TITLE

# A novel description of the *Vastus lateralis* morphology of the Temminck's Ground Pangolin *(Manis temminckii)*

AUTHORS

Kathryn Merle van Boom[1,2], Dorothy Breed[3], Alix Hughes[2], Dee Blackhurst[4], Tertius Abraham Kohn[1,2,5]

AFFILIATIONS

[1] Department of Medical Bioscience, Faculty of Natural Sciences, University of the Western Cape, Cape Town, South Africa

[2] Division of Exercise Science and Sports Medicine, Department of Human Biology, University of Cape Town, Cape Town, South Africa

[3] Biodiversity Management Branch, Environmental Management Department, City of Cape Town, 53 Berkley Road, Maitland, 6504, South Africa

[4] Division of Chemical Pathology, Department of Pathology, University of Cape Town, Cape Town, South Africa

[5] Centre for Veterinary Wildlife Studies, Faculty of Veterinary Science, University of Pretoria, South Africa

RUNNING TITLE

Pangolin skeletal muscle characteristics

CORRESPONDING AUTHOR

Tertius A. Kohn

ORCID ID: 0000-0003-4152-6319

Department of Medical Bioscience, Faculty of Natural Sciences, University of the Western Cape, South Africa

Email: tkohn@uwc.ac.za

Tel: +27 21 959 2189

Fax: +27 21 959 3125

## ABSTRACT

The pangolin (*Manidae* family*)* is the world's most trafficked animal, yet very little is known about its physiology and metabolism primarily due to its inconspicuous and solitary nature. Skeletal muscle samples from the *Vastus lateralis* were collected *postmortem* from a single female Temminck's ground pangolin (*Manis temminckii). S*amples were analyzed for fiber type composition, fiber size and myosin heavy chain isoform content. The oxidative and glycolytic metabolic capacity was determined through citrate synthase, 3-hydroxyacetyl co A dehydrogenase, creatine kinase, lactate dehydrogenase, phosphofructokinase and glycogen phosphorylase enzyme activities. Lastly, antioxidant capacity was determined through superoxide dismutase and catalase enzyme activities, and the total antioxidant capacity. The pangolin metabolic profile was then compared to other endurance and non-endurance mammals, in which data was standardized relative to human endurance athletes in order to provide context. Slow twitch type I fibers, rich in mitochondria were the predominant fiber type within the pangolin indicating a reliance on oxidative derived energy from fats and carbohydrates. This suggests that the pangolin has a high endurance capability when compared to other wild animals and human endurance athletes. This is the first study to investigate the skeletal muscle physiology and metabolism of any pangolin species, in an attempt to further understand this endangered animal and aid with conservation efforts.

## KEYWORDS

Skeletal muscle, metabolism, mitochondria, oxidative stress, wildlife

# INTRODUCTION

The pangolin (*Manidae* family) is the most trafficked animal in the world with an estimated 895,000 pangolins trafficked between 2000 and 2019 primarily from Africa to Asia (Challender et al., 2020), which has sadly left them on the brink of extinction. The 2020 COVID-19 pandemic has re-ignited the public interest in pangolins as they were implicated as an intermediary host for the transmission of SARS-CoV-2 to humans, although this was later refuted (Frutos et al., 2020; Heighton and Gaubert, 2021). Considering the strict regulations surrounding their conservation, surprisingly few studies have investigated their physiology with notably less representation of the African species (Heighton and Gaubert, 2021).

The Temminck's ground pangolin (*Manis temminckii*, also called *Smutsia temminckii*) is a unique terrestrial mammal covered with keratinous scales as opposed to hair (Skinner and Chimimba, 2006). This inconspicuous, predominantly solitary animal is one of eight pangolin species (Pietersen et al., 2019). Temminck's ground pangolin is the most widespread of the African pangolin species and occurs from eastern to southern Africa, typically inhabiting savanna woodlands in low-lying regions with moderate shrubs (Pietersen et al., 2019).

Male pangolins range in length between 0.93 to 1.29 m with a body mass between 10.7 to 15.9 kg, while the females range between 0.77 to 1.04 m in length and 4.6 to 10.1 kg in mass (Skinner and Chimimba, 2006). Pangolins are solitary foragers eating an exclusively ant and termite diet, and are considered to move at a slow, steady pace on their hindlimbs (Pietersen et al., 2016; Skinner and Chimimba, 2006). When under threat, pangolins curl themselves into a tight ball with their scales providing very effective protection against predators (such as leopards), this along with their elusive nature, result in humans being their most notable threat (Pietersen et al., 2019; Skinner and Chimimba, 2006).

According to the International Union for Conservation of Nature (IUCN), pangolins are listed as vulnerable with the decline in population primarily due to the overexploitation of their body parts and scales for spiritual rituals - in particular the use in Traditional Chinese Medicine (Pietersen et al., 2019). In 2017, all eight pangolin species were moved from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II to Appendix I, which affords them legislative protection in the form of international trade bans (Challender and Waterman, 2017). Sadly, this has not prevented the illegal trade of pangolins and/or pangolin parts (Challender and Waterman, 2017). Historically, pangolins have received very little conservation efforts and investment, but this has changed in the past two decades due to the growing number of species linked to illegal trade (Challender and Waterman, 2017). However, there are still significant knowledge gaps within pangolin research primarily in areas such as veterinary care, immunology, education and ethnozoology (Heighton and Gaubert, 2021).

There have been some studies investigating the anatomy and morphology of limb muscles, masticatory muscles, digestive organs and enzymes, and reproductive organs of primarily the Asian pangolin species (Akmal et al., 2019; Kawashima et al., 2015; Zhang et al., 2019). The unique tongue and its associated structures seem to be the most widely studied aspect of the pangolin (Abayomi et al., 2009; Doran and Allbrook, 1973). However, to date, there are no studies that have investigated physiology and metabolism of the ground pangolin, specifically that of the skeletal muscle.

Skeletal muscle is a heterogeneous tissue with varying contractile, metabolic and functional properties which are largely dependent on the myosin heavy chain isoform (MHC) which is expressed – either MHC I, IIA or IIX (Bottinelli, 2001; Kohn et al., 2011a; Schiaffino and Reggiani, 2011). This in turn gives rise to type I, IIA and IIX muscle fibers respectively (Schiaffino and Reggiani, 2011). Type I fibers are slow twitch fibers with a high oxidative capacity, high mitochondrial content, small cross- sectional area and are fatigue resistant. In contrast, type IIX fibers are fast, powerful fibers dependent on glycolytic metabolism and with large cross- sectional areas (Kohn et al., 2011a; Schiaffino and Reggiani, 2011). Type IIA fibers exhibit a mixture of both type I and type IIX characteristics. Muscle fiber types and characteristics differ between species and show varying degrees of plasticity in response to physiological stressors like exercise, therefore, fiber type alone does not solely dictate the metabolic properties of the muscle (Daugaard et al., 2000; Kohn et al., 2011a, 2007; Schiaffino and Reggiani, 2011). Enzyme markers, such as lactate dehydrogenase and citrate synthase, can be used as indicators of the glycolytic or oxidative capacity of the muscle (Kohn et al., 2011a). Similarly, enzymes such as superoxide dismutase (SOD) provide an indication of the antioxidant capacity of the muscle (Hohl et al., 2020).

The Temminck's ground pangolin (order Pholidota) is more closely related to the order Carnivora (which includes felid and canid species) than the order Xenarthra (which includes anteaters, armadillos and sloths) with which it shares similar characteristics, indicating a convergent evolution (Du Toit et al., 2014). Within felid and canid families, there is notable skeletal muscle diversity with many species such as lions, cheetahs and caracals exhibiting higher proportions of type IIX fibers and higher glycolytic capacity (Kohn et al., 2011a; Williams et al., 1997), while other species, such as the domestic dog, have higher proportions of oxidative type IIA fibers (Acevedo and Rivero, 2006; Toniolo et al., 2007). The armadillo and sloth, which share similar external structures or feeding patterns to the pangolin, exhibited higher proportions of type IIA and IIX and type I and IIA fibers, respectively (Olson et al., 2016; Spainhower et al., 2018, 2021). As no muscle data currently exist for the pangolin, comparing their parameters to other species, including antelope that harbor high endurance capabilities, and others (e.g. Carnivora and Xenarthra), would provide a better understanding of muscle function.

We were fortunate to have obtained a muscle sample from a recently deceased Temminck's ground pangolin that had been obtained by the authorities. The aim of this study was therefore to provide insight into the skeletal muscle characteristic of the *Vastus lateralis* in the pangolin. Specifically, the muscle fiber composition, metabolic profile and antioxidant status of the pangolin will be compared to that of human endurance athletes as well as other mammals. Due to the slow movement and defensive strategies employed by the pangolin, we hypothesize a higher proportion of slower type I and IIA muscle fibers as well as a higher oxidative capacity when compared to other species.

## METHODS

### *Animal sampling*

This study included a single (n=1) adult female Temminck's ground pangolin (*Manis temminckii)* that was killed by a motor vehicle in the Western Cape province of South Africa. The pangolin weighed 8 kg and the exact age could not be determined. This animal is not indigenous to this region and authorities suspected that the animal was released by poachers to avoid prosecution or accidentally escaped from captivity. Unfortunately, this animal was hit by a motorist and succumbed to her wounds. An example of a Temminck's ground pangolin is shown in Figure 1.

Skeletal muscle tissue of the *Vastus lateralis* was opportunistically collected *post mortem*, divided into smaller pieces (±5 mm in diameter) and rapidly frozen in liquid nitrogen. Samples were stored at -87°C until analyses. The results were compared to human endurance athletes for which ethical approval was already obtained (Webster et al., 2020). As the focus of our laboratory is comparative muscle physiology, endurance athletes have been used as a reference point especially for their high oxidative capacity. Enzyme activity comparisons were also made with previously published literature on two-toed and three-toed sloths (Spainhower et al., 2021), caracal, lion and blesbok (Kohn, 2014; Kohn et al., 2011a). These species were selected based on their phylogenetic relatedness to the pangolin (specifically the lion, caracal and sloths) and diverse skeletal muscle capabilities (e.g. endurance and sprinting ability).

### *Permits*

A research permit (CN44-31-5381) from CapeNature (the official institute for biodiversity conservation in the Western Cape, South Africa) to collect skeletal muscle samples from wild animals that have been killed for various reasons (such as roadkill) was obtained. The Biodiversity Management Branch, Department of Environmental Management at the City of Cape Town, holds a CapeNature permit (CN39-59-4243) that allows possession of the carcass of an endangered animal and donated the muscle samples to Prof Tertius Kohn at the University of the Western Cape.

### *Analyses*

#### *Fiber type and cross-sectional area*

The fiber type and cross-sectional area (CSA) were determined using immunohistochemistry as described by Dada *et al.*, (2018). Briefly, frozen sections were blocked with 5% bovine serum albumin (BSA) dissolved in 0.1 M phosphate buffered saline (PBS) pH 7.4 and incubated overnight at 4˚C with primary antibodies, diluted 1:50 in PBS (Developmental Studies Hybridoma Bank, Iowa, USA) against MHC I (BA-D5; IgG2b), MHC IIA (SC-71; IgG1) and MHC IIX (6H1, IgM). After a brief wash in PBS, the sections were incubated at room temperature for two hours with secondary antibodies (1:250) that recognized the various immunoglobulin forms (AMCA goat anti-mouse IgG2b; AlexaFlour 488 goat anti-mouse IgG1, and Cy3 goat anti-mouse IgM – Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The slides were dried and mounted with Mowiol containing antifade. Fibers were identified as type I, IIA and IIX (Figure 2A) and expressed as a percentage of the total number of fibers, while the CSA of 50 fibers per fiber type was determined using ImageJ (version 1.50e, Maryland, USA).

### *MHC isoform content*

The MHC isoform content was determined by the methods described by Kohn *et al.* (2011a). Briefly, a small piece of muscle was homogenized in 0.1 M PBS, pH 7.3 in a ratio of 1:100 (for every 1 mg muscle, 100 μl buffer was added) using a glass homogenizer and then sonicated. These homogenates were used to determine the enzyme activities representing the various muscle metabolic pathways. After the protein concentrations were determined (Bradford, 1976), samples were diluted with sample buffer (5% β-MEtOH, 2.5% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8 and 0.1% bromophenol blue). Samples then underwent SDS-PAGE to separate the MHC isoforms (Figure 2B). The relative MHC isoforms proportions were determined using the Un-Scan-It Software package (Silk Scientific Corporation, Utah).

### *Metabolic and antioxidant enzyme assays*

Enzyme activities that serve as markers of the various pathways were determined fluorometrically at 25°C, with an excitation and emission wavelength of 340nm and 460nm, respectively (Hohl et al., 2020; Kohn et al., 2011a). Citrate synthase (CS) and 3-hydroxyacyl acetyl Co enzyme A dehydrogenase (3HAD), act as marker enzymes of the Kreb's cycle and β-oxidation, respectively – and therefore indicators of oxidative metabolism. The markers of oxygen independent metabolism, and more rapid energy production, include lactate dehydrogenase (LDH), phosphofructokinase (PFK), creatine kinase (CK) and glycogen phosphorylase (PHOS). A standard NADH or NADPH curve was generated at known concentrations and used to determine the maximum enzyme activity expressed as μmol/min/g protein.

Markers of antioxidant capacity (SOD, catalase and ORAC) were determined as described by Hohl *et al.* (2020). Briefly, SOD activity, expressed as U/g protein, where U was determined using xanthine oxidase to produce oxygen radicals, was measured at an absorbance of 550 nm. Catalase (CAT) activity, expressed as nmol $H_2O_2$/min/g protein, was determined by the decrease in hydrogen peroxide at an absorbance of 240 nm. The total antioxidant capacity is represented by the Oxygen Radical Absorbance Capacity (ORAC), which measures the decay of fluorescein by fluorescence detection at an excitation and emission wavelength of 485 nm and 520 nm, respectively. ORAC values were calculated relative to a Trolox (Vitamin E analogue) standard and expressed as μmol/g protein.

*Normalization of comparative metabolic enzyme data*

In this study, metabolic enzyme data was obtained fluorometrically while comparative data from other species was measured using a spectrophotometric method at an absorbance wavelength of 340nm or 412nm and expressed as µmol/min/g wet weight (Kohn, 2014; Kohn et al., 2011a; Spainhower et al., 2021).

Therefore, because of methodological differences between studies, human endurance athletes were selected as the reference point (or known constant) as metabolic enzyme data are readily available for both fluorometric and spectrophotometric methods (Kohn et al., 2011a; Webster et al., 2020). The enzyme activities of the wild animals were not predicted from the human values, but measured in the present and previous studies, as described above. However, the endurance athletes were used to normalize the data of the wild animals to a fold difference (i.e. species-specific enzyme activity divided by human enzyme activity) to allow comparison between species (depicted in Figure 3 and the supplementary data).

## RESULTS

The pangolin *Vastus lateralis* comprised primarily of slow twitch fibers, where immunohistochemistry amounted to 72% type I and 28% type IIA fibers, and no IIX fibers (Figure 2A). These findings were echoed by the MHC isoform content showing that this muscle contained 86% MHC I and 14% MHC IIA, and no detectable MHC IIX (Figure 2B). The CSAs of the type I and IIA fibers were 5048 ± 1166 $\mu m^2$ and 2869 ± 416 $\mu m^2$, respectively. The various metabolic enzyme activities and antioxidant capacity are presented in Table 1. 3HAD and CS activities, indicating oxidative capacity, were 225 and 195 µmol/min/g protein respectively. While the activities of LDH (568 µmol/min/g protein), PFK (46 µmol/min/g protein), CK (9138 µmol/min/g protein) and PHOS (40 µmol/min/g protein) were indicative of glycolytic capacity or rapid energy production. The enzyme activities for markers of oxidative stress defense were SOD (5488 U/g protein), CAT (18 nmol/min/g protein) and ORAC (112 µmol/g protein). The metabolic enzyme activity of the pangolin relative to endurance human athletes and other species are shown in Figure 3 and the supplementary tables.

## DISCUSSION

This is the first study to investigate the MHC isoform content, fiber type composition, metabolic enzyme activity and antioxidant capacity in the *Vastus lateralis* of the Temminck's ground pangolin. Due to the small sample size (n = 1), the results are descriptive in nature as no statistical analyses were possible.

The MHC and fiber type composition both show a predominance in MHC I expression and type I fibers, respectively, with no MHC IIX or type IIX fibers present. This high proportion of slow fibers highlights the potential endurance capacity of pangolins, as human endurance athletes and antelope species also exhibit higher proportions of these fiber types (Kohn, 2014; Kohn et al., 2007). Extensive studies conducted on various fore- and hindlimb muscles of the three-toed (*Bradypus variegatus*) and two-toed (*Choloepus hoffmanni*) sloth, revealed a predominance in type I fiber expression (Spainhower et al., 2018, 2021). No type IIX fibers were found in any of the 12 muscle groups analyzed. Specifically, the *Vastus lateralis* of the three-toed and two-toed sloth contained 100% and 62% type I fibers, respectively (Spainhower et al., 2021).

The CSAs of the fiber types appear to be different in the pangolin where type I and IIA fibers respectively measured 5048 ± 1166 $\mu m^2$ and 2869 ± 416 $\mu m^2$. This finding contradicts the normal skeletal muscle paradigms where type I fibers are usually similar to or even smaller in size than type IIA and IIX fibers. However, previous studies have shown that the predominant fiber type may have the largest CSA (Spainhower et al., 2018, 2021). Additionally, pangolins display a steady, bipedal locomotion and the fiber size distribution may be indicative of hindlimb function (Kohn et al., 2011a; Spainhower et al., 2018). The larger type I CSA would also explain the larger percentage of MHC I content determined from SDS-PAGE - larger fibers harbor more contractile proteins, resulting in a larger MHC band. Comparatively, the CSAs of the fibers from the *Vastus lateralis* of human middle distance endurance runners range from approximately 4000 to 5600 $\mu m^2$ (I) and 4500 to 6700 $\mu m^2$ (IIA), while the fiber CSA of lions averaged 2014 µm (I) and 2005 $\mu m^2$ (IIA) (Kohn et al., 2011a, 2011c, 2007). In mixed breed domestic dogs, the CSA of the *Triceps brachii,* which were similar to that of the lion, averaged 2218 $\mu m^2$ (I) and 1446 $\mu m^2$ (IIA) (Acevedo and Rivero, 2006), and the type I fibers from the *Vastus lateralis* of the sloth averaged approximately 1900 $\mu m^2$. However, across all the muscle functional groups of the three-toed and two-toed sloth, the variation in CSAs between the muscle groups ranged between 425 to 7787 $\mu m^2$ (type I) and 326 to 5211 $\mu m^2$ (type IIA) in the hindlimb muscles (Spainhower et al., 2021). The type I fibers of the *Vastus lateralis* of the pangolin is thus similar to human endurance athletes while the CSA of type IIA fibers aligns more with that of dogs, lions and sloths. However, it would be challenging to draw any conclusion as CSA differs between species, indicating large genetic variation (Kohn et al., 2011a)

The marker enzymes for the oxidative capacity in the pangolin were exceptionally high, far exceeding what has been normally found in trained humans and wild antelope (Kohn, 2014; Kohn et al., 2007). According to Table 1, 3HAD and CS were ~2.5 and ~3.2x higher than endurance athletes, suggesting that the pangolin greatly relies on oxidative and fat metabolism. The CK and LDH activities of the pangolin were similar to that found in endurance athletes, while their PFK and phosphorylase activities were low, indicating a lower glycolytic capacity than the endurance trained humans. Importantly, fiber type alone does not determine oxidative or glycolytic capacity of muscles with function and lifestyle playing important roles. This has been shown in various species, such as black wildebeest and dogs, which have a high oxidative capacity, but a lower proportion of type I fibers (Acevedo and Rivero, 2006; Kohn et al., 2011b; Kohn, 2014).

When the enzyme activities were normalized to the values obtained from endurance athletes (Figure 3), the CS and 3HAD activities of the pangolin were 16 and 14-fold greater, respectively, than the three toed sloth, whereas those from the two toed sloth and blesbok were between 2 and ~4 fold lower.

Similarly, the pangolin's oxidative capacities were greatly elevated compared to the two felids, lion and caracal, ranging from 6 to approximately 12-fold higher. In contrast, the pangolin had the lowest LDH activity with its CK activity only marginally exceeding that of the two and three toed sloth. The PFK activity of the pangolin was similar to the caracal and lion, while the phosphorylase activity was considerably lower.

The seemingly higher 3HAD and CS activities of the pangolin compared to the sloth, lion and other wild animals, and the higher values compared to the endurance athletes, indicates a reliance on β-oxidation and oxidative metabolism (Kohn et al., 2011a; Spainhower et al., 2018). This may be linked to their foraging behavior as well as their home ranges of 6.5 ± 5.9 km$^2$ for adults and 6.1 ± 4.0 km$^2$ for juveniles, which is relatively large when considering the size and limited mobility of the pangolin (Pietersen et al., 2016, 2014). Pangolins are usually slow movers and will only move at a brisk pace on their hindlimbs when under stress, anecdotally reaching speeds of 5 km/h, and will curl into a tight ball to protect themselves from predators (Skinner and Chimimba, 2006). The low LDH, PFK, CK and glycogen phosphorylase activities compared to other wild animals, supports the potential need for endurance over rapid energy production in the movements and lifestyle of the pangolin.

Hohl *et al.* (2020) investigated the antioxidant enzyme activities in six wild antelope species and found that these species have higher SOD activity compared to laboratory rats, while the rats had a higher CAT activity compared to the wild antelope. Although in the current study the antioxidant activity was expressed in different units, this equates to a much lower SOD activity in the pangolin compared with the antelope, while the SOD and CAT activities was similar to the rat. The pangolin's SOD, CAT and ORAC activity seemed lower than the endurance athlete. Antioxidants are vital in countering the harmful effects of oxidative damage caused by reactive oxygen species that are produced during metabolic processes, and this field is becoming increasingly important in understanding the role of environmental and behavioral stressors in wild and captive animals (Costantini et al., 2017; Hohl et al., 2020). Given that this pangolin was being trafficked by poachers, it was likely under considerable stress, and the lower levels of antioxidant enzymes and antioxidant capacity (compared to the human) may indicate that these enzymes did not have the time to upregulate in response to the stress or that the pangolin was able to tolerate the oxidative stress experienced (Costantini et al., 2017). This pattern was seen in free-ranging cheetahs that were physically restrained for 24 hours, in which they had higher levels of blood oxidative damage markers but no increase in antioxidant enzyme activities (Costantini et al., 2017). However, no direct measure of stress or oxidative stress was investigated in the pangolin.

## CONCLUSION

The MHC isoform content, fiber type composition and metabolic and antioxidant enzyme activity was investigated in the *Vastus lateralis* of an individual pangolin. The pangolin has a predominance of type I fibers which aligns with the higher CS and 3HAD activity and indicates a high oxidative capacity. This supports the potential need for endurance in the movement and lifestyle of the pangolin. Although only one individual was analyzed in the current study, to the authors knowledge, this is the first paper to investigate skeletal muscle physiology and metabolism of the Temminck’s ground pangolin and indeed any pangolin species. Pangolins are seldom seen and considered rare and threatened. Every opportunity should be used to further understand these vulnerable and highly trafficked species, in an attempt to aid with conservation efforts.

## ACKNOWLEDGEMENTS

The authors would like to thank the staff of CapeNature and the City of Cape Town for their continued support in research to better understand wildlife.

### *Author contributions*

Conceptualization and design: TAK; Sample collection: DB; Sample analysis: KvB, TAK, AH, DMB; Data analysis: KvB, TAK; Writing: KvB; Reviewing, editing and approval of final manuscript: all authors.

### *Funding*

KvB received an MSc Scholarship from the South African National Research Foundation. TAK was the recipient of the Tim and Marilyn Noakes Sports Science Postdoctoral Fellowship while conducting the research.

### *Conflict of interest*

The authors declare that they have no conflict of interest.

### *Corresponding author's declaration*

Conflict of Interest: The authors declare that they have no conflict of interest.

## REFERENCES

Abayomi, T.A., Ofusori, D.A., Ayoka, O.A., Odukoya, S.A., Omotoso, E.O., Amegor, F.O., Ajayi, S.A., Ojo, G.B., Oluwayinka, O.P., 2009. A Comparative Histological Study of the Tongue of Rat (Rattus Norvegicus), Bat (Eidolon Helvum) and Pangolin (Manis Tricuspis). Int. J. Morphol. 27, 1111–1119. https://doi.org/10.4067/s0717-95022009000400026

Acevedo, L.M., Rivero, J.-L.L., 2006. New insights into skeletal muscle fibre types in the dog with particular focus towards hybrid myosin phenotypes. Cell Tissue Res. 323, 283–303.

Akmal, Y., Nisa, C., Novelina, S., 2019. Anatomy of the Male Reproductive Organs of Javan Pangolin (Manis javanica) 13–14. https://doi.org/10.31219/osf.io/q4z6k

Bottinelli, R., 2001. Functional heterogeneity of mammalian single muscle fibres: Do myosin isoforms tell the whole story? Pflugers Arch. Eur. J. Physiol. 443, 6–17. https://doi.org/10.1007/s004240100700

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Challender, D., Waterman, C., 2017. Implementation of CITES decisions 17.239 b) and 17.240 on Pangolins (Manis spp.). CITES Secr. 128.

Challender, D.W.S., Heinrich, S., Shepherd, C.R., Katsis, L.K.D., 2020. International trade and trafficking in pangolins, 1900–2019, in: Pangolins. Elsevier, pp. 259–276.

Costantini, D., Wachter, B., Melzheimer, J., Czirják, G., 2017. Socioecological and environmental predictors of physiological stress markers in a threatened feline species. Conserv. Physiol. 5, 1–14. https://doi.org/10.1093/conphys/cox069

Dada, S., Henning, F., Feldmann, D.C., Kohn, T.A., 2018. Baboon (Papio ursinus) single fibre contractile properties are similar to that of trained humans. J. Muscle Res. Cell Motil. 39, 189–199.

Daugaard, J.R., Nielsen, J.N., Kristiansen, S., Andersen, J.L., Hargreaves, M., Richter, E.A., 2000. Fiber type-specific expression of GLUT4 in human skeletal muscle: Influence of exercise training. Diabetes 49, 1092–1095. https://doi.org/10.2337/diabetes.49.7.1092

Doran, G.A., Allbrook, D.B., 1973. The tongue and associated structures in two species of African pangolins, Manis gigantea and Manis tricuspis. J. Mammal. 54, 887–899. https://doi.org/10.2307/1379083

Du Toit, Z., Grobler, J.P., Kotzé, A., Jansen, R., Brettschneider, H., Dalton, D.L., 2014. The complete mitochondrial genome of Temminck's ground pangolin (Smutsia temminckii; Smuts, 1832) and phylogenetic position of the Pholidota (Weber, 1904). Gene 551, 49–54. https://doi.org/10.1016/j.gene.2014.08.040

Frutos, R., Serra-Cobo, J., Chen, T., Devaux, C.A., 2020. COVID-19: Time to exonerate the pangolin from the transmission of SARS-CoV-2 to humans. Infect. Genet. Evol. 84, 104493.

Heighton, S.P., Gaubert, P., 2021. A timely systematic review on pangolin research, commercialization, and popularization to identify knowledge gaps and produce conservation guidelines. Biol. Conserv. 256, 109042.

Hohl, R., Blackhurst, D.M., Donaldson, B., van Boom, K.M., Kohn, T.A., 2020. Wild antelope skeletal muscle antioxidant enzyme activities do not correlate with muscle fibre type or oxidative metabolism. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 242, 110638. https://doi.org/10.1016/j.cbpa.2019.110638

Kawashima, T., Thorington, R.W., Bohaska, P.W., Chen, Y.J., Sato, F., 2015. Anatomy of Shoulder Girdle Muscle Modifications and Walking Adaptation in the Scaly Chinese Pangolin (Manis Pentadactyla Pentadactyla: Pholidota) Compared with the Partially Osteoderm-Clad Armadillos (Dasypodidae). Anat. Rec. 298, 1217–1236. https://doi.org/10.1002/ar.23170

Kohn, T.A., 2014. Insights into the skeletal muscle characteristics of three southern African antelope species. Biol. Open 3, 1037–1044.

Kohn, T.A., Burroughs, R., Hartman, M.J., Noakes, T.D., 2011a. Fiber type and metabolic characteristics of lion (Panthera leo), caracal (Caracal caracal) and human skeletal muscle. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 159, 125–133. https://doi.org/10.1016/j.cbpa.2011.02.006

Kohn, T.A., Curry, J.W., Noakes, T.D., 2011b. Black wildebeest skeletal muscle exhibits high oxidative capacity and a high proportion of type IIx fibres. J. Exp. Biol. 214, 4041–4047. https://doi.org/10.1242/jeb.061572

Kohn, T.A., Essén-Gustavsson, B., Myburgh, K.H., 2011c. Specific muscle adaptations in type II fibers after high-intensity interval training of well-trained runners. Scand. J. Med. Sci. Sport. 21, 765–772. https://doi.org/10.1111/j.1600-0838.2010.01136.x

Kohn, T.A., Essén-Gustavsson, B., Myburgh, K.H., 2007. Do skeletal muscle phenotypic characteristics of Xhosa and Caucasian endurance runners differ when matched for training and racing distances? J. Appl. Physiol. 103, 932–940. https://doi.org/10.1152/japplphysiol.01221.2006

Olson, R.A., Womble, M.D., Thomas, D.R., Glenn, Z.D., Butcher, M.T., 2016. Functional Morphology of the Forelimb of the Nine-Banded Armadillo (Dasypus novemcinctus): Comparative Perspectives on the Myology of Dasypodidae. J. Mamm. Evol. 23, 49–69. https://doi.org/10.1007/s10914-015-9299-4

Pietersen, D., Jansen, R., Connelly, E., 2019. Smutsia temminckii. IUCN Red List Threat. Species 2019 8235. https://doi.org/10.2305/IUCN.UK.2019-3.RLTS.T12765A123585768.en

Pietersen, D.W., McKechnie, A.E., Jansen, R., 2014. Home range, habitat selection and activity patterns of an arid-zone population of Temminck's ground pangolins, Smutsia temminckii . African Zool. 49, 265–276. https://doi.org/10.1080/15627020.2014.11407642

Pietersen, D.W., Symes, C.T., Woodborne, S., McKechnie, A.E., Jansen, R., 2016. Diet and prey selectivity of the specialist myrmecophage, Temminck's ground pangolin. J. Zool. 298, 198–208.

Schiaffino, S., Reggiani, C., 2011. Fiber types in Mammalian skeletal muscles. Physiol. Rev. 91, 1447–1531. https://doi.org/10.1152/physrev.00031.2010

Skinner, J.D., Chimimba, C.T., 2006. The mammals of the Southern African subregion. Cambridge University Press, Cape Town.

Spainhower, K.B., Cliffe, R.N., Metz, A.K., Barkett, E.M., Kiraly, P.M., Thomas, D.R., Kennedy, S.J., Avey-Arroyo, J.A., Butcher, M.T., 2018. Cheap labor: Myosin fiber type expression and enzyme activity in the forelimb musculature of sloths (Pilosa: Xenarthra). J. Appl. Physiol. 125, 799–811. https://doi.org/10.1152/japplphysiol.01118.2017

Spainhower, K.B., Metz, A.K., Yusuf, A.R.S., Johnson, L.E., Avey-Arroyo, J.A., Butcher, M.T., 2021. Coming to grips with life upside down: how myosin fiber type and metabolic properties of sloth hindlimb muscles contribute to suspensory function. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 191, 207–224. https://doi.org/10.1007/s00360-020-01325-x

Toniolo, L., Maccatrozzo, L., Patruno, M., Pavan, E., Caliaro, F., Rossi, R., Rinaldi, C., Canepari, M., Reggiani, C., Mascarello, F., 2007. Fiber types in canine muscles: myosin isoform expression and functional characterization. Am. J. Physiol. Physiol. 292, C1915–C1926.

Webster, C.C., Van Boom, K.M., Armino, N., Larmuth, K., Noakes, T.D., Smith, J.A., Kohn, T.A., 2020. Reduced glucose tolerance and skeletal muscle GLUT4 and IRS1 content in cyclists habituated to a long-term low-carbohydrate, high-fat diet. Int. J. Sport Nutr. Exerc. Metab. 30, 210–217. https://doi.org/10.1123/ijsnem.2019-0359

Williams, T.M., Dobson, G.P., Mathieu-Costello, O., Morsbach, D., Worley, M.B., Phillips, J.A., 1997. Skeletal muscle histology and biochemistry of an elite sprinter, the African cheetah. J. Comp. Physiol. - B Biochem. Syst. Environ. Physiol. 167, 527–535. https://doi.org/10.1007/s003600050105

Zhang, F., Xu, N., Yu, Y., Wu, S., Li, S., Wang, W., 2019. Expression Profile of the Digestive Enzymes of Manis javanica Reveals Its Adaptation to Diet Specialization. ACS Omega 4, 19925–19933. https://doi.org/10.1021/acsomega.9b02845

FIGURE LEGENDS

*Figure 1:* A wild Temminck's ground pangolin - photo courtesy of Trevor Hardaker.

*Figure 2: (A)* Immunohistochemical stain of the *Vastus lateralis* muscle in a pangolin. The blue fluorescence indicates type I fibers and the green fluorescence indicates type IIA fibers. The scale bar represents 100 µm. (B) SDS-PAGE image depicting the relative intensities of MHC isoforms in human and pangolin *Vastus lateralis* skeletal muscle.

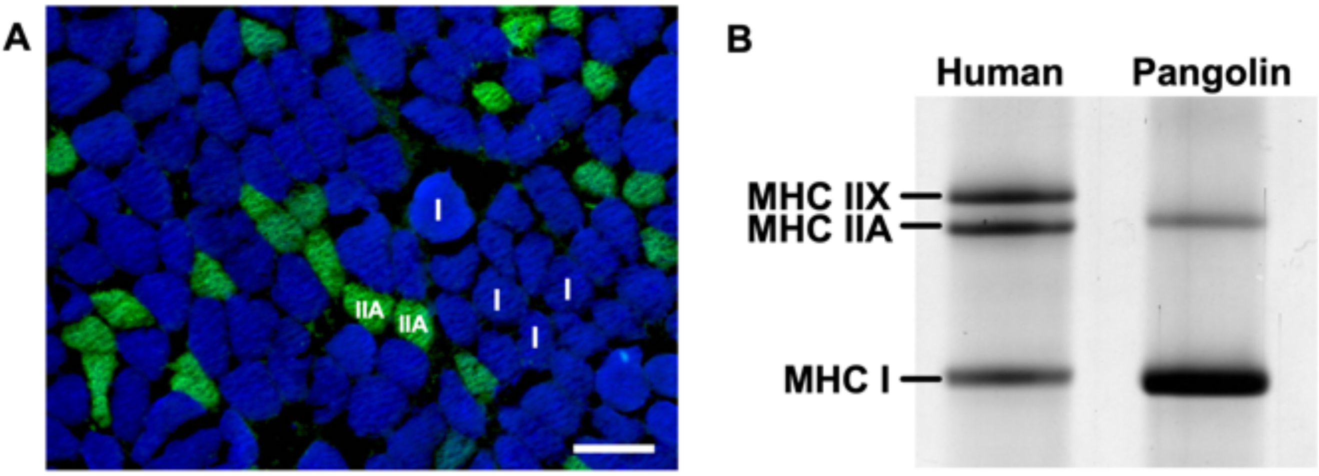
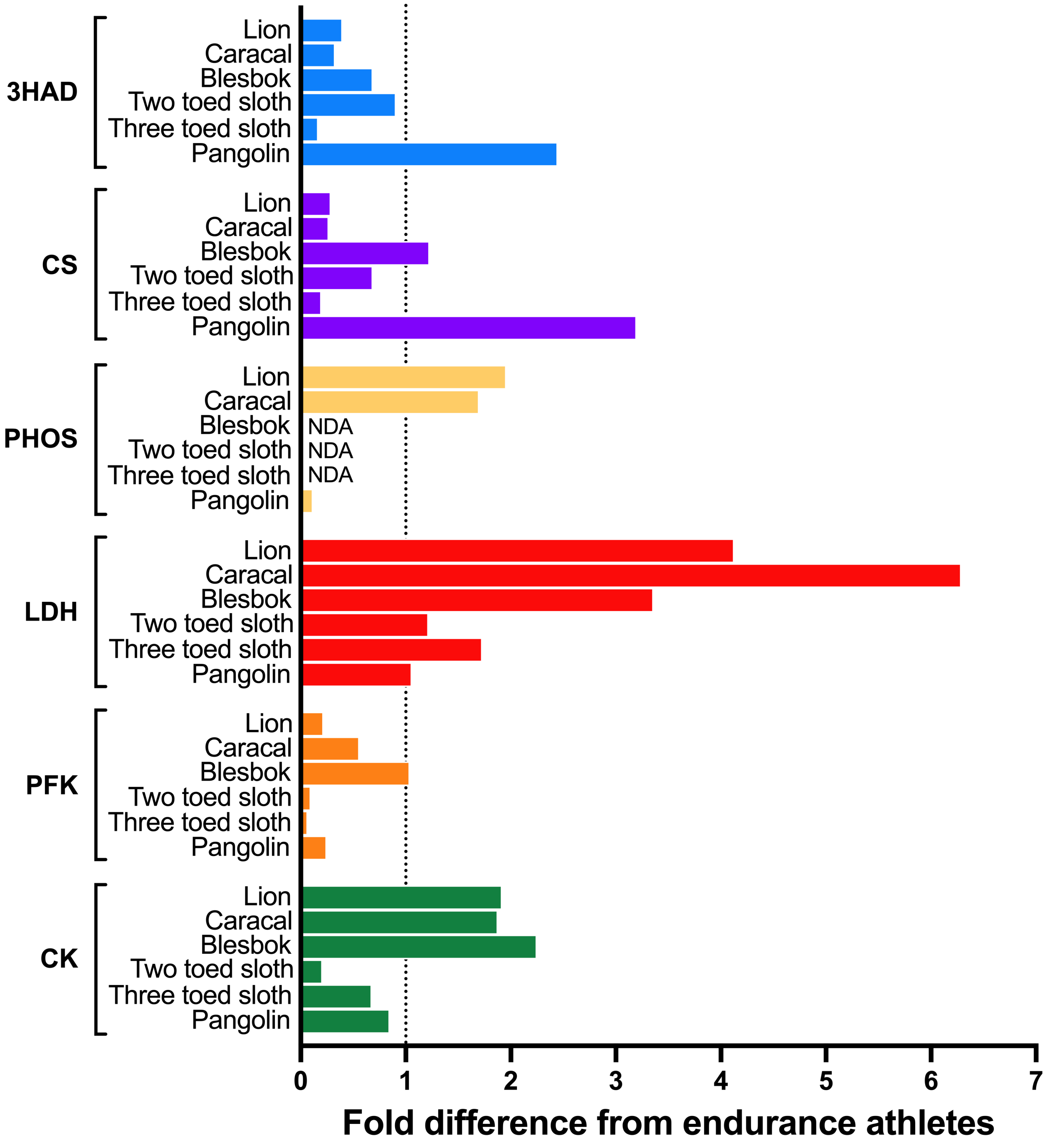


*Figure 3:* Metabolic enzyme activities of the *Vastus lateralis* of various species compared to human endurance athletes. The enzyme activities of the animals were normalized to the activities found in human endurance athletes (represented by the vertical dotted line). 3HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; CK, creatine kinase; PHOS, glycogen phosphorylase. The data of the human endurance athletes, sloths, antelope, lion and caracal are from published literature (Kohn et al., 2011a; Spainhower et al., 2021; Webster et al., 2020). No data were available (NDA) for blesbok, two-toed and three-toed sloth PHOS activities. Specific values are available in the supplementary data.



## Supplementary Data for Fig. 3

**Supplementary Table 1**: Enzyme activities and fold difference for markers of oxidative and oxygen independent (glycolytic) markers in the *Vastus lateralis* of the Temminck's ground pangolin and comparative endurance human athlete using fluorometric methods. Data obtained from the current study and a subset of data based on Webster et al. (2020).

| | **Enzyme activity (µmol/min/g protein)** | | **Fold difference from endurance human*** |
|---|---|---|---|
| **Enzyme** | **Pangolin** | **Endurance Human#** | |
| LDH | 568 | 536 | 1.06 |
| 3HAD | 225 | 92 | 2.45 |
| CS | 195 | 61 | 3.20 |
| PFK | 46 | 183 | 0.25 |
| CK | 9138 | 10754 | 0.85 |
| PHOS | 40 | 337 | 0.12 |

**Fold difference is calculated as pangolin enzyme activity/human enzyme activity. # Endurance human athlete is the reference point for each enzyme activity fold difference.*

*3HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; CK, creatine kinase; PHOS, glycogen phosphorylase;*

**Supplementary Table 2:** Enzyme activities and fold difference for markers of oxidative and oxygen independent (glycolytic) markers in the *Vastus lateralis* of the various species and comparative endurance human athlete using spectrophotometric methods. Data obtained from previous publications (Kohn et al., 2011; Kohn, 2014; Spainhower et al., 2021).

| | **Enzyme activity (µmol/min/g ww)** | | | | | | **Fold difference for each species from endurance human*** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Enzyme** | **Caracal** | **Lion** | **Three toed sloth** | **Two toed sloth** | **Blesbok (antelope)** | **Endurance Human#** | **Caracal** | **Lion** | **Three toed sloth** | **Two toed sloth** | **Blesbok (antelope)** |
| LDH | 346 | 227 | 95 | 67 | 185 | 55 | 6.29 | 4.13 | 1.73 | 1.22 | 3.36 |
| 3HAD | 2 | 2 | 1 | 5 | 4 | 5 | 0.33 | 0.40 | 0.17 | 0.91 | 0.69 |
| CS | 6 | 7 | 5 | 16 | 28 | 23 | 0.27 | 0.29 | 0.20 | 0.69 | 1.23 |
| PFK | 15 | 6 | 1 | 2 | 28 | 27 | 0.56 | 0.22 | 0.04 | 0.07 | 1.04 |
| CK | 274 | 281 | 100 | 31 | 328 | 146 | 1.88 | 1.92 | 0.68 | 0.21 | 2.25 |
| PHOS | 39 | 45 | NA | NA | NA | 23 | 1.70 | 1.96 | NA | NA | NA |

**Fold difference is calculated as various species enzyme activity/human enzyme activity. #Endurance human athlete is the reference point for the enzyme activity fold difference of each species.*

*3HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; CK, creatine kinase; PHOS, glycogen phosphorylase; ww, wet weight; NA, data not available.*