Hind foot drumming: Myosin heavy chain muscle fibre distribution in the hind limb muscles of three African mole-rat species (Bathyergidae)

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DECLARATIONS:

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Author contributions
Lauren Sahd performed the analysis and drafted the manuscript. Narusa Doubell helped with sectioning and staining of the samples and edited the manuscript. Nigel Bennett provided the samples and edited the manuscript. Sanet Kotzé was the principal investigator, designed the project and edited the manuscript.

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The authors have no conflict of interest to declare.

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ABSTRACT

Hind foot drumming as a form of seismic signalling plays a pivotal role in the communication of various mammalian species including Bathyergidae (African mole-rats). The aim of the present study was to histologically determine if the action of hind foot drumming would influence the number of type II fibres present in the hind limb muscles of two drumming (Georychus capensis and Bathyergus suillus) and one non-drumming (Cryptomys hottentotus natalensis) bathyergid species. Twenty-one frozen muscles of each species were selected for the purpose of mid-belly cryostat sections. These sections were immunohistochemically labelled for myosin heavy chain slow muscle fibres (MHC-I). In addition, oxidative capacity was determined by means of histochemical staining. A high percentage of fast type II muscle fibres was found in all the functional muscle groups, although there were no statistical differences between the drumming and non-drumming species. Bathyergus suillus had significantly fewer type II fibres in mm. semitendinosus, gluteofemoralis, tibialis cranialis, plantaris and the medial head of m. gastrocnemius compared to the other two species. In all three species, the majority of the muscle fibres in all functional muscle groups demonstrated low oxidative capacity which correlated with the expression of type II muscle fibres. It therefore seems likely that the number of type II muscle fibres in the hind limb muscles of the Bathyergidae species studied here is more influenced by either body size or digging strategy rather than being an adaptation for hind foot drumming.

Keywords: Muscle fibre typing; Seismic signalling; Immunohistochemistry

INTRODUCTION

Seismic signals play a vital role in the communication of various animals including the family Bathyergidae (Randall, 2010, 2014). African mole-rats (Bathyergidae) are subterranean rodents that display variable sociality including solitary, social and eusocial species (Bennett & Faulkes, 2000; Faulkes & Bennett, 2013). As these subterranean rodents rarely leave their burrow systems, several bathyergid species use hind foot drumming to produce seismic signals for communication during territorial and courtship behaviour (Bennett & Faulkes, 2000; Bennett*.* Maree & Faulkes*,* 2006). Hind foot drumming in these quadrupedal mole-rats is facilitated by the rapid flexion and extension of the hip and knee joints of a single or alternating hind limb (Randall, 2014). The present study focused on two drumming and one non-drumming subterranean bathyergid species namely, *Georychus capensis, Bathyergus suillus* and *Cryptomys hottentotus natalensis*, respectively.

Georychus capensis (Cape mole-rat; Pallas, 1778) is a mid-sized, chisel tooth digging member of the bathyergid family with a mean body mass of 180 g, displaying no apparent sexual dimorphism. This species is native to South Africa and occurs predominantly in the Western Cape, but there are relict populations in Natal and Mpumalanga (Bennett, Maree & Faulkes, 2006). As a solitary member of the family Bathyergidae, it is known to be highly territorial and aggressive towards members of different as well as the same species (Bennett & Jarvis, 1988). They engage in hind foot drumming to alert others of their presence and prevent interaction (Bennett & Jarvis, 1988). Males and females drum at different frequencies and speeds with males drumming at 26 beats per second while the females drum at 15 beats per second. During the breeding season male *G. capensis* can drum continuously for up to two minutes (Bennett & Jarvis, 1988; Narins, Reichman, Jarvis & Lewis, 1992; Bennett & Faulkes, 2000; Bennett *et al.,* 2006).

Bathyergus suillus (Cape dune mole-rat; Schreber, 1782) is the largest member of the family Bathyergidae, with males weighing up to two kilograms, and one of the only bathyergid species

to use scratch digging for burrow excavation (Bennett *et al.,* 2009). This species is indigenous to South Africa and only occurs in the mesic (moderate levels of rainfall and moisture) regions of the western and southern Cape near the coastline (Bennett *et al.*, 2009; Thomas *et al.*, 2012). *Bathyergus suillus* is a solitary species that is known to be hostile and aggressive towards other conspecifics. They engage in drumming behaviour to announce their presence to conspecifics (Hart, O'Riain, Jarvis & Bennett, 2006). No rate of hind foot drumming has been determined in this species but during courtship very fast drumming has been reported, especially in males (Bennett & Faulkes, 2000, Hart *et al*., 2006)

Cryptomys hottentotus natalensis (Natal mole-rat; Roberts, 1913) is a sub-species of the species complex *Cryptomys hottentotus* (common mole-rat). This is a relatively small bathyergid species with a mean body mass of 106 g in males and 88 g in females (Jarvis & Bennett, 1991). This species is predominantly found in Kwa-Zulu Natal and Mpumalanga, South Africa (Hart, Bennett, Malpaux, Chimimba & Oosthuizen, 2004) in a variety of different soil types. The soil types they prefer are in mesic regions and vary between hard soils to sandy soils found along riverbanks (Bennett & Faulkes, 2000). *Cryptomys hottentotus natalensis* is a social member of the family Bathyergidae, living in colonies of between 8 and 16 individuals (Hickman, 1979; Bennett & Faulkes, 2000). Seismic signalling in the form of foot drumming has not been reported in this subspecies. However, occasional foot thumping has been reported in the genus *Cryptomys* (Lacey, Patton & Cameron, 2000).

The myosin heavy chain (MHC) isoforms expressed in individual muscle fibres influence the contractile properties of the muscle fibre including the myosin ATPase activity, filament sliding velocity, the output power of the fibre as well as the contraction velocity of the fibre (Bottinelli, Canepari, Reggiani & Stienen, 1994; Schiaffino & Reggiani, 2011). There are four main isoforms expressed in adult mammalian skeletal muscles namely MHC-I, IIa, IIb and IIx. (Quiroz-Rothe & Rivero, 2001, Acevedo & Rivero, 2006, Hyatt, Roy Rugg & Talmadge, 2010). Myosin heavy chain I fibres are slow contracting, whereas MHC IIa, IIb and IIx fibres contract much faster and more powerfully than MHC-I (Bottinelli, 2001). Myosin heavy chain isoform IIb results in rapidly contracting fibres and are commonly found in the limb muscles of small mammals such as rats and mice. These MHC IIb fibres are seldom found in limb musculature of larger animals but may be expressed in ocular muscles. However, adult marsupials and xenarthans do express MHC IIb fibres in their skeletal muscles (Kohn & Myburgh, 2007, Toniolo, Cancellara, Maccatrozzo, Patruno, Mascarello & Reggiani, 2008; Spainhower, Cliffe, Metz et al., 2018). The fibre type composition of individual muscles varies within groups of the same species as well as different species. Where some species such as sloths (Xenarthra; Spainhower, Metz, Yusuf, Johnson, Avey-Arroyo & Butcher, 2021) and Japanese moles (*Mogera* species; Ichikawa, Matsu, Higurashi, et al. 2019) express a single MHC isoform within a whole muscle, other species express all four muscle isoforms with variations in proportion in a single muscle. These differences in muscle fibre phenotype can be attributed to sex, age, hormonal, neural and phylogenetic influences (Novák, Zacharová & Soukup, 2010; Schiaffino & Reggiani, 2011).

Extensor muscles of the knee and ankle joints have a dual functionality, being both postural (anti-gravity) and propulsive (Eng *et al*., 2008). For example, in several mammalian species the m. vastus intermedius has more slow contracting type I fibres that are fatigue resistant for postural movements, while the m. vastus medialis or lateralis has more fast contracting type II fibres for powerful propulsive movements. A similar pattern is expressed in m. soleus which has more type I fibres when compared to m. gastrocnemius. The above examples have been observed in the saltatorial five-toed jerboa (Jouffroy, Medina, Renous & Gasc, 2003), leaping

primates (Jouffroy & Medina, 1996; Jouffroy, Stern, Medina & Larson, 1999) and the rat (Armstrong & Phelps 1984; Eng *et al* 2008). In contrast, ankle flexor muscles such m. tibialis cranialis almost exclusively express type II fibres in various small mammals such as the rat and rabbit (Sartorius, Lu, Acakpo-Satchivi, Jacobson, Byrnes & Leinwand, 1998; Jouffroy *et al*., 2003).

When muscle fibre composition differs between the superficial and deep layers of skeletal muscle, it is termed regionalisation of muscle fibres. Typically, the percentage of type I fibres is higher in deep layers of muscles while the opposite is observed with type II fibres (Kernell, 1998; Eng *et al.,* 2008). Regionalisation is more apparent in extensor muscles compared to flexor muscles (Armstrong, Saubert, Seeherman, & Taylor, 1982). Furthermore, a correlation between fibre type regionalisation and joint stabilisation has been observed in the forelimb muscles of two small mammals (northern tree shrew and common yellow-toothed cavy; von Mering & Fischer, 1999). Selective recruitment of muscle fibres within these regionalised muscles has been observed in electromyographic studies (English, 1984, Hoffer, Loeb, Sugano, Marks, O'Donovan & Pratt, 1987).

The energy metabolism of muscle fibres is conventionally considered to be related to their MHC isoform content. Traditionally it was believed that fast MHC-II fibre types rely more on glycolytic metabolic pathways, whereas slow MHC-1 fibres are often reported to utilize oxidative cellular metabolism exclusively (Kohn & Myburgh, 2007; Schiaffino & Reggiani, 2011). Nicotinamide adenine dinucleotide (NADH) staining intensity has been used as a measure of muscle oxidative capacity in various studies (Kohn, Burroughs, Hartman & Noakes, 2011a; Kohn, Curry & Noakes 2011b; Curry, Hohl, Noakes, Kohn, 2012). Additionally, these studies on muscle enzyme activity suggest a lack of correlation between MHC isoform expression and muscle energy metabolism (Kohn *et al.,* 2011a,b; Curry *et al.,* 2012). Oxidative capacity is related to fatigue resistance in muscle fibres. However, oxidative capacity can be altered in response to environmental and functional stimuli without changes to the MHC isoform content (Gollnick, Riedy, Quitinskie & Bertocci, 1985). More recently, it has been found that fast MHC isoforms may have different oxidative capacities across different muscles (Bloemberg and Quadrilatero, 2012).

The fibre type composition of the hind limb muscles in the rat (*Rattus norvegicus)* has been determined in various studies (Ariano, Armstrong & Edgerton, 1973; Armstrong & Phelps 1984; Eng *et al* 2008) as well as in the mouse (*Mus musculus*; Burkholder, Fingado, Baron & Liber, 1994). These studies indicated that rats and mice have high proportions of type II muscle fibres in their hind limbs. However, in African mole-rats, little is known regarding how limb muscle fibre type or metabolism is influenced by function and/or behaviour, and *vice versa.*

In the present study it was hypothesised that the two drumming species will have more type II fibres in the hind limb muscles, specifically in the hip and knee flexors and extensors, compared to the non-drumming species. The rationale is that these muscle groups are thought to be the major muscles involved with hind foot drumming based on muscle architecture differences observed between the drumming and non-drumming mole-rat species (Sahd, Bennett & Kotzé, 2021). It is also hypothesised that the muscle fibres of the hind limb would have low oxidative capacity due to their hypoxic subterranean environment. Additionally, prolonged periods of contraction of the hind limbs during drumming may not be necessary as drumming typically lasts two minutes which would not require a fatigue resistant oxidative metabolism in these muscles. Therefore, the present study aimed to determine if the action of hind foot drumming influences the number of MHC-I and MHC-II muscle fibres in the hind limb muscles of three

African mole-rat species as demonstrated using immunohistochemistry. Additionally, the present study aimed to investigate the oxidative capacity of the hind limb muscles of these African mole-rats.

MATERIALS AND METHODS

Sample

Frozen hind limbs of a total of 17 animals from three species, *G. capensis* (the Cape mole-rat; n=6 females), *B. suillus* (the Cape-dune mole-rat; n=5 [2 males, 3 females]) and *C. h. natalensis* (the Natal mole-rat; n=6 [2 males, 4 females]) were analysed. No animals were specifically captured or killed for the present study but were obtained from previous, unrelated, ethically cleared studies (Table 1). Ethical approval for the use of the specimens was obtained from the Stellenbosch University Research Ethics Committee: Animal Care and Use (SU-ACUM 16-00005) and the University of Pretoria Animal Ethics Committee (ECO79-17). Twenty-one hind limb muscles (Table 2) were selected for muscle fibre typing based on their functional muscle groups and observed statistical differences in muscle architecture parameters (eg. physiological cross-sectional area) between the species studied (Sahd, Bennett & Kotzé, 2021). Musculus soleus of *B. suillius* was excluded since this muscle could only be found intact in two samples due to damage prior to receipt of the specimens. Musculus gracilis posticus was only harvested from *C. h. natalensis* as it was absent in the other two species (Sahd, Bennett & Kotzé, 2019)

TABLE 1 Species information including ethical clearance, capture site, and mean body mass

Species	Ethical approval	*n*	Capture site	Mean body mass (g)
Georychus capensis	University of Johannesburg: 215086650-10/09/15	6	Darling, Western cape	217.88 ± 26.15
Bathyergus suillus	Stellenbosch University: 10NP_VAN01	5	Darling, Western cape	538.08 ± 227.07
Cryptomys hottentotus natalensis	University of Pretoria: ECO0070-14	6	Glengarry, Kwa-Zulu Natal	90.00 ± 16.81

TABLE 2 Muscle functional groups and abbreviations

Muscle group	Muscle
Hip flexors (HF)	M. iliacus (I) M. pectineus (P) M. rectus femoris (RF)
Hip extensors (HE)	M. gluteus superficialis (GS) M. gluteus medius (GMED) M. semitendinosus (ST) M. biceps femoris (BFAN) M. semimembranosus (SM) M. gluteofemoralis (GF)
Hip rotators (studied as individual muscles as there is only one muscle in the group)	M. piriformis (PI)
Hip adductors (studied as individual muscles as there is only one muscle in the group for the drumming species)	M. gracilis anticus (GA) M. gracilis posticus (only in *C.h. natalensis*; GP)
Knee extensors (KE)	M. vastus lateralis (VL) M. vastus medialis (VM) M. rectus femoris (RF)
Knee flexors (KF)	M. semitendinosus (ST) M. biceps femoris (BFAN) M. semimembranosus (SM) M. gracilis anticus (GA) M. gastrocnemius (GCM, GCL) M. plantaris (PLA)
Ankle flexors (AF)	M. tibialis cranialis (TA) M. Extensor digitorum longus (EDL) M. Extensor hallucis longus (EHL)
Ankle extensors (AE)	M. gastrocnemius (GCM, GCL) M. soleus (S) M. plantaris (PLA)

Note: The muscle names and groups are based on Sahd, Bennett & Kotzé, 2019.

Muscle harvesting

Frozen hind limbs were partially thawed (to ease dissection and prevent damage) and skinned. Individual muscles were collected from origin to insertion, placed onto a piece of cork and surrounded with tissue freezing medium (*OCT, Leica Biosystems: Wetzlar, Germany*). The prepared muscles were snap frozen in liquid nitrogen (-170°C) and stored in a -80°C freezer overnight to preserve the structure of the muscle. Mid-belly sections of the muscles were then collected on the following day, mounted onto cork with tissue freezing medium and sprayed with Pellox freezing spray (*Cell Path Services, Midrand, South Africa*) to keep the tissue sections frozen while sectioning took place.

Sectioning and staining

Two mid-belly serial cross sections (7µm thick) of each muscle were made using a Leica Cryostat (*Leica Biosystems: Wetzlar, Germany*) at -15°C. Each section was transferred onto a positively charged microscope slide. Three different muscles were placed onto a single microscope slide as outlined in Figure 1. The prepared slides were stored in a freezer at -20°C prior to staining. The frozen slides were allowed to reach room temperature before staining.

RF VM VL	GS GMED PI	ST BFAN SM
1	2	3
GF GA GP	P I S	TA EDL EHL
4	5	6
GCM GCL PLA	Control	
7	8	

FIGURE 1 Slide layouts for the placement of muscle sections

One of each slide was labelled immunohistochemically for myosin heavy chain slow (MHCs) using the NovoCastra antibody (CAS: NCL-MHCs; LOT: 6065928; *Leica Biosystems: Wetzlar, Germany*) as the primary antibody. Positive staining was detected using a DAB Novolink™ Polymer detection system (CAS: RE7150-CE; LOT: 6029001; *Leica Biosystems: Wetzlar, Germany*), similar to the protocol detailed by Kalmar, Blanco & Greensmith (2012). Slides were washed in TRIS buffered saline (TBS; pH 7.6) for two increments of five minutes each after which a Novopen (NCL-Pen; *Leica Biosystems: Wetzlar, Germany*) reagent pen was used to circle the individual tissue sections to ensure the reagents were restricted to the desired area. Fifty µl of the primary antibody (MHCs; 1:40 dilution with TRIS) was applied to each tissue section, after which the slides were placed in a plastic humidity chamber and incubated at 37°C for one hour in a TC2323 SHEL LAB CO_2 Incubator (*Sheldon Manufacturing Inc.: Cornelius, Oregon, United States*). The slides were washed in TBS for two increments of five minutes each between steps. Thereafter, 50 µl of Novalink ™ post primary antibody (was used as the secondary antibody) was applied to each tissue section and incubated in a plastic humidity chamber for 15 minutes at room temperature. Novalink™ Polymer was applied to each section and incubated for 15 minutes at room temperature. Fifty µl of DAB solution (made up of 1:50 dilution of Novalink™ DAB chromogen in Novalink™ DAB substrate buffer) was applied to each tissue section and incubated for five minutes at room temperature. Slides were washed in tap water, counterstained with Meyer's haematoxylin for one minute and rinsed in running tap water for five minutes. The slides were dehydrated in a series of ethanol (70%, 96%, 96%, 99%, 99%), cleared in xylene and mounted using DPX mounting media. A cross section of the m. biceps femoris of a rat was used as a positive control for all runs. The positive control was reacted with the primary and secondary antibodies in isolation as well as together to test the individual and combined reactivity of the primary and secondary antibodies before staining the mole-rat samples. An example of the positive staining is illustrated in Figure 2.

The second muscle section in a subset of the sample (three specimens per species) was stained with NADH (*Sigma- Aldrich* CAS:104809-32-7) and nitroblue tetrazolium salt (*Sigma-Aldrich* CAS: 298-83-9) dissolved in TRIS buffer (pH 7.4) for 30 minutes at 37℃ (Curry et al., 2012; Novikoff et al., 1961) in a TC2323 SHEL LAB CO_2 Incubator (*Sheldon Manufacturing Inc.: Cornelius, Oregon, United States*). After incubation, the slides were rinsed in distilled water and mounted with glycerine jelly.

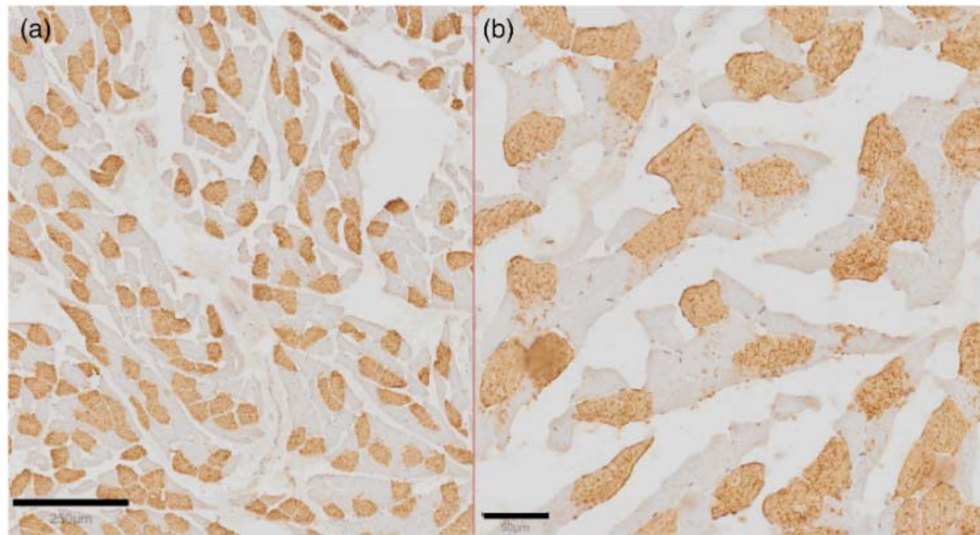

FIGURE 2 Examples of the positive staining in m. gracilis anticus of *Bathyergus suillus* at two different magnifications (a) bar = 250 μm and (b) bar = 50 μm

Visualisation and quantification

Whole slide images were obtained using an Olympus V120-100L (*Olympus Corporation, Tokyo, Japan*) slide scanner at the University of Cape Town, Pathology Learning Centre. Images were taken with the 40X objective, with a final resolution of 16 µm per pixel. Qupath version 2.0.m11 (Bankhead, Loughrey, Fernández et al., 2017) was used to quantify the percentage of positively stained fibres per muscle cross section and classify the muscles fibres based on staining intensity for the NADH. Before analysis the stain vectors of each slide were estimated in the Qupath programme. All analysis was performed three times per muscle to check the precision of the automated quantification and classification (the same or similar results were recorded with each analysis). The polygon tool was used to select the whole muscle tissue area (avoiding areas where the tissue was folded or longitudinal) after which the positive cell detection function was used to automatically determine the percentage of type I fibres present. Settings are detailed in the Supplementary information 1. The remaining fibres that did not stain positively, were considered to be type II fibres. (The total number of tissue detections and the derived number of fibres analysed per individual animal are detailed in Supplementary information 2).

To quantify differences in oxidative capacity between fibre types, the polygon tool was used to select the whole muscle tissue area (avoiding areas where the tissue was folded or longitudinal). Subsequently, the cell detection function was used to provide a detection space for cell classification. The cell intensity threshold setting was then used to classify the muscle fibres based on the haematoxylin optical density image (Supplementary information 1) similar to that described by Curry *et al*., (2012). The thresholds per slide were automatically determined by the Qupath software based on the staining intensity of the individual slide. This setting counted the number of detections per threshold and was converted into a percentage of all positive detections (detections of tissue). The percentage of light, dark and medium fibres was calculated per muscle group per species.

Statistical analysis

Descriptive statistics including the mean and standard deviation were reported per species. One-way analysis of variance (ANOVA) was used to determine significant differences between

species. Fischer's Least Significant Difference (LSD) *post-hoc* test was used to determine the p-values. Statistically significant results were determined with a $p < 0.05$. All statistical analyses were performed using Statistica 13.5 (*TIBCO software, Palo Alto, California, USA*). Graphs were created in ggplot2 (Wickham, 2016) in R (R core team, 2013).

RESULTS

The percentages of the type I and type II muscle fibres for each muscle per species are detailed in Table 3. *Georychus capensis* had the most type II fibres compared to the other two species where all the individual muscles expressed greater than 50 % type II muscle fibres. In the functional muscle groups of *G. capensis* had more than 70% type II muscle fibres in all muscle groups except for the ankle extensors. The ankle extensors had the least type II muscle fibres in all three species (Fig 3). *Bathyergus suillus* had significantly fewer type II fibres in the knee flexor muscle group compared to either *G. capensis* or *C. h. natalensis* (F=4.778, p=0.03). Additionally, *B. suillus* had significantly fewer type II muscle fibres in the ankle flexor muscle group compared to *G. capensis* (F=4.887, p=0.03). However, both these muscle groups in *B. suillus* expressed more than 60% type II muscle fibres.

TABLE 3 The muscle fiber composition (%) of slow (type I) and fast (type II) fibres in the hind limb muscles of *Georychus capensis*, *Bathyergus suillus*, and *Cryptomys hottentotus natalensis* (fibers that did not stain positively were considered fast)

	Georychus capensis		Bathyergus suillus		Cryptomys hottentotus natalensis	
Muscle	Slow (i)	Fast (ii)	Slow (i)	Fast (ii)	Slow (i)	Fast (ii)
Rectus femoris	15.25 ± 5.21	84.75 ± 5.21	19.65 ± 14.54	80.35 ± 14.54	16.44 ± 6.92	83.56 ± 6.92
Vastus medialis	10.91 ± 8.33	89.09 ± 8.33	40.87 ± 22.93	59.13 ± 22.93	29.69 ± 21.50	70.31 ± 21.50
Vastus lateralis	29.31 ± 22.21	70.69 ± 22.21	33.10 ± 23.92	66.84 ± 23.92	22.70 ± 12.30	77.30 ± 12.30
Gluteus superficialis	9.16 ± 5.30	90.84 ± 5.30	26.83 ± 15.06	73.17 ± 15.06	23.62 ± 19.11	76.38 ± 19.11
Gluteus medius	28.97 ± 10.53	71.03 ± 10.53	43.14 ± 21.72	56.86 ± 21.72	39.36 ± 10.94	60.64 ± 10.94
Piriformis	26.89 ± 9.84	73.11 ± 9.84	38.78 ± 24.63	61.22 ± 24.63	26.60 ± 17.35	73.40 ± 17.35
Semitendinosus	12.18 ± 3.69[a]	87.82 ± 3.69	37.75 ± 4.13[b]	62.25 ± 4.13	16.60 ± 10.28[a]	83.40 ± 10.28
Biceps femoris cranial head	2.73 ± 2.43	97.27 ± 2.43	9.19 ± 10.89	90.81 ± 10.89	13.09 ± 29.55	86.91 ± 29.55
Semimembranosus	40.28 ± 10.88	59.72 ± 10.88	49.37 ± 18.84	50.63 ± 18.84	43.88 ± 9.00	56.12 ± 9.00
Gluteofemoralis	5.89 ± 3.72[a]	94.11 ± 3.72	19.00 ± 6.34[b]	81.00 ± 6.34	1.94 ± 2.39[a]	98.06 ± 2.39
Gracilis anticus	14.63 ± 9.22	85.37 ± 9.22	34.08 ± 18.95	65.92 ± 18.95	14.54 ± 7.57	85.46 ± 7.57
Gracilis posticus	–	–	–	–	10.43 ± 11.35	89.57 ± 11.35
Pectineus	48.51 ± 25.13	51.49 ± 25.13	57.18 ± 22.78	42.82 ± 22.78	56.84 ± 23.52	43.16 ± 23.52
Iliacus	9.11 ± 4.97	90.89 ± 4.97	24.75 ± 18.06	75.25 ± 18.06	24.85 ± 15.70	75.15 ± 15.70
Tibialis cranialis	10.71 ± 7.30[a]	89.29 ± 7.30	29.52 ± 11.02[b]	71.94 ± 11.02	11.84 ± 5.96[a]	88.16 ± 5.96
Extensor digitorum longus	15.72 ± 12.73	84.28 ± 12.73	25.03 ± 14.26	74.97 ± 14.26	16.53 ± 12.18	83.47 ± 12.18
Extensor hallucis longus	11.69 ± 5.17[a]	88.31 ± 5.17	45.15 ± 18.67[b]	54.85 ± 18.67	31.78 ± 17.74[b]	68.22 ± 17.74
Gastrocnemius medial head	16.24 ± 10.00[a]	83.76 ± 10.00	50.68 ± 14.28[b]	49.32 ± 14.48	25.96 ± 14.03[a]	74.04 ± 14.03
Gastrocnemius lateral head	29.95 ± 8.76	70.05 ± 8.76	39.11 ± 17.47	60.89 ± 17.47	29.72 ± 7.83	70.28 ± 7.83
Plantaris	34.14 ± 14.45[a]	65.86 ± 14.45	54.72 ± 14.53[a]	45.28 ± 14.53	23.15 ± 8.80[b]	76.85 ± 8.80
Soleus	47.14 ± 29.49	52.86 ± 29.49	–	–	50.67 ± 33.13	49.33 ± 33. 13

Note: Differing superscript letters denote significant differences between species $p < .05$.

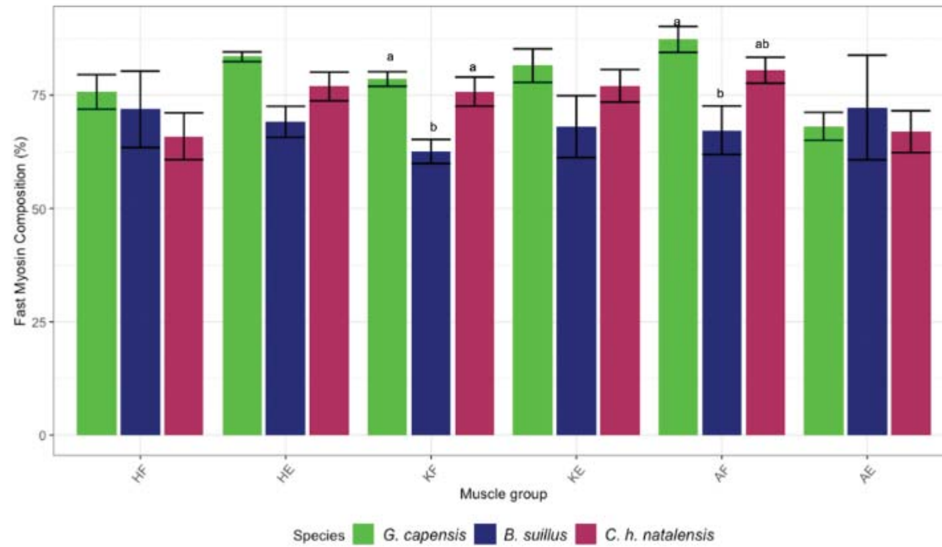

FIGURE 3 The mean percentage of fast myosin muscle fibres of the functional muscle groups in *Georychus capensis* (green), *Bathyergus suillus* (blue), and *Cryptomys hottentotus natalensis* (maroon). Muscle group abbreviations: AE, ankle extensors; AF, ankle flexors; HE, hip extensors; HF, hip flexors; KE, knee extensors; KF, knee flexors. Differing superscript letters indicate significant differences between species $p < .05$. The error bars denote the standard error

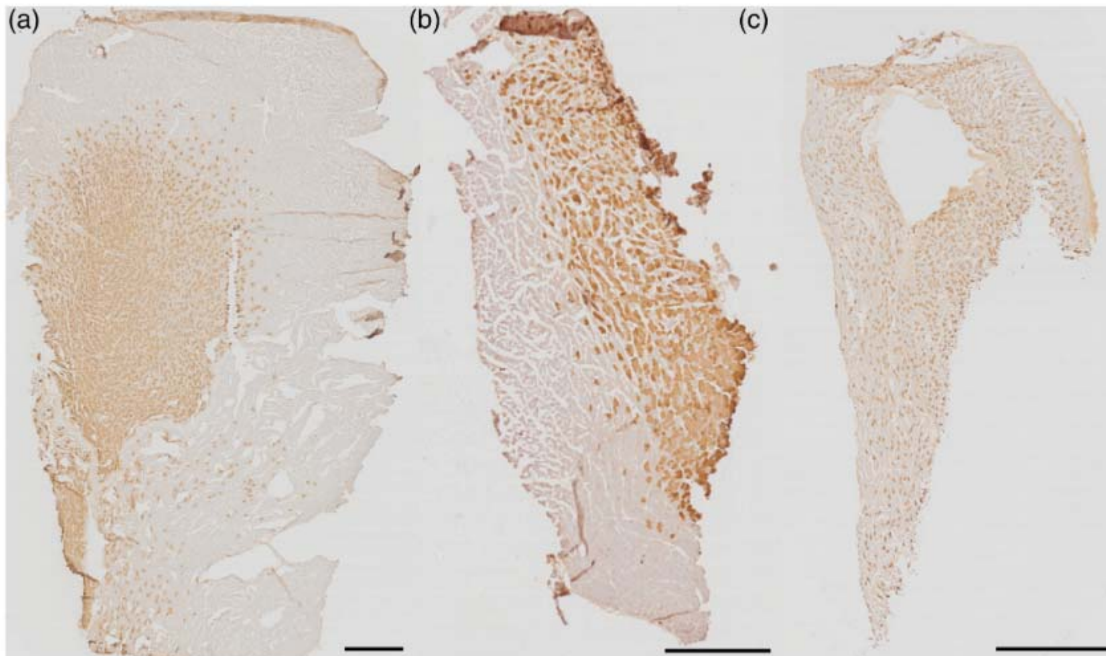

FIGURE 4 Muscles displaying regionalization of muscle fiber types (a, b) compared to a muscle with no regionalization (c). (a) Musculus rectus femoris of *Bathyergus suillus* as a representative of all three species bar = 1 mm. (b) Musculus semitendinosus of *Georychus capensis* as representative of all three species bar = 1 mm. (c) Musculus gluteus superficialis of *Bathyergus suillus* as representative of all three species and non-regionalized muscles bar = 2 mm

While all three species predominantly expressed type II muscle fibres in all the individual muscles, *B. suillus* had significantly fewer fast fibres in mm. semitendinosus and gluteofemoralis compared to either *G. capensis* or *C. h. natalensis* (F=20.898, p<0.01; F=23.009, p<0.01, respectively). Additionally, *B. suillus* had significantly fewer type II fibres in the m. tibialis cranialis (F=7.4261, p<0.01), the medial head of m. gastrocnemius (F=7.5568,

$p<0.01$) and m. plantaris ($F=6.4571$, $p=0.01$) compared to either *G. capensis* or *C. h. natalensis*. In the m. extensor hallucis longus, *Georychus capensis* had significantly more fast fibres compared to both *B. suillus* and *C. h natalensis* ($F=7.3112$, $p<0.01$). Regionalisation of the muscle fibres was observed in only two muscles, namely, mm. rectus femoris and semitendinosus (Fig 4) in all the individual animals in all three species.

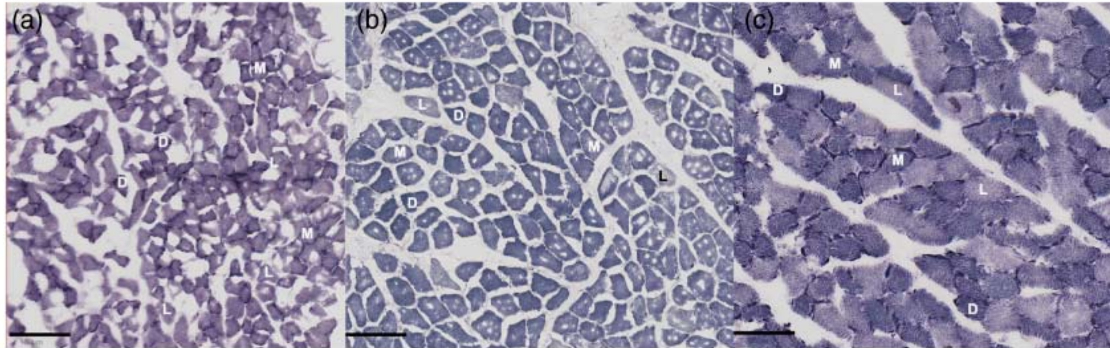

FIGURE 5 The m. semitendinosus of (a) *Georychus capensis*, (b) *Bathyergus suilus*, and (c) *Cryptomys hottentotus natalensis* stained with NADH (to evaluate oxidative capacity) to demonstrate the staining intensity of each species. Darkly stained fibers (D), medium stained fibers (M), and lightly stained fibers (L). Bar = 100 μm

The NADH staining (to indicate oxidative capacity) intensity varied between species with *C. h. natalensis* staining the darkest of the three species and *B. suillus* the lightest (Fig. 5). The percentage of light (low oxidative capacity), medium (moderate oxidative capacity) and dark (high oxidative capacity) of the functional muscle groups of the three species is illustrated in Figure 6. All functional groups in all three species had less than 20% darkly stained muscle fibres, less than 30% medium stained fibres and more than 60% lightly stained muscle fibres. Fibres that stained positively for MHCs did not necessarily have a high oxidative capacity (dark stain with NADH) when compared on serial sections but rather had light to intermediate staining. This was particularly evident in *B. suillus* which had several muscles with nearly a 50% slow MHC-1 expression and yet its fibres had the greatest anaerobic potential (or lowest oxidative potential). This emphasised that the oxidative capacity of the muscle fibres varied regardless of the myosin heavy chain expression (Fig. 7).

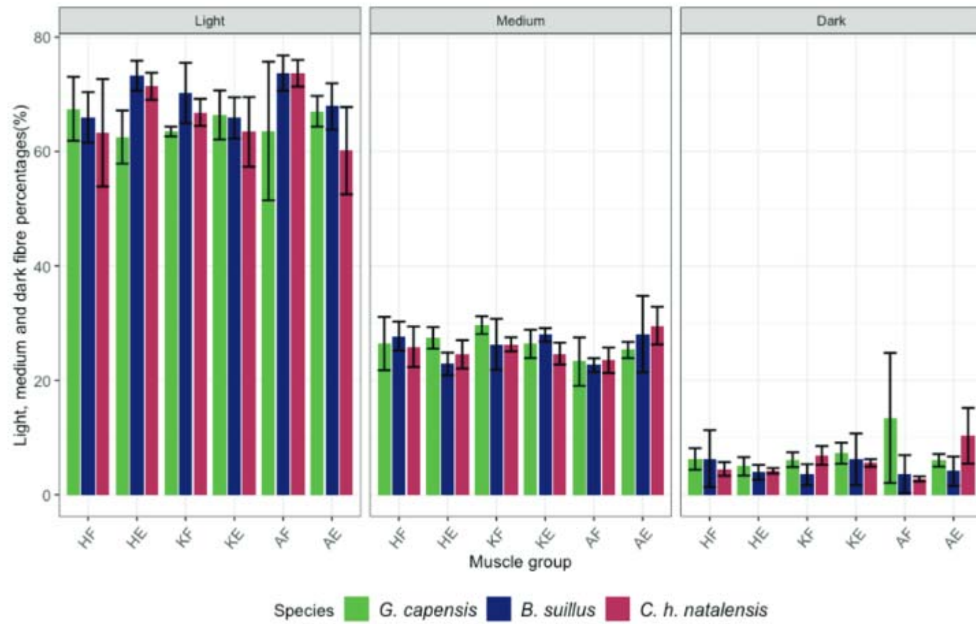

FIGURE 6 The mean (±*SE*) percentage of the light, medium and dark stained fibers as an indication of oxidative capacity in the functional muscles groups of *Georychus capensis* (green), *Bathyergus suillus* (blue), and *Cryptomys hottentotus natalensis* (maroon). Muscle group abbreviations: AE, ankle extensors; AF, ankle flexors; HE, hip extensors; HF, hip flexors; KE, knee extensors; KF, knee flexors

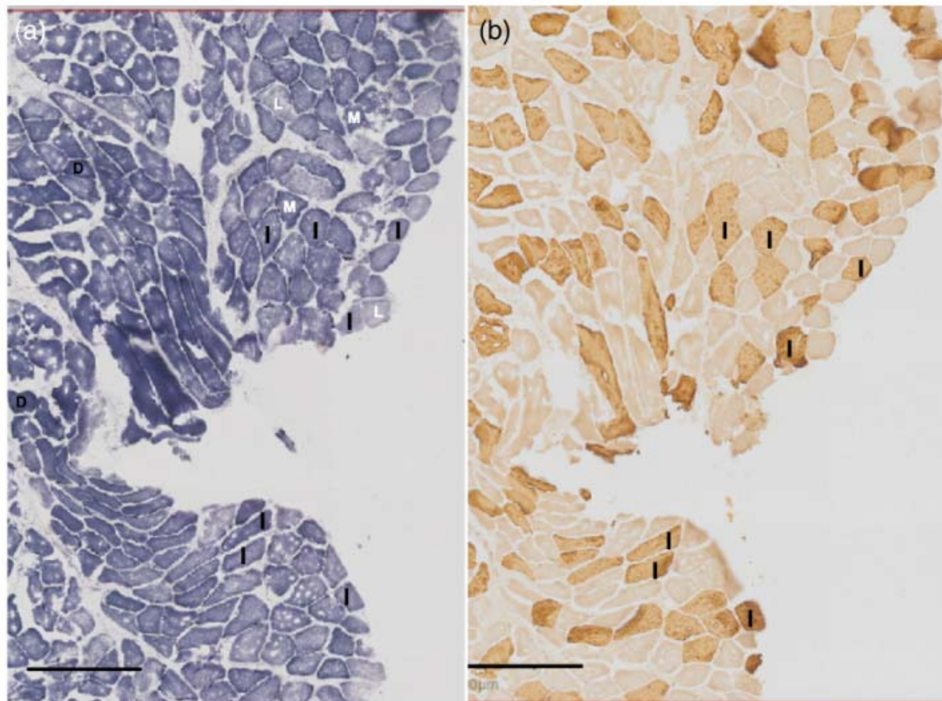

FIGURE 7 Two serial cross sections (5 μm apart) of m. semitendinosus of *Bathyergus suillus*, as representative of all three species, stained with (a) NADH and (b) MHCs. This illustrates how the fibers which stained positively for MHCs did not necessarily have a high oxidative capacity (stain darkly on the NADH) but rather had light to intermediate staining. Type I muscle fiber (I), darkly stained fibers (D), medium stained fibers (M), and lightly stained fibers (L). Bar = 200 μm

DISCUSSION

Various studies have hypothesised that the fibre type composition of a muscle coincides with its specific functional demands in a species (Suzuki & Tamate, 1988; Suzuki, 1990; Kanatous, Di Michele, Cowan, & Davis 1999; Singh, Melis, Richmond & Scott, 2002). However, Álvarez & Pérez (2019) as well as two recent studies in sloths (Spainhower *et al.,* 2018, 2021) state that functional adaptations to morphology may be overruled by phylogeny and ecological demands. The present study aimed to determine if the functional demands of hind foot drumming would influence the number of type I and type II MHC isoform muscle fibres expressed in the hind limb muscles in two drumming and one non-drumming African mole-rat species.

The three mole-rat species studied here typically had fewer type II muscle fibres in all the muscles analysed compared to those reported in other terrestrial rodents such as the rat (Ariano *et al*, 1973; Armstrong & Phelps, 1984, Eng *et al*, 2008), mouse (Burkholder *et al*, 1994) and guinea pig (Ariano *et al*, 1973). However, the three African mole-rat species in the present study still expressed a predominantly type II muscle fibre phenotype in their hind limb muscles with only a few exceptions. This may be due to phylogenetic differences between the rodent species as myosin isoform expression can be constrained by phylogeny (Novák *et al,* 2010). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis in two non-drumming, subterranean Japanese mole species revealed that both species only had type II fibres in their front and hind limb muscles (Ichikawa *et al.* 2019). Avivi, Band, Joel, Shenzer & Coleman (2009) demonstrated that the m. gastrocnemius muscles in the subterranean, non-drumming blind mole rat (*Spalax ehrenbergi*) had mainly type II muscle fibres which is consistent with the results in the present study. Additionally, Avivi *et al.,* (2009) observed that the overall muscle oxidative capacity in the blind mole rat (*Spalax ehrenbergi*) was intermediate and low, similar to that of the muscles observed in the present study. The low amount of highly oxidative fibres in all three species studied here could be an adaptation to compensate for the low oxygen levels in their subterranean environments (Bennett & Faulkes, 2000) by relying more heavily on anaerobic muscle metabolism (Essén-Gustavsson and Henriksson, 1984; Kohn, Burroughs, Hartman & Noakes, 2011). The low oxidative capacity expressed in the functional muscle groups of the three species studied here also corresponds with the expression of predominantly type II muscle fibres in the functional groups. Therefore, it is unlikely that hind foot drumming may have caused adaptations to either the MHC isoform expressed or oxidative capacity of the muscle fibres.

An experimental study on several hind limb muscles in mice which were exposed to running exercise regimes (continued fast contractions of the muscle) determined that the muscle fibre type composition was not altered but that muscle fibre hypertrophy observed histologically, was rather the adaptive response to exercise. However, no gross muscle hypertrophy was reported (Glaser, You, Zhang & Medler, 2009). Similar to running exercise regimes, hind foot drumming in the African mole-rat species studied here may cause changes to individual muscle fibre size instead of altering or influencing the number of type II fibres. Furthermore, the lack of changes on the MHC isoform expression seen in the tail of opossum emphasises that MHC expression is likely constrained by phylogeny (Thomas, Chadwell, Walker, Budde, VandeBerg & Butcher, 2017).

Regionalisation in fibre-type proportions has been observed in various mammalian species (Wang & Kernell, 2001) which may suggest that regionalisation is necessitated by different functional needs in a single muscle. Typically, there are more type I fibres in the deeper layers of a muscle, while the opposite is observed for type II fibres.

(Kernell, 1998; Eng *et al.,* 2008). This pattern was observed in two muscles namely, the mm. rectus femoris and semitendinosus in all three species studied here. Regionalisation may provide a biomechanical advantage during a period where rapid contraction of the fast muscle fibres is needed as it could indicate that the intramuscular architecture is optimised for fast contraction (Walmsley *et al.,* 1978). However, the observed regionalisation might not be an adaptation for hind foot drumming as it was observed in all three species and not just the two drumming species and only in two muscles, albeit that m. semitendinosus plays an important role in hind foot drumming (Sahd *et al.,* 2020). Therefore, regionalisation may rather be an adaptation for joint stabilisation (von Mering & Fischer, 1999) during burrowing, when the mole-rats brace themselves with their hind limbs to prevent backward movements (Hildebrand, 1985; Samuels & Van Valkenburgh, 2008).

Additionally, *G. capensis* had more than 70% type II fibres in all the functional muscle groups except for the ankle extensors. The hip and knee extensors of *G. capensis* had more than 75% type II MHC expression. Furthermore, mm. iliacus (hip flexor), gluteus superficialis and (hip extensor) had more than 90% expression of type II muscle fibres in *G. capensis* compared to the other two species which had approximately 70% expression of type II muscle fibres in these same muscles. As hind foot drumming in these mole-rats is facilitated by the rapid flexion and extension of the hip and knee joints of a single or alternating hind limb (Randall, 2014), this arrangement of the muscle fibres in the hip and knee extensors would enable muscles to contract faster than in the other two species. This would allow for the attainment of the rapid speed of drumming reported in *G. capensis* (Van Sandwyk & Bennett, 2005). The drumming duration of *G. capensis* is approximately two minutes during the breeding season and in small bursts in territorial interactions (Bennett & Faulkes, 2000; Bennett *et al.,* 2006), therefore sustained prolonged periods of rapid contraction may not be necessary and this is reflected in the low to intermediate oxidative capacity (low fatigue resistance) expressed by the muscle groups in *G capensis.*

Kleiber's law states that energy metabolism for unit body mass is inversely proportional to body size (Kleiber, 1947). Thus, body size plays a key role in the determination of the energy demands placed on skeletal muscles. Small mammals typically have more type II muscle fibres in their skeletal muscles compared to large mammals that tend to have more type I muscle fibres (Schiaffino & Reggiani, 2011). The three mole-rat species seem to follow this pattern. However, the type II muscle fibre composition of several muscles was significantly lower in *B. suillus* compared to the other two species studied. This difference could be attributed to the large body size of *B suillus* compared to *G. capensis* and *C. h. natalensis* (Table 2). *Bathyergus suillus* is the largest member of the family Bathyergidae (weighing up to 2 kg) and therefore has different energetic demands to the skeletal muscles for moving their relatively large body frames (Schiaffino & Reggiani, 2011). This trend of fewer numbers of type II fibres in skeletal muscles was similarly observed in the forelimb muscles of groundhogs which are also large rodents (4.7±0.8 kg; Rupert, Rose, Organ & Butcher, 2015). As both *G. capensis* and *B. suillus* engage in hind foot drumming, the difference in body size between these species may overrule adaptations for hind foot drumming. Additionally, differences in digging strategies between the species could have affected the number of type II muscle fibres. The scratch digging *B. suillus* use their hind limbs for stabilisation during the digging process (Hildebrand, 1985; Samuels & Van Valkenburgh, 2008), while the chisel tooth digging *G. capensis* and *C. h. natalensis* use their forelimbs in addition to their hind limbs to brace themselves while burrowing (Bennett & Faulkes, 2000; Bennett, Maree & Faulkes 2006). This more substantial

stabilisation role of the hind limb during burrowing in *B. suillus* may require more sustained slow contractions resulting in the lower expression of type II muscle fibres.

Limitations

Due to the unavailability of fresh samples, frozen samples had to be thawed and then snap frozen which may have resulted in freezing injury and thus affecting true percentages of fibre types. Care was taken to only partially thaw the specimens to limit freezing injury and histologically, little evidence of such freezing injuries could be observed. The exact age and health status of the specimens were not known as the animals were wild caught which possibly influenced the results. The present study did not differentiate between the types of fast fibres. As literature on mole-rat fibre typing of skeletal muscle is depauperate, the present study serves as a baseline study for future research.

CONCLUSION

The present study hypothesised that the two drumming species (*G. capensis* and *B. suillus*) would have more type II fibres compared to the non-drumming species in the hip and knee flexors and extensors, to allow for rapid hind limb drumming. This was partially true as *G. capensis* had the most type II muscle fibres overall in the individual muscles of the hind limb and no functional muscle group had less than a 68% expression of type II fibres in this species. In contrast, the drumming *B. suillus* had the least type II muscle fibres in all the muscles studied, of which six significant differences were observed compared to the other two species. This study further hypothesised that the muscle fibres of the hind limb muscles of African mole-rats would have low oxidative capacity which was confirmed, as over 60% of all the functional muscle groups fibres stained light in all three species. This finding coincided with the predominant expression of type II muscle fibres in all three species. Additionally, the low oxidative capacity of the hind limb muscle in the two drumming species may indicate that two minutes of drumming may not be prolonged enough to require more oxidative metabolism to resist fatigue. As no statistically significant differences were observed between the two drumming species and the non-drumming species, it seems likely that the body size, or the digging strategy of Bathyergidae may have a larger influence on the number of type II fibres in the hind limb muscles than reflecting their ability to drum.

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