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Histological and morphometric analysis of skeletal muscle in some vertebrates

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Abstract

The skeletal system is primarily driven by the skeletal muscles to produce kinematic movements. The study evaluates the histological and morphometric properties of skeletal muscle in *Clarias gariepenus* (*Cl. gariepinus*), *Bufo bufo* (*B. bufo*), *Agama agama* (*A. agama*), *Columba livia domestica* (*C. domestica*) and *Rattus rattus* (*R. rattus*). The study was carried in order to relate the similarities and differences of skeletal muscles in these species with evolutionary trend. The epaxial muscle of *Cl. Gariepinus*, the biceps femoris muscle of *B. bufo*, *R. rattus,* puboischiotibialis of *A. agama,* and pectoral muscle from *C. domestica* were removed and assessed grossly for physical appearance then processed for histological analysis. The diameters of the muscle fibers were measured and one-way analysis of variance was used to compare the differences. The muscles of *Cl. gariepinus*, *B. bufo* and *A. agama* appeared whitish with scanty fusiform nucleus and large intermuscular space. However, the muscles of *C. domestica* and *R. rattus* appeared red with distinct round nucleus and small intermuscular space. No significant difference ($P>0.05$) was observed in the muscle diameter of *Cl. gariepinus* (8.86±0.13µm) compared to *B. bufo* (8.25±0.27µm). The muscle diameter of *A. agama* (10.18±0.25µm) was significantly higher ($P<0.05$) relative to *Cl. gariepinus* (8.86±0.13µm), *B. bufo* (8.25±0.27µm), *C. domestica* (3.38±0.13µm) and *R. rattus* (4.66±0.15µm). Conclusively, non-tetrapod vertebrates (*Cl. gariepinus*, *B. bufo,* and *A. agama*) have simple, white-colored skeletal muscle with few flat-shaped nuclei and large fiber diameters while higher vertebrates (*C. domestica* and *R. rattus*) have complex, red-colored skeletal muscle with numerous oval-shaped nucleus and small fiber diameter.

Keywords: *Clarias gariepenus*, muscles fiber, nucleus, *Rattus rattus*, vertebrates

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INTRODUCTION

The skeletal system is primarily driven by the skeletal muscles to produce kinematic movements (Buehler *et al.,* 2021). skeletal muscles are
derived from the embryonic mesodermal germ layer in a process known as myogenesis (Tani *et al*., 2020). They are striated muscles, which can be controlled voluntarily by the somatic nervous system, and consist of the cells of myocytes, fibroblasts, and adipocytes (Mukund and Subramaniam, 2020). In humans, skeletal muscles constitute 40% and 30% of total body mass in men and women, respectively (Karagounis and Hawley, 2011; Mengeste *et al*., 2021). In fishes, skeletal muscles contribute 34-48 % of the total body mass while in most mammals, they constitute 45-55% of the total body mass (Periasamy *et al*., 2017; Csapo *et al.,* 2020). The functions of skeletal muscles may vary in different species, including maintaining posture, regulating movement, diet-induced thermogenesis, and temperature homeostasis (Csapo *et al*., 2020). Skeletal muscles are believed to play important role in metabolism and can switch from carbohydrates to fatty acid utilization during prolonged exercise (Periasamy *et al*., 2017). Different criteria are used to classify muscles, they include color, location, neural control, embryonic origin, and microscopic appearance (Kardong, 2012). The color-based convention is the simplest but has to be done in conjunction with other classification methods for a better understanding of the muscle complexity.
Skeletal muscles' extracellular matrix consists of glycoprotein, elastin, proteoglycans, and collagen. Glycoproteins are proteins-carbohydrate complex. Elastin is the protein that gives muscle tissues and organs elasticity and resilience while proteoglycans give tissues the ability to withstand compressional forces (Gamblin *et al.,* 2009; Halper and Kjaer, 2014; Kristensen and Karsdal, 2016; Martínez *et al*., 2020). Collagen is the major structural protein in skeletal muscle and accounts for 1-10% of dry muscle mass (Gillies and Lieber, 2011). They provide structural support for extracellular space, serve as nerve conduits for nerve gaps and also enhance cellular interaction in adhesion and mobility (Faroni *et al*., 2014; Elanga *et al.,* 2022). Phylogenetically, all vertebrates evolved from Agnathans (jawless fishes) which are regarded as the first vertebrate (Conlon, 2013). Hence, their system becomes more complex across the evolutionary tree from Agnathans, Gnathostomes (jawed fishes), amphibians, reptiles, birds, and mammals (Donoghue and Purnell, 2009; Jones *et al.,* 2019). Because skeletal muscles play important roles in diet-induced thermogenesis and temperature homeostasis, their structural orientation might differ as a result of diet, environment, and lifestyle (Dauncey and Ingram, 1988). Structural orientation entails the orientation (strike, dip, and tilt of major axes), aspect ratio, and physiological property trends of an organ, system, or organism (Lelievre and Oldenberg, 2009; Mahrous *et al.,* 2021). Previous studies have related the structure and function of skeletal muscle in humans, pigs, rodents, and fishes; attributing that diet, aging, disuse, and/or disease as the major causes of altered muscle structure in humans and other mammals (Gillies and Lieber, 2011; Granic *et al.,* 2019; Csapo *et al*., 2020). Even with the increasing number of studies on skeletal muscle classification in vertebrates, few information is available on the histological, morphometric, and color-based classification of skeletal muscles in all the classes of vertebrae and how these features are related to function and evolutionary trends (Peters, 1989; Williams *et al*., 1997). Scott *et al*., (2001) highlighted several methods of skeletal muscles fiber classification including morphological, physiological, histochemical and biochemical. They also explained how different methods disagree and the advantages of each method. Hence, there is a need for more studies relating to the structure of skeletal muscles in all the vertebrate classes and how they are related to evolutionary trend. Therefore, the current study aimed to evaluate the histological and morphometric properties of skeletal muscles of *Clarias gariepinus* (African Catfish), *Bufo bufo* (Common Toad), and *Agama agama* (Lizard), *Columbia livia domestica* (domestic Pigeon), and *Rattus rattus* (Rat). It intends to be a continuation of the previous studies and to relate the structures of skeletal muscle in these vertebrates with evolutionary trends.

MATERIAL AND METHODS

Experimental animals

Three individuals of the following *Clarias gariepinus*, *Bufo bufo*, *Agama agama*, *Columba livia domestica,* and *Rattus rattus* were used for the study. *Clarias gariepinus* (*Siluriformes*) and *Bufo bufo* (*Anura*) were caught from Lake Alau in Maiduguri, Nigeria by local fishermen. The *Agama agama* (*Squamata*) were captured from a garden on the University of Maiduguri campus by the authors. *Columba livia domestica*

(*Columbiformes*) were purchased from the Monday market in Maiduguri while *Rattus-rattus* (*Rodentia*) were bought from the Department of Biochemistry, University of Maiduguri. The rats were bred at the Department of Biochemistry for research purposes while the other animals were kept in the animal house for 72 hours before the experiment.

Ethical approval

The research was approved by the Department of Human Anatomy ethical committee, University of Maiduguri (UM/HA/UGP19.20-115) and conducted following the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animals were anesthetized with ketamine injection before dissection and efforts were made to minimize suffering.

Surgical procedure

An incision was made in the right thigh regions of *Agama agama*, *Bufo bufo*, and *Rattus rattus*. The puboischiotibialis of *Agama agama* were excised and the biceps femoris of *Bufo bufo*, and *Rattus rattus* were excised. The pectoral muscles of *Columba livia domestica* and epaxial muscle of *Clarias gariepenus* were also excised. Color-based changes in the muscles were observed and reported. All the muscles were fixed in 10% neutral buffered formalin for 18 hours, dehydrated in graded alcohol (70%, 90%, & 100% ethanol) for 3 minutes each, embedded in paraffin wax, and sectioned at 5µm with a rotary microtome (Leica RM2125 Rotary Microtome). The sections were made halfway between the muscle origin and belly in all the samples for consistency. Tissue sections were stained with Hematoxylin and Eosin (H & E) and mounted with DPX. Micrographs were taken using a microscope camera (MBJX-ISCOPE, Los Angeles) at x400 magnification. The sizes of muscles fiber (diameter) were measured using a standardized ocular micrometer. Five microscopic slides were used for muscle diameter measurement in each group and 10 measurements were made on each slide.

Statistical analysis

Data were analyzed using GraphPad Prism 9. One-way analysis of variance was used to compare muscle fiber diameters of different animals and statistical significance was considered at 95% confidence interval. The results of muscle fiber diameter were presented as mean ± standard error of the mean (SEM).

RESULTS

Color-based classification

It was observed that Bufo bufo and Agama agama muscles were whitish. *Clarias gariepenus* muscles are also white with some strips of red fibers running along its length (Figure 1A-1C). The muscle of *Columbia livia domestica* and *Rattus rattus* were observed to be red (Figure 1D, 1E).

Histological description

The micrographs of *Clarias gariepinus, Bufo bufo* and *Agama agama* muscles revealed less distinct and scanty flat or fusiform-shaped nucleus, large inter-muscular spaces, and lacking definitive intramuscular connective tissues i.e., no perimysium (Figure 2A, 2B). The muscle bundles are smaller relative to *Agama agama* muscle bundles but larger than the *Columbia livia domestica,* and *Rattus rattus* muscle bundles. The shape of *Clarias gariepinus,* and *Bufo bufo* muscle bundles ranges from spherical to triangular (Figure 2A, 2B). The muscles of Agama agama also showed a scanty fusiform-shaped nucleus with a smaller inter-muscular septum compared to *Clarias gariepinus,* and *Bufo bufo*. The muscle bundles of *Agama agama* are larger compared to *Clarias gariepinus, Bufo bufo*, *Columbia livia domestica,* and *Rattus rattus* muscle bundles. The inter-muscular space of the Agama agama muscle is also devoid of perimysium (Figure 2C). However, the muscles tissue of *Columba livia domestica,* and *Rattus rattus,* showed multiple and distinct oval or round-shaped nucleus with smaller inter-muscular spaced and definitive muscle fascicle (distinct perimysium and endomysium). The bundles are round to fusiform shaped and closely packed together (Figure 2D, 2E).

Results of statistical analysis

Agama agama (10.18±0.25 µm) had a significantly higher ($P<0.05$) muscle fiber diameter relative to *Clarias gariepenus* (8.86±0.13 µm), *Bufo bufo* (8.25±0.27 µm), *Rattus rattus* (4.66±0.15 µm), and *Columbia livia domestica* (3.38±0.13 µm) and see Figure 3. The muscle fiber diameter of *Clarias gariepenus* (8.86±0.13 µm) was not significantly changed relative to the *Bufo bufo*

(8.25±0.27 µm) at P>0.05 (Figure 3). The muscle fiber diameter of *Rattus rattus* (4.66±0.15 µm), and *Columbia livia domestica* (3.38±0.13 µm) and were significantly lower (P<0.05) compared to *Clarias gariepenus* (8.86±0.13 µm) and *Bufo bufo* (8.25±0.27 µm) see Figure 3.

DISCUSSION

The present study has shown a gradual increase in the complexity of skeletal muscle from the simple form in *Siluriformes* to the more complex form in *Rodentia*. The changes include the shapes, size of muscle bundles, and the orientation of muscle fibers, nuclei abundance, and color change from white to red. These findings suggest that a phylogenetic relationship exists among the clades. The present study showed the skeletal muscle of *Clarias gariepenus*, *Bufo bufo,* and *Agama agama* were white with strips of red fibers running along the length of *Clarias gariepenus* muscle. Earlier studies reported that white muscle fibers were dominant in the lower class of vertebrates (Luna *et al.,* 2015; Wu *et al.,* 2018). On the other hand, *Columba livia domestica* and *Rattus rattus* muscles are red. Previously, it was reported that red muscle fibers are found in most birds and mammals (Schmidt *et al.,* 2015; Meyers and McFarland, 2016; Holecek and Mucida, 2017). Red muscles are usually associated with the maintenance of posture in mammals and flight in most birds (Rosser *et al.,* 1994; Meyers and Stakebake, 2005). The ability of red muscles to maintain posture in mammals, enhance and sustain flight in some birds, and endure fatigue in both species might be due to the presence of numerous capillaries, myoglobin, and mitochondria for proper oxygen supply and cellular respiration. This might be associated with the numerous nuclei observed in the muscle fiber of *Columba livia domestica* and *Rattus rattus* in the present study.

The muscles of lower vertebrates were reported to have less capillaries and myoglobin, they undergo glycolytic metabolism and are known as fast twitch muscles. They are also considered as fatigue intolerant muscles (Wu et al., 2018). This could be due to the few capillaries and myoglobin leading to low oxygen uptake. Hence, reduced metabolism for long-term sustenance and endurance. However, the muscles contraction rate is fast, as seen in snakes (Moon and Gans, 1998). The present study reports a few nuclei in the muscle fiber of *Clarias gariepenus, Bufo bufo,* and *Agama agama*. This might be associated with the reduced metabolic rate of muscles in these clades. The muscles of higher vertebrates (Aves, and Mammals) are oxidative muscles having rich capillaries, myoglobin, and mitochondria and are known as slow twitch/fatigue tolerant muscles (Portner, 2011). A structure has the required form and function before the biological role it will eventually serve. This affirms the concept of gradual change in design and biological role, where a structure or part works in an organism and how it serves to serves in to an environment (Kardong, 2012).

In the present study, tissue section of skeletal muscle revealed intramuscular connective tissue (endomysium) in all the vertebrates. A previous study reported that fishes use the myoseptal tendon which helps them to send a force of contraction from the trunk muscle to horizontal septum (Gemballa *et al.,* 2003). However, this is not so with perimysium, as *Bufo bufo, and Agama agama* showed less distinct perimysium while *Clarias gariepenus* lack a true perimysium (a collagen fiber in proteoglycan matrix that surrounds muscles bundles and separates them into groups (Purslow, 1989; 2020). Intramuscular connective tissue distribution varies significantly between muscles with different functions. They also vary among different clades (Kjaer, 2004; Purslaw, 1999). Intramuscular connective tissues have a wide range of functions that include providing a location for fat deposits, patterning muscle development and innervation, the transmission of contractile and erectile forces, and muscle tension stretch (Purslaw, 2020).

Clarias gariepenus, Bufo bufo, and *Agama agama* were seen to have larger skeletal muscle fiber diameters compared to *Columba livia domestica* and *Rattus-rattus*. Previous research also showed that fast twitch or glycolytic muscles (mostly found in lower vertebrates) have a larger fiber diameter when compared to the slow twitch which is mostly found among the higher vertebrates (Wu *et al.,* 2018). However, another study demonstrated a significantly larger tongue muscle thickness in *Bufo bufo* relative to *Agama agama* (Ishaya *et al*., 2022). This may be correlated with their biological activities as it relates to energy production, maintenance of posture in birds and mammals, and grasping tongue found in amphibians and reptiles.

CONCLUSION

The study is limited to color-based classification, histological and morphometric characteristics of skeletal muscle. Hence, the conclusion is based on these features. The current study revealed that *Clarias gariepinus*, *Bufo bufo*, and *Agama agama* have white muscle, lacking definitive intramuscular connective tissues with large muscle diameter and few fusiform-shaped nuclei. *Columba livia domestica* and *Rattus rattus* have red muscle with distinct intramuscular connective tissues, smaller muscle diameter, and numerous oval to round-shaped nuclei. The complexity of *Columba livia domestica* and *Rattus rattus* muscles are largely related to the abundant nuclei and distinct intramuscular connective tissues. The increasing complexity of muscles in these vertebrates may be related to the evolutionary trend from lower to higher class.

Conflict of interest

The authors declare that there are no conflicts of interest to report.

Author contributions

MSJ was involved in protocol/project development, data collection/analysis, manuscript writing. DNI was involved in protocol/project development, data analysis, manuscript writing/editing while AMOO was involved in protocol/project development, data analysis and manuscript editing.

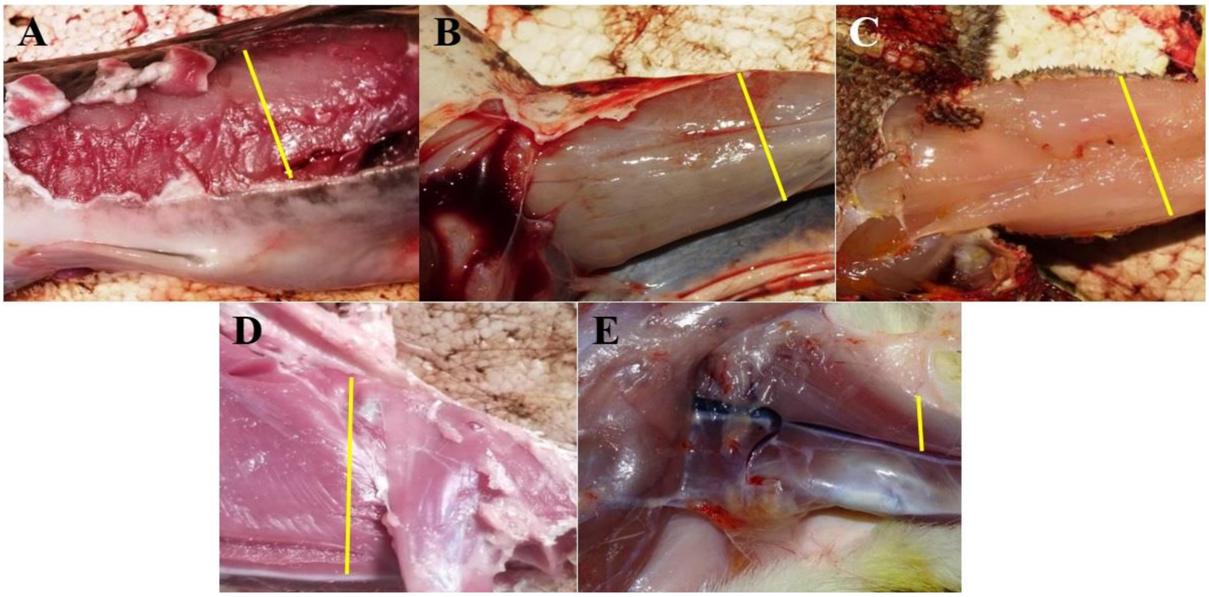

Figure 1. Color-based classification of epaxial muscles of *Clarias gariepenus* (A), biceps femoris muscles of *Bufo bufo* (B), and *Rattus rattus* (E), puboischiotibialis muscles of *Agama agama* (C), and pectoral muscles of *Columbu livia domestica* (D). The yellow lines indicate positions where histological sections were made.

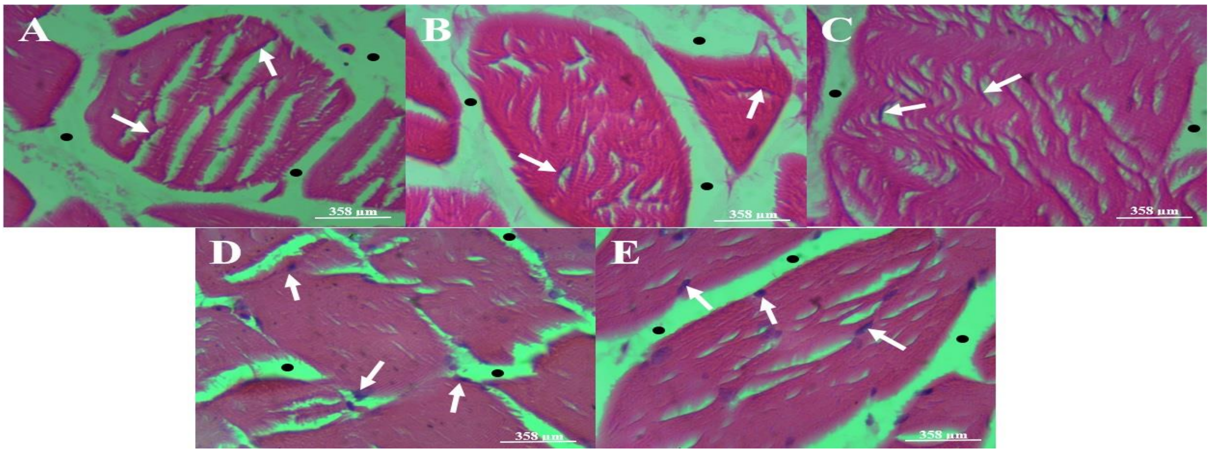

Figure 2. Histological features of epaxial muscles of *Clarias gariepenus* (A), biceps femoris muscles of *Bufo bufo* (B), and *Rattus rattus* (E), puboischiotibialis muscles of *Agama agama* (C), and pectoral muscles of *Columbu livia domestica* (D) showing nucleus (white arrows) and inter-muscular spaces (black dots). H&E x400

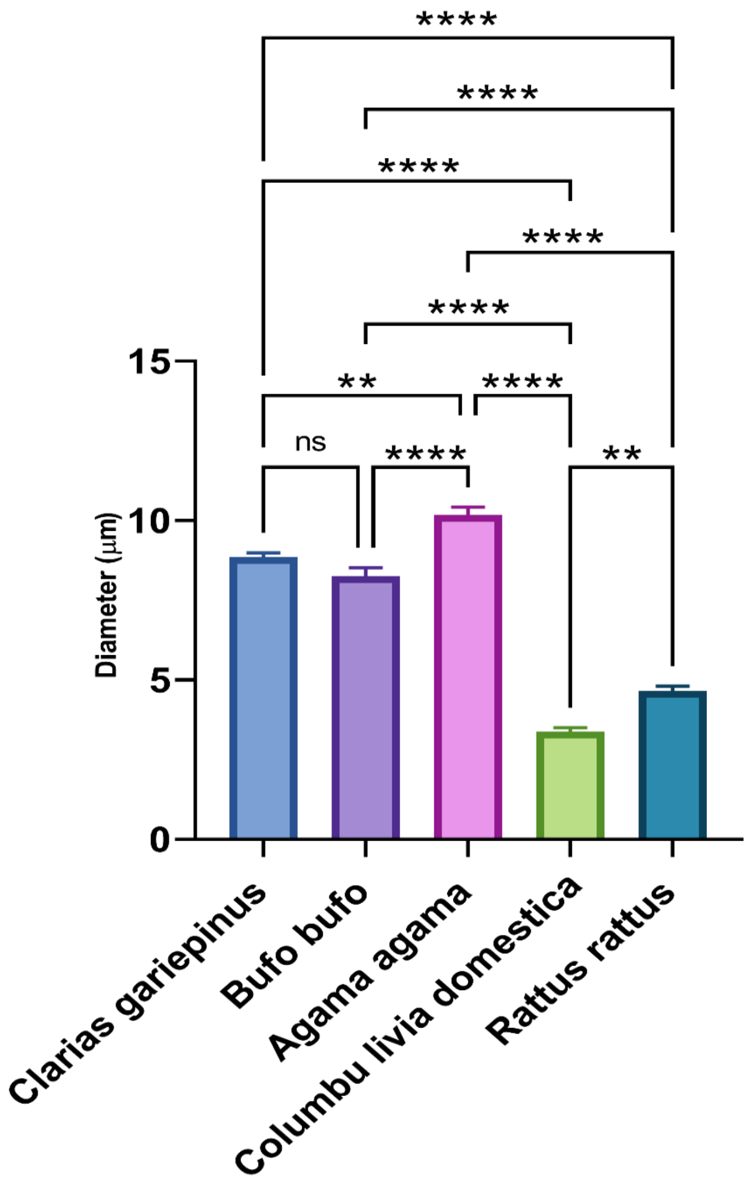

Figure 3. Muscle fibre diameters of *Clarias gariepenus*, *Bufo bufo*, *Agama agama*, *Columbu livia domestica*, and *Rattus rattus*. Values were presented as mean ± SEM. SEM= standard error of mean, ns= not significant, **=P<0.05, ****=P<0.0001, n=3

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