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Original Article

Ovarian morphology and histology of the golden tree snake *Chrysopelea ornata* (Shaw, 1802)

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Abstract

In this study, we aimed to describe the morphology of ovaries, and used histological approaches to define the ovary morphology and oogenic follicles, including the proportion of atresia of the golden tree snake, *Chrysopelea ornata* (Shaw, 1802). Morphological observations showed that the ovaries contained different stages of follicles, suggesting active ovarian activities during sexual maturation of the female snake. Half of the follicles in a paired ovary, where the oogenesis took place, were previtellogenic follicles. Multiple layered structures of the follicular cells in the previtellogenic follicles could be categorized into at least three distinguishable cell types based on cell size and shape including small cells, intermediate cells, and pyriform cells. The previtellogenic follicles continuously developed into early vitellogenic follicles where more uptake of yolk granules could be observed. In addition, ovaries also contained the atresia (atretic follicles) showing the irregular follicles and sign of yolk degeneration. The atresia can be found in about 37% of the total follicles. For the first time, this study focused on the ovarian histology and proportions of follicular types in the golden tree snake.

Keywords: folliculogenesis, golden tree snake, histology, oogenesis, reproductive system

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1. Introduction

The knowledge on morphology and histology of reproductive system in reptile is necessary for a better understanding of vertebrate reproductive biology, adaptations, reproductive cycle, and life-history strategies. The reproductive system of female snakes is complex and could be classified into two parts including the elongated ovarian tissues and the oviducts (Jacobson, 2007; Shanbhag, 2002; Thongboon *et al.*, 2020). It is well known that the differentiating follicles of oocyte and folliculogenesis in snakes are similar to birds and other reptiles (Guraya, 1989). A particular ovarian event such as the atresia or atretic follicle with the breakdown of its follicular layers in the snake has been widely observed (Guraya, 1965; Thongboon *et al.*, 2020), as a major determining and limiting factor to achieve the succession of *in vitro* production of snake embryos. It was indicated that the low number of atretic follicles resulted in an increase in the offspring and reproductive efficiency.

The golden tree snake, *Chrysopelea ornata* (Shaw, 1802), belongs to the family Colubridae, which is widely distributed in Southeast Asia, including Thailand (Hawkeswood, 2017; Nurngsomsri, 2015). Regarding reproductive modes, the golden tree snake is an oviparous snake (IUCN Bangladesh, 2015). The distribution, external morphology and taxonomy of the golden tree snake *C. ornata* are well documented (Nurngsomsri, 2015); however, certain internal anatomy, particularly, the reproductive system, remains unknown. The histological approach is thus required to better understand the ovarian structure as well as the proportion of follicular types and oocytes in *C. ornata*.

2. Materials and Methods

Regarding samples in this study, five females of *Chrysopelea ornata* with 67.55±0.86 cm of the average snout-vent length (SVL) were randomly obtained from Khohong Mountain, Songkla province (7° 0' 25.5" N; 100° 29' 54.5" E) in Thailand from January to June 2017. All specimens were captured by hand incorporating with a torch at night time. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1723001).

All snake samples were longitudinally dissected. The morphology of the snake reproductive features was observed under a stereomicroscope (Leica 750; Leica Camera AG, Wetzler, Germany). After that, their whole bodies were fixed by immersion in Bouin's solution for 24 hrs and processed using standard histological procedures (Presnell and Schreibman, 1997; Suvarna, Layton, & Bancroft, 2013). Three sections of 4-μm-thick paraffin-embedded blocks were obtained and stained with Harris's haematoxylin and eosin (H&E) (Presnell & Schreibman, 1997; Suvarna *et al.*, 2013). The H&E stained sections were examined to visualize the ovarian structures and oogenesis using the guidelines according to previously established criteria (Uribe, Gonzalez-Porter, Palmer, & Guillette1998, Siegel, Miralles, Chabarria, & Aldridge, 2011, Thongboon *et al.* 2020). The photomicrographs were taken by using a Leica DM750 light microscope with a digital camera. Three histological slides were used to determine the size and features of the oocyte, which were quantified using the Image J system. (National Institute of Health, EUA). Also, the oocyte proportion were counted from histological slides and calculated by the formula: Number of each oocyte stage x 100/total number of oocytes.

3. Results and Discussion

3.1 Morphology of female reproductive system of the golden tree snake

The reproductive system of the golden snake *C. ornata* was comprised of a paired elongate ovaries and oviducts (Figures 1A-2B). The elongated ovaries were attached to the kidney through the mesentery (Figure 2A), as commonly seen in lizards (Guraya, 1989) and other snakes (Thonhboon *et al.*, 2020). The snake ovary produced eight to nine oocytes (or ovarian follicles), as shown in Figure 2B. Therefore, a large cluster of oocytes was seen at once (Figure 1). The small and oval-shaped previtellogenic follicles were identified within the ovary (Figure 2B). This feature was similar to what found in the Texas blind snake *Leptotyphlops dulcis*, the Western blind snake *Leptotyphlops humilis*, the Angola blind snake *Typhlops angolensis* (Fox & Dessauer, 1962) and the grass snake *Natrix natrix* (Gadow, 1987). In this study, the snakes were collected from the same season. However, the reproductive cycle of the golden tree snake *C. ornata* has not been previously documented in Thailand and should be further studied to understand different proportion of follicles within the ovary in response to seasons.

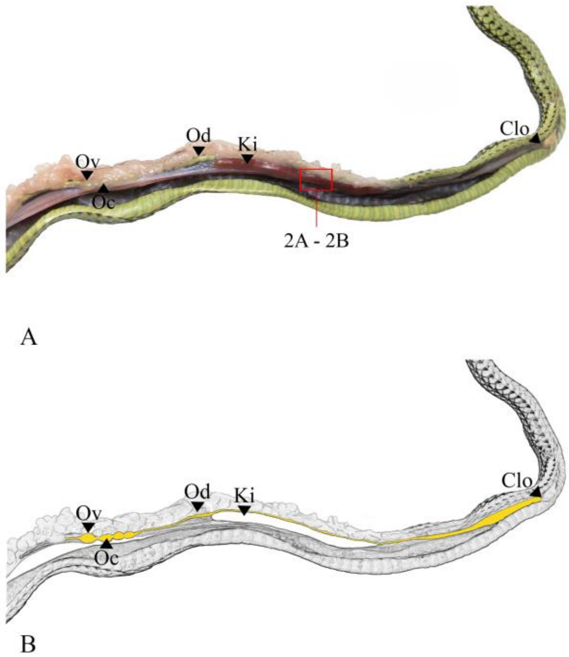

Figure 1. External morphology (A) and schematic diagram (B) of the ovary in longitudinal view of the golden tree snake *Chrysopelea ornata*. The elongated ovary (Ov) is composed of differentiating oocytes (Oc). Abbreviations: Clo = cloaca, Ki = kidney, Od = oviduct

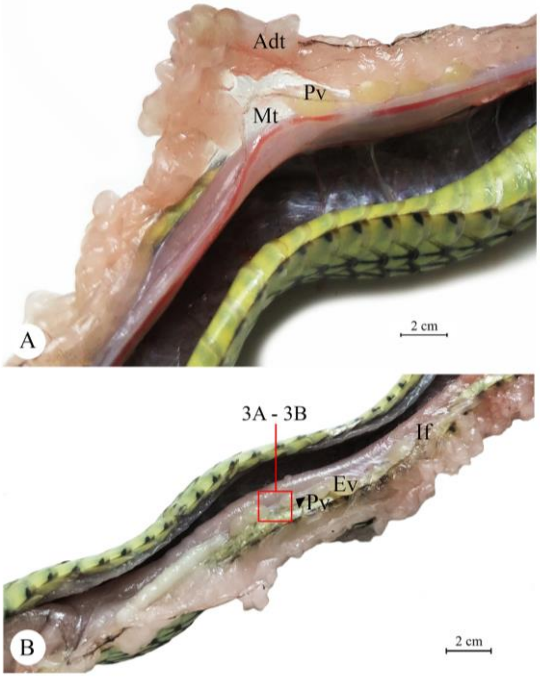

Figure 2. Magnified and longitudinal view of the ovary and surrounding tissues of the golden tree snake *Chrysopelea ornata*. A: Photograph showing prominent previtellogenic follicles (Pv) embedded within the adipose tissue (Adt) and ventrally attached by the mesentery (Mt). B: Photograph showing both previtellogenic follicles (Pv) and early vitellogenic follicles (Ev) found along the ovary length. The funnel-shaped infundibulum (If) close to the ovary was also observed.

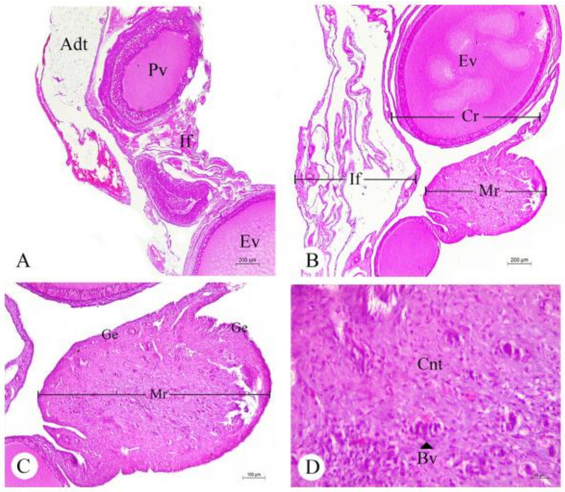

Figure 3. Photomicrographs showing the ovarian structure of the golden tree snake *Chrysopelea ornata*. A: The ovarian structure (longitudinal section) showing the previtellogenic follicles (Pv) and early vitellogenic follicles (Ev). B: The ovarian structure composed by two regions: cortex region (Cr) and medullar region (Mr). The cortex region contained different stages of follicles such as early vitellogenic follicle (Ev). C-D: The cortex region (Cr) was surrounded by the thin layer of germinal epithelium (Ge). Within the medullar region, it contained connective tissue (Cnt) and blood vessels (Bv). Abbreviation: Adt = adipose tissue. Staining method: A-D, Harris's haematoxylin and eosin (H&E)

3.2 Ovarian structure and oogenic follicles

According to the longitudinal section, our observation showed that the ovary was close to adipose tissue and infundibulum (Figure 3A). It is known that the multiple oogonia (contained a large central nucleus and about 10-15 µm in diameter) also existed as cell clusters but they were rarely observed in this study. It is suggested that the presence of oogonial proliferation was commonly found during the embryonic development and continues in adulthood in some types of reptiles (Lessman, 1999) for example *Podarcis sicula* (Machado-Santos *et al.*, 2015).

The snake ovaries were generally divided into two regions called medulla and cortex regions, as shown in Uribe *et al.* (1998), Siegel *et al.* (2011) and Ramírez-Pinilla *et al.* (2015). These defined regions were also found from histological observation in the ovary of *C. ornata* (Figure 3B). Several differentiating follicles of oocytes were concentrated within the cortex regions (Figure 3C). The medulla region carries vascularized connective tissues containing small arteries and capillaries (Figure 3D).

In this study, the ovary was considered an asynchronous ovarian type, in which the oocytes could be classified into three different follicles based on cell shape, folliculogenesis, size of the cells and histological staining including previtellogenic follicles (Pv), early and late vitellogenic follicles (Ev and Lv), and atretic follicles or follicular atresia (At).

3.3 Previtellogenic follicles

During the primary growth phase, the previtellogenic follicles (400-500 µm in diameter) were observed mostly in this study (51.7% proportion). The oval nucleus enlarged and was surrounded by the acidophilic cytoplasm (Figure 4A). An exclusive feature of this stage was the increase in the follicular epithelium (Figure 4A). Three distinct layers of the follicular epithelium included small cells, intermediate cells, and pyriform cells were identified (Figure 4A). These characters were largely similar to those described for the follicles of the long-tailed skinks *Mabuya* sp. (Ramírez-Pinilla *et al.*, 2015) and the monocled cobra *Naja kaouthia* (Tumkiratiwong, Meesuk, Chanhome, & Aoqphol, 2012), but multilayered epithelium up to 4-6 layers were documented in the green lizard *Anolis carolinensis* (Laughran, Larsen, & Schroeder, 1981) and the Mexican spiny-tailed iguana *Ctenosaura pectinata* (Uribe *et al.*, 1996).

The size of small cells was 5.57±0.13 µm in diameter and its arrangement was closely located near the basal lamina of the oocytes. The round-to-oval nuclei of the small cells were easily found and surrounded by lightly granular cytoplasm (Figure 4A). It was reported that the small cells were differentiated into spherical intermediate cells. The diameter of intermediate cells with 8.45±0.08 µm was observed. The intermediate cells had a large nucleus and were surrounded by eosinophilic cytoplasm (Figure 4A), similar to what found in the northern alligator lizard *Elgaria coeruleus* (Klosterman, 1987), the Italian wall lizard *Podarcis siculus* (Filosa, Taddei, & Andreuccetti, 1979) and other lizards (Ramírez-Pinilla *et al.*, 2015).

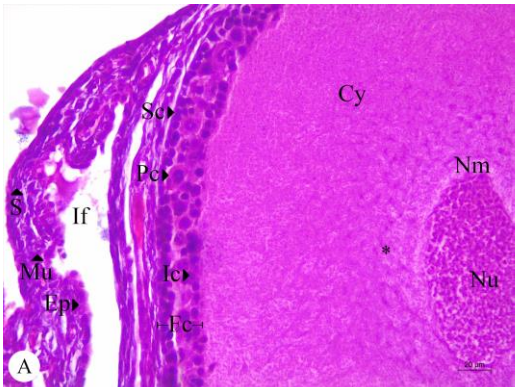

Figure 4. Magnified photomicrograph showing a previtellogenic follicle and its follicular layer (Fc) of the golden tree snake *Chrysopelea ornata*. This follicle showed the oval nucleus (Nu) with the prominent nuclear membrane (Nm). The heterogenous content (asterisk) was also found within the cytoplasm (Cy) close to the nuclear membrane (Nm). The follicular cell layer composed by small cells (Sc), intermediate cells (Ic), and pyriform cells (Pc). Infundibulum (If) contained three layers including epithelium (Ep), muscularis (Mu) and serosa (S). Staining method: Harris's haematoxylin and eosin (H&E)

Pyriform cell sizes were increased having 16.10±0.71 µm in diameter (Figure 4A). These cells probably differentiated from the small cells (Betz, 1963; Boyd, 1940). The pyriform cells had a large nucleus surrounded with a pale cytoplasm. As follicular development progressed, a thin layer of the zona pellucida-like structure in the previtellogenic follicles were found (Figure 4A), as similarly reported in other lizards (Ibrahim & Wilson, 1989; Maurizii & Taddei, 2012). Previous observation suggested that the important function of the pyriform cells was observed, playing an indirect role in the regulation of vitellogenin production (Betz, 1963, Goldberg, 1970, Saha, Manna, & Sircara, 1984).

3.4 Vitellogenic follicles

During the second growth phase, vitellogenic follicles were classified into the early and late vitellogenic follicles in snakes (Thongboon *et al.*, 2020); however, the early vitellogenic follicle was only found in this study (Figure 5). According to Santos *et al.* (2015), it indicated that lizards from different seasons exhibited different proportion of follicular phases. The presence of only early vitellogenic phase in this study might be hence a result of seasons during sampling collection.

The early vitellogenic follicles were shown approximately 11.1% of the follicles in the ovary and were characterized by an increase in size, about 1.44±3.77 cm in diameter. The multilayered follicular epithelium was identified (Figure 5A, 5C). This follicle stage began to accumulate the small and acidophilic yolk granules in the cytoplasm (Figure 5B), as generally found in the lizard oocyte development (Ho *et al.*, 1982). It is well known that the synthesis of yolk granules took place in the hepatocyte. The yolk granules are then transported into the bloodstream and delivered into the oocytes (Limatola & Filosa 1989; Tokarz, 1977). This yolk granule synthesis and uptake pathway is regulated by the estrogen (estradiol-17β), which is essential to

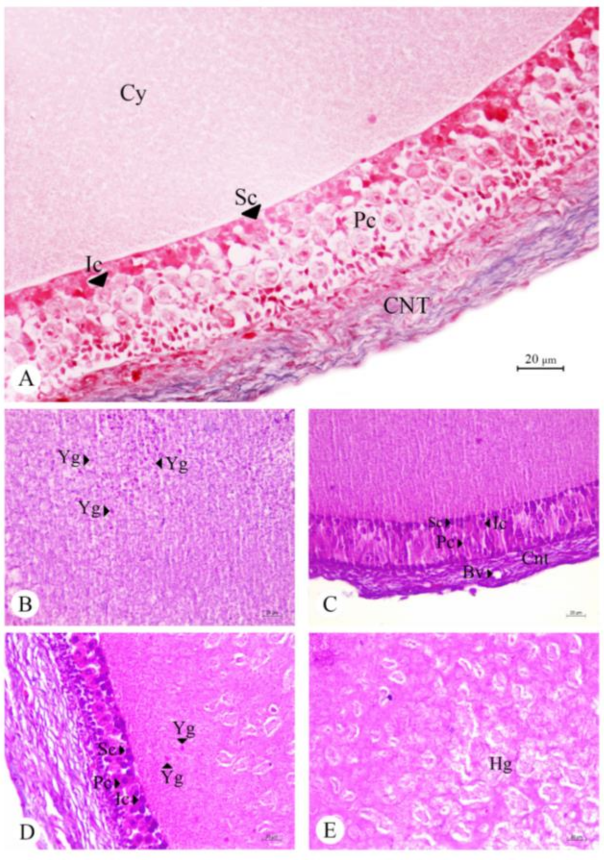

Figure 5. Photomicrographs showing the early vitellogenic follicles and its follicular layers (Fc) of the golden tree snake *Chrysopelea ornata*. A-C: The follicle was surrounded by a layer of connective tissue (CNT). At the beginning of early vitellogenic follicle, the yolk granules (Yg) were firstly accumulated. The follicular cell layers were clearly developed in this stage of follicle, compared to the previtellogenic follicle. The follicular layers were composed of small cells (Sc), intermediate cells (Ic), and pyriform cells (Pc). Later, the early vitellogenic follicle with the formation of heterogenous substance (Hg) was observed. Staining methods: A, Masson Trichrome; B-C, Harris's haematoxylin and eosin. Abbreviations: Bv = blood vessel, CNT = connective tissue, Cy = cytoplasm, Yg = yolk granule

control vertebrate reproductive activities (Tokarz, 1977; Morales, Baerga-Santin, & Cordero-López, 1996; Edwards, Jones, & Davies, 2002). It is generally accepted that the yolk deposits help to support embryo development (Varma, 1970; Uribe, Omana, Quintero, and Guillette, 1995). The multilayered follicular epithelium was continuously developed, especially in pyriform cells (Figure 5D), which in agreement with previous observation in *Hemidactylus mabouia* (Moodley & Van wyk, 2007). The formation of heterogenous substance (Hg) in the ooplasm was also found (Figure 5E).

3.5 Atretic follicles

Numerous atretic follicles were observed in ovarian structures up to approximately 37% of total follicles. The follicular atresia was irregular in shape and contained fragmented follicular cell layers in the previtellogenic follicles (Figures 6A). The key feature of the atresia included vacuolization (presence of number vacuoles) in the follicles, granulosa layer/follicular layers were disorganized (Figure 6B) and cell types cannot be clearly distinguished and importantly the presence of yolk granule degeneration (Figure 5C). Irregular shape of follicular cell was observed in Figure 6D. This atresia morphology was also reported in lizard reproductive system (Guraya & Varma 1976; Gouder, Nadkarni, & Appaswamy 1979; Ciarcia, Paolucci, & and Di Fiore, 1993; Norris, 2007; Saidapur, 1978). In addition, it has been shown that the appearance of atretic follicles plays a pivotal role to control the clutch sizes (Me´ndez de la Cruz, Guillette, & Villagran Santa Cruz, 1993) and limit the number of eggs during a particular reproductive cycle through digestion and removal of ooplasma content (Hernandez-Franyutti, Uribe, & Guillette, 2005). The presence of high number of the follicular atresia in the golden tree snake might be its reproductive mechanism to control the number of eggs in response to specific environmental conditions or particular seasons, as shown in Santos *et al.* (2015). Further investigation on the golden tree snake ovary from different seasons might help us to understand more on the reproductive cycles of this rainforest snake.

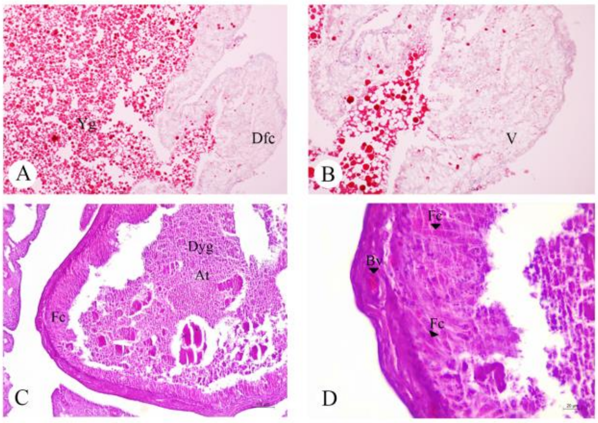

Figure 6. Photomicrographs showing atresia or atretic follicle in the ovary of the golden tree snake *Chrysopelea ornata*. A-B: Disorganization of the follicular layers (Dfc) containing various size of vacuoles (V). C-D: the atresia (At) exhibited degeneration of yolk granules (Dyg) and irregular follicular cell layers. Abbreviations: Bv = blood vessel, Fc = follicle cell, Yg = yolk granule. Staining methods: A-B, Masson Trichrome; C-D, Harris's haematoxylin and eosin

4. Conclusions

The structures of the female reproductive system in the golden tree snake *C. ornata* were observed and reported for the first time in this study, in which all structures were corresponded to the general pattern identified in the oviparous snakes, in particular asynchronous oocyte/follicle development. However, the reproductive cycle related to its physiology of the golden tree snake should be examined in further studies to understand the effect of seasonal changes to the ultrastructure and proportion of follicles and oocytes.

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