

## Microanatomy of the Testes and Male Genital Ducts of Banded Krait *Bungarus fasciatus* (Schneider, 1801)

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### Abstract

The Banded krait is a species of venomous snakes that ranks among the top 5 in Thailand, with a significance role as a predator for controlling other animals. Despite numerous studies on the ecology, the microanatomy of body systems has still not reported clearly. The present study was to investigate the microanatomy of the testes and male genital ducts of Banded krait that collected from Phatthalung Province, Thailand. The snakes were collected after anesthetized, and their testes and genital ducts were fixed in Bouin's fixative. The reproductive organs were collected during July to October 2022, and were sectioned of 5- $\mu$ m-thick pieces for staining with Harris's hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), alcian blue (AB) pH 2.5 and pH 1.0, bromophenol blue (BB) and Masson's trichrome (MT). The testes contained the seminiferous tubules with 7 stages of germ cells: (I) type A spermatogonium, (II) type B spermatogonium, (III) primary spermatocyte, (IV) secondary spermatocyte, (V) round spermatid, (VI) elongated spermatid and (VII) spermatozoa. Besides, Sertoli and Leydig's cells were appeared in the seminiferous tubules and the interstitial area, respectively. During the sampling period, the spermatozoa were observed in the seminiferous tubules and genital ducts. The extra-testicular rete testis, ductuli efferentes and ductus epididymis were embedded in the epididymal sheath. The epithelial cells of rete testis, ductus epididymis, as well as ductus deferens possessed microvilli, whereas those of the ductuli efferentes composed of cilia. The epithelial cells of all portions showed the positive staining with PAS and BB due to the presence of neutral glycoproteins and proteins, respectively, and the negative staining for AB pH 2.5 and pH 1.0 because of the absence of acidic glycoproteins.

**Keywords:** Banded krait, Histochemistry, Histology, Male genital duct, Reproductive system, Testis

### Introduction

The reproductive system is responsible for producing gametes and sex steroid hormones, with functions relating to an individual's fitness and species persistence [1,2] and offspring production [3]. The male reproductive system of snakes is located in the abdominal cavity, consisting of 2 elongated testicular masses and male genital ducts [4]. The testes undergo spermatogenesis to produce the final stage of germ cell development, resulting in spermatozoa, which must pass the male genital ducts for releasing into the copulatory organs or hemipenis. These male genital ducts consist of the rete testis, ductuli efferentes, ductus epididymis and ductus deferens [5].

Morphologically and histologically, the germinal epithelium in the seminiferous tubules of reptiles shows the similar patterns to those of birds and mammals [6], but it differs from those of fishes and

amphibians. In fishes and amphibians, germ cells undergo a single cohort development inside cysts (some researchers call spermatocysts). Eventually, mature cysts rupture, releasing spermatozoa into the lumen of lobules or tubules [7]. However, in amniotes (reptiles, birds and mammals), the development of germ cells occurs inside seminiferous tubules and progresses into advanced stages from the basement membrane to the lumen, where spermatozoa are released [7].

In the past, numerous studies have focused on the gross morphology, microanatomy and reproductive biology of various snake species, including *Elaphe climacophora* [8], *Eryx jayakari* [9], *Agkistrodon piscivorus leucostoma* [7], *Seminatrix pygaea* [10], *Boiga irregularis* [11], *Zaocys dhumnades* [12], *Pelamis platurus* [13], *Philodryas patagoniensis* [2], *Chrysopelea ornata* [14] and *Epicrates cenchria* [15]. However, currently, there is a dearth of studies on some reptilian species.

The Krait or Banded krait, scientifically named *Bungarus fasciatus* (Schneider, 1801), belongs to the family Elapidae, a large snake family. Its body features the segmented appearance with black and yellow bands, where each segment shows approximately the same size. This pattern extends to both the upper and lower parts, although the abdominal section tends to be lighter in color. Vertebrae projecting from the upper back give the triangular shaped-body [16]. The Banded krait inhabits various regions of Thailand, especially, the areas utilized by humans such as community and farm, causing many issues [17]. It was reported that the human-related incidents (fatalities), have contributed to the increase in the mortality rate of Banded krait, leading to the decline in its population.

Presently, numerous researchers give much interest in various aspects of the snake's biology, including its habitat, diet and lifestyle [18]. However, there remains a dearth of knowledge regarding its histology, therefore, the microanatomy of testes and male genital ducts of *Bungarus fasciatus* (Schneider, 1801) was deeply explored. The findings may be beneficial to other research disciplines such as reproductive biology and pathology. Furthermore, studying the body systems of reptiles serves as a valuable model for the research in physiology, functionality and tissue abnormality.

## Materials and methods

Five animals, Banded krait snake, were collected from Pa Phayom District, Phatthalung Province, Thailand, between July and October 2022. The Banded kraits were transported to the laboratory and kept in wooden boxes for 3 days. Subsequently, the snakes were anesthetized by injecting 3 - 5 mL of 10 % sodium pentobarbital in 70 % ethanol into the peritoneal cavity. After the unconsciousness, an incision was made by dissection from the cloaca to the head. Subsequently, the testes and male genital ducts were separated by surgery and fixed in Bouin's fixative for 48 h. Afterward, they were photographed, and the reproductive organs were processed using basic paraffin methods (consisting of dehydration, clearing, infiltration and embedding) and cut to obtain sections 5- $\mu$ m-thick, stained with Harris's hematoxylin and eosin (H&E) to study general characteristics, periodic acid Schiff's-hematoxylin (PAS-H) for neutral glycoproteins/mucopolysaccharides, alcian blue (AB) pH 2.5 - nuclear fast red (NF) for carboxylated acid glycoproteins/mucopolysaccharides, AB pH 1.0 - NF for sulfated acid glycoproteins/mucopolysaccharides, bromophenol blue (BB) for general proteins and Masson's trichrome (MT) for connective tissue and muscle [19]. The results were viewed using a compound microscope (Olympus CX31), and pictures were taken with a digital camera (Cannon Eos 700D). The mean of each parameter was obtained from size measurements using ImageJ software analysis (cell size, nuclear length, nuclear width, epithelial cell height, lumen width and tubular width). The treatment of animals was carried out under animal use authorization, COA No. TSU 2022-002, IACUC No. 0002.

## Results and discussion

### Gross anatomy

The reproductive system of Banded krait was arranged in the parallel side of the digestive system, kidneys and ureters, presenting of 2 testicles and 2 sides of male genital ducts (**Figure 1**). The left and right testicles represented the average size of  $1.65 \pm 0.18$  and  $1.67 \pm 0.14$  cm, respectively (**Table 1**). It was found that the right testis was positioned higher or anteriorly compared to the left testis, according to the *C. ornata* [14]. Laterally positioned to the testes, the male genital ducts were long-coiled with running to the lower part of the body, attached to the ventral side of the kidney, and finally opened into the cloaca (**Figure 1**). In a fresh condition, the testes and male genital ducts were creamy-white in color.

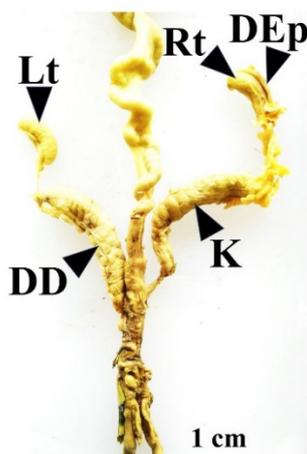
### Microanatomy

#### The testes

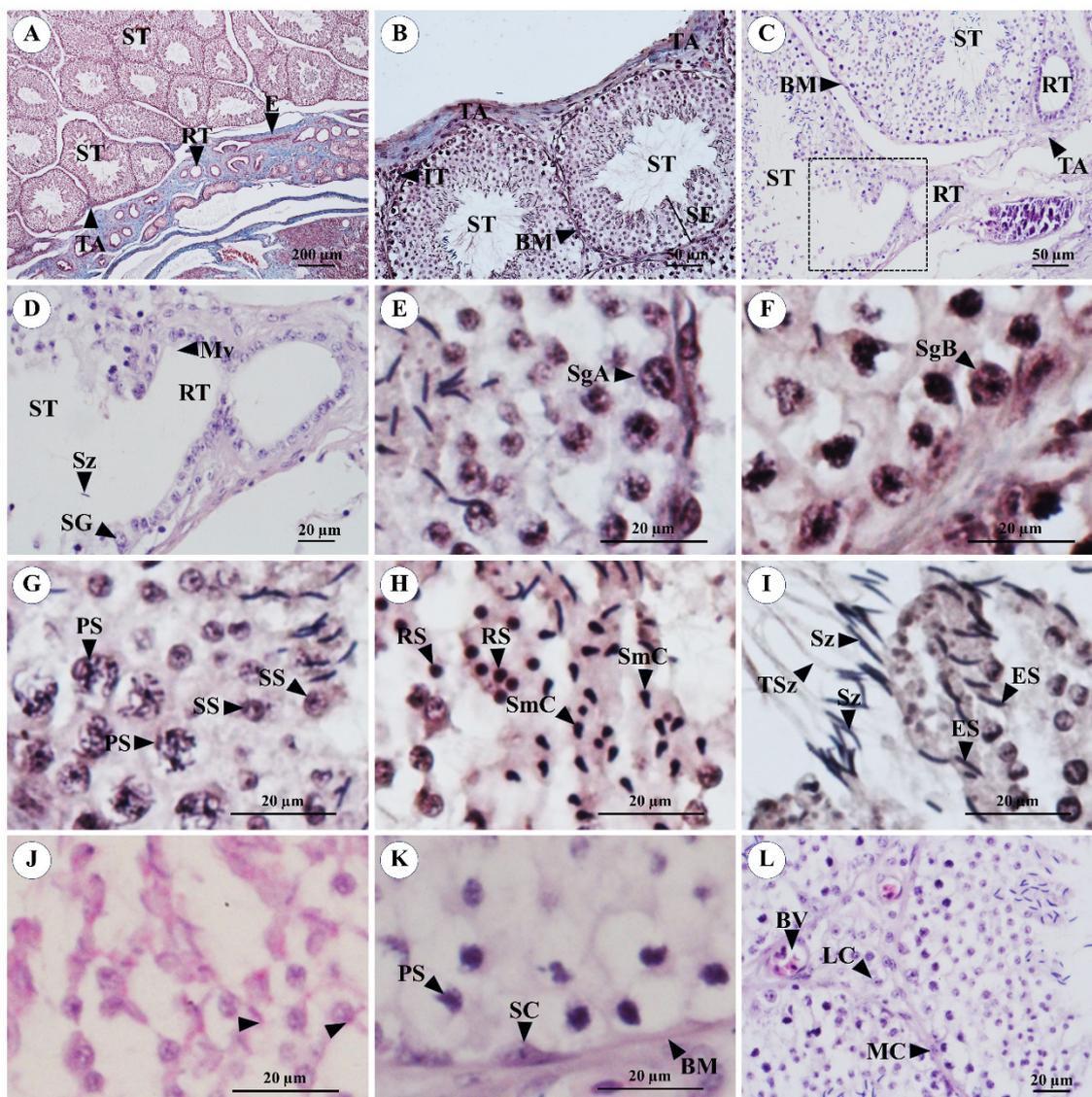
The testis was surrounded by the tunica albuginea, a thin connective tissue composed of collagen fibers and fibrocytes (**Figures 2(A) and 2(B)**), corresponding to other reptiles [20]. Additionally, blood vessels were inserted in the tunica albuginea, while smooth muscle was not appeared. At the posterior side of the testes, the tunica albuginea thickened, inserting into the testis and forming the septa that enclosed the seminiferous tubules. The seminiferous tubules exhibited long-coiled tubes with the observed spermatogenesis and spermiogenesis (**Figure 2(A)**). The regions between 2 and 3 seminiferous tubules contained the interstitial areas, consisting of loosely-connective tissue, blood vessels and Leydig's cells or interstitial cells (**Figure 2(B)**). At the posterior side of testes, the connection of seminiferous tubules and rete testis was observed, presenting the change from the stratified germinal epithelium to the simple cuboidal epithelium with microvilli of rete testis, as clearly seen in **Figures 2(C) and 2(D)**.

**Table 1** Mean ( $\bar{x}$ ) and standard deviation (S.D.) of testes size, seminiferous tubule width and seminiferous epithelium height of Banded Krait.

	Testes size (cm) (n = 5)		Seminiferous tubule width ( $\mu\text{m}$ ) (n = 30)	Seminiferous epithelium height ( $\mu\text{m}$ ) (n = 30)
	Left testis	Right testis		
$\bar{x}$	1.65	1.67	276.90	81.44
S.D.	0.18	0.14	31.87	11.42



**Figure 1** The gross anatomy of the reproductive system of Banded Krait, where key structures include DD: Ductus deferens, DEp: Ductus epididymis, K: Kidney, Lt: Left testis and Rt: Right testis.



**Figure 2** The histology and histochemistry of the testes of Banded Krait, where (A) Overview of the testis and epididymal region, seminiferous tubules (ST), tunica albuginea (TA) and rete testis (RT) embedded in the epididymal sheath (E). (B) Seminiferous epithelium (SE) lining the seminiferous tubules, along with interstitial tissue (IT). (C-D) Junction where the seminiferous tubules merge with the rete testis with D providing an enlarged view of the inset box in picture C. (E) Type A spermatogonium (SgA). (F) Type B spermatogonium (SgB). (G) Primary spermatocyte (PS) and secondary spermatocyte (SS). (H) Round spermatid (RS) and spermiogenesis cells (SmC). (I) Elongated spermatid (ES) and spermatozoa (Sz). (J) Positive staining with PAS at the acrosomal formation of round and elongated spermatids (arrowheads). (K) Sertoli cell (SC). (L) Leydig's cell (LC) and myoid cell (MC). BM: Basement membrane, BV: Blood vessel, Mv: Microvilli, SG: Spermatogonium, TSz: Tail of spermatozoa. (A-B, E-I: MT staining) (C-D, J-L: H&E staining).

The seminiferous tubule contained the seminiferous epithelium, also called germinal epithelium, consisting of germ cells and somatic Sertoli cells (**Figure 2(B)**). The average height of the seminiferous epithelium was  $81.44 \pm 11.42 \mu\text{m}$ , and the seminiferous tubule width was  $276.90 \pm 31.87 \mu\text{m}$  (**Table 1**).

Histological observations revealed that the Sertoli cells had irregular nuclei often with 1 or 2 nucleoli, and were located near the basement membrane (**Figure 2(K)**). Meanwhile, the germ cells were arranged in the successive layers from the basement membrane to the lumen of the seminiferous tubule. Based on cell size, shape, position in the seminiferous tubule and staining characteristics, the male germ cells could be categorized into 7 stages of development: (I) type A spermatogonium, (II) type B spermatogonium, (III) primary spermatocyte, (IV) secondary spermatocyte, (V) round spermatid, (VI) elongated spermatid and (VII) spermatozoa. The characteristics of each germ cell of Banded krait, were similar to those of other snakes such as *E. jayakari* [9].

Each stage of germ cells exhibited the following characteristics: (I) type A spermatogonium was located near the basement membrane of seminiferous tubule and had the cell size of  $8.96 \pm 0.76 \mu\text{m}$  (**Table 2**). This cell had the oval-shaped and large central nucleus, and 1 or 2 nucleoli. Its cytoplasm stained basophilic dye (**Figure 2(E)**). Type A spermatogonium transformed into type B spermatogonium and divided to maintain the population of spermatogonium for producing the next generation of gametes. (II) Type B spermatogonium was located in the same position as type A spermatogonium, but it had the rounder nucleus and lacked a prominent nucleolus (**Figure 2(F)**). Type B spermatogonium had the cell size of  $11.42 \pm 1.84 \mu\text{m}$  (**Table 2**). Type B spermatogonium developed into (III) primary spermatocyte, which was positioned nearer to the lumen than both types of spermatogonium and had the cell size of  $12.30 \pm 0.67 \mu\text{m}$  (**Table 2**). Its nucleus was round in shape, and its cytoplasm stained pink with eosin. Furthermore, the chromatin condensation at different stages of prophase I of meiosis I could be observed inside the nucleus (**Figure 2(G)**). (IV) Secondary spermatocyte, a type of germ cells smaller than the primary spermatocyte, was found near the lumen and had the cell size of  $5.68 \pm 0.50 \mu\text{m}$  (**Table 2**). The nucleus stained intensely purple with hematoxylin due to the chromatin condensation, and small amount of pink-stained cytoplasm surrounded the nucleus (**Figure 2(G)**). The number of secondary spermatocytes observed, was low, and similar to the findings in other studies [9]. Gribbins [21] stated that secondary spermatocytes had the short-lived stage, resulting in rarity in tissue sections. (V) Round spermatid, the cell located near the lumen, was smaller than the secondary spermatocyte, and had the cell size of  $3.28 \pm 0.20 \mu\text{m}$  (**Table 2**). The small nucleus was densely packed with chromatin, surrounded by the minimal cytoplasm and stained intensely purple and brown with H&E and MT, respectively (**Figure 2(H)**). Additionally, this cell began to form an acrosomal vesicle, which was noticeable as a tiny spot of pink staining with PAS (**Figure 2(J)**). The transformation from the round to elongated spermatids occurred in the region near the lumen. (VI) Elongated spermatid had the elongated nucleus and tail (**Figure 2(I)**). An acrosomal cap appeared, stained pink or magenta by PAS (**Figure 2(J)**). Elongated spermatids were located nearer to the lumen than the round spermatids with the head embedded in the cytoplasm of Sertoli cells, and the tail pointing toward the lumen. The nucleus of elongated spermatids stained more intensely than that of the round spermatids (**Figure 2(I)**). (VII) Spermatozoa were embedded in the cytoplasm of Sertoli cells and freely floated in the lumen of seminiferous tubules. The main difference between elongated spermatids and spermatozoa was their staining characteristics with the latter showing more intense staining with H&E and MT than the former. The nucleus lengths of elongated spermatids and spermatozoa were  $5.56 \pm 0.24$  and  $6.55 \pm 0.50 \mu\text{m}$ , respectively (**Figure 2(I)**) and the nucleus width of elongated spermatids and spermatozoa were  $1.12 \pm 0.11$  and  $0.76 \pm 0.10 \mu\text{m}$ , respectively (**Table 2**).

The meiotic cells and the transformation of spermatids into spermatozoa occurred similarly to other snakes. Spermatogenic cells undergo meiotic division and develop from the boundary to the lumen of the seminiferous tubule. Spermatogonia are pre-meiotic cells, while primary and secondary spermatocytes are meiotic cells. Spermatids undergo spermiogenesis to form spermatozoa, involving acrosomal formation, nuclear elongation, tail formation and chromatin condensation [7] and sloughing off excess cytoplasm [22].

In addition, surrounding the germ cells, the cytoplasm of Sertoli cells showed pink with staining by eosin, but did not clearly indicate the boundary of the Sertoli cell. The functions of Sertoli cells are nutrition, support, formation of the blood-testis barrier [23] and phagocytosis of excess cytoplasm [22]. Between the seminiferous tubules, the observed Leydig's cells had the large round nucleus with 1 or 2 nucleoli. The cytoplasm of these cells showed pink with staining by eosin, and some cells contained small lipid droplets (**Figure 2(L)**). The size of Leydig's cell was  $7.98 \pm 0.88 \mu\text{m}$  (**Table 2**). Other studies stated that the inactive spermatogenesis period of the *S. pygaea* and the *Agkistrodon contortrix* found many lipid droplets in the cytoplasm of Leydig's cells, whereas during active spermatogenesis, fat droplets were not found because the lipid droplets were used as the substrate for steroid hormone synthesis or testosterone production [24]. Additionally, Loebens *et al.* [2] suggested that the hypertrophy of Leydig's cells is related to the peak of testosterone synthesis and spermatogenesis. However, the previous studies showed the variation of lipid droplets in Leydig's cell during active and inactive periods in each species of order squamata, snakes [9] and lizards [25]. Besides, the appearances of large round nucleus and pinked-cytoplasm with staining by eosin were obtained for the active Leydig's cell, whereas the inactive period displayed flattened nucleus, small amount of lipid droplets and sparse Leydig's cells at interstitial areas [25]. As a result, the observed small lipid droplets, large round nucleus coinciding with spermatozoa in the lumen of seminiferous tubules and male genital ducts, were describable that during this period, the Banded krait was undergoing active spermatogenesis.

Near the basement membrane of the seminiferous tubule, myoid cells were observed, which had the flattened or oval nucleus and surrounded the outside of the seminiferous tubule (**Figure 2(L)**). The myoid cells or myofibroblasts, found in the present study were similar to those observed in the *Thamnophis sauritus* [21] and the *S. pygaea* [26]. Although they had been referred by different names such as myofibroblasts in the *A. contortrix* [24] and myoid cells in the *S. pygaea* [26], these cells were the same myoid cells [24]. They stated that the myofibroblasts contained the actin in cytoplasm, which could contract to aid in the release of spermatozoa from the seminiferous tubule. Whereas, Aire [27] suggested that the myoid cells or myofibroblasts in snakes are similar to the myofibroblasts in birds, which have the function in the release of spermatozoa.

**Table 2** Mean ( $\bar{x}$ ) and standard deviation (S.D.) of cell size ( $\mu\text{m}$ ) for various cell types including type A spermatogonium (SgA), type B spermatogonium (SgB), primary spermatocyte (PS), secondary spermatocyte (SS), round spermatid (RS), elongated spermatid (ES), spermatozoa (Sz), Leydig's cell (LC), nuclear length (NL) and nuclear width (NW) (n = 30).

	Cell size									
	SgA	SgB	PS	SS	RS	ES		Sz		LC
						NL	NW	NL	NW	
( $\bar{x}$ )	8.96	11.42	12.30	5.68	3.28	5.56	1.12	6.55	0.76	7.98
S.D.	0.76	1.84	0.67	0.50	0.20	0.24	0.11	0.50	0.10	0.88

### ***The male genital ducts***

The genital ducts of Banded krait were located both inside and outside the testes, running alongside the digestive system, kidneys and ureters. The male genital duct comprised of the rete testis, ductuli efferentes, ductus epididymis and ductus deferens. These results were similar to those of other snakes, such as the *Z. dhumnades* [12] and the *C. ornata* [14]. Considering the position of the rete testis, it could be divided into 2 parts: Intra-testicular rete testis, which was the tube that located inside the testis (**Figure**

2(C)), and extra-testicular rete testis, which was located in the epididymal sheath (**Figure 2(A)**). Based on the histological characteristics, the ductuli efferentes could be divided into 2 parts: Proximal and distal ductuli efferentes. For the ductus epididymis was dividable into 3 parts: proximal, middle and distal ductus epididymis. The extra-testicular rete testis, ductuli efferentes and ductus epididymis were embedded in the epididymal sheath, which was loosely-connective tissue comprising of collagen fibers and blood vessels. This character was similar to other snakes such as the *S. pygaea* [10] and the *P. platurus* [13], but differentiated from that of the *A. piscivorus* [28], which possessed the external rete testis due to the merging of the seminiferous tubule with the epididymal sheath. Siegel *et al.* [28] stated that the epididymal sheath is the modification of serosa.

### *Rete testis*

At the posterior side of testes, seminiferous tubules opened into the rete testis (**Figures 2(C) and 2(D)**), which was the first tube connecting each other like a network. Both of the observed intra-testicular and extra-testicular rete testis, were similar to those of the *A. piscivorus* [28] and the *S. pygaea* [10]. The epithelium of rete testis was lined by simple squamous to cuboidal cells with short microvilli. The oval nuclei contained 1 or 2 nucleoli (**Figures 3(A) - 3(E)**). These results were similar to those observed in the *S. pygaea* [10] and the *P. platurus* [13]. The presence of microvilli on the apical surface of epithelial cells could be indicative that these ducts were consistent to the absorption and secretion [10,13]. In addition, histochemistry results showed the positive staining with PAS (**Figure 3(B)**) and BB (**Figure 3(E)**) but negative staining with AB pH 2.5 (**Figure 3(C)**) and AB pH 1.0 (**Figure 3(D)**) of the epithelial cells. These positive staining results were similar to those observed in the *S. pygaea* [10], where Sever [10] suggested that vesicles in the cytoplasm of epithelium cell may involve in the absorption and exocytosis. Additionally, some sections represented spermatozoa in the central lumen. The epithelium cell height of the rete testis was  $7.14 \pm 1.32 \mu\text{m}$ , the lumen width was  $68.50 \pm 10.16 \mu\text{m}$ , and the tubule width was  $96.56 \pm 4.16 \mu\text{m}$  (**Table 3**).

### *Ductuli efferent*

Ductuli efferentes were the tubes that connected the extra-rete testis to the ductus epididymis. The transition from the rete testis to the ductuli efferentes was the change from the simple cuboidal epithelium with microvilli to the ciliated simple cuboidal epithelium (**Figure 3(B)**). The epithelium of the ductuli efferentes was similar to those of the *S. pygaea* [10] and the *P. platurus* [13]. Upon observing histological characters, the ductuli efferentes could be divided into 2 regions, namely proximal (**Figures 3(G) - 3(J)**) and distal ductuli efferentes (**Figures 3(K) - 3(O)**). The epithelium of both regions was the ciliated simple cuboidal epithelium, consisting of 2 cell types: ciliated and non-ciliated cells (**Figures 3(F) - 3(O)**). The difference between the proximal and distal ductuli efferentes was due to the epithelium cell height, the lumen width, and the tubule width. In the proximal region, the epithelium cell height was  $17.37 \pm 1.32 \mu\text{m}$ , lumen width was  $47.37 \pm 7.28 \mu\text{m}$  and tubule width was  $67.75 \pm 4.61 \mu\text{m}$ , whereas in the distal region, the epithelium cell height was  $11.06 \pm 0.76 \mu\text{m}$ , lumen width was  $17.86 \pm 2.44 \mu\text{m}$  and tubule width was  $45.96 \pm 2.65 \mu\text{m}$  (**Table 3**). Additionally, the diameter of the lumen in both regions was narrower than that of the rete testis, and in some sections, the free-floating spermatozoa could be found in the lumen.

The ciliated cells showed the positive staining with PAS at the basal part of the cilia (**Figures 3(G) and 3(L)**) but negative staining with AB pH 2.5 (**Figures 3(H) and 3(M)**), AB pH 1.0 (**Figures 3(I) and 3(N)**) and BB (**Figures 3(J) and 3(O)**). Meanwhile, the ductuli efferentes were the only portion that had cilia lining the lumen, which functioned in spermatozoa movement and luminal content transport [29]. The non-ciliated cells showed the positive staining with PAS and BB but negative staining with AB pH 2.5 and

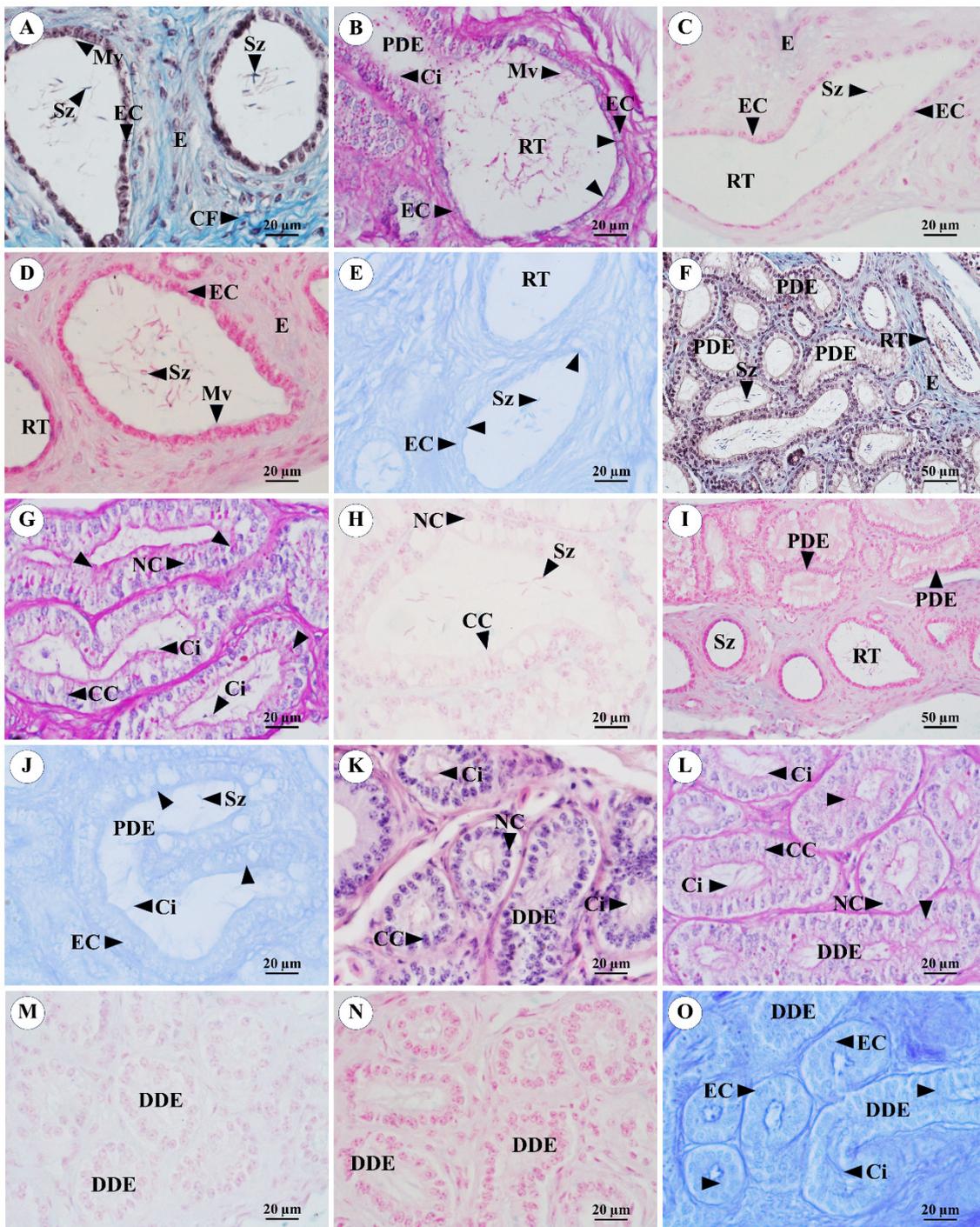
AB pH 1.0, which could be indicative that their secretion consisted of neutral glycoproteins and proteins, respectively. These results were similar to those found in the *S. pygaea* [10], the *P. platurus* [13] and the *A. piscivorus* [28]. Hermo and Robaire [30]; Sever and Freeborn [13], stated that the content released from ductuli efferentes found in mammals consists of glycoconjugates and enzymes, involving in spermatozoa maturation.

**Table 3** Mean ( $\bar{x}$ ) and standard deviation (S.D.) of the measurements ( $\mu\text{m}$ ) of rete testis, and proximal and distal ductuli efferentes (PDE and DDE, respectively) in the Banded Krait, where measurements include epithelial cell height (ECH), lumen width (LW) and tubular width (TW) (n = 30).

	Rete testis			Ductuli efferentes					
	ECH	LW	TW	PDE			DDE		
ECH				LW	TW	ECH	LW	TW	
$\bar{x}$	7.14	68.50	96.56	17.37	47.37	67.75	11.06	17.86	45.96
S.D.	1.32	10.16	4.16	1.32	7.28	4.61	0.76	2.44	2.65

#### *Ductus epididymis*

The transition from the ductuli efferentes to the ductus epididymis was verified by the change of the ciliated simple cuboidal epithelium into the pseudostratified tall columnar epithelium with microvilli (**Figure 4(A)**). This was similar to the observations in *S. pygaea* [10] and *Z. dhumnades* [12]. Based on histological characteristics, namely epithelial cell height, luminal and tubule widths, the ductus epididymis could be subdivided into 3 portions: proximal, middle and distal ductus epididymis. In other studies of lizards, the ductus epididymis was divided into 4 regions (initial segment, caput, corpus and cauda epididymis) [31,32], while in some studies, such division was not feasible [13]. In **Figures 4(C) - 4(O)**, from proximal to distal epididymis, the lumen width was larger and the epithelial cell height was lower. Each portion of the ductus epithelium consisted of 2 cell types: basal and principal cells (**Figures 4(C) - 4(O)**). Basal cells were located near the basement membrane and had oval-flattened nuclei. Whereas principal cells had basal and oval nuclei with microvilli, protruding into the lumen and containing small secretory granules. These granules of principal cells showed the positive staining with PAS (**Figures 4(C), 4(H) and 4(L)**) and BB (**Figures 4(F), 4(K) and 4(O)**) but negative staining with AB pH 2.5 (**Figures 4(D), 4(I) and 4(M)**) and AB pH 1.0 (**Figures 4(E), 4(J) and 4(N)**), indicating to the secretion of neutral carbohydrates and proteins corresponding to those observed in the *S. pygaea* [33]. In contrast, other studies in lizards reported lipid secretion [34], as well as protein and glycoprotein secretions [35]. Additionally, all portions of the epididymal lumen contained free spermatozoa that demonstrated by the staining of spermatozoa heads with black and intense blue with MT and hematoxylin, respectively.



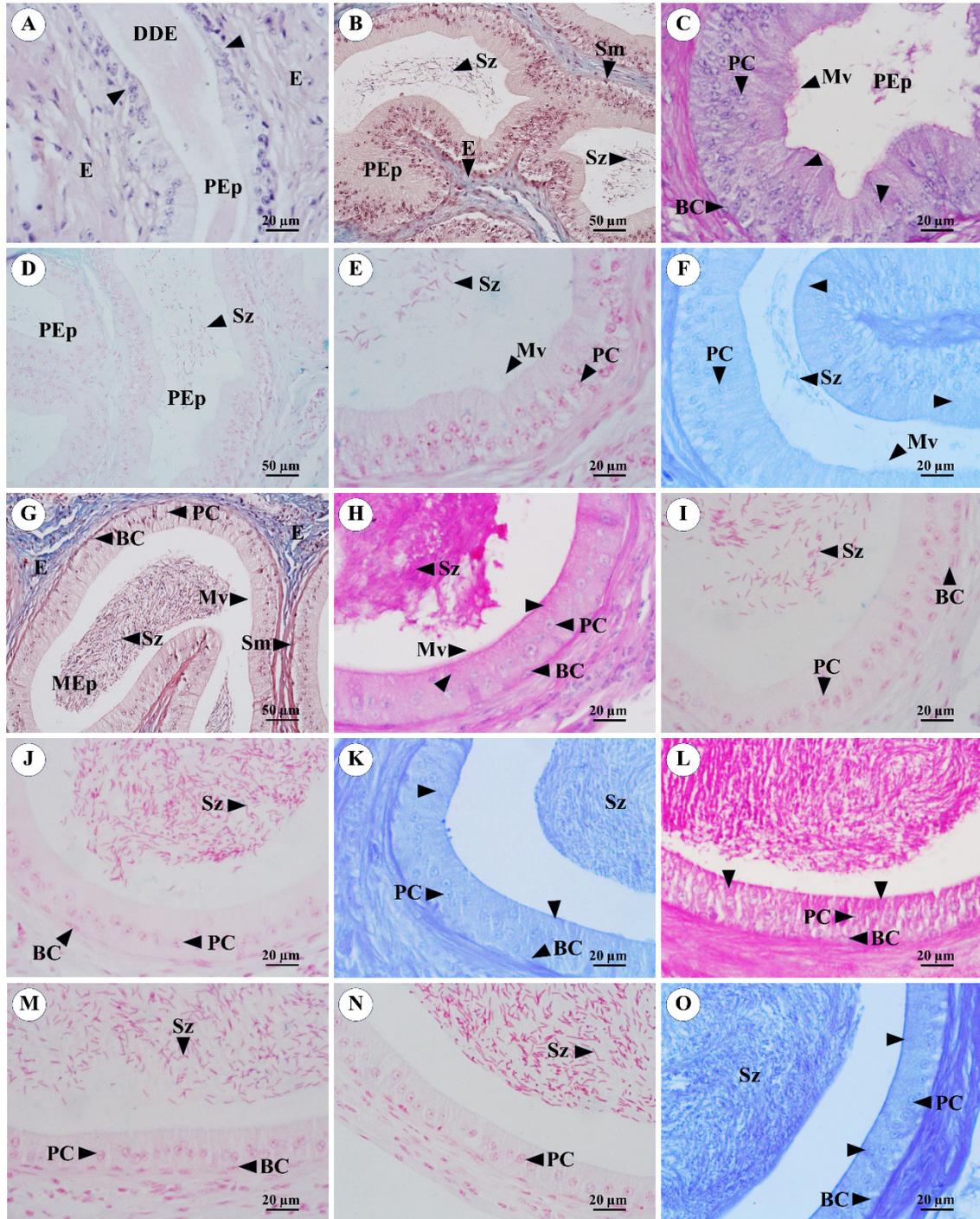
**Figure 3** (A-E) Histology and histochemistry of the rete testis (RT) of Banded krait. (F-J) Histology and histochemistry of the proximal ductuli efferentes (PDE) and (K-O) Histology and histochemistry of the distal ductuli efferentes (DDE). CC: Ciliated cell, CF: Collagen fiber, Ci: Cilia, E: Epididymal sheath, EC: Epithelial cell, Mv: Microvilli, NC: Non-ciliated cell, RT: Rete testis, Sz: Spermatozoa, arrowhead in B, G and L: PAS-positive staining granules, arrowhead in E, J and O: BB-positive staining granules. (A and F: MT staining) (B, G and L: PAS staining) (C, H and M: AB pH 2.5 staining) (D, I and N: AB pH 1.0 staining) (E, J and O: BB staining) (K: H&E staining).

The histological characteristics altered across each portion, in the proximal epididymis, the epithelium was the pseudostratified tall columnar, exhibiting the variation in cell height, resulting in the star-shaped lumen. Several microvilli were found on the apical surface of the principal cells (**Figures 4(B) - 4(F)**). The epithelial cell height in the proximal portion was  $55.60 \pm 4.21 \mu\text{m}$ , the lumen width was  $89.75 \pm 6.99 \mu\text{m}$ , and the tubule width was  $202.07 \pm 19.65 \mu\text{m}$  (**Table 4**). In contrast, the middle epididymis exhibited the uniform epithelial cell height, resulting in the rounded lumen, and the shorter epithelial cells than those in the proximal epididymis (**Figures 4(G) - 4(K)**). The epithelial cell height in the middle portion was  $36.40 \pm 2.67 \mu\text{m}$ , the lumen width was  $143.63 \pm 8.84 \mu\text{m}$ , and the tubule width was  $217.69 \pm 16.08 \mu\text{m}$  (**Table 4**). The distal epididymis was lined by the pseudostratified cuboidal epithelium. The amounts of microvilli and granules in the principal cells were sparse in this portion, and the shortest epithelial cells were found relative to 2 other portions of ductus epididymis (**Figures 4(L) - 4(O)**). The epithelial cell height in the distal portion was  $22.61 \pm 1.52 \mu\text{m}$ , the lumen width was  $222.77 \pm 18.87 \mu\text{m}$ , and the tubule width was  $296.30 \pm 41.44 \mu\text{m}$  (**Table 4**).

Based on the histological features of the ductus epididymis, the proximal epididymis with its pseudostratified tall columnar epithelium and numerous microvilli on the apical surface of principal cells, served as the primary site for reabsorption and secretion. Conversely, the distal epididymis with its pseudostratified cuboidal epithelium, and the fewer microvilli, as well as the largest lumen and tubule width than those of the proximal and middle epididymis, indicating to the primarily function in spermatozoa storage.

**Table 4** Mean ( $\bar{x}$ ) and standard deviation (S.D.) of measurements ( $\mu\text{m}$ ) for the proximal ductus epididymis (PEp), middle ductus epididymis (MEp), distal ductus epididymis (DEp), as well as ductus deferens of Banded krait. The measurements include epithelial cell height (ECH), lumen width (LW) and tubular width (TW) (n = 30).

	Ductus epididymis									Ductus deferens		
	PEp			MEp			DEp			ECH	LW	TW
	ECH	LW	TW	ECH	LW	TW	ECH	LW	TW			
$\bar{x}$	55.60	89.75	202.07	36.40	143.63	217.69	22.61	222.77	296.30	10.00	351.93	385.89
S.D.	4.21	6.99	19.65	2.67	8.84	16.08	1.52	18.87	41.44	0.72	36.39	39.00

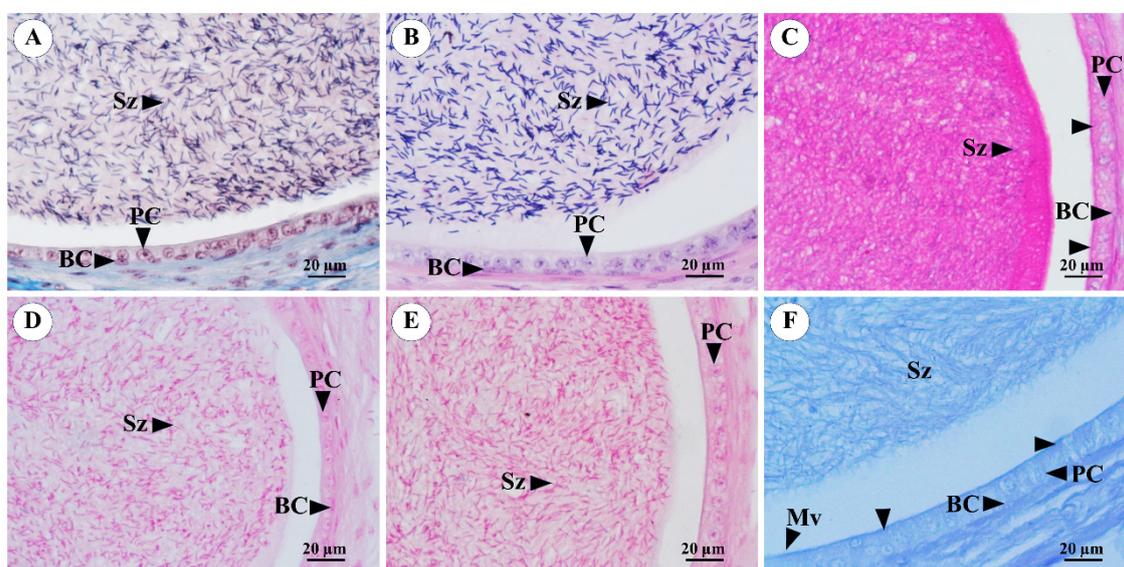


**Figure 4** (A) Junction (arrowhead) between the distal ductus efferentes (DDE) and proximal ductus epididymis (PEp) of Banded krait. (B-F) Histology and histochemistry of the proximal ductus epididymis. (G-K) Histology and histochemistry of the middle ductus epididymis (MEp). (L-O) Histology and histochemistry of the distal ductus epididymis (DEp). BC: Basal cell, E: Epididymal sheath, Mv: Microvilli, PC: Principal cell, Sm: Smooth muscle, Sz: Spermatozoa. Arrowheads in C, H and L indicate PAS-positive staining granules. Arrowheads in F, K and O indicate BB-positive staining granules. (A: H&E staining) (B and G: MT staining) (C, H and L: PAS staining) (D, I and M: AB pH 2.5 staining) (E, J and N: AB pH 1.0 staining) (F, K and O: BB staining).

### *Ductus deferens*

The ductus deferens was the final portion of the genital duct, where the transition from the ductus epididymis to the ductus deferens was unclear. The ductus deferens was lined by simple-pseudostratified low columnar-cuboidal epithelium. The epithelial cells contained fewer microvilli and smaller secretory granules (**Figures 5(A) - 5(F)**). These results were indicative that the ductus deferens played the key role in reabsorption, corresponding to Sever [33] that the function of this duct was luminal fluid absorption. The secretory granules showed the positive staining with PAS (**Figure 5(C)**) and BB (**Figure 5(F)**) but negative staining with AB pH 2.5 (**Figure 5(D)**) and AB pH 1.0 (**Figure 5(E)**). These results could be indicative that this portion was to synthesize neutral carbohydrates and proteins, according to the findings in the *A. piscivorus* [28], but different from the *S. pygaea* [33], whereas the epithelium reacted positively with AB but showed negative staining with PAS and BB. The epithelial cell height of the ductus deferens was  $10.00 \pm 0.72 \mu\text{m}$ , the lumen width was  $351.93 \pm 36.39 \mu\text{m}$ , and the tubule width was  $385.89 \pm 39.00 \mu\text{m}$  (**Table 4**), making this tube the widest among all portions. Additionally, throughout the length of this duct, the floating spermatozoa were found in the lumen.

Overall, the observed free spermatozoa in the lumen of seminiferous tubules and male genital ducts, were indicative that the sampling period was during the breeding season or active spermatogenesis period. The ductus epididymis and ductus deferens were identified as the main sites for spermatozoa storage before ejaculation, corresponding to the reports in the *S. pygaea* [33], the *B. irregularis* [11], the *Z. dhumnades* [12], the *A. piscivorus* [28] and the *P. patagoniensis* [2]. However, Gang *et al.* [12] suggested that the ductus deferens is the main region for both spermatozoa storage and long-term storage [36]. Furthermore, the muscularis layer in the wall of genital ducts helps to ejaculate spermatozoa to the hemipenis.



**Figure 5** (A-F) Histology and histochemistry of the ductus deferens of Banded krait. BC: Basal cell, Mv: Microvilli, PC: Principal cell, Sz: Spermatozoa. Arrowhead in C indicates PAS-positive staining granules. Arrowhead in F indicates BB-positive staining granules. (A: MT staining) (B: H&E staining) (C: PAS staining) (D: AB pH 2.5 staining) (E: AB pH 1.0 staining) (F: BB staining).

## Conclusions

The reproductive system of Banded krait was similar to those of other snakes, consisting of the elongated testes for germ cell production and development. The type and stages of germ cell development were similar to those observed in other reports using the compound microscope studies. The spermatozoa moved through male genital ducts, which were divided into the rete testes, ductuli efferentes, ductus epididymis and ductus deferens. Although some portions resembled those of other snakes, histological results of the ductus epididymis could be divided into 3 portions: proximal, middle and distal ductus epididymis. Furthermore, during the collection of the animals, the spermatozoa were observed in the seminiferous tubules and in every region of the male genital ducts, indicating that the Banded krait exhibited the active spermatogenesis and breeding season during these months. This work may provide a valuable basic model for studying the reproductive system in other reptiles or vertebrates and histopathology.

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