CSIRO PUBLISHING

Reproduction, Fertility and Development
https://doi.org/10.1071/RD20293

Steroidogenesis during prenatal testicular development in Spix's cavy *Galea spixii*

A. C. Santos[A], *A. J. Conley*[B], *M. F. Oliveira*[C] *and A. C. Assis Neto* [A,D]

[A]School of Veterinary Medicine and Animal Science, University of Sao Paulo. Av. Prof. Dr. Orlando de Marques Paiva, 87; ZC 05508 270; São Paulo – Brazil.
[B]Population Health & Reproduction, School of Veterinary Medicine, University of California, 3223 VM3B, Davis, CA 95616, USA.
[C]Department of Animal Science, Federal Rural University of Semiarid. Av. Francisco Mota, 572, 59625 900, Mossoro, Rio Grande do Norte, Brazil.
[D]Corresponding author. Email: antonioassis@usp.br

Abstract. Spix's cavy is a potentially good experimental model for research on reproductive biology and sexual development. The aim of the present study was to evaluate the ontogeny of the steroidogenic enzymes involved in testicular androgen synthesis during prenatal development. Testes were investigated on Days 25, 30, 40 and >50 of gestation. Immunohistochemistry and immunoblotting were used to establish the site and relative amount of androgenic enzymes, including 5α-reductase, cytosolic 17β-hydroxysteroid dehydrogenase (17β-HSDI) and mitochondrial microsomal 3β-hydroxysteroid dehydrogenase (3β-HSDII), throughout prenatal development. The testicular parenchyma began to organise on Day 25 of gestation, with the development of recognisable testicular cords. The mesonephros was established after Day 25 of gestation and the ducts differentiated to form the epididymis, as testicular cords were beginning to proliferate and the interstitium to organise by Day 30 of gestation, continuing thereafter. The androgen-synthesising enzymes 5α-reductase, 17β-HSDI and 3β-HSDII were evident in Leydig cells as they differentiated at all subsequent gestational ages studied. In addition, immunoblotting showed an increase in immunoreactivity for the enzymes at Days 30 and 40 of gestation ($P < 0.05$) and a decrease at Day 50 of gestation ($P < 0.05$). It is concluded that the increase in androgenic enzymes in Leydig cells coincides with the functional differentiation of the testes, and with the stabilisation and differentiation of mesonephric ducts forming the epididymis.

Keywords: androgens, experimental models, gonads, rodents, steroidogenic enzymes.

Received 7 November 2020, accepted 28 January 2021, published online 9 March 2021

Introduction

Recent studies on the reproductive biology of Spix's cavy *Galea spixii* (Wagler, 1831) suggest that this species may be an excellent model for experimental research into reproductive development (Oliveira *et al.* 2008, 2012; Santos *et al.* 2012, 2017*a*, 2017*b*). In females, the duration of the oestrous cycle was determined and the vaginal tissue was found to have the capacity to synthesise and metabolise steroids locally throughout the phases of the oestrous cycle (Santos *et al.* 2017*a*). Other studies have shown that the female genitalia have some characteristics of male development, including urethral canalisation of the genital tubercle and clitoris (Santos *et al.* 2014*a*, 2018). To date, most attention has focused on spermatogenesis in males because of the interest in developing reproductive technologies in this species and in establishing captive breeding programs (Santos *et al.* 2012, 2014*b*, 2015, 2017*b*). Puberty in Spix's cavy occurs at around 45 days of postnatal life (Santos *et al.* 2012). Previous studies have shown that enzymes involved in sex steroid synthesis, such as cytochromes P450 17α-hydroxylase/17,20-lyase (P450c17) and aromatase (P450arom), which are responsible for the conversion of progestagens to androgens and androgens to oestrogens respectively, are present in the testes at all stages of postnatal sexual development (immature, prepubertal, pubertal and postpubertal; Santos *et al.* 2017*b*). By Day 25 of gestation, differentiation of the gonads in males and females has already started, followed by the internal and external genitalia (Santos *et al.* 2018).

The development of the genital organs and spermatogenesis in males is directly affected by androgen synthesis and expression of the androgen receptor in target tissues (Weng *et al.* 2005; Arnold 2009; Nakamura 2010; Song *et al.* 2012; Santos *et al.* 2017*b*). Basic understanding of sexual differentiation in mammals is based on the work of Jost (1965) and Phoenix *et al.* (1959). Since then, new concepts have

emerged regarding the genetic determinates of sexual differentiation in mammals from experimental models (Arnold 2009; Arnold and Chen 2009; Nakamura 2010; Cunha *et al.* 2014; Morselli *et al.* 2016). Knowledge about the process regulating the hormonal mechanisms that modulate this sexual differentiation is still under investigation, because there are species differences in the sources of sex steroids and even the pathways by which they are synthesised (Conley and Bird 1997; Wilson *et al.* 2003). This subject has remains of interest because intersexuality and the morphological diversity of the phenotype of genital organs is under continual study (Tannour-Louet *et al.* 2014; Zhao *et al.* 2017). In addition, some authors have focused on the effect of maternal diet on testicular development and sex steroid synthesis (Mossa *et al.* 2018).

Due to the challenges of conducting human studies (Cunha *et al.* 2016), animal models remain of continued interest. As a basis for the study of sexual differentiation, many researchers have focused on hormone sources during prenatal testicular development in different mammals (Zurita *et al.* 2003; Weng *et al.* 2005; Browne *et al.* 2006; Antonio-Rubio *et al.* 2011; Song *et al.* 2012). In cavies, the postnatal steroidogenic processes (Santos *et al.* 2017*b*) and the morphological aspects of sexual differentiation (Santos *et al.* 2018) have been reported. Thus, in order to complement knowledge regarding the complexity of the steroidogenic processes involving androgen synthesis during prenatal development, the objective of this work was to evaluate the immunolocalisation of steroidogenic enzymes involved in the production of androgens during testicular development in Spix's cavy.

Materials and methods

Animals

Pregnancies were established by pairing 10 female with 10 male *G. spixii*. Seven fetuses at each stage of development (i.e. at Days 25, 30, 40 and 50–52 of gestation) were collected and processed, as described below. All specimens used in the study came from the Wild Animal Multiplication Center of the Federal Rural University of the Semiarid, located in Mossoró (Brazil). The collections were authorised by the Brazilian Institute of Environment (1478912/2011) and the research was authorised by the Ethics Committee of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (Protocol 2923/2013).

Animals were distributed in 2.5-m^2 boxes ($n = 1$ female and 1 male per box) with a sand floor, fenced with wire mesh and covered with an asbestos tile. Mounds of grass were made available for the females to make their nests. Cavies were fed using a commercial rabbit feed, corn and fruits typical of the region, with water provided *ad libitum*.

From the first day of pairing, cavies were monitored daily by exfoliative vaginal cytology. Copulation was confirmed by the presence of spermatozoa in vaginal smears transferred to microscopic slides and stained by the rapid-panoptic method (Laborclin). The time of detection of spermatozoa in the vaginal smear was designated as Day 0 of gestation and fetuses were collected on Days 25, 30, 40 and >50 of gestation near parturition (range 50–52 days of gestation). Collections from Day 25 of gestation were based on the previously reported timing of the initiation of sexual differentiation in this species (Santos *et al.* 2015). At each stage of gestation, pregnant females were anaesthetised with xylazine hydrochloride 2% (40 mg kg^{-1}, i.m.) and ketamine hydrochloride 1% (60 mg kg^{-1}, i.m.) and then killed by administration of 2.5% sodium thiopental (60 mg kg^{-1}) via intracardiac injection.

Light microscopy and immunohistochemistry

All tissue samples from gonads from fetuses and adult testicles were fixed in 4% paraformaldehyde buffer solution for 48 h and then dehydrated in increasing concentrations of ethanol (50–100%) before being cleared in xylol for subsequent paraffin embedding. Thereafter, 5 μm slices were obtained using a Leica RM2165 microtome. For light microscopy, samples were stained with haematoxylin and eosin and observed and photographed under an Olympus BX61VS microscope. For immunohistochemistry, tissue samples were fixed in 4% paraformaldehyde buffer solution for 48 h and then dehydrated in increasing concentrations of ethanol (50–100%) before being cleared in xylol for subsequent paraffin embedding. Thereafter, 5 μm slices were obtained using a Leica RM2165 microtome. Then, samples were cleared and dehydrated before antigen retrieval. Antigen retrieval was performed by boiling the samples in citrate buffer in a microwave. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in a dark room for 20 min at room temperature (20°C), and samples were then blocked with Protein Block (Vector Laboratories). The samples were then incubated for 16 h with primary polyclonal antibodies (5α-reductase, 3β-HSDII and 17β-HSDI) at 4°C.

The negative controls for the 5α-reductase and mitochondrial microsomal 3β-hydroxysteroid dehydrogenase (3β-HSDII) antisera used a blocking synthetic peptide produced by RHEA-BIOTECH. As a negative control for cytosolic 17β-hydroxysteroid dehydrogenase (17β-HSDI), phosphate buffer was used instead of the primary antibody. Adult testes were used for comparison with fetal tissues and as a positive control. Immunoreactivity was detected using the biotinylated secondary antibody Immpress Universal Kit (Vector Laboratories). The samples were then washed in phosphate buffer solution and developed with IMMPACT DAB (Vector Laboratories). The slides were stained with haematoxylin and mounted with glue and coverslips for observation and photographic documentation under an Olympus BX61VS microscope.

Collection of material for immunoblotting

To analyse the presence of and quantify immunoreactivity for steroidogenic enzymes, fetal and adult testes were frozen in nitrogen and stored at −80°C until analysis. Adult testes were used for comparison with fetal tissues and as a positive control.

Protein extraction for immunoblotting

Fragments of fetal and adult testes were placed in tubes (Falcon) and homogenised using a Polytron homogeniser (PT 3000 KINEMATICA; Brinkman) in hypotonic lysis buffer containing 10 nM Tris-HCl and 5.5 nM EDTA, pH 7.4, in the presence of a

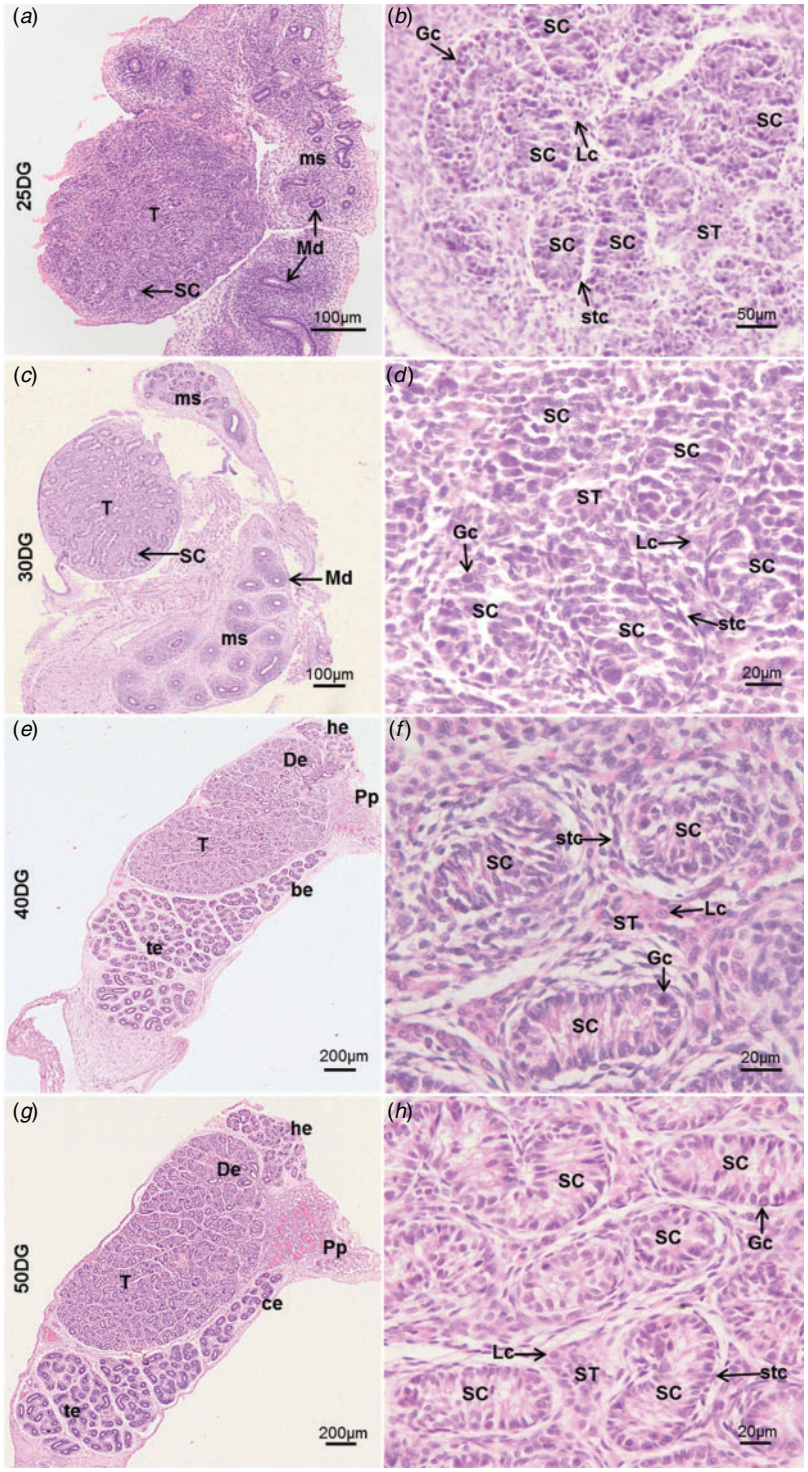

Fig. 1. Morphological organisation of the testes on Days 25 (*a*, *b*), 30 (*c*, *d*), 40 (*e*, *f*) and 50 (*g*, *h*) of gestation (haematoxylin and eosin staining). De, efferent ductules; DG, days of gestation; Gc, gonocytes; Lc, Leydig cells; Md, mesonephric duct; ms, mesonephros; Pp, pampiniform plexus; SC, seminiferous cord; ST, stroma; stc, support cells; T, testis.

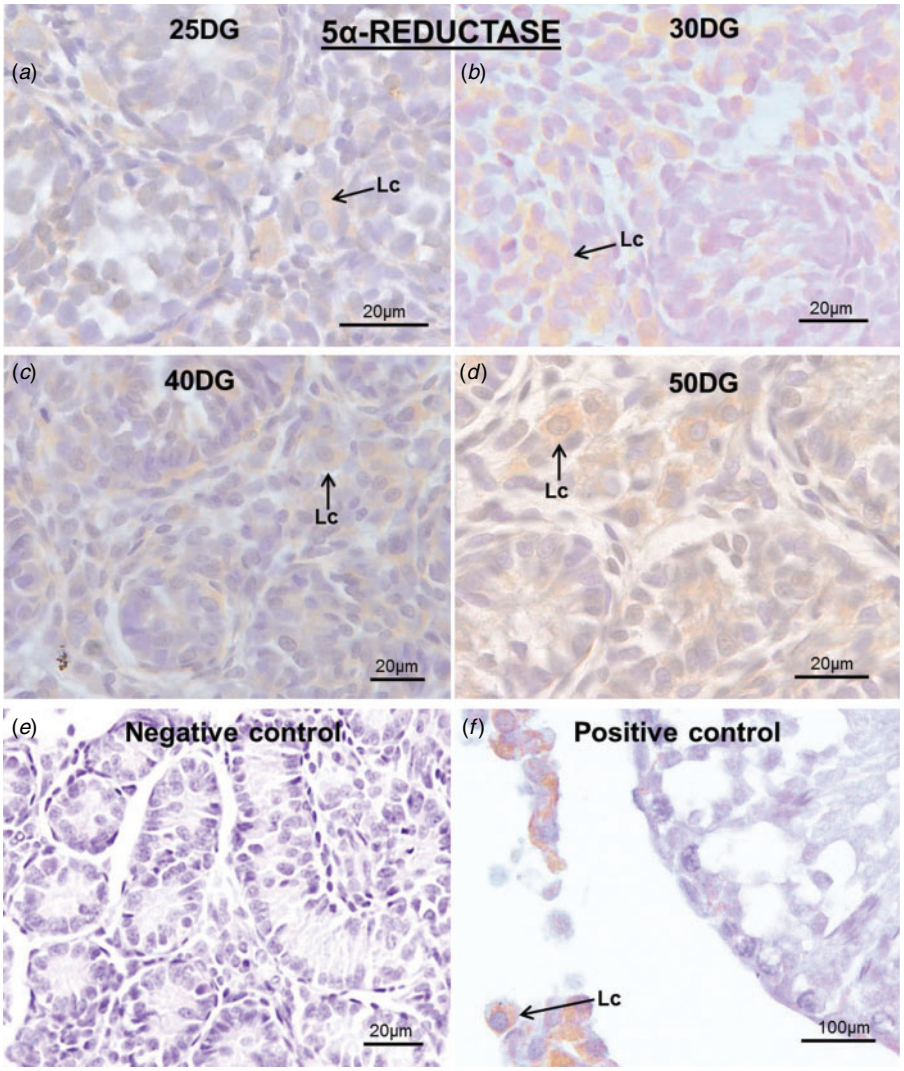

Fig. 2. Immunolocalisation of 5α-reductase in the developing testis on Days 25, 30, 40 and 50 of gestation (DG). Note the immunoreactivity in Leydig cells (Lc) in the fetal testes and in the adult testis used as positive control.

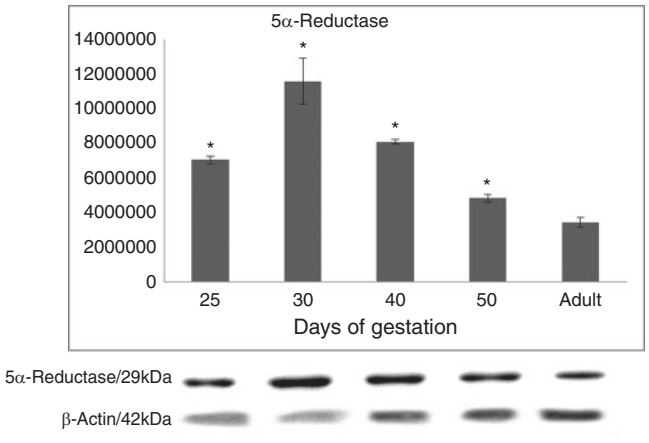

Fig. 3. Chemiluminescence quantification of 5α-reductase (in arbitrary units). Data are the mean ± s.d. $*P < 0.05$ compared with adult testes (Tukey's test).

mixture of protease inhibitors (20 μg mL^{-1} benzamidine, 1 μg mL^{-1} pepstatin, 0.5 μg mL^{-1} leupeptin, 0.1 μg mL^{-1} apoprotein and 100 μg mL^{-1} phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 1000*g* for 10 min at 4°C and the supernatant was transferred to 1.5-mL tubes and stored at −20°C. The total protein concentration of was analysed according to the method of Bradford (1976) using a commercially available kit (Protein Assay Kit; Bio-Rad). Spectrophotometer measurements obtained at 595 nm for samples were compared against a standard curve constructed for albumin.

Immunoblotting

Total proteins (20 μg) from each sample were denatured by treatment with 20 μL Laemmli buffer (15% glycerol, 0.05 M Tris, 0.055 bromophenol blue, 9% sodium dodecyl sulfate (SDS)) containing 6% β-mercaptoethanol and heated at 95°C for 5 min. The samples were then electrophoretically separated

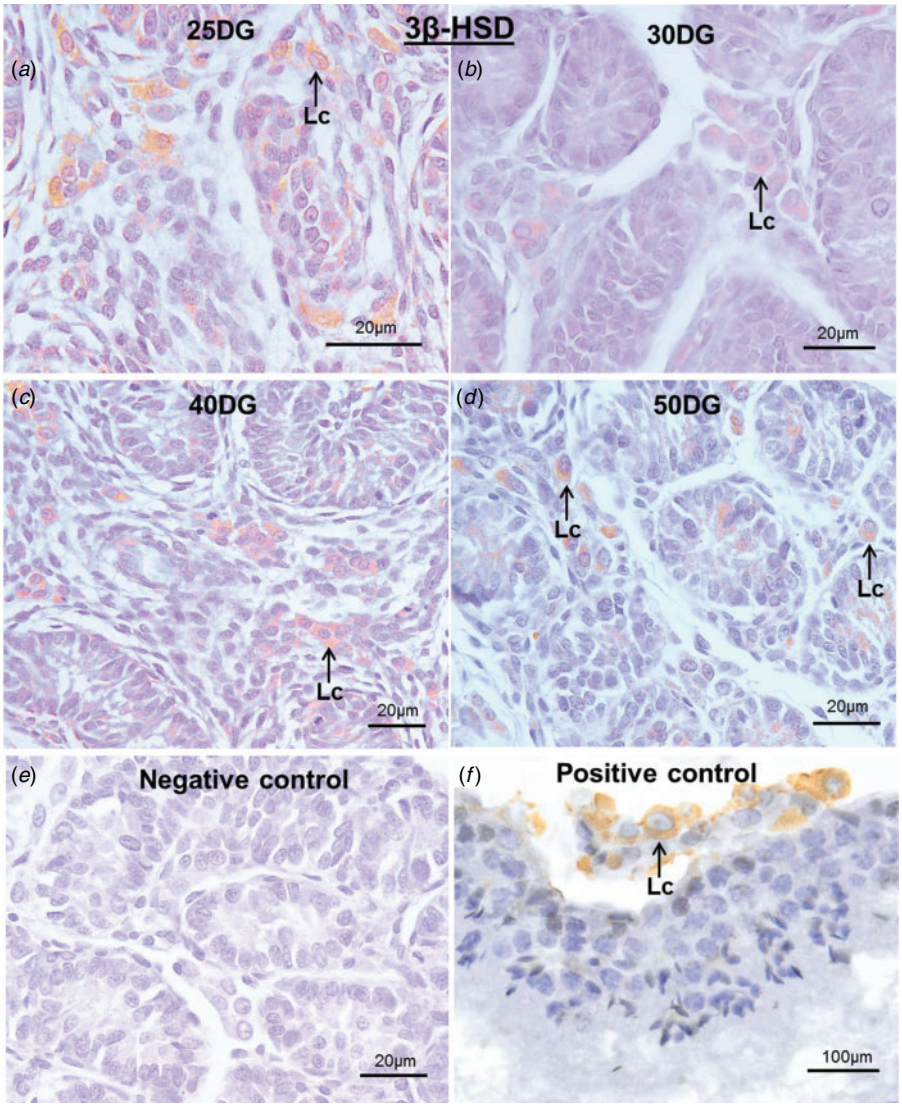

Fig. 4. Immunolocalisation of 3β-HSDII in the fetal testes on Days 25, 30, 40 and 50 of gestation (DG). Note the immunoreactivity in Leydig cells (Lc) in the fetal and adult testes (positive control).

on an SDS–polyacrylamide gel. After separation, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) under a constant current of 120 mA for 2 h at 4°C and immersed in Tris-HCl buffer (Tris 12.5 mM, 100 mM glycine, 1% SDS and 20% methanol). After transfer, non-specific antigen sites were blocked by Tris-buffered saline Tween-20 (TBST; 1% Tween 20) and skimmed milk (5%) for 2 h at room temperature (20–25°C) with constant agitation. Samples were then incubated with primary antibodies diluted in TBST and 3% albumin at 4°C for 12 h with constant shaking.

After incubation with primary antisera (5α-reductase, 3β-HSDII and 17β-HSDI), the samples were incubated with peroxidase-conjugated secondary antibody diluted 1:1000 (anti-mouse or anti-rabbit IgG, depending on the specificity of the primary antibody; Bio-Rad) in TBST with 1% albumin for 2 h at room temperature with constant stirring. Conjugated β-actin, anti-mouse (Bio-Rad), diluted 1:1000 in TBST with 1% albumin for 30 min (time of incubation), was used as an endogenous control. The signal was detected by adding substrate to CLARITY peroxidase (Bio-Rad) for 5 min, which produced a luminescence reaction at the antibody-binding site. Samples transferred to PVDF membranes were analysed using a CHEMI-DOC apparatus (Bio-Rad). Bands corresponding to each protein studied were quantified using Image Laboratory 4.01 software (Bio-Rad) and the expression of proteins of interest was quantified in arbitrary units (AU) in relation to β-actin.

Statistical analysis of protein quantification by immunoblotting

The amount of testis proteins was compared between different age groups. Variance analysis using GraphPad InStat was used to obtain the mean ± s.d. of samples of the same age. The

Cramér–von Mises test was used to determine the normality of distribution and homoscedasticity of variables. Tukey's test was used for comparisons between different age groups.

Results

Morphological development of the testes

At Day 25 of gestation it was possible to observe testicular cords in the periphery of the testicular parenchyma. No lumen was present in the testicular cords. Two types of undifferentiated cells, the gonocytes and supporting cells, were found. In the stroma, Leydig cells were easily visible. The mesonephros had ducts scattered throughout the parenchyma (Fig. 1*a*, *b*).

At Day 30 of gestation the testicular cords were more evident and present throughout the testicular parenchyma. Gonocytes were present in these cords, surrounded by undifferentiated support cells. Leydig cells were easily visible in the unorganised stroma. At this stage, it was not yet possible to distinguish the caput, corpus and cauda epididymidis differentiating from the mesonephros (Fig. 1*c*, *d*).

At Day 40 of gestation, it was possible to observe more organised testicular cords. The testicular stroma began to become more organised and delimited between the testicular cords. During this period, the gonocytes and undifferentiated support cells were observed in the testicular cords. Leydig cells were present in the stroma. The mesonephric ducts had differentiated into the epididymis. During this period, it was possible to establish a regional division of the epididymis (Fig. 1*e*, *f*).

After Day 50 of gestation, testicular cords were present throughout the testis and the stroma between them was more compact. Gonocytes and supporting cells formed the testicular cords. Leydig cells from the stroma were organised at the border of the testicular cords. During this period, the epididymis was divided into the caput, corpus and cauda (Fig. 1*g*, *h*).

Immunohistochemistry and immunoblotting of the testes

Immunological techniques for the detection of steroidogenic enzymes at Days 25, 30, 40 and >50 of gestation revealed that 5α-reductase was present in the testis in all periods studied. This enzyme was present in the Leydig cells of the developing testes and in the adult testis tissue, used as a positive control (Fig. 2). An immunoreactive band at 29 kDa was detectable by western immunoblotting. Immunoblotting also demonstrated that 5α-reductase levels were higher in the developing testes on Day 30 of gestation ($P < 0.05$), with lower levels on Days 40 and >50 of gestation ($P < 0.05$ for both). 5α-Reductase levels in the adult testis were lower level than in the fetal testes, regardless of gestational age (Fig. 3).

3β-HSDII was present in Leydig cells at all ages studied. Antibody specificity was confirmed by detection of the enzyme in the Leydig cells of the adult testis (Fig. 4). An immunoreactive band was detected at 42 kDa, as suggested by manufacturer (RHEABIOTEC, Campinas, SP, Brazil). 3β-HSDII was detected at higher levels in the developing testes at Day 30 of gestation ($P < 0.05$). 3β-HSDII levels were stable up to Day 40 of gestation ($P > 0.05$), then decreased at >Day 50 of gestation ($P < 0.05$). The adult testis had higher 3β-HSDII levels that the fetal testes, regardless of gestational age (Fig. 5).

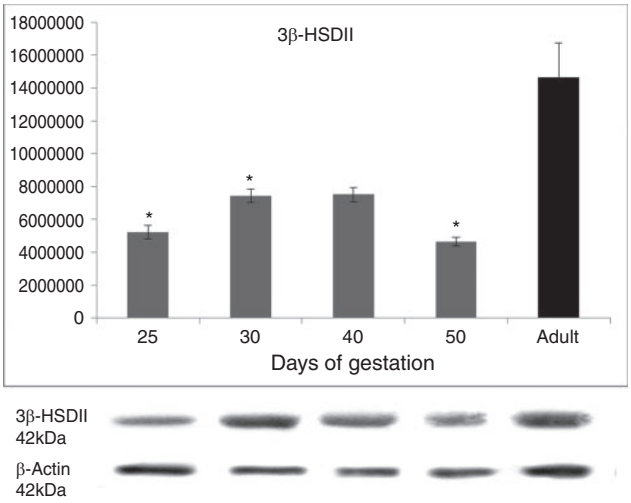

Fig. 5. Chemiluminescence quantification 3β-HSDII (in arbitrary units). Data are the mean ± s.d. *$P < 0.05$ compared with adult testes (Tukey's test).

17β-HSDI was detected in Leydig cells at all ages studied. Leydig cells from adult testis were also immunoreactive to the antibody used, confirming its specificity (Fig. 6). An immunoreactive band was detected at 35 kDa. 17β-HSDI levels were higher on Day 30 than on Day 25 of gestation ($P < 0.05$). There was a slight decrease in 17β-HSDI levels on Day 40 of gestation ($P < 0.05$) and another more evident decrease at the end of gestation. The adult testis had higher 17β-HSDI levels than all fetal testes, regardless of gestational age (Fig. 7).

Discussion

This is the first study to describe the expression of enzymes necessary for testosterone synthesis in Spix's cavy during the prenatal period. The results increase our knowledge of the development of fetal testicular testosterone synthesis necessary for masculinisation and defeminisation of males. Prior studies on testicular steroidogenesis in this species investigated the period of development from birth to puberty (Santos *et al.* 2017*b*). The results of the present study, together with those published previously (Santos *et al.* 2018), demonstrate that Leydig cell differentiation involves the expression of all enzymes necessary for testosterone and dihydrotestosterone synthesis, including 3β-HSDII, 17β-HSDI and 5α-reductase, during the development of the testes. The detection of higher enzyme levels by immunoblotting on Days 30 and 40 of gestation coincides with the periods of greater testicular differentiation and with the differentiation of the epididymis. Thus, our results agree with those of Song *et al.* (2012), who stated that androgens produced in the developing testes function in the differentiation and formation of the internal genitalia through a local action. Murashima *et al.* (2015) added that androgens are also responsible for virilisation of the external genitalia in males.

The location of enzymes involved in androgen synthesis in Leydig cells is consistent with the study conducted in Spix's cavy during the postnatal period (Santos *et al.* 2017*b*) and in

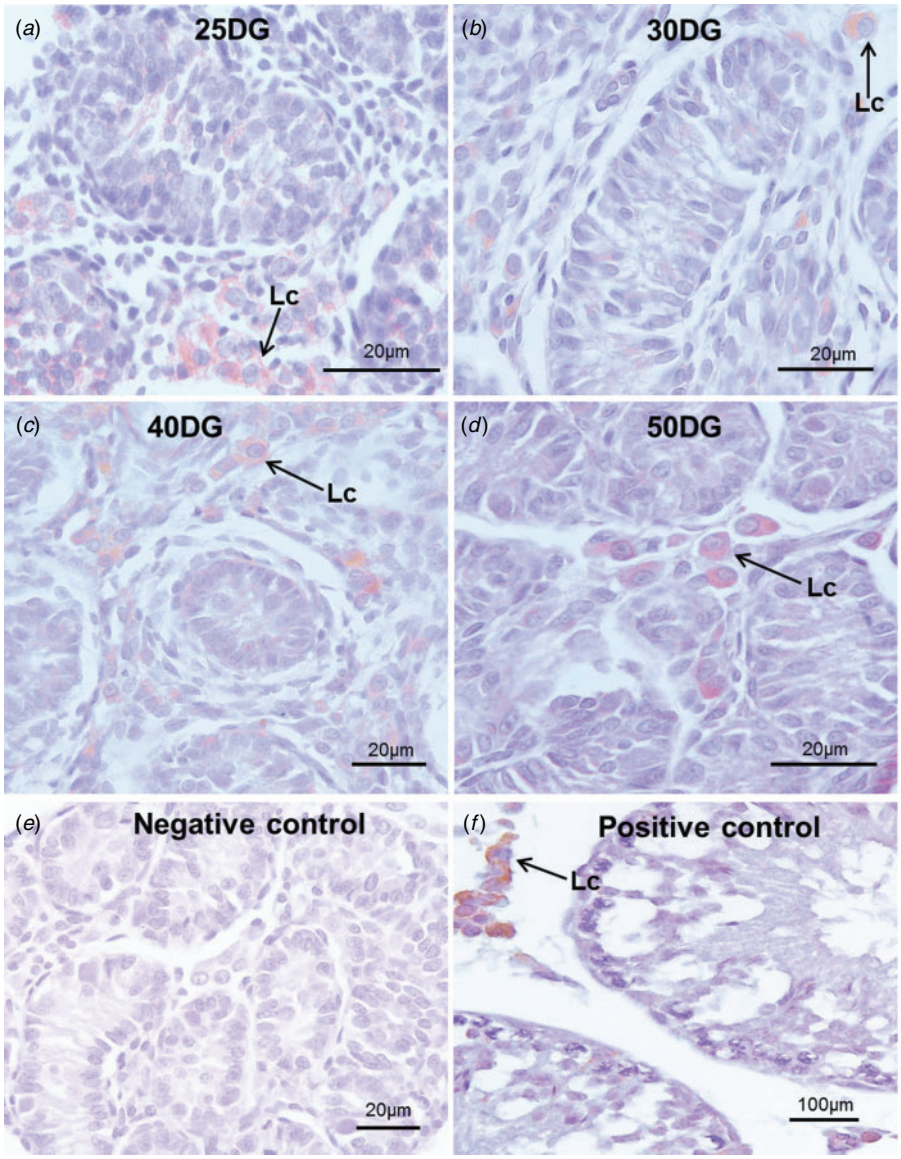

Fig. 6. Immunolocalisation of 17β-HSDI in the fetal testes on Days 25, 30, 40 and 50 of gestation (DG). Note the immunoreactivity in Leydig cells (Lc) in the fetal and adult testes (positive control).

other mammalian species, confirming that androgens are synthesised in the Leydig cells. Supporting cells and germ cells also express cytochrome P450c17 and P450 aromatase in Spix's cavy from birth to puberty (Santos *et al.* 2017*b*). The same immunolocalisation in the seminiferous epithelium was observed in the wild squirrel and wild raccoon dog (Qiang *et al.* 2003; Zhang *et al.* 2010).

The increased detection of 17β-HSDI in Leydig cells was coincident with stabilisation of the mesonephric ducts and presumably increased testosterone synthesis. The production of testosterone is required for the maintenance of the mesonephric ducts and their differentiation into the epididymis, as well as for the virilisation of the male external genitalia in mice (Song *et al.* 2012), domestic animals Sinowatz (2010) and humans (Moore and Persaud 2008). To the best of our knowledge, 17β-HSDI expression in the fetal testis has not previously described in any species.

In Spix's cavy, the increased detection of 5α-reductase in Leydig cells coincided with the organisation of the testicular cords and testicular interstitium. The higher 5α-reductase levels coincide with the virilisation period of the male external genitalia and channelling of the urethra in the genital tubercle to form the penis on Day 30 of gestation in Spix's cavy (Santos *et al.* 2018). Using cell culture, Murono *et al.* (1994) demonstrated that during the differentiation of Leydig cell precursors into mature Leydig cells, testosterone is rapidly converted by 5α-reductase into dihydrotestosterone. 5α-Reductase promotes sexual differentiation by converting testosterone to

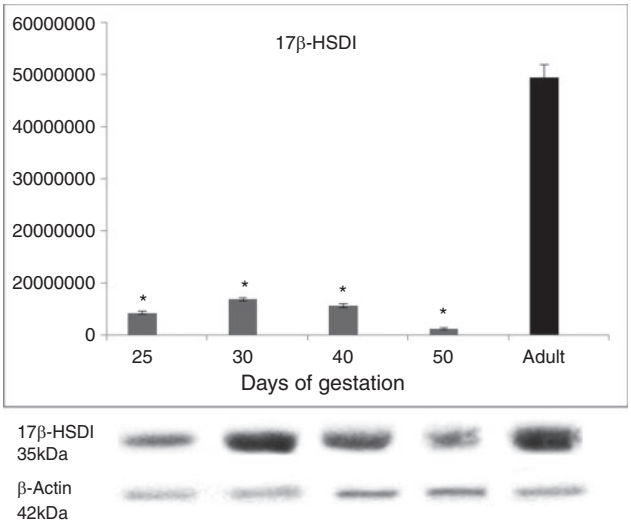

Fig. 7. Chemiluminescence quantification of 17β-HSDI (in arbitrary units). Data are the mean ± s.d. $*P < 0.05$ compared with adult testes (Tukey's test).

dihydrotestosterone, which is two- to threefold more potent than testosterone and is essential for masculinisation of the male external genitalia (Nakamura 2010; Murashima *et al.* 2015).

Lachance *et al.* (1990) demonstrated in human tissue cells that 3β-HSDI uses several potential substrates, catalysing the transformation of pregnenolone into progesterone, 17α-hydroxypregnenolone into 17α-hydroxyprogesterone, dehydroepiandrosterone into 4-androstenedione, and 5-androstene-3β,17β-diol into testosterone, which also fuels 5α-dihydrotestosterone synthesis and is therefore essential in the biosynthesis of all these classes of steroid hormones. In the present study, the highest levels of 3β-HSDII in Leydig cells were detected on Days 30 and 40 of gestation, suggesting an increase in the synthesis of sex steroid hormones. A similar expression profile was demonstrated in mice in the prenatal period (Antonio-Rubio *et al.* 2011). The increase of 3β-HSDII on Days 30–40 of gestation coincides with the period of greater tissue differentiation, suggesting that its presence is related to the tissue organisation of the developing testes and tubular tract. We cannot state exactly which hormones were produced in the present study, because 3β-HSDII oxidises several different substrates, as demonstrated by Lachance *et al.* (1990), but Murono *et al.* (1994) demonstrated the participation of 3β-HSDII in the differentiation of Leydig cells in culture.

The involvement of fetal and neonatal Sertoli cells in steroidogenesis has been demonstrated in rats, specifically with regard to oestrogen synthesis (Dorrington and Armstrong 1975; Weniger 1993). Oestradiol synthesis by the rat testes is stimulated by both LH and testosterone (Weniger 1993), but declines with increasing developmental age (Tsai-Morris *et al.* 1985). These data suggest that Leydig and Sertoli cells can contribute to and synergise in steroid synthesis by the testis during fetal development and after birth. Distinct lineages of Leydig cells with different steroidogenic potential populate the testes in fetal versus adult life (Shima and Morohashi 2017). Whether Sertoli cell differentiation represents a similar phenomenon is unknown, but testicular development is more complex than once thought and has been investigated in too few species. Expanding such studies to other rodents, and other mammalian species, will likely shed light on the general importance of such observations.

Conclusion

5α-Reductase, 17β-HSDI and 3β-HSDII were present in the Leydig cells of Spix's cavy in all ages studied. At Days 30 and 40 of gestation there was an increase in these enzymes, but at the end of gestation their levels decreased. The increase in the presence of androgenic enzymes coincides with the greater differentiation of the testes and with the stabilisation and tissue differentiation of the mesonephros to form the epididymis.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank the Foundation for Research Support of the State of São Paulo (FAPESP) for funding this study (FAPESP process 2013/02535-9/2016/24040-0).

References

Antonio-Rubio, N. R., Guerrero-Estévez, S. M., Lira-Romero, E., and Moreno-Mendoza, N. (2011). Expression of 3β-HSD1 and P450 Aromatase enzymes during mouse gonad differentiation. *J. Mol. Histol.* **42**, 535–543. doi:10.1007/S10735-011-9358-Y

Arnold, A. P. (2009). The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. *Horm. Behav.* **55**, 570–578. doi:10.1016/J.YHBEH.2009.03.011

Arnold, A. P., and Chen, X. (2009). What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues? *Front. Neuroendocrinol.* **30**, 1–9. doi:10.1016/J.YFRNE.2008.11.001

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254. doi:10.1016/0003-2697(76)90527-3

Browne, P., Place, N. J., Vidal, J. D., Moore, I. T., Cunha, G. R., Glickman, S. E., and Conley, A. J. (2006). Endocrine differentiation of fetal ovaries and testes of the spotted hyena (*Crocuta crocuta*): timing of androgen-independent versus androgen-driven genital development. *Reproduction* **132**, 649–659. doi:10.1530/REP.1.01120

Conley, A. J., and Bird, I. M. (1997). The role of cytochrome P450 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the delta 5 and delta 4 pathways of steroidogenesis in mammals. *Biol. Reprod.* **56**, 789–799. doi:10.1095/BIOLREPROD56.4.789

Cunha, G. R., Risbridger, G., Wang, H., Place, N. J., Grumbach, M., Cunha, T. J., Weldele, M., Conley, A. J., Barcellos, D., Agarwal, S., Bhargava, A., Drea, C., Hammond, G. L., Baskin, L. S., and Glickman, S. E. (2014). Development of the external genitalia: Perspectives from the spotted hyena (Crocuta crocuta). *Differentiation* **87**, 4–22. doi:10.1016/J.DIFF.2013.12.003

Cunha, G., Overland, M., Li, Y., Cao, M., Shen, J., Sinclair, A., and Baskin, L. (2016). Methods for studying human organogenesis. *Differentiation* **91**, 10–14. doi:10.1016/J.DIFF.2015.10.005

Dorrington, J. H., and Armstrong, D. T. (1975). Follicle-stimulating hormone stimulates estradiol-17beta synthesis in cultured Sertoli cells.

Proc. Natl. Acad. Sci. USA **72**, 2677–2681. doi:10.1073/PNAS.72.7.2677

Jost, A. (1965). Gonadal hormones in the sex differentiation of the mammalian fetus. In 'Organogenesis'. (Eds R. L. Urpsrung and H. Dehaan.) pp. 611–628. (Holt, Rinehart and Winston: New York, NY.)

Lachance, Y., Luu-The, V., Labrie, C., Simard, J., Dumont, M., Launoit, Y., Guqin, S., Leblanc, G., and Labrie, F. (1990). Characterization of human 3β Hydroxysteroid Dehydrogenase/Δ 5-Δ 4-Isomerase gene and its expression in mammalian cells. *J. Biol. Chem.* **265**, 20469–20475. doi:10.1016/S0021-9258(17)30528-8

Moore, K., and Persaud, T. V. N. The developing human: clinically oriented embryology. 8th ed. Philadelphia: Saunders/Elsevier; 2008:287–327.

Morselli, E., Frank, A. P., Santos, R. S., Fátima, L. A., Palmer, B. F., and Clegg, D. J. (2016). Sex and gender: critical variables in pre-clinical and clinical medical research. *Cell Metab.* **24**, 203–209. doi:10.1016/J.CMET.2016.07.017

Mossa, F., Bebbere, D., Ledda, A., Burrai, G. P., Chebli, I., Antuofermo, E., Ledda, S., Cannas, A., Fancello, F., and Atzori, A. S. (2018). Testicular development in male lambs prenatally exposed to a high-starch diet. *Mol. Reprod. Dev.* **85**, 406–416. doi:10.1002/MRD.22974

Murashima, A., Kishigami, S., Thomson, A., and Yamada, G. (2015). Androgens and mammalian male reproductive tract development. *Biochim. Biophys. Acta* **1849**, 163–170. doi:10.1016/J.BBAGRM.2014.05.020

Murono, E. P., Washburn, A. L., and Goforth, D. P. (1994). Enhanced stimulation of 5-α Reductase activity in cultured Leydig Cell precursors by Human Chorionic Gonadotropin. *J. Steroid Biochem. Mol. Biol.* **48**, 377–384. doi:10.1016/0960-0760(94)90078-7

Nakamura, M. (2010). The mechanism of sex determination in vertebrates-Are sex steroids the key-factor? *J. Exp. Zool. A Ecol. Genet. Physiol.* **313A**, 381–398. doi:10.1002/JEZ.616

Oliveira, M. F., Mess, A., Ambrósio, C. E., Dantas, C. A. G., Favaron, P. O., and Miglino, M. A. (2008). Chorioallantoic Placentation in *Galea Spixii* (Rodentia, Caviomorpha, Caviidae). *Reprod. Biol. Endocrinol.* **6**, 39. doi:10.1186/1477-7827-6-39

Oliveira, M. F., Vale, A. M., Favaron, P. O., Vasconcelos, B. G., Oliveira, G. B., Miglino, M. A., and Mess, A. (2012). Development of yolk sac inversion in *Galea spixii* and *Cavia porcellus* (Rodentia, Caviidae). *Placenta* **33**, 878–881. doi:10.1016/J.PLACENTA.2012.06.019

Phoenix, C. H., Goy, R. W., Gerall, A. A., and Young, W. C. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology* **65**, 369–382. doi:10.1210/ENDO-65-3-369

Qiang, W., Murase, T., and Tsubota, T. (2003). Seasonal changes in spermatogenesis and testicular steroidogenesis in wild male raccoon dogs (*Nyctereutes procynoides*). *J. Vet. Med. Sci.* **65**, 1087–1092. doi:10.1292/JVMS.65.1087

Santos, P. R. S., Oliveira, M. F., Silva, A. R., and Assis-Neto, A. C. (2012). Development of spermatogenesis in captive bred Spix's yellot-toothed (*Galea spixii*, Wagler, 1831). *Reprod. Fertil. Dev.* **24**, 877–885. doi:10.1071/RD12015

Santos, A. C., Oliveira, M. F., Viana, D. C., and Assis-Neto, A. C. (2014*a*). Sexual differentiation of the external genitalia in embryos and fetuses in the Spix's yellow toothed cavy (*Galea spixii*). *Placenta* **35**, A25. doi:10.1016/J.PLACENTA.2014.06.085

Santos, P. R. S., Oliveira, M. F., Arroyo, M. A. M., Silva, A. R., Rici, R. E. G., Miglino, M. A., and Assis-Neto, A. C. (2014*b*). Ultrastructure of spermatogenesis in Spix's Yellow-toothed cavy (*Galea spixii*). *Reproduction* **147**, 13–19. doi:10.1530/REP-13-0452

Santos, A. C., Viana, D. C., Bertassoli, B. M., Oliveira, G. B., Oliveira, D. M., Oliveira, M. F., Miglino, M. A., and Assis-Neto, A. C. (2015). Characterization of the estrous cycle in *Galea spixii* (Wagler, 1831). *Pesqui. Vet. Bras.* **35**, 89–94. doi:10.1590/S0100-736X2015000100017

Santos, A. C., Conley, A. J., Oliveira, M. F., Oliveira, G. B., Viana, D. C., and Assis-Neto, A. C. (2017*a*). Immunolocalization of steroidogenic enzymes in the vaginal mucous of *Galea spixii* during the estrous cycle. *Reprod. Biol. Endocrinol.* **15**, 30. doi:10.1186/S12958-017-0248-3

Santos, P. R. S., Oliveira, F. D., Arroyo, M. A. M., Oliveira, M. F., Castelucci, P., Conley, A. J., and Assis-Neto, A. C. (2017*b*). Steroidogenesis during postnatal testicular development of *Galea Spixii*. *Reproduction* **154**, 645–652. doi:10.1530/REP-17-0075

Santos, A. C., Conley, A. J., Oliveira, M. F., and Assis-Neto, A. C. (2018). Development of urogenital system in the Spix cavy: a model for studies on sexual differentiation. *Differentiation* **101**, 25–38. doi:10.1016/J.DIFF.2018.04.001

Shima, Y., and Morohashi, K. I. (2017). Leydig progenitor cells in fetal testis. *Mol. Cell. Endocrinol.* **445**, 55–64. doi:10.1016/J.MCE.2016.12.006

Sinowatz, F. Development of the urogenital system. In Hyttel P, Sinowatz F, Vejlsted M (eds), Essential of domestic animal embryology. Edinburgh-London-New York-Oxford-Philadelphia-St Louis-Sydney-Toronto: Saunders-Elsevier; 2010:252–283.

Song, C. H., Gong, E.-Y., Park, J. S., and Lee, K. (2012). Testicular steroidogenesis is locally regulated by androgen via suppression of Nur77. *Biochem. Biophys. Res. Commun.* **422**, 327–332. doi:10.1016/J.BBRC.2012.04.161

Tannour-Louet, M., Han, S., Louet, J.-F., Zhang, B., Romero, K., Addai, J., Sahin, A., Cheung, S. W., and Lamb, D. J. (2014). Increased gene copy number of VAMP7 disrupts human male urogenital development through altered estrogen action. *Nat. Med.* **20**, 715–724. doi:10.1038/NM.3580

Tsai-Morris, C. H., Aquilano, D. R., and Dufau, M. L. (1985). Cellular localization of rat testicular aromatase activity during development. *Endocrinology* **116**(1), 38–46. doi:10.1210/ENDO-116-1-38

Weng, Q., Medan, M. S., Ren, L.-Q., Watanabe, G., Tsubota, T., and Taya, K. (2005). Immunolocalization of steroidogenic enzymes in the fetal, neonatal e adult testis of the Shiba goat. *Exp. Anim.* **54**, 451–455. doi:10.1538/EXPANIM.54.451

Weniger, J. P. (1993). Estrogen production by fetal rat gonads. *J Steroid Biochem Mol Biol.* **44**, 459–462. doi:10.1016/0960-0760(93)90250-Z

Wilson, J. D., Auchus, R. J., Leihy, M. W., Guryev, O. L., Estabrook, R. W., Osborn, S. M., Shaw, G., and Renfree, M. B. (2003). 5alpha-androstane-3alpha,17beta-diol is formed in Tammar wallaby pouch young testes by a pathway involving 5alpha-pregnane-3alpha,17alpha-diol-20-one as a key intermediate. *Endocrinology* **144**, 575–580. doi:10.1210/EN.2002-220721

Zhang, H., Sheng, X., Xu, H., Li, X., Xu, H., Zhang, M., Li, B., Xu, M., Weng, Q., Zang, Z., and 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. doi:10.1262/JRD.09-078T

Zhao, F., Franco, H. L., Rodriguez, K. F., Brown, P. R., Tsai, M.-J., Tsai, S. Y., and Yao, H. H.-C. (2017). Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFII in mice. *Science* **357**, 717–720. doi:10.1126/SCIENCE.AAI9136

Zurita, F., Barrionuevo, F. J., Berta, P., Ortega, E., Burgos, M., and Jiménez, R. (2003). Abnormal sex - duct development in female moles: the role of anti-Müllerian hormone and testosterone. *Int. J. Dev. Biol.* **47**, 451–458.

Handling Editor: James Cummins