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STRUCTURE OF THE TESTIS OF *RANA TIGERINA* AND ITS CHANGES DURING DEVELOPMENT AND SEASONAL VARIATION

PRAPEE SRETARUGSA[a], VIMONWAN NAKIEM[b], PRASERT SOBHON[a], JITTIPAN CHAVADEJ[a], MALEEYA KRUATRACHUE[c] AND E. SUCHART UPATHAM[c]

[a] *Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*

[b] *Department of Anatomy, Faculty of Public Health, Burapha University, Chonburi, Thailand.*

[c] *Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*

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ABSTRACT

Testes of one- to fourteen - month - old Rana tigerina*, a native rice-field frogs of Thailand, were studied by light microscopy. Based on the nuclear characteristics, 12 stages of male germ cells could be identified in the seminiferous tubules. Primary and secondary spermatogonia are the earliest germ cells that show large euchromatic nuclei with prominent nucleoli. Primary spermatocytes consist of five stages, namely, leptotene, zygotene, pachytene, diplotene and metaphase 1 spermatocytes. Secondary spermatocytes have blocks of highly condensed heterochromatin attached to the nuclear envelopes resembling a "clock-face" pattern. There are three stages of spermatids : the early, the middle and the late stages. The cytoplasm of the late stage spermatid becomes highly vacuolated and starts to degenerate. In a fully mature spermatozoon, the nucleus becomes highly elongated, and chromatin completely condensed. Spermatozoa are embedded in the cytoplasm of Sertoli cells. At each stage of division and differentiation, a clone of cells derived from a single spermatogonium is surrounded by processes of folliculo-Sertoli cells. Leydig cells are found between seminiferous tubules. During development, testis with hollow sex cords appears during the second month. Definite seminiferous tubules are formed during the third month. Spermatogonia are present in the epithelium of seminiferous tubules during the early part of the fourth month. Active spermatogenesis begins during the fifth month, and full production of spermatozoa could be detected from the sixth month onwards. In adult male frogs, there are abundant spermatozoa and round spermatids in seminiferous tubules during the breeding period (from March to September); while during the non-breeding period (from October to February), such cells are much fewer in number. Moreover, the late stages of germ cells are also detached from seminiferous epithelium.*

INTRODUCTION

The identification of cells in the developing testes of anurans have been investigated by many researchers, and *Xenopus laevis* is the anuran of choice for most experiments. In light microscopic investigations, the germ cells in testes of *X. laevis* have been classified into 11 stages based on premeiotic DNA synthesis, appearance, and sizes[1]. These stages were primary spermatogonia (1°Sg), secondary spermatogonia (2°Sg), five stages of primary spermatocytes (1°Sc): leptotene spermatocyte (LSc), zygotene spermatocyte (ZSc), pachytene spermatocyte (PSc), diplotene spermatocyte (DSc), secondary spermatocyte (2°Sc), early spermatid (ESt), middle or round spermatid (RSt), late spermatid and spermatozoa (Sz). In addition, autoradiographic studies with tritiated thymidine were employed to determine the durations of various cell types in *X. laevis*: the longest was 12 days in pachytene stage, and the shortest was one day for diplotene stage; and that one day was needed for second meiotic division to

complete[1]. Similarly, in *Rana esculenta*, the longest duration was in pachytene stage which took 12 days, and the shortest duration was one day for diplotene and secondary spermatocyte stages[2]. The duration of spermatogenesis, from primary spermatogonium to the formation of spermatozoa was 41 days[2]. In a toad, *Bufo arenarum*, the germ cells have been classified by using both light and electron microscopes into eight stages, and these stages were 1°Sg, 2°Sg, 1°Sc, 2°Sc, ESt, RSt, LSt and Sz[3]. In comparison to other species of anurans, the classification of germ cells in the testes in *Rana tigerina* is still lacking. Hence, the primary purpose of the present study is to classify the stages of germ cells in the frogs' testes based on their morphological characteristics.

In most amphibians, bipotential gonad primordia finally differentiate into either testes or ovaries before metamorphosis; however in some, remnants of opposite sex cells, eg. spermatocytes or oocytes still persist beyond this point into juvenile and adult stages. In such cases, subsequent differentiation of the tissues may result in bisexual stages with both ovarian and testicular tissues present within the same individual[3]. Such a situation is not uncommon in many species of anuran, such as *Rana temporaria, R. esculenta, Bufo bufo, Hynobius retardatus* and *Ambystoma maculatum*[4]. So far, there is no data on the development of testes of *R. tigerina*. Hence, another aim of the present investigation is to study the development of testes in this species.

Reproductive patterns of most animals particularly the lower vertebrates are correlated with the climatic conditions prevailing in the habitats. In equatorial habitats with a constant warm and humid climate, amphibians may reproduce throughout the year, for examples, a frog, *Rana erythraea* in Borneo[5], and a toad, *Bufo melanostictus* in Singapore and Indonesia[6,7]. In regions with distinctive wet and dry seasons such as southern India or Southeast Asia, the main breeding period coincides with the height of monsoon rain during August-October. In tropical country such as Thailand which also has distinctive wet and dry seasons, its native amphibians, including *R. tigerina*, are expected to be seasonal breeders. Therefore, another aim of the present study is to identify the duration of the breeding period and the morphological changes in testes that accompany this seasonal variation.

MATERIALS AND METHODS

1. Experimental animals

R. tigerina were cultured in cement tanks at Faculty of Science, Mahidol University. They were maintained on an approximately 12 hours light/dark cycle, at 25°-35°C, with the relative humidity ranging from 80 to 100%. The culture water was changed at alternate days. The frogs were fed once daily with pelleted feed in the afternoon.

2. Light microscopic study

2.1 Classification of germ cells in the testis

Mature male frogs aged more than 12 months old were anesthesized by being placed in an ice bath for 5-10 minutes, or until they became immobile. The testes were removed and further processed for light microscopic observation.

The specimens were fixed in Bouin's solution for three hours, then the tissues were washed in several changes of 70% alcohol in order to remove the fixative. Specimens were further dehydrated through increasing concentrations of ethyl alcohol at 80, 95 and 100%, consecutively, for 15 minutes each, then infiltrated with dioxane, and embedded in paraffin.

Five - to six - micron - thick sections were cut, deparaffinized and stained with Harris's hematoxylin and eosin, and examined under an Olympus light microscope BH-2.

2.2 Development of the testis

Young frogs aged from 1 upto 14 months old were used in this study. At least eight frogs were taken at the end of each month for histological studies. The testes were removed and processed in the same manner as described in section 2.1.

2.3 Changes of the testis during seasonal variation

Fully mature frogs aged more than 12 months old were used in this study. The testes were removed from the frogs at the end of each month throughout the year, and processed for light microscopic observation as described in section 2.1.

RESULTS

Classification of various stages of cells in spermatogenesis

The histological sections of testis (Fig1A) illustrate that each testicular lobule contains several convoluted seminiferous tubules which is surrounded by a thick basement membrane. The seminiferous epithelium consists of two groups of cells, namely, spermatogonic cells and folliculo-Sertoli cells. The interstitial areas are filled with Leydig cells, blood and lymphatic vessels, and connective tissues. Based on nuclear characteristics and cell size, the male germ cells in the testes of *R. tigerina* can be divided into 12 stages.

1. Primary spermatogonia (1°Sg)

Early 1°Sg are large round cells, that constitute a high proportion of the cells of the seminiferous tubules, especially in immature frogs. They generally rest on the basement membrane, and contain round or ovoid nuclei with fine and mostly euchromatin material (Fig.1B). The size of the nucleus is around 10-11 μm. The nuclei also possess one or two prominent nucleoli. The cytoplasm is generally lightly stained. Frequently, cells with large bilobed nuclei could be found, which may be the spermatogonia cells that are undergoing nuclear division.

2. Secondary spermatogonia (2°Sg)

2°Sg are round but smaller than 1°Sg, and can be distinguished from 1°Sg by their smaller nuclei with diameters around 6-8 μm. The nuclei contain small blocks of heterochromatin distributed along the nuclear envelopes, and the nucleoli remain prominent. 2°Sg usually exist in groups of 2 or 4 cells which are still lying close to the basement membrane. Each group is surrounded by processes of follicular cells (Fig. 1B).

3. Leptotene spermatocytes (LSc)

These cells are larger than 2°Sg and have round shape. They have large round nuclei with a thin rim of cytoplasm. The nuclear size is averaging about 7-8 μm in diameter. Chromatin that begins to condense into small blocks is distributed evenly throughout the nucleus. Nucleoli cannot be detected at this stage. LSc are located toward the lumen of the seminiferous tubules, and seldom touch the basement membrane. In addition, they are grouped in large clusters of more than four cells, and each cluster is surrounded by follicular cells (Fig. 1B).

4. Zygotene spermatocytes (ZSc)

The distinguishing features of ZSc from LSc are the changes in size and color of the nuclei, which become slightly smaller than those of LSc; while the heterochromatin blocks

become larger and denser than those of LSc (Fig.1B).

5. Pachytene spermatocytes (PSc)

The nuclei of PSc are still round and about 6-7 μm in size. The chromatin becomes condensed into long and thick fibers that are intertwined into loops that resemble a "bouquet pattern". The number of cells in each cluster increases, and each cluster is still enclosed by follicular cells (Fig.1E).

6. Diplotene spermatocytes (DSc)

The general characteristics of the cells in this stage resemble those of PSc (Fig.1E). The size of the nuclei is about 5-7 μm. However, DSc decrease in size and their nuclei show chromatin fibers with increasing thickness. They are also fewer in numbers in comparison to PSc.

7. Diakinetic (Dia) and metaphase spermatocytes (MSc)

The diakinetic spermatocytes have thick chromosomes that are arranged close to the equatorial region; they rapidly turn into the metaphase spermatocytes whose chromosomes are aligned along the equatorial plate. The nuclear boundary of MSc disappears. Both Dia and MSc are so few and transient that they are rarely observed within the seminiferous tubules (Fig.1C).

8. Secondary spermatocytes (2°Sc)

These cells arise after the first meiotic division. They pass through this stage rapidly, and are, therefore, seldom observed in histological sections. The identifiable characteristic is the appearance of a "cartwheel" or a "clock-face" chromatin pattern in the nucleus which arises due to the coarse clumping of chromatin along the nuclear envelope (Fig.1C).

9. Early spermatids (ESt)

These cells arise from the division of secondary spermatocytes. ESt markedly decrease in size, and they are comparatively fewer in number in comparison to other spermatid stages within the tubules. They still have round nuclei which are also reduced in size to approximately 4-5 μm in diameter, and become eccentrically located within the cells. The nuclei are deeply stained due to chromatin condensation over one-half, while the rest of the nuclei appears very lightly stained (Fig.1E).

10. Round (or middle) spermatids (RSt)

RSt are the most numerous cells in the tubules, probably due to their long duration. Their nuclei remain round and reduced to about 5 μm in diameter. The chromatin becomes evenly condensed and deeply stained throughout the nucleus. The cytoplasm is lightest in comparison to other cell types (Fig.1E).

11. Late spermatids (LSt)

The nuclei of LSt undergo elongation and further reduced in size during the transformation into spermatozoa. Chromatin becomes completely condensed throughout the nucleus. They usually lie close to the lumen of the seminiferous tubules (Fig.1C), and are grouped in clusters.

12. Spermatozoa (Sz)

The mature spermatozoa possess highly elongated heads and tails. The head contains an ellipsoid nucleus with completely condensed chromatin which is deeply stained. The heads of spermatozoa appear to be arranged in array and embedded themselves in the apical cytoplasm of Sertoli cells (Fig1D), while their tails point toward the seminiferous lumen.

Development of the testis

The definitive testis could be observed when the frog is around one month old (Figs.2A,B). It appears as a small ovoid organ closely attached to the ventral surface of the kidney. The testis is enclosed within the inner layer of connective tissue capsule, which is in turn covered by an outer layer of peritoneal membrane. Within the newly formed testis, the principal cell type is primordial germ cells with large nuclei. Most of them are undergoing mitotic division. Primary and secondary germinal cavities are observed in testis of one-month-old frogs. Primary germinal cavity is filled with mesenchyme cells whereas the secondary germinal cavity is a distinct cavity. The testis of two-month-old frogs exhibits the formation of hollow sex cords (Fig.2C). On the third month, these hollow sex cords differentiate into definite seminiferous tubules whose walls become thicken and start to contain 1°Sg. Some oogonia are also observed (Fig.2D). On the fourth month, seminiferous tubules become enlarged and their epithelia contain 1°Sg, 2°Sg, and most of late stage germ cells and few spermatozoa (Fig.2E). From the fifth to sixth months, the testis enlarges rapidly together with the massive cellular proliferation (Figs.3A,B). Spermatozoa which start to appear in the fourth month, begin to increase rapidly in number during the fifth to sixth months (Fig.3A), while the tubules also contain a large number of spermatocytes and round spermatids (Fig.3B). By the end of the six month, the testis assumes similar appearance as that of the fully mature frogs with age more than one year old (Fig.3C,D).

Seasonal variation of the testis

The seasonal changes are classified into four periods based on the structural and physiological changes of testis as well as the body and testicular weights, which undergo changes throughout the year as shown in Table 1.

The testes were small and weighed the least during the post-breeding period. The testis during these months contains relatively very few cells, most of which are spermatogonia and early spermatocytes with few round spermatids, and only few spermatozoa remain. During the non-breeding period, the number of the late stage germ cells, especially round spermatids and spermatozoa drastically decreases as well as the body and testicular weights. Some tubules are dilated and exhibit desquamation of their epithelium; and some even show a complete breakdown (Figs.4B,C,D).

Spermatogenetic activity is reactivated in February to March, which is designated as the pre-breeding period. There are a concomitant increase in the body weight and the size and weight of the testes. The early germ cells proliferate rapidly and transform into spermatozoa during April to September, which is designated as the breeding period. The lumen of thick

Table 1. Testicular and body weight of *R. tigerina* during seasonal variation.

Period (month)	Body weight (g) (Mean ± SEM)	Testicular weight (g) (Mean ± SEM)
Pre-breeding (Feb-Mar.)	126.9 ± 4.69	0.24 ± 0.05
Breeding (Apr.-Sept.)	142.4 ± 4.0	0.49 ± 0.14
Post-breeding(Oct.-Nov.)	132.4 ± 6.75	0.10 ± 0.004
Non-breeding (Dec.-Jan.)	93.9 ± 13.5	0.06 ± 0.01

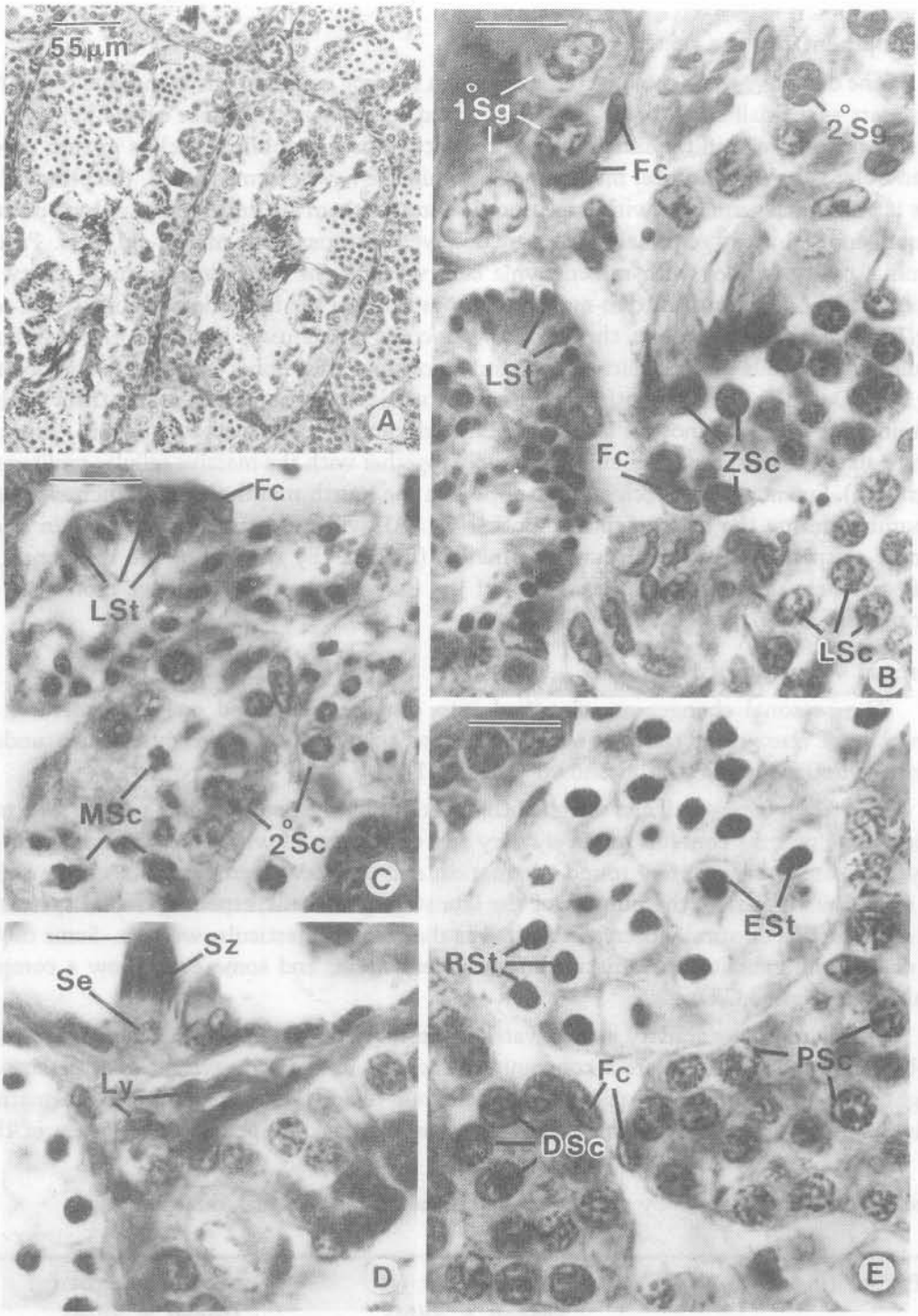

Fig.1. A : Seminiferous tubules, illustrating various stages of spermatogenic cells.
B-E : High magnification of seminiferous tubules, showing primary spermatogonia (1°Sg), secondary spermatogonia (2°Sg), leptotene spermatocyte (LSc), zygotene spermatocyte (ZSc), pachytene spermatocyte (PSc), diplotene spermatocyte (DSc), metaphase spermatocyte (MSc), secondary spermatocyte (2°Sc), early spermatid (ESt), round spermatid (RSt), late spermatid (LSt), spermatozoa (Sz), follicular cell (Fc), Sertoli cell (Se) and Leydig cell (Ly). Bar = 15 µm.

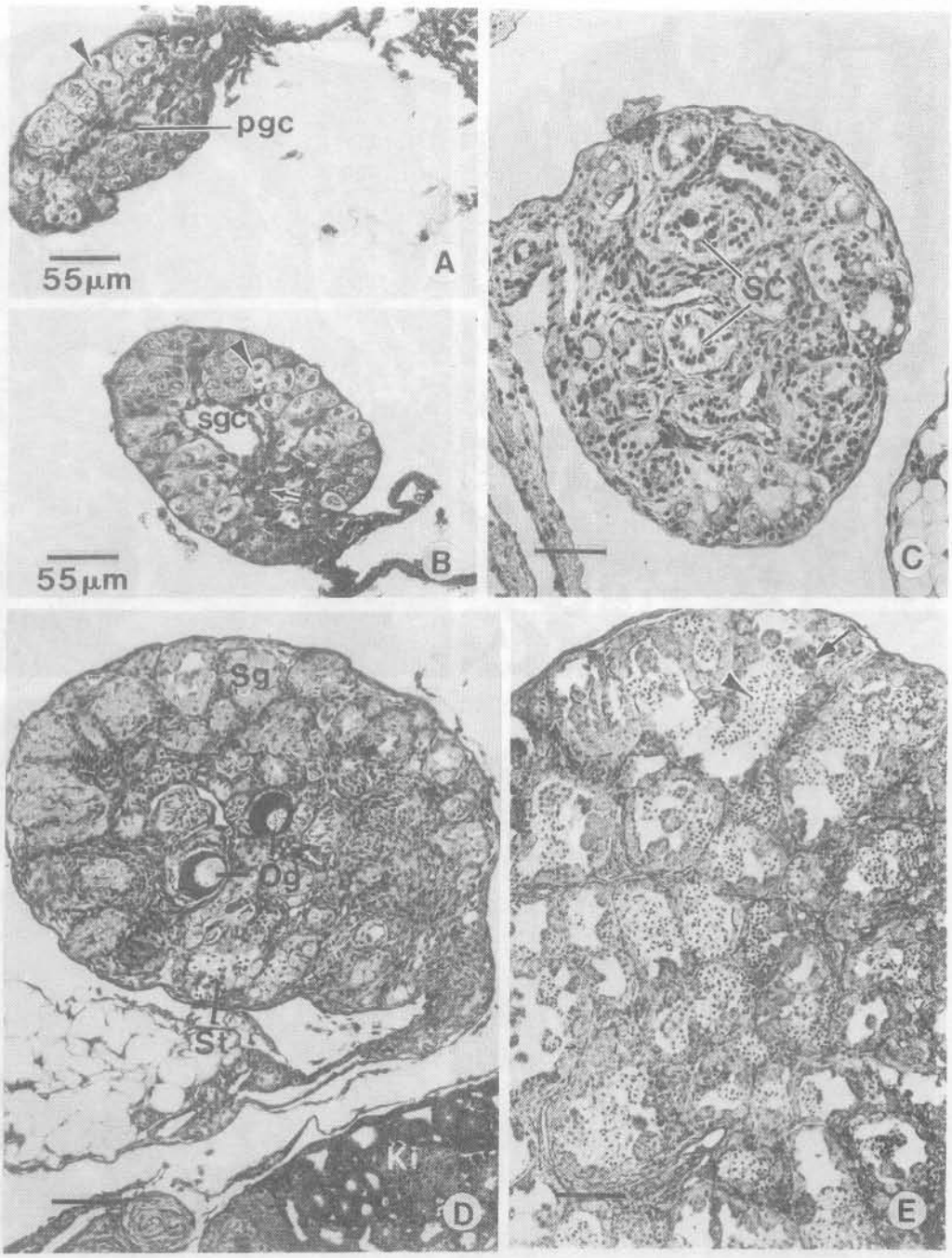

Fig.2. A,B : Testes of one-month-old frogs showing mitotic division of primordial germ cells (arrowheads), primary and secondary germinal cavities (pgc, sgc) and mesenchyme cells (arrow).

C : Testis of two-month-old frog, showing hollow sex cord (SC).

D : Testis of three-month-old frog, showing definitive seminiferous tubule which contains spermatogonia (Sg) and spermatids (St). Some oogonia (Og) are present. Ki = kidney.

E : Testis of four-month-old frog, showing late stage spermatids (arrowhead) and some spermatozoa (arrow). C,D,E : bar = 110 μm.

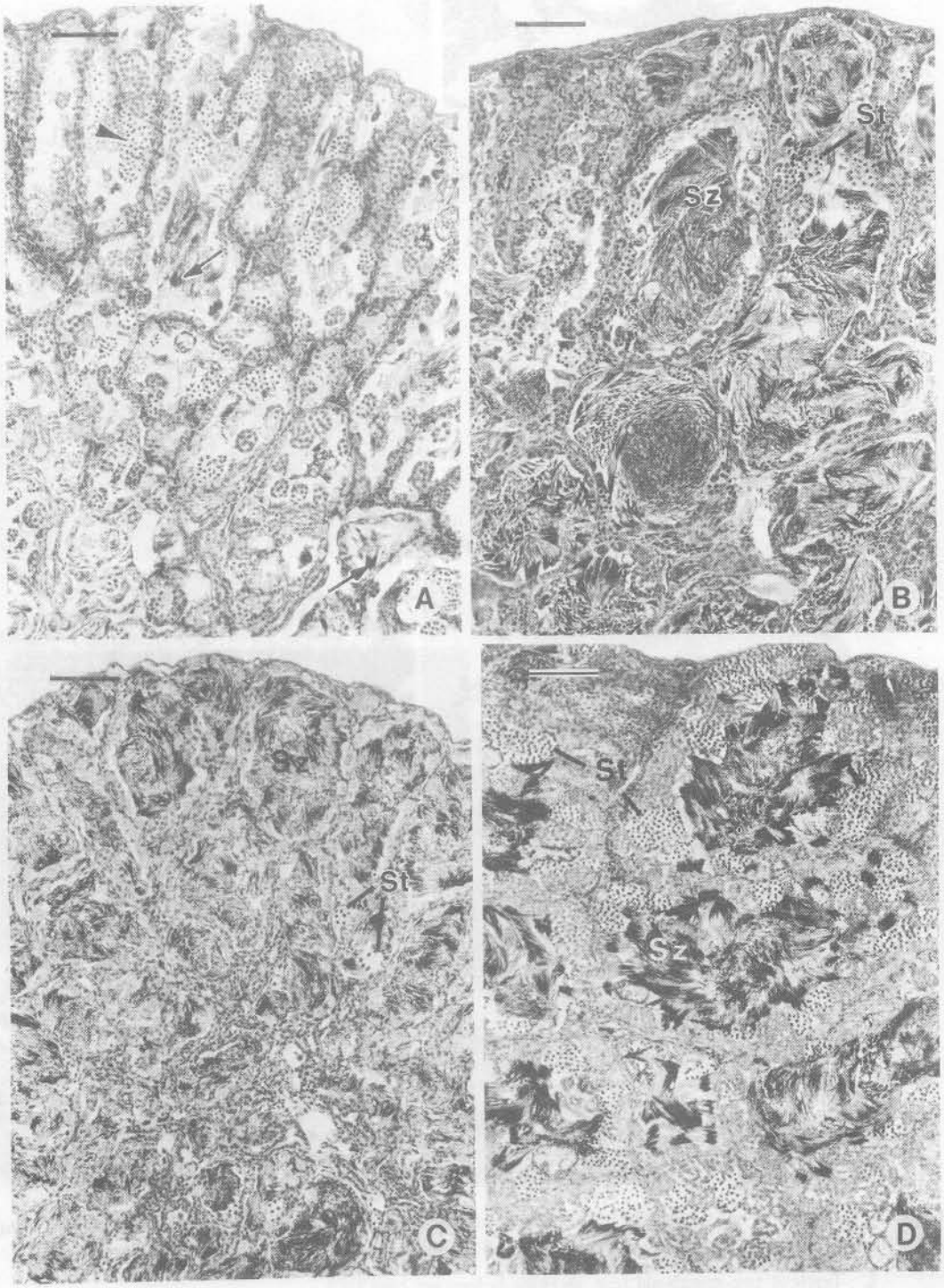

Fig.3. A : Testis of five-month-old frog. Note the increase in number of spermatids (arrowhead) and spermatozoa (arrows). Bar = 110 μm.

B-D : Testes of six-, eight-, and fourteen-month-old frogs, showing a large number of spermatids (St) and spermatozoa (Sz) which continue to be produced from six months onward. Bar = 110 μm.

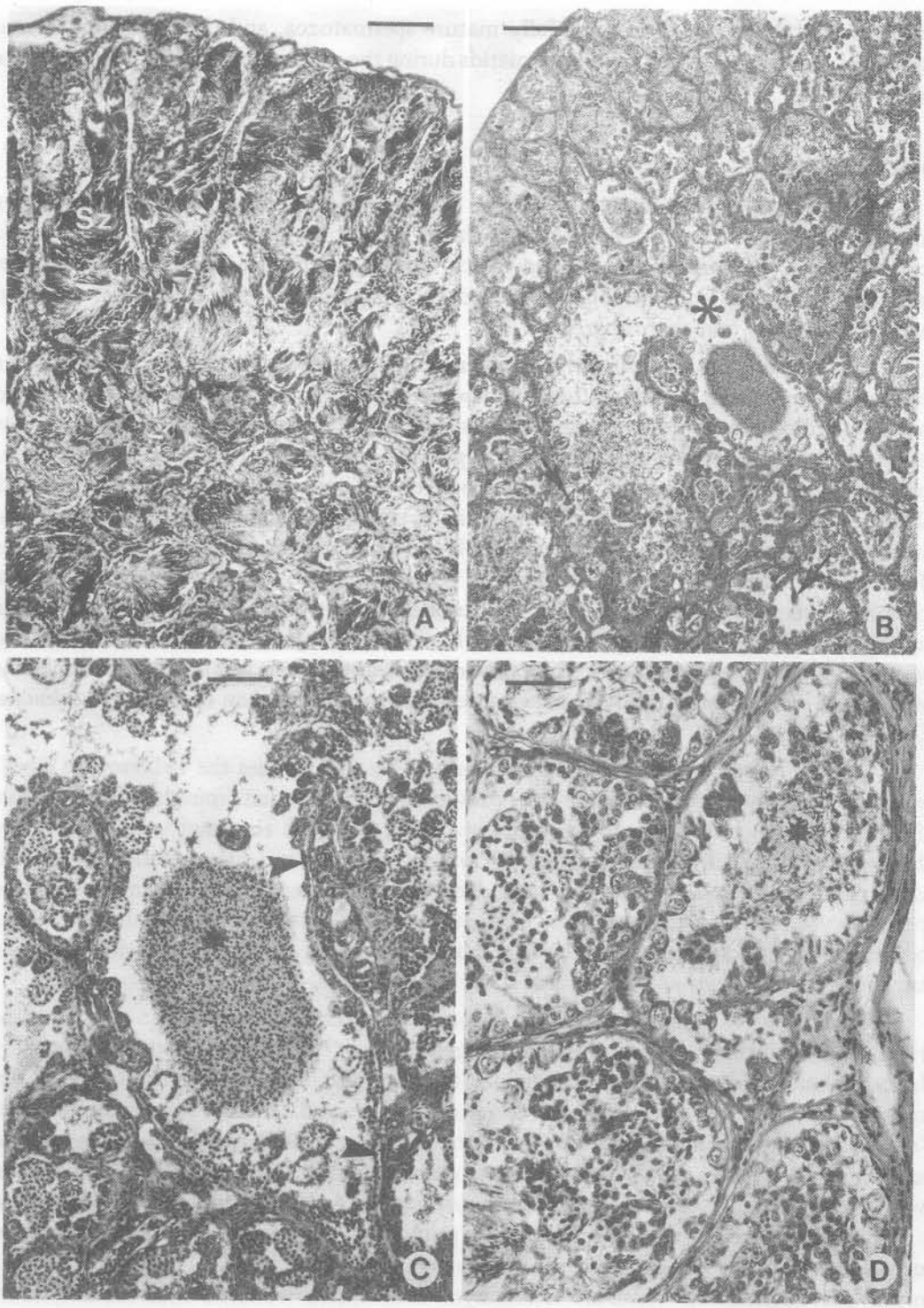

Fig.4. A : Testis of frog during the breeding period, showing the thick-walled seminiferous tubules which contain a large number of spermatozoa (Sz).

B-D: Testes of frogs during the non-breeding period, revealing a small number of spermatozoa (arrows) in the thin-walled semiferous tubules. Some tubules are dilated and broken (asterisk). The epithelium is thin

seminiferous tubules are filled with fully mature spermatozoa, and the epithelium contains numerous spermatocytes and round spermatids during the mid-breeding period which is around July (Fig.4A).

DISCUSSION

In the testis of fully mature *R. tigerina*, 12 stages of germ cells could be identified based on the nuclear characteristics and the cell shape. These stages are primary and secondary spermatogonia, primary spermatocytes which consist of five stages (leptotene, zygotene, pachytene, diplotene and metaphase), secondary spermatocytes, three stages of spermatids (early, round, late) and spermatozoa.

In comparison to *R tigerina* in the present study, eleven stages of male germ cells in the testes of *X. laevis* have been identified[1]. These investigators used autoradiographic technique to label germ cells with ^{3}H thymidine, and calculated the duration of each cell stage. It was found that the durations of leptotene, zygotene, pachytene, diplotene and metaphase spermatocytes were 4,6,12,1 and 1 days, respectively. Spermiogenesis was completed within 12 days. Moreover, the duration of premeiotic S stage was calculated to be 6-7 days[1]. In *R. esculenta*, eleven stages of male germ cells had been found[2], a situation comparable to the study of *X. laevis*[1]. However, it was noted that the durations of leptotene spermatocyte and spermiogenesis were longer in *X. laevis*. For urodele species, such as, *Triturus vulgaris*, similar cell stages could be identified as in anurans[8]. However, durations of the cells in various stages might vary; for example, the durations for premeiotic spermatogonia and leptotene spermatocytes were longer, while that of pachytene spermatocytes was shorter than those of *R. esculenta* and *X. laevis*.

In *R. tigerina*, there has not yet been any study that applies the radioisotope labelling technique to study the cellular durations. However, it appears that from the relative numbers of germ cells present in the tubules at any one time, the most abundant cells are both types of spermatogonia, pachytene spermatocytes, round spermatids and differentiating spermatozoa; while the least abundant cells are secondary spermatocytes, diplotene and metaphase 1 spermatocytes. The relative numbers of these cells present in the tubules should indirectly indicate the length of duration for the cells transit to the next stages, which could be relatively long for the former group of cells and short for the latter.

Up to now, there has not yet been a common agreement on the origin of the testicular tissues of amphibians. It was suggested that genital cords were derived from the kidney[9]. On the other hand, there have been suggestions that the primordial germ cells of urodele testes might be derived from mesoderm, and those of anurans and salientia were from endoderm[4]. Testis primordium in *Rana sylvatica* consists of the inner medullary surrounded by the outer cortical regions; the former was thought to be derived from the tissue of metanephric blastema, while the latter might be derived from peritoneal covering of the genital ridge[10]. In *R. tigerina*, a group of primordial germ cells, similar to primary spermatogonia, appears at the antero-medial region of the kidney when the frog is about one month old. This group of cells are mixed with stromal cells which are mesenchyme-like. Definitive testes were observed in four-month-old frogs. Initially, primary spermatogonia and mesenchyme-like stromal cells are mixed within the same mass that is surrounded by a fibrous capsule. Later, hollow sex cords are formed within the mass and turned into seminiferous tubules. Before the fifth month, spermatogonia and spermatocytes are the dominant cells in the seminiferous epithelium. Spermiogenesis may occur promptly between the fifth to sixth months at which time spermatids,

especially round spermatids, could be readily observed. Thereafter, mature spermatozoa increase rapidly until they reach the maximum number around 12 months. Therefore, the puberty in *R. tigerina* is around the sixth month and complete sexual maturity is attained around the twelfth month.

One of the most remarkable characteristics of amphibian is the cyclical changes of testes in consonant with the variation in environmental and seasonal conditions. Particularly, the trend in seasonal breeding correlates with climatic cycle in temperature and precipitation. *Rana pipiens berlandieri* and *Pseudacris clarkii* show a tendency toward two breeding seasons in spring and autumn[11]. In some species of *Bufo*, living in a more temperate climates, a biennial pattern of reproduction also sometimes occurs[12] . If there is a climatic change in the tropics, breeding may not occur every year, as in *Rana pretiosa*[13]. In tropical regions where rain occurs in all months of the year, breeding is typically non-seasonal and spawning can occur throughout the year[14]. The spermatogenetic cycles of many tropical and subtropical species show continuous production of spermatozoa throughout the year, for example, in *Bufo paracnemis, B. arenarum, B. melanostictus* and *Rana hexadactyla*[15]. In contrast, some temperate species possess strictly seasonal reproductive cycle; spermatogenesis becomes depressed during the cooler winter months[16].

In the central and upper parts of Thailand, where the wet and dry seasons are quite distinct, *R. tigerina* shows seasonal changes in reproduction. During the non-breeding period (December to January), the seminiferous tubules consist of early stages of spermatocytes with only a few round spermatids. In the breeding season (April to September), the production of spermatids and spermatozoa come in to full steam. Such cyclical change may be dependent on the availability of gonadotropins. It was demonstrated that during the discontinuous cycle in *R. temporaria*, the germ cells are never refractory to gonadotropic hormones. Spermatogenetic activity can be stimulated in the seasonally quiescent males by the administration of exogenous gonadotropins or elevated environmental temperatures[15]. The toads, *B. bufo*, and green frogs, *R. esculenta*, are other well-studied examples which show similar responses[12,16]. When spermatogenetic activity resumes, it proceeds at a high rate for a limited period, and the seminiferous tubules become rapidly stocked with spermatozoa which are discharged during the succeeding breeding period. In *R. tigerina*, it was found that the maximum numbers of spermatozoa and round spermatids occur during the breeding period. It seems, therefore, that the frog's physiology is well adapted to the amount of rain, and may be also to the duration of sunlight, which are different during the wet rainy season in comparison to the dry winter months. Such homeostasis could be regulated by gonadotropin from the pituitary gland, which in turn, is controlled by the upper brain centers.

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บทคัดย่อ

การศึกษาโครงสร้างและการพัฒนาของต่อมท่ออัณฑะของกบนา *Rana tigerina* อายุ 1-14 เดือนด้วยวิธีทางกล้องจุลทรรศน์ธรรมดาพบว่า เซลล์สืบพันธุ์ในต่อมอัณฑะสามารถแบ่งออกเป็น 12 ระยะโดยพิจารณาลักษณะของนิวเคลียส ได้แก่ เซลล์สเปอร์มาโทโกเนียมขั้นต้นและขั้นที่สองซึ่งมีนิวเคลียสขนาดใหญ่และมียูโครมาทินเป็นส่วนมาก เซลล์สเปอร์มาโทไซต์แบ่งเป็นห้าระยะได้แก่ เลปโททีน ไซโกทีน แพไคทีน ดิโพลทีน และสเปอร์มาโทไซต์ในเมทาเฟส 1 เซลล์ในระยะเลปโททีนมีการขดตัวของเฮเทอโรโครมาทินเพิ่มขึ้นและกระจายทั่วไป เซลล์ในระยะแพไคทีนมีการไขว้ไปมาของเส้นโครมาทินลักษณะคล้ายซ่อดอกไม้ ส่วนเซลล์สเปอร์มาโทไซต์ขั้นที่สองมีการขดตัวของก้อนเฮเทอโรโครมาทินกระจายเป็นลักษณะคล้ายหน้าปัทม์นาฬิกา เซลล์สเปอร์มาทิดมีสามระยะ คือ ระยะต้น ระยะกลาง และระยะปลาย ระยะสุดท้ายคือเซลล์อสุจิซึ่งมีนิวเคลียสลักษณะเรียวยาวและมีการขดตัวของเส้นใยโครมาทินอย่างแน่นทึบ เซลล์สืบพันธุ์ทุกระยะถูกโอบรอบด้วยเซลล์ฟอลลิคิวโล-เซอร์โทไล

ในการศึกษาการพัฒนาของต่อมอัณฑะพบว่า ต่อมอัณฑะของกบนาเริ่มปรากฏเมื่อกบมีอายุได้สองเดือน และมีเซลล์สเปอร์มาโทโกเนียมปรากฏเมื่อต้นเดือนที่สาม ในเดือนที่สี่อัณฑะมีการขยายขนาดและมีการพัฒนาของเซลล์อย่างรวดเร็ว และเมื่อกบอายุหกเดือนจะพบเซลล์อสุจิจำนวนมากคั่งอยู่ในหลอดอสุจิ

ในการศึกษาการเปลี่ยนแปลงของต่อมอัณฑะตามฤดูกาล พบว่า ในฤดูกาลผสมพันธุ์ (เมษายน-กันยายน) พบเซลล์อสุจิที่สมบูรณ์และเซลล์สเปอร์มาทิดระยะกลางที่มีนิวเคลียสกลมเป็นจำนวนมาก ส่วนในระยะนอกฤดูผสมพันธุ์ (ตุลา-กุมภาพันธ์) นั้น จะพบเซลล์เหล่านี้น้อยลง เซลล์ที่ปรากฏส่วนใหญ่เป็นเซลล์สืบพันธุ์ชั้นต้น ส่วนใหญ่ของเซลล์สืบพันธุ์ระยะท้ายๆ หลุดออกจากหลอดอสุจิ