Journal of Reproduction and Development, Vol. 56, No. 3, 2010

—Original Article—

Seasonal Changes in Spermatogenesis and Immunolocalization of Cytochrome P450 17α-Hydroxylase/c17-20 Lyase and Cytochrome P450 Aromatase in the Wild Male Ground Squirrel (*Citellus dauricus Brandt*)

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Abstract. The purpose of this study was to investigate seasonal changes of spermatogenesis and the cellular localization of P450c17 and P450arom in wild male ground squirrels during the breeding and non-breeding seasons. The testicular weight, testicular size and score count of spermatogenesis from April to September were measured, and histological and immunohistochemical observations of testicular tissues were performed in wild male ground squirrels. In addition, total proteins were extracted from testicular tissue in the breeding and non-breeding seasons and were used for Western blotting analysis for P450c17 and P450arom. There were marked variations in testicular weight, testicular size and score count of spermatogenesis from the breeding season (April) to the non-breeding season (September). Histologically, spermatogonia, primary spermatocytes, secondary spermatocytes and spermatozoa were identified in the breeding season (April). Immunolocalization of P450c17 was detected in Leydig cells and spermatozoa during the breeding season and was only found in Leydig cells during the non-breeding season. The positive signals of P450c17 by Western blotting were both observed in the breeding and non-breeding seasons. Immunolocalization of P450arom was observed in Leydig cells, Sertoli cells and all types of spermatogenic cells including mature-phase spermatozoa in the breeding season, while immunoreactivity for P450arom was not present in the testis of the non-breeding season. With P450arom antibody, a band was also only detected in the breeding season by Western blotting. These results suggest that the seasonal changes in testicular weight and size are correlated with spermatogenesis and immunolocalization of P450c17 and P450arom, and androgen and estrogen may play an important role in the spermatogenesis and testicular recrudescence and regression process.

Key words: Ground squirrel, Immunolocalization, P450c17, P450arom, Spermatogenesis

(**J. Reprod. Dev. 56:** 297–302, 2010)

The reproductive strategies of seasonal breeders are adaptations to annual changes in the environment, and they minimize the animals' energetic efforts for reproduction. Mature males show synchronized cycles of testicular growth and involution between the breeding and non-breeding periods [1]. Seasonal changes of testicular steroidogenesis and spermatogenesis in wild animals have been reported in many species, including American black bears [2], Japanese raccoon dogs [3], Japanese black bears [4], Northern fur seals [5] and polar bears [6]. In these wild animals, immunohistochemical studies on testicular tissues have demonstrated that the distributions of immunoreactivities for steroidogenic enzymes in testicular tissue are associated with seasons and species.

It is well known that normal testicular development and maintenance of spermatogenesis are controlled by gonadotropins and testosterone, whose effects are modulated by locally-produced factors, and among them, with estrogen being the main factor involved [7]. Testosterone and estrogen biosynthesis is catalyzed by a member of the cytochrome P450 superfamily, namely cytochrome P450 17α-hydroxylase/c17–20 lyase (P450c17, the product of *CYP17A1* gene) and aromatase cytochrome P450 (P450arom, the product of *CYP19* gene), respectively [8]. Immunolocalization of P450c17 and P450arom in the testes has been reported in numerous species, including American black bears [2], raccoon dogs [3], Shiba goats [9], brown bears [10], black bears [4, 11], Göttingen miniature pigs [12], rams [13], bank voles [14], rats [15, 16], mice [17] and ground squirrels [18]. Although the immunolocalization of P450c17 and P450arom has been detected in some wild animals and experimental rodents, there are few reports on wild rodents.

The wild male ground squirrel (*Citellus dauricus Brandt*) is a typical seasonal breeder and has a breeding season from April to May that is followed by a long period of sexual dormancy from June to March [18]. Studies in ground squirrels have shown that ground squirrels actively forage, breed and play for about six months in late spring, summer and early fall and hibernate during

Received: May 8, 2009
Accepted: January 12, 2010
Published online in J-STAGE: March 3, 2010

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the remainder of the year [18]. The breeding season of the wild ground squirrel begins soon after emergence from hibernation [19]. Although several observations about reproduction in ground squirrels have been reported recently [18–20], such as seasonal changes in spermatogenesis and immunolocalization of inhibin/activin subunits in wild male ground squirrels [21], there are still many limitations in the understanding of the mechanisms of reproduction, especially the role of steroidogenic enzymes in the spermatogenesis and testicular recrudescence and regression process. The aim of the present study was to investigate the seasonal changes of spermatogenesis and immunoreactivity of P450c17 and P450arom in testicular tissues during the breeding and non-breeding seasons and to elucidate the relationship between steroidogenic enzymes (P450c17 and P450arom) and reproductive function in wild male ground squirrels.

Materials and Methods

Animals

Forty-six wild male ground squirrels that were thought to be adult based on their body weights (242–412 g) were captured from April to September of 2008 in Hebei Province, PR China. The testes were excised from each body, and testicular weight was measured using scales; testicular size was expressed in mm based on measurement (length × width × height)$^{1/3}$ after necropsy. Each obtained testis was cut into 2 portions; one portion was fixed in 4% paraformaldehyde (Sigma Chemical, St. Louis, MO, USA) in 0.05 M PBS (pH 7.4) for histological and immunohistochemical observations, and the other portion was immediately stored at –20 C until it was used for Western blotting detection.

Histology

Testicular samples were dehydrated in ethanol series and embedded in paraffin wax. Serial sections (4 μm) were mounted on slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA). Some sections were stained with hematoxylin-eosin (HE) for observations of general histology.

Evaluation of spermatogenesis

The score count for determining the stage of spermatogenesis [22] was modified and used to evaluate spermatogenesis. The modified criteria of the score count, principally based on the most advanced spermatogenetic cells, were as follows: Score 1, spermatogonia only; Score 2, no cells further than primary spermatocytes; Score 3, no cells further than secondary spermatocytes; Score 4, no cells further than round spermatids; Score 5, no cells further than mature-phase spermatozoa. Counting was usually performed while the whole sample was examined under a light microscope using a low (×100) and then high (×400) magnification.

Immunohistochemistry

Fourteen pairs of testes obtained from wild ground squirrels in April (n=4), May (n=3), August (n=3) and September (n=4) were used for immunohistochemistry. The serial sections of testes were incubated with 10% normal goat serum to reduce background staining caused by the secondary antibody. The sections were then incubated with primary antibody (1:2000) raised against porcine testicular P450c17 [23] and human placental cytochrome P450 aromatase (P450arom) [24] for 12 h at room temperature. Thereafter, the sections were incubated with the secondary antibody, goat anti-rabbit lgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin^{TM} staining kit (Sigma) and then visualized with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 ml of 0.05 mol Tris-HCl buffer (pH 7.6) plus 30 μl H_2O_2. Finally, the reacted sections were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The control sections were treated with normal rabbit serum (Sigma) instead of the primary antiserum.

Western blotting

Testicular tissue was weighed and diced into small pieces using a clean razor blade. The tissue was homogenized in a homogenizer containing 300 μl of 10 mg/ml PMSF stock and incubated on ice for 30 min while maintaining the temperature at 4 C throughout all the procedures. Homogenates were centrifuged at 12,000 × *g* for 10 min at 4 C. Protein extracts (25 μg) were mixed with an equal volume of 2 × Laemmli sample buffer. Equal amounts of each sample were loaded and run on a 12% SDS-PAGE gel at 18 V/cm and transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The membranes were blocked in 3% BSA for 1 h at room temperature. Primary incubation of the membranes was carried out using a 1:1000 dilution of rabbit anti-P450c17 antibody (1:1000 dilution) or rabbit anti-P450arom antibody (1:1000 dilution) for 60 min. Secondary incubation of the membrane was then carried out using a 1:1000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase for 60 min. Finally, the membrane was colored with 25 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 25 ml TBS-T buffer (0.02 M Tris, 0.137 M NaCl and 0.1% Tween-20, pH 7.6) plus 3 μl H_2O_2.

Statistical analysis

Mean values (± SD) were calculated and analyzed using 1-way ANOVA. Duncan's multiple comparison test was used for detection of significant differences using the SPSS computer package.

Results

Testicular weight and size

The seasonal changes in testicular weight and size in the wild male ground squirrels are shown in Fig. 1a and b, respectively. The largest values of testicular weight and size were found during the breeding season in April and May, respectively. The smallest values of testicular weight and size were found during the non-breeding season in July and August, respectively. There were significant seasonal changes in testicular weight and size between the breeding and non-breeding seasons ($P<0.05$).

Histology and score count of spermatogenesis

A number of seminiferous tubules and interstitial connective tissue were observed in the testes of the wild male ground squirrels examined. The most prominent cells in the interstitium were Ley-

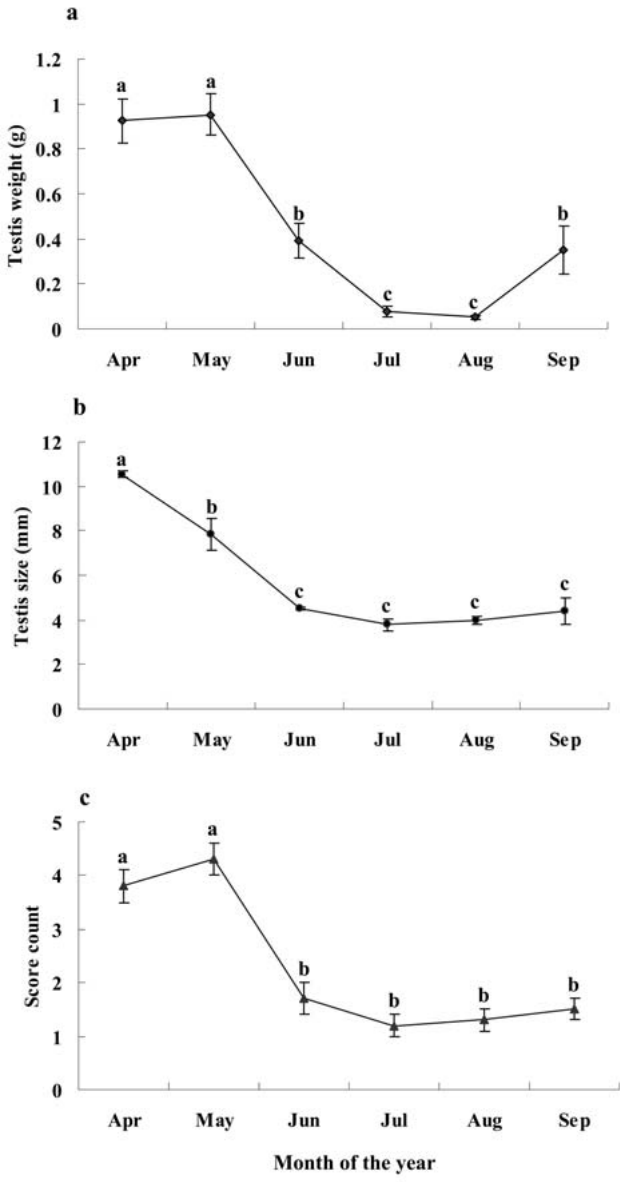


Fig. 1. Seasonal changes in testicular weight (a), testicular size (b) and score count of spermatogenesis (c) in wild male ground squirrels. Different letters denote statistically significant values ($P<0.05$).

dig cells. The spermatogenic cell types changed between the breeding and non-breeding season; in the breeding season, spermatogonia, primary spermatocytes, secondary spermatocytes and spermatozoa were identified based on the histological appearance of the seminiferous epithelium (Fig. 2a). In the non-breeding season, only spermatogonia and primary spermatocytes were observed (Fig. 2b). Marked seasonal changes of score count of spermatogenesis from April to September attest to the changes of histological appearance of seminiferous epithelium during this period (Fig. 1c).

Immunohistochemistry

Immunohistochemistry for P450c17 and P450arom was performed in the testes during the breeding and non-breeding seasons, respectively (Table. 1 and Fig. 3). Marked seasonal changes in the immunolocalization of P450c17 and P450arom were observed in the breeding season. P450c17 was immunolocalized intensely in Leydig cells and spermatozoa during the breeding season (Fig. 3a) and weakly in Leydig cells during the non-breeding season (Fig. 3b). The most extensive immunostaining of P450arom was present in Leydig cells, Sertoli cells, spermatogonia, all kinds of spermatocytes and spermatozoa during the breeding season (Fig. 3c). No immunohistochemical reaction for P450arom was observed in testicular tissues during the non-breeding season (Fig. 3d). No immunostaining was detected in control sections when normal rabbit serum was substituted for the primary antibody (Fig. 3e).

Western blotting

The results of Western blotting analysis for P450c17 and P450arom in the testes of the breeding and non-breeding seasons are shown in Fig. 4a and b, respectively. A positive signal of P450c17 was detected in protein extracted from testes in April (Fig. 4a, lane 1) and September (Fig. 4a, lane 2). Water was used for the negative control (Fig. 4a, lane 3). With the P450c17 antibody, the major bands detected in testis migrated to a position at about 57 kDa. A positive signal of P450arom was detected in protein extracted from testes in April (Fig. 4b, lane 1). Meanwhile, the protein extracted from a whole ovary in the breeding season was used as a positive control (Fig. 4b, lane 3). With the P450arom antibody, the major bands detected in the testes and ovary migrated to a position at about 55 kDa.

Discussion

The present study demonstrated that a seasonal change in testicular weight and size occurred in the wild ground squirrels, and higher values of testicular weight and size were found in the breeding season. Seasonal changes of spermatogenesis and immunolocalization of P450c17 and P450arom in the wild male ground squirrels were also observed in this study. Specific bands of about 57 kDa for P450c17 and 55 kDa for P450arom were detected in the testis of the breeding season, while only the band for P450c17 was found in the non-breeding season by Western blotting analysis. These findings suggest that seasonal alterations including those in testicular weight and size in wild male ground squirrels are closely associated with spermatogenesis and immunoreactivity of P450c17 and P450arom.

In the present study, the testicular weights and sizes of the wild ground squirrels decreased significantly from the breeding season to the non-breeding season. In the histological appearance of the seminiferous epithelium, spermatocytes and plenty of spermatic cells were observed in the seminiferous tubules of testes in the breeding season, while no spermatocytes and spermatic cells were found in the seminiferous tubules of testes in the non-breeding season. The seasonal change in score count of spermatogenesis was positively correlated with the changes of histological appearance of the seminiferous epithelium. These findings are similar to those

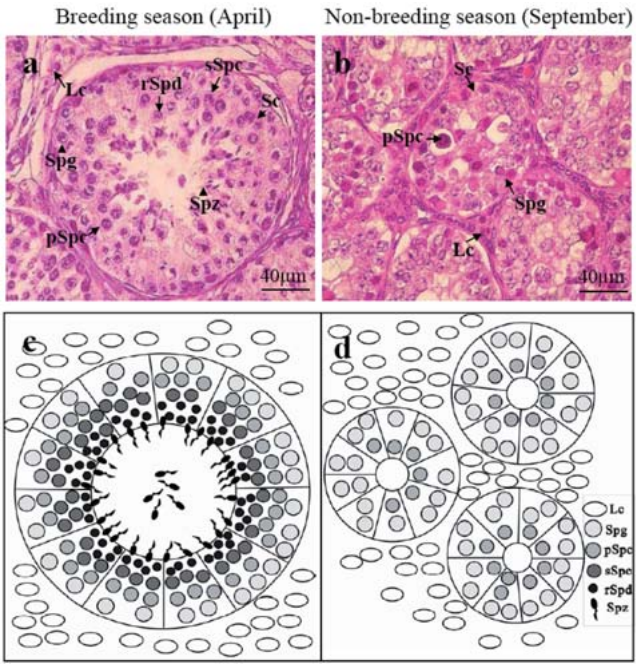


Fig. 2

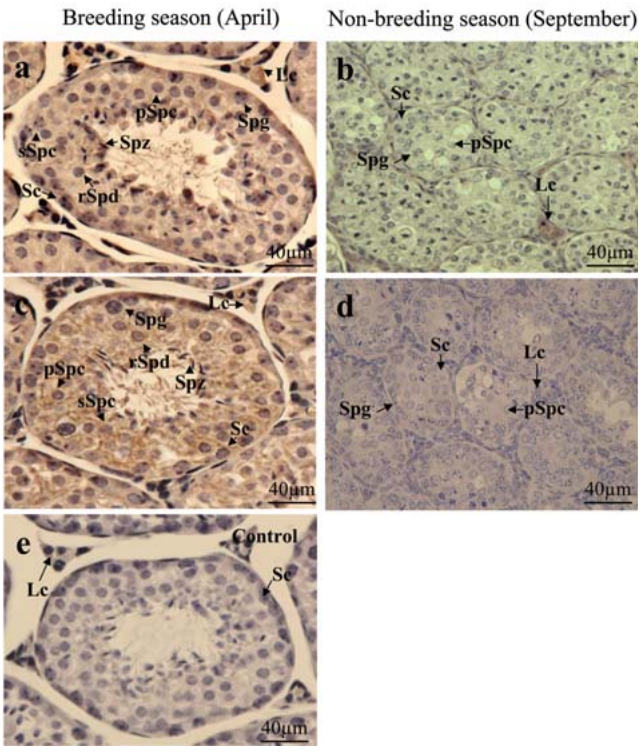
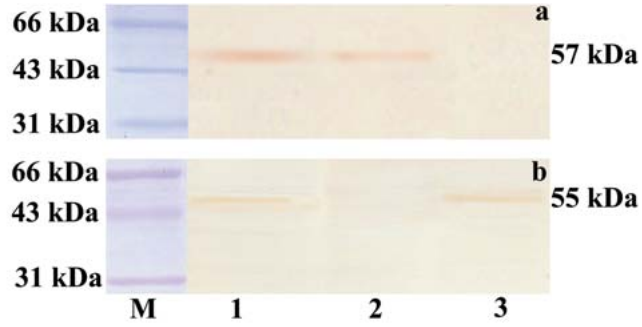


Fig. 3



	Lane M	Lane 1	Lane 2	Lane 3
a	Marker	April 15th Testicular extract	September 15th Testicular extract	Water
b	Marker	April 15th Testicular extract	September 15th Testicular extract	April 15th Ovarian extract

Fig. 4.

observed in other mammals, such as American black bears [2], raccoon dogs [3], Japanese black bears [4], roe deer [25] and horses [26, 27]. In the American black bear, testicular size changes seasonally, and the largest size is observed in the breeding season, with the entire spermatogenic cell population from spermatogonia to spermatozoa present in seminiferous tubules [2]. In wild raccoon dogs, the seasonal changes in the mean score count of spermatogenesis reach the maximum in the breeding season [3]. In our previous study, the diameters of the seminiferous tubules of wild ground squirrels were significantly higher in the breeding season than in the non-breeding season [21]. Together with all the above results, it appears that the cyclical alteration in the growth and involution of the testes may be universal in wild mammals.

P450c17 is the key enzyme that translates progestins to andro-

Fig. 2. Histology of the seminiferous epithelium in the wild male ground squirrels in the breeding season (a; April) and non-breeding season (b; September), and the scheme of seasonal changes in the testis of the wild male ground squirrel (c, d). Seminiferous tubules contain germ cells ranging from spermatogonia (Spg) to spermatids (Spd) in April (a). Seminiferous tubules have a small diameter and only contain spermatogonia (Spg) and primary spermatocytes (pSpc) in September (b). Lc, Leydig cell; Sc, Sertoli cell; Spg, spermatogonia; Spc, spermatocytes; pSpc, primary spermatocytes; sSpc, secondary spermatocytes; rSpd, round spermatids; Spz, spermatozoon. Bar: 40 μm.

Fig. 3. Immunolocalization of P450 17α-hydroxylase cytochrome/c17–20 lyase (P450c17) and cytochrome P450 aromatase (P450arom) in the testes of wild male ground squirrels in the breeding season (a, c; April) and non-breeding season (b, d; September). Immunostaining for P450c17 was found in Leydig cells and spermatozoa in the breeding season (a) and was only found in Leydig cells in the non-breeding season (b). Immunoreactivity for P450arom is distributed in Leydig cells, Sertoli cells, spermatogonia, all kinds of spermatocytes and spermatozoa in the breeding season (c), and no immunostaining of P450arom was observed in the non-breeding season (d). No immunostaining was detected in control sections in which normal rabbit serum was substituted for the primary antibody (e). Lc, Leydig cell; Sc, Sertoli cell; Spg, spermatogonia; pSpc, primary spermatocytes; sSpc, secondary spermatocytes; rSpd, round spermatids; Spz, spermatozoon. Bar: 40 μm.

Fig. 4. Western blotting of cytochrome P450 17α-hydroxylase/c17–20 lyase and cytochrome P450 aromatase in the testes of wild male ground squirrels, respectively (a, b). The left side shows the 12% range prestained SDS-PAGE standards (lane M). Total protein was extracted from the whole testis of the breeding season (lane 1) and the whole testis of the non-breeding season (lane 2). Water was used for the negative control in the Western blotting of P450c17 (a, lane 3). The extractive from an ovary of a wild female ground squirrel was used as the positive control in the Western blotting of P450arom (b, lane 3).

Table 1. Immunolocalization of P450c17 and P450arom in the testes of wild ground squirrels based on the breeding and non-breeding seasons

	Date of sampling	Numbers of wild ground squirrels	P450c17	P450arom
Breeding season	April 15th 2008	4	Lc, Spd	Lc, Sc, Spg, pSpc, sSpc, Spd
	May 1st 2008	3	Lc, Spd	Lc, Sc, Spg, pSpc, sSpc, Spd
Non-breeding season	August 24th 2008	3	Lc	Positive staining was not observed
	September 15th 2008	4	Lc	Positive staining was not observed

Lc, Leydig cell; Sc, Sertoli cell; Spg, spermatogonia; pSpc, primary spermatocytes; sSpc, secondary spermatocytes; Spz, spermazoa.

gens. In this study, P450c17 positive staining was observed in Leydig cells and spermatozoa of the wild male ground squirrels during the breeding season and was only found weakly in Leydig cells during the non-breeding season. According to the results of P450c17 immunoreactivity, the ability to synthesize androgen of wild male ground squirrels testes, which is manifested not only in the breeding season but also in the non-breeding season, rises to the maximum in the breeding season and then decreases during the non-breeding season. Furthermore, the presence of P450c17 in spermatozoa in the breeding season shows that the spermatozoa are another important source of androgens in the breeding season. Previous studies have shown that androgens produced by Leydig cells and spermatozoa play an important role in regulating and maintaining spermatogenesis, such as the conversion of round spermatids to elongated ones and preventing cell apoptosis in androgen-dependent tissue [28, 29]. In a previous study of roe deer testes, immunoreactivity of P450c17, which has a positive correlation with seasonal changes of the expression of INSL3 (Leydig cell-derived insulin-like factor) in Leydig cells, was present in Leydig cells in the breeding season but was not detected in the non-breeding season, and the results of that study suggested that P450c17 might cooperate with INSL3 to affect spermatogonial division and the dedifferentiation of Leydig cells [30]. Our previous study showed that the inhibin α and inhibin/activin βB subunits were expressed in Leydig cells and Sertoli cells in the breeding season, suggested that these growth factors might cooperate with steroid hormones and play an autocrine or paracrine role in the differentiation of spermatogenic cells [21]. Combined with the distribution and role of inhibin/activin subunits and P450c17 in the testes of wild ground squirrels, we suggest that P450c17 might interact with inhibin/activin subunits to regulate the process of spermatogenesis and increase the weight and size of testes during the breeding season in wild male ground squirrels.

The role of estrogen in spermatogenesis has been studied in recent years [31–34]. Although the concentration of estrogen was not measured in this study, the estrogen-synthesis enzyme (P450arom) was detected in the testicular tissues. Immunohistochemistry and Western blotting of P450arom in testes were first observed in the wild male ground squirrels. The most extensive immunostaining of P450arom was present in Leydig cells, Sertoli cells, spermatogonia, all kinds of spermatocytes and spermatozoa during the breeding season. These findings showed that P450arom produced by Leydig cells, Sertoli cells and germ cells had a positive correlation with fully developed spermatogenesis, and these cells containing P450arom may be the main sources of the estrogen that may play a role in positively regulating the process of spermatogenesis in wild male ground squirrels. A previous study suggested that Leydig cells may produce estrogen in an autocrine or paracrine fashion in the testis to control self-development [35]. Moreover, germ cell development relies on a highly coordinated interaction with Sertoli cells. Germ cells and Sertoli cells can communicate directly via ligand/receptor-mediated interactions or paracrine factors [31]. Estrogen has a stimulatory effect on spermatogenesis in *hpg* mice [32] and prevents germ cell apoptosis [33, 34]. Therefore, taking the numerical and morphological changes of germ cells in the process of spermatogenesis from the breeding season to the non-breeding season into account, our results suggested that estrogen produced by Leydig cells, Sertoli cells and germ cells may affect the production and apoptosis of germ cells during the spermatogenesis process, and the presence of P450arom in Sertoli cells and germ cells accompanied with testicular recrudescence also supports the view that P450arom and estrogen may play a role in re-initiating spermatogenesis [2]. In short, the present results suggested that although Sertoli cells do not increase in number in the breeding season, their capacities for protein secretion (P450arom, androgen binding protein and inhibin) and for support of increased numbers of germ cells as in spermatogenesis become more efficient and result in the variations of weight and size of the testes in terms of morphology. In addition, the fact that no immunoreactivity of P450arom was detected in the testes of the non-breeding season by immunohistochemistry and Western blotting analysis is in agreement with the end of the process of spermatogenesis, which implied that the ability to synthesize estrogen might be very weak in the non-breeding season. Accordingly, the present results suggest that estrogen might not be necessary for normal testis function in the non-breeding season in wild ground squirrels, which is after the process of spermatogenesis.

In summary, the seasonal changes in testicular weight and size in wild male ground squirrels are correlated with spermatogenesis and immunoreactivity of P450c17 and P450arom; androgens and estrogens may play an important role in the spermatogenesis and testicular recrudescence and regression process.

Acknowledgments

This study is supported by a Grant-in-Aid from The Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry of China and

Program for Changjiang Scholars and Innovative Research Team in Universities (IRT0607) from China and a Grant-in-Aid from the National Natural Science Foundation of China (NSFC; No. 30670261).

References

1. **Bronson FH, Heidemann PD**. Seasonal regulation of reproduction in mammals. *In*: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1994: 541–583.
2. **Tsubota T, Howell-Skalla L, Nitta H, Osawa Y, Mason JI, Meiers PG, Nelson RA, Bahr JM**. Seasonal changes in spermatogenesis and testicular steroidogenesis in the male black bear (*Ursus americanus*). *J Reprod Fertil* 1997; 109: 21–27.
3. **Weng Q, Murase T, Tsubota T**. Seasonal changes in spermatogenesis and testicular steroidogenesis in wild male raccoon dogs. *J Vet Med Sci* 2003; 65: 1087–1092.
4. **Komatsu T, Tsubota T, Yamamoto Y, Atoji Y, Suzuki Y**. Seasonal changes in the immunolocalization of steroidogenic enzymes in the testis of the Japanese black bear (*Ursus thibetanus japonicus*). *J Vet Med Sci* 1997; 59: 521–529.
5. **Tsubota T, Nagashima T, Kohyama K, Murase T, Kita I**. Seasonal changes in testicular steroidogenesis and spermatogenesis in a Northern fur seal, *Callorhinus ursinus*. *J Reprod Dev* 2001; 47: 415–420.
6. **Howell-Skalla, Cattet MRL, Ramsay MA, Bahr JM**. Seasonal changes in testicular size and serum LH, prolactin and testosterone concentrations in male polar bears (*Ursus maritimus*). *Reproduction* 2002; 123: 729–733.
7. **Carreau S, Genissel C, Bilinska B, Levallet J**. The oestrogen sources in the testis and the reproductive tract of the male. *Inter J Androl* 1999; 22: 211–213.
8. **Payne AH, Hales DB**. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* 2004; 25: 947–970.
9. **Weng Q, Medan MS, Ren L, Watanabe G, Tsubota T, Taya K**. Immunolocalization of steroidogenic enzymes in the fetal, neonatal and adult testis of the Shiba goat. *Exp Anim* 2005; 54: 451–454.
10. **Tsubota T, Nitta H, Osawa Y, Mason IJ, Kita I, Tiba T, Bahr JM**. $P450_{c17}$ and $P450_{arom}$ in the Hokkaido brown bear (*Ursus arctos yesoensis*) testis. *Gen Comp Endocrinol* 1993; 92: 439–444.
11. **Okano T, Murase T, Tsubota T**. Spermatogenesis, serum testosterone levels and immunolocalization of steroidogenic enzymes in the wild male Japanese black bear (*Ursus thibetanus japonicus*). *J Vet Med Sci* 2003; 65: 1093–1099.
12. **Weng Q, Medan MS, Watanabe G, Tsubota T, Tanioka Y, Taya K**. Immunolocalization of steroidogenic enzymes P450scc, 3betaHSD, P450c17, and P450arom in Göttingen miniature pig testes. *J Reprod Dev* 2005; 51: 299–304.
13. **Bilinska B, Lesniak M, Schmalz B**. Are ovine Leydig cells able to aromatize androgens? *Reprod Fertil Dev* 1997; 9: 193–199.
14. **Bilinska B, Schmalz-Fraczek B, Kotula M, Carreau S**. Photoperiod-dependent capability of androgen aromatization and the role of estrogens in the bank vole testis visualized by means of immunohistochemistry. *Mol Cell Endocrinol* 2001; 178: 189–198.
15. **Pelletier G, Li S, Luu-The V, Tremblay Y, Belanger A, Labrie F**. Immunoelectron microscopic localization of three key steroidogenic enzymes (cytochrome P450scc, 3 β-hydroxysteroid dehydrogenase and cytochrome P450c17) in rat adrenal cortex and gonads. *J Endocrinol* 2001; 171: 373–383.
16. **Carpino A, Pezzi V, Rago V, Bilinska B, Ando S**. Immunolocalization of cytochrome P450 aromatase in rat testis during postnatal development. *Tissue Cell* 2001; 33: 349–353.
17. **Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K, Bahr JM**. Germ cells of the mouse testis express P450 aromatase. *Endocrinology* 1993; 132: 1396–1401.
18. **Pudney J, Canick JA, Clifford NM, Knapp JB, Callard GV**. Location of enzymes of androgen and estrogen biosynthesis in the testis of the ground squirrel. *Biol Reprod* 1985; 33: 971–980.
19. **Nunes S, Pelz KM, Muecke EM, Holekamp KE, Zucker I**. Plasma glucocorticoid concentrations and body mass in ground squirrels: seasonal variation and circannual organization. *Gen Comp Endocrinol* 2006; 146: 136–143.
20. **Holekamp KE, Talamantes F**. Seasonal variation in circulating testosterone and oestrogens of wild-caught California ground squirrels. *J Reprod Fertil* 1991; 93: 415–425.
21. **Sheng X, Zhang HL, Zhang W, Song MS, Zhang MY, Li B, Weng Q, Watanabe G, Taya K**. Seasonal changes in spermatogenesis and immunolocalization of inhibin/activin subunits in the wild male ground squirrel (*Citellus dauricus Brandt*). *J Reprod Dev* 2008; 54: 460–464.
22. **Johnsen SG**. Testicular biopsy score count—a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones* 1970; 1: 2–25.
23. **Hales DB, Sha L, Payne AH**. Testosterone inhibits cAMP-induced *de novo* synthesis of Leydig cell cytochrome P45017α by an androgen receptor-mediated mechanism. *J Biol Chem* 1987; 262: 11200–11206.
24. **Kitawaki J, Yoshida N, Osawa Y**. An enzyme-linked immunosorbent assay for quantitation of aromatase cytochrome P450. *Endocrinology* 1989; 124: 1417–1423.
25. **Blottner S, Hingst O, Meyer HHD**. Seasonal spermatogenesis and testosterone production in roe deer (*Capreolus capreolus*). *J Reprod Fertil* 1996; 108: 299–305.
26. **Johnson L**. Seasonal differences in equine spermatogenesis. *Biol Reprod* 1991; 44: 284–291.
27. **Nagata S, Tsunoda N, Nagamine N, Tanaka Y, Taniyama H, Nambo Y, Watanabe G, Taya K**. Testicular inhibin in the stallion: cellular source and seasonal changes in its secretion. *Biol Reprod* 1998; 59: 62–68.
28. **Tenniswood MP, Guenette RS, Mooibroek M, Wong P, Welsh JE**. Active cell eath in hormone-dependent tissue. *Cancer Metastasis Rev* 1992; 11: 197–220.
29. **Thomposon EB**. Apoptosis and steroid hormones. *Mol Endocrinol* 1994; 11: 665–673.
30. **Sabine HK, Schön J, Kehlen A, Blottner S, Klonisch T**. Seasonal expression of INSL3 and *Lgr8/Insl3* receptor transcripts indicates variable differentiation of Leydig cells in the roe deer testis. *Biol Reprod* 2004; 71: 1079–1087.
31. **Liza O'donnell, Kirsten MR, Margaret EJ, Evan RS**. Estrogen and spermatogenesis. *Endocr Rev* 2001; 22: 289–318.
32. **Ebling FJ, Brooks AN, Cronin AS, Ford H, Kerr JB**. Estrogenic induction of spermatogenesis in the hypogonadal mouse. *Endocrinology* 2000; 141: 2861–2869.
33. **Pentikainen V, Erkkila K, Suomalainen L, Parviven M, Dunkel L**. Estradiol acts as a germ cell survival factor in the human testis *in vitro*. *J Clin Endocrinol Metab* 2000; 85: 2057–2067.
34. **Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER**. Impairment of spermatogenesis in mice lacking a functional aromatase (*cpy 19*) gene. *Proc Natl Acad Sci USA* 1999; 96: 7986–7991.
35. **Abney TO, Myers RB**. 17β-Estradiol inhibition of Leydig cell regeneration in the ethane dimethylsulfonate-treated mature rat. *J Androl* 1991; 12: 295–304.