Korean J Biol Sci 1: 99-105, 1997

Testicular Cycles in the Korean Frogs: Annual Spermatogenic Patterns, Seasonal Changes in the Steroidogenic Competence, and Responsiveness to Gonadotropins *in vitro*

Sun Kun Ko[2], Hae Mook Kang[1, 3], Jung Woo Kim[4], and Hyuk Bang Kwon[1]*

Department of Biology and [1]Hormone Research Center, Chonnam National University, Kwangju 500-757, Korea; [2]Department of Biology, Honam University, Kwangju 502-791, Korea; [3]Department of Genetic Engineering, Chongju University, Chongju, Chungbuk 360-764, Korea; [4]Department of Biology, Seonam University, Namwon, Chonbuk 590-170, Korea.

Key Words:
Testicular cycle
Spermatogenesis
Testosterone
Gonadotropin
Amphibians

Using three species of Korean frogs (*Rana dybowskii*, *R. rugosa* and *R. nigromaculata*), the annual spermatogenic pattern, the seasonal changes in the steroidogenic competence, and responsiveness of testis to gonadotropins in terms of testosterone secretion *in vitro* were examined. The spermatogenic pattern of *R. dybowskii* was classified as a discontinuous type since spermatogenesis stops completely after spawning in late winter (February) until mid-summer (July). In contrast, the pattern of *R. nigromaculata* and *R. rugosa* was classified as a potent continuous type since sperm was always present in the seminiferous tubules all year round. In all three species, the levels of testicular testosterone and that of testosterone secreted by testis following *in vitro* culture were very low in late summer (August), but increased thereafter until winter (hibernation period). Interestingly, responsiveness of testis in vitro to gonadotropins in terms of testosterone secretion increased markedly in November (early hibernation period). Specifically, bullfrog LH was more effective than FSH in stimulating the secretion of testosterone by frog testis in vitro during hibernation period. This fact suggests that testosterone secretion by testis during hibernation is at least regulated by the pituitary gonadotropin rather than environmental factors. Taken together, the data presented here suggest that testicular cycles of three species of Korean frogs are closely linked to their females breeding cycles, and are eventually controlled by various environmental cues.

Most lower vertebrates have an annual reproductive cycle. In males, the testis weight increases markedly during the period of active spermatogenesis and decreases after breeding season. These cyclical fluctuations are in part due to the changes in the rate of androgen biosynthesis by the Leydig cells in testis. The androgen synthesis was known to be controlled by the level of the tropic gonadotropins in circulation and by the responsiveness of Leydig cells to luteinizing hormone (LH) (for reviews, Lofts, 1984; Whittier and Crew, 1987; Hardy et al., 1992)

In amphibians, testicular cycles were analyzed by examining the changes in testis weight, distribution of gonadal cells, spermatogenic pattern, and seasonal variations of testosterone secretion. Many investigators have reported that the amphibian testicular cycle was influenced by various environmental factors such as temperature and photoperiod, as observed in other seasonal breeding vertebrates through the hypothalamo-pituitary-gonadal axis (for review, Paniagua et al., 1990).

Particularly, in temperate amphibians, spermatogenesis was known to occur most actively just prior to breeding season, and to completely stop or depress for certain period after breeding period. Thus, spermatogenic patterns of male frogs well coincide with oocyte growth and spawning of female frogs. The seasonal fluctuation of testosterone levels has been well described in several amphibians such as *R. catesbeiana* (Licht et al., 1983), *R. esculenta* (Pierantoni et al., 1984a), *Ambystoma tigrium* (Norris et al., 1985), *R. perezi* (Delgado et al., 1989), and *Bufo japonicus* (Itoh et al., 1990). As observed in other vertebrates, synthesis of testosterone is presumed to be controlled by the hypothalamus-pituitary-gonadal axis in *Rana*. In *R. esculenta*, Fasano et al. (1988; 1993) reported that the seasonal fluctuation of gonadotropin-releasing hormone (GnRH) is well correlated with the plasma testo-

* To whom correspondence should be addressed.
Tel: 82-62-520-6874, Fax: 82-62-520-7786.

sterone level. It was also well known that testicular testosterone level increased in parallel with the increase in the cell number, the nuclear size of interstitial cells, and the cellular organelles in the testicular interstitial tissues (Hardy et al., 1992). However, limited information is available for gonadotropin action on testis function in amphibians and only two species of animals, *Bufo japonicus* (Itoh et al., 1990) and *R. catesbeiana* (Licht et al., 1983), were thoroughly investigated. In these studies, levels of gonadotropins and androgen did not exhibit the overall reciprocal relationship, which was expected from the feed back mechanism of pituitary-gonadal axis. In toad, however, plasma follicle stimulating hormone (FSH) level was found to be associated with testicular weight and plasma LH level with plasma androgen levels. However, the regulation mechanism of the biosynthesis and secretion of testicular testosterone by gonadotropins in most temperate frogs was not clearly understood.

In order to assess the role of gonadotropins in regulating the testicular activity and to elucidate the regulatory mechanism of breeding cycle in frogs, we tried to analyze the annual testicular cycle of Korean frogs by examining spermatogenic patterns, steroidogenic competence of testis, and responsiveness of testis to gonadotropins using three species of Korean frogs.

Materials and Methods

Animals

Male frogs of three species (*R. dybowskii*, *R. nigromaculata* and *R. rugosa*) were collected year-round from fields in the Chonnam area, a south-western part of Korean peninsula. Frogs were collected at least more than once per month. Frogs were kept in plastic boxes containing tap water and kept in a room without heating and lighting. Male frogs of R. nigromaculata were kept under soil during hibernation period. The habitat, breeding season, and reproduction pattern of three frogs were described in detail elsewhere (Kwon et al., 1989, 1991; Yoo et al., 1995).

Classification of spermatogenic stages

The testis was fixed in 4% neutral buffered paraformaldehyde (NBP) for 24 - 48 h, embedded in paraplast (Sigma), and sectioned into 5 - 6 μm with a rotatory microtome (American Optical). The sections were stained with Delafield's hematoxylin and eosin. By microscopical observations, the gonadal cells in seminiferous tubules were divided into five classes depending on the formation of gonadal cysts, cell number in cysts and the nuclear size of spermatogenetic cells as described previously (Rastogi et al., 1983). The five classes are the following: primary spermatogonia (PSG), secondary spermatogonia (SSG), primary spermatocyte (PSC), secondary spermatocyte (SSC), and spermatid (ST). PSG was located individually at the basal membrane in the seminiferous tubule and did not form a cyst. Nuclear size of PSG was in the range of 15 - 21 μm in diameter. Two to 8 cells of SSG were contained in a cyst and the nuclear size was in the range of 9 - 12 μm in diameter. Nine to 12 cells of PSG were contained in a cyst and nuclear size was in the range of 6 - 9 μm. 17 - 32 cells of SSC (4.5 - 6 μm) were contained in a cyst. Finally, ST having 1.2 - 3 μm of nuclear size was presented in a cyst consisting of 33 - 64 cells. Based on this grouping, seminiferous tubule of testis were arbitrarily categorized into five stages (I~V) according to the distribution pattern of spermatogonia, gonadal cysts containing spermatogenic cells, and sperm in seminiferous tubules of testis. Characteristics of each stage are described in detail in Table 1.

Testis culture in vitro

Testis isolated from adult male was thoroughly rinsed with Amphibians Ringer (AR) to remove blood debris. The rinsed testis was sliced into a small size (1 × 1 × 2 mm) under a stereomicroscope (Kobayashi et al., 1989). The testis slices were put into 24-well culture dish (Nunc) containing 2 ml of AR per well and cultured in a shaking-incubator (25℃, 80 oscillations/min). Frog pituitary homogenate (FPH) was

Table 1. Classification of spermatogenic stage in the seminiferous tubules of testis

Class	Spermatogenic stage	Character
I	Immature	Gonadal cysts are filled with spermatogonia cells and the number of cysts increases steadily. Spermatocytes and sperms are not observed in seminiferous tubules.
II	Early spermatogenesis	Spermatogenic cells of all developing stages and few sperms appear, but seminiferous lumen is not formed yet. Number of spermatocyte and spermatid increases but spermatogonia decrease.
III	Late spermatogenesis	All developing spermatogenic cells are present abundantly and the lumens are already formed into seminiferous tubules. However, sperms do not move into lumen.
IV	Spermiation	Lumens are filled with sperms, and a few of the developing gonadal cysts remain in seminiferous tubules.
V	Post-spawning	Most of sperms disappeared and the some residual sperms are found in the seminiferous lumen. Some of new developing primary spermatogonia cells begin to appear.

prepared and the doses of FPH were chosen on the basis of our previous data obtained from several species of Rana (Kwon and Shuetz, 1985; Kwon et al., 1989). The purified bullfrog LH and FSH were a kind gift of Dr. S. Ishii (Waseda University, Tokyo, Japan)

Radioimmunoassay of testosterone

The levels of testosterone secreted by testis slices during culture in media or testicular testosterone were measured by radioimmunoassay. Culture media were saved and kept in a deep freezer (-30℃) until assayed. Testosterone in testis was extracted by employing the procedures described by Pierantoni et al. (1984a). Briefly, testis tissue was homogenized with an ultrasonicator at 20 KHz for 1 min in methanol. After centrifugation of 10,000×g at 4℃ for 20 min, the supernatant was collected, freeze-dried and stored in -30℃. Media were assayed directly without further purification. The lyophilized methanol extracts were reconstituted using gelatin phosphate-buffered saline (GPBS).

Radioimmunoassay procedures were adapted from the method utilized in previous studies (Kwon et al., 1989; 1991). Labeled testosterone (1, 2, 6, 7-^{3}H-testosterone, 98 Ci/mmole) were purchased from Amersham. The T antiserum was produced and qualified by Dr. Y. D. Yoon (Hanyang University). The T antiserum cross-reacts 14% with 5α-dihydrotestosterone, 6% with 5α-androstenediol, 0.8% with androstenedione. Validation of testosterone RIA for frog gonads was described elsewhere (Kwon et al., 1991). Each sample was quantified for tritium using a liquid scintillation counter (Packard tri-carb 1500). Routinely, two sets of steroid standards (1 - 500 pg) were included in each assay. The lower limit of assay sensitivity was 5 pg/testis.

Statistical analysis of data was carried out by using analysis of variance (ANOVA) or student's t-test.

Results

Testicular cycle

To understand the annual changes of spermatogenic activity in the three species of Korean frogs, we analyzed the annual testicular cycle based on the spermatogenic pattern. The stages of spermatogenesis were classified into 5 stages as described in Table 1 and the results are summarized in Fig. 1.

In *R. dybowskii*, the primary and the secondary spermatogonia in the seminiferous tubule (immature stage, stage I) appeared in August and the early spermatogenesis (stage II) occurred in September and October. The late spermatogenesis (stage III) began to occur at October, and was most active in November. The spermiation (stage IV) began to occur in November and was very active until March. Spermatogenesis stopped completely in April and this immature stage continued until August when new round of spermatogenesis started.

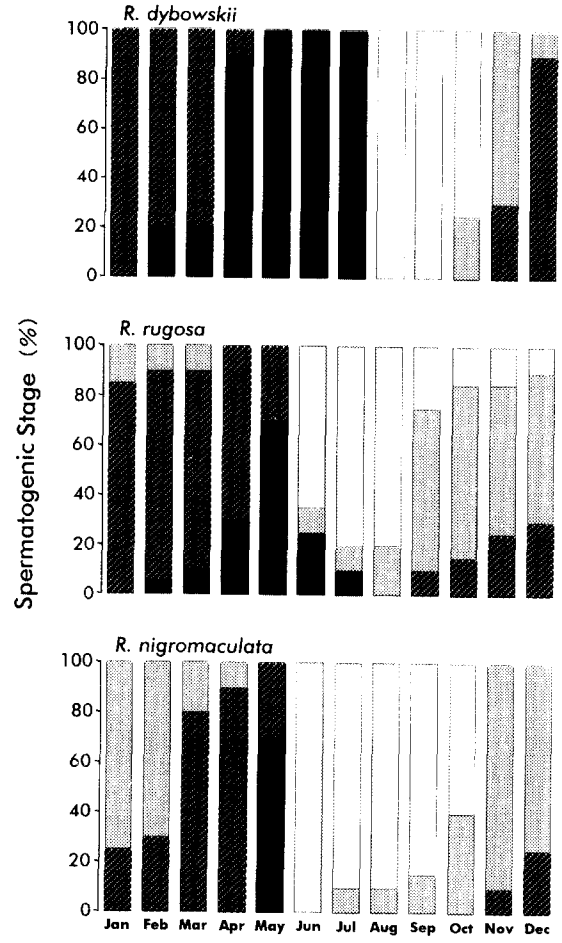

Fig. 1. Annual changes of spermatogenic stages in the three species of frogs. Three species of male frogs (*R. dybowskii*, *R. rugosa*, and *R. nigromaculata*) were collected through the year. Spermatogenesis in the seminiferous tubules was examined and then divided into five classes of spermatogenic stages according to Table I. Data were obtained from 5 observations per animal using at least three frogs for each month (at least, n=15) and expressed as percentage. □: Stage I, immature stage ; ▯: Stage II, early spermatogenic stage; ▯: Stage III, ▨: Stage IV, spermiation stage; ■: Stage V, post-spawning stage.

In *R. nigromaculata* and *R. rugosa*, the immature stage was not observed and the early spermatogenesis stages started just after post-spawning period (May) and continued until August or September. Late spermatogenesis occurred in September in *R. rugosa* and in November in *R. nigromaculata*. The spermiation started at early hibernation and continued until their breeding season. *R. nigromaculata* showed a short post-spawning period in May. In contrast, *R. rugosa* exhibited a long spawning period (February through July), indicating that *R. rugosa* had a relatively long breeding season.

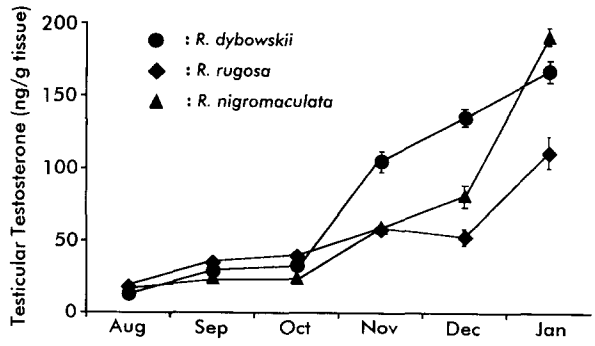

Fig. 2. Seasonal fluctuations of testicular testosterone levels in three species of Korean male frogs. Frogs were collected each month from August to January and the contents of testicular testosterone were measured by radioimmunoassay. Each point represents average ng testosterone per gram of testis tissue (mean ± SEM, 4 - 5 animal per month)

Taken together, the data suggest that *R. nigromaculata* and *R. rugosa* have a "potent continuous spermatogenesis pattern", in which sperm is always observed within the seminiferous tubules all year round, and *R. dybowskii* has a discontinuous type because spermatogenesis completely stops after spawning (March) until August.

Steroid secretion by testis obtained at different seasons in vitro.

To elucidate the control mechanism of testicular cycle in the Korean frogs, we examined the ability of testosterone production by the testis *in vitro* and their responsiveness to gonadotropins in terms of testosterone secretion. In all three species of frogs, testes were obtained from frogs collected in late summer through winter when spermatogenesis occurred very actively.

Initially, testicular testosterone was measured with frogs collected in each month (Fig. 2). The amount of testosterone was low until October and began to increase markedly from November (early hibernation), and consistently increased to high levels in January (Fig. 2). In general, testicular testosterone increased in parallel with the progress of later spermatogenesis and spermiation as depicted in Fig. 1.

Testosterone secretion by the testis slices *in vitro* in response to FPH was examined using frogs collected in August and November. As shown in Fig. 3, the levels of testosterone in medium increased markedly by 20 h of culture. Interestingly, testis slices obtained in August secreted relatively low levels of testosterone and did not respond to FPH stimulation. In contrast, testis slices obtained in November secreted higher levels of testosterone in response to FPH than control. Thus, it is evident that FPH stimulated the secretion of testosterone by testis slices obtained in November, but not obtained in August. A similar pattern of testosterone secretion

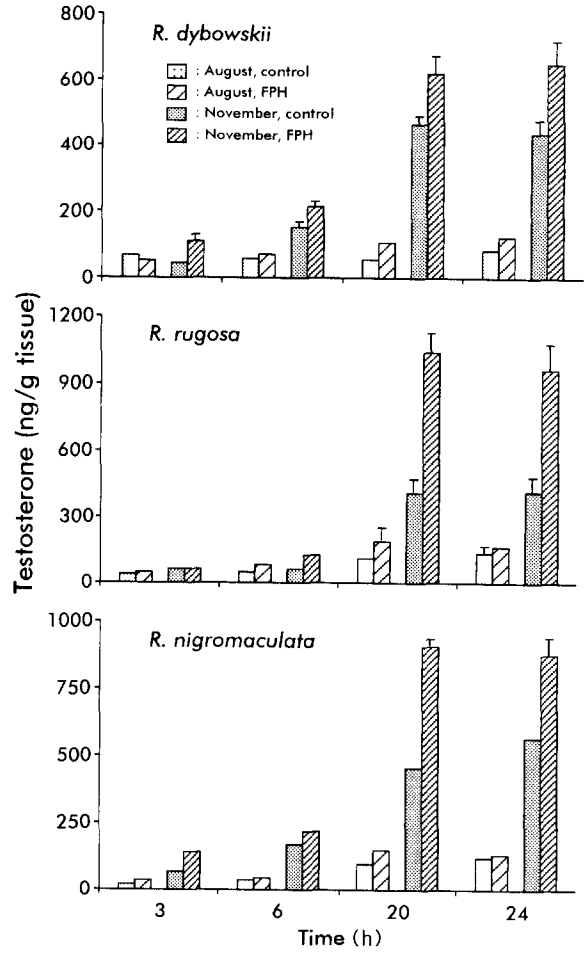

Fig. 3. Time course of testosterone secretion by testis following *in vitro* culture. The testis slices were obtained in August and December, and incubated in AR in the presence or absence of FPH (0.1 gland/ml). At designated time points, the culture media were collected and the levels of testosterone were assayed. Each point indicates the mean of three experiments.

was observed in the three species of frogs although the levels of testosterone secreted varied among species (Fig. 3).

On the basis of the above results, changes of responsiveness of testis to different doses of FPH were examined with the three species of frogs. Testes were obtained from frogs collected between August - December and cultured for 20 h in the presence of various doses of FPH. After culture, levels of testosterone in medium were measured. Testosterone levels secreted by the testis were very low until October in all the three species of frogs. However, testis slices obtained in November secreted higher levels of testosterone and those in December secreted highest levels of the steroid in response to various doses of FPH (Fig. 4). Interestingly, those testis slices obtained earlier than November did not secrete testosterone in response to FPH. However, testis obtained in December secreted much higher

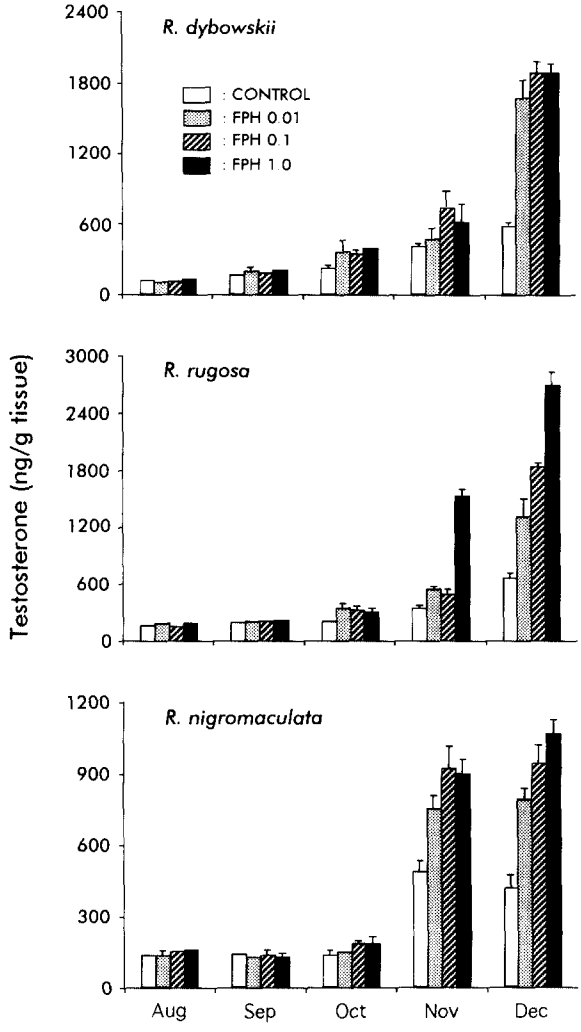

Fig. 4. Seasonal changes in the steroidogenic competence and responsiveness of testis to gonadotropin in the secretion of testosterone. Testis slices were obtained from three species frogs during the period through August to December. Testis slice were cultured for 20 h in AR in the presence of various doses of FPH (0.01-1 gland/ml). The levels of testosterone in media were measured by radioimmunoassay. Each bar in the figure represents average (mean + SEM) ng testosterone per g tissue (n=3).

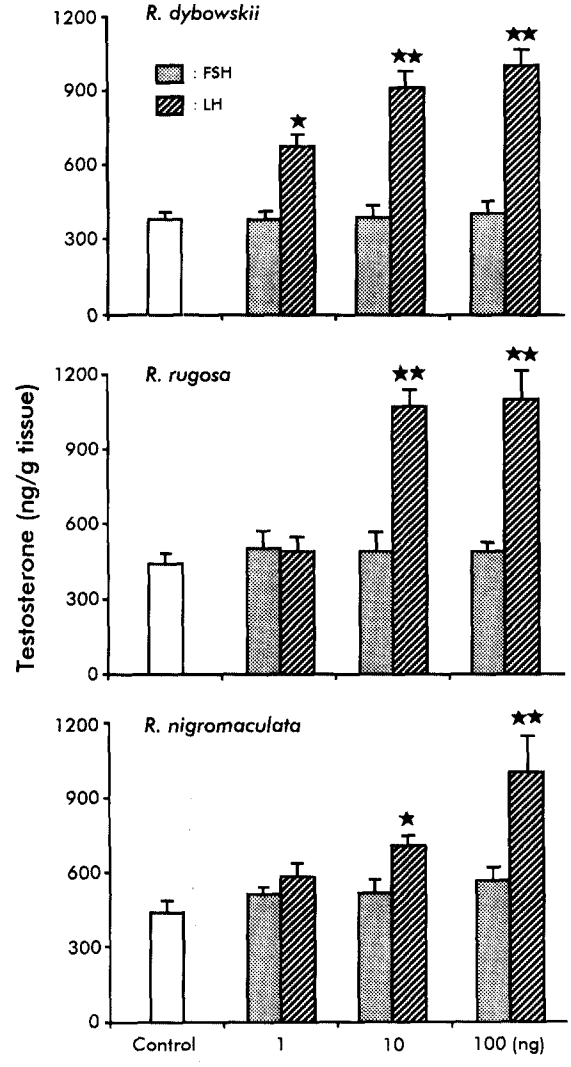

Fig. 5. Effects of bullfrog FSH and LH on the secretion of testosterone by testis slice culture *in vitro*. Testis slices were obtained from frogs collected in December and incubated for 20 h in AR in the presence of various doses of bullfrog FSH and LH (1-100 ng/ml). After culture, the levels of testosterone in medium are measured. Each bar represents average (mean + SEM) ng of testosterone per g tissue.
★: $P < 0.05$, ★★: $P < 0.01$.

levels of testosterone than control in response to FPH in a does-dependent manner (Fig. 4). Thus, the data indicate that the ability of testosterone production and secretion of testis varied seasonally and that the responsiveness of the testis to gonadotropin also changes seasonally. Particularly, it is notable that testis obtained in November or December markedly secreted testosterone in response to FPH when later spermatogenesis and spermiation occurred actively.

To assess the role of gonadotrpins (LH, FSH) in the secretion of testosterone, testis slices were cultured in the presence of a purified bullfrog LH or FSH, and the steroid levels in medium were measured (Fig. 5). In all three species of frogs, the bullfrog FSH failed to stimulate the secretion of testosterone, but the bullfrog LH significantly stimulated the secretion of testosterone in a does-dependent manner ($P < 0.05$ or $P < 0.01$) (Fig. 5). Thus it is evident that LH, but not FSH, stimulated production of testosterone by testes in the three frogs.

Discussion

Testicular cycles of Korean male frogs

In the present study, it was demonstrated that all the three Korean frogs (*R. dybowskii, R. rugosa* and *R. nigromaculata*) exhibited a typical testicular cycle of temperate zone frogs. Thus, it is evident that their spermatogenic patterns and breeding cycles are well

adapted to the environmental conditions of their habitat. In general, amphibian spermatogenesis begins from the primary spermatogonia and is followed by the formation of gonadal cysts containing the secondary spermatogonia. Thereafter, spermatogenesis in cysts is synchronized depending on environmental factors (van Tienhoven, 1983). In many amphibian species, annual spermatogenic cycles were found to be dependent on seasonal changes in environment such as temperature and photoperiod (Lofts, 1984; Whittier and Crew, 1987 ; Delgado et al., 1992)

In *R. dybowskii*, after spawning in February or early March, spermatogenesis stopped completely (post-spawning stage) for several months, then started again from late summer (August), indicating that the quiescent period of spermatogenesis existed after spawning (Fig. 1). In fact, no primary spermatogonia cells were observed in the seminiferous tubules during this period. This pattern has been defined as a discontinuous spermatogenic type as seen in a temperate frog, *R. temporaria* (Lofts, 1974). In contrast, *R. rugosa* and *R. nigromaculata* exhibited a continuous spermatogenic pattern although the amount of specific gonadal cells at certain periods varied between them. Thus, these two species of frogs did not exhibit a quiescent period of spermatogenesis through the year. This type of spermatogenesis has been reported in other temperate amphibians such as *R. esculenta* and *Bufo japonicus* (Rastogi et al., 1976; Moriguchi and Iwasawa, 1987).

Analysis of testicular cycle made it possible to estimate the exact breeding season of the three species of Korean frogs: May in *R. nigromaculata*, February in *R. dybowskii*, and May - July in *R. rugosa*. These breeding seasons well coincide with those estimated on the bases female reproduction (Kwon et al., 1989, 1991; Yoo et al., 1995). Therefore, testicular cycle is closely linked to the female ovarian cycle in amphibians.

Testicular cycle and testosterone

In three species of Korean frogs, levels of testicular testosterone and the ability of testis to secrete testosterone *in vitro* were found to increase markedly in November (Figs. 2 and 3). Moreover, responsiveness of testis *in vitro* to gonadotropin increased markedly at this season (Fig. 4). These data suggest that the initial stages of spermatogenesis occurs independent of testicular testosterone, and that the later spermatogenesis and spermiation are controlled by the steroid.

Similarly, in temperate amphibians, such as *R. esculenta* and *R. perezi*, it has been shown that the maximal levels of testicular testosterone appeared during hibernation and very low levels appeared during active period. These seasonal changes of testicular testosterone seem to be under the controls of pituitary gonadotropin and environmental factors such as temperature or photoperiod (for review, see Paniagua, 1990). Although the seasonal fluctuation of testicular testosterone was known to reflect the number of interstitial cells, the fluctuation did not reflect directly the spermatogenic activity (Pierantoni et al., 1984a,b; Delgado et al., 1989; Itoh et al., 1990).

Low temperatures and short light periods were found to increase the plasma testosterone level of frogs collected at early autumn or winter but not of frogs collected at summer (Iela et al., 1980). Therefore, both temperature and light seem to influence on the testicular activity. This idea is in accord with our data showing that testicular testosterone increased in November in Korean frogs.

The fact that gonadotropin stimulates the androgen production by testis in frog was originally described by Muller (1977). Thereafter, several investigators have shown that the stimulation of gonadotropin is not prerequisite for testicular functions. Several investigators have examined the relationship between annual patterns of gonadotropin secretion and plasma androgen level in amphibians (Licht et al., 1883; Itoh et al., 1990). But they failed to find out a constant positive correlation between the gonadotropins and gonadal activities (plasma testosterone level). Thus it is likely that some other factors are involved in the regulation of testicular androgen secretion in amphibians.

Since Lofts (1961) has suggested the presence of both FSH and LH in amphibian gonadotropins, this two type of gonadotropins were purified in *R. catesbeiana* (Licht and Papkoff, 1976; Takahashi and Hanaoka, 1981), *Bufo japonicus* (Takada and Ishii, 1986), and *R. Pipiens* (Farmer et al., 1977). Bullfrog LH was shown to have the potency to stimulate androgen secretion in the frog testis and bullfrog FSH to stimulate spermatogenesis in *R. pipiens* (Licht and Papkoff, 1976). In Ambystoma, it was shown that FSH was responsible for spermatogenesis and LH for the secretion of androgen (Norris et al., 1985). This is in accord with our data indicating that bullfrog LH but not FSH stimulated the secretion of testicular testosterone *in vitro* (Fig. 5). Interestingly, testis responded to gonadotropin only from November. Possibly, the increased responsiveness of testis to LH is closely linked to the appearance or increase of LH receptors in testis. Further studies are needed to address this possibility.

In summary, testicular cycles of three Korean frogs were analyzed in detail for the first time in this study. The data presented here gave us the information about the progress of spermatogenesis through the year in Korean frogs. In addition, it was found that testicular testosterone were high in hibernation period and responsiveness of testis to gonadotropin exhibited seasonal variation, and testi-

cular cycles were closely linked to female ovarian cycles.

Acknowledgements

This work was supported in part by the Ministry of Education (BSRI 95-4425) and by Korea Science and Engineering Foundation through Hormone Research Center (HRC-96-0101)

References

Delgado MJ, Alonso-Go'mez AL, and Alonso-Bedate M (1992) Role of environmental temperature and photoperiod in regulation of seasonal testicular activity in the frog, *Rana perezi*. *Can J Physiol Pharmacol* 70: 1348-1352.

Delgado MJ, Gutierrez P, and Alonso-Bedate M (1989) Seasonal cycles in testicular activity in the frog, *Rana perezi*. *Gen Comp Endocrinol* 73: 1-11.

Farmer SW, Licht P, Papkoff H, and Daniels E (1977) Purification of gonadotropins in the Leopard frog (*Rana pipiens*). *Gen Comp Endocrinol* 32: 158-162.

Fasano S, Minucci S, Pierantoni R, Fasolo A, Matteo LD, Basile C, Varriali B, and Chieffi G (1988) Hypothalamo-hypophysis and testicular GnRH control of gonadal activity in the frog, *Rana esculenta*: Seasonal GnRH profiles and annual variations *in vitro* androgen output by pituitary stimulated testes. *Gen Comp Endocrinol* 70: 31-34.

Fasano S, Goos HJ, Jassen C, and Pieratoni R (1993) Two GnRH fluctuate in correlation with androgen levels in the male frog *Rana esculenta*. *J Exp Zool* 266: 277-283.

Hardy MP, Sprando RL, and Ewing LL (1992) Leydig cell renewal in testes of seasonally breeding animals. *J Exp Zool* 261: 161-171.

Iela L, Pierantoni R, and Rastogi RK (1980) Effect of temperature and light on the production of androgens in the male *Rana esculenta*. *Experientia* 36: 256.

Itoh M, Inoue M, and Ishii S (1990) Annual cycle of pituitary and plasma gonadotropins and plasma sex steroids in wild population of the toad, *Bufo japonicus*. *Gen Comp Endocrinol* 78: 242-253.

Kobayashi T, Oshimi A, and Iwasawa H (1989) Development of an *in vitro* spermiation system in the frog, *Rana nigromaculata*. *Zool Sci* 6: 1027-1032.

Kwon HB, Choi HH, Ahn RS, and Yoon YD (1991) Steroid production by amphibian (*Rana nigromaculata*) ovarian follicles at different developmental stages. *J Exp Zool* 260: 66-73.

Kwon HB, Lim YK, Choi MJ, and Ahn RS (1989) Spontaneous maturation of follicular oocytes in *Rana dybowskii in vitro*: Seasonal influences, progesterone production, and involvement of cAMP. *J Exp Zool* 252: 109-199.

Kwon BH and Schuetz AW (1985) Role of cAMP in modulating intrafollicular progesterone levels and oocyte maturation in amphibians (*Rana pipiens*). *Dev Biol* 117: 354-364.

Licht P, McCreery BR, and Pang R (1983) Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. *Gen Comp Endocrinol* 94: 1587-1594.

Licht P, and Papkoff H (1976) Separation of two distinct gonadotropins from the pituitary gland of the bullfrog, *Rana catesbeiana*. *Endocrinology* 94: 1587-1594.

Lofts B (1961) The effects of follicle-stimulating hormone and luteinizing hormone on the testis of hypophysectomized frogs (*Rana temporaria*). *Gen Comp Endocrinol* 1: 179-189.

Lofts B (1974) Physiology of the Amphibia, Academic Press, New York, Vol 2, pp 107-247.

Lofts B (1984) Amphibia. In: Laming GE (ed), Marshal's Physiology of Reproduction. Reproductive Cycles in Vertebrates. Churchill-Living Press, Edinburgh, Vol 1, pp 127-205.

Matteo LD, Minucci S, Fasano S, Pierantoni R, Varriale B, and Chieffi G (1988) A gonadotropin-releasing hormone (GnRH) antagonist decreases androgen production and spermatogonial multiplication in frog (*Rana esculenta*): Indirect evidence for the existence of GnRH or GnRH-like material receptors in the hypophysis and testis. *Endocrinology* 122: 62-67.

McCreery BR, Licht P, Barnes R, Rivier JE, and Vale WW (1982) Actions of agonistic analogs of gonadotropin releasing hormone (GnRH) in the bullfrog *Rana catesbeiana*. *Gen Comp Endocrinol* 46: 511-520.

Moriguchi Y and Iwasawa H (1987) Annual changes in male reproductive organs in *Bufo japonicus formosus*: Histological observation. *Gen Comp Endocrinol* 6: 115-120.

Muller CH (1977) *In vitro* stimulation of 5α-dihydrotestosterone and testosterone secretion from bullfrog testis by nonmammalian and mammalian gonadotropins. *Gen Comp Endocrinol* 33: 109-121.

Norris DO, Norman MF, Pancak MK, and Duvall D (1985) Seasonal variation in spermatogenesis, testicular weight, vasa deferentia, and androgen levels in neotenic male tiger salamanders, *Ambystoma tigrium*. *Gen Comp Endocrinol* 60: 51-57.

Paniagua R, Frail B, and Saez FJ (1990) Effects of photoperiod and temperature on testicular function in amphibians. *Histol Histophotol* 5: 365-378.

Pierantoni RL, Iela M, D'Istra M, Fasano S, Rastogi RK, and Delrio G (1984a) Seasonal testosterone profile and testicular responsiveness to pituitary factors and gonadotropin releasing hormone during two different phases of the sexual cycle of the frog (*Rana esculenta*). *J Endocrinol* 102: 387-392.

Pierantoni RL, Fasano S, Matteo LD, Minucci S, Varriela B, and Chieffi G (1984b) Stimulatory effect of a GnRH agonist (Buserelin) in *in vitro* and *in vivo* testosterone production by the frog (*Rana esculenta*) testis. *Mol Cell Endocrinol* 38: 215-223.

Rastogi RK (1976) Seasonal cycle in anuran (amphibia) testis: The endocrine and environmental controls. *Bull Zool Agrar Bachic* 43: 151-172.

Rastogi RK, Iela L, Di Meglio M, Di Matteo L, Minucci S, and Izzo-Vitiello I (1983) Initiation and kinetics profiles of spermatogenesis in the frog, *Rana esculenta* (Amphibia). *J Zool London* 201: 515-525.

Tanaka K and Ishii S (1986) Isolation of gonadotropin of the toad, *Bufo japonicus*. *Zool Sci* 3: 971-990.

Takahashi H and Hanaoka Y (1981) Isolation and characterization of multiple components of basic gonadotropin from bullfrog (*Rana catesbeiana*). pituitary gland. *J Biochem* 90: 1333-1340.

Van Tienhoven A (1983) Reproductive Physiology of Vertebrates. 2nd ed. Cornell University Press, New York. pp 137-169.

Whittier JM and Crews D (1987) Seasonal reproduction patterns and control. In: Norris DO and Jones RE (eds), Hormones and Reproduction in Fishes, Amphibians and Reptiles, Plenum Press, New York, pp 385-409.

Yoo MS, Ra CH, Kim JY, Kang SG, and Kwon HB (1995) Reproductive cycle and maturation induction of oocytes in Rana rugosa. *Korean J Zool* 38: 96-105.

[Received December 16, 1996; accepted February 6, 1997]