at 340 nm or 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, under substrate saturation and in the absence of inhibitory conditions. All reactions were initiated by addition of substrate. Results were expressed in μmol min^{-1} g^{-1} wet muscle mass. Established enzyme protocols were used (Bergmeyer, 1983), with minor modifications as follows:

PK (E.C. 2.7.1.40) – 100 mmol l^{-1} imidazole (pH 7.0), 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 2.5 mmol l^{-1} ADP, 0.02 mmol l^{-1} fructose-1,6-biphosphate ($F_{1,6}P_2$), 0.15 mmol l^{-1} NADH, 12 U ml^{-1} LDH, muscle sample homogenate and 3.6 mmol l^{-1} phospho(enol)pyruvate (omitted for control);

LDH (E.C. 1.1.1.27) – 100 mmol l^{-1} imidazole (pH 7.0), 5 mmol l^{-1} dithiothreitol (DTT), 15 mmol l^{-1} NADH, muscle sample homogenate and 1 mmol l^{-1} pyruvate (omitted for control);

CS (E.C. 4.1.3.7) – 50 mmol l^{-1} Tris (pH 8.0), 0.1 mmol l^{-1} DTNB, 0.2 mmol l^{-1} acetyl-CoA, muscle sample homogenate and 0.9 mmol l^{-1} oxalacetate (omitted for control).

Statistics

Conventional analyses were used after evaluating the suitability of data for parametric approaches. To avoid statistical problems related to the parametric evaluation of ratios, the count of fibres of a given type was analysed in the context of an analysis of covariance (ANCOVA), using total fibre count as a covariate. To test hypotheses regarding differentiation of muscle type along the longitudinal axis of the muscle, fibre count of a given type per muscle region was nested within reaction type (mATPase and $NADH^+$) in an ANCOVA.

Results

Fig. 3 illustrates key aspects of the digging behaviour for *L. microcephalum*. The soil compression stroke starts with the shovelled snout positioned virtually perpendicular to the longitudinal axis of the body (Fig. 3A). A brief and forward shovelling movement of the head then detaches a portion of the substrate in front of the animal (Fig. 3B,C), and an upward movement compresses the soil until the snout approximately parallels the longitudinal body axis (Fig. 3D). The substrate is compacted and attached to the upper wall of the tunnel, allowing an increase in tunnel length. A tunnel dug this way is approximately the same diameter as the animal. The contracting muscles increase the diameter of the animal behind the head and apparently help to secure the body against the tunnel walls, improving the ability to apply force to the substrate. Once a new empty space is available, the animal extends the head to occupy it, fastens the proximal part of the body into the new place and gradually repositions the rest of the body.

Forces developed

L. microcephalum were able to produce forces approaching 24 N during simulated digging trials. Although the sample size was small, the amount of force produced by individuals was clearly and positively correlated with head width (Fig. 4; $P<0.01$).

Overall characteristics of the digging muscle

The longissimus dorsi of *L. microcephalum* connects dorsally to a thick dorsal tendon and ventrally to the rib cage by an array of connective tissue. Its diameter decreases at the distal part and it is composed of symmetrical pennated fibres that originate bilaterally at the dorsal tendon, with a pennation angle that ranges from 20 to 35°, and run diagonally and

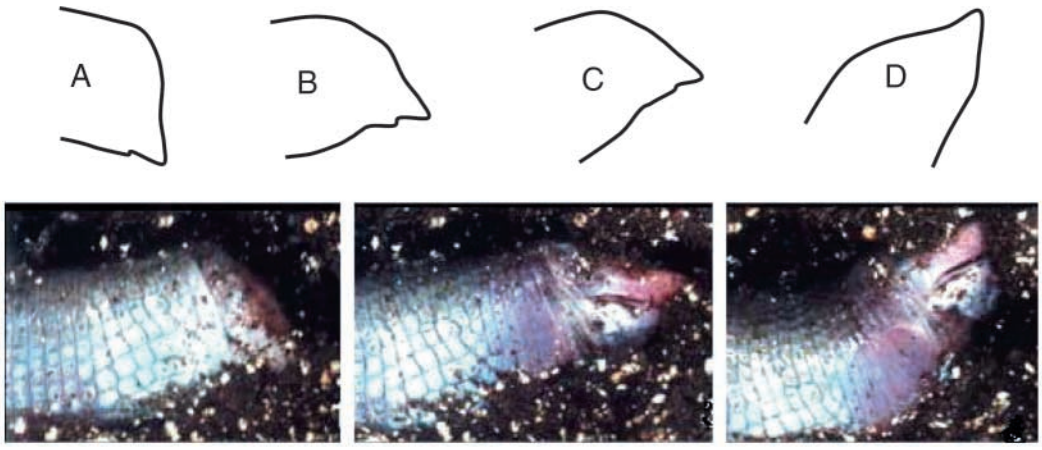

Fig. 3. Sketches A–D, outlined from video and photograph series, illustrate a soil compression stroke in *Leposternon microcephalum*.

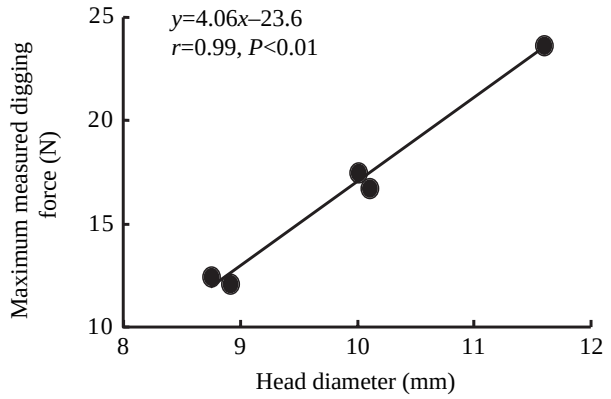

Fig. 4. Maximum forces produced as a function of head diameter in *Leposternon microcephalum*.

backwards to the ribs. Fibres are well organized in fascicles and joined together by a thin layer of loose connective tissue that becomes more dense when proximal to the tendon. The fibres are either polygonal or round and exhibit an acidophilus sarcoplasm and a central or peripheral basophilic nucleus. The muscle of all individuals changed in colour longitudinally, being dark red proximally and becoming lighter distally. Detailed illustrations of amphisbaenian muscle and skeletal morphology can be found elsewhere (Gans, 1973; Gasc, 1981).

Fibre morphological and biochemical profile

Two main fibre types were observed (Fig. 5). The NADH reaction allowed classification of fibres as strongly oxidative or moderately oxidative, whereas the ATPase reaction indicated the presence of both fast and slow fibres. Fibres identified as either fast or moderately oxidative were far more common than alternative types at any muscle section. Fibres identified as either fast or moderately oxidative appeared to be the same and were far more common than alternative types at any muscle section. The counts for both reaction types were similar (ANCOVA, $F_{1,22}$=0.034, P=0.854). The proportion of alternative fibre types (slow or highly oxidative) was somewhat higher in the proximal part than in other parts of the muscle, but this difference was clearly non-significant (nested ANCOVA, $F_{4,22}$=0.647, P=0.635). Despite limited histochemical differentiation, fibres exhibited remarkable morphological and biochemical differences along the longitudinal axes of the dorsal muscle. At the proximal part, fibres exhibited small areas (918±302 μm^2, mean ± S.D.) in comparison with fibres at the distal part (1606±491 μm^2; $F_{1,997}$=713, P<0.001; see Fig. 5). Despite the inability of the histochemical approach to identify differences in the metabolic profile of fibres along the longitudinal axis of the digging muscle, proximal fibres had a distinct biochemical profile. Proximal fibres were characterized by a significantly higher activity of CS and a significantly lower activity of PK, in comparison with either medial or distal parts (Fig. 6). The activity of LDH did not differ among muscle sections despite much greater absolute values in comparison with other enzymes.

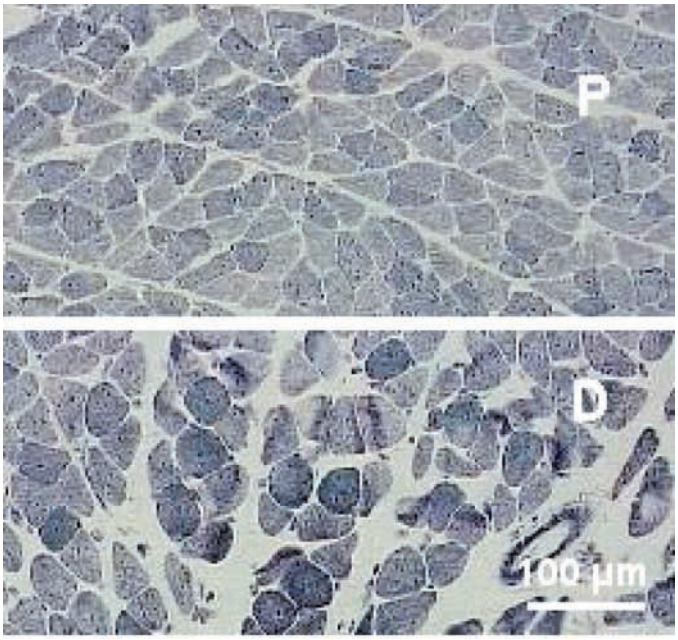

Fig. 5. Typical fibre composition in proximal (P) and distal (D) muscle sections stained with the NADH-TR reaction.

Implications of a model of work during head-first digging

Amphisbaenians must compress portions of substrate to an exiguous thickness to open and increase the length of a tunnel of similar diameter to that of the body. We developed a mechanical model (see details in the Appendix) that addresses changes in the amount of work (w_1) required to compress substrate in head-first diggers as their diameter changes. Our

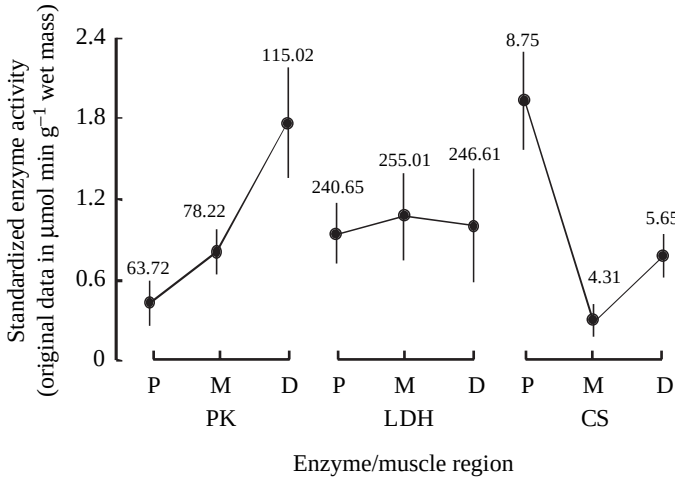

Fig. 6. Metabolic differentiation along the longitudinal axis in the digging muscle of *Leposternon microcephalum*. The vertical axes present standardized values to facilitate comparisons of enzyme ratios. The numbers above the symbols are actual measured values. P, proximal; M, medial; D, distal (P≠D for PK and CS, also P≠M for CS; ANOVA P<0.05).

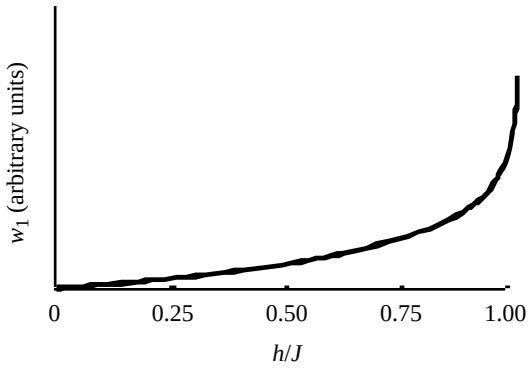

Fig. 7. Tendency of increase in w_1 as the ratio h/J increases (details in text and Appendix).

analysis leads to the following proportionality, in which k_0 is a constant of compressibility of granular soils, h is the diameter of the animal, and J is a linear measure related to the minimum thickness of a given type of substrate when it is compressed:

$$w_1 \xleftrightarrow{\text{proportional}} -k_0 \ln\left(1 - \frac{h}{J}\right). \quad (1)$$

The ratio h/J indicates how thick the layer J would be when expressed as a fraction of the diameter of an animal. The relationship between w_1 and h/J, then, addresses the amount of work required by an amphisbaenian of a given size to open a tunnel of body diameter by compressing detached substrate to an insubstantial thickness. The parameter J will increase with animal diameter because the amount of substrate to be compressed will also increase, and higher forces will be required for the head upstroke while digging. The key point is that the work required for compression increases exponentially after a given animal diameter (h), thus imposing great physical challenges to animals of large diameters (Fig. 7).

Discussion

The purpose of this work was to study the kinematics and physiology of digging in amphisbaenians to better understand muscle performance in tubular, head-first digging organisms. We addressed this issue from various perspectives, including animal morphometrics, muscle morphology and muscle metabolic characteristics. For morphometric analyses, both our empirical data (Fig. 4) and the morphometric model suggest that an allometric component affects digging forces. A minimum cross-sectional area is required to allow the absolute forces required for digging, and larger animals produce larger forces. As animal diameter increases (ontogenetically or evolutionarily), however, the pressure exerted by unit of cross-sectional area does not increase proportionally. Consequently, the cost of digging increases and the force enhancement due to a larger diameter would be insufficient to match the concomitant increase in the cost of digging. Head-first diggers, then, experience evolutionary constraints on body diameter, and a greater total muscle mass is possible only by increasing body length. The conclusions of our model and data are compatible with the observation that the largest known amphisbaenians, living or fossil, are only ~3 cm in diameter (Gans, 1977) and that the most specialized burrowing species exhibit small diameters and increased elongation of the body (Gans, 1968), two traits that must reduce relative effort during digging. The largest amphisbaenids, indeed, are pigmented dorsally and are also active on the forest floor surface, two traits that suggest less specialization to a fossorial life.

Various aspects of muscle and fibre morphology enhance force and work production in the longissimus dorsi of *L. microcephalum*. First, pennation increases the effective cross-sectional area of the muscle, hence increasing force production achieved at a given whole-muscle shortening velocity as muscle fibre velocity decreases as a proportion of whole-muscle velocity (for a review, see Lieber and Fridén, 2000). Second, the curvilinear shape of the muscle probably allows for the elongation of fibres diagonally and distally, following the tubular shape of the body. Fibre elongation would also result in increased forces (see fig. 9b in Lieber and Fridén, 2000). Third, an annular fibre distribution will result, during contraction, in increased radial coelomic pressures in the body and in proximal body stiffening; two events that grant appropriate dispersion of reaction forces through the body of the animal during a soil-compression stroke.

According to the histochemical tests conducted, the longissimus dorsi of *L. microcephalum* mainly exhibits fibres that are both fast and moderately oxidative, and fibre type proportion remains fairly constant along the longitudinal axis. This finding contrasts with the distinct change in biochemical profile derived from the enzymatic analysis and suggests that metabolic differentiation occurs within the dominant type of fibres. It is also possible, however, that improved histochemical tools for amphisbaenian muscle might, in the future, allow for higher resolution of fibre typing. The dominance of moderately oxidative fibres is ecologically relevant, because strictly glycolytic fibres, despite high power production, are probably inappropriate for sustained digging as they are prone to fatigue. Strictly aerobic fibres, on the other hand, would enhance sustained activity but would be comparatively less powerful and would be more sensitive to oxygen stress, an ecological trait that might affect some amphisbaenians (oxygen availability has never been measured in amphisbaenian underground environments). The fibres identified as fast and moderately oxidative are usually characterized by a relatively high capacity for both oxidative and glycolytic carbohydrate usage (Hochachka and Somero, 2002); their presence apparently reflects a compromise in the functional properties of the muscle and allows for the production of sustained and significant forces in various behavioural and ecological contexts related to a fossorial life.

The biochemical analysis allows for interesting insights regarding metabolic specialization in the longissimus dorsi of *L. microcephalum*. First, this muscle exhibits low activity of LDH in comparison with truly specialized glycolytic muscles in ectotherms (for example: Baldwin et al., 1995; Bennett and

Dawson, 1972; Mendiola et al., 1991; Miller et al., 1993; Somero and Childress, 1980). Indeed, the glycolytic capacity of the longissimus dorsi of *L. microcephalum* is somewhat lower than that of the white portion of leg muscles associated with locomotion in some lizards (Bennett and Dawson, 1972; Garland et al., 1987; Gleeson, 1983), snakes (Wilkinson and Nemeth, 1989) and crocodiles (Baldwin et al., 1995). However, in the present study, PK/LDH ratios ranged from 0.27 (proximal) to 0.55 (distal), values that suggest relatively high glycolytic fluxes and a high capacity to incorporate carbohydrates into oxidative pathways (Hochachka et al., 1983). Although this muscle does not classify as highly aerobic, CS activity in the proximal part is high in comparison with that of the skeletal muscles of other reptiles (Bennett and Dawson, 1972; Garland et al., 1987; Gleeson, 1983). Additionally, the CS/LDH ratio, an estimator of the reliance of muscle on aerobic pathways (Hochachka et al., 1983), changes from 0.023 (distal) to 0.036 (proximal); the latter value is comparable with that of ectotherm aerobic muscles, such as the red muscle of tuna fish (~0.04, Guppy et al., 1979) or the vastus lateralis of high elevation humans (~0.04; Hochachka et al., 1983; Kayser et al., 1996). The oxidative capacity of the muscle seems quite enhanced at the proximal region, which is also characterized by an apparent increase in myoglobin concentration, increase in fibre density and decrease in fibre diameter. A smaller fibre area probably allows for more intricate capillary network, facilitates oxygen diffusion and enhances fine-tuned movements of the head.

The unambiguous trend towards improved aerobic capacity at the proximal part of the longissimus dorsi of *Leposternon microcephalum*, coupled with apparently enhanced oxygen transportation and storage, strongly suggests functional differentiation of the muscle along the longitudinal axes. The functional significance of the muscle's metabolic heterogeneity is probably related to differential participation of parts of the muscle in the head soil-compression stroke. Our model suggests that, given the elastic properties of the substrate, increased contraction time, higher endurance and less power are required along most of the soil-compression stroke. By contrast, short-term and high-power contractions are required for final soil compression. So, one interpretation is that proximal fibres, more aerobic and able to withstand fatigue, are particularly relevant for initial soil compression, whereas distal fibres, more glycolytic in nature, are recruited to compensate for the higher power requirement of final soil compression. Additionally, the relative importance of different parts of the muscle, and the probability of recruitment, might vary as the animal moves through different substrates. The fibres further back in the muscle might gain importance when substrate viscosity increases. Additionally, proximal fibres are probably used more often and in a more diverse array of behavioural contexts, as amphisbaenians use their heads for prey capture and territorial defence.

In conclusion, to build underground galleries, the digging muscles of *Leposternon microcephalum* must be designed to produce forceful, powerful and repeated contractions of the head. These functional requirements pose problems that might be further complicated by the unsuitability of highly aerobic physiological solutions due to power requirement and perhaps oxygen stress. Similar problems are probably experienced by head-first diggers in other taxa. Digging muscles are restricted to a tubular body shape, and force production cannot be indefinitely enhanced by increasing muscle (= body) diameter because the work required for digging also increases with animal diameter, and at a non-linear rate. For the species studied, the solutions to these problems have been: (1) maintenance of small body diameters and increased muscle mass by elongation of the body; (2) enhancement of force production through intense muscle fibre pennation and fibre elongation around the body axis; additionally, a pennate muscle design is probably important to allow for partial contraction of the muscle, otherwise the inactive part would be stretched by the active part, decreasing the magnitude of the external forces produced; (3) reliance on fast and moderately oxidative fibres, able to use both aerobic and oxygen-independent pathways, and that apparently enable animals to modulate carbohydrate metabolism according to oxygen availability and energetic demands; and (4) muscular metabolic heterogeneity along the longitudinal axes. Muscular metabolic heterogeneity is quite unusual in ectotherm vertebrates and is possibly a finely tuned mechanism to maintain energy homeostasis during digging, itself a heterogeneous process in terms of force requirements. Regarding biochemistry, the digging muscles of this and perhaps other amphisbaenian species do not depart from what has been observed in other squamate skeletal muscles. However, our integrative study indicates that the exploitation of a highly specialized habitat in amphisbaenids is based on a complex set of morphological and physiological muscle traits that is probably very ancient in the lineage of vertebrate evolution.

Appendix. A model of work during head-first digging

The main assumptions in this model are (1) digging amphisbaenians do not remove soil and (2) the tunnel opened is equal in diameter to the animal (h, where h is the mean diameter of the animal body). Our analysis starts by noticing that along the soil compression stroke, the head movement comprises an arc of 90° or $\pi/2$ rad. The volume V of detached soil is proportional to the volume of a doughnut-shaped figure (torus) generated by the head of the animal during an upstroke (the head is assumed to behave as a rotating circle of radius h). Under this general scenario:

$$V = \int_{h_0}^{h} k_1 h \, \mathrm{d}h = k_1 h^2 , \qquad \text{(A1)}$$

where k_1 is a constant specific to the cross-sectional area of the torus, and h_0 is the initial point of the head before the upper stroke movement. Notice that we integrate the mean diameter of the animal in h, because this is the distance covered by the

head (see assumption 2 above). Now, given that granular soils behave similarly to compressible fluids (Imhoff et al., 2000; O'Sullivan and Simota, 1995), the product pV is a constant and receives the name constant of compressibility of the soil, k_0. Then:

$$p = \frac{k_0}{V} = \frac{k_0}{k_1}\, h^{-2}, \qquad \text{(A2)}$$

where the second equality comes from V in equation A1.

The minimum estimate of the work component related to the soil compression w_1 is defined by the first law of thermodynamics, considering no heat exchange. Then:

$$\mathrm{d}w_1 = -p\mathrm{d}V \Leftrightarrow w_1 = -\int p\mathrm{d}V\,. \qquad \text{(A3)}$$

The minus sign in the above equation indicates that work is applied to the volume. From equation A2, and using the technique of change of variables to integrate equation A3 in h (instead of in V), we obtain:

$$w_1 = -\int_{h_0}^{h} \frac{k_0}{k_1}\, h^{-2} 2k_1 h\, \mathrm{d}h = -2k_0 \ln\left(\frac{h}{h_0}\right). \qquad \text{(A4)}$$

Here, h_0 and h are, respectively, linear dimensions related to the volume of the substrate before and after compression, for which we have no information. However, if we assume a linear magnitude J to be related to the maximum possible compression applicable to an amount of a given substrate, then the maximum volume displaced would be proportional to J:

$$V_{\max} = K \cdot J^{\mathrm{n}}, \qquad \text{(A5)}$$

where K is a characteristic constant of shape, and n determines the degree of dependence of the volume on the linear magnitude J. Similarly, the volume actually displaced by the head movement is:

$$V_{\mathrm{act}} = K \cdot (J - h)^{\mathrm{n}}\,. \qquad \text{(A6)}$$

Thus, the ratio of actual:maximum volume is given by:

$$\frac{V_{\mathrm{act}}}{V_{\max}} = \left(\frac{J-h}{J}\right)^{\mathrm{n}} = \left(1 - \frac{h}{J}\right)^{\mathrm{n}}. \qquad \text{(A7)}$$

Finally, we return to the minimum estimate of the work component w_1 and relate it to the linear dimension of the animal (i.e. the mean body diameter h), and the following relation of proportionality expressed in Equation 1 (main text) applies:

$$w_1 \xleftrightarrow{\text{proportional}} -k_0 \ln\left(1 - \frac{h}{J}\right). \qquad \text{(A8)}$$

Notice that if h tends to zero, the work of compression tends to zero as well. As h rises, the work required grows and tends to minus infinity in a non-linear way. The compression is limited to J, and, as a consequence, at some value of body diameter h, the increase in required work for digging would surpass the augmented force due to a higher cross-sectional area of the muscles, related to h^2 (see Fig. 7).

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