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Seasonal variations of aromatase and estrogen receptors expression in the testis of free-ranging sand rats

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ABSTRACT

An increasing number of studies revealed the importance of estrogen in male reproduction. However, most research was conducted in laboratory rodents subjected to standardized environmental conditions. Therefore, seasonal regulations of estrogen pathways remain poorly understood under natural conditions. Using immunohistochemistry, the expression of several molecules involved in the functioning of testis (i.e. 17-β estradiol [E2], P450 aromatase, estrogen receptors ESR1, ESR2, and GPER1 [also known as GPR30]) were investigated in free-ranging fat sand rats, *Psammomys obesus*, during the breeding and resting seasons. Leydig cells showed a strong immunoreactivity for aromatase in the testis sampled during the breeding season only; however, E2, ESR1, ESR2 and GPER1 were present during both seasons. Sertoli cells showed a positive signal for E2 and ESR2 during the breeding season; though, all molecules, except GPER1, were present during the resting season. Spermatogonia were reactive for E2, ESR2 and GPER1 during the breeding season and for ESR1 and GPER1 during the resting season. During both seasons, spermatocytes-I presented a moderate reactivity for E2, ESR1, ESR2 and a strong reactivity for GPER1; aromatase was detected during the resting season only. Spermatids and spermatozoa were present exclusively during breeding season and were reactive for all molecules; except round spermatids that were negative for aromatase. The functioning of the testis depends on finely tuned stimulation and inhibition systems. Our results suggest that differential expression of aromatase, ESR1, ESR2, and GPER1 across cells types is involved in the seasonal activation/inactivation cycle of spermatogenesis in a free-ranging species.

1. Introduction

The testis is a multifaceted organ that exerts exocrine and endocrine reproductive functions, notably spermatogenesis and steroidogenesis. Both functions are interacting intimately to maintain fertility. The production of sperm is under hormonal regulations of the hypothalamo–pituitary–gonadal axis: testicular activity is under the control of LH and FSH that are in turn under the control of GnRH, with different feedback loops among the endocrine systems involved. This complex regulatory system enables organisms to adjust their reproductive effort (e.g. sperm production, development of secondary sexual characteristics, sexual behaviors) to environmental conditions and to their physiological status (Willmer et al., 2005). Thus subtle regulations of reproductive functions determine fertility but also influence individual fitness and population viability. Sexual steroids occupy a central role in these regulations. In male vertebrates, testosterone secreted by the Leydig cells (induced by LH-stimulation) is critical for spermatogenesis and it stimulates other reproductive traits (McLachlan et al., 1996). Consequently, this major androgen steroid attracted considerable attention, detailed information is available in many animal species (Goldey and van Anders, 2015). For example, besides the classical roles exerted on secondary sexual characteristics, the testis testosterone

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maintains the blood–testis barrier (Meng et al., 2005), induces meiosis and postmeiotic development of germ cells (Dohle et al., 2003; Holdcraft and Braun, 2004) and inhibits germ cell apoptosis (Singh et al., 1995).

However, different studies revealed the importance of estrogen hormones in males, notably in the functioning of the epididymis; but this hormone also influences the hypothalamo–pituitary–testis axis and the production of sperm through the modulation of the activity of the Sertoli, Leydig and germ cells (Hess et al., 1997; O'Donnell et al., 2001). Estrogens are notably involved in spermatogonial stem cell division (Miura et al., 1999), initiation and maintenance of spermatogenesis (Ebling et al., 2000), and they promote the survival of germ cells (Pentikäinen et al., 2000). Estrogen biosynthesis is catalyzed by a microsomal P450 aromatase complex responsible for the irreversible transformation of androgens into estrogens (Payne and Hales, 2004). In the reproductive tract of immature males, estrogens are essentially produced by Sertoli cells (Van der Molen et al., 1981), whereas in mature individuals they are found in germ cells, spermatozoa and Leydig cells (Payne et al., 1976; Levallet et al., 1998b; Carreau et al., 2006, 2007a,b). Two different nuclear estrogen receptors (ER) have been identified (ESR1, ESR2) with similar affinities for estradiol (Kuiper et al., 1997). The localization of estrogen receptors in testicular cells varies depending on the species, developmental stages of the cells and types of receptors (Abney, 1999; O'Donnell et al., 2001; Hess and Carnes, 2004). Recently, a new receptor that binds estradiol has been described: the G protein-coupled estrogen receptor (GPER) also named G protein-coupled receptor 30 (GPR30, also known as GPER1). This receptor mediates the 17β-estradiol activation of various downstream signaling pathways and it exerts multiple roles in the cell physiology. Using ligands such as ER antagonist fulvestrant (ICI 182,780) it has been shown that GPER can be found in a large range of human and rodent tissues, more precisely in the endoplasmic reticulum compartment (Sirianni et al., 2008; Prossnitz and Barton, 2009). Importantly, 17β-estradiol may have differential effects when activating ESR (nuclear) versus GPER (non-nuclear) receptors. For example, the selective activation of GPER has a rapid anti-apoptotic effect in cultured Sertoli cells, whereas delayed cell proliferation can occur under ESR1 activation in other cells (this response takes time from gene activation to protein production). Nevertheless, these two different regulatory pathways are partly overlapping and they may involve cross-talk between the two receptors (Lucas et al., 2010).

Overall, a better understanding of the reproductive endocrinology of mammals necessitates considering both androgen and estrogen regulations in females and in males (Pelletier et al., 2000; Zhou et al., 2002). Although most comparative studies focused on circulating levels of sex steroids in mammals (Bronson, 1989), examination of the localization of their respective receptors is equally essential to accurately identify their main sites of action. Assessment of the localization of the major enzymes involved in steroidogenesis is also important (Payne and Hales, 2004). Research on these issues concentrated in laboratory rodents (Couse and Korach, 1999). Yet, histological studies of male reproductive tracts showed considerable variations among closely related rodent species (mice vs rats) in the localization and in the expression of sex steroid receptors for example (Zhou et al., 2002). Therefore, inferring possible regulation during the reproductive cycle in free ranging species of rodents from the limited laboratory studies available might be speculative. Especially when the strong variability in life history traits exhibited across the high phylogenetic diversity of mammals is considered (Bromham et al., 1996). Information gathered in free-ranging species of mammals subjected to seasonal variations is needed to assess the general validity of the results obtained in strains of rodents that have escaped natural selection over many generations (Schön and Blottner, 2008; Oliveira et al., 2009; Beguelini et al., 2014; Zarzycka et al., 2016).

Captivity investigations performed in seasonal breeder rodents (voles, hamsters) showed that moderate exposure to estradiol prompted gonadal recrudescence and spermatogenesis in individuals maintained under unfavorable photoperiodic regime (Pak et al., 2002; Gancarczyk et al., 2004). In domestic stallions (pseudo-seasonal breeders) capacitation and acrosome reaction were independent from a marked seasonal expression of estrogen receptors (ESR1, ESR2 and GPER), suggesting that upstream processes such as epididymal maturation were more important (Gautier et al., 2016). These captivity studies indicate that seasonal variations represent an appropriate system to identify and thus assess the roles of estrogens in the functioning of the testis in free ranging animals, as shown in a tropical bat species (Beguelini et al., 2014).

In this study, we examined adult males of the fat sand rats (*Psammomys obesus*) during the reproductive cycle. This species lives in an extremely arid environment characterized by drastic seasonal changes of the climatic conditions; breeding period occurs from autumn through early spring; a non-breeding resting phase takes place from late spring through summer. This contrasted seasonal pattern offers an opportunity to compare reproductive traits between sexually active versus inactive individuals. The reproductive physiology of fat sand rat has been intensively studied throughout the whole annual cycle, providing baseline information to examine possible changes in the expression of molecules involved in the estrogenic dependent functioning of testis. Seasonal variations of the genital tract (e.g. including seminal vesicles), of the histology of the testis (including indicators of hormonal activity), and of plasma levels of testosterone have been described (Khammar and Brudieux, 1984, Khammar, 1987; Gernigon et al., 1991; Gernigon, 1992; Menad, 2008, 2015; Menad et al., 2014). During the annual cycle, the testis of male fat sand rat undergoes profound changes. In the active period, sperm production is abundant and Leydig cells have a highly developed endoplasmic reticulum (a key substratum for GPER1). In the resting period, there is a reduction in the diameter of the seminiferous tubules, a stoppage of the spermatogenesis, and a regression of the endoplasmic reticulum of the Leydig cells associated with an accumulation of the lipids in the Leydig cells. Regarding testicular contents of androgens (ng/g of testis), high values were observed in autumn and in winter (testosterone: 7.6 ± 1.1; androstenedione: 0.76 ± 0.11) whereas low values were reported in early summer (June) (testosterone: 1.5 ± 0.3; androstenedione: 0.20 ± 0.05) with raising values in late July (Khammar and Brudieux, 1984). Annual variations of the testosterone metabolic clearance rate (liters/24 h/100 g body wt) were parallel to the changes of testicular androgens concentrations; clearance peaking in winter (6.7 ± 0.7) and decreasing in June (3.2 ± 0.3). Presumably this contrasted pattern of androgen production during the testis cycle should be reflected by marked seasonal differences in the expression of endocrine receptors.

The main aim of this study was to provide information on estrogen regulation in the testis in males of a wild species subjected to natural seasonal fluctuations. Indeed, although potential important roles of estrogen are suspected in free-ranging males of mammals, current information is limited to individuals maintained in captivity (Hamster, bank vole, roe deer), and two free-ranging bat species (Schön and Blottner, 2008; Oliveira et al., 2009; Beguelini et al., 2014; Zarzycka et al., 2016). In this study, we focused on the testis and on the localization of estradiol (E2), estrogen receptors (ESR1, ESR2, and GPER1) and of the P450 aromatase. Considering the meager level of scientific knowledge on these issues in wild species, we addressed a simple question. Do the expression and the localization of the main estrogen receptors and P450 aromatase vary among the main cell types (Leydig, Sertoli, germ cells) and during the breeding cycle?

2. Material and methods

2.1. Animals and samples

The fat sand rat (*Psammomys obesus*) is a diurnal rodent that lives in

the North-West of the Algerian Sahara. This species has been used to study the seasonal changes in the reproductive cycle of arid desert rodents (Khammar, 1987; Gernigon et al., 1991; Menad et al., 2014) and as a model to examine several metabolic disorders in humans (Donath et al., 1999).

Sixteen adult males (mean body mass 145 ± 3.36 g) were trapped in the region of Béni Abbès (30°07′N 2°10′W) during the breeding ($N = 8$) and during resting ($N = 8$) seasons. They were euthanized in the morning, 48 h after capture. The testes were quickly excised, weighed, fixed in Bouin's solution, dehydrated in increasing concentrations of ethanol (70%, 95%, and 100%), cleaned in toluene, and embedded in paraffin. All experiments were carried out in compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) following an approval by the local Ethical Committee of the Houari Boumediene University of Sciences and Technology, Algeria.

2.2. Histology

Testes were cut in 5 µm thick sagittal sections with a Leitz vertical microtome and mounted on histological slides or on “Super Frost” glass slides for immunohistochemistry. After hydration, the sections were stained with Masson's trichrome (Martoja, 1967; Gabe, 1968).

2.3. Immunohistochemistry

The expression/presence of aromatase, estradiol, and estrogen receptors (ER: ESR1, ESR2, and GPER1) was analyzed by immunohistochemistry. Sections were deparaffined with cyclohexane and rehydrated with decreasing concentrations of ethanol. The slides were then washed in tap water for 10 min. For antigen retrieval (ER and GPER1), the slides were incubated at 95 °C in a 10 mM sodium citrate solution (H-3300, pH 6.0) for 45 min (for ESR1 and ESR2 analysis) or 30 min (for GPER1). After heating, the slides were left to cool down for 20 min and then washed in distilled water. Endogenous peroxidase activity was quenched with a 3% peroxidase solution for 20 min followed by two baths in distilled water. Sections were encircled using a DakoPen (Dako, USA) and incubated with a 10% normal goat serum (S-1000) for 1 h at room temperature to block non-specific binding sites. Afterwards, the slides were incubated with primary rabbit polyclonal antibodies against human aromatase (ab3504, Abcam plc, Cambridge, UK), estradiol (AB924, Millipore, CHEMICON), ESR1 (H-184:sc-7207, Santa Cruz Biotechnology, USA), ESR2 (H-150:sc-8974, Santa Cruz Biotechnology, USA) and GPER1 (ab39742, Abcam plc, Cambridge, UK). Antibodies against aromatase, estradiol were used as 1:200 dilutions in PBS, and ESR1, ESR2 and GPER1 were used as 1:50. Next, tissue sections were incubated during 1 h with aromatase, estradiol and GPER1 antibodies or overnight at 4 °C in a wet chamber for ESR1 and ESR2. Then, the slides were washed in PBS solution and incubated with corresponding secondary biotinylated antibodies (Anti-Mouse IgG/Rabbit IgG; BA-1400, Vectastain Universal) for 1 h in a wet chamber. After rinsing three times in PBS for 5 min, the slides were incubated with a streptavidin-biotin-peroxydase complex for 1 h. Each tissue section was washed in PBS and stained by the AEC (Amino-Ethyl-Carbazole; Vector Laboratories, SK-4200) for aromatase and estradiol or the DAB chromogen (3,3′-diaminobenzidine, kit for peroxidase; Vector Laboratories) for ESR1, ESR2 and GPER1. The reaction was stopped by rinsing the samples in a PBS solution. Tissue sections were then counterstained with hematoxylin (Hematoxylin QS, H-3404; Vector Laboratories, Burlingame, CA, USA) for 1 min, then mounted using VectaMount (AQ Aqueous Mounting Medium, H-5501) for aromatase and estradiol or dehydrated and preserved using the Permount mounting medium (Fisher Scientific, USA) for ESR1, ESR2 and GPER1. The immunostaining was observed under the Nikon Eclipse E 400 light microscope fitted with the Nikon DXM 1200 digital camera. The intensity of the immunostaining was scored as null (−), weakly positive (+), moderately positive (++) or strongly positive (+++) by two independent observers blinded to the antigen type under analysis.

2.4. Analyses

Using data from immunohistochemistry can pose technical difficulties. Notably, various limitations have been identified ranging from the selection of antibody panels to the ability of the observers that interpret and quantify the information of the pictures (de Matos et al., 2010). Yet, we emphasize that our main goal was to compare results obtained during two different seasons while using a fully standardized protocol. Consequently, possible limitations caused by non-optimal applicability of immunohistochemistry applied equally in all samples, and thus did not impede our ability to detect seasonal differences.

Nonetheless, we provide additional information regarding several important technical issues. The specificity of the antibodies was further tested using the western blot technique on the epididymis. For ESR1, ESR2, and GPER1 we respectively obtained a molecular weight 64 kDa, 55 kDa and 55 kDa; enabling us to partly further discriminate the different receptors examined. Moreover, the specificity of the antiserum provided by the supplier (rabbit anti-estradiol polyclonal antibody, AB924, LV1464029) has been evaluated for its ability to give a positive and specific immunostaining of estradiol-containing cells in human mammary gland carcinomas and Sertoli Leydig tumors of the ovary using both the immunoperoxidase and immunofluorescence procedures (© 2016 Merck KGaA; Kercmar et al., 2014). Negative controls have also been used: samples were incubated with normal goat serum instead of using the primary antibody; they systematically provided negative responses. Regarding the quantification of immunostaining, densitometry provided parallel results than the visual estimate performed by the two independent observers. Yet, the visual estimate quantification was simple (i.e. four categories) and thus was conservative.

3. Results

3.1. Histology

During the breeding season, germ cell lineages were observed in the seminal epithelium (Fig. 1A). At the basal side, spermatogonia (spg) presented a well stained chromatin. Above spermatogonia, voluminous spermatocytes (spc I) showed a chromatin organized in clusters. Near the lumen of the seminiferous tube, the spermatids were either round (rsd) or elongated (esd). Within the lumen, spermatozoa presented a filamentous appearance. The Sertoli cells extended across the whole thickness of the epithelium, exhibiting a basal triangular nucleus with a central nucleolus. In the interstitial tissue, Leydig cells were either dispersed or aggregated in clusters.

During the resting season, spermatogenesis was blocked at the stage of spermatocyte I, spermatids and spermatozoa were absent (Fig. 1B).

3.2. Immunohistochemistry

All results of immunohistochemistry for 17-β estradiol, aromatase, ESR1, ESR2 and GPER1 are summarized in Table 1.

3.3. Aromatase

During the breeding season, a strong immunoexpression of P450 aromatase was localized in the cytoplasm of Leydig cells (Fig. 1C, D). In the periphery of seminiferous tubule, smooth muscle cells were positive. The cytoplasm of elongated spermatids and spermatozoa showed an intense and a moderate immunohistochemical reaction respectively. Sertoli cells were negative.

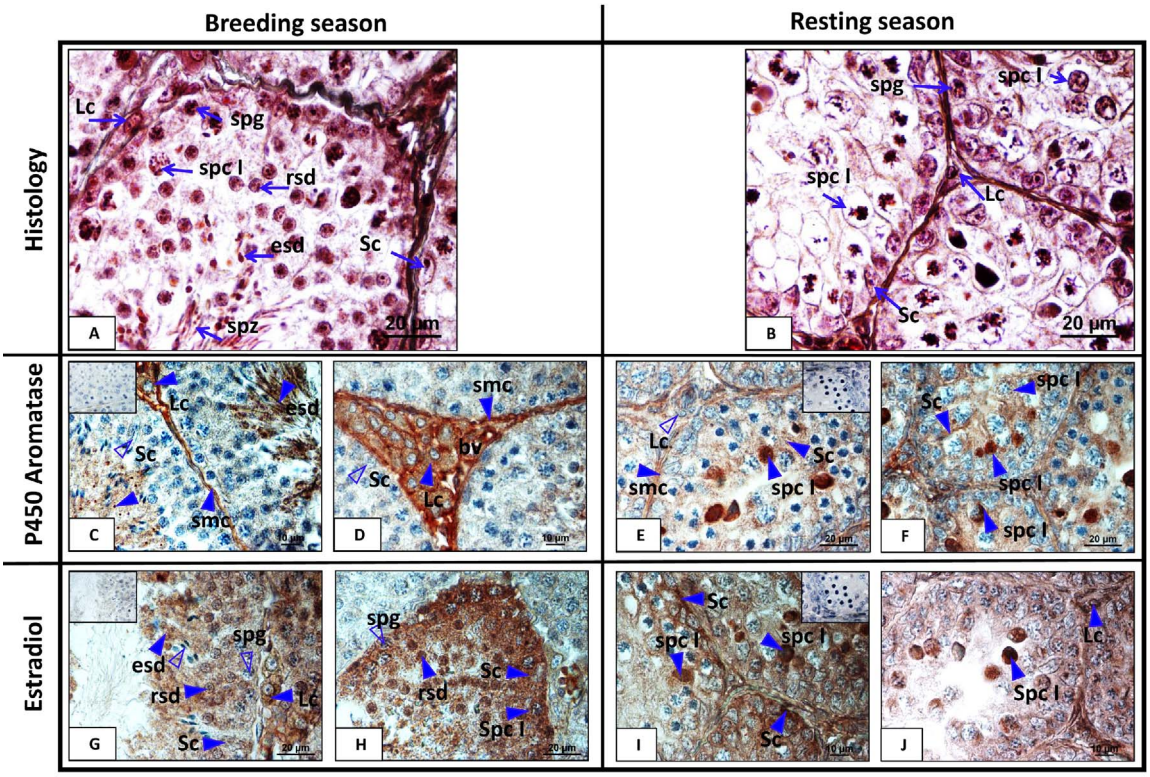

Fig. 1. Histology and immunohistochemistry of P450 aromatase and 17-beta estradiol in the testis of the sand rat *Psammomys obesus*. Panels 1A and 1B illustrate the histological differences during the breeding versus and resting season. (1A): in the wall of the seminiferous tubule, all germ cells are present. Spermatogonia and spermatocytes I are present with a colorful chromatin and a mound chromatin respectively. The spermatids, localized to the lumen of the seminiferous tube are either round or elongated. Sertoli cells occupy the whole thickness of the epithelium; the basal nuclei with triangular shapes and central nucleolus are visible. In the interstitial tissue, Leydig cells are present. (1B): the cellular mosaic is not fully developed, spermatogenesis is blocked at the stage of spermatocyte I. Spermatids and sperm are absent. In interstitial tissue, Leydig cells are still present. Panels 1C, 1D, 1E, and 1F illustrate P450 aromatase immunohistochemistry results. During the breeding season (1C and 1D) an intense immunoexpression of aromatase is localized in Leydig cells and elongated spermatids. At the wall of the seminiferous tubule, smooth muscle cells are positive. During resting season (1E and 1F), immunohistochemical signal is mainly localized in cytoplasm of spermatocytes I. A Moderate immunoreaction is localized in the cytoplasm of the Sertoli cell, spermatogonia and the smooth muscle cells. Nuclei of Leydig cells are negative and cytoplasm is positive or negative. Panels 1G, 1H, 1I and 1J illustrate the results regarding the immunohistochemistry of 17-beta estradiol. During the breeding season (1G and 1H), the immunoreactivity of the 17-beta estradiol is visible in germ cells and in the cytoplasm of Sertoli. Immunohistochemical staining was absent in the nuclei of spermatogonia and of elongated spermatids, contrarily to the cytoplasm of spermatocyte I, cytoplasm of elongated spermatids and the nuclei of round spermatids that display an intense immunoreactivity. Leydig cells show in the cytoplasm a strong immunohistochemical reaction. During the resting season (1I and 1J), immunostaining of 17 beta estradiol was localized mainly in the cytoplasm and nucleus of spermatocytes I. A strong immunoreaction was observed in basal cytoplasm of Sertoli cells. The cytoplasm of the Leydig cells was positive. No immunostaining was observed in the controls (insert). The full arrow indicates staining, the hollow arrow indicates a lack of staining. Legends for cell types: spg stands for spermatogonia, spcI for spermatocyte I, rsd for round spermatid, esd for elongated spermatid, Sc for Sertoli cells, Lc for Leydig cells, and smc for smooth muscle cell.

Table 1
Immunolocalization of aromatase, E2, ESR1, ESR2 and GPER1 in testis of sand rat (*Psammomys obesus*) in breeding and resting season.

		Breeding season				Resting season					
		Aro	E2	ESR1	ESR2	GPER1	Aro	E2	ESR1	ESR2	GPER1
Leydig cell	N	−	−	+	+ + +	+ + +	−	−	+ +/+	+ +/−	+ + +
	C	+ + +	+ + +	+ + +	+ + +	+ + +	+	+ +	+/−	−	+ + +
Sertoli cell	N	−	+	−	+ +/−	−	−	−	+ + +	−	−
	C	−	+ +	−	−	−	+ +	+ + + (basal)	+	+ + (basal)	−
Spg	N	−	−	+/−	+ + +	+ + +/−	−	−	+ + +	−	−
	C	−	+ +	−	+	−	+	−	−	−	−
spcI	N	−	−	+ +/−	+ +	−	−	+ + +/−	+ +/−	−	+ + +/−
	C	−	+ +	+/−	+ +	+ + +	+ +	+ + +	+ +/−	+ +	+ + +
rsd	N	−	+ + +/+ +	+ +	+ + +	−/+ +	/	/	/	/	/
	C	−	−	+	−	−/+ +	/	/	/	/	/
esd	N	−	−	−	−	+ +	/	/	/	/	/
	C	+ + +	+ + +	+ + +	+ + +	+	/	/	/	/	/
spz	N	+ +	+	+	+ +	+ +	/	/	/	/	/
smc	N	+ +	+/−	+	+	+ + +/−	+	+	+ +	+	−

spg, spermatogonia; spcI, spermatocyte I; rsd, round spermatid; esd, elongated spermatid; spz, sperm; smc, smooth muscle cell; N, nucleus; C, cytoplasm.

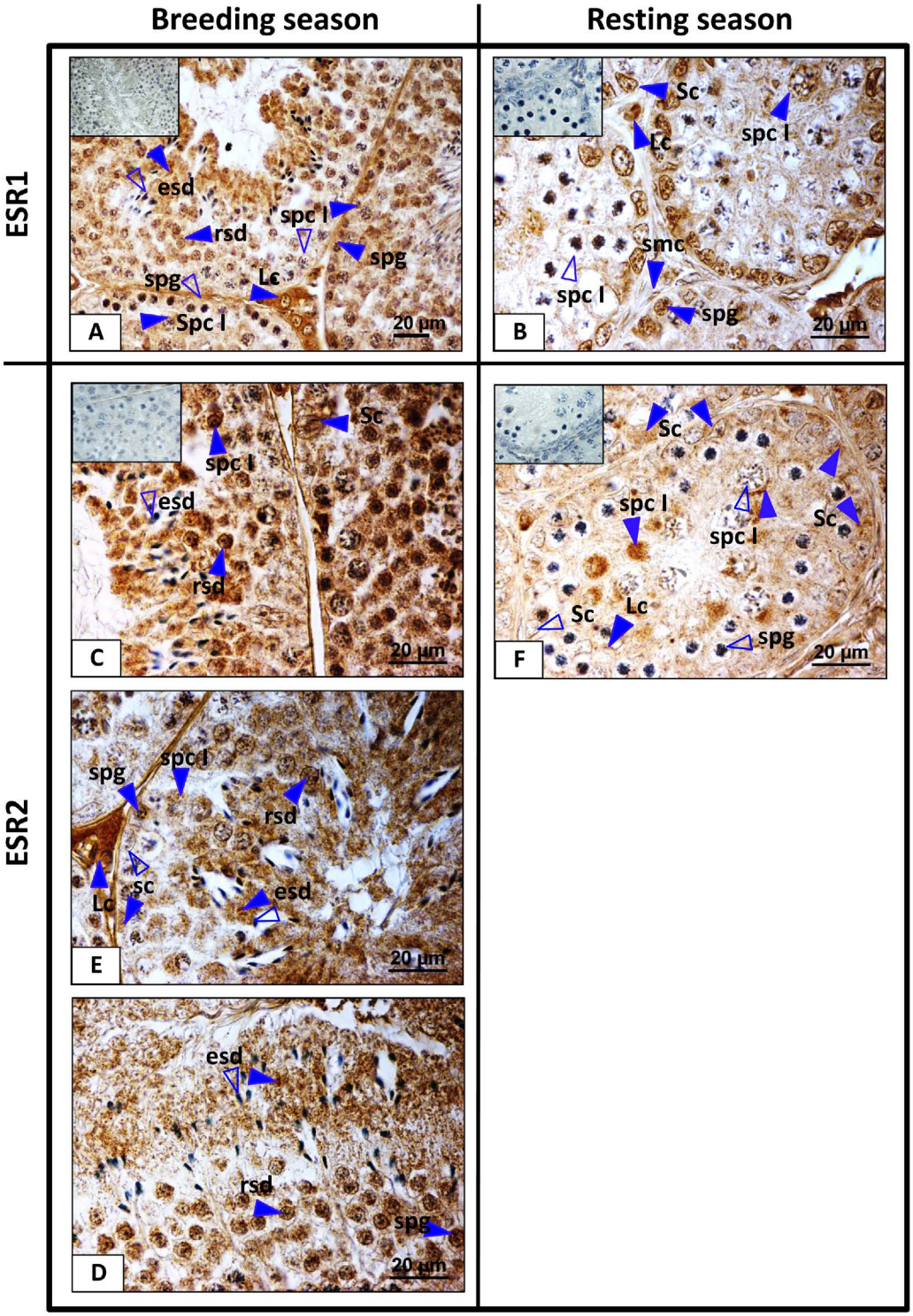

Fig. 2. Immunohistochemistry of ESR1 and ESR2 in the testis of the sand rat *Psammomys obesus*. Panels 2A and 2B illustrate immunohistochemistry of ESR1. During the breeding season (2A) the immunoreactivity of ESR1 was localized in the cytoplasm of Leydig cells and the nuclei of round spermatid. The nuclei of spermatocytes I were positive or negative contrarily to elongated spermatids that remained negative. During resting season (2B), the immunohistochemical staining of ESR1 was visible in the nuclei of Sertoli cells, Leydig cells and spermatogonia. Spermatocytes I were positive or negative. Panels 2C, 2D, 2E, and 2F provide results on the immunohistochemistry of ESR2. During the breeding season (2C, 2D and 2E), the immunoexpression of ESR2 showed an ubiquitous distribution in germ, Sertoli, and Leydig cells. In germ cells, the immunoreactivity was moderate in cytoplasm and nuclei of spermatocytes I and strong in nuclei of round spermatids. The elongated spermatids did not exhibit nuclear immunoreactivity in most cases. During resting season (2F), the cytoplasm of spermatocytes I was stained, the Sertoli cells exhibited a positive immunoreactivity in their basal cytoplasm. Controls were negative (insert). Legends for arrows and cells types as in Fig. 1.

During the resting season, immunohistochemical signal of P450 aromatase was mainly localized in the cytoplasm of spermatocytes I and Sertoli cells (Fig. 1E, F). The cytoplasm of Leydig cells showed a weak immunoreaction.

3.4. 17-β Estradiol

During the breeding season, a positive immunoreactivity of 17-β estradiol was observed in germ cells and in the cytoplasm and nuclei of

the Sertoli cells (Fig. 1G, H). No immunohistochemical staining was found in the nuclei of spermatogonia, spermatocytes I and elongated spermatids. However, the label was intense in the nuclei of round spermatids. The cytoplasm of Leydig cells showed a strong immunohistochemical reaction.

During the resting season, the immunostaining of 17-β estradiol was localized mainly in the cytoplasm of spermatocytes I and Leydig cells (Fig. 1I, J). The cytoplasm of Sertoli cells was positive essentially in their basal part.

3.5. ESR1

During the breeding season, a strong immunoreactvity of ESR1 was localized in the cytoplasm of Leydig cells and elongated spermatids (Fig. 2A). The nuclei of spermatocytes I and round spermatids showed a moderate immunoreaction. Several spermatocytes I did not exhibit any immunostaining.

During the resting season, an intense immunohistochemical staining of ESR1 was observed in the nuclei of Sertoli cells and in the nuclei of spermatogonia (Fig. 2B). The nucleus and cytoplasm of some spermatocytes I were positive, but most were negative. The nucleus of Leydig cells showed a moderate staining.

3.6. ESR2

During the breeding season, the immunoexpression of ESR2 showed an ubiquitous distribution in the germ, Sertoli and Leydig cells (Fig. 2C–E). The cytoplasm and nucleus of germ cells and spermatocytes I was moderately immunoreactive; but we found a strong signal in the nucleus of rounded spermatids, and in the cytoplasm of elongated spermatids. The nucleus of elongated spermatids provided an inconstant signal, either positive or negative.

During the resting season, the cytoplasm of Leydig cells and spermatocytes I was moderately for ESR2 (Fig. 2F). Many Leydig cells were negative. Sertoli cells showed a moderate immunoreactivity localized in the basal cytoplasm.

3.7. GPER1

During the breeding season, a strong immunoreactivity of GPER1 was localized in the cytoplasm of spermatocytes I and in the nucleus of some spermatogonia (Fig. 3A–C). A moderate immunostaining of GPER1 was localized in the nucleus of elongated spermatids, sperms and in some round spermatids. The cytoplasm and nucleus of Leydig cells showed a strong immunoreaction. Sertoli cells remained negative.

During the resting season, an intense immunoreactivity of GPER1 was localized in the nucleus and the cytoplasm of spermatocytes I and of Leydig cells; in contrast Sertoli cells and spermatogonia remained negative (Fig. 3D, E). Many spermatocytes I showed a strong immunoreactivity of their cytoplasm and nucleus, with the exception of few cells that were negative.

4. Discussion

All the molecules examined in this study that were presumably involved in the reproduction of male sand rats (i.e. E2, P450 estradiol aromatase, ESR1, ESR2, and GPER1) were actually localized in the testis of the individuals sampled in the field. More precisely, the respective distribution revealed by specific positive immunostaining of each different molecule examined corresponded well to the expected patterns. For instance, a strong positive signal was observed in the cytoplasm of the Leydig cells for the P450 aromatase during the breeding season, precisely where and when steroidogenesis peaks in the sand rat (Khammar and Brudieux, 1984). Nuclear receptors (ESR1 and ESR2) were essentially observed in the nucleus of the cells studied while the non-genomic receptor (GPER1) was generally detected in the cytoplasmic compartment. Overall, despite the inherent limitations associated with the use of immunohistochemistry, our results enabled us to assess possible seasonal patterns in the expression of important effectors involved in the estrogenic regulation of the testis.

Strong differences were observed between the males captured during the breeding season compared to those captured during the non-breeding (resting) season. Consequently, these results concur with previous studies to suggest that estrogen regulations are involved in the seasonal functioning of the testis in free-ranging vertebrates (Schön and Blottner, 2008; Oliveira et al., 2009; Caneguim et al., 2013; Beguelini et al., 2014; Zarzycka et al., 2016). Yet we emphasize that in most previous studies, individuals were maintained in captivity under artificial conditions during various periods; very few investigations were based on the examination of free-ranging individuals directly sampled in the field (Oliveira et al., 2009). Therefore our study provides a significant link between laboratory and field results. Below we briefly review our main results following a simple order from the Leydig cells to the germ cell lineage.

In Leydig cells, E2, ESR1, ESR2 and GPER1 were observed both during the breeding and the resting seasons. However, the aromatase reactivity was marked during the reproductive season only. This seasonal effect could be androgen-dependent. In the sand rat, the breeding season is characterized by a strong elevation of serum levels of testosterone (Khammar, 1987; Gernigon et al., 1991; Gernigon, 1992; Gernigon et al., 1994; Menad, 2008, 2015; Menad et al., 2014). Androgens are aromatized into estrogens by the P450 aromatase (Payne et al., 1976; Simpson et al., 1994). An increasing P450 aromatase activity might be associated with increasing testosterone production, resulting in an overall increase of E2 production. On the other hand, E2 binds to estrogen receptors (ESR1, ESR2 and GPER1) and can modulate the activity of the Leydig cell (Saunders et al., 1998; Pelletier and El-Alfy, 2000). But the exact modulatory patterns remain unknown.

According to Li et al. (2015) E2 sustains spermatogenesis, notably by promoting the division and the survival of the germ cells via autocrine and paracrine mechanisms. In the roe deer, stallion, and several rodents, estrogens are positively involved in sperm production (Gancarczyk et al., 2004; Schön and Blottner, 2008; Zhang et al., 2010; Gautier et al., 2016). Experimental studies showed that estrogen stimulation is particularly important for the reactivation of spermatogenesis at the onset of the breeding season. Indeed, in transgenic hypogonadal mice (hpg, that congenitally lack gonadotropin) treated with exogenous estrogen for 70 days, a full quantitative restoration of spermatogenesis was observed (Ebling et al., 2000). Moreover, E2 can exert a positive feedback by increasing the release of FSH in males (Ebling et al., 2000). However, the general effect of exogenous E2 on the pituitary is inhibitory; cascading effects with decreasing LH secretion, lower intra-testicular testosterone levels and thus diminution of spermatogenesis have been documented in adult mice (O'Shaughnessy, 2014). In the Wistar rat, low doses of bisphenol A (an agent that binds to estrogen receptors, interferes with LH receptor, and aromatase expression) can perturb the hypothalamic–pituitary axis, produce hypogonadism and compromise sperm production (Wisniewski et al., 2015).

During the resting season, the aromatase staining was low in the Leydig cells suggesting a reduced production of E2. Perhaps that the strong immunoreactivity of E2 found in the Leydig cells originated from another source such as the Sertoli cells? The presence of ESR1, ESR2 and GPER1 in the Leydig cells, both during the breeding and resting seasons, suggests that this cell is a target of E2 during the whole reproductive cycle through autocrine and/or paracrine complex mechanisms and possibly temporal variations in the sensitivity of the hypothalamo–pituitary axis (Yen and Tsai, 1971). But the higher level of expression observed during the breeding season suggests that increasing levels of E2 essentially stimulate sperm production as observed in other mammalian species.

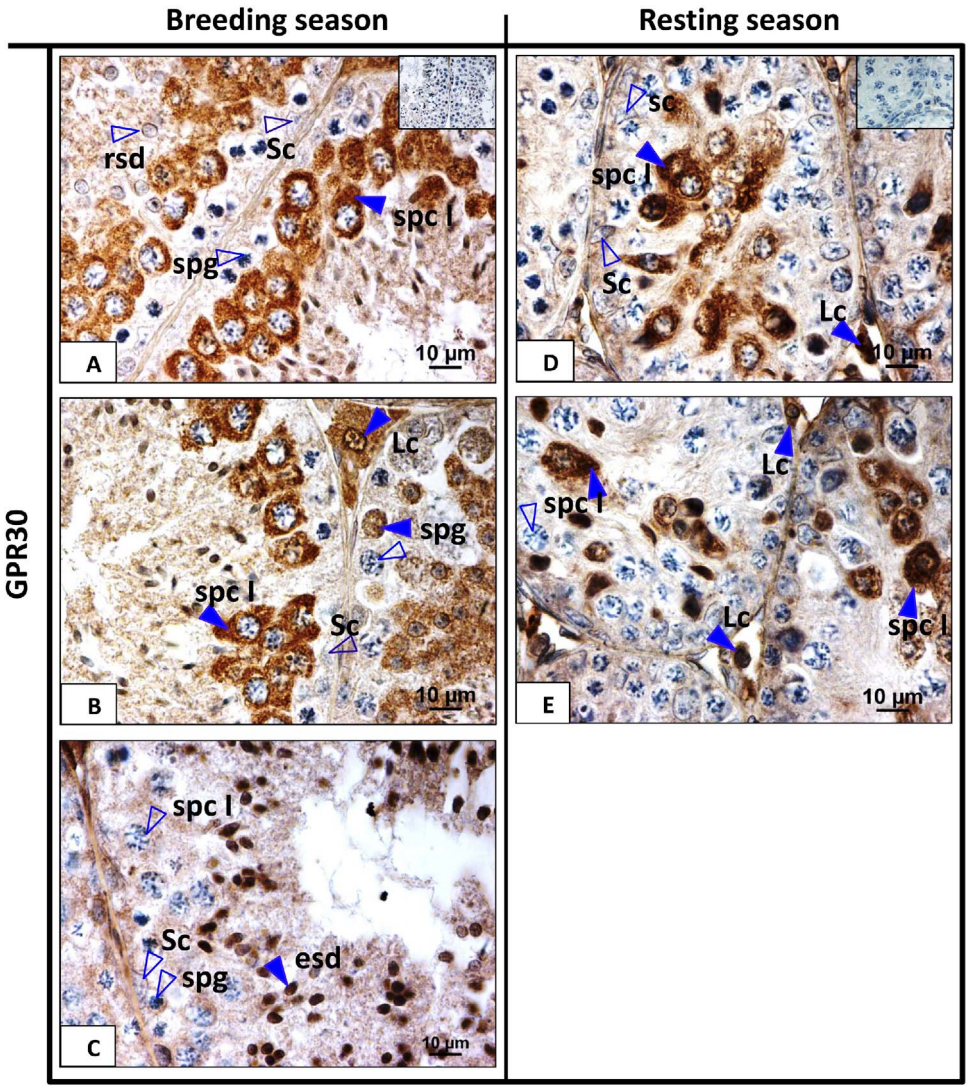

Fig. 3. Immunohistochemistry of GPER1 in testis of sand rat *Psammomys obesus*. During the breeding season (panels 3A, 3B and 3C), GPER1 immunoreactivity was localized in the cytoplasm of spermatocytes I and in some nuclei of spermatogonia and round spermatids. The immunostaining of GPER1 was observed also in the nuclei and cytoplasm of Leydig cells and elongated spermatids contrarily to Sertoli cells which are negative. During the resting season (3D and 3E), a strong immunoreaction was present in spermatocytes I and in Leydig cells

In Sertoli cells, positive signals for E2 and ESR2 were observed during both seasons, P450 aromatase and ESR1 were found during the resting season only, and GPER1 was not detected. ERs are expressed in the Sertoli cells in humans and in immature rats (Lucas et al., 2008, 2010; Filipiak et al., 2012). The amount of aromatase transcripts remains very low in the Sertoli cells of adult rats (Levallet and Carreau, 1997). Sertoli cells are also influenced by androgens, but their respective role relative to E2 in the regulatory mechanisms of spermatogenesis is not yet understood. Nonetheless, the activation of spermatogenesis during the breeding season versus blockage at the stage of spermatocyte I during the resting season might be partly controlled by androgens and E2. The stimulation of ESR1 by E2 is necessary for the functional maturation of the Sertoli cells that occupy a central position in spermatogenesis. The role of aromatase is crucial; indeed in mutant mice deficient for this enzyme Sertoli cells and spermatogenesis functioning are not effective (Smith et al., 2015).

In all germ cell types, the three ER (ESR1, ESR2 and GPER1) were observed during the breeding season. However, during the resting season (when only two types of germ cells were present), spermatogonia exhibited an immunostaining for ESR1 and GPER1, while spermatocytes I were positive for the three ER. González et al. (2012) suggested that the distribution of ER is species specific and varies during the development of the germ cells. In the germ cells of the sand rat, P450 aromatase was localized exclusively in elongated spermatids and sperm during the breeding season. However, during the resting season, this enzyme exhibited a weak immunoreactivity in the spermatogonia and spermatocytes I respectively. The presence of the P450 aromatase in the male germ cells was first detected in the mouse (Nitta et al., 1993). Subsequently, the presence of this enzyme was found in diverse cell types and in different species. For example it was found in all testicular cells of Sprague-Dawley rats, excepted in the peritubular myoid cells, and in the germ cells of different species of mammals and birds (Saez et al., 1989; Tsubota et al., 1993; Kwon et al., 1995; Almadhidi et al., 1995; Janulis et al., 1998; Silandre et al., 2007). In adult rats, germ cells are an important source of estrogens sustained by intensive aromatase activity sometimes representing half of the total cell activity (Levallet et al., 1998a). However, the level of expression of aromatase is not an exact indicator of cellular activity (Levallet et al., 1998b) and the P450 aromatase activity is negatively controlled by germ cells (Boitani et al., 1981). Whatever the case the ubiquity of ER in germ cells suggest important estrogenic functions.

In spermatogonia, a moderate immunoresponse of E2 and a strong immunoreaction of both ESR2 and GPER1 were observed during the breeding season. However, during the resting season, the immunoreac-

tion of ESR2 disappeared while a strong ESR1 and GPER1 staining persisted. ER have been found in human spermatogonia (Mäkinen et al., 2001; Saunders et al., 2001) and ESR1 in the bat *Myotis nigricans* during the reproductive season (Beguelini et al., 2014). Nuclear ESR1 are involved in the setting of spermatogenesis at puberty and are considered as biomarkers of fertility (Aquila and De Amicis, 2014).

In spermatocytes I, a moderate immunoreactivity of ESR1, ESR2 and a strong immunoreaction of GPER1 during both seasons were observed. Low doses of E2 stimulate aromatase expression and early spermatogenesis in male voles exposed to short photoperiod treatment (Gancarczyk et al., 2004). Conversely, high doses of E2 alter testicular structure and induce tubular apoptosis.

In round and elongated spermatids, a positive immunoreactivity for aromatase, E2, and ER was localized, except for P450 aromatase in round spermatids. E2 is essential for the differentiation of the spermatids (Robertson et al., 2002) and for the maturation of sperm (Lubahn et al., 1988). This suggests a functional implication of E2 in spermiogenesis. The biogenesis of the acrosome is an estrogen-dependent process in interaction with androgen receptors (O'Shaughnessy, 2014; Smith et al., 2015).

In sperm, we found a positive signal for the P450 aromatase, E2, and ER. The amounts of transcript and P450 aromatase activity decrease in immotile compared to motile sperm in humans (Carreau et al., 2012). Human spermatozoa also express GPER1 (Franco et al., 2011). Thus, E2 is possibly involved in sperm motility, stimulating carbohydrate metabolism (G6PDH production) and lipid metabolism (increasing lipase activity and β oxidation) (Aquila and De Amicis, 2014). Different studies showed that E2 is of paramount importance for sperm quality (Müller et al., 2012; Arkoun et al., 2014; O'Shaughnessy, 2014; Gautier et al., 2016).

Although these results provide a complex picture regarding possible roles of estradiol in the germ cells and in sperm characteristic, previous studies indicate that this steroid exerts essentially stimulating effects on spermatogenesis, but this outcome might well be dose dependent. Indeed, E2 is essential for the maturation of the spermatocytes (Robertson et al., 2002). In vitro, E2 prevents the apoptosis of germ cells within human seminiferous tubules, even in the absence of gonadotropins (Pentikäinen et al., 2000; Delbès et al., 2004; Mishra and Shaha, 2005). Further, E2 stimulates the mitotic division of spermatogonia (Pierantoni et al., 2009). In Sprague-Dawley rats, E2 induces the expression of genes involved in the regulation of cell proliferation/apoptosis balance (Chimento et al., 2010), and GPER1 might be involved too (Sirianni et al., 2008). Other in vivo studies demonstrated that E2 caused germ cell apoptosis in immature and adult rats (Blanco-Rodríguez and Martínez-García, 1997; Walczak-Jedrzejowska et al., 2007). The expression of cyclin A1 and B1 mRNA, two cell cycle regulators, was down-regulated by E2 and G1 (the selective GPER1 agonist) while an up-regulation of pro-apoptotic factor Bax was observed under similar conditions (Chimento et al., 2010). These investigations demonstrated that E2, working through both ESR1 and/or GPER1, activated the rapid EGFR/ERK/c-Jun pathway in pachytene spermatocytes, modulating the expression of genes involved in the balance between cell proliferation and apoptosis (Chimento et al., 2010). Likely, a specific balance between cell proliferation and cell death via apoptotic pathways, as well as the drastic changes in cell morphology, is due to the presence of a highly organized network of cell cycle regulatory mechanisms during this stage of spermatogenesis, possibly partly influenced by E2 and ER.

However, recent results obtained in the toad showed that E2 did not influence the proliferation of testicular cells throughout the year, but influenced apoptosis during the breeding season (Scaia et al., 2015). In another seasonal breeder, the male black bear, the presence of aromatase was reported at the beginning of testicular recrudescence, first in Sertoli cells, and then in round and elongated spermatids during the mating season (Tsubota et al., 1997). In the Siberian hamster, estrogens induce the initiation of spermatogenesis, independently of FSH in photo-regressed adult males (Pak et al., 2002). These results indicate that E2 exerts major roles in the spermatogenesis of seasonal breeding species.

Regarding our results, we hypothesize that the seasonal differences in the expression of aromatase and ER distribution may explain contrasted effects on spermatogenesis. A proliferative effect of E2 on Sertoli cells, spermatogonia, and a stimulatory effect on other cell types mediated by ESR2 and GPER1 may occur during the breeding season. Conversely, an inhibitory effect of E2 mediated by ESR1, possibly reinforced by apoptosis, may occur during the resting season.

We acknowledge that this simplistic scenario cannot fully account for other complex regulations. For instance, the effects of E2 on different receptors can be pleiotropic since mutants for ESR1 (ERαKO), ESR2 (ERβKO) and GPER1 (GPER1KO) exhibit different phenotypes. Further, the implication of another receptor, the Aryl Hydrocarbon Receptor (AHR) in seasonal testis activity was recently described in the bank vole (*Myodes glareolus*) (Zarzycka et al., 2016). In this species, AHR and GPER1 are expressed in testes of actively reproducing voles and are photoperiod-dependent. Thus, AHR expression may promote signaling pathways that involve both photoperiod and estrogens, whereas GPER1 signaling might be limited to estrogen responses. This suggests that in this species, a differential activity of signaling molecules mediates a signal transduction predominantly via AHR and secondarily via GPER1.

5. Conclusions

Although our results revealed that E2 and ER are likely major effectors in the reproductive cycle of free-ranging male sand rats, our study brings more questions than responses. E2, P450 aromatase, ESR1, ESR2 and GPER1 exhibited various non-univocal immunohistochemistry patterns in the Leydig, Sertoli and germ cells. While the most marked expressions were observed during the breeding season, positive signals were nonetheless observed during the non-breeding resting period. This suggests that complex regulations occur locally in the testes. Further studies are needed to examine seasonal changes in circulating levels of sex steroids, E2 notably, to better understand more global regulations of the reproductive cycle. Yet, our study provides a baseline to better identify and localize the sites of action of these hormones.

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References

Abney, T.O., 1999. The potential roles of estrogens in regulating Leydig cell development and function: a review. Steroids 64, 610–617.

Almadhidi, J., Seralini, G.E., Fresnel, J., Silberzahn, P., Gaillard, J.L., 1995. Immunohistochemical localization of cytochrome P450 aromatase in equine gonads. J. Histochem. Cytochem. 43, 571–577.

Aquila, S., De Amicis, F., 2014. Steroid receptors and their ligands: effects on male gamete functions. Exp. Cell Res. 328, 303–313. http://dx.doi.org/10.1016/j.yexcr.2014.07.015.

Arkoun, B., Gautier, C., Delalande, C., Barrier-Battut, I., Guénon, I., Goux, D., Bouraïma-Lelong, H., 2014. Stallion spermatozoa: putative target of estrogens; presence of the estrogen receptors ESR1, ESR2 and identification of the estrogen-membrane receptor

GPER. Gen. Comp. Endocrinol. 200, 35–43. http://dx.doi.org/10.1016/j.ygcen.2014.02.016.
Beguelini, M.R., Falleiros, L.R., Góes, R.M., Rahal, P., Morielle-Versute, E., Taboga, S.R., 2014. Differential expression of aromatase, estrogen receptor alpha and 17β-HSD associated with the processes of total testicular regression and recrudescence in the bat *Myotis nigricans* (Chiroptera: Vespertilionidae). Gen. Comp. Endocrinol. 201, 53–64. http://dx.doi.org/10.1016/j.ygcen.2014.03.044.
Blanco-Rodríguez, J., Martínez-García, C., 1997. Apoptosis pattern elicited by oestradiol treatment of the seminiferous epithelium of the adult rat. J. Reprod. Fertil. 110, 61–70.
Boitani, C., Ritzén, E.M., Parvinen, M., 1981. Inhibition of rat Sertoli cell aromatase by factor(s) secreted specifically at spermatogenic stages VII and VIII. Mol. Cell. Endocrinol. 23, 11–22.
Bromham, L., Rambaut, A., Harvey, P.H., 1996. Determinants of rate variation in mammalian DNA sequence evolution. J. Mol. Evol. 43, 610–621. http://dx.doi.org/10.1007/BF02202109.
Bronson, F.H., 1989. Mammalian Reproductive Biology. University of Chicago Press.
Caneguim, B.H., da Luz, J.S., Valentini, S.R., Cerri, P.S., Sasso-Cerri, E., 2013. Immunoexpression of aromatase and estrogen receptors β in stem spermatogonia of bullfrogs indicates a role of estrogen in the seasonal spermatogonial mitotic activity. Gen. Comp. Endocrinol. 182, 65–72. http://dx.doi.org/10.1016/j.ygcen.2012.12.002.
Carreau, S., Silandre, D., Bourguiba, S., Hamden, K., Said, L., Lambard, S., Galeraud-Denis, I., Delalande, C., 2007a. Estrogens and male reproduction: a new concept. Braz. J. Med. Biol. Res. 40, 761–768. http://dx.doi.org/10.1590/S0100-879X2007000600003.
Carreau, S., Bouraima-Lelong, H., Delalande, C., 2012. Estrogen, a female hormone involved in spermatogenesis. Adv. Med. Sci. 57, 31–36. http://dx.doi.org/10.2478/v10039-012-0005-y.
Carreau, S., Silandre, D., Bourguiba, S., Hamden, K., Said, L., Lambard, S., Galeraud-Denis, I., Delalande, C., 2007b. Estrogens and male reproduction: a new concept. Braz. J. Med. Biol. Res. 40, 761–768.
Carreau, S., Delalande, C., Silandre, D., Bourguiba, S., Lambard, S., 2006. Aromatase and estrogen receptors in male reproduction. Mol. Cell. Endocrinol. 246, 65–68. http://dx.doi.org/10.1016/j.mce.2005.11.021.
Chimento, A., Sirianni, R., Delalande, C., Silandre, D., Bois, C., Andò, S., Maggiolini, M., Carreau, S., Pezzi, V., 2010. 17β-Estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. Mol. Cell. Endocrinol. 320, 136–144. http://dx.doi.org/10.1016/j.mce.2010.01.035.
Couse, J.F., Korach, K.S., 1999. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. 20, 358–417. http://dx.doi.org/10.1210/edrv.20.3.0370.
de Matos, L.L., Trufelli, D.C., de Matos, M.G.L., da Silva Pinhal, M.A., 2010. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomark. Insights 5, 9–20.
Delbès, G., Levacher, C., Pairault, C., Racine, C., Duquenne, C., Krust, A., Habert, R., 2004. Estrogen receptor beta-mediated inhibition of male germ cell line development in mice by endogenous estrogens during perinatal life. Endocrinology 145, 3395–3403. http://dx.doi.org/10.1210/en.2003-1479.
Dohle, G.R., Smit, M., Weber, R.F.A., 2003. Androgens and male fertility. World J. Urol. 21, 341–345. http://dx.doi.org/10.1007/s00345-003-0365-9.
Donath, M.Y., Gross, D.J., Cerasi, E., Kaiser, N., 1999. Hyperglycemia-induced beta-cell apoptosis in pancreatic islets of *Psammomys obesus* during development of diabetes. Diabetes 48.
Ebling, F.J., Brooks, A.N., Cronin, A.S., Ford, H., Kerr, J.B., 2000. Estrogenic induction of spermatogenesis in the hypogonadal mouse. Endocrinology 141, 2861–2869. http://dx.doi.org/10.1210/endo.141.8.7596.
Filipiak, E., Suliborska, D., Laszczynska, M., Walczak-Jedrzejowska, R., Oszukowska, E., Marchlewska, K., Kula, K., Slowikowska-Hilczer, J., 2012. Estrogen receptor alpha localization in the testes of men with normal spermatogenesis. Folia Histochem. Cytobiol. 50, 340–345. http://dx.doi.org/10.5603/19743.
Franco, R., Boscia, F., Gigantino, V., Marra, L., Esposito, F., Ferrara, D., Pariante, P., Botti, G., Caraglia, M., Minucci, S., Chieffi, P., 2011. GPR30 is overexpressed in post-puberal testicular germ cell tumors. Cancer Biol. Ther. 11, 609–613.
Gabe, M., 1968. Techniques Histologiques. Masson, Paris 1113 pp.
Gancarczyk, M., Paziewska-Hejmej, A., Carreau, S., Tabarowski, Z., Bilińska, B., 2004. Dose- and photoperiod-dependent effects of 17β-estradiol and the anti-estrogen ICI 182,780 on testicular structure, acceleration of spermatogenesis, and aromatase immunoexpression in immature bank voles. Acta Histochem. 106, 269–278. http://dx.doi.org/10.1016/j.acthis.2004.04.002.
Gautier, C., Barrier-Battut, I., Guénon, I., Goux, D., Delalande, C., Bouraïma-Lelong, H., 2016. Implication of the estrogen receptors GPER, ESR1, ESR2 in post-testicular maturations of equine spermatozoa. Gen. Comp. Endocrinol. 233, 100–108. http://dx.doi.org/10.1016/j.ygcen.2016.05.022.
Gernigon, Malaprade D., Mesbah, A., Lécher, P.T., 1991. Aspects cytologiques du testicule et de l'épididyme du rat des sables (*Psammomys obesus*) dans son biotope. In: Chabaud, R. (Ed.), Le Rongeur et l'Espace, pp. 129–142.
Gernigon, T., 1992. Etudes cytologiques et biochimiques des variations saisonnières de l'appareil génital mâle d'un rongeur saharien diurne, le rat des sables (*Psammomys obesus*). Thèse Dr. Alger USTHB 180 pp.
Gernigon, T., Berger, M., Lécher, P., 1994. Seasonal variations in the ultrastructure and production of androgen-dependent proteins in the seminal vesicles of a Saharian rodent (*Psammomys obesus*). J. Endocrinol. 142, 37–46.
González, C.R., Muscarsel Isla, M.L., Leopardo, N.P., Willis, M.A., Dorfman, V.B., Vitullo, A.D., 2012. Expression of androgen receptor, estrogen receptors alpha and beta and aromatase in the fetal, perinatal, prepubertal and adult testes of the South American plains Vizcacha, *Lagostomus maximus* (Mammalia, Rodentia). J. Reprod. Dev. 58, 629–635.
Goldey, K.L., van Anders, S.M., 2015. Sexual Modulation of Testosterone: Insights for Humans from Across Species. Adapt. Hum. Behav. Physiol. 1, 93–123. http://dx.doi.org/10.1007/s40750-014-0005-1.
Hess, R.A., Carnes, K., 2004. The role of estrogen in testis and the male reproductive tract: a review and species comparison. Anim. Reprod. 1, 5–30.
Hess, R.A., Bunick, D., Lee, K.-H., Bahr, J., Taylor, J.A., Korach, K.S., Lubahn, D.B., 1997. A role for oestrogens in the male reproductive system. Nature 390, 509–512. http://dx.doi.org/10.1038/37352.
Holdcraft, R.W., Braun, R.E., 2004. Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. Development 131, 459–467. http://dx.doi.org/10.1242/dev.00957.
Janulis, L., Bahr, J.M., Hess, R.A., Janssen, S., Osawa, Y., Bunick, D., 1998. Rat testicular germ cells and epididymal sperm contain active P450 aromatase. J. Androl. 19, 65–71.
Kercmar, J., Tobet, S.A., Majdic, G., 2014. Social isolation during puberty affects female sexual behavior in mice. Front. Behav. Neurosci. 8, 337. http://dx.doi.org/10.3389/fnbeh.2014.00337.
Khammar, F., 1987. Variations saisonnières de l'activité endocrine du testicule de deux espèces de rongeurs désertiques, le Rat des sables (*Psammomys obesus*) et la gerbille (*Gerbillus gerbillus*). Thèse Dr. Alger USTHB.
Khammar, F., Brudieux, R., 1984. Seasonal changes in testicular contents of testosterone and androstenedione and in the metabolic clearance rate of testosterone in the sand rat (*Psammomys obesus*). J. Reprod. Fertil. 71, 235–241.
Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., Gustafsson, J.A., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138, 863–870. http://dx.doi.org/10.1210/endo.138.3.4979.
Kwon, S., Hess, R.A., Bunick, D., Nitta, H., Janulis, L., Osawa, Y., Bahr, J.M., 1995. Rooster testicular germ cells and epididymal sperm contain P450 aromatase. Biol. Reprod. 53, 1259–1264.
Levallet, J., Bilinska, B., Mittre, H., Genissel, C., Fresnel, J., Carreau, S., 1998b. Expression and immunolocalization of functional cytochrome P450 aromatase in mature rat testicular cells. Biol. Reprod. 58, 919–926.
Levallet, J., Carreau, S., 1997. [In vitro gene expression of aromatase in rat testicular cells]. Comptes rendus l'Académie des Sci. Série III, Sci. la vie 320, 123–129.
Levallet, J., Mittre, H., Delarue, B., Carreau, S., 1998a. Alternative splicing events in the coding region of the cytochrome P450 aromatase gene in male rat germ cells. J. Mol. Endocrinol. 20, 305–312. http://dx.doi.org/10.1677/jme.0.0200305.
Li, Q., Zhang, F., Zhang, S., Sheng, X., Han, X., Weng, Q., Yuan, Z., 2015. Seasonal expression of androgen receptor, aromatase, and estrogen receptor alpha and beta in the testis of the wild ground squirrel (*Citellus dauricus* Brandt). Eur. J. Histochem. 59, 2456. http://dx.doi.org/10.4081/ejh.2015.2456.
Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S., Wilson, E.M., 1988. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 240, 327–330.
Lucas, T.F.G., Royer, C., Siu, E.R., Lazari, M.F.M., Porto, C.S., 2010. Expression and signaling of G protein-coupled estrogen receptor 1 (GPER) in rat Sertoli cells. Biol. Reprod. 83, 307–317. http://dx.doi.org/10.1095/biolreprod.110.084160.
Lucas, T.F.G., Siu, E.R., Esteves, C.A., Monteiro, H.P., Oliveira, C.A., Porto, C.S., Lazari, M.F.M., 2008. 17beta-estradiol induces the translocation of the estrogen receptors ESR1 and ESR2 to the cell membrane, MAPK3/1 phosphorylation and proliferation of cultured immature rat Sertoli cells. Biol. Reprod. 78, 101–114. http://dx.doi.org/10.1095/biolreprod.107.063909.
Mäkinen, S., Mäkelä, S., Weihua, Z., Warner, M., Rosenlund, B., Salmi, S., Hovatta, O., Gustafsson, J.A., 2001. Localization of oestrogen receptors alpha and beta in human testis. Mol. Hum. Reprod. 7, 497–503.
Martoja, R., 1967. Initiation aux techniques de l'histologie animale. In: Martoja, R., Martoja-Pierson, M. (Eds.), Masson, Paris 343 pp.
McLachlan, R.I., Wreford, N.G., O'Donnell, L., de Kretser, D.M., Robertson, D.M., 1996. The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. J. Endocrinol. 148, 1–9. http://dx.doi.org/10.1677/JOE.0.1480001.
Menad, R., 2015. Régulation fonctionnelle de l'épididyme d'un rongeur déserticole. Thèse Dr. Alger 173 pp.
Menad, R., 2008. Régionalisation structurale et fonctionnelle de l'épididyme d'un rongeur déserticole diurne. 117 pp.
Menad, R., Smaï, S., Moudilou, E., Khammar, F., Exbrayat, J.-M., Gernigon-Spychalowicz, T., 2014. Immunolocalization of estrogen and androgen receptors in the caput epididymidis of the fat sand rat (*Psammomys obesus*): effects of seasonal variations, castration and efferent duct ligation. Acta Histochem. 116, 559–569. http://dx.doi.org/10.1016/j.acthis.2013.11.004.
Meng, J., Holdcraft, R.W., Shima, J.E., Griswold, M.D., Braun, R.E., 2005. Androgens regulate the permeability of the blood–testis barrier. Proc. Natl. Acad. Sci. U. S. A. 102, 16696–16700. http://dx.doi.org/10.1073/pnas.0506084102.
Mishra, D.P., Shaha, C., 2005. Estrogen-induced spermatogenic cell apoptosis occurs via the mitochondrial pathway: role of superoxide and nitric oxide. J. Biol. Chem. 280, 6181–6196. http://dx.doi.org/10.1074/jbc.M405970200.
Miura, T., Miura, C., Ohta, T., Nader, M.R., Todo, T., Yamauchi, K., 1999. Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys. Res. Commun. 264, 230–234. http://dx.doi.org/10.1006/bbrc.1999.1494.
Müller, G., Martino-Andrade, A.J., Santos, A.S., Reghelin, A.L., Garcia, D.M., Sant'Ana, G.R., Spercoski, K.M., Meyer, K.B., Torres, S.M., Silva Junior, V.A., Morais, R.N., 2012. Testicular testosterone: estradiol ratio in domestic cats and its relationship to spermatogenesis and epididymal sperm morphology. Theriogenology 78, 1224–1234. http://dx.doi.org/10.1016/j.theriogenology.2012.05.018.

Nitta, H., Bunick, D., Hess, R.A., Janulis, L., Newton, S.C., Millette, C.F., Osawa, Y., Shizuta, Y., Toda, K., Bahr, J.M., 1993. Germ cells of the mouse testis express P450 aromatase. Endocrinology 132, 1396–1401. http://dx.doi.org/10.1210/endo.132.3.8440194.

O'Donnell, L., Robertson, K.M., Jones, M.E., Simpson, E.R., 2001. Estrogen and spermatogenesis. Endocr. Rev. 22, 289–318. http://dx.doi.org/10.1210/edrv.22.3.0431.

Oliveira, R.L., Oliveira, A.G., Mahecha, G.A.B., Nogueira, J.C., Oliveira, C.A., 2009. Distribution of estrogen receptors (ERα and ERβ) and androgen receptor in the testis of big fruit-eating bat *Artibeus lituratus* is cell- and stage-specific and increases during gonadal regression. Gen. Comp. Endocrinol. 161, 283–292. http://dx.doi.org/10.1016/j.ygcen.2009.01.019.

O'Shaughnessy, P.J., 2014. Hormonal control of germ cell development and spermatogenesis. Semin. Cell Dev. Biol. 29, 55–65. http://dx.doi.org/10.1016/j.semcdb.2014.02.010.

Pak, T.R., Lynch, G.R., Tsai, P.-S., 2002. Estrogen accelerates gonadal recrudescence in photo-regressed male Siberian hamsters. Endocrinology 143, 4131–4134. http://dx.doi.org/10.1210/en.2002-220569.

Payne, A.H., Kelch, R.P., Musich, S.S., Halpern, M.E., 1976. Intratesticular site of aromatization in the human. J. Clin. Endocrinol. Metab. 42, 1081–1087. http://dx.doi.org/10.1210/jcem-42-6-1081.

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25, 947–970. http://dx.doi.org/10.1210/er.2003-0030.

Pelletier, G., El-Alfy, M., 2000. Immunocytochemical localization of estrogen receptors alpha and beta in the human reproductive organs. J. Clin. Endocrinol. Metab. 85, 4835–4840. http://dx.doi.org/10.1210/jcem.85.12.7029.

Pelletier, G., Labrie, C., Labrie, F., 2000. Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. J. Endocrinol. 165, 359–370. http://dx.doi.org/10.1677/JOE.0.1650359.

Pentikäinen, V., Erkkilä, K., Suomalainen, L., Parvinen, M., Dunkel, L., 2000. Estradiol acts as a germ cell survival factor in the human testis in vitro. J. Clin. Endocrinol. Metab. 85, 2057–2067. http://dx.doi.org/10.1210/jcem.85.5.6600.

Pierantoni, R., Cobellis, G., Meccariello, R., Cacciola, G., Chianese, R., Chioccarelli, T., Fasano, S., 2009. Testicular gonadotropin-releasing hormone activity, progression of spermatogenesis, and sperm transport in vertebrates. Ann. N. Y. Acad. Sci. 1163, 279–291. http://dx.doi.org/10.1111/j.1749-6632.2008.03617.x.

Prossnitz, E.R., Barton, M., 2009. Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER. Prostaglandins Other Lipid Mediat. 89, 89–97. http://dx.doi.org/10.1016/j.prostaglandins.2009.05.001.

Robertson, K.M., O'Donnell, L., Simpson, E.R., Jones, M.E.E., 2002. The phenotype of the aromatase knockout mouse reveals dietary phytoestrogens impact significantly on testis function. Endocrinology 143, 2913–2921. http://dx.doi.org/10.1210/endo.143.8.8957.

Saez, J.M., Sanchez, P., Berthelon, M.C., Avallet, O., 1989. Regulation of pig Leydig cell aromatase activity by gonadotropins and Sertoli cells. Biol. Reprod. 41, 813–820. http://dx.doi.org/10.1095/biolreprod41.5.813.

Saunders, P.T., Sharpe, R.M., Williams, K., Macpherson, S., Urquart, H., Irvine, D.S., Millar, M.R., 2001. Differential expression of oestrogen receptor alpha and beta proteins in the testes and male reproductive system of human and non-human primates. Mol. Hum. Reprod. 7, 227–236.

Saunders, P., Fisher, J., Sharpe, R., Millar, M., 1998. Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis. J. Endocrinol. 156, R13–R17. http://dx.doi.org/10.1677/joe.0.156R013.

Scaia, M.F., Volonteri, M.C., Czuchlej, S.C., Ceballos, N.R., 2015. Effect of estradiol on apoptosis, proliferation and steroidogenic enzymes in the testes of the toad *Rhinella arenarum* (Amphibia, Anura). Gen. Comp. Endocrinol. 221, 244–254. http://dx.doi.org/10.1016/j.ygcen.2014.12.016.

Schön, J., Blottner, S., 2008. Estrogens are involved in seasonal regulation of spermatogenesis and sperm maturation in roe deer (*Capreolus capreolus*). Gen. Comp. Endocrinol. 159, 257–263. http://dx.doi.org/10.1016/j.ygcen.2008.09.008.

Silandre, D., Delalande, C., Durand, P., Carreau, S., 2007. Three promoters PII, PI.f, and PI.tr direct the expression of aromatase (cyp19) gene in male rat germ cells. J. Mol. Endocrinol. 39, 169–181. http://dx.doi.org/10.1677/JME-07-0046.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15, 342–355. http://dx.doi.org/10.1210/edrv-15-3-342.

Singh, J., O'Neill, C., Handelsman, D.J., 1995. Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. Endocrinology 136, 5311–5321. http://dx.doi.org/10.1210/endo.136.12.7588276.

Sirianni, R., Chimento, A., Ruggiero, C., De Luca, A., Lappano, R., Andò, S., Maggiolini, M., Pezzi, V., 2008. The novel estrogen receptor, G protein-coupled receptor 30, mediates the proliferative effects induced by 17beta-estradiol on mouse spermatogonial GC-1 cell line. Endocrinology 149, 5043–5051. http://dx.doi.org/10.1210/en.2007-1593.

Smith, L.B., Walker, W.H., O'Donnell, L., 2015. Hormonal regulation of spermatogenesis through Sertoli cells by androgens and estrogens. Sertoli Cell Biol. http://dx.doi.org/10.1016/B978-0-12-417047-6.00006-5.

Tsubota, T., Howell-Skalla, L., Nitta, H., Osawa, Y., Mason, J.I., Meiers, P.G., Nelson, R.A., Bahr, J.M., 1997. Seasonal changes in spermatogenesis and testicular steroidogenesis in the male black bear *Ursus americanus*. J. Reprod. Fertil. 109, 21–27.

Tsubota, T., Nitta, H., Osawa, Y., Mason, J.I., Kita, I., Tiba, T., Bahr, J.M., 1993. Immunolocalization of steroidogenic enzymes, P450scc, 3 beta-HSD, P450c17, and P450arom in the Hokkaido brown bear (*Ursus arctos* yesoensis) testis. Gen. Comp. Endocrinol. 92, 439–444. http://dx.doi.org/10.1006/gcen.1993.1180.

Van der Molen, H.J., Brinkmann, A.O., de Jong, F.H., Rommerts, F.F., 1981. Testicular oestrogens. J. Endocrinol. 89 (Suppl), 33P–46P.

Walczak-Jedrzejowska, R., Slowikowska-Hilczer, J., Marchlewsk, K., Oszukowska, E., Kula, K., 2007. During seminiferous tubule maturation testosterone and synergistic action of FSH with estradiol support germ cell survival while estradiol alone has pro-apoptotic effect. Folia Histochem. Cytobiol. 45 (Suppl. 1), S59–S64.

Willmer, P., Stone, G., Johnston, I., 2005. Environmental Physiology of Animals. Blackwell Publishinghttp://dx.doi.org/10.1007/s13398-014-0173-7.2.

Wisniewski, P., Romano, R.M., Kizys, M.M.L., Oliveira, K.C., Kasamatsu, T., Giannocco, G., Chiamolera, M.I., Dias-da-Silva, M.R., Romano, M.A., 2015. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic–pituitary–testicular axis. Toxicology 329, 1–9. http://dx.doi.org/10.1016/j.tox.2015.01.002.

Yen, S.S.C., Tsai, C.C., 1971. The biphasic pattern in the feedback action of ethinyl estradiol on the release of pituitary FSH and LH. J. Clin. Endocrinol. Metab. 33, 882–887. http://dx.doi.org/10.1210/jcem-33-6-882.

Zarzycka, M., Gorowska-Wojtowicz, E., Tworzydlo, W., Klak, A., Kozub, K., Hejmej, A., Bilinska, B., Kotula-Balak, M., 2016. Are aryl hydrocarbon receptor and G-protein-coupled receptor 30 involved in the regulation of seasonal testis activity in photosensitive rodent—the bank vole (*Myodes glareolus*)? Theriogenology 86http://dx.doi.org/10.1016/j.theriogenology.2016.02.019. 674.e1–686.e1.

Zhang, H., Sheng, X., Hu, X., Li, X., Xu, H., Zhang, M., Li, B., Xu, M., Weng, Q., Zhang, Z., Taya, K., 2010. Seasonal changes in spermatogenesis and immunolocalization of cytochrome P450 17alpha-hydroxylase/c17-20 lyase and cytochrome P450 aromatase in the wild male ground squirrel (*Citellus dauricus* Brandt). J. Reprod. Dev. 56, 297–302.

Zhou, Q., Nie, R., Prins, G.S., Saunders, P.T.K., Katzenellenbogen, B.S., Hess, R.A., 2002. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J. Androl. 23, 870–881. http://dx.doi.org/10.1002/j.1939-4640.2002.tb02345.x.