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Histomorphological and Morphometric Analysis of the Tongue in the Agama Lizard (*Agama Agama*), Toad (*Bufo Bufo*) and Rabbit (*Oryctolagus Cuniculus Domesticus*)

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ABSTRACT

Introduction: Comparative morphology elucidates the differences and similarities between organs and tissues in organisms. This study related the functional and nutritional qualities of the tongue with the histological and morphometric parameters on the dorsal lingual surface of the tongue in the Agama lizard, Toad and Rabbit.

Material and Methods: Histologic and Morphometric studies were carried out to determine epithelial and lamina propria thickness and skeletal muscle fiber thickness in each animal, data obtained from these measurements were statistically analyzed and P<0.05 was considered as significant.

Results: The rabbit tongue demonstrated the thickest epithelium with the length measuring 1212.09 µm2 which was significantly thicker when compared to the lizard's tongue at P<0.001. The epithelium of the lizard's tongue was found to be significantly thicker than the toad (P<0.001) and the thickness of the lamina propria in the toad was widest at 2654.28 µm2 when compared to the lizard and rabbit with a significance at P<0.001. This layer was thinnest in the rabbit (696.62 µm2). The toad had the thickest muscle fiber diameter (180.99 µm2) which was significant at P<0.001 when compared to lizard (75.50 µm2) and rabbit (125.60 µm2).

Conclusions: Histological arrangement and structure of the tongue in the selected vertebrates corresponds to dietary requirements and function.

Keywords: Comparative Anatomy; Tongue; Histology; Toad; Agama Lizard; Rabbit.

Introduction

Comparative anatomy is the study of the body structures of different species of animals in order to understand the adaptive changes they have undergone in the course of evolution[1]. It investigates the homologies or inherited similarities among organisms in bone structure and in other parts of the body [2]. Every organism's anatomical structure gradually adapts to suit their respective habitat by natural selection and mutation[3]. Comparative morphology elucidates the differences and similarities between organs and tissues in organisms.

The common Agama, red-headed rock Agama, or rainbow Agama (*Agama agama*) is a species of lizard from the kingdom Animalia, family Agamidae, phylum Chordata, class Reptilia, order Squamata, suborder Iguanla and genus Agama found in most of sub-Saharan Africa[4]. The size of Agama lizards vary from 13 to 30 cm (5.1 to 11.8 in) in total length [5]. Males are typically 7.5–12.5 cm (3–5 inches) longer than the average female. The Agama lizard can be identified as having a white underside, brown back limbs and a tail with a light stripe down the middle. The striped tail possesses about six to seven dark patches along its side. Females, adolescents and subordinate males have an olive green head, while a dominant male has a blue colored body and a yellow tail. Agama lizards are primarily insectivores, but they have been known to eat small mammals, small reptiles, and vegetation such as flowers, grasses and fruits[4,6-7].

Rabbits are small mammals in the kingdom Animalia, family *Leporidae*, phylum Chordata, subphylum Vertebrata, class Mammalia, order *Lagomorpha* and genre *Oryctolagus cuniculus*. They have long ears, short fluffy tails, and strong large hind legs [8]. They have two pairs of sharp incisors (front teeth), one pair on top and one pair on the bottom and also have two peg teeth behind the top incisors. Their teeth are specifically adapted for gnawing and grow continuously throughout their lives. *Oryctolagus cuniculus domesticus* vary tremendously in color, fur type, size and general appearance. The size of the rabbit ranges in weight from 2 to 16 pounds (1 to 7 kilograms), depending on breed[9]. Rabbits are herbivores. Their diet includes grasses, clover and some cruciferous plants. They are opportunistic feeders who also eat fruits, seeds, buds, and tree barks[8,9-10].

The common toad is an amphibian of the kingdom Animalia, family *Bufonidae*, phylum *Chordata*, class amphibian, order Anura, genus Bufo, and specie

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Bufo bufo[11]. The common toad can reach a length of 15cm. Females are normally stouter than their male counterparts. The toad's head is broad with a wide mouth below the terminal snout which has two small nostrils. They have bulbous protruding eyes with yellow irises and horizontal slit shaped pupils and they have no teeth. The head joins the body without a noticeable neck and there is no external vocal sac. Their body is broad and squat positioned close to the ground. The fore limbs are short with the toes of the fore feet turning inwards. Skin of the toad is dry and covered with small wart like lumps and the color is fairly uniform shade of brown. They have no tail. The common toad tends to be sexually dimorphic with the females being browner and males greyer [12]. The underside is a dirty white speckled with grey and black patches. Toads eat invertebrates such as insects, larvae, spiders, slugs and worms which they catch using their sticky prehensile tongues. Larger toads may also ingest slow worms, small grass snakes and harvest mice which are swallowed alive[13].

The tongue is considered to be an analogous structure being similar in different species and serving the same or similar functions in these organisms[2]. The tongue plays an important role in feeding[12]. Some animals such as frogs and toads have tongues that are specially adapted for catching prey[7,13]. Amphibians are remarkable in having a tongue that is propelled from the mouth, impacts upon a prey, adhere to it and pull the prey into the buccal cavity[14]. This role is noteworthy in reptiles as they lizard tongue is adapted to carry out several functions including prey prehension[15], prey transport, moistening and swallowing[12,16,17], in some species, the tongue possesses lingual protrusions that are used to detect species, kin and sex recognition as well courtship[18]. Taste buds which are present in the papillae of rabbits are specialized for perceiving chemical stimuli and play a role in taste transduction. The intrinsic layer muscle of the tongue are responsible for vigorous and precise movements such as prehension, lapping, grooming and manipulation of food within the mouth and speech articulation[8].

The present study was designed to relate the functional and nutritional characteristics of the tongue with the histological and morphometric parameters on the dorsal lingual surface of the tongue in the selected species.

Materials and Method

Animal Husbandry

Three Rabbits (*Oryctolagus cuniculus*), Toads (*Bufo bufo*) and Agama Lizards (*Agama agama*) were obtained for the current study from the local market and were allowed to acclimatize for two days in the animal house under standard temperature and fed with animal feed appropriate to their feeding needs.

Animal Sacrifice

The animals were brought to the animal house and injected using 0.2ml/kg ketamine hydrochloride (Abhirami Pharmachem, India) on the left thigh of each animal to induce an unconscious state. The animals were then restrained on a flat board using pins to secure their forelimbs. The tongue was accessed from the oral cavity by separating the mandible from the maxilla and freeing up the tongue from the floor of the oral cavity. The extrinsic tongue muscles were dissected out and the tongues were removed and rinsed in normal saline to remove blood and tissue residue.

Tissue Processing

A section was taken on the anterior third of the tongue of all three animals and then fixed in 10% formaldehyde to prevent putrefaction and autolysis. The tongue tissue was then dehydrated using increasing concentrations of alcohol and then cleared with xylene before being impregnated with paraffin wax and then sectioned to 5µm in thickness and then stained with Heamatoxylin and Eosin. Three animals were sacrificed per group and ten histological slides per animal were processed with sections being taken per 8 slices. A total of ten sections were taken per animal and photographed.

Histological Analysis

The micrographs of the histological sections were obtained using an Amscope light microscope (MBJX-ISCOPE, Los Angeles) which was fitted with a digital camera (M500, X 64, version 3.7). The micrographs were observed under several magnifications (40X, 100X and 400X) to photograph the entire thickness of the tongue as well as partial areas of the histological sections.

Morphometric Analysis

Morphometric analysis of the micrographs was done using computerized image analysis system ImageJ 1.53a (Wayne Rasband National Institutes of Health, USA, Java 18.0_112). The stage micrometer was used to calibrate imageJ using the same objective and pixel resolution as the micrographs being measured. In each animal, the following measurements were taken:

i. thickness of the epithelial layer (µm2) which was measured from stratum basale to stratum corneum along the length of the tissue

ii. thickness of lamina propria (µm2) was measured from the basement membrane of stratum basale to the muscular layer

iii. skeletal muscle fiber thickness (µm2) was determined by from its longitudinal orientation.

Statistical Analysis

The data obtained from these measurements were analyzed using GraphPad Prism 9.1.0. $P<0.05$ was considered significant.

Ethical Consideration

During the research work, the Authors adhered to the ethical guidelines outlined by the University of Maiduguri Research and Ethical Committee, and the National Institutes of Health (NIH) guide for the CARE and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and conformed to Directive 2010/63/EU. The present research was approved by the Ethical Committee of the Department of Human Anatomy, University of Maiduguri, with code number UM/HA/UGP 19.20-096.

Results

Morphometric Analysis of the Tongue

The rabbit tongue demonstrated the thickest epithelium as the length measured 1212.09 μm^2 which was significantly thicker when compared to the lizard's tongue at P<0.001. However, the epithelium of the lizard's tongue was found to be statistically significantly thicker than that of the toad (P<0.001) [Figure 1]. Additionally, the thickness of the lamina propria in the toad however was widest at 2654.28 μm^2 when compared to the lizard and rabbit with a significance at P<0.001. Although this layer was thinnest in the rabbit with a thickness of 696.62 μm^2 (Figure 2). The toad had the thickest muscle fiber diameter (180.99 μm^2) which was also significant at P<0.001 when compared to the lizard (75.50 μm^2) and rabbit (125.60 μm^2) [Figure 3].

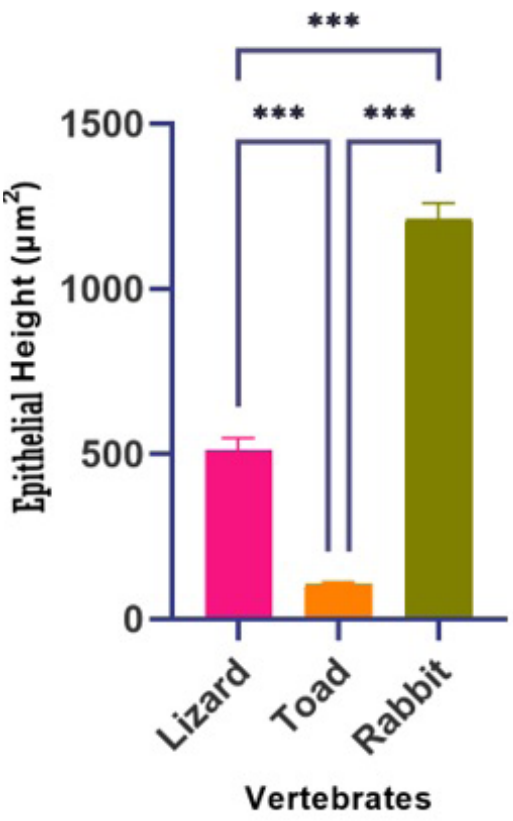

Figure 1. showing the epidermal thickness in the Lizard, Toad and Rabbit. Data presented as mean ±SEM, one way ANOVA, ***p <0.001, n=3.

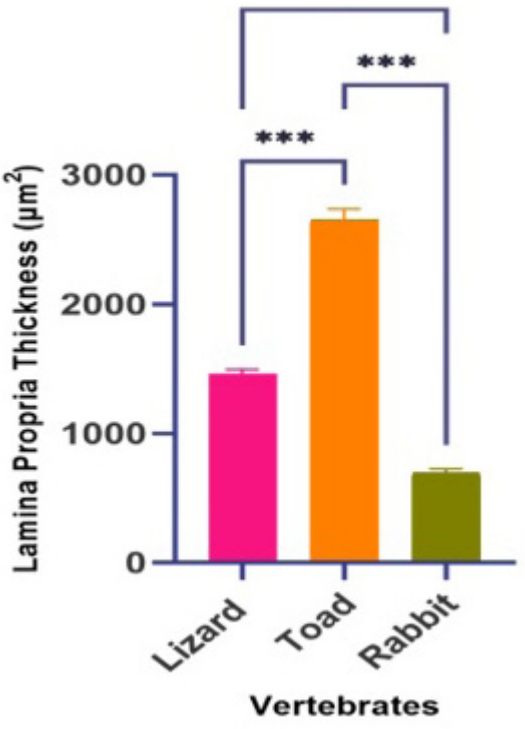

Figure 2. showing the thickness of the lamina propria in the Lizard, Toad and Rabbit. Data presented as mean ±SEM, one way ANOVA, ***p <0.001, n=3.

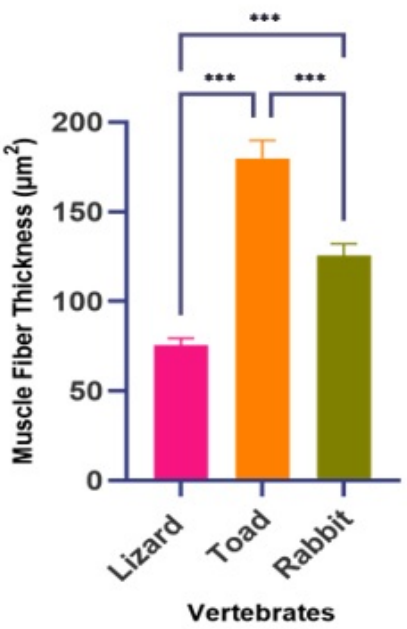

Figure 3. depicting the muscle fiber thickness in the Lizard, Toad and Rabbit. Data presented as mean ±SEM, one way ANOVA, ***p <0.001, n=3.

Histological Analysis of the Tongue

In the micrograph depicting the epithelium and lamina propria of the tongue in the Agama lizard (Figures 4A and 5A), the epithelial lining consisted of stratified layers of cells with basal layers composed of low cuboidal cells. The layers that lie above the basal layer were made up of polygonal cells that had

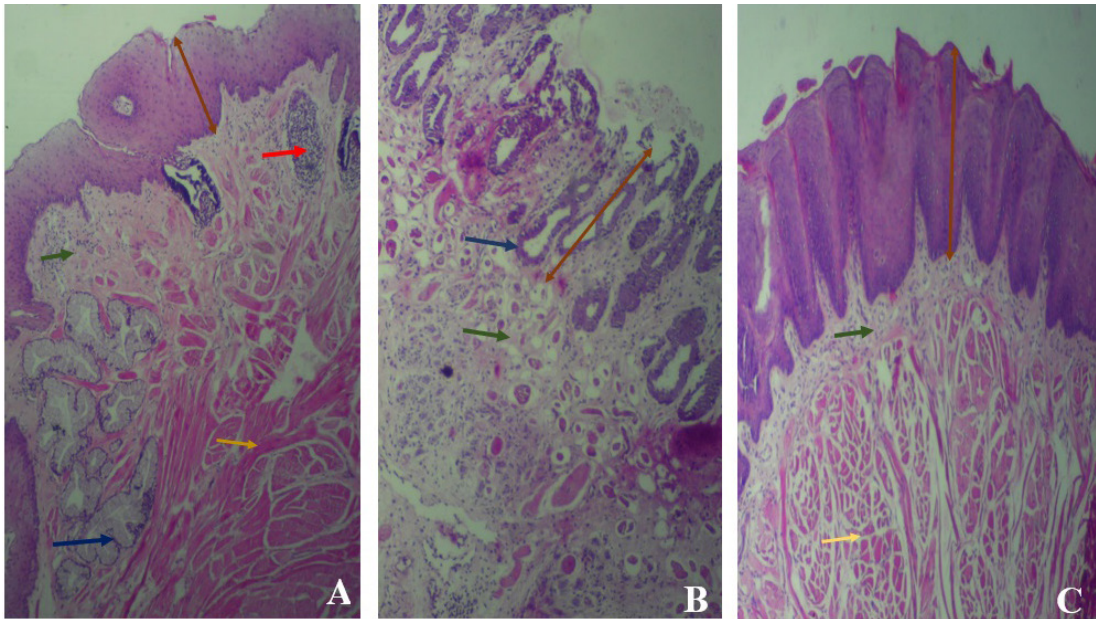

Figure 4. showing the cross section of the tongue in Agama Lizard (A), Toad (B) and Rabbit (C). Orange arrow – epidermis, red arrow – lymphoid aggregation, blue arrow – lingual gland, green arrow – dermis. H & E X100.

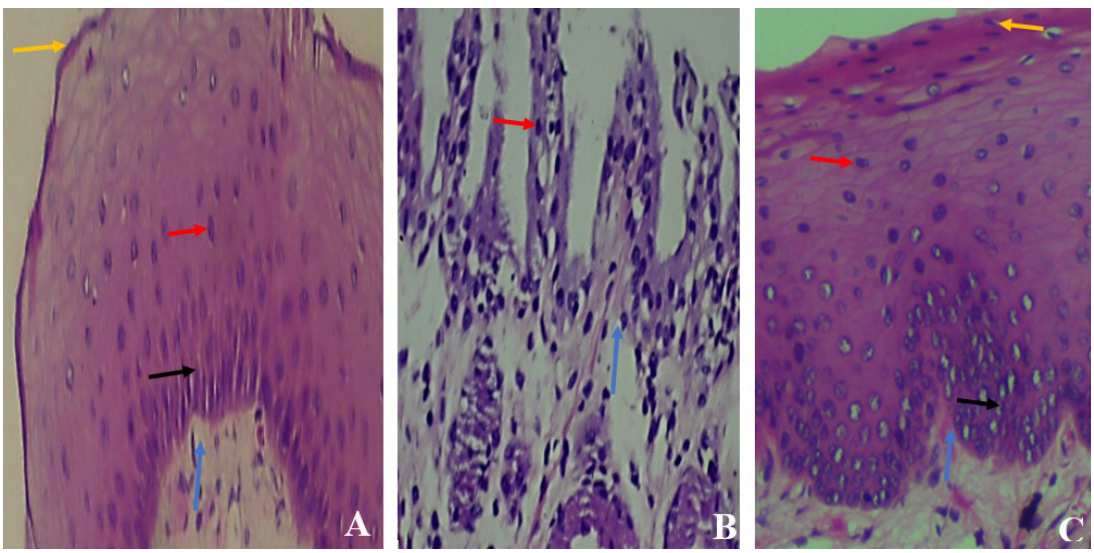

Figure 5. showing the epithelial lining of the tongue in the Agama Lizard (A), Toad (B) and Rabbit (C) showing the arrangement of cells which differ according to species. In the lizard, the cells are low columnar in the Agama Lizard and Rabbit (black arrow). Dermal pappilae also exist in the Agama Lizard and Rabbit (blue arrow). The dermal papillae is not so pronounced in the Toad. Red arrows show polyhedral keratinocytes in the epidermis of all animals. The surface of the epidermis in all three animals showed different levels of keratinization (yellow). H and E X400.

rounded nuclei at the basal region and then flattened nuclei as the cells neared the surface. The cytoplasm of the cells also displayed a flattened shape compared to the cells in stratum spinosum. The uppermost layer showed a condensation of squamous cells (Figure 5A) which was stained darkly. The micrograph of the toad showed the absence of stratified layers of squamous cells as observed in the Agama lizard and in the Rabbit. The layer above the lamina propria in the toad was covered with pseudostratified columnar epithelium and there was an abundance of simple tubular glands extending from the epithelium into the lamina propria. The surface of the tongue showed the presence of numerous filiform papillae which had the appearance of forked processes with a lamina propria core (Figure 5B). The micrograph of the tongue in the rabbit showed thicker epithelium than observed in the agama lizard. The cells at the basal layer were numerous and was thrown into numerous folds (papillae). The basal layer contained numerous cells and the outline of the cells at this later were difficult to decipher. The cells in stratum spinosum were polygonal with centrally located nuclei which were rounded in shape and basophilic. The keratinocytes retained their nuclei as they migrated towards the superficial layer and stratum corneum consisted of squamous cells which were nucleated. The nuclei however were flattened but still centrally placed (Figure 5C).

The lamina propria of the tongue in the agama lizard showed the presence of numerous lymphoid aggregates in the sub-epithelial regions scattered liberally around. Also present were several compound

tubule-alveolar glands (Figure 4A). There was a rich supply of blood capillaries in this region as well as connective tissue fibers. Slips of muscle fibers also extended into the lamina propria, the connective tissue was areolar in nature and fibroblasts as well as lymphocytes were found dispersed in the connective tissue (Figure 6A). The lamina propria of the tongue in the toad was broad, forming an expanse between the epithelial lining above and the muscular layer below it. It consisted of an areolar connective tissue which was interspersed with several simple columnar glands. There was also several capillaries as well as scattered fibroblasts as evidenced by the presence flattened nuclei and similarly, lymphocytes which had rounded nuclei. Slips of muscle fibers were also seen extending into the lamina propria (Figure 6B). The lamina propria in the micrograph representing the tongue of the rabbit showed the narrowest lamina propria. There were numerous ridges formed at this boundary and also, there was an abundance of blood vessels and connective tissue cells which extended into the dermal ridges. There were no observed slips of muscle extending into the lamina propria nor lymphoid follicles found present in this space (Figure 6C).

The muscular layer of the tongue in the agama lizard showed fascicles of muscle fibers thrown into different orientations. Each fascicle was surrounded by perimysium which also carried vascular supply too the muscle fiber. The longitudinal fibers showed striations and nuclei that were peripherally located (Figure 7A). The muscle fibers were thicker in the toad and the fibers also had a striated appearance and numerous peripheral nuclei. The perimysium was more robust and encapsulated the fascicles (Figure 7B). The muscle layer of the tongue in the rabbit showed fibers that were also oriented in many directions. The longitudinal fascicles were interposed around the transverse bundles. Individual fibers also showed striations and peripherally located nuclei similar to the fibers found in the agama lizard and toad (Figure 7C).

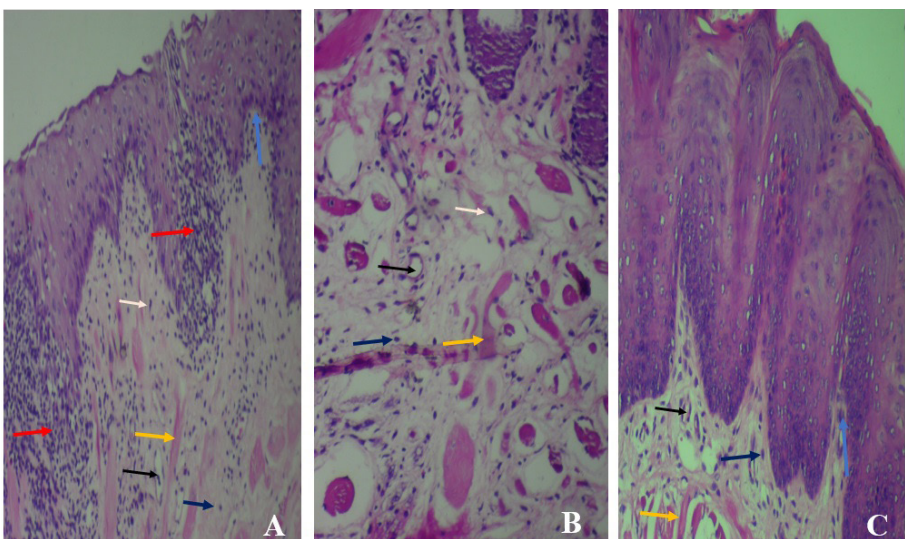

Figure 6. showing lamina propria in the Agama Lizard (A), Toad (B) and Rabbit (C) dermal papillae (blue arrows), Lymphoid aggregation (red arrow), lymphocytes (dark blue arrow) fibroblasts (pink arrow) slips of muscle fibers extending into the dermis (yellow arrow) and blood vessels (black arrow) H & E X100

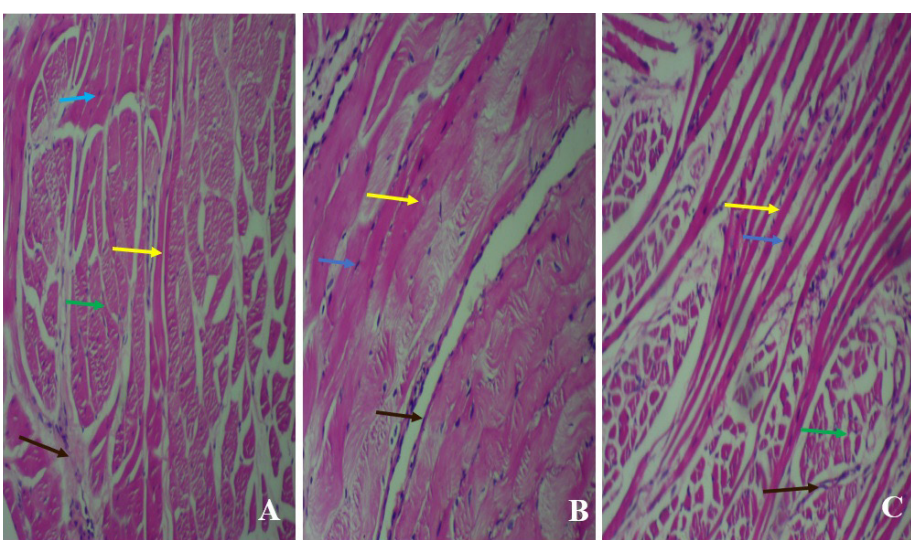

Figure 7. showing the muscle layer in the lizard (A), Toad (B) and Rabbit (C) showing the longitudinal (yellow arrow), transverse (yellow) muscle fibers with their peripherally oriented nuclei (blue arrow) and perimysium (brown arrow) surrounding muscle fascicles. H & E X100

Discussion

The tongue is an organ located in the oral cavity that plays various roles in many vertebrates. Like many organs, the structure of the tongue is adapted to suit the dietary needs of the organism. The tongue in the Agama Lizard, Toad and Lizard in the present study were arranged in three continuous layers which consists of a mucous-lined lingual epithelium bearing lingual papillae, lamina propria which is connective tissue core deep to the epithelial lining and a muscular layer which is deep to the lamina propria. The dorsal surface of the tongue bears taste papillae which may or may not have taste buds.

There are three types of gustatory papillae on the tongue in the rabbit, these are vallate, foliate and fungiform papillae; fungiform papillae are usually observed on tongues of amphibians[18-19] and reptiles[20]. In the present study, several lingual papillae were observed on this mucous layer of the tongue of the Agama lizard, predominantly fungiform papillae and fewer filiform papillae were also identified, however, taste buds were not found on these papillae. This finding is in agreement with work carried out by[20,12] also reported the presence of numerous lingual papillae in the dorsal surface of the Lizard tongue. The presence and shape of the lingual papillae in the tongue are adapted to possibly help in food intake and processing[20]. In the toad, several filiform papillae were found on the lingual surface of the tongue[12] also reported the presence of filiform papillae and mucous glands amongst the papillae in the epithelial lining which is consistent with the present study. However, taste discs which are specialized taste organs which are described as being convex discs with a thick layer of mucous cells separated by apical sheet prolongations of wing cells were not observed in the present study, neither were the epithelial cells of the filiform papillae ciliated. The surface of the rabbit tongue was roughened with the presence of several papillae. Fungiform and filiform papillae were observed with a lamina propria core. No taste buds were observed in the current study[21] also reported the presence of numerous fungiform papillae but did not report any taste buds in the lingual papillae.reported the existence of not only filiform and fungiform papillae in the dorsal lingual surface but also circumvallate and foliate papillae which were arranged in two rows on the dorsal surface of the tongue. The coarseness of the dorsal surface of the tongue due to the presence of taste buds serves to increase surface area and also plays an important role in taste sensations in the rabbit[22] stated that the density and roughness of the papillae assists the in grooming and movement of food towards the pharynx as well as provide an additional protection for the tongue.

In the current study, the tongue in the Agama lizard presented a layer of keratinized lingual epithelium which was arranged in layers of squamous cells with lingual papillae on the superficial surface of the mucous membrane on the dorsal surface of the tongue. These adaptations are suited for the dietary needs of the lizard which are varied but mostly insectivorous, although vegetation also forms a large part of the diet of some lizards[23]. The keratinized epithelium plays a protective part, preventing abrasion on the lingual surface of the tongue during feeding. This was also found in the lingual epithelium of the rabbit where there was a layer of thickened epithelium which had a cornified surface layer although, the coarseness could vary depending on the type and degree of contact with food[8]. The lingual epithelium in toads did not comprise of stratified epithelium but consisted of columnar or low columnar cells and several glands. This is consistent with studies conducted by[17] who reported that the epithelium was covered by a non-keratinized layer of cells which were pseudostratified and ciliated at the apical border. In the present study however, ciliated apical cells were not observed. The epithelium of the toad is adapted for food recognition and recognition of prey[24]. The morphometric analysis of the current study confirms the histological observations in the micrographs with the epithelial lining of the rabbit was thicker than in the Lizard and Toad.

The Agama lizard uses its tongue as a prehensile object during feeding and consequently used for food capture. There is a requirement of a muscular tongue to carry out these movements which include well-formed extrinsic and intrinsic lingual muscles. In the present work, the micrograph featuring the Agama lizard tongue showed muscle fiber bundles which were oriented in several orientations along the entire thickness of the tongue mostly observed as longitudinal and transverse arrangements[23] observed the same arrangement in the tongue of Agama lizard however, it was reported that in addition to the transverse and longitudinal arrangement of muscles, there was a ring muscle in the posterior-most region of the tongue which surrounds the lingual process of the hyoid bone[23] also recorded that the muscle fiber bundles were surrounded with connective tissue which was well developed dorsally and was invaded by glandular tissue dorsally and laterally. These observations were not present in the present study as the serous glands observed were localized within the lamina propria and did not invade into the muscular layer. There was also numerous lymphoid follicles located in the lamina propria which was not reported in the study mentioned above, the lymphoid aggregations may play a role in immune functions. The toad. The rabbit tongue muscles in the current study was robust and consisted of muscle bundles oriented in transverse and longitudinal orientation which aids in a varied mobility of the tongue necessary for phonating, chewing, and swallowing. This is also as proven by[17] who stated that the tongue muscles in the rabbit were so organized

to enable the dorsum of the tongue to harden for pressing food during mastication as well as shifting the food backwards to facilitate swallowing. The toad muscle is well formed to facilitate the feeding habits of the frog which involves it being propelled from the mouth and to capture prey and pull it back into the oral cavity. The underlying lamina propria tissue bed is made up of areolar connective tissue which is wider when compared to the other vertebrates in the current study which allows the tongue to slide in and out of the oral cavity

Conclusion

The structure of the tongue relates to the feeding patterns of the animal in the current study. The tongue has a basic arrangement consisting of the lingual epithelium changing from stratified squamous epithelium in the lizard and rabbit and a simple epithelium in the toad. The muscular layer was well developed in the toad when compared to the rabbit and lizard to enable the thrusting motion of the tongue used in prey capture.

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