

**Full Paper**

**Morphology and histology of female reproductive tract of the dog-faced water snake *Cerberus rynchops* (Schneider, 1799)**

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**Abstract:** The development of viviparity, live-bearing, is an ill-defined evolutionary event that is considered to have emerged in several lineages of reptiles. Therefore, the knowledge from a wide variety of viviparous species will offer better insight into understanding the reproductive adaptations associated with the development of viviparity. The dog-faced water snake, *Cerberus rynchops*, belongs to the family Homalopsidae that is considered to be viviparous, but detailed reproductive features are still poorly known for this species. In this study we investigated the female reproductive system of mature *C. rynchops* using histological techniques. A paired ovary was anchored to the kidney in this species. Eight to nine ovarian follicles were observed and classified as previtellogenic follicles, vitellogenic follicles and atretic follicles. The oviductal structure of *C. rynchops* was divided into four regions, namely infundibulum, uterine tube, uterus and vagina, and all oviductal regions were covered with ciliated and non-ciliated cuboidal epithelial cells and surrounded by connective tissue of submucosa, a thick layer of the muscularis and serosa. In the uterus we observed the uterine glands, which are commonly present in viviparous snakes.

**Keywords:** Oogenesis, dog-faced water snake, reproductive tract, histology, Gulf of Thailand

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## INTRODUCTION

The order Squamata is a diverse group of reptiles that contains more than 7,700 living species of snakes and lizards [1]. Viviparity (live-bearing) has been exclusively observed in several snakes [2, 3] and lizards [4, 5], but is not a common reproductive mode (15–20%) in this order [1]. The sporadic distribution of viviparity led to the widely-accepted hypothesis that viviparity has evolved independently multiple times in reptiles, and multidisciplinary approaches have facilitated our understanding of its evolutionary significance [6]. For example, the accumulation of behavioral data enabled a detailed mapping of reproductive modes, demonstrating that viviparity is adaptive to cold climates [7]. Phylogenetic studies identified several clades in which viviparity was recently evolved [8]. However, descriptive and mechanistic studies, especially detailed morphological analysis, are still limited to a small number of species [4-6, 9-12].

The transition of oviparity to viviparity requires fundamental changes in the reproductive system. Given the multiple evolutionary origins of viviparity, the knowledge from a wide variety of viviparous reptile species will offer a better insight into the understanding of the reproductive adaptations associated with changes in reproductive modes. In general, the female reproductive system of snakes is composed of oviducts and ovarian tissues as revealed by morphological studies over 50 years [2, 13]. The oviduct is classified into four distinct regions, namely the infundibulum, uterine tube, uterus and vagina; these four regions are commonly found in squamates [14, 15]. The oviduct is connected to the cloacal urodaeum at the oviductal-cloaca junction [9]. Uribe et al. [5] studied the oviductal-cloaca junction in a snake, *Toluca lineata*, and further classified it into anterior and posterior regions with differential histology. Siegel et al. [1] reviewed the terminology of the squamate oviduct and suggested the following names: anterior infundibulum, posterior infundibulum (sperm storage area), glandular uterus and non-glandular uterus.

The dog-faced water snake (*Cerberus rynchops*) belongs to the family Homalopsidae. All 35 species in this family are considered to be viviparous [2]. *C. rynchops* is a common homalopsid viviparous species that has been recorded in Australia, New Guinea, Southeast Asia and Sri Lanka [16]. A number of researchers (e.g. [1, 17]) have investigated the population structure and genetic diversity of *C. rynchops*, but only few studies have addressed the reproductive characteristics of this species [18-20]. Several review articles described the reproductive features of snakes in Homalopsidae and other families [e.g., *Leptotyphlops dulcis*, *L. humilis*, *Ramphotyphlops braminus* and *Typhlops angolensis* [21]; *Typhlops punctatus*, *Bungarus fasciatus* and *Vipera aspis* [22]]. However, these studies included only overall reproductive features and lack detailed histological analyses. In this study we provide the overall anatomical and microscopic morphology of the female reproductive system of *C. rynchops* using histochemical techniques for a better understanding of the reproductive biology of this ecologically important snake.

## MATERIALS AND METHODS

Five dead females of *Cerberus rynchops* with an average snout-vent length (SVL) of  $64 \pm 0.56$  cm were generously donated from Pranburi local fisheries in the Paknam Pranburi Estuary, Thailand, in January 2016. *C. rynchops* is not a protected species. It is a standard practice for local fishermen to kill these snakes after catch since they are predators of fish, and these snakes are also consumed by humans. Owing to the unique manner in which these snake specimens were obtained, the present study did not require the approval by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University.

The female reproductive systems were dissected and fixed in Davidson's fixative for about 24 - 36 hr. The reproductive organs were anatomically observed in Ringer's solution under a stereomicroscope (Leica 750, Leica Camera AG, Germany). They were subsequently processed using standard histological procedures [23, 24]. Tissue fragments of particular regions including ovary and reproductive tract were dehydrated in ethanol and cleared in toluene before embedding in paraffin, and then conventional paraffin sections were cut at 4- $\mu$ m thickness and stained with Harris's haematoxylin and eosin (H&E) and Masson's trichrome (MT) (to detect the connective tissue and collagen fibre) or periodic acid-Schiff (PAS) (to detect glycoproteins) [23, 24]. The histological sections of the female reproductive system of *C. rynchops* were photomicrographed under a Leica DM750 light microscope using the guidelines of Uribe et al. [5] and Siegel et al. [15].

## RESULTS AND DISCUSSION

### Gross Anatomy of Reproductive System

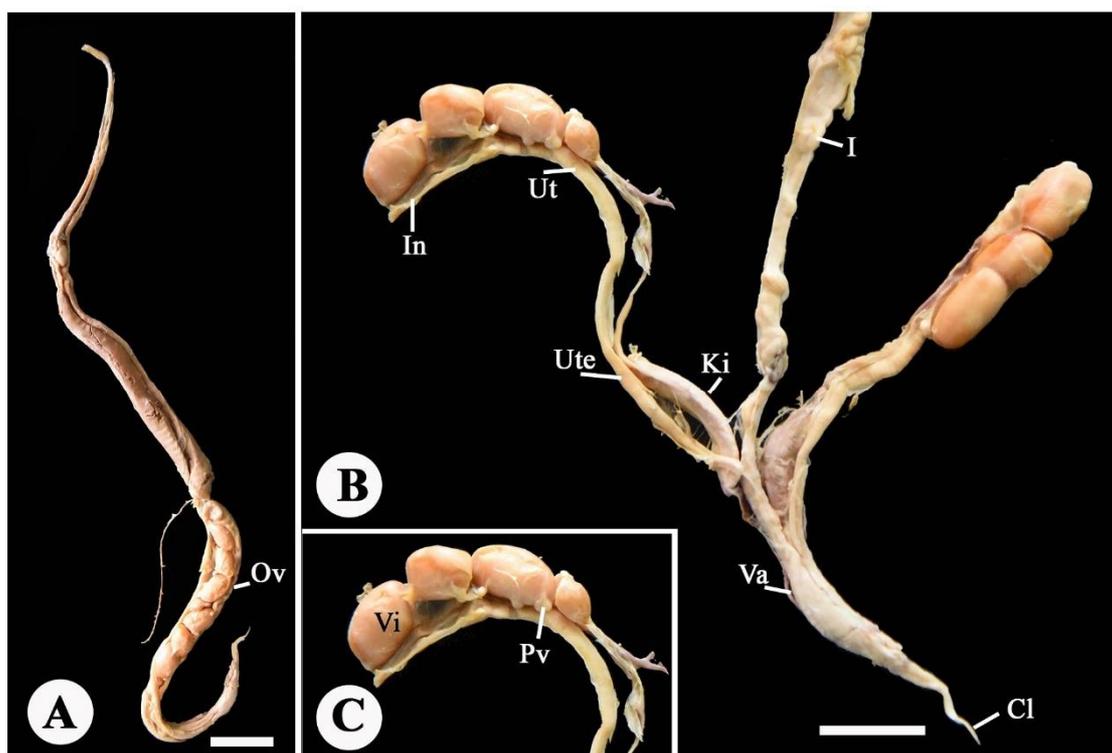
All five specimens show similar gross anatomy of the female reproductive system, which is composed of the ovaries and oviducts (Figure 1A). The elongated bodies of paired ovaries are anchored to the kidney (Figure 1B) as commonly seen in lizards [19]. Each ovary has eight to nine ovarian follicles (Figures 1B,1C) and they are connected to the ovary by loose connective tissues. The anterior regions of the ovary are connected by the infundibulum, and both oviducts open to the cloaca at the posterior region (Figure 1B). The right oviduct is more elongated than the left oviduct (Figure 1B). The small and oval-shaped pre-vitellogenic follicles are located around vitellogenic follicles (Figure 1C). Similar female reproductive features have been reported for *Leptotyphlops dulcis*, *L. humilis*, *Typhlops angolensis* [21] and *N. natrix* [25]. Histological details of the ovary and oviduct are described below.

### Ovarian Histology and Oogenic Follicles

The timing and duration of sexual maturity of *C. rynchops* in Thailand has not been well documented, thus requiring further investigations. The gross anatomy indicated that the five *C. rynchops* specimens examined in this study were sexually mature adults with mature ovaries (Figure 1). A previous study on *C. rynchops* in Malaysia found no evidence of seasonal reproduction, whereas other Cambodian homalopsid snakes were reported to mate in December to January [26]. It was therefore not surprising that all of our specimens caught in January had mature ovaries in a similar range of previous report.

As shown in Figures 2A and 2C, each ovary is covered by a thin layer that consists of the surface ovarian epithelium and tunica albuginea, which is a dense connective tissue. The ovaries are divided into two regions, viz. cortex and medulla (Figure 2B). The cortex region contains different stages of oogenic follicles (Figure 2A), similar to some lizards [27]. The stromal region is surrounded by vascularised connective tissues (Figure 2C).

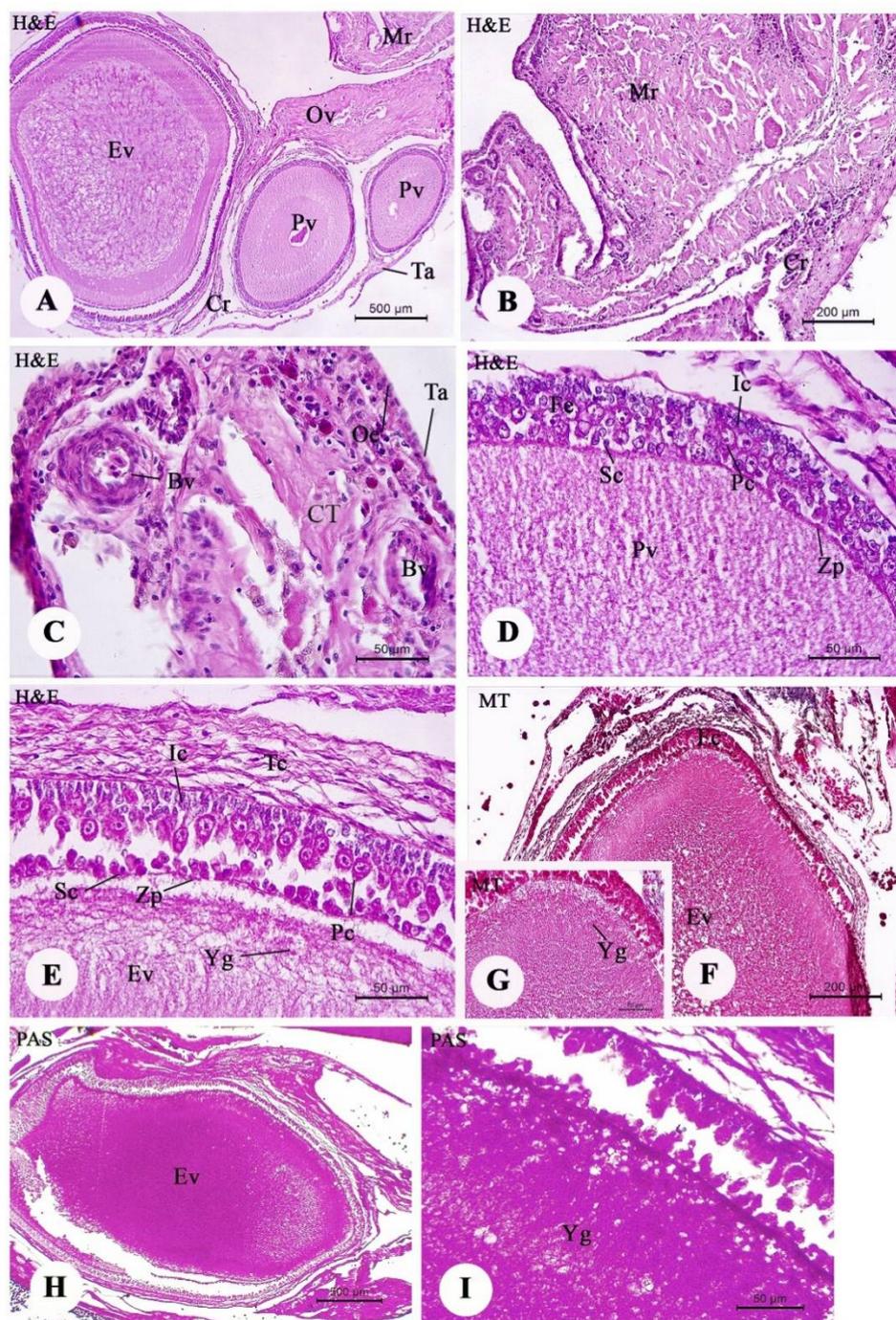
Oocytes in the cortex region of *C. rynchops* are classified into three different stages (previtellogenic, vitellogenic and atretic follicles) based on size, histological staining features and folliculogenesis. The feature of each stage is described in more detail below.



**Figure 1.** General morphology of female reproductive system of *Cerberus rynchops*: (A, B) Elongated body of paired ovaries (Ov); (C) Enlarged view of ovary. Oocytes at previtellogenic follicle (Pv) and vitellogenic follicle (Vi) stages are observed. [Cl = cloaca, I = intestine, In = infundibulum, Ki = kidney, Ut = uterine tube, Ute = Uterus, Va = vagina. Scale bar A = 2 cm; B = 1 cm]

### Previtellogenic Follicles

The previtellogenic follicles are about 500  $\mu\text{m}$  in diameter (Figure 2D). The nucleus is large with a diameter of about 300  $\mu\text{m}$  (data not shown), likely due to increased lampbrush chromosome activity [19]. The follicular epithelium is clearly multilayered and consists of three distinct cell layers: small, intermediate and pyriform cells. These observations are similar to that seen in a lizard, *Mabuya* sp. [27]. However, the follicular epithelium of *C. rynchops* differs from those found in lizards: four and six layers of follicular epithelium were found in *Anolis carolinensis* [28] and *Ctenosaura pectinata* [29] respectively. It is noted that we observed disorganisation of ovarian structures in some regions, possibly due to post-mortem changes associated with the mode of sample collection and handling prior to our receipt of samples.



**Figure 2.** Light photomicrographs of ovaries with previtellogenic and early vitellogenic follicles of *Cerberus rynchops*: (A) Ovary structure surrounded by tunica albuginea (Ta); (B) Cortex and medulla regions of ovary; (C) Vascularised connective tissues; (D) Previtellogenic follicles; (E-I) Early vitellogenic follicles (Ev). [Bv = blood vessels, CT = connective tissue, Cr = cortex region, Fc = follicular cell, Ic = intermediate cell, Mr = medulla region, Oc = ovarian epithelium, Pc = pyriform cells, Pv = previtellogenic follicle, Sc = small cell, Tc = theca cell, Yg = yolk granules, Zp = zona pelucida]. Note: A-E = stained with H&E; F, G = stained with MT; H, I = stained with PAS

The small cells of the follicular epithelium (about 5  $\mu\text{m}$  in diameter) have considerably irregular shapes (Figure 2E). These cells are exclusively located near the basal lamina of the oocyte. We assume that these small cells are differentiating into spherical intermediate cells, which are about

10 µm in diameter, as previously proposed in *Gerrhonotus coeruleus* [30] and *Lacerta sicula* [31] and some other lizards [27]. The round nucleus of the small cell containing a prominent nucleolus is surrounded by a lightly stained granular cytoplasm.

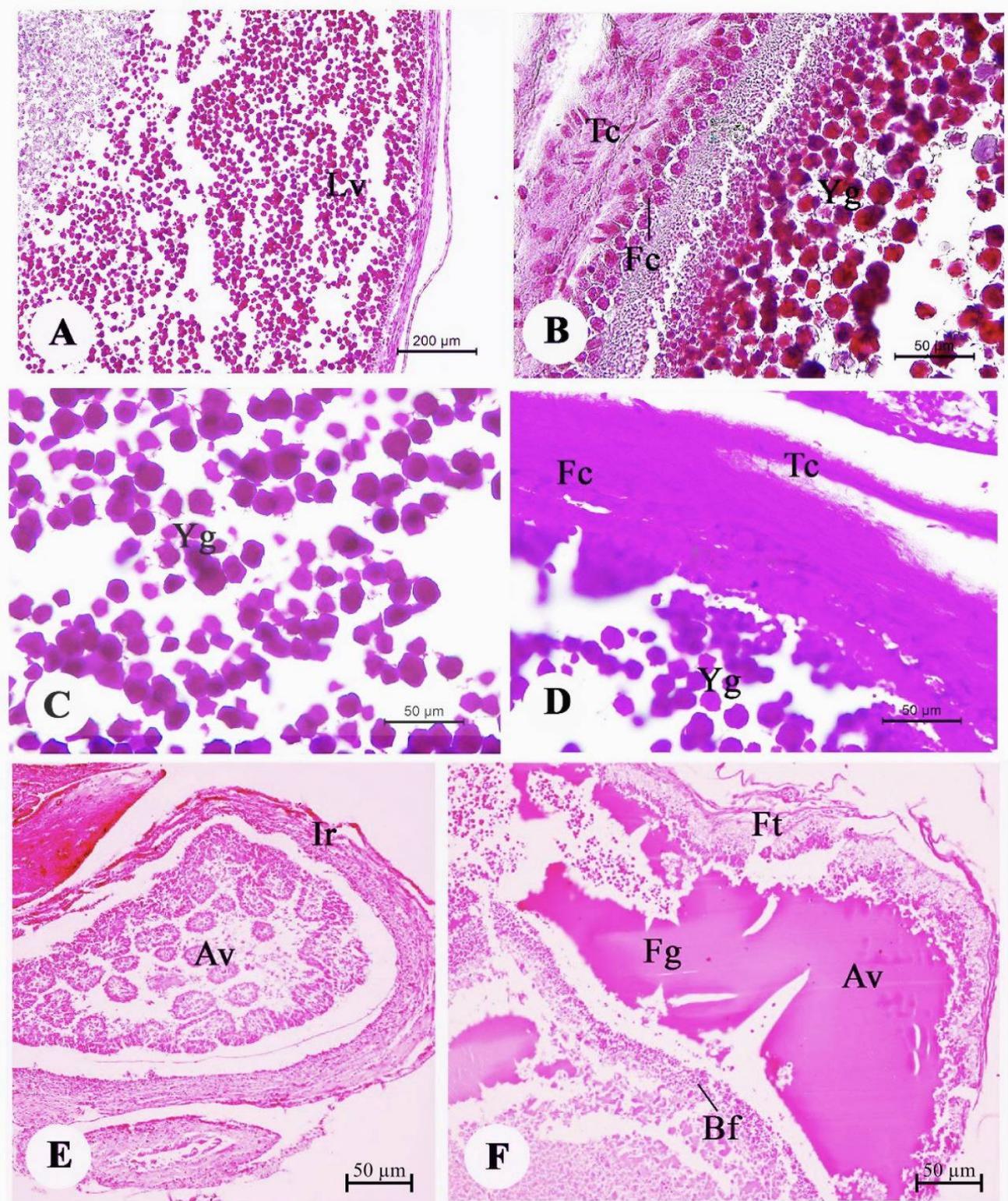
Pyriform cells are spherical and have the largest diameter of about 15 µm (Figure 2E). The nuclei of these cells contain clumps of heterochromatin and prominent nucleoli; relatively pale cytoplasm is seen around the nuclei of these cells. Previous ultramicroscopic observations have shown that the cytoplasm of pyriform cells is enriched with several membrane organelles such as mitochondria, granular endoplasmic reticulum and well-developed Golgi complexes [32,30,33]; these features are impossible to conclusively identify with light microscopy. The important function of the pyriform cells is to produce macromolecules such as ribosomes, DNA and RNA that are transferred to the oocyte through intercellular bridges [34-36] during the vitellogenic process [35]. It is likely that the macromolecular transfer had already occurred in samples seen here and resulted in the pale colour of the cytoplasm in H&E staining in this study.

Another notable feature of the previtellogenic follicles is the presence of a striated layer of the zona pellucida, about 2 µm in thickness, around the oocyte (Figure 2D). The thickness of zona pellucida varies from 0.5 to 5 µm. This type of striated layer of the zona pellucida has been commonly found in lizards [30, 37, 38].

### **Vitellogenic Follicles**

Vitellogenic follicles are further classified into early and late vitellogenic follicles (Figures 2E–2G). Early vitellogenic follicles increase their size to about 1.0 cm in diameter. These early vitellogenic follicles are easily identified by small and spherical yolk granules that are strongly stained with acidophilic stain of the ooplasm (Figure 2E). All yolk granules also show intense staining reactivity with MT and PAS staining (Figures 2G-2I), which enables direct observation of yolk accumulation patterns; the accumulation of yolk granules begins at the oocyte periphery (Figures 2E, 2I), which is also observed during oocyte development in some lizards [39]. This is probably because major yolk proteins are transported from the liver. It is well known that yolk uptake starts during meiotic arrest [40, 41]. During the yolk uptake, yolk proteins are synthesised by vitellogenesis in the liver, secreted to the blood stream and finally incorporated into oocytes. This pathway is regulated by estradiols, especially estradiol 17β, which play a critical role in the hepatic synthesis of vitellogenin, a major yolk protein [42-44].

In the late vitellogenic stage, cell diameter increases up to 1.5 cm in association with a progressive increase in the size and number of yolk granules (Figures 3A–3C). In contrast, there is a decrease in the size of the follicular and theca cells (Figures 3B–3D). The zona pellucida is rarely seen at this stage. These observations suggest that changes in the follicular cells and zona pellucida are related to the uptake of yolk granules. The primary component of yolk granule is formed from the precursor protein vitellogenin, which is synthesised in the liver and then secreted into the blood stream. Consequently, vitellogenin uptake and yolk formation in the ooplasm of the oocyte are associated with the decrease in size of the surrounding structures [20]. This type of embryonic development has been reported in a lizard *Anolis carolinensis* [28].



**Figure 3.** Light photomicrographs of late vitellogenic follicles and atretic follicle of *Cerberus rynchops*: (A-D) Late vitellogenic follicles (Lv); (E-F) Atretic follicles (Av). [Fc = follicular cell, Bf = breakdown of follicle, Tc = theca cell, Yg = yolk granules, Zp = zona pelucida, Ir = peripheral region of atretic follicle in irregular shape, Ft = fragmentation of zona pellucida]. Note: A, B = stained with MT; C, D = stained with PAS; E, F = stained with H&E

### Atretic Follicles

The corpora lutea is generally formed by differentiated follicular cells [2], but it was not observed in any sections from the five snake specimens used in this study. In marked contrast, atretic follicles, another sign of ovarian maturation and breakdown, were seen in late vitellogenic stages along the ovary (Figures 3E-3F). The atretic follicles are irregular in shape (Figure 3E). Fusion of yolk granules was observed in peripheral areas of the ooplasm. The zona pellucida was also fragmented in atretic follicles at the late vitellogenic stage. These observations of atretic follicles indicate that our snake specimens were sexually mature, in agreement with other investigators [45-47]. Atretic follicles have been previously suggested to play a role in the proximate control of clutch size, but this has not yet been demonstrated in many lizard species [48].

### Oviduct Histology

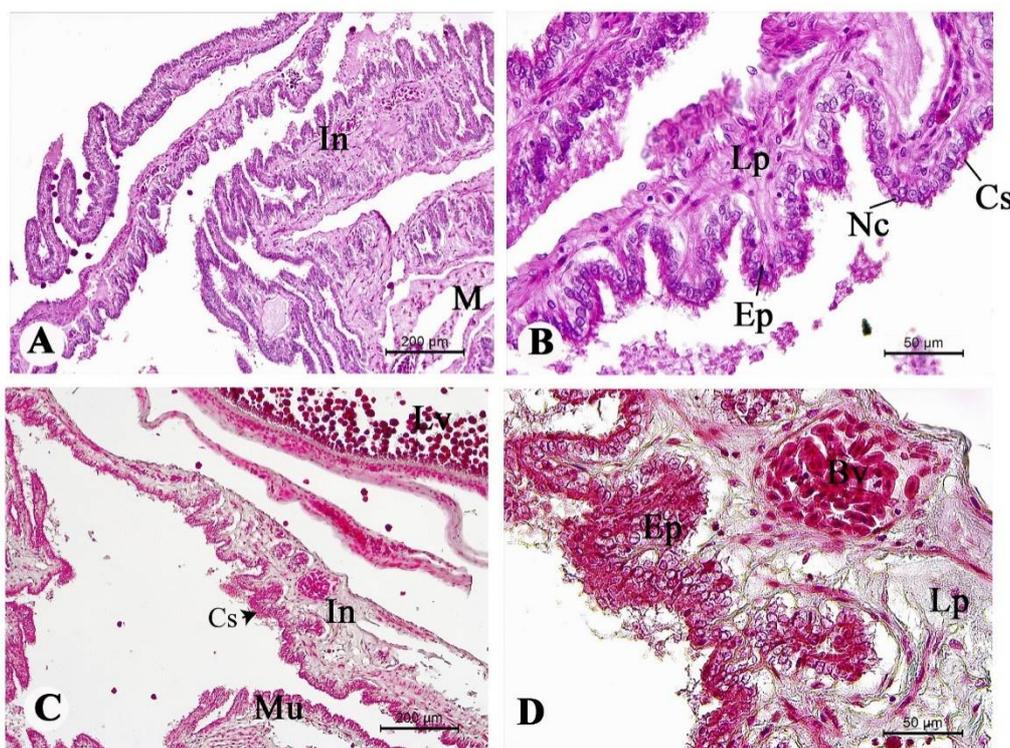
The oviduct is classified into four regions: the infundibulum, uterine tube, uterus and vagina, based on histological characteristics, as investigated in several documents [15, 49]. In some lizards the uterine tube and infundibulum are combined [50, 51], but *C. rynchops* has the four regions. Sometimes the infundibulum is further classified into anterior and posterior regions, whereas the uterus is classified into glandular and non-glandular regions. Longitudinal observations further showed that these oviductal regions have four tissue layers, viz. mucosa, submucosa, muscularis and serosa, from inside to outside.

### Infundibulum

The infundibulum of *C. rynchops* contains finger-like irregular folds of the mucosal layer (Figure 4A), which project in the anterior direction. The irregular mucosal folding likely allows the infundibulum to expand when eggs enter and pass through. The epithelial layer of the infundibulum is ciliated and covered with cuboidal epithelium (Figure 4B). The precise function of this epithelial layer remains unclear; however, Ramírez-Pinilla et al. [27] suggested that it might provide the site of fertilisation in reptiles. Epithelial cells of *C. rynchops* are ciliated (Figures 4B, 4C) and have small nuclei located in the centre of the cell, as observed in many other reptiles [5, 50]. The cilia would facilitate movement of sperms towards the anterior infundibulum [9]. Neither the glands nor sperms were observed in the posterior infundibulum of our specimens. Blood vessels are scattered in the connective tissue throughout the lamina propria and submucosal layers (Figure 4D).

### Uterine Tube

The uterine tube shows similar histological features to the infundibulum. It is composed of four layers, i.e. mucosa, submucosa, muscularis and serosa (Figure 5A). Slight differences between the uterine tube and infundibulum include: 1) only the epithelial layer of the uterine tube consists of non-ciliated simple cuboidal epithelium (Figure 5B), and 2) the oviductal wall is thicker than that of the infundibulum (about 100 µm vs. 500 µm; Figures 5B, 5D, 5F). The surface of the epithelium positively reacts with PAS, indicating an accumulation of glycoproteins (Figure 5E). One unexpected and interesting observation is the presence of an unidentified parasite in the uterine tubes in all samples examined (Figures 5A, 5C). The exact nature of the parasite remains to be elucidated, but a working hypothesis is that the parasite belongs to *Trichinella* spp, since it shares similar features to parasites observed in other animals such as *Crocodylus niloticus* [52] and other vertebrates [53, 54].



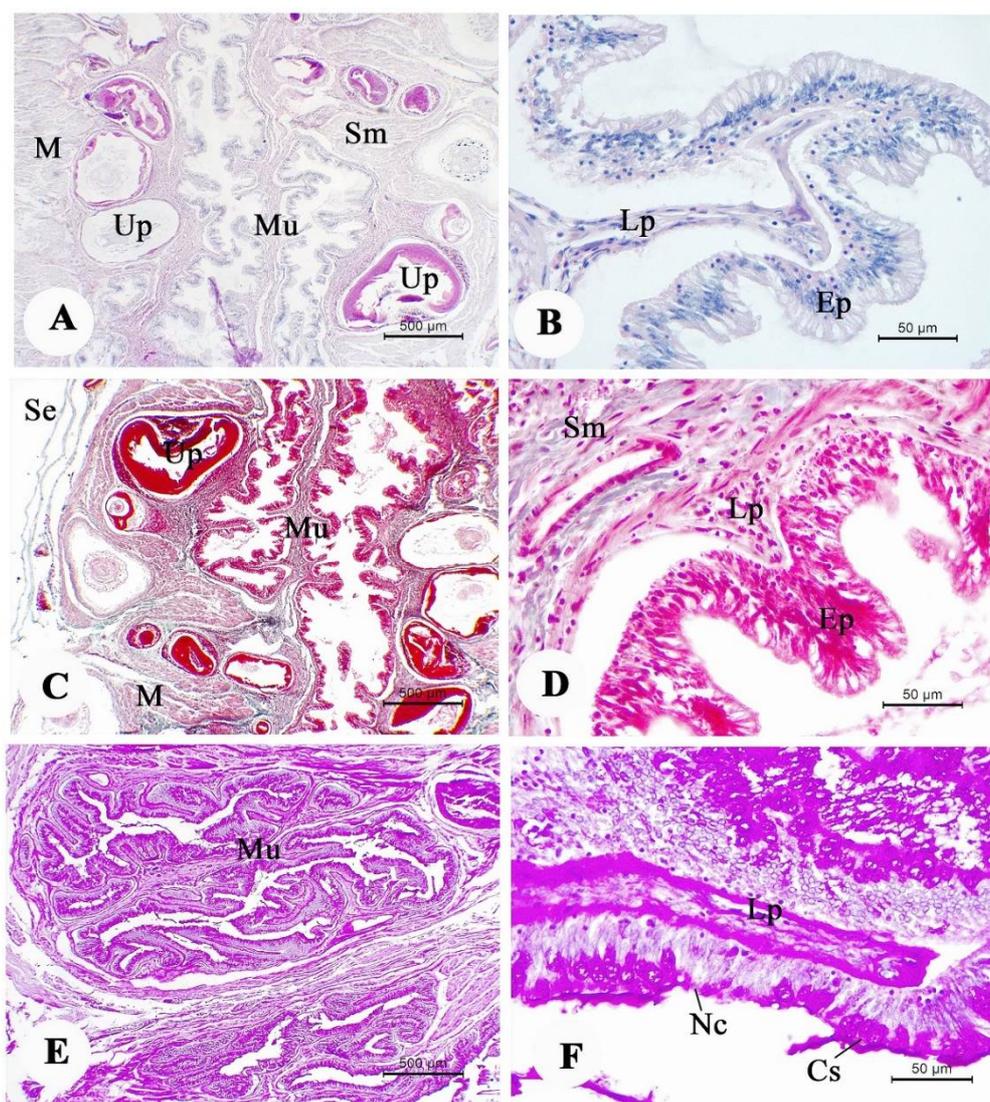
**Figure 4.** Light photomicrographs of infundibulum of *Cerberus rynchops*: (A) Overall histological features of infundibulum (In) with prominent muscularis (M); (B-D) Epithelial layer (Ep) of mucosa (Mu) in infundibulum has non-ciliated simple cuboidal epithelium (Nc) and ciliated simple cuboidal epithelium (Cs) cells. [Bv = blood vessel, Lp = lamina propria] Note: A, B = stained with H&E; C, D = stained with MT

## Uterus

A few irregular folds of the mucosa were observed in the uterus (Figures 6A, 6B). The mucosal epithelium is covered with ciliated and non-ciliated cuboidal epithelium cells (Figures 6B, 6D, 6F). The uterus also exhibits glandular developments (i.e. uterine glands); each gland is well-developed and scattered along the mucosal layer (Figures 6A-6F). The uterine glands are covered by a simple cuboidal epithelium and contain secretory granules (Figure 6F). The uterine glands are present mostly in oviparous snakes, as pointed out in an earlier report [27]. The gland cells contain centrally-located nuclei with heterochromatic materials (Figures 6E, 6F). The epithelial cells of this gland also show both reduced cytoplasm and few secretory granules. Muscularis layers are located around the mucosal layer of the glands (Figures 6C, 6E), but they are slightly thicker (350  $\mu\text{m}$  in diameter) than those in the uterine tube (200  $\mu\text{m}$ ).

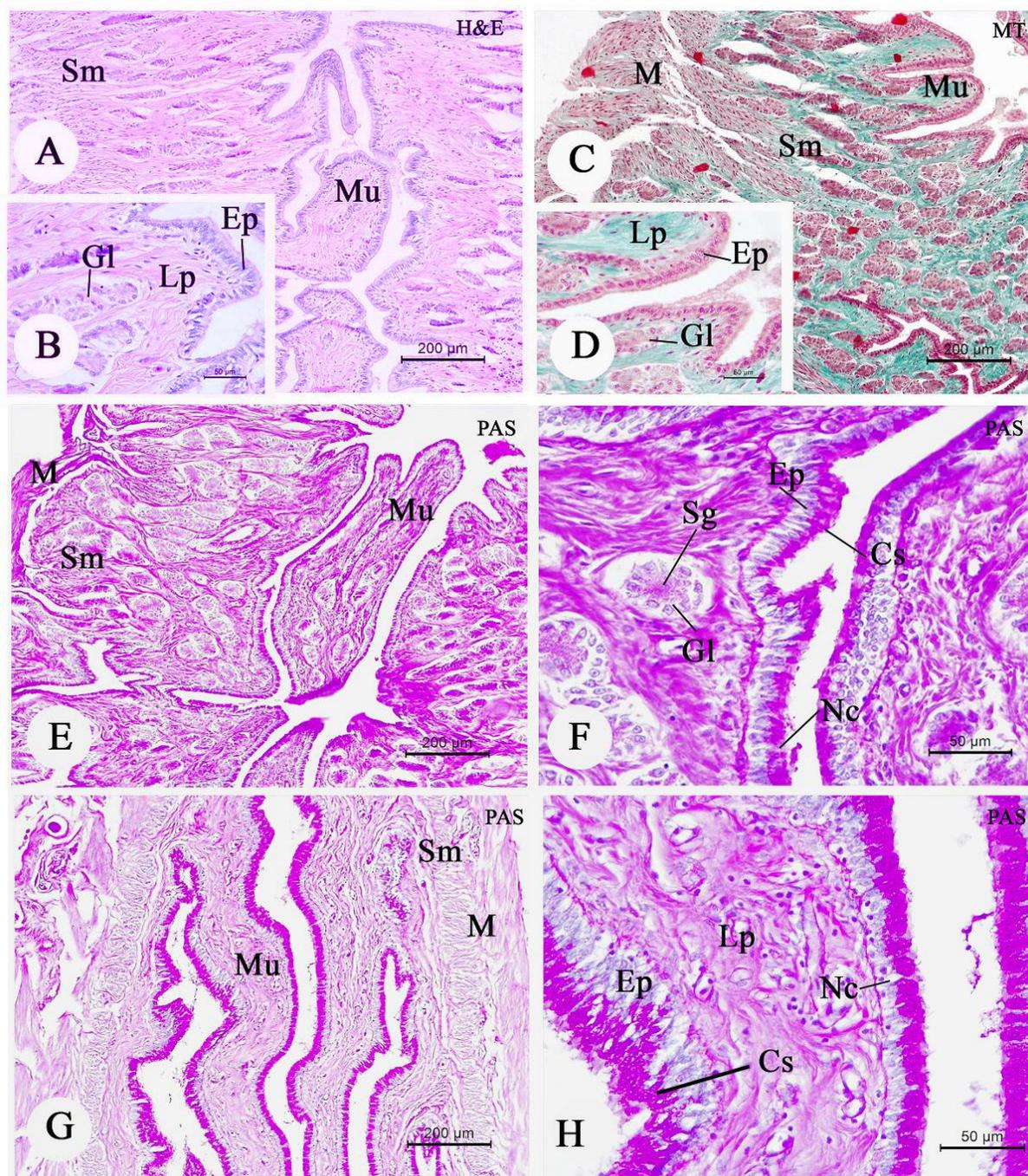
## Vagina

The vagina is a thick-walled tube characterised by elongated, thick and longitudinal mucosal folds (Figure 6G). The mucosal epithelium is composed of tall (about 25  $\mu\text{m}$ ) ciliated and non-ciliated columnar epithelial cells (Figure 6H). The apical surface of the epithelial layer is lightly stained with PAS (Figure 6H). The vagina also has thick muscle layers (Figure 6G), which likely serve as a sphincter for egg retention during gestation and egg movement into the oviposition. A layer of simple squamous epithelium was also found in the serosa.

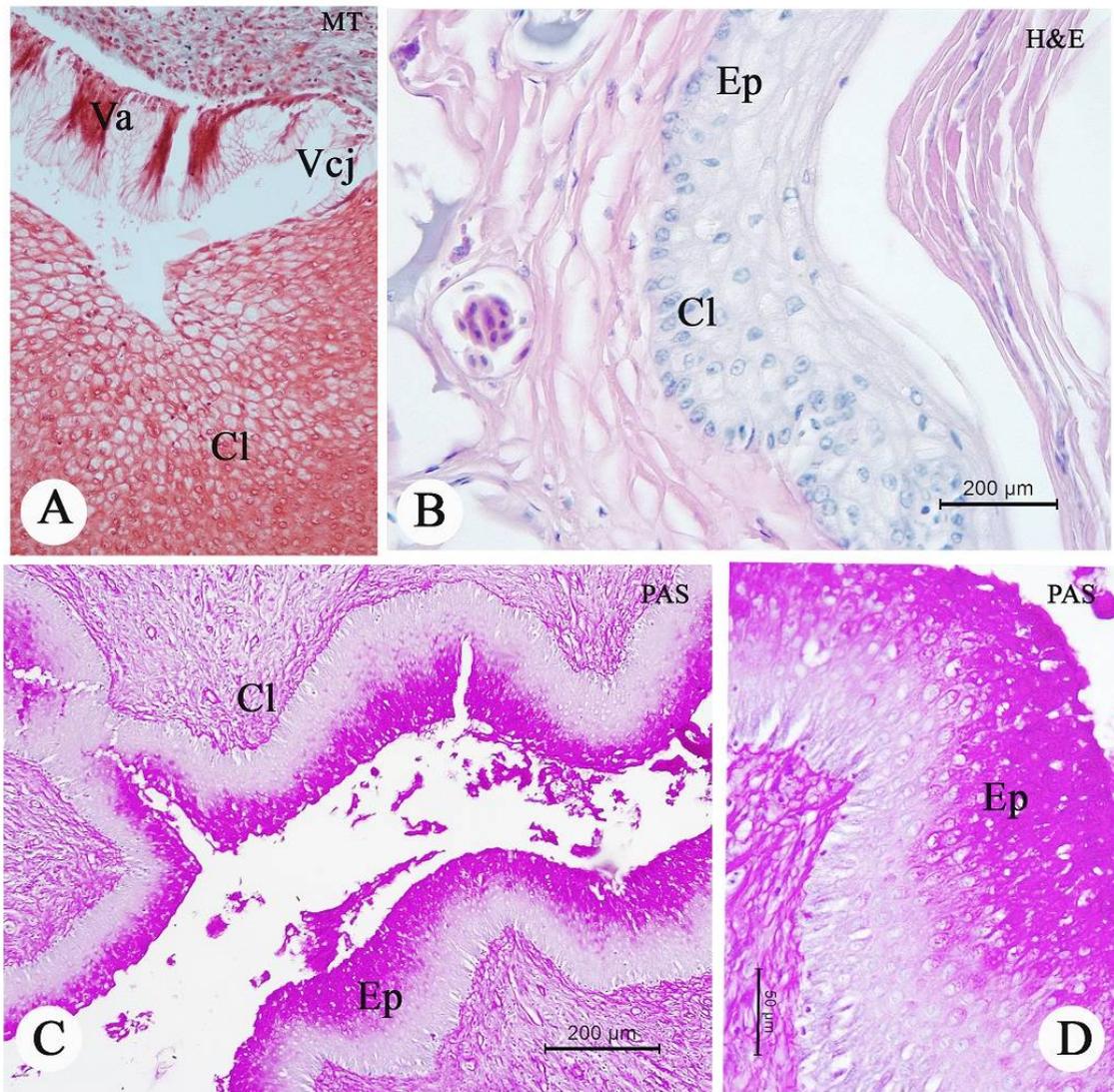


**Figure 5.** Light photomicrographs of uterine tube of *Cerberus rynchops*: (A-D) Overall histology of uterine tube composed of four layers, i.e. mucosa (Mu), submucosa (Sm), muscularis (M) and serosa (Se), unidentified parasites (Up) seen in muscularis; (E,F) Epithelium of mucosa (Mu) was covered by non-ciliated simple cuboidal epithelium (Nc) and ciliated simple cuboidal epithelium (Cs) cells. [Ep = epithelial layer, Lp = lamina propria] Note: A, B = stained with H&E; C, D = stained with MT; E, F = stained with PAS

The virginal-cloacal junction (Figure 7A) was identified by changes in morphological features of the epithelial layers; the morphological transition in *C. rynchops* is similar to those observed in another lizard *Ctenosaura pectinata* [5, 29]. The vaginal region is covered by simple columnar and non-ciliated epithelium cells (Figure 7A). The epithelium is stratified, having 4 to 8 polyhedral cell layers (Figure 7B). The surface of this epithelium also positively reacts with PAS, indicating the presence of glycoprotein (Figure 7D); this observation is consistent with that in another snake *Toluca lineata* [5]. The connective tissue and muscle layers are better defined in the vaginal-cloacal junction than in other regions.



**Figure 6.** Light photomicrographs of uterus and vagina of *Cerberus rynchops*: (A-F) Overall histology of uterus. Epithelial layer (Ep) of mucosa (Mu) is covered by ciliated simple cuboidal epithelium (CS) and non-ciliated simple cuboidal epithelium (Nc) cells. Lamina propria (Lp) of uterus is contained in several glands (Gl); (G, H) Vaginal structure. Epithelial layer (Ep) of mucosa (Mu) is covered by ciliated simple cuboidal epithelium (CS) and non-ciliated simple cuboidal epithelium (Nc) cells. [Lp = lamina propria, M = muscularis, Sm = submucosa, Sg = secretory granules of the gland] Note: A, B = stained with H&E; C, D = stained with MT; E-H = stained with PAS



**Figure 7.** Light photomicrographs of the vaginal-cloaca junction (Vcj) of *Cerberus rynchops*: (A) Structure between vagina (Va) and cloaca (Cl); (B-D) Epithelial layer (Ep) of cloaca (Cl) is stratified, having 4 to 8 polyhedral cell layers. Note: A = stained with MT; B = stained with H&E; C, D = stained with PAS

## CONCLUSIONS

This study has examined the morphology and histological features of the female reproductive system of a snake, *C. rynchops*, which is considered to be viviparous, a placement/designation that has to date not yet been supported with empirical detailed morphological investigation. Viviparity has evolved many times during the evolution of reptiles, and therefore precise features of reproductive systems of viviparous reptiles might be different depending on species. Determination of the seasonal reproductive cycle and behavioral observations of captured *C. rynchops* would be required. These studies would be more beneficial if combined with studies on neuroendocrine fluctuations and evolution.

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