

## Reproductive Morphology of the Golden Tree Snake *Chrysopelea ornata* (Serpentes: Colubridae) from Ao Nang, Krabi Province, Thailand

(Morfologi Pembiakan Ular Pokok Emas *Chrysopelea ornata* (Serpentes: Colubridae) dari Ao Nang, Wilayah Krabi, Thailand)

THANAPHON LIKHITYUNGYUEN<sup>1</sup>, RUJIKORN PIMSORN<sup>1</sup>, TANATPON PROMPA<sup>2</sup>, MONGKOL PONGSUCHART<sup>1</sup> & PANAS TUMKIRATIWONG<sup>1\*</sup>

<sup>1</sup>Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

<sup>2</sup>Department of Anatomy, Faculty of Veterinary, Kasetsart University, Bangkok 10900, Thailand

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### ABSTRACT

We monitored the reproductive morphology of the female and male Golden tree snake, *Chrysopelea ornata*. The females and males were collected during a year from Ao Nang, Muang Krabi, Krabi province, a southern part of Thailand. We investigated the female and male reproductive systems via anatomical and histological approaches. The results demonstrated that the reproductive tracts of females and males were located above the kidney and its right tract was more anteriorly than the left tract. Ovarian follicles were classified into the previtellogenic, early vitellogenic, and late vitellogenic follicles. Previtellogenic follicles were contained three types of cells in the multi-layered granulosa layer: small, intermediate, and large pyriform cells. This such layer becomes the single layer in which the pyriform cells were disappeared in the vitellogenic follicles. Various stages including corpora lutea, gestation, oviposition, and birth were all observed in this study. The oviductal structure of the Golden tree snake was divided into four regions: anterior and posterior infundibulum, uterus, and vagina. The anterior oviductal wall was covered by ciliated and non-ciliated cuboidal epithelial cells and a thin muscularis layer while its posterior portion was contained by ciliated and non-ciliated cuboidal and columnar epithelial cell, and a thick muscularis layer. We found the tubular ciliated glands in the posterior infundibulum. Additionally, the hypertrophied uterine glands in the uterus were observed during the vitellogenic stage. The seminiferous tubules were active simultaneously with the hypertrophied sexual segment of the kidney.

Keywords: *Chrysopelea ornata*; flying snake; ovarian follicle; reproductive tract; testis

### ABSTRAK

Pemantauan morfologi pembiakan ular pokok emas betina dan jantan, *Chrysopelea ornata* telah dilakukan. Ular betina dan jantan telah dikumpulkan selama setahun dari Ao Nang, Muang Krabi, wilayah Krabi, bahagian selatan Thailand. Kajian sistem pembiakan ular betina dan jantan melalui pendekatan anatomi dan histologi. Keputusan menunjukkan bahawa saluran pembiakan betina dan jantan terletak di atas buah pinggang dan saluran kanan lebih anterior daripada saluran kiri. Folikel ovari dikelaskan kepada folikel previtelogen, vitelogen awal dan vitelogen lewat. Folikel previtelogen mengandungi tiga jenis sel dalam lapisan granulosa berbilang lapisan: sel piriform kecil, pertengahan dan besar. Lapisan sedemikian menjadi satu lapisan kerana sel-sel piriform telah hilang dalam folikel vitelogen. Pelbagai peringkat termasuk corpora lutea, kehamilan, oviposisi dan kelahiran diperhatikan dalam kajian ini. Struktur oviduktus ular pokok emas dibahagikan kepada empat kawasan: infundibulum anterior dan posterior, rahim dan faraj. Dinding oviduktus anterior dilitupi oleh sel epitelium kuboid bersilium dan tidak bersilium serta lapisan muskularis nipis manakala bahagian belakangnya mengandungi sel epitelium kuboid dan kolumnar bersilium dan tidak bersilium serta lapisan otot tebal. Kami mendapati tiub kelenjar bersilium di infundibulum posterior. Di samping itu, kelenjar rahim hipertrofi dalam rahim diperhatikan semasa peringkat vitelogenik. Tubul seminifer aktif serentak dengan segmen seksual hipertrofi buah pinggang.

Kata kunci: *Chrysopelea ornata*; folikel ovari; saluran pembiakan; testis; ular terbang

## INTRODUCTION

The Golden tree snake (*Chrysopelea ornata* Shaw 1802) is common in all regions of Thailand and often found in house buildings. The Genus *Chrysopelea* distributes over almost tropical part of southeast Asia (Bulian 1997) and contains 5 species (Socha 2011), including *C. ornata* Shaw 1802; *C. paradisi* Boie 1827; *C. pelias* Linnaeus 1758; *C. rhodopleuron* Fischer 1880; *C. aprobanica* Smith 1943, of which the first three species are restricted to Thailand. A few natural historical studies were available to such this species, especially information on the reproductive biology of this species was little available as far as it was concerned. For example, it belongs to oviparous and polyandrous species with its copulation period being from January to April in Surat Thani province, a southern part of Thailand (Bulian 1997).

We were interested in the female and male reproductive systems of the Golden tree snake. Typically, the reproductive system of female snakes contains a paired ovary and oviduct connecting to a cloacal opening. The terminology of several oviductal portions was a controversial issue among reptilian groups (Rojas et al. 2015). Blackburn (1998) demonstrated that the oviduct was anatomically divided into three different parts, namely infundibulum, uterus, and vagina in an oviparous Horned lizard, *Phrynosoma cornutum*. However, with a histological approach, the oviduct can be divided into several different portions, including the infundibulum, the uterine tube (glandular region), the isthmus (aglandular segment), the uterus (shell-forming region), and the vagina (cervix) in the gekkonid species, *Hoplodactylus maculatus* (Girling 2002). Anatomically, the oviductal regions of the Common garter snake, *Thamnophis sirtalis* (Blackburn 1998), and the Crowned snake, *Tantilla coronata* (Aldridge 1992) can also be divided roughly into three distinct regions: infundibulum, uterus, and vagina as a Horned lizard, *Phrynosoma cornutum*. However, based on the histological approach, the oviductal regions of the Crowned snake can be allocated into several different segments: namely infundibulum, seminal receptacle at the infundibulum-uterus junction, anterior and posterior uterus, and vagina (Aldridge 1992). The several distinguishable regions mentioned above are not recognized in all reptilian species and additional regions may also be included (Aldridge 1992). Rojas et al. (2015) additionally demonstrated that in the Patagonia green racer, *Philodryas patagoniensis*, there are two portions of infundibulum, anterior and posterior infundibulum. The latter one functions as a sperm storage

site in both pregnant and non-pregnant snakes of this species. This species also stores sperm at utero-vaginal junction (UVJ) (Rojas et al. 2015). In Military ground snake, *Erythrolamprus miliaris*, its uterine part is also contained both glandular and non-glandular regions (Rojas et al. 2017).

Grossly, the male reproductive system of typical snakes is comprised of two elongated oval testes, ductus deferens, and hemipenis (Tumkiratiwong et al. 2012). Histologically, the seminiferous tubules pass spermatozoa sequentially through rete testis, ductuli efferentes, ductus epididymis, and ductus deferens (Trauth & Sever 2011). In male squamates, the sexual segment of the kidney (SSK) is another unique characteristic of their reproductive system. SSK is a male accessory sexual gland that passes secretions into the ureter (Trauth & Sever 2011). SSK has a secretory function, and its secretions are mixed with the semen to be transmitted into the female tract during copulation (Aldridge et al. 2011). It undergoes hypertrophy and secretes materials that help maintain sperm stored in the vas deferens prior to ejaculation (Aldridge et al. 2011). It is the largest at the time of spermatogenesis in the Common garter snake, *Thamnophis sirtalis* (Bishop 1959). In some species, it may contribute secretions to the formation of the copulatory plug produced to block the female cloaca after copulation and prevent the entry of sperm from another male (Norris & Carr 2013).

In this study, we monitored the anatomical and histological reproductive morphologies of the female and male Golden tree snake *Chrysopelea ornata* from Ao Nang, Krabi Province, Thailand.

## MATERIALS AND METHODS

## ANIMAL COLLECTION

We collected 24 adult females during January - December 2019 and 10 adult males during May - November 2019 of the Golden tree snake from Ao Nang, Muang Krabi, Krabi province, a southern part of Thailand. The identification of gender was based on the external morphology in which the female's head and body size are larger and greener in color than those of the male. Each snake was captured and placed in a plastic box, then transported to the Physiology Laboratory Division at the Department of Zoology, Faculty of Science, Kasetsart University. This study was approved by the Kasetsart University Institutional Animal Care and Use Committee and found to be in accordance with the guidelines of animal care and use under the Ethical

Review Board of the Office of National Research Council of Thailand (NRCT) for the conduction of the scientific research under ID# ACKU63-SCI-019.

#### STUDY ON THE INTERNAL MORPHOLOGY OF REPRODUCTIVE ORGANS

The individual snake was killed by the freezing method according to Al-Sadoon and Kandeal (2015) in freezer of the household refrigerator for approximately 30 min based on our trial and error for this species. And immediately followed by dissecting ventrally to monitor the gross anatomy of the male and female reproductive system. Female and male reproductive organs were excised and preserved for further investigation on their internal morphologies of the reproductive organs via the histological approach.

#### STUDY ON THE HISTOLOGICAL REPRODUCTIVE ORGANS AND MALE SEXUAL SEGMENT OF THE KIDNEY

The female and male reproductive organs and also male kidney were removed and fixed in a 10% neutral buffered formalin for 48 h. The samples were processed by paraffin technique (Luna 1968) and the tissues were cut in section to 5  $\mu$ m in thickness by using a MICROM rotary microtome model HM 335 E (69190 Walldorf, Germany). The sections were stained with hematoxylin and eosin (H&E stain).

### RESULT

#### EXTERNAL MORPHOLOGY OF FEMALE AND MALE REPRODUCTIVE SYSTEMS

The ovaries of female Golden tree snakes were located above the kidneys. The right one was more anterior to the left one. The ovaries were connected to the elongated oviducts that were opened to the cloacal opening. Anatomically, the oviductal part is divided into three regions: infundibulum, uterus, and vagina (Figure 1(A)). The ovarian follicles were classified into two stages: one, the smaller white previtellogenic follicle (Figure 1(A) and 2(A)), and the other, the larger yellow vitellogenic follicle to which more capillaries were supplied (Figure 1(B) and 2(B)). The infundibulum was the most anterior portion of the oviduct. It was subdivided into anterior and posterior parts (Figure 1(A)). The former was a funnel-shaped portion and the latter is folding appearances. The uterus that connected to the posterior infundibulum had more diameter than that of the posterior infundibulum.

Eggs were found in the uterus via ultrasonography of a snake collected in January (Figure 1(C)). The vagina was elongated that continued from the uterus and has less diameter than that of the uterus. Its posterior end was open into the cloacal opening. We also found a snake laying eggs in June and another snake giving birth in September. We summarized the developmental stages from follicles into the eggs (Figure 2(A)-2(D)).

The testes of the male Golden tree snake were elongated oval bodies and located above the kidney. The right testis was positioned more anteriorly than the left one. The testes were connected to ductus deferens which run parallel to the kidney. Both of them were opened into the cloaca and connected to the long and spinous hemipenes which functioned as an intromittent organ to transmit sperms into the female reproductive tract (Figure 1(D)).

#### HISTOLOGICAL ALTERATIONS IN OVARIAN FOLLICLE

With the histological approach, the ovarian follicles were classified into two stages: Previtellogenic follicle (PVF) (Figure 3(A)) and vitellogenic follicle (VF). The latter was subdivided into the early vitellogenic follicle (eVF) and the late vitellogenic follicle (LVF). The follicle was composed of the oocyte surrounded by the internal granulosa and external thecal layers. The PVF contained the multilayered granulosa in which three types of cells were contained: the small cell (S), the intermediate cell (I), and the pyriform cell (P) (Figure 3(B)). The S and I appeared as a spherical shape and the P were the largest unique flask-like shape. We found the PVF throughout the year.

In VF, the granulosa layer was progressively degenerated into a single layer in which the pyriform cells were disappeared. In eVF, the small yolk granules were abundant in the ooplasm (Figure 3(C) and 3(D)) of snakes collected in April, May, and December. In the LVF, yolk granules in the ooplasm were increased in both their size and number (Figure 3(E)) in snakes sampled in February and December. Additionally, we observed corpus luteum (CL) (Figure 3(F)), composed of luteal cells that were surrounded by thecal cells in the snake collected in June.

#### HISTOLOGICAL ALTERATIONS IN OVIDUCTAL TRACT

The oviduct was classified into four regions based on the histological morphology: Anterior infundibulum, posterior infundibulum, uterus, and vagina. The anterior

infundibulum had characteristics of a thin wall of simple ciliated and non-ciliated cuboidal epithelial cells and the mucosa was arranged into finger-like folding (Figure 4(A)). The posterior infundibulum was an irregular folded structure similar to its anterior region but had a thicker muscular layer than the anterior region and consisted of numerous tubular ciliated glands in lamina propria (Figure 4(B)).

The uterus was characterized by a slightly folding with simple ciliated and non-ciliated columnar epithelial cells which were increased in the number of layers especially at the posterior uterus. The muscular layer was thicker than that of the infundibulum with outer longitudinal and inner circular appearances. The main character of this region was abundant in uterine glands (Figure 4(C)). In the vitellogenic stage, the uterine glands were hypertrophied and abundant in secretory granules (Figure 4(D)).

The vagina had a long-folded structure with well-developed lamina propria and muscular layers. The epithelial cells of the anterior region were simple ciliated and non-ciliated columnar cells (Figure 4(E)) while its posterior region contained pseudostratified ciliated and non-ciliated columnar cells. The cloaca was covered with non-ciliated stratified epithelial cells (Figure 4(F)).

#### HISTOLOGICAL ALTERATIONS IN SEMINIFEROUS TUBULES AND SEXUAL SEGMENT OF KIDNEY

With anatomical and histological testicular studied, spermatozoa were produced in seminiferous tubules (ST) and then, were transported into rete testes, ductuli efferentes, ductus epididymis, and ductus deferens, respectively (Figure 5(A) and 5(B)). The epithelial cell of ST consisted of Sertoli cells and various stages of germ cells. Spermatogonium was located at basal of its tubule and followed by primary spermatocyte, secondary spermatocyte, early spermatid, and late spermatid, respectively. The active spermatozoa were released into the lumen of the ST (Figure 5(C)) to complete the spermiation process. The interstitial tissues were spread throughout ST, which was composed of blood vessels and Leydig cells. The spermatozoa were found densely in ductus epididymis (Figure 5(B)) and ductus deferens (Figure 5(D)) as shown in the snake sampled in May. We found the arrested ST which was reduced in diameter of the tubules and layer of the seminiferous epithelium (Figure 5(E)) as demonstrated in the snake collected in September.

The sexual segments of the kidney (SSK) were characterized by the simple columnar epithelial cells with

the nucleus at the basal region and by its larger diameter as compared to that of the renal tubules. The SSK was hypertrophied with the numerous secretory granules located at the apical region in the Golden tree snake sampled in June (Figure 6(A) and 6(B)) as compared to the regressed SSK with both the reduced diameter of SSK and the secretory granules sampled in October (Figure 6(C) and 6(D)).

#### DISCUSSION

The female reproductive system of the Golden tree snake comprised a paired ovary connected to a paired oviduct which was divided into three main regions; infundibulum with the anterior and posterior subdivision, uterus, and vagina as seen, for example, in the Common garter snake (*Thamnophis sirtalis*) (Halpert et al. 1982). The right side of its reproductive tract is located more anteriorly than the left one, which was similar to most snakes, such as the Monocled cobra (*Naja kaouthia*) (Tumkiratiwong et al. 2012), the Patagonia green racer (*Philodryas patagoniensis*) (Rojas et al. 2015), and the Dog-faced water snake (*Cerberus rynchops*) (Thongboon et al. 2020). Such a pattern mentioned above supported an optimal arrangement of space in the coelomic cavity during pregnancy (Perkins & Palmer 1996). We observed that the PVF contains multi-layered granulosa in which distinct shapes of pyriform cells appeared. Andreuccetti (1992) showed that once differentiated, pyriform cells exhibit abundant ribosomes, Golgi membranes, vacuoles, mitochondria, and lipid droplets, which all of them extend to the apex of the cell at the level of the intercellular bridge, suggesting that its cellular components may be directly transferred to the oocyte. These pyriform cells become disappeared in the granulosa layer whenever turned into the VF as found in this Golden tree snake. In the eVF, the number of such layers was reduced while fat droplets appeared in the oocyte inside the granulosa layer. In the IVF, the increases in size and the number of fat droplets were found in single-layered granulosa as the result of the vitellogenic process under the action of estradiol which stimulates the process of incorporation of the liver-synthesized vitellogenin into the oocyte (Morales et al. 1996). Granulosa and theca cells accumulated with lipids become differentiated into corpora lutea (Bragdon 1952) as found in a Golden tree snake sampled in June.

Following ovulation, the oocytes are firstly moved into the anterior infundibulum, then the posterior one which both portions were distinguished based on the anatomical and histological morphology. The anterior



part enfolds the oocyte, and its ciliated epithelial cell positions the oocyte into it (Girling 2002) whereas the epithelial cell of the posterior part is also composed of ciliated cells which functions as the site of fertilization (Rojas et al. 2015). In several snake species such as *P. patagoniensis* (Rojas et al. 2015), the Military ground snake (*Erythrolamprus miliaris*) (Rojas et al. 2017), the Sand-dunes blackhead (*Apostolepis gaboi*) (Braz et al. 2019), and the Amazonian lancehead (*Bothrops atrox*) (Silva et al. 2019), such a region functions as sperm receptacles to store sperm. In this study, we did not found sperm inside sperm receptacles. We found numerous tubular ciliated glands that are projected into the lumen of the posterior infundibulum. Rojas et al. (2017) described that this gland plays a role in

transporting sperms into sperm receptacles. The uterus is a portion of eggshell production (Girling 2002; Perkins & Palmer 1996). We demonstrated that the main character of this region was composed of numerous uterine glands, which underwent hypertrophied in the vitellogenic females, similar to most oviparous snakes, such as the Ring-necked snake (*Diadophis punctatus*) (Perkins & Palmer 1996) and *P. patagoniensis* (Rojas et al. 2015). In addition, it had a thick muscular layer to expel the egg during oviposition (Girling 2002). Their muscular layers of a vagina were well developed for egg retention during gestation, and for eggs movement during oviposition (Thongboon et al. 2020).

According to our investigations on histological alternations in seminiferous tubules (ST) and sexual

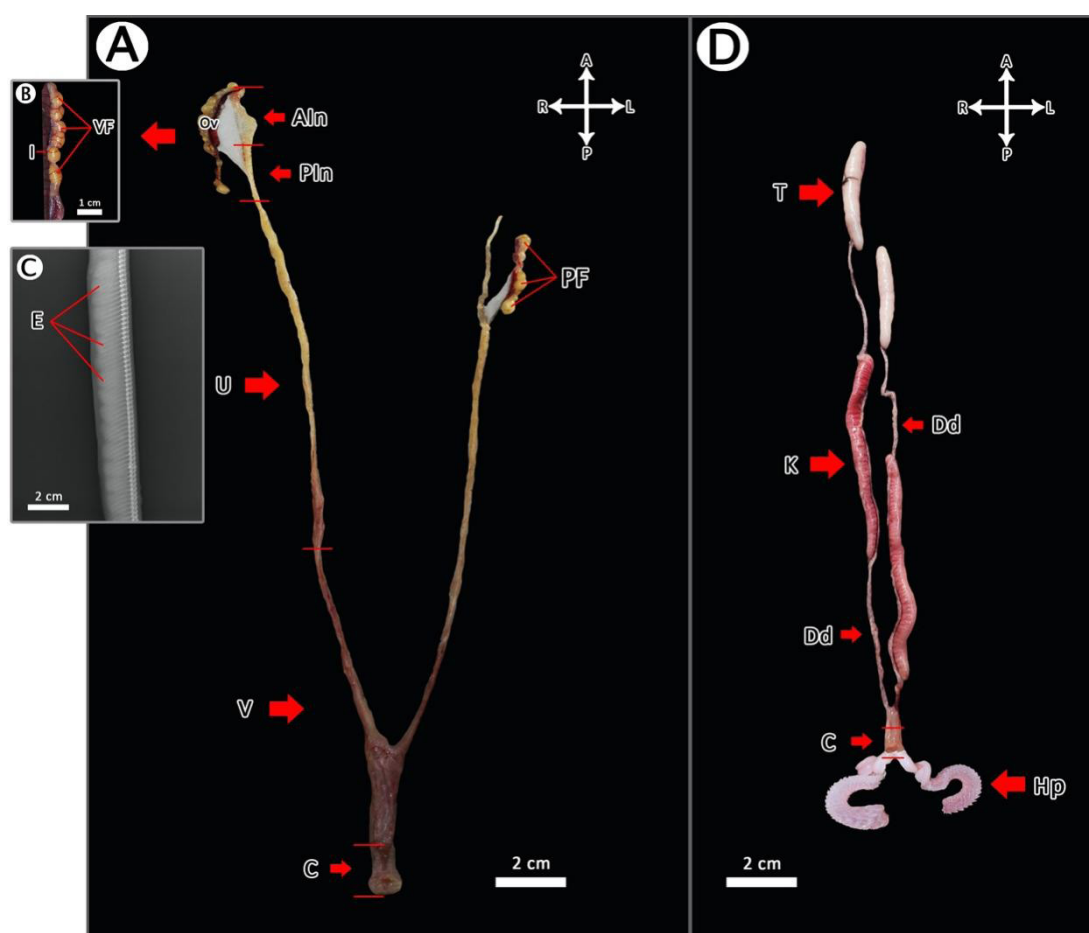


FIGURE 1. Internal reproductive organs of Golden tree snake: A, female internal reproductive organs; B, vitellogenic follicles; C, uterine eggs; and D, male internal reproductive organs

A, anterior; Aln, anterior infundibulum; C, cloaca; Dd, ductus deferens; E, eggs; Hp, hemipenis; I, intestine; K, kidney; L, left; Ov, ovary; P, posterior; PF, previtellogenic follicles; Pln, posterior infundibulum; R, right; T, testis; U, uterus; V, vagina; and VF, vitellogenic follicles

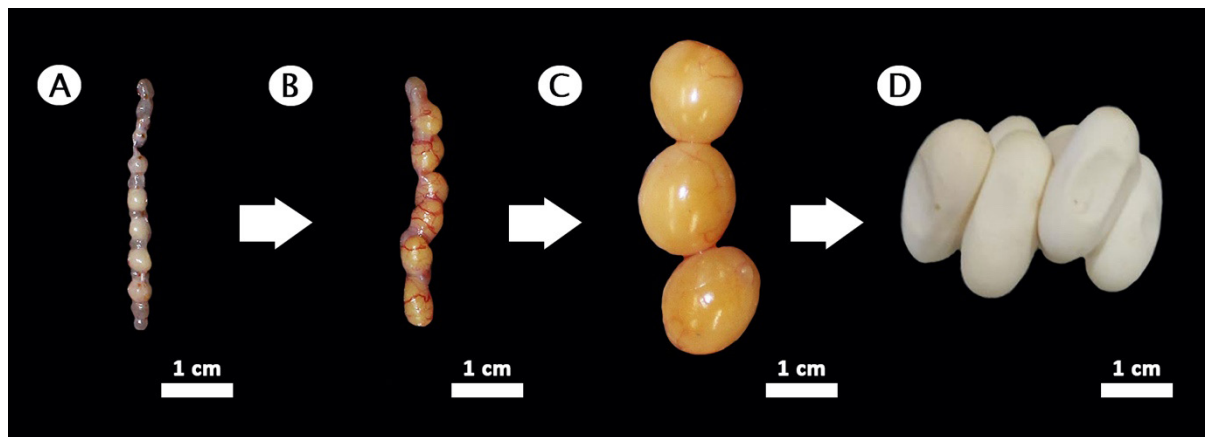


FIGURE 2. The development from previtellogenic follicles into eggs of the Golden tree snake

A, previtellogenic follicles; B and C, the vitellogenic follicles; D, eggs

segments of the kidney (SSK) of the male Golden tree snake, both ST and SSK were hypertrophied as shown in snakes sampled in May, June, July, and November, however, this was not theoretical assertion as there was no morphometric and statistical data caused by few male snake samples during a year. Meesook et al. (2016) stated that timings in which both ST and SSK of *Calotes* spp. were active spermatogenic and hypertrophied stages have simultaneously occurred. In other words, timings in which they showed the arrested spermatogenesis and regressed SSK were in accordance with those of the regressed testes (Meesook et al. 2016). In the male *T. sirtalis*, its testes were spermatogenically active at the same time as the hypertrophied SSK and in the active reproductive event, the diameter of the SSK tubule was five times greater than that of the SSK tubule compared to the inactive reproductive event (Bishop 1959). However, developed SSK had been reported in the female Rainbow whiptail lizard, Genus *Cnemidophorus* (Del Conte 1972; Del Conte & Tamayo 1973) and the Ground skink (*Scincella laterale*) (Sever & Hopkins 2005), suggesting that such females secreted a low level of spontaneous androgens which caused SSK development (Del Conte & Tamayo 1973; Sever & Hopkins 2005). In this study, we did not

monitor the alterations in female SSK. The hypertrophied SSK was synchronous with both androgen secretion and spermatogenic activity (Sever & Hopkins 2005). The SSK of sexually active squamates underwent hypertrophy via the action of androgens (Norris 2007). In this study, we did not investigate the SSK alteration and androgen secretion. We found regressed SSK in male Golden tree snakes in August, September, and October. However, we could not assert the regressed state in SSK due to the shortage of male samples. Meesook et al. (2016) demonstrated that in *Calotes* spp., the hypertrophied and the regressed SSK were changed seasonally and synchronously with the active spermatogenic event and the spermatogenic arrest, respectively. SSK secretions form a copulatory plug that adheres to the female's cloaca following copulation to occlude oviductal openings in the Iberian rock lizard (*Lacerta monticola*) but such a plug neither did prevent the subsequent mating nor did reduce the female's attractiveness (Moreira & Birkhead 2003). Srivastava and Thapliyal (1965) demonstrated that in the Chequered water snake (*Natrix piscator*), recrudescence and regression of spermatogenesis are seasonal changes while the Leydig cells and SSK showed acyclic changes.

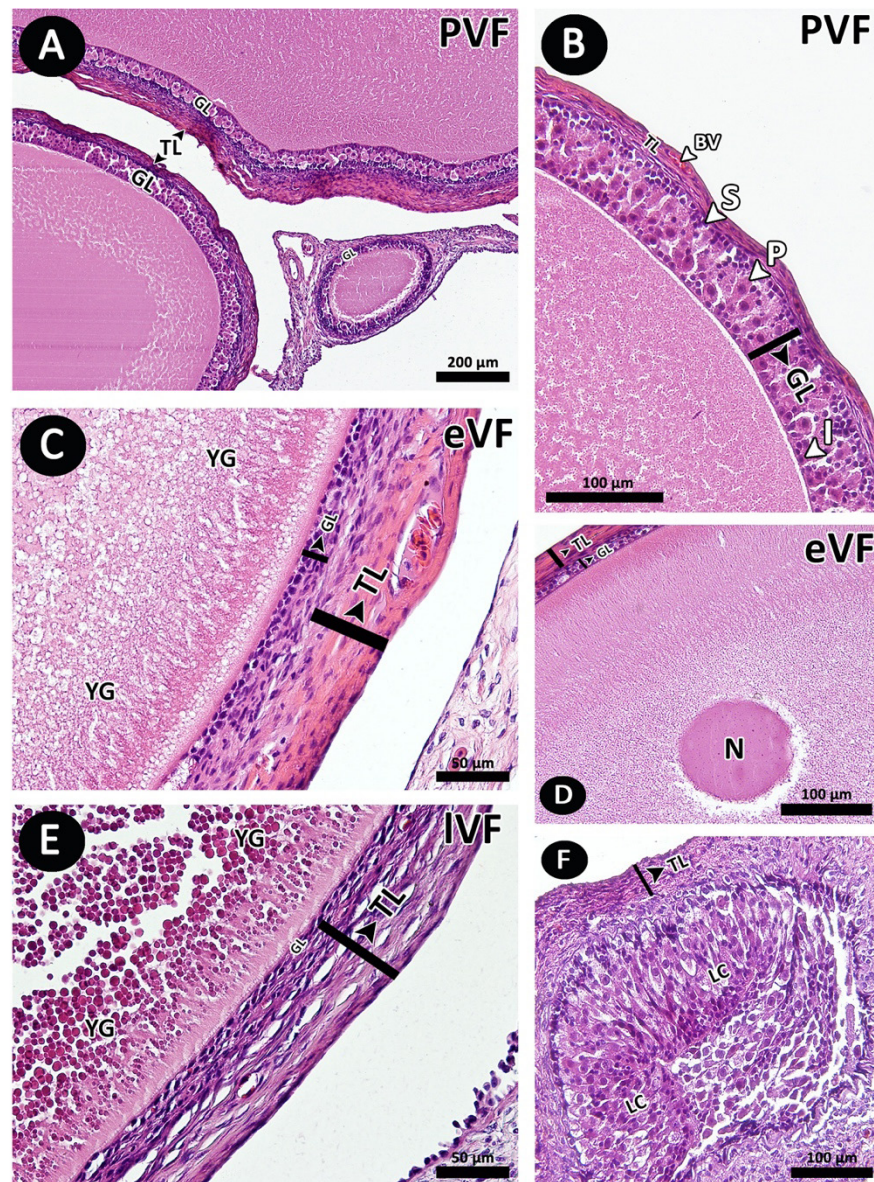


FIGURE 3. Transverse sections of follicles of the Golden tree snake: A and B, previtellogenic follicles of snake in February; C and D, early vitellogenic follicle of snake in April; E, late vitellogenic follicle of snake in February; and F corpus luteum of snake in June

BV, blood vessel; eVF, early vitellogenic follicle; GL, granulosa layer; I, intermediate cell; LC, luteal cells; IVF, late vitellogenic follicle; N, nucleus; P, pyriform cell; PVF, previtellogenic follicle; S, small cell; TL, theca layer; and YG, yolk granules



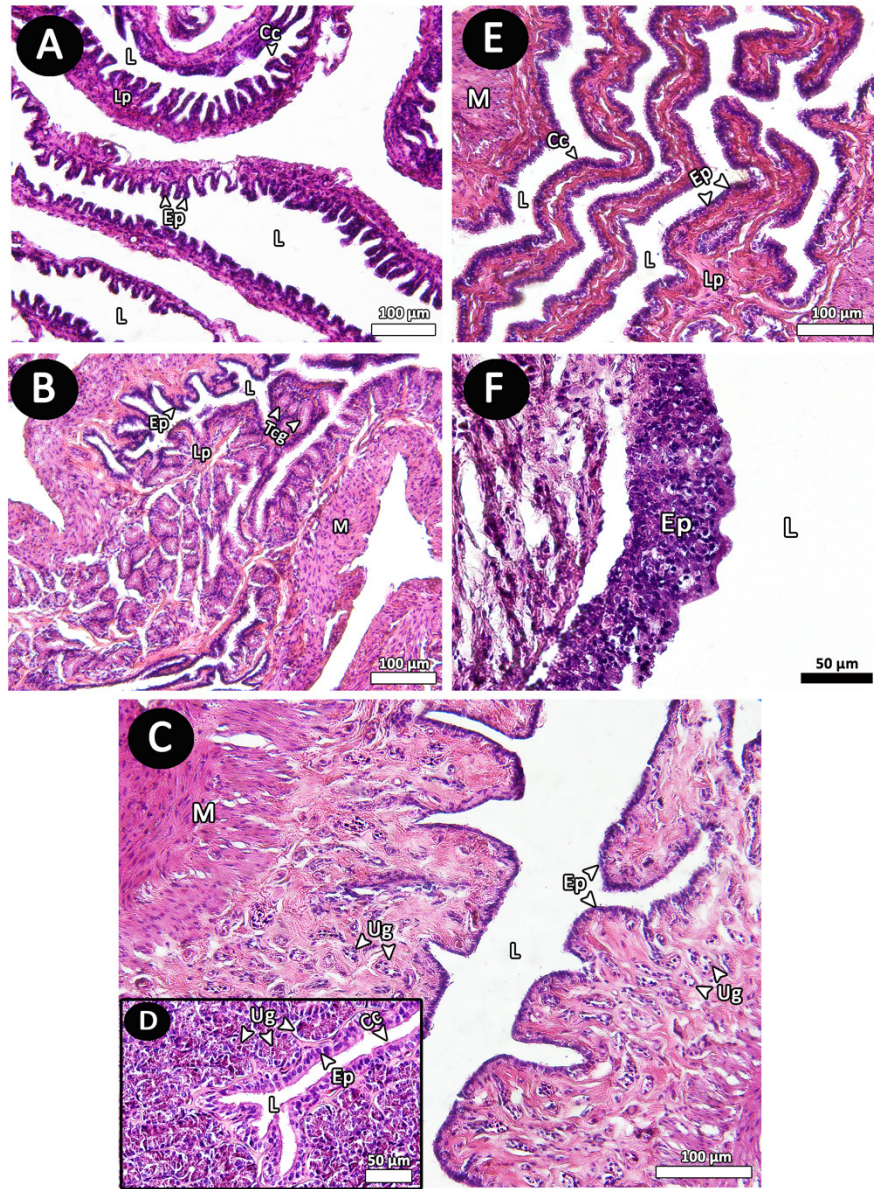


FIGURE 4. Transverse sections of the female reproductive tracts of the Golden tree snake: A, anterior infundibulum; B, posterior infundibulum; C, uterus of the previtellogenic female; D, uterus of vitellogenic female; E, vagina; and F, cloacal opening

Cc, ciliated cell; Tcg, tubular ciliated gland; Ep, epithelial cell; L, lumen; Lp, lamina propria; M, muscularis; and Ug, uterine gland



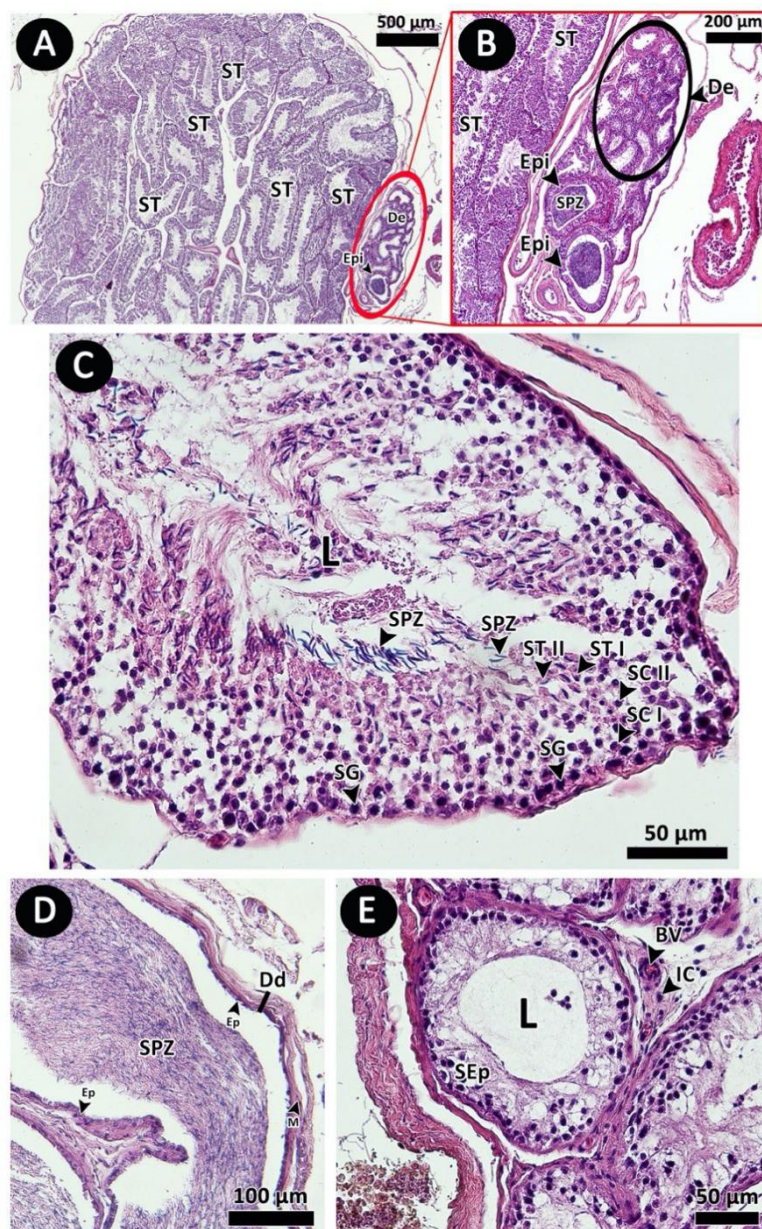


FIGURE 5. Transverse sections of the male reproductive system of the Golden tree snake: A, cross section of the testis; B, testicular ducts; C, spermatogenesis in seminiferous tubule in May; D, spermatozoa in ductus deferens in May; and E, testicular regression in September

BV, blood vessel; Dd, ductus deferens; De, ductuli efferentes;  
 Ep, epithelial cell; Epi, ductus epididymis; IC, interstitial cells; L, lumen; M, muscular layer; SG, spermatogonium;  
 SEp, seminiferous epithelium; ST, seminiferous tubule; SC I, primary spermatocyte; SC II, secondary spermatocyte; ST  
 I, early spermatid; ST II, late spermatid; and SPZ, spermatozoa



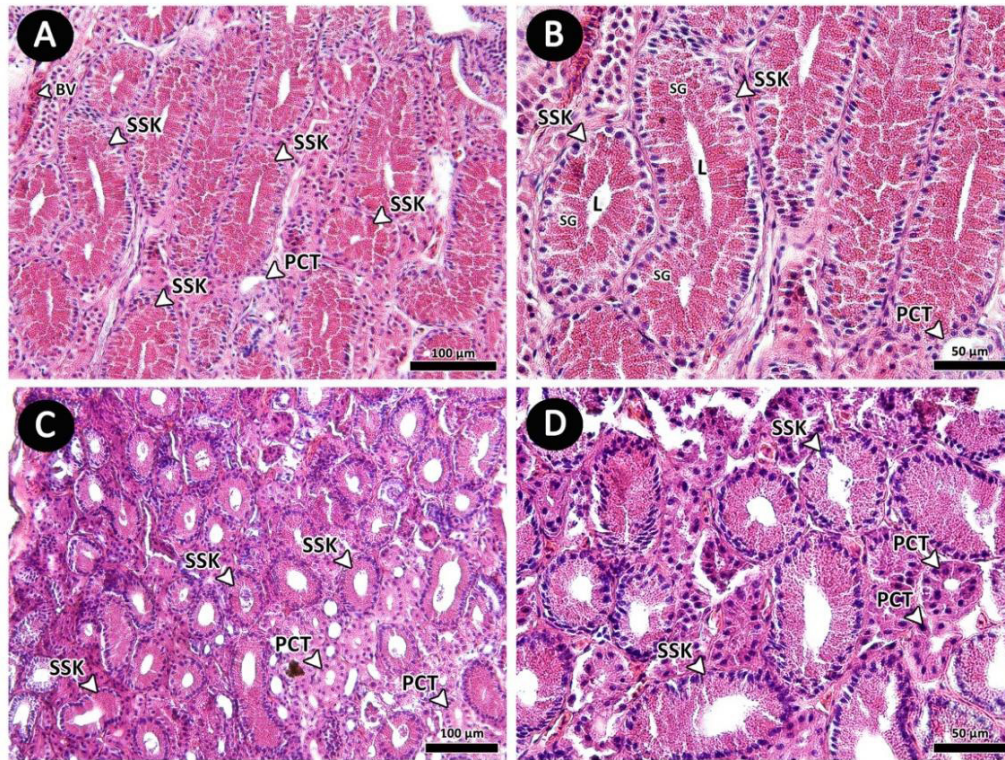


FIGURE 6. Transverse sections of the kidney of the Golden tree snake: A, hypertrophied SSK in June; B, higher magnification of A; C, regressed SSK in October; and D, higher magnification of C

BV, blood vessel; L, lumen; PCT, proximal convoluted tubule; SG, secretory granules; and SSK, sexual segment of the kidney

#### CONCLUSION

Three types of follicles including PVF, eVF, and IVF were contained inside ovaries. Pyriform cells were appeared in the PVF and degenerated in the VF. Yolk granules in VF were increased in both numbers and sizes. The simple ciliated and non-ciliated cuboidal epithelial cells and the finger-like folding mucosa lined the anterior infundibulum as the posterior one but the latter having a thicker muscular layer and numerous tubular ciliated glands in lamina propria. The simple ciliated and non-ciliated columnar epithelial cells were found in the uterus with abundances of the uterine glands which were hypertrophied in vitellogenic female. The well-developed lamina propria and muscular layers occurred primarily in the vagina and the epithelial cells of its anterior region were a simple ciliated and non-ciliated columnar while its posterior one was pseudostratified columnar. The lumen of ductus epididymis and ductus deferens were accumulated with abundant spermatozoa

and the hypertrophied SSK with the numerous secretory granules were demonstrated in the active testes in the Golden tree snake, *Chrysopelea ornata*.

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\*Corresponding author; email: fscipnt@ku.ac.th