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# Structure and Development of the Testis of Bullfrog, *Rana catesbeiana,* and Their Changes during Seasonal Variation

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**ABSTRACT** The testes of fully mature bullfrogs, *Rana catesbeiana,* were studied by light microscope. The germ cells in the developing testis can be classified into 12 stages based on nuclear characteristics. Primary(type A) and secondary(type B) spermatogonia are the earliest germ cells, with the former showing large and completely euchromatic nuclei with prominent nucleoli and the latter with small blocks of heterochromatin distributed along the nuclear envelope. Spermatocytes consist of five stages; namely, leptotene, zygotene, pachytene, diplotene and diakinesis metaphase spermatocytes. Succeeding stages show increasing condensation of chromatin : from the coarse fibers, that are evenly distributed throughout the nucleus in leptotene stage to the highly condensed blocks of heterochromatin in pachytene and diplotene stages. Nucleoli are not detected in any stages. Secondary spermatocytes have blocks of completely condensed heterochromatin attaching to the nuclear envelopes. There are three stages of spermatids: the early stage shows coarse chromatin granules occurs evenly over the nucleus, the middle stage has increased chromatin condensation over the entire oval nucleus while the nuclear size decreases. The late stage exhibits completely condensed chromatin in an elongated nucleus, and its cytoplasm becomes highly vacuolized and starts to degenerate. In fully mature spermatozoa, the nucleus becomes highly elongated and chromatin completely condensed. During development of the testis, sex cords in putative testis appear in two-month-old frogs. Seminiferous tubules containing primary spermatogonia appear in the definitive testis of four-month-old frogs while spermatocytes are present in five-month-old frogs. Spermiogenesis and full production of spermatozoa could be detected from the seventh month onwards. The frogs become fully mature about sixteen months when their testes undergo cyclical change. During breeding period (April-September), there are abundant spermatozoa, round spermatids in seminiferous tubules, while during non-breeding period (October-March), such cells are much fewer in number and most remaining cells are spermatogonia and primary spermatocytes.

KEYWORDS: *Rana catesbeiana*, spermatogenesis, male germ cells, testicular development.

## INTRODUCTION

Amphibian male germ cells, especially in *Xenopus laevis*, have been studied by many investigators, using transmission electron microscope to investigate nuclear and cytoplasmic characteristics of various spermatogenetic stages, including spermatogonia, spermatocytes as well as cells in spermiogenesis.[1,2] $H^3$-thymidine labelling and autoradiography have also been used for detecting the duration of the *X. leavis* male germ cells cultured in serum free media. Results showed that the time required for the premeiotic S-phase spermatogonia to develop to late zygotene spermatocyte,that later changed to spermatid, were 14 days and 28 days, respectively.[3] Labelling with $H^3$-thymidine was also performed *in vivo* to determine the duration of cells in meiotic prophase and spermiogenesis in *X. laevis*. And it has been shown that spermatocyte spent four days in leptotene, six days in zygotene, one day in diplotene, one day in meiotic phase; and 12 days was required for the completion of spermiogenesis.[2] In *Rana pipiens*, male germ cells could be divided into nine stages, i.e., spermatogonia; spermatocytes which were divided into leptotene, zygotene, pachytene, diplotene, diakinesis; secondary spermatocytes; spermatids and spermatozoa.[4] In addition, cells in spermiogenesis were classified into five stages based on nuclear characteristics.[5] In bullfrogs, *Rana catesbeiana*, which is the species indigenous in North America, the stages of spermatogenesis and spermiogenesis have not yet

been studied in details . Thus, one of the primary purposes of this experiment is to classify various stages of germ cells in this species of frogs. Furthermore, bullfrogs had been imported and commercially cultured in Thailand for a number of years. As Thailand is a tropical country with distinctive wet and dry seasons the maturation and cyclical change of the testis may be quite different from frogs reared in North America.Therefore, the other aims of this study are to investigate maturation of the testis during the frogs' development, their breeding period, as well as the histological changes in testis that accompany seasonal variations.

## Materials and Methods

**1. Experimental animals**

*R. catesbeiana* were cultured in cement tanks at Faculty of Science, Mahidol University. They were maintained in an approximately 12 hour light/dark cycle, at 25°-35°C, with the relative humidity ranging from 45 to 95%. The culture water was changed at alternate days. The frogs were fed daily with pelleted feed in the afternoon.

**2. Light microscopic study**

Mature male frogs aged more than 18 months old were anesthesized by placing in an ice bath for 5-10 minutes or until they became immobile. Then the spinal cords were pitched and the frogs were decapitated. Testes were dissected and immediately fixed in cold 4% glutaraldehyde plus 2% paraformaldehyde in 0.1 M phosphate buffer, and post-fixed in cold 1% Osmium tetroxide in 0.1 M phosphate buffer. After fixation specimens were washed and dehydrated in ethanol and embedded in Araldite 502. Semithin sections were cut at 0.5-1 µm and stained with methylene blue for light microscopic observations.

For paraffin procedure, the dissected testes were fixed in Bouin's fluid and dehydrated through increasing concentrations of ethyl alcohol, and embedded in paraffin. Five to six-micron-thick sections were deparaffinized and stained with Harris's Haematoxylin and Eosin, and examined with an Olympus light microscope BH-2

**3. Development of testes**

Young frogs aged 1, 2, 3 up to 18 months old were bred and reared in cement tanks with food and general conditions as stated previously. At least ten frogs from each age group were used in this study. The testes were processed using paraffin procedure as previously described in section 2.

**4. Changes of testes during seasonal variation**

Fully mature frogs aged more than 18 months old were used in this study. The testes were removed from the frogs at the end of each month throughout the year, and the testicular tissue was prepared using paraffin technique for light microscopic observation as described in section 2.

## Results

**1. Classification of spermatogenic cells**

In semithin sections, the male germ cells of bullfrogs, *R. catesbeiana*, can be distinguished into 12 stages based on nuclear characteristics and sizes. Spermatogenesis in the frog takes place within follicular structures called spermatocysts that rest upon the basement membrane of the seminiferous tubules (Fig 1).

*1.1 Primary spermatogonia (1°Sg)*

Primary spermatogonia is the first stage germ cells in seminiferous tubules, and they constitute a high proportion of the cell population in the tubules of immature frogs. The cells are large and round in shape, and have round or oval nuclei with fine and mostly euchromatic material. They are generally located close to the basement membranes of seminiferous tubules. The size of the nucleus is about 10-13 µm. Frequently, cells with large bilobed nuclei could be found which may be spermatogonial cells that are undergoing nuclear division. Each nucleus also possesses one or two nucleoli which are very prominent. The cytoplasm is generally lightly stained (Fig 1B).

*1.2 Secondary spermatogonia (2°Sg)*

Secondary spermatogonia are round, but in comparison to 1°Sg they are smaller cells whose nuclear diameter is about 9-12 µm. They can be distinguished from primary spermatogonia by the presence of small blocks of heterochromatin along the nuclear envelopes, and those that are scattered all over the nuclei which tend to have smaller-size. The nucleoli are still prominent. Each group of 2 Sg usually consists of 2 or 4 cells surrounded by follicular cells' processes which still lie close to the basement membrane (Fig 1C).

*1.3 Leptotene spermatocytes (LSc)*

These cells are round and larger than 2°Sg, and have large round nuclei with diameter about 11-

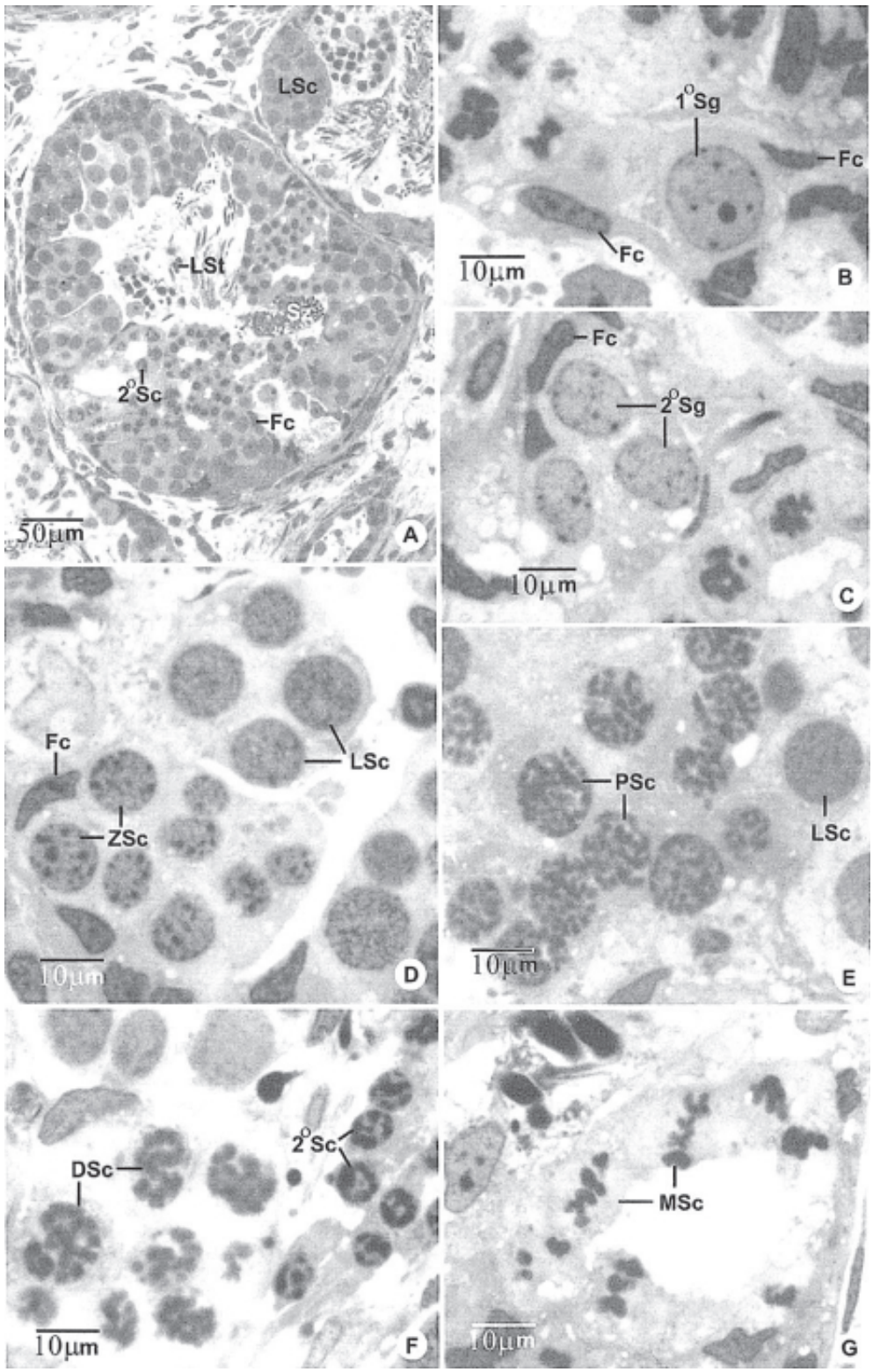


**Fig 1.** A) Seminiferous tubules, illustrating various stages of spermatogenic cells. B-G) High magnification of seminiferous tubules, showing primary spermatogonia (1°Sg), secondary spermatogonia (2°Sg), leptotene spermatocyte (LSc), zygotene spermatocyte (ZSc), pachytene spermatocyte (PSc), diplotene spermatocyte (DSc), metaphase spermatocyte (MSc), secondary spermatocyte (2°Sc), and follicular cell (Fc).

13 µm with a thin rim cytoplasm. Their chromatin begins to condense into loosely arranged blocks which are distributed evenly throughout the nucleus. In contrast to 2°Sg, there is no heterochromatin blocks along the inner surface of the nuclear envelope. Small nucleoli are still present in this stage. Group of leptotene spermatocytes form large clusters of more than four cells each, that are surrounded by follicular cells (Fig. 1D). Leptotene spermatocytes are usually located towards the lumen of seminiferous tubules and seldom touch the basement membrane.

*1.4 Zygotene spermatocytes (ZSc)*

The discriminating features of zygotene spermatocytes are the increased condensation of chromatin blocks and the change in size of the nuclei, which become smaller than that of leptotene spermatocyte. The heterochromatin blocks gradually become larger and perceptibly denser than in previous stage (Fig 1D).

*1.5 Pachytene spermatocytes (PSc)*

The nuclei of these cells are still round and about 10-12 µm in size. The chromatin becomes condensed into long thick cords, that are intertwined into loops resembling "bouguet pattern". These cells are still aggregated in clusters, each of which is still surrounded by processes of follicular cells (Fig 1E).

*1.6 Diplotene spermatocytes (DSc)*

The general appearance of the cells in this stage is similar to PSc; however, the heterochromatin cords become denser and larger. Nuclear diameter is about 10-11 µm, and thus the nucleus appears smaller than in PSc. They are also fewer in number in comparison to PSc (Fig 1F).

*1.7 Diakinetic (Dia) and Metaphase spermatocytes (MSc)*

The cells in these stages show thick chromosomes that are arranged close together in Dia, and later move to the equatorial region in MSc, when the nuclear boundaries disappear. They are so few and transient that they are rarely observed within the seminiferous tubules (Fig 1G).

*1.8 Secondary spermatocytes (2°Sc)*

These cells arise after the first meiotic division, and the nuclear diameter becomes smaller. Dense blocks of heterochromatin distributed in cart-wheel or clock-faced pattern within the nucleus arise from the coarse clumping of chromatin along the nuclear envelope (Fig 1F).

*1.9 Early spermatids (ESt)*

ESt arise after the second meiotic division, and they are markedly decreased in size and number within the tubules. They still have round nuclei which are also reduced in size to approximately 8-9 µm in diameter. The condensation of chromatin occurs evenly over the nucleus and the nucleus becomes eccentrically located within the cell. A small dark spot in the cytoplasm which may represent the Golgi complex can occasionally be seen at this stage of spermatid (Fig 2A).

*1.10 Middle or round spermatids (RSt)*

The nuclei of these cells show higher degree of chromatin condensation than in previous stages, and the condensation occurs uniformly throughout the nuclei which are reduced in diameter to about 6-7 µm. Each nucleus tends to be oval in shape. A small dark bar adhered to the nucleus which may represent the centrioles can be found. RSt is the most numerous cells in the tubules, probably due to their long duration (Fig 2B).

*1.11 Late spermatids (LSt)*

The nucleus of this late stage spermatid is characteristically reduced in size and begins to elongate. Chromatin becomes completely condensed throughout the nucleus. The cells usually move close to the lumen of seminiferous tubules, while they are stilled grouped together in clusters (Fig 2C).

*1.12 Spermatozoa (Sz)*

The mature spermatozoa exhibit highly elongated heads and tails. The head contains an ellipsoid nucleus with completely condensed chromatin which is deeply stained. Heads of several spermatozoa appear to be arranged in array that are embedded in the apical cytoplasm of Sertoli cells, while their tails point toward the seminiferous lumen (Fig 2D).

Cells with varying size located in clusters between the seminiferous tubules are the interstitial cells (Ic). Those that lie at the periphery of the clusters usually have spindle shape, and are closely associated with the blood vessels (Fig 2E).

**2. Development of testes**

Putative testis could be observed as a small ovoid organ on the ventral surface of the kidneys when the frogs are around two months old. The testis is enclosed by peritoneal membrane and its vas deferens opens into the ureter.

Within the newly formed testis, the only principal

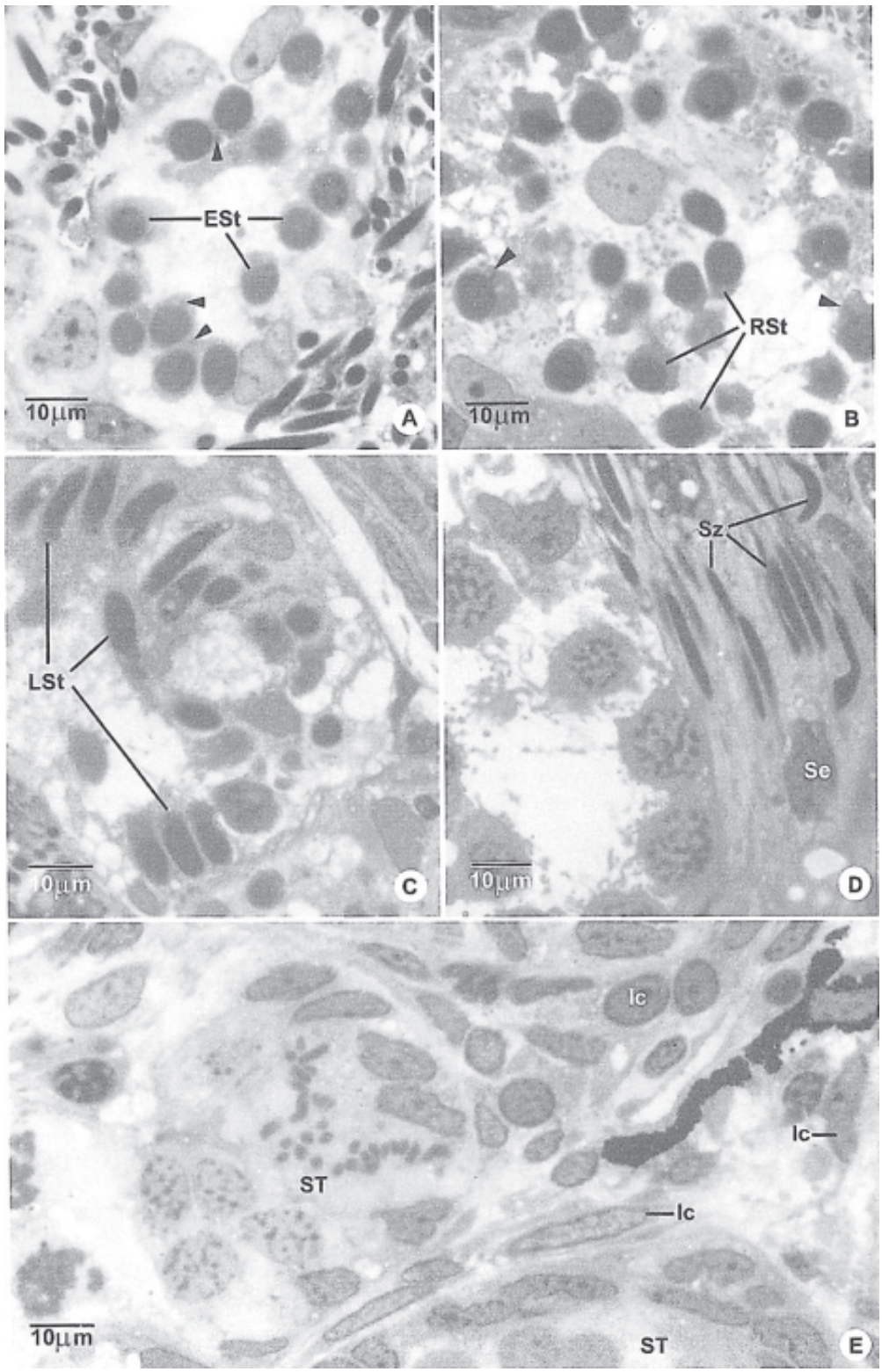


**Fig 2.** A-D) High magnification of seminiferous tubules, showing early spermatid (ESt), round spermatid (RSt), late spermatid (LSt), spermatozoa (Sz), and Sertoli cell (Se).In E section of the area between seminiferous tubules (ST), showing interstitial cells (Ic).

cell type is stromal cells with large round nuclei and spindle-shaped cytoplasm, characteristics which resemble undifferentiated mesenchymal cells. During 2-4 months, this cell type predominates in the sex cords located at the center, while some spermatogonia are present in those at the periphery (Fig 3A-C). Around the fourth month, all sex cords are enlarged and primary spermatogonia become dominant cell type.

In the fifth month, the seminiferous tubules become definitive entities and their walls start to contain other cell stages, including mainly primary spermatocytes.(Fig 3D). Spermatids could be identified in seven-month-old frogs and afterwards. Spermatozoa start to appear at the end of the seventh month (Fig 4A). By the end of the sixteenth month, the testis assumes similar appearance and cellular association as that of the fully mature male frogs (Fig 4B-D).

**3. Changes of testes during seasonal variation**

Table 1. shows the changes in the body and testicular weights of adult male frogs collected over the period of 12 months. During post-breeding season (September-November), the testis shows decrease in weight, and the deepest drop is observed at the end of September or the beginning of October. During this period, the testis contains relatively few cell nests, and spermatogenetic activity is drastically decreased. Subsequently, testicular weights start to increase slightly in non-breeding period (December-January) when some early germ cells start to replenish the tubules which may still appear dilated and exhibit certain degree of degeneration of the epithelium (Fig 5B-D).

Spermatogenetic activities is clearly reactivated in February to March, which is designated as pre-breeding period. The testicular weights significantly increase in comparison to earlier periods, and there is a rapid increase in the number of cell nests . Most of these cell nests transform into spermatozoa during April to September which is the breeding period. The maximum testicular weight is observed in May, then it starts to decrease gradually until it reaches the minimum in September. During the breeding period, thick seminiferous epithelium contains numerous spermatocytes and round spermatids, while lumen are filled with fully mature spermatozoa. The maximal spermatogenetic activity is observed during the mid-breeding period around June (Fig 5A).

## Discussion

**Germ cell classification**

In *R. catesbeiana*, the changes of nuclear characteristics could be used to divide germ cell into pre-mitotic stages which are composed of primary(typeA) and secondary(typeB) spermatogonia. The former is distinguished by the presence of completely euchromatic nuclei while the latter by the presence of small heterochromatin clumps along the nuclear envelope and nuclear center . After these cells enter meiosis I, the successive daughter cells consist of leptotene, zygotene, pachytene, diplotene and diakinesis- metaphase stage primary spermatocytes, respectively. Primary spermatocytes are characterized by the increased clumping of heterochromatin blocks or cords, that begin as loosely packed bodies of chromatin fibers in leptotene stage to the highly condensed heterochromatic cords in pachytene and diplotene stages, when they become intertwined to form bouquet-liked pattern.

When meiosis I is completed, the daughter cells

**Table 1.** Seasonal changes in the average body and testicular weights in *R.catesbeiana* during various months of the year.

| Month | n | Body Weight (g) Mean ± SD | Testicular Weight (g) Mean ± SD | %Testicular Weight/Body Weight (GSI) |
|---|---|---|---|---|
| December-January | 5 | 263.03 ± 36.05 | 0.40 ± 0.06 | 0.15 |
| February | 8 | 257.81 ± 41.04 | 0.40 ± 0.11 | 0.15 |
| March | 8 | 249.02 ± 32.68 | 0.39 ± 0.07 | 0.16 |
| April | 7 | 309.85 ± 29.46 | 0.49 ± 0.09 | 0.16 |
| May | 4 | 341.91 ± 110.07 | 0.63 ± 0.2 | 0.18 |
| June | 5 | 324.19 ± 85.2 | 0.50 ± 0.13 | 0.15 |
| July | 5 | 380.02 ± 51.95 | 0.58 ± 0.07 | 0.15 |
| August | 5 | 292.19 ± 11.48 | 0.33 ± 0.12 | 0.11 |
| September | 4 | 270.84 ± 29 | 0.28 ± 0.03 | 0.11 |
| October-November | 7 | 249.87 ± 18.85 | 0.32 ± 0.04 | 0.13 |

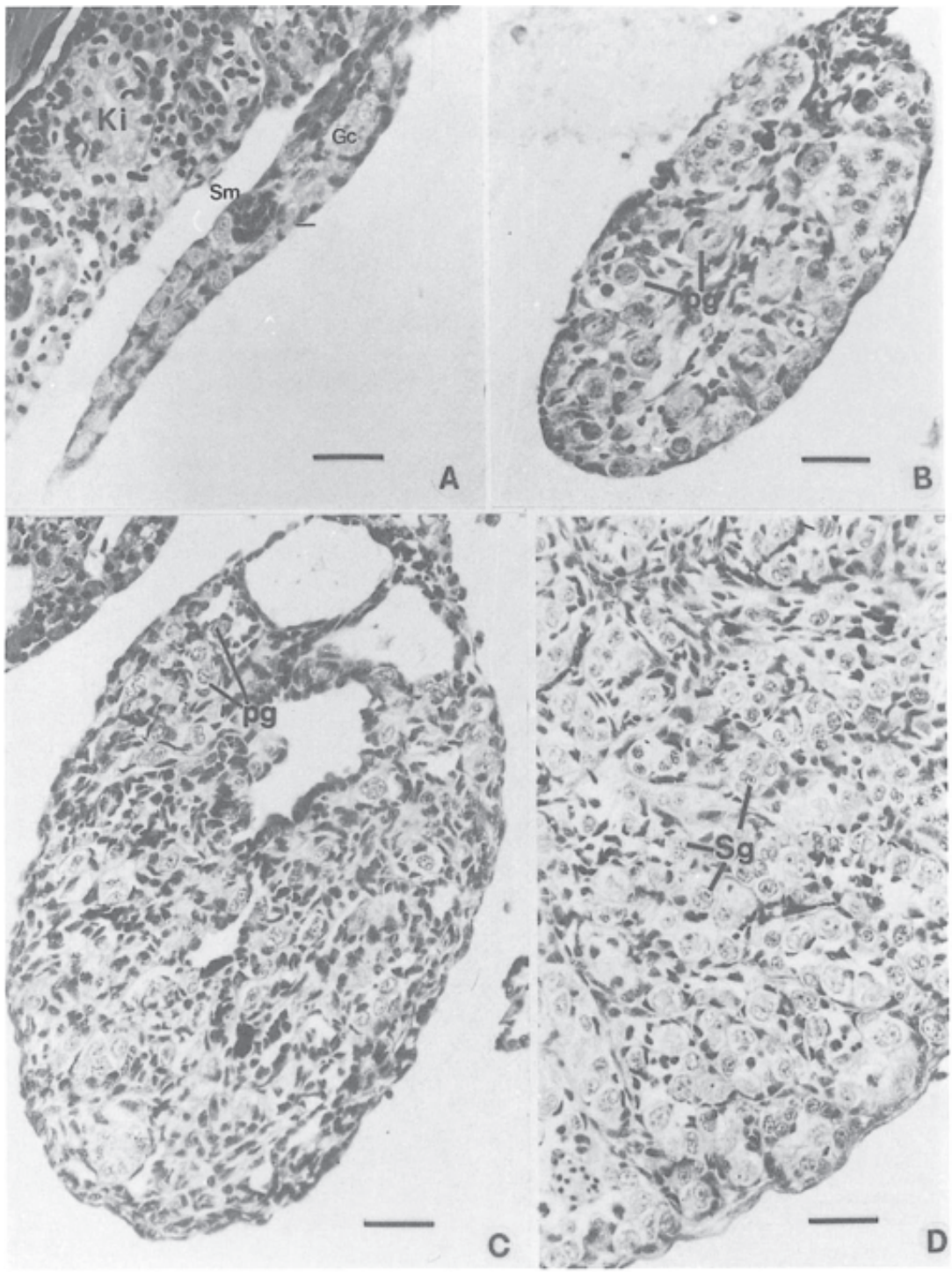


**Fig 3.** A) Testis of one-month-old frog located ventral to kidney (Ki), illustrating germ cells (Gc) and stromal cells (Sm). B) Testis of two-month-old frog, illustrating the appearance of primary germ cells (pg). C) Testis of four-month-old frog, illustrating a large number of primordium germ cells (pg). D) Testis of five-month-old frog, illustrating the formation of seminiferous tubules containing mostly early stages of germ cells. Sg = spermatogonia, Bar = 55 µm.

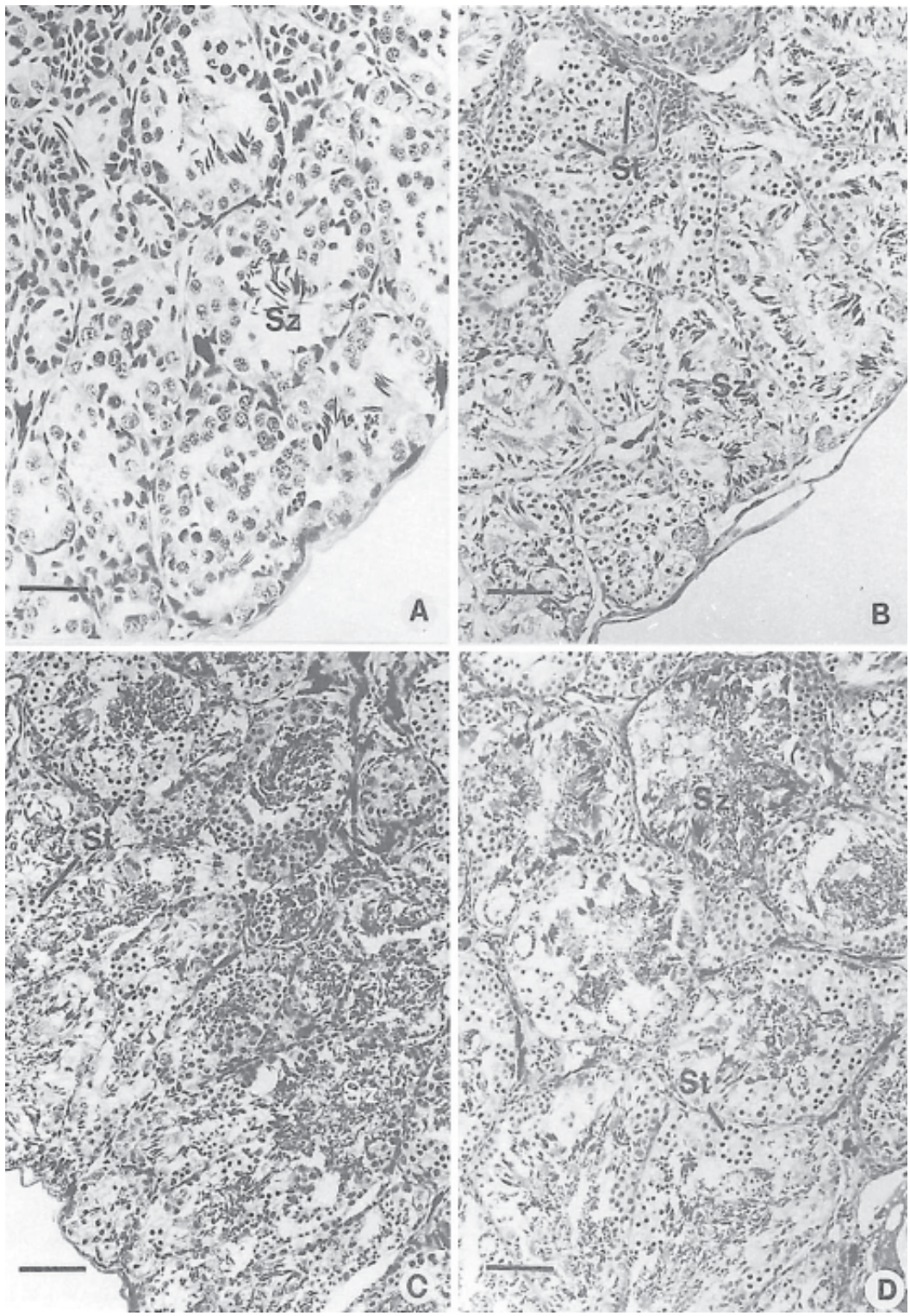


**Fig 4.** A,B) Testis of eight-and ten-month-old frogs, illustrating definitive seminiferous tubules which contain various stages of spermatogenic cells, abundant spermatids (St) and spermatozoa (Sz). Bar = 55 µm. C,D) Testis of twelve -and fourteen-month-old frogs, illustrating every stages of germ cells and a full number of spermatids (St) and spermatozoa (Sz). Bar = 110 µm.

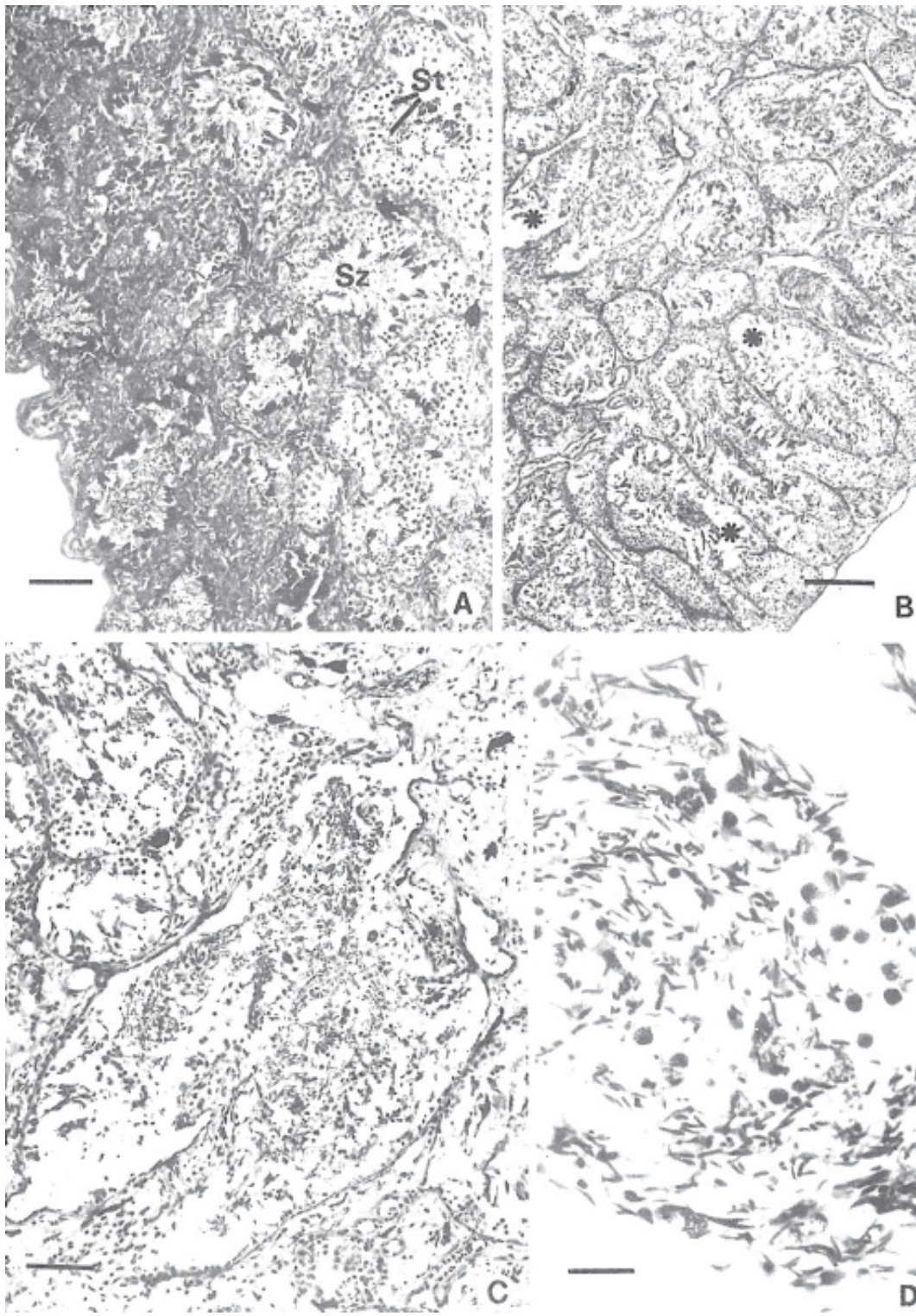


**Fig 5.** A) Testis of frog during breeding period, illustrating the thick- walled seminiferous tubules containing various stages of germ cell, especially a large number of spermatozoa (Sz) and spermatids (St). Bar = 110 µm. B,C) Testes of frog during non- breeding period, illustrating the desquamation of late stages of germ cells in the lumen of seminiferous tubules (asterisk). Some tubules are dilated and epithelium are thin ( arrow head) and some break down (arrow). B : bar = 300 µm, C : bar = 110µm. D) High magnification at the lumen of seminiferous tubule, demonstrating the late stages of detached germ cells. D : bar = 55 µm

are much reduced in size. Haploid secondary spermatocytes show nucleus with heterochromatin clumping along the nuclear periphery in a "cart-wheel" pattern. These cells pass through meiosis II division, and consequently, transform into spermatids which are divided into early, middle and late spermatids. In early spermatid stage, the chromatin granules or fibers become tightly packed within the round nuclei. These nuclei begin to transform into more elongated shape and are simultaneously reduced in size in middle and late spermatids, respectively. In addition, the small dark bar appearing in the cytoplasm could represent the beginning of the tail formation. After the condensation of chromatin and transformation of the nuclei are completed, the spermatozoa are derived.

In comparison to *R. catesbeiana* observed in the present study, Kalt[2] and Callard *et al*[6] have studied testes of male *X. laevis* and found 11 stages of germ cells. They employed $H_3$-thymidine labelling and calculated the durations of various cell stages, including leptotene, zygotene, pachytene, diplotene and metaphase spermatocytes, whose durations were found to be 4, 6, 12, 1 and 1 days, respectively; and spermiogenesis needed 12 days to complete the process. In the toad, *B. arenarum*, Houssay[7] could divide male germ cells into eight stages: namely two spermatogonia stages, spermatocyte I, spermatocyte II, three spermatid stages and spermatozoa. Afterwards, Rastogi *et al*.[8] studied adult male *Rana esculenta* maintained at 18°C, and found 11 stages of spermatogenic cells, a situation comparable to the study of *X. laevis* by Kalt.[2] However, it was noted that leptotene stage and spermiogenesis required longer periods than in *R. esculenta*. For a urodele species, *Triturus vulgaris*, kept at 16°C, there were similar cell stages as identified in anurans. However, the duration of primary spermatocyte was longer while that of pachytene stage was shorter than both in *X. laevis* and *R. esculenta*.[9]

Radioisotope-labelling studies to determine the durations of male germ cells in *R. catesbeiana* have not yet been carried out. However, the relative durations of various cell stages could be inferred from the light microscopic observations. It is generally assumed that at any instant cells with longer duration should be present in more numerous number, while the scarce cells observed in each section should reflect the short duration or quick passage through that stage. From this generalization, it is suggested that spermatogonia, pachytene spermatocytes, middle stage spermatids and differentiating spermatozoa had long durations; while secondary spermatocytes, diplotene and diakinesis-metaphase 1 spermatocytes have comparatively short durations.

**Development of testes**

Up to now, there have been many reports on the development of the testis. However, there have not yet been a common agreement on the origin of the testicular tissues in amphibians. Noble[10] suggested that testes were derived from the kidney, while Spengel[11] and Brauer[12] found that testes apparently developed from a separate pair of gonadal ridges. Testis primordium as shown in *R. sylvatica,* consists of both medulla and cortex; and the inner medullary region was thought to be derived from the peritoneal covering of the genital ridge.[13] Sexual differentiation of gonads were thought to be controlled by two classes of substances: corticin that is localized in the cortex and stimulates the differentiation of female system, and medullarin which is localized in the medulla and stimulates the differentiation of male system. The predomination of the influence of one over the other was suggested to result in sexual differentiation of the gonads.[13]

In *R. catesbeiana*, a few large primordial germ cells could be observed in one-month-old frogs; and these cells were intermingled with mesenchymal cells in the region of putative testis or ovary. When the frogs were two months old, the sex cords appear in the gonads; afterwards, the definitive testis, which is ascertained by the presence of spermatogonia, could be discriminated in the fourth month, and spermatocytes appeared in five-month-old frogs. Seminiferous tubules with thick epithelium consisting of all stages of germ cells appeared on the seventh month, when there were abundant spermatids, especially middle or round stage, and a few spermatozoa. Thereafter, the number of mature spermatozoa increased gradually until they reached the maximum around the sixteenth month. Therefore, it appears that the sexual turning point or "puberty" in *R. catesbeiana* reared in Thailand climate is around seven months old, and the complete sexual maturation is attained about sixteen months.

**Seasonal variation**

Reproductive activities of most amphibians are greatly susceptible to environmental fluctuations, thus most of them exhibit markedly seasonal testicular cycle. Rastogi *et al*.[14] studied various environmental influences which could alter the characteristics of internal morphology of the testis

in *R. esculenta,* and found that these influences consist of rainfall, temperature and photoperiod. These factors cause cyclical external morphological changes as well as internal changes of the testis. For example, the toad, *B. arenarum* has maximum development of the thumbpad and testicular weight in spring, when the germ cells and spermatozoa were also markedly increased.[15] *Rana ridibunda*, with continuous type spermatogenesis, show significantly increased number of spermatids during breeding season around April to June, while in winter, the number of spermatocytes was decreased and reach minimum in the coldest month.[16]

In the central and upper parts of Thailand, where the wet and dry seasons are quite distinct, *R. catesbeiana* show responses to seasonal change in both external appearance and reproductive capacity. During the breeding season (April-September), the secondary male characteristics such as thick greyish thumbpad are exhibited while the seminiferous tubules' production of spermatids and spermatozoa also come in full steam. In the non-breeding period (December-January), these tubules consist mainly of early spermatocytes with only few round spermatids; and some tubules are dilated and exhibit desquamation of the epithelium. In the pre-breeding period (February-May) there are numerous primary spermatocytes and spermatogonia in preparation for subsequent development, while during the post-breeding period (October-November) there appear spaces or vacuoles in the tubules from ruptured cell nests. Such cyclical change may depend on the availability of gonadotropins, as has been demonstrated in *Rana temporaria* that exogenous administration of these hormones or the elevation of environmental temperature could stimulate recrudescence of spermatogenetic activity in the seasonally quiescent males.[17] The toad, *Bufo bufo*[18], and greenfrog, *R. esculenta*[19], were the other two well-studied species which showed similar cycles to seasonal variation. Another evidence supporting the response of the testis to seasonal changes is the gonadosomatic index (GSI) which correlates the changes of body weight and testicular weight. Kao *et al*.[20] showed that GSI in *Rana rugulosa* exhibited the rising phase in hibernation and pre-breeding season (January-March), while the maximum GSI were observed in the breeding period (April-June). On the other hand, GSI sharply decreased in the late breeding period (June-early July) and remained at the low level in post-breeding period (August-October). In *R. catesbeiana*, the changes of GSI also exhibited similar pattern as those of *R. rugulosa* and it is interesting to note that in this species the reactivated spermatogenesis actually begins even before the rainy season which usually starts around middle of May. Furthermore decline of spermatogenesis commences before the end of rainy season which is around the end of October. Therefore, the present study demonstrated that *R. catesbeiana* does exhibit cyclical changes of the testis in response to seasonal variation.

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