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Seasonal expressions of androgen receptor, P450arom and estrogen receptors in the epididymis of the wild ground squirrel (*Citellus dauricus* Brandt)

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ABSTRACT

The aim of this study was to investigate the seasonal expressions of androgen receptor (AR), estrogen receptors alpha and beta (ERα and ERβ) and aromatase cytochrome P450 (P450arom) in the epididymis of the wild ground squirrel. Histologically, the epididymis was with larger duct diameter and cell population during the breeding season. AR was presented in the peritubular smooth muscle cells and epithelial cells in the whole epididymis with stronger staining in the breeding period. P450arom was intensely localized in epithelial cells and spermatozoa during the breeding season, absent in the non-breeding season and moderately stained in pre-hibernation. During the breeding season, ERα was intensely expressed in epithelial cytoplasm and/or nucleus, whereas in the non-breeding season and pre-hibernation, weaker staining signal was found in nucleus of epithelial cells. ERβ was absent in the entire annual cycle by immunohistochemical and Real-time PCR detection. The mRNA levels of AR, P450arom and ERα were higher in the epididymis of the breeding season when compared to those of the non-breeding season and pre-hibernation. Taken together, these results suggest that epididymis of the wild ground squirrel is a primary target for androgen and estrogen, and the expression of P450arom represents that epididymis may be a potential source of estrogen.

1. Introduction

The mammalian epididymis is essential for sperm maturation, including acquisition of motility and capacity to fertilize an oocyte (Dacheux and Dacheux, 2014). It happens during spermatozoa transit through the epididymal ducts, which can be roughly divided into caput, corpus and cauda regions based on structural and functional parameters (Cornwall, 2009). Because spermatozoa are almost sythetically inactive, the maturation processes involve the interaction of spermatozoa and micro-environment primarily created by epithelial cells of epididymal epithelium (Cornwall, 2009). The epididymal epithelial cells are comprised of 5 major cell types: principal, basal, clear, apical, and halo cells, of which principal cells make up the majority of the population (Arrighi, 2014). Epithelial cells play different roles in epididymal functions, cells within each region of the epididymis have a unique transcriptome and distinct functions (Cornwall, 2009). In addition, principal cells line the lumen of the epididymis forming a blood-epididymis barrier to control the exchange of molecules between blood and epididymal lumens (Gregory and Cyr, 2014). Altogether, the epididymis provides a critical environment for sperm maturation, and due to the hormone-dependent feature, the epididymal function is mainly under maintenance of androgen and estrogen (Breton et al., 2016; Shayu et al., 2007).

The testicular Leydig cells are main source of epididymal androgen and estrogen. In mature adults, androgen is produced in testicular Leydig cells under stimulation of luteinizing hormone (Menon and Menon, 2012), and a portion of androgen is irreversible converted into estrogen by cytochrome P450 aromatase (P450arom) (Li et al., 2015). The transfer of testicular androgen and estrogen to epididymis was mainly proceeded by androgen binding protein and testicular luminal fluids respectively (Balbontin and Bustos-Obregon, 1989; Pearl et al., 2007). In the meantime, the epididymis was proved to be a source of estrogen by detectable P450arom localized in epididymis (Shayu and Rao, 2006). Androgen and estrogen exert their physiological roles via nuclear receptors. Unlike androgen which acts exclusively through androgen receptor (AR), estrogen function is mediated through

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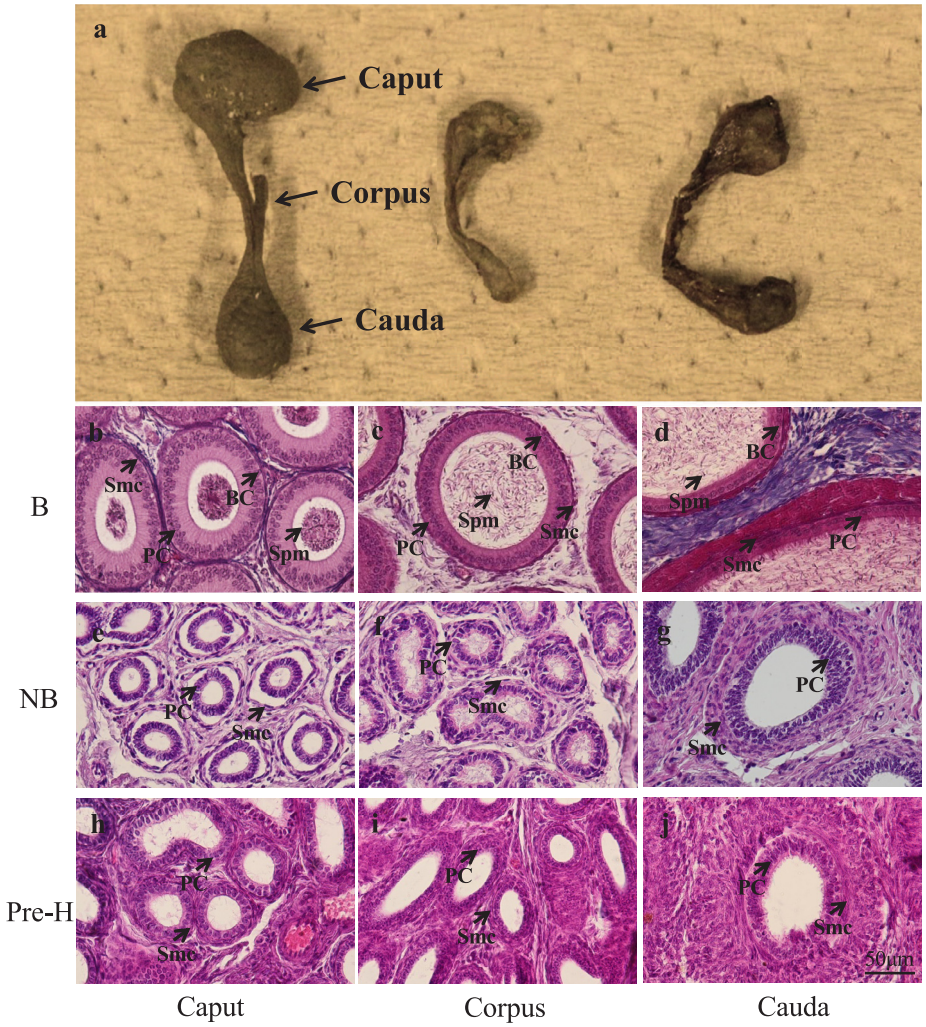

Fig. 1. Seasonal changes of epididymal histology in the breeding season (a, left), non-breeding seasons (a, middle) and pre-hibernation (a, right). The b–j were HE staining of tubules of epididymis (400×) during the breeding (b–d), the non-breeding seasons (e–g) and pre-hibernation (h–j), respectively. Abbreviations: BC, basal cells; PC, principal cells; Smc, smooth muscle cells; Spm, spermatozoa; B, the breeding season; NB, the non-breeding season; P, pre-hibernation. Bar represents 50 μm.

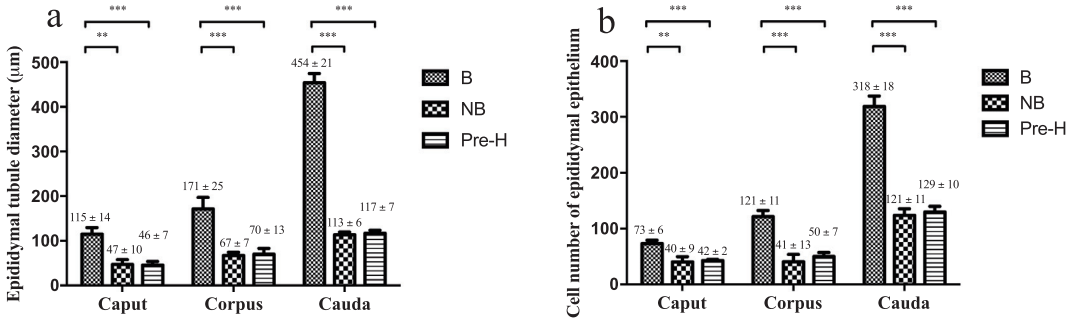

Fig. 2. Cell population of each epididymal segments in the breeding season, the non-breeding season and pre-hibernation. Numbers represent means ± SD of lumen diameter or cell population for 3 wild ground squirrel individuals. Means within the columns marked with marks indicate significant difference (* represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$).

estrogen receptor α and β (ERα and ERβ). AR is localized in most cell types of the male reproductive tissues with pivotal action (Murashima et al., 2015). However, the ER subtypes are in general differentially expressed. While ERα is predominantly expressed in reproductive tissues, and is involved in the regulation of reproduction, ERβ is expressed in other tracts including urogenital tract, immune system and cardiovascular system etc. (Pavao and Traish, 2001; Pelletier, 2000). Nevertheless, together, AR and ERs are essential for the normal development and functional maintenance of male reproductive organs (Cooke et al., 2017; Murashima et al., 2015).

Annual activities of the wild ground squirrels are strictly managed during the annual cycle. They have a short reproductive period in Spring which was from April to May, followed by a static non-reproductive period from June to August, and a long hibernating period from October to the next Spring (Dobson et al., 1992; Zhang et al., 2016). Our published results have found that testicular and epididymal morphology of the wild ground squirrel in the annual cycle is variable among different reproductive status throughout the year (Sheng et al.,

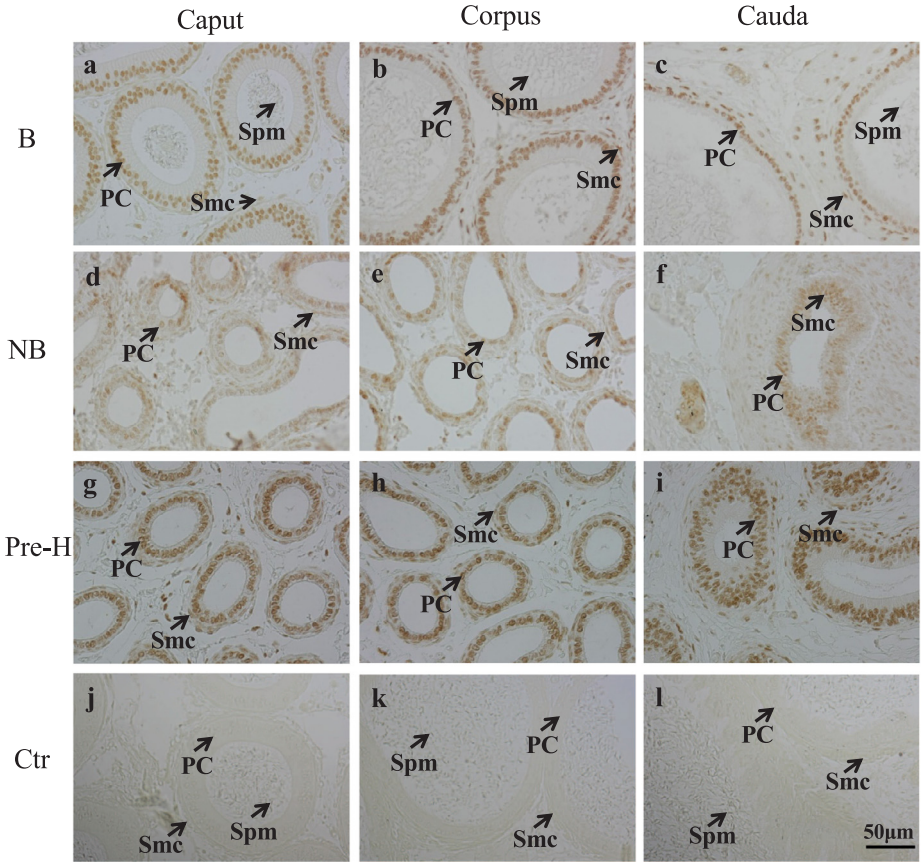

Fig. 3. Immunohistochemical results of AR in the epididymis of the wild ground squirrel during the breeding (B), non-breeding seasons (NB) and pre-hibernation (P). The first (a–c), second (d–f) and third (g–i) row represent immunostaining in the breeding, the non-breeding seasons and pre-hibernation, respectively. The last row (j–l) represents negative control of three seasons. Abbreviations: PC, principal cells; Smc, smooth muscle cells; Spm, spermatozoa. Bar represents 50 μm.

2008; Zhang et al., 2013). Besides, we previously reported that AR, P450arom, ERα and ERβ in testicular tissues was related to changes of testicular activity during the breeding, non-breeding and pre-hibernation seasons (Li et al., 2015). However, systematic and comparative examinations concerning the epididymal morphology and distribution of AR, P450arom and ERs have not been carried out in this species. To provide preliminary insight of relation between steroid hormones and epididymal functions throughout the reproductive cycle of the wild ground squirrels, we investigated the immunohistochemical localizations of AR, P450arom, ERα and ERβ as well as their mRNA expression patterns in epididymis during the breeding, non-breeding and pre-hibernation seasons.

2. Materials and methods

2.1. Animals

The wild ground squirrels were captured by box traps in September 24th, 2012 (pre-hibernation, n = 12) and in April 13th (breeding season, n = 15) and June 27th (non-breeding season, n = 10), 2013 in Hebei Province, China. All procedures were carried out in accordance with the Policy on the Care and Use of Animals by the Ethical Committee, Beijing Forestry University and approved by the Department of Agriculture of Hebei Province, China (JNZF11/2007). An overdose of pentobarbital (BioDee Co., Beijing, China) was applied afterwards for euthanasia. Epididymal tissues were extracted quickly after necropsy. The epididymides were cut, weighed and measured. One side of the epididymis was fixed in 4% paraformaldehyde buffered in 0.05 M PBS (pH 7.4) for histological and immunohistochemical observations, and the other side was frozen in liquid nitrogen immediately and stored at −80 °C until used for RNA isolation.

2.2. Antibodies

The following primary antibodies, including rabbit polyclonal anti-AR (sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-ERα (sc-542, Santa Cruz Biotechnology), rabbit polyclonal anti-ERβ (sc-8974, Santa Cruz Biotechnology), rabbit polyclonal anti-P450arom (derived from human P450arom C-terminus, sc-30086, Santa Cruz Biotechnology) were used in the present study. The dilution of AR, ERα, ERβ and P450arom antibodies for immunohistochemistry were all 1:500. The specificity of AR, ERα, ERβ and P450arom antibodies have been described in our previous studies in wild ground squirrel (Li et al., 2015).

2.3. Histology and morphology

The epididymal samples were dehydrated by a certain concentration of ethanol and embedded in paraffin wax. Serial sections (5 μm) were dried on glass slides coated with ploy-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). Sections were stained with hematoxylin-eosin (HE) for general histological and morphological observations.

All pictures were captured by a digital camera (EOS450D, Canon, Japan) attached to a light microscope (BX51, Olympus, Japan) under a 400× magnification. Cell population of caput, corpus and cauda epididymis was counted from HE staining sections (n = 10) of three wild ground squirrel indivisuals. Five epididymal tubules of each HE staining section were chosen randomly to count total cell numbers within the tubules. The results were sumed and averaged to indicate the total cell number of each segment of epididymis from different seasons.

2.4. Immunohistochemistry

Epididymal serial paraffin sections were incubated with 10% normal goat serum (C-0005, Beijing Biosynthesis Biotechnology CO.

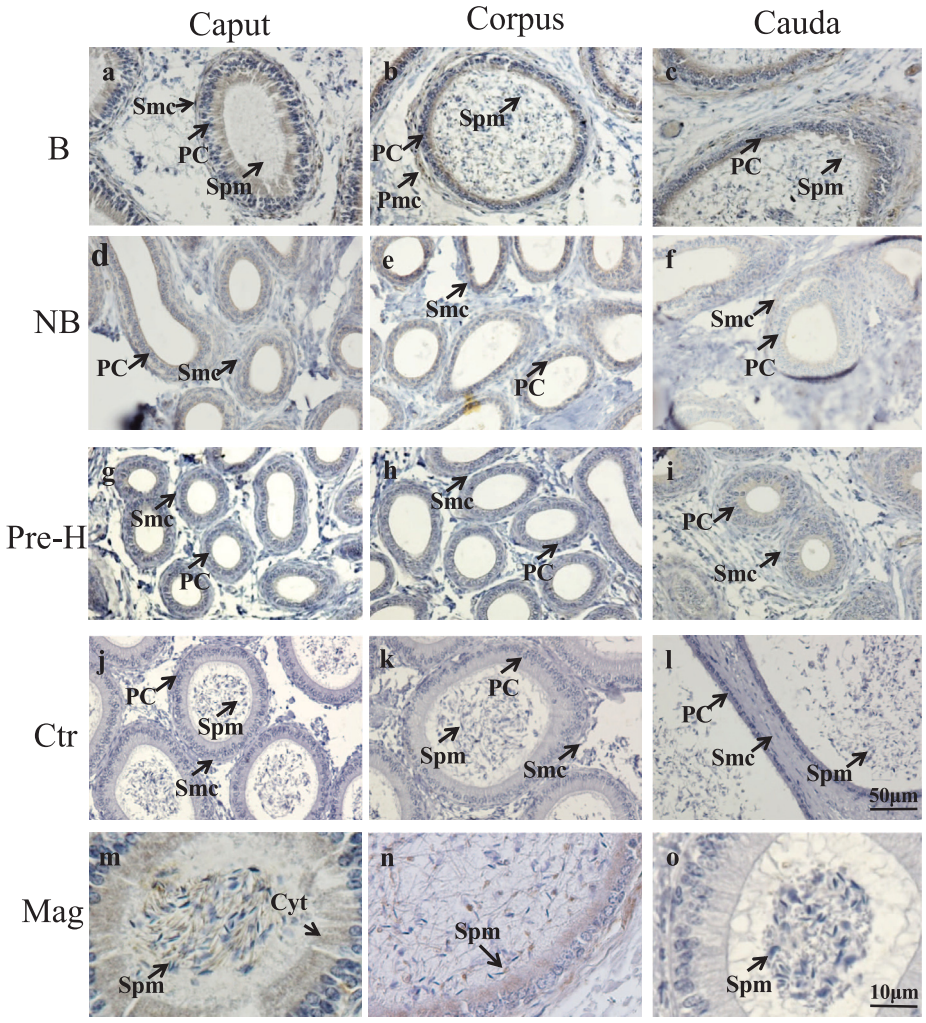

Fig. 4. Immunohistochemical results of P450arom in the epididymis of the wild ground squirrel during the breeding (B), non-breeding seasons (NB) and pre-hibernation (P). The first (a–c), second (d–f) and third (g–i) row represent immunostaining in the breeding, the non-breeding seasons and pre-hibernation, respectively. The forth row (j–l) represents negative control of three seasons. The last row represents the magnification of P450arom expression (m–n are positive staining, o is negative control). Abbreviations: PC, principal cells; Smc, smooth muscle cells; Spm, spermatozoa. Bars represent 50 μm in a–l, 10 μm in m–o.

LTD, Beijing, China) to reduce background staining. The sections were then incubated with primary antibody for 12 h at 4 °C. Subsequent incubations with the secondary antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using SP Kit (Rabbit) (SP-0023, Beijing Biosynthesis Biotechnology CO. LTD, Beijing, China) was performed, followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 ml of 0.05 M Tris-HCl buffer, pH 7.6, plus 30 μl H_2O_2. Control sections were treated with normal goat serum instead of the primary antisera. The immunostained slides were scanned using the software Image-Pro Plus 4.5 (Media Cybernetics, Rockville, MD, USA) at 40× magnification. The immunohistochemical staining was determined as positive (+), strong positive (++), very strong positive (+++), and negative (−). Staining that was weak but higher than control was set as positive (+); the highest intensity staining was set as very strong positive (+++); staining intensity between + and +++ was set as strong positive (++).

2.5. Total RNA isolation

Total RNA was isolated from epididymal tissues of the wild ground squirrels using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Approximately 0.1 g of each epididymal region (caput, corpus and cauda) were thawed and immediately homogenized in 1 ml of TRIzol™ Reagent. The homogenate was incubated for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes. After the addition of 0.2 ml of chloroform, the mixture was vigorously shaken for 15 s at room temperature and centrifuged at 12,000*g* for 20 min at 4 °C. The aqueous phase was then transferred to RNase free tube and added 500 μl of isopropanol. Then the sample was kept for 10 min at room temperature. RNA was precipitated by centrifugation at 12,000*g* for 20 min at 4 °C. The RNA pellet was washed twice with 70% ethanol and dissolved in 30 μl of diethylprocarbonate-treated water. The integrity and concentration of total RNA were tested by gel electrophoresis and spectrophotometer respectively, and the total RNA was diluted to 500 ng/μl.

2.6. Real-time PCR

The first-strand cDNA from total RNA was synthesized using StarScript II First-strand cDNA Synthesis Mix (GenStar, Beijing, China). The 20 μl of reaction mixture contained 2 μl of total RNA, 1 μl of Oligo (dT) $_{18}$, 1 μl of StarScript II RT Mix, 10 μl of 2× reaction mix, 6 μl of diethylprocarbonate-ddH_2O. Realtime PCR was utilised to quantify targeted cDNAs using an ABI PRISM 7900HT system (Applied Biosystems, Foster City, CA, USA). The primers used in this study were described in our previous publication listed (Li et al., 2015). PCR was carried out using ABI PRISM® 7500 Fast Real-Time PCR System (Applied Biosystems) according to manufacturer's instruction, and levels of

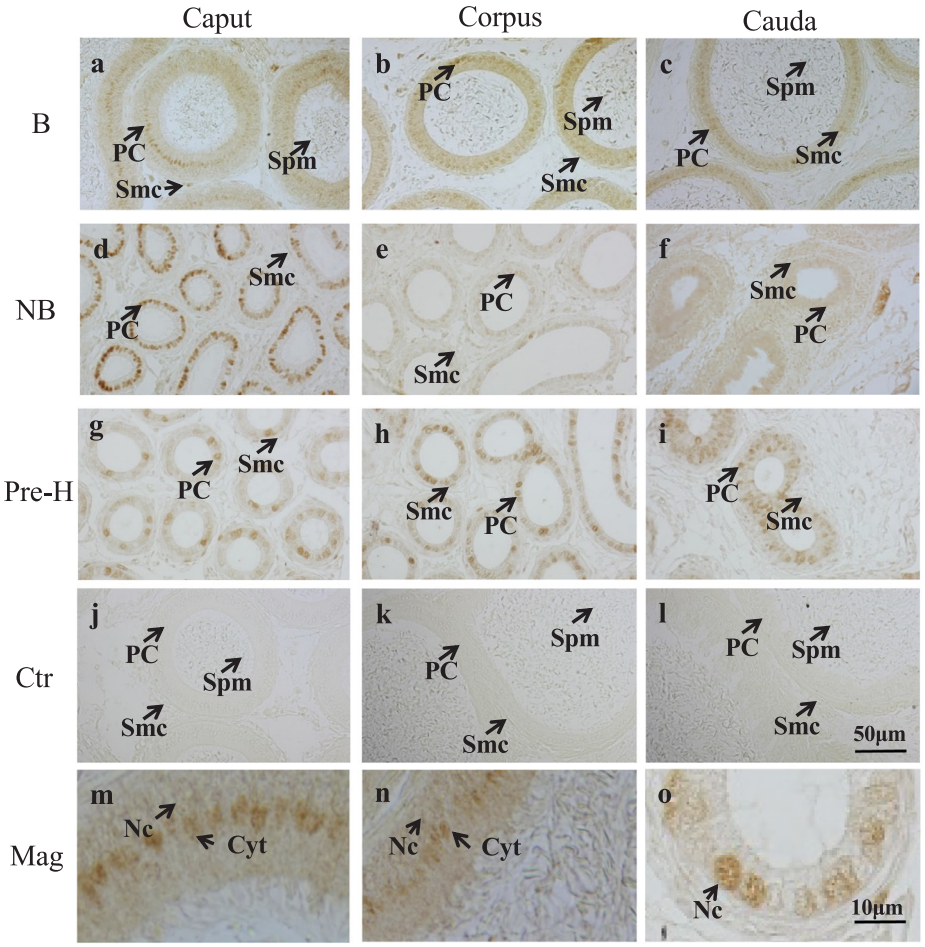

Fig. 5. Immunohistochemical results of ERα in the epididymis of the wild ground squirrel during the breeding (B), non-breeding seasons (NB) and pre-hibernation (P). The first (a–c), second (d–f) and third (g–i) row represent immunostaining in the breeding, the non-breeding seasons and pre-hibernation, respectively. The forth row (j–l) represents negative control of three seasons. The last row represents the magnification of ERα expression (m–o). Abbreviations: PC, principal cells; Smc, smooth muscle cells; Spm, spermatozoa. Bars represent 50 μm in a–l, 10 μm in m–o.

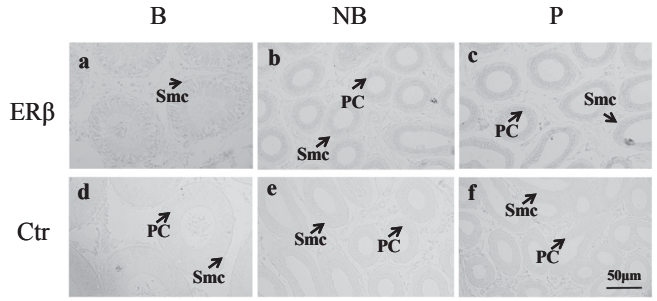

Fig. 6. Immunohistochemical results of ER-β in the epididymis of the wild ground squirrel during the breeding (B), non-breeding seasons (NB) and pre-hibernation (P). The first row represents immunostaining. The second row (d–f) represents negative control. Abbreviations: PC, principal cells; Smc, smooth muscle cells; Spm, spermatozoa. Bar represents 50 μm. Immunohistochemical localization of AR, ERα, ERβ and P450arom in epididymal cells of the wild ground squirrel.

each target mRNA relative to β-actin mRNA were determined using the $2^{-\Delta\Delta CT}$ method. All quantitative reactions were subjected to 95 °C for 30 sec, followed by 35 cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 10 s. Melting curve analysis was applied to all reactions to ensure homogeneity of the reaction product. In addition, the amplified size was checked by electrophoresis and then sequenced. The purified PCR products were ligated into pCR 2.1-TOPO (Invitrogen) and the ligation products were used to transform the competent Escherichia coli using TOPO TA Cloning Kit (Invitrogen). Plasmids were extracted from the bacteria and positive clones containing the proper insert were sequenced in both directions using Thermo Sequenase II Dye Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech, Amersham, UK) with an automatic sequencing system (ABI PRISM 377, Applied Biosystems Japan, Tokyo, Japan).

2.7. Statistical analysis

Statistical comparisons were made with the Student's *t*-test and one-way ANOVA followed by Tukey's test by using Graphpad prism software (Version 5.0, GraphPad Software, Inc., San Diego, USA). A value of $P < 0.05$ was considered indication of statistical significance.

3. Results

3.1. Histology and morphology

The histological and morphological differences of epididymis in different seasons were shown in Figs. 1 and 2. The size of epididymis was larger in the breeding season, and the mass was bigger in pre-hibernation than that of the non-breeding season (Fig. 1 a). Principle cells and basal cells were presented in the breeding season, and the epididymal lumens were filled with sperm, however, sperm and basal cells were absent in the non-breeding season (Fig. 1 b–j). The diameter of

Table 1
Immunohistochemical localization of AR, ERα, ERβ and P450arom in epididymal regions and cells of the wild ground squirrel.

	AR			ERα			ERβ			P450arom		
	B	NB	P	B	NB	P	B	NB	P	B	NB	P
Caput	+++	++	++	++	++	++	−	−	−	++	++	++
Corpus	++	+	++	+++	+	++	−	−	−	+	+	−
Cauda	+++	++	+++	+++	++	++	−	−	−	−	−	+
Sperm	−	/	/	−	/	/	−	/	/	+++	/	/

B, breeding season: NB, nonbreeding season; P, pre-hibernation; −, negative staining; +, positive staining; ++, strong positive staining; +++, very strong positive staining; /, No significant similarity with other species.

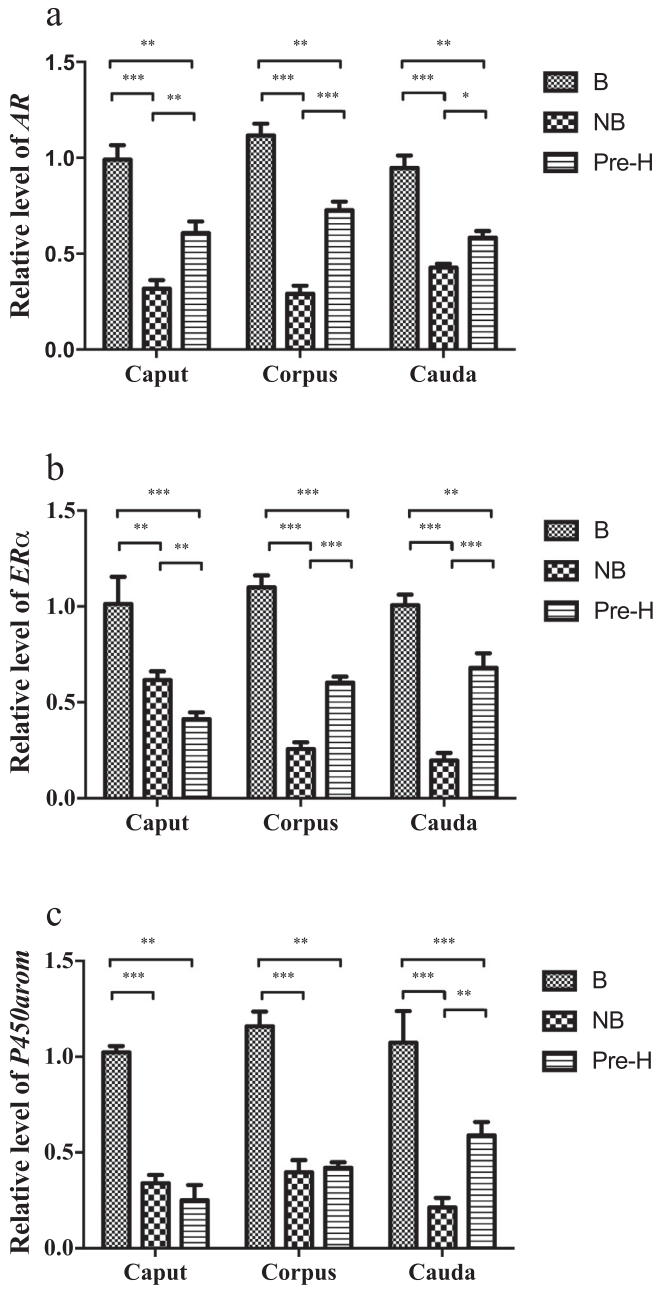

Fig. 7. Expression level of *AR* (a), *ERα* (b) and *P450arom* (c) mRNA level during the annual reproductive cycle. Bars represent means ± SD for 5 independent experiments (n = 5 per season for Real-time PCR). Means within the columns marked with marks indicate significant difference (* represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$).

epididymal tubules during the breeding season was significantly larger than the other periods, the caudal diameter in the breeding season was almost 4-fold compared to the non-breeding season (Fig. 2 a). Meanwhile, there were marked differences of cell population during these three periods. In the reproductive stage, the cell numbers were significantly greater than the non-breeding period and pre-hibernation (Fig. 2 b). Similar to tubules diameter, the cell population in the caudal epididymis of the breeding season was over 3-fold greater than those in the non-breeding season (Fig. 2 b).

3.2. Immunolocalizations of AR, P450arom and ERα in the epididymis of the wild ground squirrel

Immunohistochemical staining of AR, P450arom, ERα and ERβ in epididymis of the breeding/ non-breeding seasons and pre-hibernation were performed (Figs. 3–6) and the result was summarized in Table 1. AR was localized in the nucleus of epithelial cells and smooth muscle cells with stronger staining in the breeding season and pre-hibernation (Fig. 3 a–i). P450arom was localized in the cytoplasm of epithelial cells in caput, corpus and cauda epididymis, the staining signal was higher during the breeding season, decreased in the non-breeding season, and a moderate rebound in pre-hibernation (Fig. 4 a–i). In the meantime, the sperm were also positively stained by P450arom antibody during the breeding season in caput and cauda epididymis (Fig. 4 m–o). During the breeding season, ERα was intensely expressed in epithelial cytoplasm and nucleus in cauda epididymis, but only cytoplasm in caput and corpus epididymis (Fig. 5 a–c). Whereas in the non-breeding season and pre-hibernation, ERα was expressed in nucleus of epithelial cells in caput with faint staining in the corpus and cauda (Fig. 5 d–i). The ERβ was absent in the entire annual cycle by immunohistochemical detection (Fig. 6 a–f). No signal was observed in the negative controls.

3.3. Expression levels of AR, P450arom and ERα mRNA in the epididymis of the wild ground squirrel

The relative abundance of *AR, P450arom, ERα* and *ERβ* mRNA were examined in the epididymis of the wild ground squirrel during the breeding, the non-breeding seasons and pre-hibernation. The expression of *AR* gene in the caput, corpus and cauda epididymis was significantly higher during the breeding season, which markedly reduced during the non-breeding season and increased in pre-hibernation (Fig. 7 a). Likewise, levels of *ERα* and *P450arom* in the breeding season were also high in all regions of epididymis, and decreased significantly during the non-breeding season (Fig. 7 b and c). Differently, the expression of *ERα* elevated in corpus and cauda, but not caput epididymis during pre-hibernation compared to the non-breeding season, while expression of *P450arom* only rised in cauda epididymis during pre-hibernation (Fig. 7 b and c). Expression of *ERβ* was negatively detected by real-time PCR.

4. Discussion

This study elucidated the expression patterns of AR, P450arom, ERα

and ERβ in epididymis of the wild ground squirrel during the breeding, non-breeding seasons and pre-hibernation. The present data illustrate that the expression levels of AR, P450arom, ERα and ERβ were correlated to morphological and functional changes of epididymis in different seasons, and indicate that androgen and estrogen may be involved in the regulation of epididymal function in the wild ground squirrel.

In this study, we found that the epididymis significantly dilated during the breeding season. It was in agreement with other seasonal reproductive species including yellowish myotis (Araujo et al., 2013), viscacha (Aguilera-Merlo et al., 2005) and fruit-eating bat (Oliveira et al., 2012) which showed larger epididymis during their reproductive season. Likewise, in the epididymis of roe deer, the caput enlargement was primarily caused by the growth of the epithelial compartment, whereas the caudal enlargement was predominantly attributed to the dilatation of the lumen which filled with epididymal fluid and spermatozoa (Schon and Blottner, 2009). This was mimic to our data which showed more cell population in the caput epididymis and larger lumen diameter with enriched spermatozoa in the cauda epididymis during the breeding season. Based on the data from different species, we may propose that the seasonal changes of epididymal morphology is a universal phenomenon in seasonal breeders to accomadate the fluctuating external environment.

Many studies demonstrated the hormonal action on epididymal function, herein contained andorgen and estrogen (Zaya et al., 2012). Our research found that during the breeding season, the expression of AR protein showed region-specific pattern in epididymis, which showed more intense expression of AR protein in the caput and corpus epididymis than those in the cauda. Similarly, in mouse, rat and monkey, the expression abundance of AR in epididymis also showed region-specific manner, which were higher in the caput and/or corpus regions (Ezer and Robaire, 2002; Yamashita, 2004; Zhang et al., 1997). Meanwhile, the expression levels of AR mRNA and protein in human were prone to decline from caput, corpus to cauda epididymis. (Ezer and Robaire, 2002). This phenomenon may indicate different function pattern of AR in caput, corpus and cauda epididymis. Besides, our results showed that the expression level of *AR* mRNA was higher in the breeding season, significantly declined in the non-breeding season and rebounded in pre-hibernation. While the spermatozoa transited through epididymal ducts, the epithelial cells secreted luminal proteins in androgen-dependent manner (Brooks, 1981), to create a micro-environment for sperm maturation (Baker et al., 2012). Taken together, the high expression level of AR in epididymis during the breeding season may facilitate function of epididymal epithelial cells.

The testicular Leydig cells were major source for estrogen biosynthesis in males by locally expressed steroidogenic P450arom (Nagata et al., 1998; Banerjee et al., 2012). However, more and more collected data demonstrated that epididymis had the capacity to synthesize estrogen as well (Shayu and Rao, 2006; Wiszniewska, 2002). The cultured epididymal epithelial cells of rat displayed features of steroidogenesis by expressing P450arom to aromatise androgen to 17β-estradiol (Wiszniewska, 2002). In our study, the localization of P450arom in the epididymal epithelial cells and sperms of the wild ground squirrel indicated that the epithelial cells and sperm might have the ability to synthesize estrogen, which was mimic to investigations in mouse and rat (Janulis et al., 1998, 1996). Meanwhile, high expression level of P450arom mRNA was detected during the breeding period, suggesting that epididymis of the wild ground squirrel synthesize more estrogen in the breeding season. Together with the present results, we suggest that epithelial cells as well as spermatozoa of epididymis are potential sources of estrogen, and locally produced estrogen may have a role in epididymal and spermatozoal function in the reproductive cycle of the wild ground squirrel.

The expression of ERs in the epididymis was varied among species. In the bonnet monkey and boar, ERα and ERβ were detectable in epididymis (Shayu et al., 2005; Pearl et al., 2007). Whereas, other species were only slightly positive to ERα. Joseph *et al.* reported that in the marmoset monkey and golden hamster, ERα was almost absent in epithelial cells of caput and cauda epididymis or with low intensity of staining signal in certain cell type (Joseph et al., 2011). Here, we only detected ERα expression, which was parallel to those findings in mouse and rat (Yamashita, 2004), bat (Oliveira et al., 2012), goat (Goyal et al., 1997) as well as human (Ergün et al., 1997), suggesting that estrogen functioned exclusively through ERα in epididymides of wild ground squirrels. Similar to the expression of AR, the segmentally different expression of ERα in the epididymis indicated that estrogen might have distinct role in the different regions of the epididymis. Lacking of functional *Esr1* caused consequences that exhibited a lower percent motility of caudal sperm, morphological anomalies of epididymis as well as defects in the luminal environment, thus led to infertility (Eddy et al., 1996; Hess et al., 2000; Joseph et al., 2010). These evidences implied that estrogen worked via ERα was critical for luminal environment and sperm maturation in epididymis. Besides the presence of ERα in the nuclei of epithelial cells, it was also detected in the cytoplasm, which indicated that ERα might not be fully activated by its ligands due to the translocation from cytoplasm to nucleus didn't occur (Kawai et al., 2005; Nguyen et al., 2009).

In conclusion, our investigation profiled the localization and expression pattern of AR, P450arom and ERs in epididymis of the wild ground squirrel during the breeding and non-breeding seasons as well as the pre-hibernation. These results delineate that the locally functional steroid hormones may be key determinants to seasonal epididymal functions of the wild ground squirrel. Our future study will investigate whether pituitary-derived gonadotropins have a role in epididymal function, and clarify the regulatory role of gonadotropins on steroid synthesis of epididymis.

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Conflicts of interest

The authors declare no conflicts of interest.

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