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Comparative Gross Morphometry and Histomorphology of Brain and Testes in Thomas's Rope Squirrel (*Funisciurus anerythrus*) and Gambian Sun Squirrel (*Heliosciurus gambianus)*

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Abstract

Squirrels have attracted many interests from many scientists as a model for research. There are several species of squirrels found across the globe; however, different species are found in specific locations. This study examined the Thomas'-Rope-Squirrel (TRS) and Gambian-Sun-Squirrel (GSS), mostly found in West Africa and Central Africa. There is paucity of information on the brain and testicular morphometry in these species. Therefore, the aim of this study was to compare the gross morphometric and histological descriptions of the brain and testes between TRS and GSS. Comparing five adult males from each species, the study found that TRS had smaller brains but significantly larger testes relative to their body size compared to GSS. Brain length differed between the species, but brain width was similar. The observed macromorphological variation in size and weight of testes could be attributed to the number of seminiferous tubules and degree of cellularity within the testis of each species. The olfactory bulb and testes of both species displayed similar histological features. In the TRS, the internal-plexiform-layer (IPL) was not easily differentiated from the mitral cell layer except when observed at a higher magnification. The subventricular-zone was evident in the TRS but not noticeable in the GSS. TRS showed a higher testicular cell density compared to the GSS. The findings from the present study show significant differences in body, brain and testicular weights of the two tree squirrels. The similarities observed in histological patterns of the organs further suggest conservation of features across rodent species.

Keywords: gross morphometry, histomorphology, brain, testes, tree squirrels

INTRODUCTION

The squirrel is a rodent that has allured many researchers with its possession of several adaptive features. Various species of squirrels are found across the globe, which have been great models for evolutionary studies. Squirrels usually differ in size, the appearance of their fur, speed and the nature of their habitat [1]. The Gambian Sun Squirrel (*Heliosciurus gambianus*) [2] and Thomas's Rope Squirrel (*Funisciurus anerythrus*) [3] are two tree squirrels commonly found in the southwestern part of Nigeria[4]. Kingdon described Thomas's Rope Squirrel (TRS) to have weights ranging from 200 g to 220 g, head and body lengths ranging from 16 cm to 23 cm and a tail length of 13 cm to 20 cm [4]. On the other hand, Gambian Sun Squirrel (GSS) weighs (between 250 g and 350 g) higher than the TRS. In addition, the head and body length (between 17 cm and 27 cm) and the tail length (between 18 cm and 26 cm) are more extended in GSS than in TRS.

The brain and testes share several molecular characteristics [5]. Though both are made up of two distinct tissues with very divergent purposes, when compared, they have several similarities extending from the genetic to histocellular aspect. The brain and testes are composed of distinct types of cells that function jointly in maintaining the rectitude and physiological state of the tissue. For example, the astrocytic and Sertoli cells provide biochemical support to the brain and testes. They do this by providing physical and nutritional support needed by neurons and germ cells, which is vital for the development and survival of these organs. Both brain and testes are high-energy-demand tissues, performing energy-demanding functions such as intellectual functions and spermatogenesis, respectively. Compared to other tissues, the brain and testes are more predisposed to oxidative assaults due to their high energy and oxygen demands [5,6].

A few decades ago, the Leydig cells of the testes were recognized as members of the neuroendocrine system [7]. The synthesis and release of a large number of biologically active substances typical for nerve and neuroendocrine cells has revealed that Leydig cells are neuroendocrine cells [6].

Previous work on the squirrel brain morphometry was described in African Striped Ground Squirrel (*Xerus erythropus*) by Abiyere *et al* [8]; however, there is dearth of information on brain morphology of GSS and TRS. Coker *et al* [9] reported testicular weights in both GSS and TRS but didn't describe morphometry of the testes in these tree squirrels. We noticed something unique about these two species of tree squirrels; the Gambian Sun Squirrels are larger but have smaller testes than Thomas's Rope Squirrels, which are smaller in body size but possess larger testes. Therefore, the study aimed to compare the gross morphometric description of both brain and testes of these two species of squirrels.

METHODS

Experimental Animals

Five (5) adult male TRS and five (5) adult male GSS were used for this comparative investigation. These squirrels were obtained by hunters within the University of Ibadan, in a dense tree area. On arrival of the squirrels, they were acclimatized for about two hours at the animal house of the Neuroscience Unit, Department of Veterinary Anatomy, University of Ibadan, to ensure that the animal was stabilized from the stress associated with moving them to the lab. The animal experiment was approved (NVRI/AEC/03/11622) by Animal Care Committee of National Veterinary Research Institute, Vom, Nigeria.

Extraction of Brain and Testes

The animals were anaesthetized with a combination of xylazine and ketamine (ketamine 100 mg/kg body weight and xylazine 10 mg/kg body weight). The animals were humanely sacrificed and the brain and testes were dissected out carefully based on methods described by Gilbert *et al.* [10] and Omirinde *et al.* [11]. The weight, length and width were measured and recorded.

Measurement of Length and Width of the Brain and Testes

Weight of the brain was obtained using Electronic Balance FA2004B (Shanghai York Instrument Co. Ltd., China) whereas length and width of the brain and testes (Figure 1) were obtained using stainless steel Electronic Digital Caliper K-319 (Kales Tool Industry & Trade Co., Ltd., China). The length of the brain is represented by a rostrocaudal distance from the olfactory bulb to the cerebellum. The width is measured as the distance between the lateral edges of the brain's cerebral hemispheres. The testes length was measured as distance from its anterior to posterior borders, while the width was determined by measuring the lateral edge distance. The measurements were recorded and subsequently used in calculating the organo-somatic index of the brain and testes by dividing organ weight (brain weight and testes weight) by body weight multiplied by 100, as described by Gilbert *et al*. [12].

$$\frac{\text{Weight of organ}}{\text{Weight of body}} \times 100$$

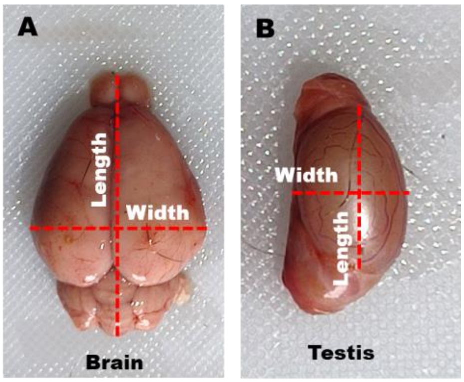

Figure 1: Photographs of the brain and testis of the squirrel showing the measurement of the length (vertical broken red lines) and width (horizontal broken red lines). **A.** The brain. **B.** The testes.

Histology and Histomorphometry

The fixed tissues of the olfactory lobe and testes were embedded in paraffin wax and sections were cut into 5 µm thick sections with microtome. Basic histological staining was carried out using Haematoxylin and Eosin (H&E) as described by Gilbert *et al.* [13]. The stained sections were visualized using microscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera.

Statistics

Data were expressed as Means ± SEM, and the differences among groups were considered significant at p-value < 0.05. Data were analyzed using Excel software. Descriptive statistics and unpaired T-tests were carried out using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego).

RESULTS

Gross Examination of the Brain and Testes

Grossly, the testes of the TRS with the smaller body were distinctly larger compared to the testes of the GSS, which has a larger body size (Figure 2A - 2D). After careful extraction of the brains, we noticed that the brains of the TRS, being a smaller species, was less than the brains of GSS, which is a larger specie (Figure 2E - 2F)

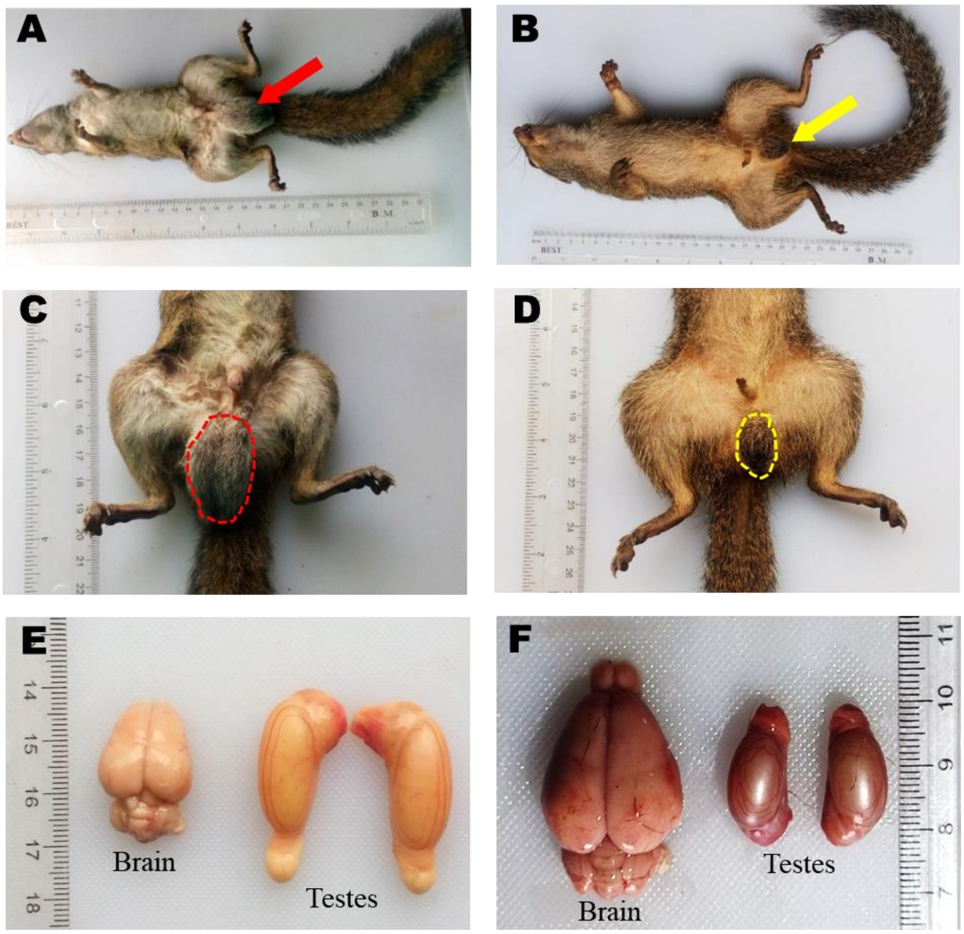

Figure 2: Photographs of TRS and GSS showing their brains and testes. **A&C**. Ventral view of TRS showing the testes (Red arrow and red-broken lines). **B&D.** The testes (yellow arrow and yellow-broken lines) of GSS. **E.** Extracted brain and testes of the TRS. **F**. Extracted brain and testes of the GSS.

Examination of Body, Brain and Testes Weights

Table 1 shows the mean body weight, the mean absolute brain weight and the mean absolute testes weight of the adult male TRS and GSS. The adult male TRS examined had mean body weight (166.60±8.30g) that is significantly less than the mean absolute body weight (250.90 ± 10.86g) of the GSS. In the TRS, the mean absolute brain weight (3.56 ± 0.23g) was significantly less than the mean absolute brain weight (5.30 ± 0.19g) of the GSS. On the other hand, the mean absolute testes weight (5.16 ± 0.29g) of the TRS was significantly larger than the mean absolute testes weight (2.18 ± 0.22g) of the GSS.

The organo-somatic indices show that the mean relative brain weight is comparable between GSS (2.14±0.88g) and TRS (2.12±0.01g). As body size increases, there is a concurrent augmentation in brain weight. However, the testes weight of TRS exhibits an exponential increase with the body weight, as evident from the mean relative testes weight (3.10±0.01g). In contrast, when we compared the mean relative testes weight of GSS (0.87±0.02g) to that of TRS, it becomes apparent that the testes in GSS do not exhibit a proportional increase in weight with body size (Figure 3A).

Table I: Mean values of body weight, relative brain weight and relative testes weight in adult males of TRS and GSS (Mean ± SEM)

Parameters (Grams, g)	**TRS (n = 5)**	**GSS (n = 5)**
Mean body weight (g)	166.60 ± 8.30	250.90 ± 10.86
Mean Absolute brain weight (g)	3.56 ± 0.23	5.30 ± 0.19
Mean Absolute Testes weight (g)	5.16 ± 0.29	2.18 ± 0.22

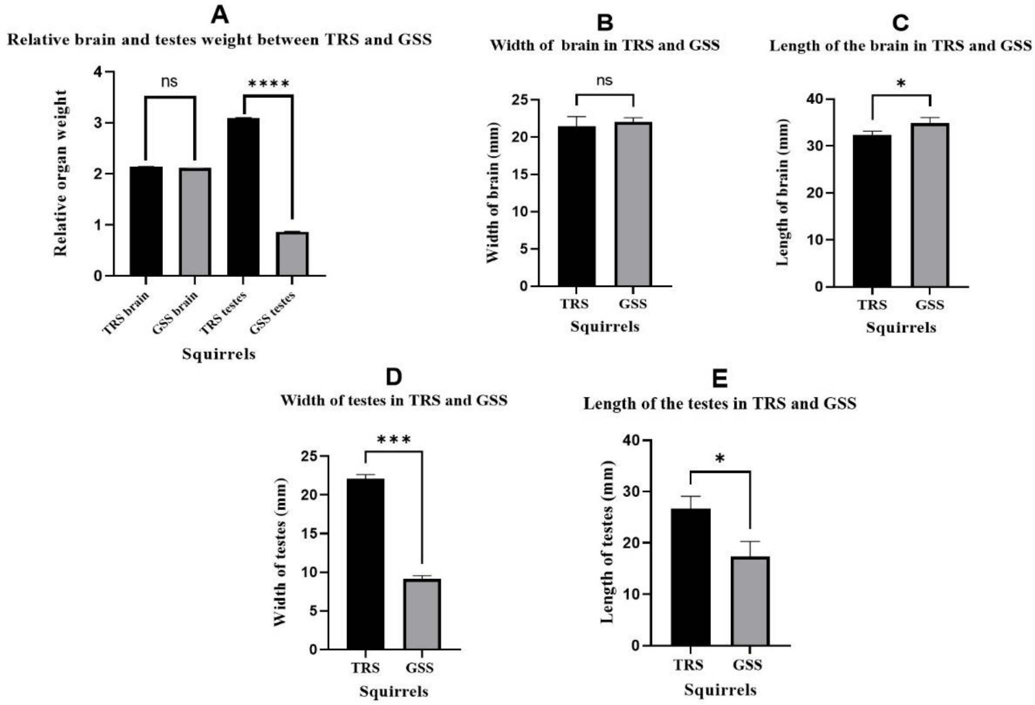

Figure 3: Bar diagrams showing comparison of brain and testes weights, lengths and widths in TRS and GSS. **A.** Showing the comparison of relative brain and testes weights between TRS and GSS. **B&C**. Showing the comparison of brain length and width between TRS and GSS. **D&E.** Showing the comparison of testes length and width between TRS and GSS. Values are expressed as means ± SEM and were analyzed using the Student t-test. * p<0.05, ** p<0.01, **** p<0.0001

Gross Morphometric Examination of the Brain and Testes

The gross morphometric investigation showed that the brain's mean length is 32.37±0.39 mm in TRS which is significantly less than the mean length reflected as 34.62±0.67 mm in GSS (Figure 3C). However, there was no statistically significant variation between the mean width recorded as 21.49±0.64 mm in the brain of TRS and mean brain width of 22.05±0.32 mm in the GSS (Figure 3B). The testes in the TRS had a mean length of 26.67±1.34 mm which was significantly greater than the 17.37±2.05 mm recorded in GSS (Figure 3E). Furthermore, the TRS testes width recorded as 11.59±0.96 mm was significantly greater than the testes width of 9.12±0.30 mm in the GSS (Figure 3D).

Histological Examination of Olfactory Bulb in the GSS and TRS

The microscopic examination in this study unveiled the layers of the olfactory bulb in both Gambian Sun Squirrel and Thomas' Rope Squirrel. These layers, when observed from the outermost to innermost regions, consist of the olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layers (MCL), internal plexiform layer (IPL), granular cell layer (GCL) and the subventricular zone (SVZ). The olfactory nerve fiber layer (Figure 4G & 4H) comprises the axons of sensory neurons from the olfactory epithelium. These fibers are densely packed and consist of thin axons that enter the olfactory bulb from the olfactory mucosa. The glomerular layer is composed of numerous acellular synaptic islands, referred to as glomeruli (Figure 4A & 4B), which are generally spherical to oval in shape. These glomeruli are arranged in one or two rows located internally to the ONL. Surrounding the glomeruli, multiple interneuron juxtaglomerular cells (JG cells) or periglomerular cells (Figure 4G & 4H) can be observed. In the external plexiform layer (EPL), there are sparsely distributed small to medium-sized multipolar tufted cells (Figure 4C & 4D). Mitral cells (Figure 4E and 4F), characterized by triangular or multipolar cell bodies, are the largest cells in the olfactory bulb and are arranged in a single row within the MCL, marking the boundary of the EPL. The granule cell layer (GCL) primarily consists of small interneuron granule cells without axons. These cells are arranged in multiple compact parallel layers (Figure 4E & 4F), although some scattered granule cells (Figure 4C & 4D) are also commonly observed among the mitral cells in the MCL. In the TRS, the IPL located between the MCL and GCL is not easily distinguishable from the MCL, but with higher magnification, the IPL becomes noticeable. The SVZ is evident in the TRS but is not visible in the GSS. Overall, the histological structure of the olfactory bulb in both the GSS and TRS is quite similar.

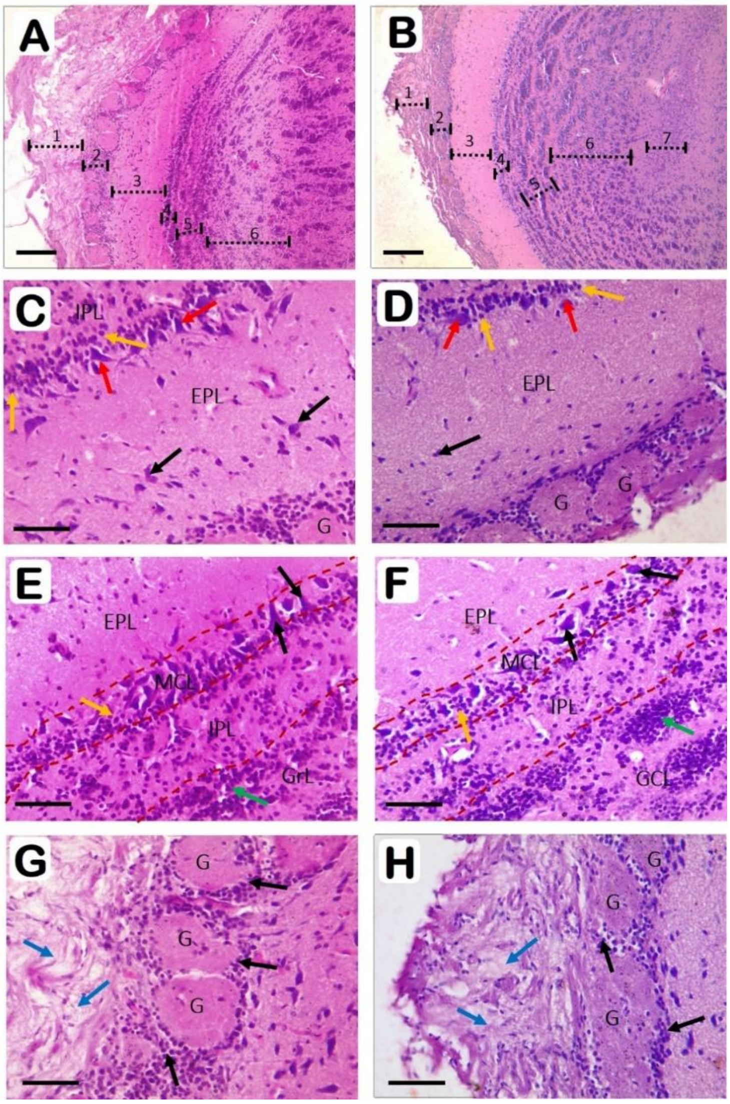

Figure 4: Longitudinal section of the main olfactory bulb of GSS (A,C,E&G) and TRS (B,D,F&H) stained with Haematoxylin and Eosin (H&E). **A&B.** Layers of olfactory bulb from superficial to deep the following layers are identified. 1. Olfactory nerve layer (ONL), 2. Glomerular layer (GL), 3. External plexiform layer (EPL), 4. Mitral layer (ML), 5. Internal plexiform layer (IPL), 6. Granular cell layer (GCL), 7. Subventricular zone (SVZ). **C,D,E&F.** Higher magnification of the olfactory bulb showing the mitral cells (red arrows) and granular cells (yellow arrows) along the MCL. Tufted cells (black arrows) were seen in the EPL. Granular cells aggregate (green arrows) were also observed in the GCL **G&H.** Higher magnification showing the glomeruli (G) which are surrounded by periglomerular or juxtaglomerular cells (J-cells) (black arrows). The axonal fibers (blue arrows) from the olfactory epithelium were observed. (Stain: H&E; Magnification: A&B x10, C,D,E,F,G&H x40; Scale bar: A&B 100 µm, C,D,E,F,G&H 50 µm)

Histological Examination of the GSS and TRS Testes

The testes of both GSS and TRS showed numerous seminiferous tubules of varying sizes covered by a fibrous testicular capsule (Figure 5A - 5D). Observed among adjacent tubules were the interstitial spaces which contain blood vessels (bv) as well as interstitial cells (ic). The germinal epithelium of the seminiferous tubules showed spermatogenic cells arranged in successive layers, from the immature spermatogonium (Sg) on the basal lamina to the primary spermatocyte (pSc) and spermatid (Sd) towards the adluminal compartment. This pattern was observed in both species; however, there was a considerable difference in the density of cells and compactness of the seminiferous tubules between TRS and GSS in that the former (Figure 5B & 5D) showed a higher cell density compared to the latter (Figure 5A & 5C).

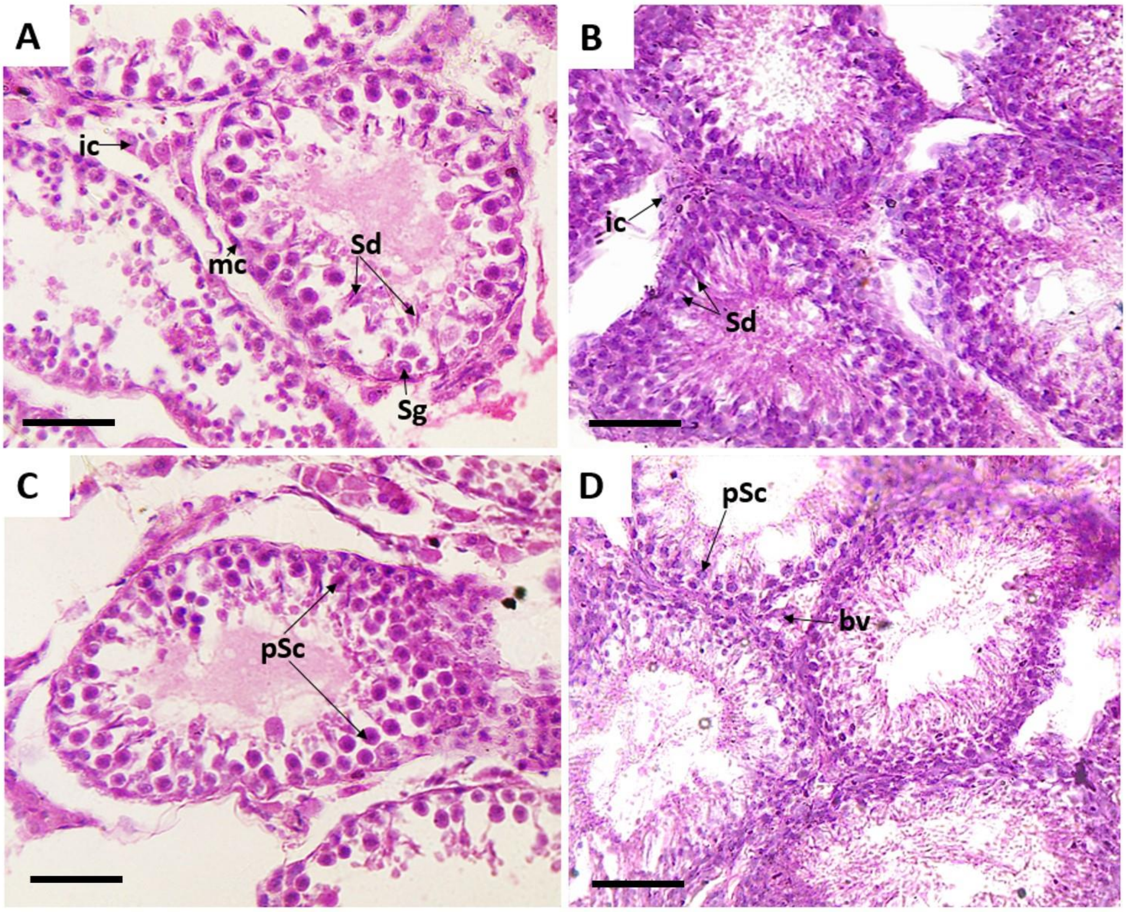

Figure 5 Photomicrographs of the testes of GSS (A & C) and TRS (B & D). **A**&**C**: showing seminiferous tubules of GSS with spermatogonium (Sg) and primary spermatocyte (pSc) located within the basal compartment while the late spermatid (Sd) was seen close to the luminal compartment. Myoid cell (mc) was also seen around the basal lamina of the seminiferous tubule as well as interstitial cell (ic) within the testicular interstitium. **B**&**D**: showing the seminiferous tubules of TRS tightly packed with spermatogenic cells at different stages of maturation. Within the seminiferous tubules were primary spermatocytes (pSc) and spermatids (Sd); interstitial cells (ic) and blood vessel (bv) present in the interstitial spaces (Stain: H&E; Magnification: A – D x40; Scale bar: A – D 50 µm)

DISCUSSION

Morphometry is notable for its use in investigating evolutionary changes and identifying developmental alterations during formation. It is also used to quantify the degree of mutations and evaluate genetic variation for single or multiple traits [1,14]. The comparisons between the current morphometric data and previous data can give an insight into the effect of the changing world on biodiversity.

The higher body weight observed in adult male Gambian Sun squirrel (GSS) as compared to adult male Thomas's Rope Squirrel (TRS), which was statistically significant, is in agreement with the report of Coker *et al.* [9]. Coker and his team reported *Heliosciurus gambianus* (Gambian Sun Squirrel) as significantly bigger than *Funisciurus anerythrus* (Thomas's Rope Squirrel) in the male and female species. Abiyere *et al.* [8] showed the mean brain weight of the African Striped Ground Squirrel (*Xerus erythropus*) to be 7.32 ± 0.25g which is higher than the brain weights of both GSS (5.30 ± 0.19g) and TRS (3.56 ± 0.23g) in our study. This may be attributed to the higher body weight (488.89 ± 7.89 g) of the ground squirrel reported by Abiyere and his team. The testes of the TRS in our study are distinctly greater in size when compared to the testes of GSS. This is similar to what was reported by Coker *et al*. [9], who showed TRS had testes that weighed higher than the GSS testes. The brain of GSS is slightly bigger than that of TRS, both in length and width, however, Abiyere *et al.* [8] reported a higher brain length in the African Striped Ground Squirrel. The brain length in the TRS, GSS and the ground squirrel is directly correlated to their body size. Both testes length and testes width parameters were higher in the TRS when compared to GSS, despite the bigger body size of GSS over TRS.

In a study by Pierce *et al.* [15] in voles (mouse-like rodent), in which among three species of voles studied, meadow volves with largest testes appeared to ejaculate more spermatozoa than pine volve having smallest. In addition, Pierce and team, suggested that difference in testes mass and total number of sperms may be related to the occurrence of multiple mating by females during a single receptive period. Huang and Johnson [16] showed how increased testes size in boars was an effective way for increasing concentration of sperm in the semen and total number of sperm per ejaculate. Huang and Johnson went further, to recommended inclusion of testes size among criteria for selecting sire lines to be used in artificial insemination activities. Kenagy and Trombulak [17] discussed hypothetical factors that may account for the evolution of testes, these factors are toxon, mode of locomotion, position of testes in the body, mating system, population density and structure and timing of mating.

Based on the histological findings, the olfactory bulb of both the GSS and TRS showed contained the olfactory nerve layer, glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer and granular cell layer. Similar results were reported in rabbits [18,19], African giant rat [20], mouse [21], rat [22]. The histoarchitectural pattern of the testes of the GSS and TRS is similar to previously described features in rodents [23,24,25,26]. The observed gross variation in testicular size and weight between GSS and TRS could be attributed to the number of seminiferous tubules as well as degree of cellularity within the testis of each species. In a population study carried out in the Botanical Garden of the University of Ibadan by Olajesu [27], it was reported that out of a sample size of eighty-seven squirrels, three species were identified. The *Funisciurus anerythrus* species represented the highest in population among the three species; *Funisciurus anerythrus* (48.7%, 20.5/km), *Heliosciurus gambianus* (33.3%, 1.5/km) *and Xerus erythropus* (18.4%, 8.5/km). Breed and Taylor [28] reported a causal relationship between relative testes size and breeding pattern in some murine rodents across Asia, Australasia and Africa. The abundance of TRS in the survey could suggest a higher reproductive capacity and mating system of this squirrel species. This seemingly correlated with the morphometric data presented in this study: TRS having significantly higher morphometric testicular values relative to GSS

In conclusion, comparative study of morphometric data among different species of squirrels provides valuable insights into their biology and reproductive strategies. Furthermore, microscopic examinations revealed similarities in the histoarchitectural patterns of the olfactory lobe and testes of TRS and GSS, suggesting conserved features across rodent species. Overall, this study underscores the importance of morphometric and histological analyses in shedding light on the complexity of evolutionary and reproductive adaptations in wildlife populations.

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AUTHOR CONTRIBUTIONS

The individuals mentioned earlier played roles in formulating, designing, executing, analyzing and producing the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

DATA AVAILABILITY

Data will be made available on reasonable request by the corresponding author.

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