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Reproductive Patterns of Captive Male and Female Monocled Cobra, *Naja kaouthia* (Lesson, 1831)

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Panas Tumkiratiwong, Worawitoo Meesuk, Lawan Chanhome, and Anchalee Aowphol (2012) Reproductive patterns of captive male and female monocled cobra, *Naja kaouthia* (Lesson, 1831). *Zoological Studies* **51**(5): 692-700. We monitored the morphology and histology of male and female reproductive systems and plasma levels of male testosterone and female estradiol to describe the reproductive pattern of captive monocled cobras (*Naja kaouthia*). Gonads were collected in May and Nov. 2007, and monthly blood samples were collected from Jan. to Dec. 2007, from captive snakes at the Queen Saovabha Memorial Institute, Bangkok, Thailand. Male testes were hypertrophic in May corresponding to spermatogenetic events and had regressed in Nov. in the absence of spermatogenetic events. We found that spermatozoa were stored in the epididymides in both May and Nov. Male testosterone levels peaked in Oct. preceding the Nov. mating time. In May, the granulosa layer of previtellogenic follicles contained 3 distinct cell types of small, intermediate, and large pyriform cells. Intermediate and large pyriform cells had disappeared from the granulosa layer of the vitellogenic follicles in Nov. Three follicular types of atretic, previtellogenic, and vitellogenic follicles were common inside the ovaries in Nov. The corpora lutea had regressed to a corpora atretica in Nov. The plasma level of female estradiol surged in Nov. coincident with the mating time and vitellogenic events. We suggest that the reproductive pattern of captive monocled cobras exhibits either postnuptial spermatogenesis or a dissociated reproductive pattern. http://zoolstud.sinica.edu.tw/Journals/51.5/692.pdf

Key words: Elapidae, Estradiol, Testosterone, Pyriform cells, Spermatogenesis.

The monocled cobra, *Naja kaouthia*, belongs to the family Elapidae, and is 1 of 4 species of cobras found in Thailand (also *N. siamensis*, *N. sumatrana*, and *Ophiophagus hannah*; Cox et al. 1998). Populations of *N. kaouthia* are actively maintained at the Queen Saovabha Memorial Institute (QSMI), Bangkok to supply healthy snakes for venom and antivenom production.

In the majority of seasonally breeding vertebrates, the expression of courtship and copulatory behavior in males is directly correlated to activation and hypertrophy of the testes, and is accompanied by an elevated levels of circulating sex steroids. This pattern is termed an associated reproductive pattern (Crews 1984 1999, Crews et al. 1984, Licht 1984, Norris 2007). In contrast, in a small number of vertebrate species, such as some turtles, snakes, and bats, males mate at a time when their gonads are quiescent and levels of circulating sex steroids are low (Garstka et al. 1982). This pattern of reproduction is referred to as a dissociated reproductive pattern (Volsøe 1944, Crews 1976 1984 1999, Lofts 1977, Licht 1984, Norris 2007). In snakes, especially in the family Colubridae, various terms have been used to describe the male reproductive patterns observed in different species. Volsøe (1944) introduced the term “prenuptial spermatogenesis” to describe

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sperm production that occurs immediately prior to mating and "postnuptial spermatogenesis" to describe sperm production that occurs after mating. Aldridge et al. (2009) introduced the term "preovulatory spermatogenesis" to describe sperm production immediately prior to ovulation and "postovulatory spermatogenesis" as sperm production which occurs after ovulation and assumed that androgenesis is associated with the development of secondary sex characters and mating behavior.

Little is known about the reproductive cycles of captive cobras. In particular, the timing of sex hormone secretion and its relation to gamete production and mating behavior are not well understood. As far as we know, no data on sex hormonal profiles of monocled cobra have been published on wild or captive animals to date. For that matter, little work of any kind has been done on monocled cobras. Because no wild monocled cobras were available to us, we studied reproductive cycles of captive snakes. We hypothesized that monocled cobras maintained in captivity would provide a good model for investigating reproductive patterns by monitoring the morphology of male and female genital systems, histological aspects of the testes and ovaries, and monthly plasma levels of male testosterone and female estradiol. We previously observed that captive monocled cobras at QSMI breed in Nov. (Chanhome et al. 2001).

MATERIALS AND METHODS

Animals

Captive-born monocled cobras are raised in captivity at the QSMI, Bangkok and maintained in wire mesh cages of 3 × 6 × 2.5 m. These snakes originated from populations in Bangkok and adjacent areas of central Thailand. They were given water ad libitum and fed laboratory mice once a week. They were exposed to ambient light cycles. We sacrificed a male and a female (Fig. 1A) in May and Nov. 2007 to investigate the general morphology of the reproductive system and histology of the testes and ovaries. We sampled blood from 8 males and 8 females once a month from Jan. to Dec. 2007 by puncturing the caudal vein with a 26-gauge needle and collecting blood in a heparinized vial. Samples were kept at 4°C until centrifugation within 2-3 h. The blood was centrifuged at 1600 xg for 10 min. Plasma was aspirated off and frozen at -79°C for later analysis for levels of plasma testosterone in males and estradiol in females.

Testicular and ovarian histology

Testes and ovaries were excised and fixed in a 10% v/v buffered neutral formalin solution, processed by the paraffin technique (Luna 1968). The tissue was cut in cross-section to 6 μm in thickness using a LEICA RM2145, (Heidelberger str. 17-19, D-69226 Nussloch Germany). Sections were stained with hematoxylin and eosin (Luna 1968). Specimens were collected in May and Nov. 2007 to represent non-breeding and breeding times (Chanhome et al. 2001).

Measurement of testosterone

The plasma level of testosterone was measured by an ^{125}I radioimmunoassay (RIA). We added a 500-μL sample of plasma to 5.0 mL of dichloromethane in a screw-top glass extraction tube. We then capped the mixture and mixed it for 60 min by gentle inversion with an end-over-end rotator, and then centrifuged the sample for 5 min at 1600 xg to separate the layers. The upper phase was aspirated without disturbing the interface. Then 2.0 ml of the lower phase was transferred to a clean 12 × 75-mm glass tube and evaporated to dryness under a gentle stream of nitrogen at 37°C. Finally, we reconstituted the extract with 200 μL of testosterone buffer. The testosterone extraction procedure was performed using a Coat A Count® Testosterone RIA Kit (Diagnostic Products, Los Angeles, CA, USA). The intra- and inter-assay variations expressed as coefficients of variation (CVs) were 8.4% and 7.9%, respectively. The approximate sensitivity of this assay was 40 pg/mL. The cross-reactivity with androstenedione was 0.5%. The spiking recovery values averaged 98.3% ± 0.6%. Dilutions of 50%, 25%, and 12.5% of the undiluted concentration of 7300 pg/mL were 3490, 1700, and 780 pg/mL, respectively.

Measurement of estradiol

The plasma level of estradiol was measured by an ^{125}I RIA. We added 250 μL of plasma to 2.0 mL of diethyl ether in a screw-top glass extraction tube, then capped and mixed it by gentle inversion with an end-over-end rotator for 30 min. It was centrifuged for 5 min at 1600 xg to separate

the layers. The lower (aqueous) phase was frozen using dry ice, and then the organic phase was decanted into another vial and evaporated to dryness under a gentle stream of nitrogen at 37°C. Finally, the extract was reconstituted with 250 μL of estradiol buffer. The estradiol extraction procedure was performed using a Coat A Count® Estradiol (TKE2) RIA kit (Diagnostic Products). The intra- and inter-assay vari ations calculated as CVs were 5.3% and 6.4%, respectively. The approximate sensitivity of this assay was 10 pg/mL. The specificity of cross-reactivity with estradiol was 0.32%. The spiking recovery values averaged 96.8% ± 3.3%. Dilutions of 50%, 25%, 12.5%, and 6.25% of the undiluted concentration of 230.9 pg/mL were 114.4, 60.0, 27.9, and 14.8 pg/mL, respectively.

Statistical analysis

Monthly male testosterone and female estradiol levels are expressed as the mean ± standard error (SE). Kolmogorov-Smirnov's test was used to determine if the data were normally distributed. Non-parametric statistics were used for the statistical analysis because not all of the data were normally distributed. Based on the ranking of the data, the Kruskal-Wallis H test was used to test for differences in male testosterone/female estradiol levels among the 12 mo, and the Mann-Whitney *U*-test was then used to compare differences in male testosterone/female estradiol levels among months. Pearson's correlation coefficients were used to determine relationships between body size and testis size/ovary size. The level of significance was set to $p < 0.01$ for all tests.

RESULTS

General morphology of male and female genital organs

The male reproductive system of the monocled cobra was comprised of 2 elongated-oval testes, vas deferens, and hemipenes. Testes were hypertrophic in May (Fig. 1B) but had regressed in Nov. (Fig. 1C). The right testis was more anterior than the left, and both opened onto the vas deferens which ran parallel to the kidney and hemipenes. Ejected hemipenes were

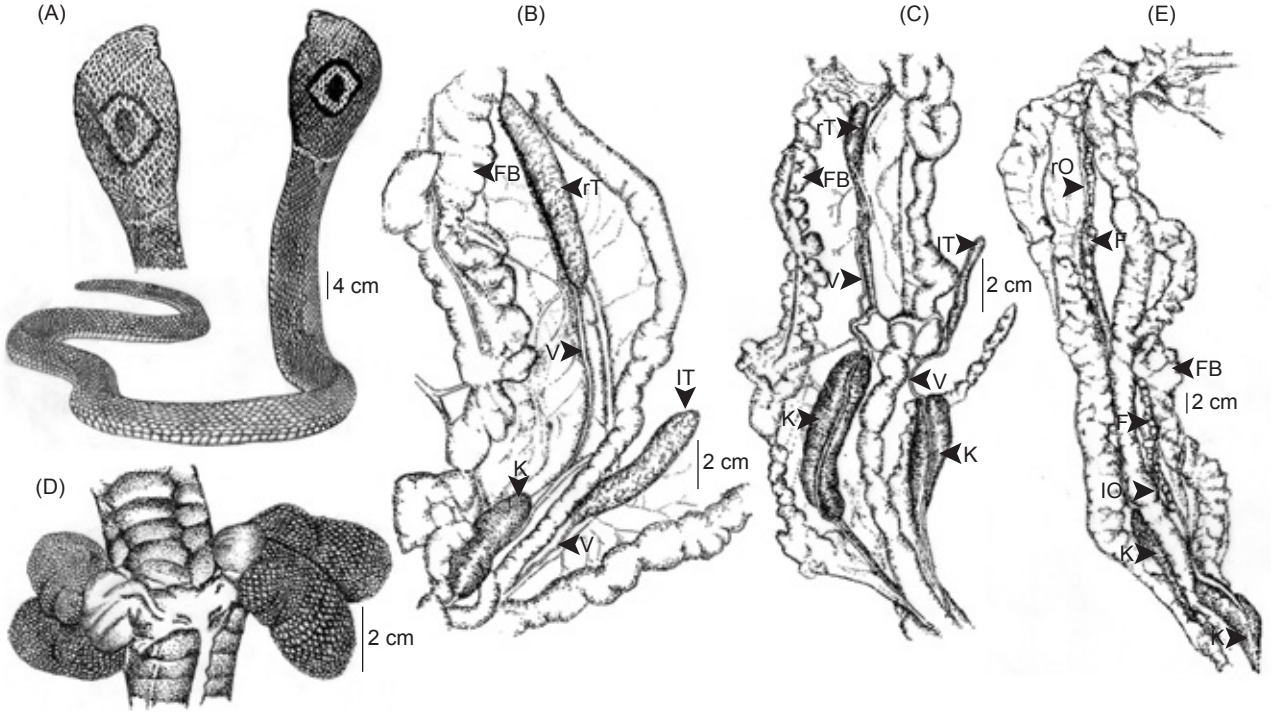

Fig. 1. Morphology of genital organs of the monocled cobra illustrated by line drawings. (A) Monocled cobra; (B) male genital organs, May; (C) male genital organs, Nov.; (D) hemipenis; and (E) female genital organs. rT, right testis; lT, left testis; V, vas deferens; K, kidney; rO, right ovary; lO, left ovary; Od, oviduct; F, follicles; FB, fat bodies. Lines were drawn from a total preparation (in ventral view).

round and covered with numerous spines (Fig. 1D). The female reproductive system consisted of 2 follicle-filled, elongate-oval ovaries; the right ovary was more anterior than the left. Follicles were organized in an array pattern (Fig. 1E) with oviducts opening onto a genital pore. The sizes of the left and right testes were positively correlated with the snout-vent length (0.651 and 0.471, respectively; Fig. 2). Follicle size was not correlated with the snout-vent length.

Testicular and ovarian histology

Male reproductive organs of the monocled cobra contained seminiferous tubules, ductus efferens, and epididymides (Fig. 3A, B). The seminiferous tubules were apparent in May (Fig. 3A, C) but had regressed in Nov. (Fig. 3B, D). Spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa were found in active seminiferous tubules of *N. kaouthia* collected in May (Fig. 3C), but they were not found in Nov. (Fig. 3D). Spermatozoa were present in the epididymides in both May and Nov. (Fig. 3E, F, respectively). Smooth muscle cells surrounded the epididymides (Fig. 3G). In Nov., female ovaries contained follicles in 3 stages: previtellogenic, vitellogenic, and atretic follicles (Fig. 4B). In May, ovaries contained only developing follicles (Fig. 4A). In May, we observed many pyriform cells in the granulosa layer (Fig. 4C), which were classified into 3 types: small, intermediate, and large (Fig. 4E). However, we observed fewer pyriform cells in the granulosa layer of vitellogenic follicles collected in Nov. (Fig. 4D) and found corpora lutea and corpora atretica follicles in Nov. (Fig. 4F).

Annual male testosterone cycle

There was an annual cycle of plasma testosterone concentrations in males (Fig. 5). Male testosterone levels significantly varied among months (Kruskal Wallis H test; $\chi^2 = 59.53$, $p < 0.01$) and between 2 each mo of all combinations of months (Mann-Whitney *U*-test, $p < 0.01$). The concentration of testosterone rose steadily from Jan. to a peak in Oct. (370 ± 60.18 pg/mL) and then sharply declined thereafter.

Annual female estradiol cycle

There was an annual cycle of plasma estradiol concentrations in females (Fig. 6), and female estradiol levels significantly varied among months (Kruskal Wallis H test; $\chi^2 = 74.23$, $p < 0.01$) and between 2 each mo of all combinations of months (Mann-Whitney *U*-test, $p < 0.01$). The estradiol concentration gradually rose from Jan. to a peak in Nov. (212 ± 20.58 pg/mL). After Nov., concentrations sharply dropped to the lowest level in Dec.

DISCUSSION

Testicular size of many snakes and lizards varies seasonally and is associated with the time of spermatogenesis (Jacobson 2007, Huang 2010). Testes of *N. kaouthia* in May were larger than those in Nov. In May, testes were hypertrophic, and more fat bodies were found in the abdomen than in Nov. Spermatogenesis was occurring in active testes in May, but they were

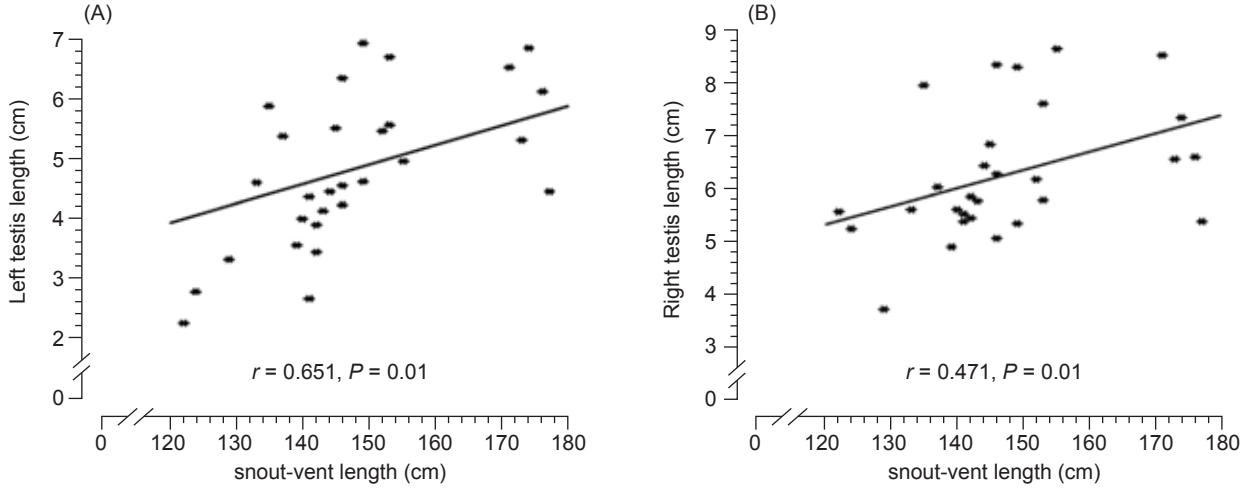

Fig. 2. Correlations between (A) left testis length and (B) right testis length with snout-vent length (cm) in the monocled cobra.

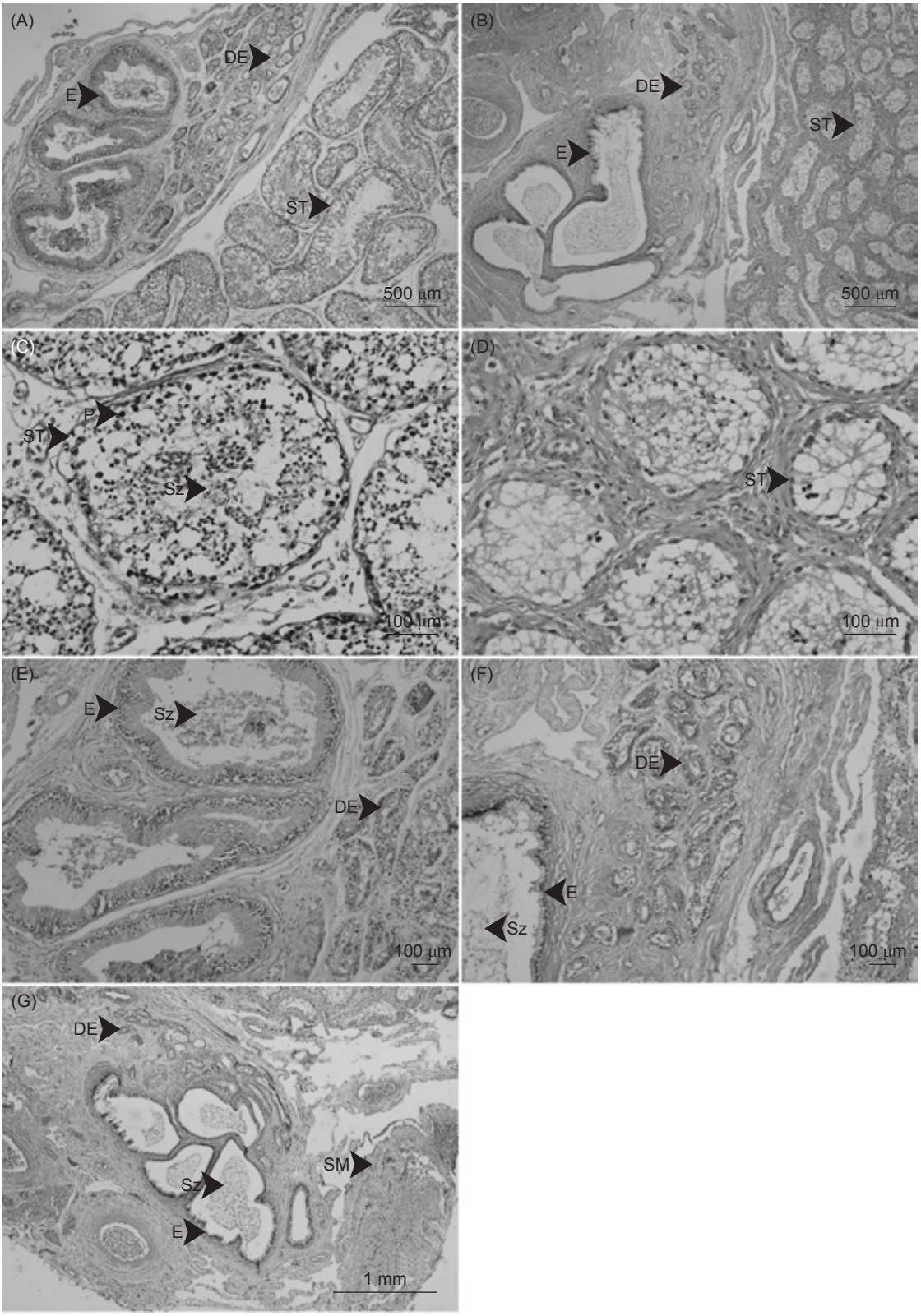

Fig. 3. Micrographs of the testis of the monocled cobra. Left panel, testis sectioned in May; right panel, sectioned in Nov. (A) Active seminiferous tubules, ductus efferens, and epididymides; (B) regressed seminiferous tubules, ductus efferens, and epididymides; (C) active spermatogenesis; (D) spermatogenetic arrest; (E, F) spermatozoa in the epididymides; and (G) smooth muscle of the epididymis. P, primary spermatocyte; Sz, spermatozoa; DE, ductus efferens; E, epididymides; ST, seminiferous tubule; SM, smooth muscle.

quiescent in Nov. We found spermatozoa stored in the epididymides. Smooth muscle was found surrounding the epididymides collected in Nov.; however, Mader (1996) reported that snakes contain no epididymides but have a vas deferens for conducting sperm to the hemipenis. We found spermatozoa in the epididymides in both May and Nov. We suggest that the epididymides, not the vas deferens, are the site at which spermatozoa are stored until the coming mating season in Nov. in *N. kaouthia*. However, sperm are stored in the vas deferens over the winter in most snakes in the family Colubridae, and spermatogenesis is initiated in summer (Aldridge et al. 2009). In soft-shelled turtles, *Trionyx sinensis*, the epididymides contain sperm throughout the entire year (Xiangkun et al.

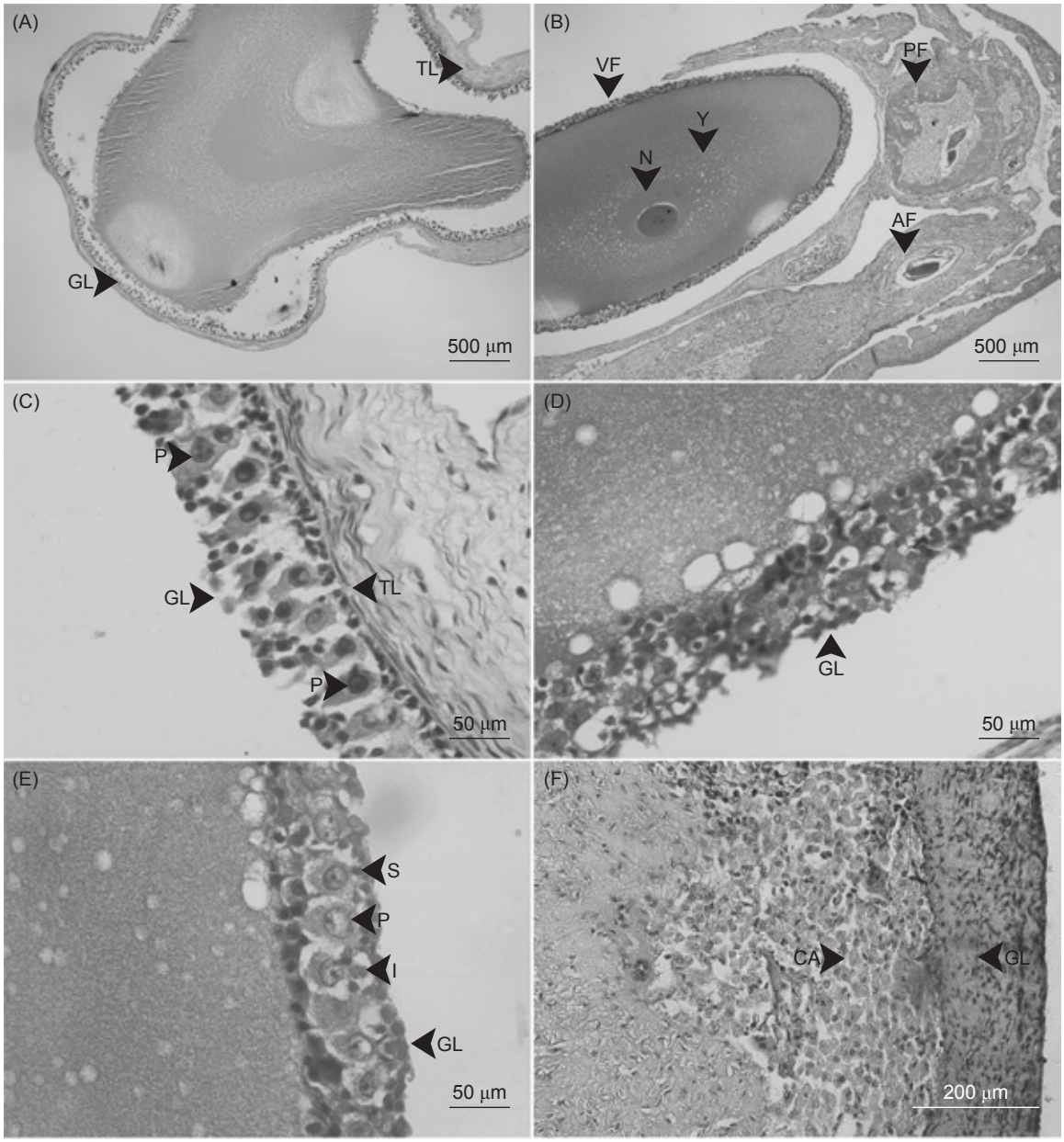

Fig. 4. Micrographs of the monocled cobra ovary. Left panel, ovary sectioned in May; right panel, sectioned in Nov. (A) A developing follicle; (B) 3 follicular types of previtellogenic, vitellogenic, and atretic follicles; (C) pyriform cells in the granulosa layer; (D) a vitellogenic follicle; (E) small, intermediate, and large pyriform cells in the granulosa layer; and (F) the corpora atretica. AF, atretic follicle; CA, corpora atretica; GL, granulosa layer; I, intermediate cell; N, nucleus; P, large pyriform cell; PF, previtellogenic follicle; S, small cell;. TL, thecal layer; VF, vitellogenic follicle; Y, yolk.

2008).

Plasma testosterone concentrations in *N. kaouthia* peaked in Oct., a month prior to the mating time in Nov. Elevated plasma testosterone levels preceded or coincided with the mating time. Similarly, Moore et al. (2000) reported that plasma testosterone increased and peaked in Sept. in garter snakes, and the level tended to be maximal 1 mo prior to the peak in estradiol concentrations in females. Aldridge et al. (2009) also reported that elevated plasma testosterone levels preceded or coincided with the mating

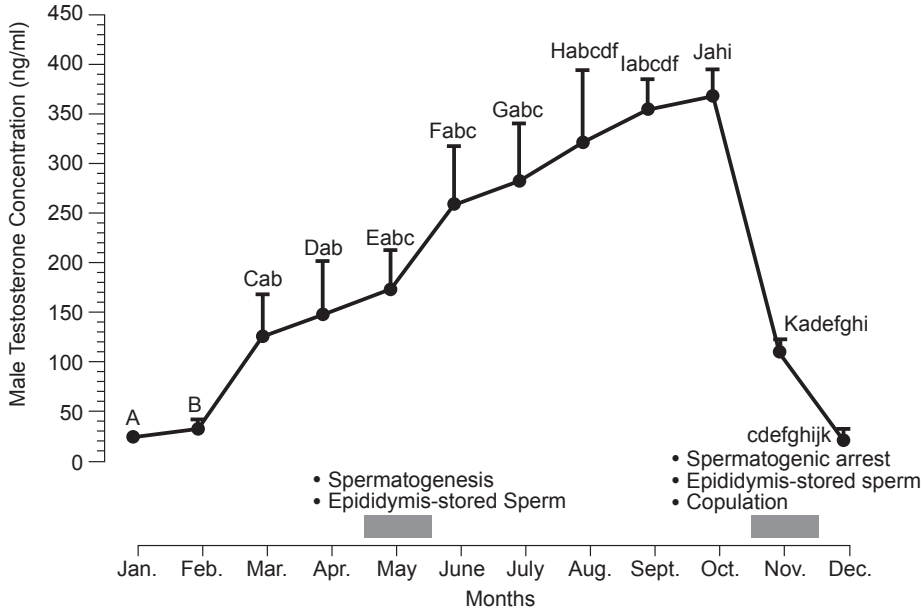

Fig. 5. Seasonal profile of male testosterone in *N. kaouthia*. Jan.-Dec., Jan. to Dec., respectively. A,a; B,b; C,c; D,d; E,e; F,f; G,g; H,h; I,i; J,j, and K,k: upper- and lowercase forms of the same letter above the standard error of each line indicate a significant difference between months ($p < 0.01$).

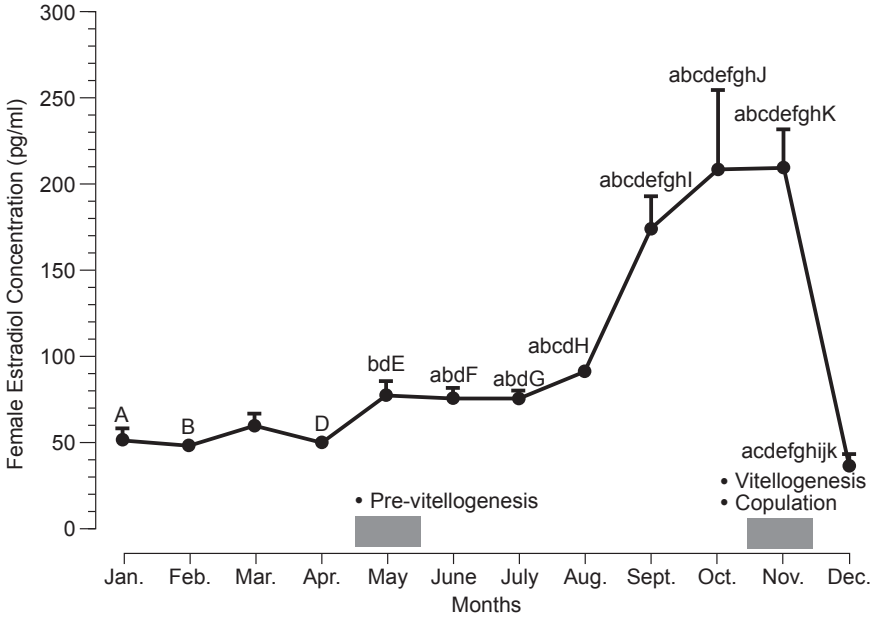

Fig. 6. Seasonal profile of female estradiol in *N. kaouthia*. Jan.-Dec., Jan. to Dec., respectively. A,a; B,b; C,c; D,d; E,e; F,f; G,g; H,h; I,i; J,j, and K,k: upper- and lowercase forms of the same letter above the standard error of each line indicate a significant difference between months ($p < 0.01$).

season in colubrids. Crews and Moore (2005) reported that mating behavior was associated with gonadal hormone secretion. The major difference between snakes with the unimodal and bimodal patterns is the site at which sperm are stored during the winter. Sperm are stored in the vas deferens in unimodal snakes and in the oviduct (and vas deferens) in bimodal snakes. In most colubrids, spermatogenesis begins after the spring mating season, a pattern described as "postnuptial spermatogenesis". There are; however, several fossorial species of snakes that occupy warmer climates of the southwestern USA, in which spermatogenesis begins before mating, a pattern termed "prenuptial spermatogenesis". The terms postnuptial spermatogenesis and dissociated reproductive cycle are synonymous and refer to species which have summer spermatogenesis following spring fertilization (Aldrige et al. 2009). The terms are independent of the mating season. Prenuptial spermatogenesis is synonymous with an associated reproductive cycle and describes species in which spermatogenesis immediately precedes spring fertilization. Aldridge et al. (2009) suggested that the terms pre- and postovulatory spermatogenesis can be used to describe the relationship between spermatogenesis and the female reproductive cycle. Krohmer et al. (1987) suggested that testosterone is associated with mating behavior and not with spermatogenesis.

Previtellogenic follicles collected in May had many pyriform cells in the granulosa layer, but these were less abundant in vitellogenic follicles collected in Nov. Andreuccetti (1992) studied the differentiation of pyriform cells and their contribution to oocyte growth in 3 lizards (*Tarentola mauritanica*, *Cordylus wittifer*, and *Platysaurus intermedius*) and 1 colubrid snake (*Coluber viridiflavus*) and revealed that pyriform cells differentiate from small follicle cells via intermediate cells after establishing an intercellular bridge with an oocyte. Once differentiated, pyriform cells display ultrastructural features indicative of synthetic activity, including abundant ribosomes, Golgi membranes, vacuoles, mitochondria, and lipid droplets. These cellular components extend to the apex of the cell at the level of the intercellular bridge, suggesting that constituents of pyriform cells may be transferred to oocytes. Pyriform cells and oocytes may fulfill similar vitellogenic functions. The establishment of an intercellular bridge may represent a crucial event in the development of an integrated system in which pyriform cells and oocyte cooperate. Norris (2007) reported that the squamate granulosa contains a unique flask-shaped cell type, a pyriform cell that is in direct contact with a developing oocyte. These cells apparently are involved with early steps in oocyte development as they either degenerate or transform into typical granulosa cells soon after the onset of vitellogenesis. As the time of ovulation approaches, granulosa cells and some thecal cells accumulate cholesterol-positive lipids, and following ovulation, proliferate and luteinize to form the corpora lutea. We found corpora lutea and corpora atretica in follicles collected in Nov. but found neither in May. Following ovulation, the corpora lutea exists only during the mating season in some reptiles such as *Iguana iguana* (Jacobson 2007). Only a few follicles that begin development reach maturity at a given time. The majority undergo atresia (Norris 2007). Therefore, atretic follicles are commonly present. Follicular atresia and formation of corpora atretica are common occurrences in reptilian ovaries as in other vertebrates (Norris 2007). Ho et al. (1982) characterized the vitellogenic cycle of 2 turtles, *Chrysemys picta* and *Sternotherus odoratus*. Plasma vitellogenin levels were positively correlated with ovarian growth and with levels of plasma estrogen during the annual reproductive cycle. Based on our histological determination and an annual female estradiol cycle that peaked in Nov., vitellogenesis occurred in Nov. but not in May, coinciding with the mating time in Nov. Moreover, Crews and Moore (2005) reported that mating behavior is also associated with gonadal hormone secretion.

Tsai and Tu (2000) pointed out that the relationship between mating and spermatogenesis, as used by Volsøe (1944), is complicated by the fact that mating can occur in summer and/or the following spring, depending on the species. They suggested that ovulation can be used instead of mating (i.e., nuptial). Aldridge et al. (2009) concurred with Tsai and Tu (2000) and suggested the terms pre- and postovulatory spermatogenesis to appropriately replace pre- and postnuptial spermatogenesis. They also added that the term postovulatory spermatogenesis is not meant to imply that the timing of spermatogenesis is linked to mating, vitellogenesis, or ovulation. Rather, that the ability to store viable sperm over long periods, in the vas deferens and/or the oviduct, has permitted the timing and length of spermatogenesis to evolve independently of vitellogenesis, the mating season, and ovulation. As a result of prolonged sperm storage, a male needs only be

prepared to mate by engaging in mating behaviors when a female enters estrus. In conclusion we suggest that the reproductive pattern of the monocled cobra, *N. kaouthia*, maintained in captivity is either postnuptial spermatogenesis or a dissociated reproductive pattern.

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