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Research paper

Postnatal testis development in the collared peccary (*Tayassu tajacu*), with emphasis on spermatogonial stem cells markers and niche

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ABSTRACT

Collared peccaries (*Tayassu tajacu*) present a unique testis cytoarchitecture, where Leydig cells (LC) are mainly located in cords around the seminiferous tubules (ST) lobes. This peculiar arrangement is very useful to better investigate and understand the role of LC in spermatogonial stem cells (SSCs) biology and niche. Recent studies from our laboratory using adult peccaries have shown that the undifferentiated type A spermatogonia (A_{und} or SSCs) are preferentially located in ST regions adjacent to the intertubular compartment without LC. Following these studies, our aims were to investigate the collared peccary postnatal testis development, from birth to adulthood, with emphasis on the establishment of LC cytoarchitecture and the SSCs niche. Our findings demonstrated that the unique LC cytoarchitecture is already present in the neonate peccary's testis, indicating that this arrangement is established during fetal development. Based on the most advanced germ cell type present at each time period evaluated, puberty (the first sperm release in the ST lumen) in this species was reached at around one year of age, being preceded by high levels of estradiol and testosterone and the end of Sertoli cell proliferation. Almost all gonocytes and SSCs expressed Nanos1, Nanos2 and GFRA1. The analysis of SSCs preferential location indicated that the establishment of SSCs niche is coincident with the occurrence of puberty. Taken together, our findings reinforced and extended the importance of the collared peccary as an animal model to investigate testis function in mammals, particularly the aspects related to testis organogenesis and the SSCs biology and niche.

1. Introduction

Testis development requires an intricate coordination of signaling and factors produced by the gonad somatic and germ cells (Ross and Capel, 2005; Svingen and Koopman, 2013). In all vertebrates investigated up to date, the Sertoli cells are the first somatic cells to differentiate in the testis, where they enclose gonocytes, forming nests that are encircled by the surrounding peritubular myoid cells, resulting in the seminiferous cords (Wilhelm et al., 2007; Yao et al., 2015). Outside these cords, mesenchymal cells differentiate into Leydig cells that colonize the interstitial compartment, initiating androgen production and genitalia masculinisation (DeFalco et al., 2011; Shima and Morohashi, 2017). In most studied mammals, Sertoli cell proliferation, which occurs from fetal life to pre-puberty, is one of the key events during testis development and growth, determining ultimately the magnitude of sperm production (Griswold, 2015; Picut et al., 2015; França et al., 2016).

The collared peccary (*Tayassu tajacu*) is an endangered suiform species with a high commercial value, especially concerning its flesh and pelt (Bodmer et al., 1997; Mayor et al., 2007). Studies in our laboratory (Costa et al., 2010) have demonstrated that this species shows a unique testis cytoarchitecture, in which the Leydig cells form cords surrounding the seminiferous tubules lobes and thus are not present in some interstitial areas between the tubules. Therefore, this particular arrangement allowed us to use the peccary as a valuable animal model to investigate spermatogonial stem cells (SSCs) biology and niche, specifically in relation to the role of Leydig cells (Campos-Junior et al., 2012, 2014).

SSCs originate from gonocytes and are represented by undifferentiated spermatogonia (A_{und}) population that consists of single (A_s), paired (A_{pr}) and aligned (A_{al}) type A spermatogonia (Nakagawa et al., 2010; Oatley and Brinster, 2012; Lord and Oatley, 2017; de Rooij, 2017). As SSCs are considered the progenitors and/or amplifying cells of spermatogenesis, the existence of these cells population is essential to

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maintain continuous sperm production and male fertility (de Rooij and Russell, 2000; Nakagawa et al., 2010; Phillips et al., 2010; de Rooij, 2017). Studies performed mainly in mice have shown that these cells present expression of self-renewal factors, such as GFRA1 (GDNF family receptor alpha-1), Nanos1 (Nanos homolog 1) and Nanos2 (Nanos homolog 2), among many others (Jaruzelska et al., 2003; Tsuda et al., 2003; Suzuki et al., 2009; Nakagawa et al., 2010; Yoshida, 2012; Campos-Junior et al., 2013; Pui and Saga, 2018). In another very important aspect related to spermatogenesis, several studies demonstrated that SSCs are preferentially located in the seminiferous epithelium adjacent to the interstitial compartment of the testis. This specific microenvironment is called “niche” and is responsible for the regulation of SSCs self-renewal, quiescence or commitment to differentiation (Chiarini-Garcia et al., 2001, 2003; Yoshida et al., 2007; Oatley and Brinster, 2012; de Rooij, 2017). The components and fine regulation of this microenvironment are currently under investigation and most studies consider the vascular network and Sertoli cells, among others somatic cells, as key players in the determination of the fate of SSCs (Ogawa et al., 2005; Yoshida et al., 2007). As the steroidogenic Leydig cells are the main component of the interstitial compartment in most species, their participation in the SSCs niche has also been investigated (Meistrich and Shetty, 2003; Shetty et al., 2016; Potter and DeFalco, 2017). Particularly, using the collared peccary as a model, studies from our laboratory demonstrated that Leydig cells are probably involved with the differentiation of A_{und}-A_{al} into type A_1 spermatogonia (Campos-Junior et al., 2012), which is considered the spermatogonial cell commited to sperm formation.

Following studies already developed in our laboratory using the collared peccary, the aims of the present work were to investigate postnatal testis development in this species, particularly the aspects related to the progression of spermatogenesis, Sertoli cells proliferation and differentiation, and the characterization of SSCs and establishment of their niche.

2. Material and methods

2.1. Animals

Twenty collared peccaries (*Tayassu tajacu*), in different ages from birth to adulthood, were used in the present study. The animals were obtained from the Federal University of Pará and EMBRAPA/PA, located in the North Region of Brazil (Amazon rainforest; 1°27′S, 48°29′W). The body and testis weights were registered for all animals and the gonadosomatic index was obtained as testes mass divided by body weight. After anesthesia with ketamine chloridate (5 mg/kg) and 0.2% acepromazine (0.2 mg/kg), testes were collected by orchiectomy in the following postnatal age periods: 0–1 months, 2–4 months, 5–7 months, 8–10 months, 11–14 months, and 24 months. Except for 11–14 months of age (n = 2), in all other age periods evaluated 3–5 animals were used per each group, being worth mentioning that it is not easy to have access to these valuable animals, particularly when a specific age is considered. All surgical procedures were performed by a veterinarian and followed approved guidelines for ethical treatment of animals (CETEA #209/2013). Testes were separated from the epididymis, weighed and cut into small fragments with a razor blade. Testis fragments were fixed by immersion in 4% buffered glutaraldehyde for 12 h or Bouin’s solution for 12 h, then routinely processed and embedded in plastic (glycol methacrylate; Leica HistoResin) or Paraplast (Sigma-Aldrich) for histological, stereological and immunohistochemistry analyses.

2.2. Testis stereology

Sections of 4-μm thickness obtained from plastic embedded testis were stained with toluidine blue. The percentage of the testis parenchyma occupied by specific testicular components was determined on images captured by light microscopy, using a 540-intersection grid from ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij/). Fifteen randomly chosen fields/images (8100 points) were scored per animal at 400× magnification. The testicular components evaluated were as follows: seminiferous cords/tubules (seminiferous epithelium, tunica propria, and lumen), Sertoli cell, Leydig cell, connective tissue and blood vessels. The volume of a specific component was determined as the product of its volume density in relation to the testis volume. For stereological calculations, the specific gravity of testis tissue was considered to be 1.0 (França and Godinho, 2003; Leal and França, 2006). To obtain a more precise measurement of testis parenchyma volume, testis capsule was excluded from testis weight (Costa et al., 2010).

Thirty round seminiferous cord/tubule diameters were measured per animal at 200× magnification, using an ocular micrometer calibrated with a stage micrometer. The mean Sertoli cell nuclear volume was determined by measuring the nuclear diameter of 20–40 nuclei per each animal and the calculation of nuclear volume (μm^3) was performed according to the formula $v = 4/3\pi r^3$, where r = nuclear diameter/2. The total number of Sertoli cells per testis was determined as follows: total number of Sertoli cells per testis = total volume of Sertoli cells in the testicular parenchyma (μm^3)/Sertoli cell nuclear volume (μm^3).

2.3. Hormone measurements

Blood samples were collected from the jugular vein of animals under surgical anesthesia. The plasma was separated by centrifugation (2.000 rpm for 10 min) and stored at −20 °C for hormone evaluation. Plasma estradiol and testosterone levels were measured by radioimmunoassay, using commercial kits (Tecsa Laboratory®, Belo Horizonte, Brazil).

2.4. Immunohistochemistry

Paraffin embedded testis sections (5 μm of thickness) were mounted on coated slides and immunostained following specific protocols, according to the antigen and antibody used (Campos-Junior et al., 2012). Briefly, after dewaxing and rehydrating, antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min after boiling in a microwave oven. Endogenous peroxidase was quenched for 30 min with 3% H_2O_2 (Sigma-Aldrich) in PBS and nonspecific binding was blocked with 10% normal serum in PBS. Primary antibodies against Ki67 (1:100; human monoclonal antibody; PharMingen, #558615), GATA-4 (1:100; mouse monoclonal antibody; Santa Cruz Biotechnology, sc-25310), Nanos1 (1:100; rabbit polyclonal antibody; Abcam, ab65203), Nanos2 (1:100; mouse polyclonal antibody; Abcam, ab169436) or GFRA1 (1:500; goat polyclonal antibody; Santa Cruz Biotechnology, sc-6157) were used and the slides were incubated overnight at 4 °C. The appropriate biotinylated secondary antibodies were applied and slides were incubated for 60 min at room temperature. Detection of signal was achieved by incubating the slides in streptavidin (Thermo Scientific, TS-125-HR) for 30 min, followed by peroxidase substrate 3,3′-diaminobenzidine (DAB, Sigma Aldrich). After this procedure, the slides were counterstained with hematoxylin (Merck), dehydrated and mounted with coverslip.

2.5. Sertoli cell phenotypic analyses

Sertoli cell maturity status was evaluated by Ki67 (proliferation marker) and GATA-4 (Sertoli and Leydig cell functional marker) staining. In order to quantify Sertoli cell proliferative activity, one thousand Sertoli cells were counted per each animal and the percentage of Ki67 positive Sertoli cells was calculated. The presence or absence of GATA-4 labelling in Sertoli cells was qualitatively analyzed throughout postnatal testis development.

2.6. Spermatogonial phenotype and niche

The quantification of germ cells expressing specific markers related to spermatogonial self-renewal (Nanos1, Nanos2, and GFRA1) was performed in the present study. For that, one thousand gonocytes or undifferentiated type A spermatogonia (A_{und}/SSCs) were counted and the percentage of labelled cells was calculated for each marker. Evaluation of the spermatogonial stem cell niche was performed according to the published literature (Campos-Junior et al., 2012, 2013). Briefly, images from 15 round seminiferous tubule cross-sections were obtained for each animal and these tubules were subdivided into three regions as follows: adjacent to another tubule (tubule-tubule [T-T]), adjacent to the interstitial compartment without Leydig cells (tubule-interstitium [T-I]), and adjacent to the interstitial compartment containing Leydig cells (tubule-Leydig cells [T-LC]). Considering 360° of tubular circumference, the number of GFRA1 + spermatogonia (A_{und}/SSCs) found in the three different regions evaluated was expressed per degree, allowing therefore an estimation of SSCs preferential location.

2.7. Statistical analysis

The obtained values were expressed as means ± SEM and data were tested for normality before statistical analysis by one-way ANOVA, followed by the Tukey post-test using GraphPad Prism (version 6; GraphPad Software Inc.). The level of significance considered was $p < 0.05$ and statistical differences were represented in relation to the previous age period investigated.

3. Results

3.1. Biometric data and progression of spermatogenesis

The collared peccary body and testis weights and the gonadosomatic index are depicted in Fig. 1. As it can be noted, the body weight showed a rapid increase from birth to 5–7 months of age and another significant growth was observed after 11–14 months of age. The testis weight increased continually up to 11–14 months of age and significantly from this period to adulthood, whereas the gonadosomatic index grew steadily from birth to 5–7 months of age with an evident acceleration after that. The unique collared peccary testis cytoarchitecture, where Leydig cells surrounds the seminiferous cord/tubule, is present since birth (Fig. 2). Regarding the most advanced germ cell type present at each time period investigated (Fig. 2), gonocytes were the only germ cell type present in the seminiferous epithelium up to 4 months of age, whereas type B spermatogonia and pachytene spermatocytes were present respectively at 5–7 and 8–10 months of age. Indicating that puberty was reached, spermiating elongated spermatids in the seminiferous epithelium and sperm in the tubular lumen were observed for the first time at 11–14 months of age.

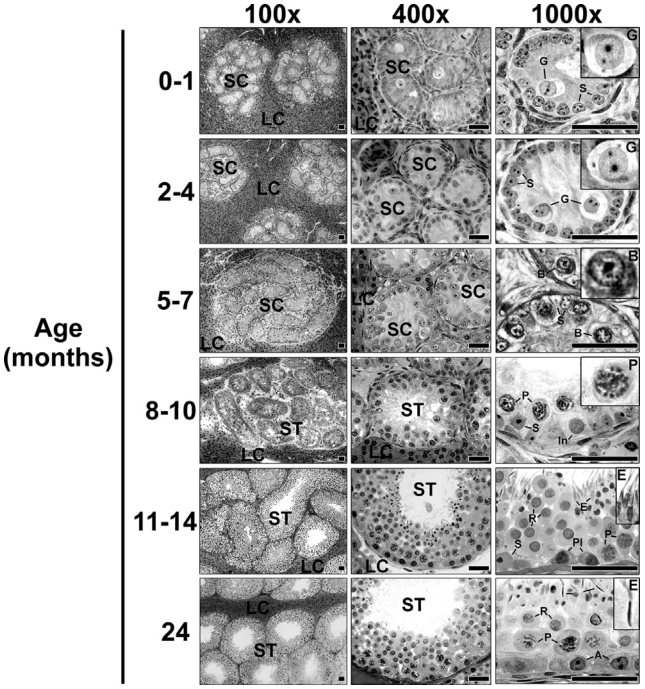

Fig. 2. Collared peccary testis cytoarchitecture and progression of spermatogenesis, from birth (0–1 month) to adulthood (24 months). Note that the unique collared peccary testis cytoarchitecture, in which Leydig cells stripes surrounds the seminiferous cord/tubule, is present since birth. The insert indicates the most advanced germ cell type observed at each time period investigated. SC = seminiferous cords; ST = seminiferous tubules; LC = Leydig cells; S = Sertoli cell; G = gonocyte; A = type A spermatogonia; In = intermediate spermatogonia; B = type B spermatogonia; Pl = pre-leptotene spermatocyte; P = pachytene spermatocyte; R = round spermatid; E = elongated spermatid. Scale bar = 40 µm.

3.2. Testis stereology and hormone levels

The cordonal/tubular diameter and the testis parenchyma components volume densities (%) are shown in Fig. 3. The seminiferous cords/tubule diameter, which is an indication of spermatogenesis progression, both qualitative and quantitatively, increased slowly and gradually from birth to 5–7 months of age, and a substantial and significant growth was observed thereafter (Fig. 3A). Although no significant differences were observed, a similar pattern was noted for the volume occupancies of the tubular compartment and the seminiferous epithelium (Fig. 3B and D). In contrast, and in overall at different degrees, the volume densities of the tunica propria, intertubular compartment, Leydig cells, blood vessels and connective tissue, were reduced from birth to 24 months of age (Fig. 3C and F–I). The first evidence of lumen formation, which is a functional marker of Sertoli cell differentiation,

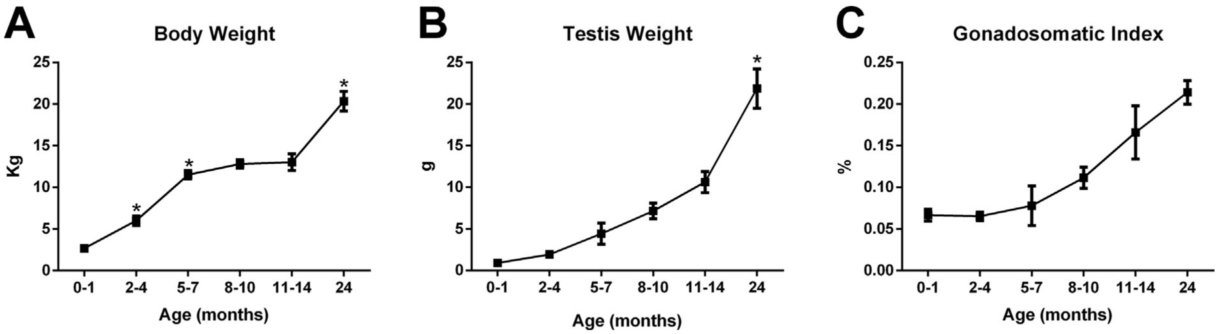

Fig. 1. Body weight (A), testis weight (B) and gonadosomatic index (C) in collared peccary along the postnatal investigated ages. As it can be observed, all parameters increase over time, particularly around one year of age. Asterisks indicate significant differences ($p < 0.05$) in relation to the previous age period investigated.

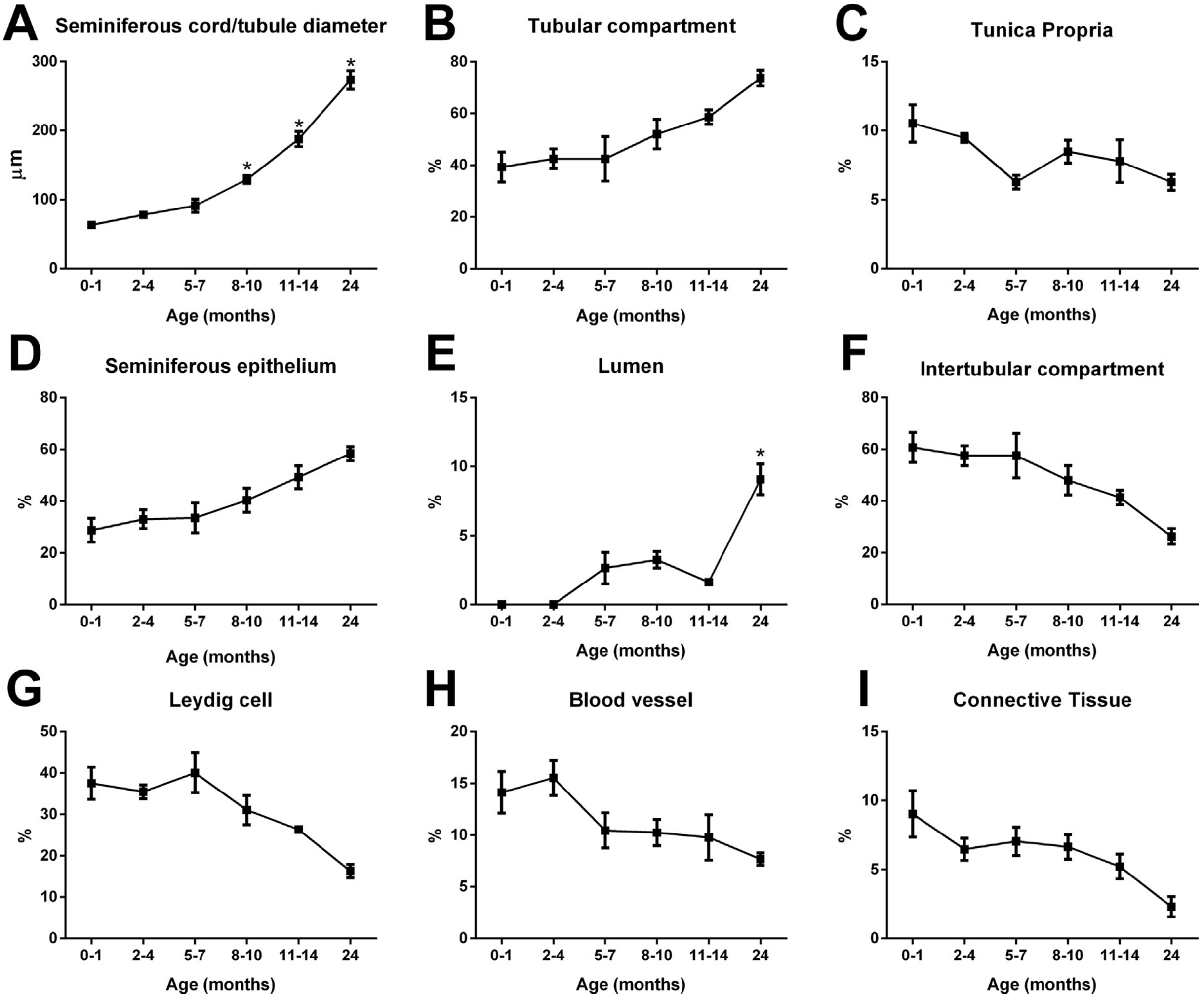

Fig. 3. Seminiferous cord/tubule diameter (A) and the volume densities of testis tubular and intertubular compartments (B; F), as well as its components (C–E; G–I), during postnatal collared peccary testis development. As it can be observed, the diameter of the cords/tubules (A) significantly increase with the progression of spermatogenesis and a similar trend is observed for the occupancy of tubular compartment (B) and seminiferous epithelium (D). An opposite trend is seen for the intertubular compartment (F) and its components (G-I), and tunica propria (C) volume densities, which decrease noticeably over time. Regarding the lumen formation (E), that is an excellent marker of Sertoli cell maturation, it initiates at 5–7 months of age, but markedly increases around one year of age. The asterisk indicates significant differences ($p < 0.05$) in relation to the previous age period investigated.

was observed at 5–7 months (Fig. 3E). However, it should be mentioned that only one animal, out of four evaluated at this age, presented luminal fluid secretion by the Sertoli cells and therefore lumen formation.

The serum hormone data are depicted in Fig. 4, and in overall both estradiol and testosterone levels presented a similar pattern with an evident peak at 8–10 months of age; however, whereas a gradual

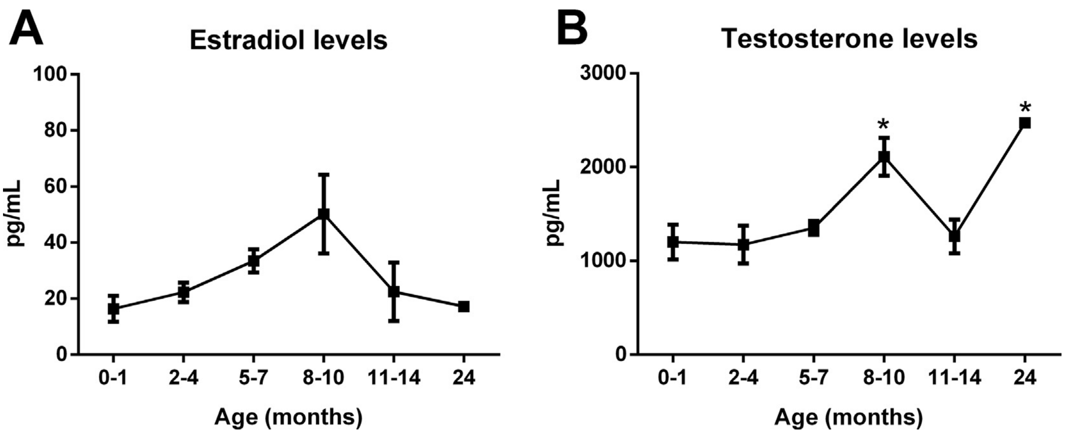

Fig. 4. Estradiol (A) and testosterone levels (B) during postnatal collared peccary testis development. As it can be noted, both hormones present a peak at 8–10 months and another testosterone peak is observed at adulthood. The asterisk denotes significant differences ($p < 0.05$) in relation to the previous age period investigated.

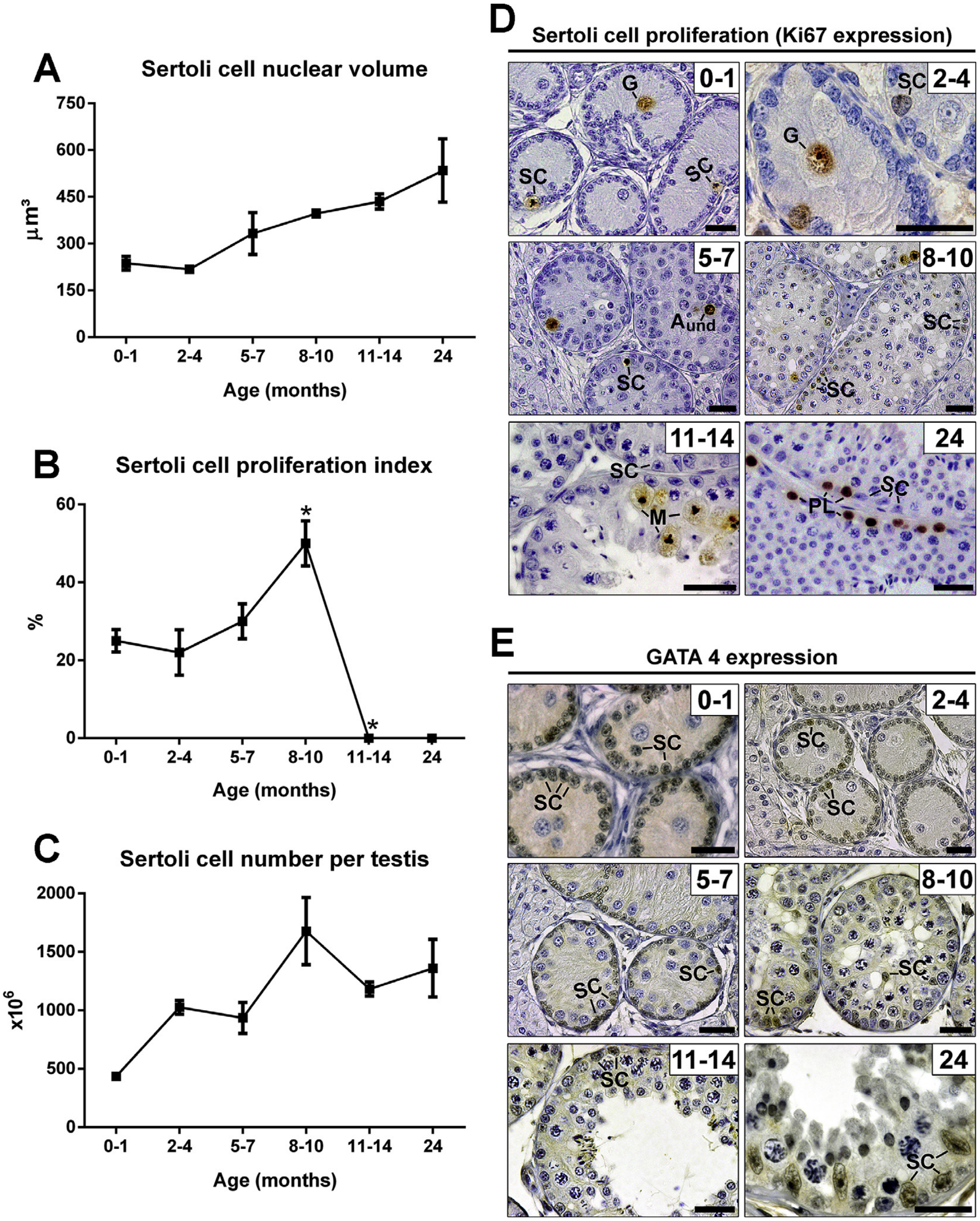

Fig. 5. Sertoli cell data during postnatal collared peccary testis development. The graphs show the values observed for Sertoli cell nuclear volume (A), proliferation index (B), and number per testis (C). Ki67 (D) and GATA-4 expression (E) were also shown during postnatal testis development. Showing its development and maturation, the Sertoli cell nuclear volume (A) grows steadily from birth to adulthood. In parallel with the proliferative marker (Ki67; D), Sertoli cell number per testis (C) increases continually up to 8–10 months of age. As expected, Sertoli cells were GATA-4 positive in all investigated ages. The asterisk indicates significant differences ($p < 0.05$) in relation to the previous ages periods investigated. SC = Sertoli cell; G = gonocyte; Aund = undifferentiated type A spermatogonia; M = meiosis; Pl = pre-leptotene spermatocyte. Scale bar = 30 μm.

decrease was observed for estradiol level thereafter (Fig. 4A), another evident peak of testosterone was noted at 24 months of age (Fig. 4B).

3.3. Sertoli cell parameters

The results obtained for Sertoli cells are shown in Fig. 5. As it can be observed, the Sertoli cell nuclear volume augmented gradually from 2 to 4 months to 24 months of age (Fig. 5A). The postnatal Sertoli cell proliferation index (Fig. 5B), measured by the presence/absence of Ki67 (Fig. 5D), reached its maximum at 8–10 months of age, decreasing drastically in such a way that only eventual Sertoli cell proliferation was noted thereafter. In accordance with these data, Sertoli cell number per testis increased gradually from birth to 8–10 months of age, tending to stabilize at the subsequent ages (Fig. 5C). GATA-4 positive Sertoli cells were present at all postnatal ages investigated (Fig. 5E), and basically all Sertoli cells were positive for this marker.

3.4. Spermatogonial stem cells phenotype and niche

The evaluation of A_{und}/SSCs phenotype confirmed the expression of classical SSCs markers in the seminiferous epithelium of collared peccaries, such as Nanos1 and Nanos2 (Fig. 6A–D) and GFRA1 (Fig. 7A

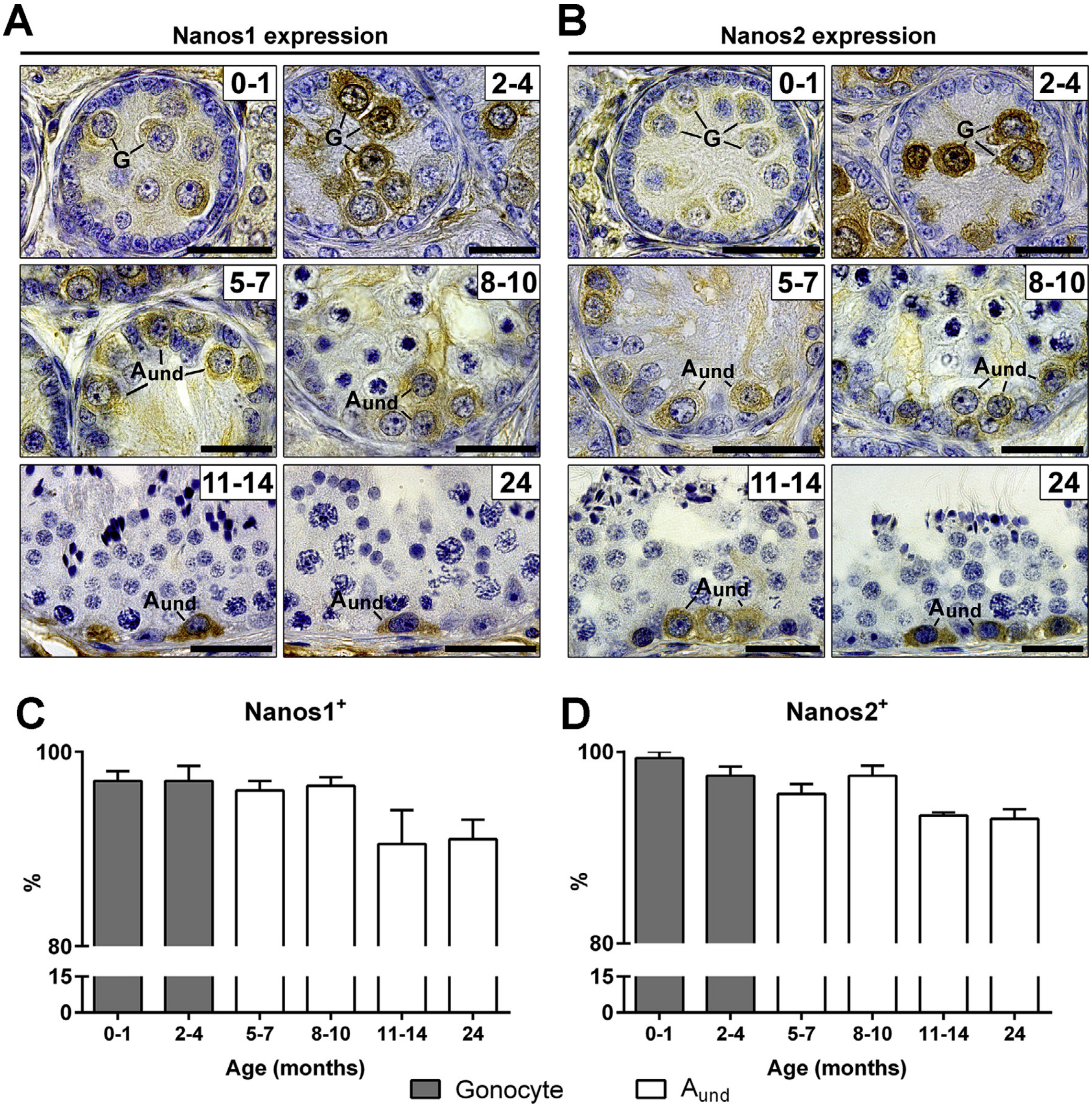

Fig. 6. Pattern of Nanos1 and Nanos2 expression in the collared peccary during postnatal testis development. Considering all ages evaluated, the expression pattern of both markers was very similar for gonocytes and spermatogonial stem cells (SSCs)/type Aund spermatogonia (A–D). SC = Sertoli cell; G = gonocyte; Aund = undifferentiated type A spermatogonia. Scale bar = 30 µm.

and B). The pattern of expression of these markers was very similar and more than 90% of gonocytes and A_{und}/SSCs expressed these proteins at all postnatal investigated ages. The two dimensional analysis of GFRA1 positive spermatogonia (A_{und}/SSCs) location showed that these cells were randomly distributed along the seminiferous cords/tubules cross sections up to 8–10 months of age. However, coinciding with the completion of spermatogenesis (11–14 months of age), SSCs were found in higher frequency nearby the intertubular compartment devoided of Leydig cells (Fig. 7C).

4. Discussion

According to previous studies developed in our laboratory, the unique testis cytoarchitecture observed for collared peccaries has proven to be very useful for investigations regarding the specific role of Leydig cells in the establishment and maintenance of SSCs niche, as well as for the SSCs differentiation (Costa et al., 2010; Campos-Junior et al., 2012, 2014). In this regard, the studies from Campos-Junior and colleagues have shown that in adult peccaries A_{und}/SSCs are preferentially located in regions adjacent to intertubular compartment without Leydig cells (Campos-Junior et al., 2012). Moreover, indicating that the collared peccary peculiar testicular cytoarchitecture is intrinsically programmed, this cytoarchitecture is re-established soon after the testis cells suspensions from pre-pubertal peccaries were xenografted under the back skin of immunodeficient mice (Campos-Junior et al., 2014). Although knowing that the access to peccary testis samples at different ages are limited, these very interesting findings led us to investigate the postnatal testis development in this species, from birth to adulthood. Therefore, we aimed to evaluate the postnatal occurrence of this unique testis cytoarchitecture, the onset and progression of spermatogenesis and, in particular, the phenotypic characterization of SSCs and their location/niche establishment.

Our results showed that the unique collared peccary Leydig cells arrangement is already present at birth, indicating that its formation may be a result of specific interactions and signaling of testicular cells during fetal development. Indeed, differentiation and early organization of testis demand an intense somatic cell interaction in order to setup correctly a functional organ (Capel, 2000; Svingen and Koopman, 2013). In this regard, fetal evaluation of collared peccaries testis development would provide new insights about the functional

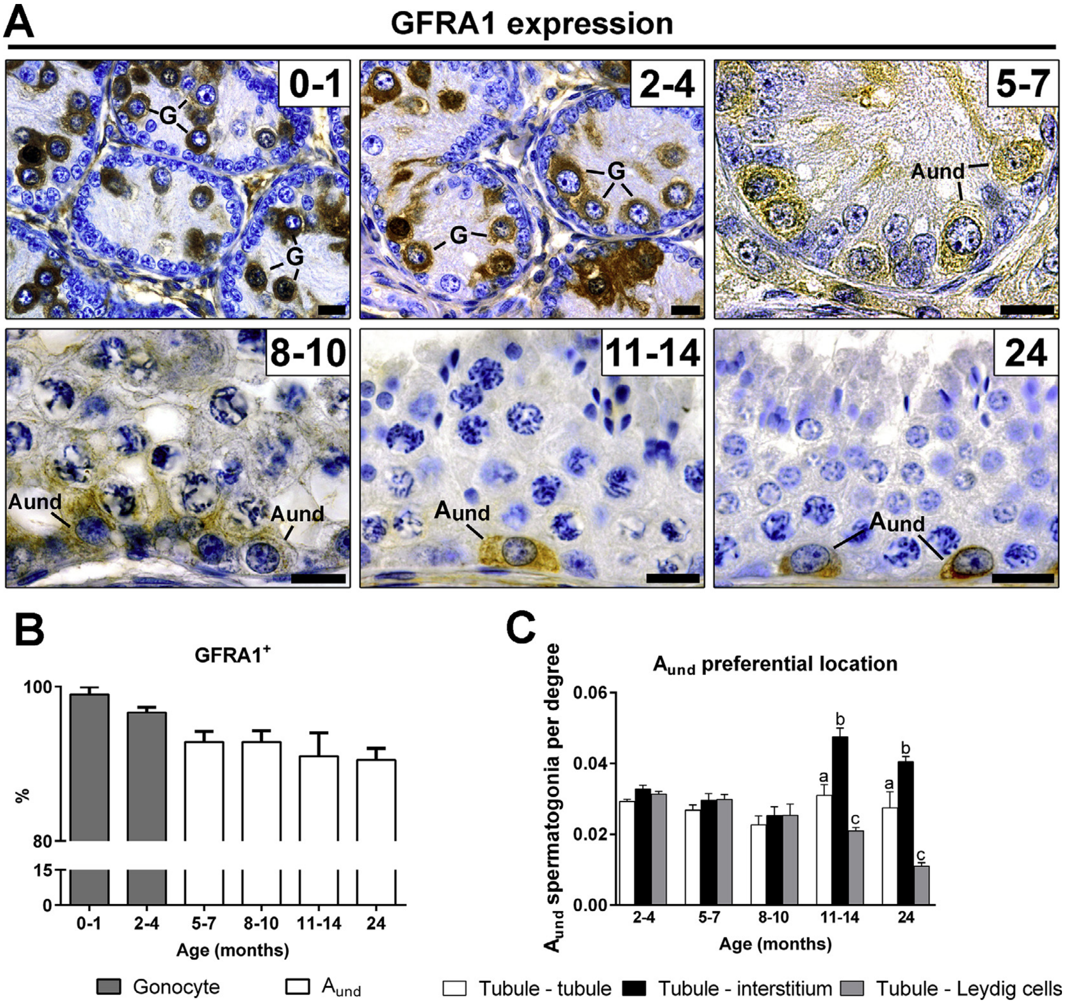

Fig. 7. Pattern of GFRA1 expression and the establishment of spermatogonial stem cell niche during postnatal testis development in the collared peccary. The expression of GFRA1 is present in all ages periods evaluated (A); and the gonocytes and spermatogonial stem cells (SSCs)/type Aund spermatogonia were found labelled for this marker (B). Very importantly, the analysis of Aund preferential location showed that the SSCs niche is established at puberty, when spermatogenesis is complete. SC = Sertoli cell; G = gonocyte; Aund = undifferentiated type A spermatogonia. Scale bar = 15 µm.

interactions among germ and somatic cells, allowing therefore a better understanding of crucial and hitherto unknown aspects of gonadal development/differentiation and function in mammals. Regarding the analysis of spermatogenesis progression during postnatal testis development, the results obtained in the present study showed that gonocytes were still present until 4 months after birth and that this primitive male germ cell differentiated into early spermatogonia at around 5 months of age, initiating therefore the spermatogenic process. This process was complete at 11–14 months of age, when elongated spermatids were seen spermiating and forming sperm. In association with the high testosterone and estradiol levels present just before this later time period, these observations indicates that, in peccaries, puberty is reached when the animals are around one year of age. Based on germ cells progression and the establishment of full spermatogenesis in the seminiferous epithelium, it could be considered that in peccaries the infantile/juvenile period extends up to seven months of age (presence of gonocytes/spermatogonia), the pre-pubertal period takes place from eight to ten months of age (presence of spermatocytes), puberty occurs at around eleven to fourteen months of age (presence of late elongated spermatids/sperm), whereas the post-pubertal period and full sexual maturity (adulthood) takes place after peccaries have reached fourteen months of age. It should be herein mentioned that, according to previous investigation performed in our laboratory, the total duration of spermatogenesis in peccaries (from SSCs to sperm) is almost two months (Costa et al., 2010). In accordance with the literature (Vergouwen et al., 1993; Avelar et al., 2010; Picut et al., 2015, 2017), these characterized periods are, in overall, in agreement with biometric and testis stereological data obtained in the present study.

Besides using a relatively small number of animals for some groups, as already commented above, considerable individual variation occurred at some specific ages for some particular parameters, such as for instance for Sertoli cell differentiation/maturation and spermatogenesis progression. This is a common finding during postnatal testis development, particularly at ages before or around puberty (França, 1987; França and Cardoso, 1988). Based on Ki67 marker, Sertoli cells proliferated until before puberty, a result that is usually observed in the literature for mammals. This very important functional aspect of testis development usually coincides with the lumen formation, extensive proliferation of spermatocytes, the formation of Sertoli cell barrier, increased androgen/steroids levels, resulting therefore in a significant increase in tubular diameter (Tarulli et al., 2012; Griswold, 2015; França et al., 2016; Lara et al., 2017). Although this key functional process of Sertoli cells is still under investigation, including ongoing studies in our laboratory (Tarulli et al., 2006; Figueiredo et al., 2016), after puberty it is considered that these cells no longer proliferate, being their population stabilized thereafter (Lucas et al., 2014; Griswold, 2015; França et al., 2016), as found in the present study. As observed for other mammalian species (Yomogida et al., 1998; Kyrönlahti et al.,

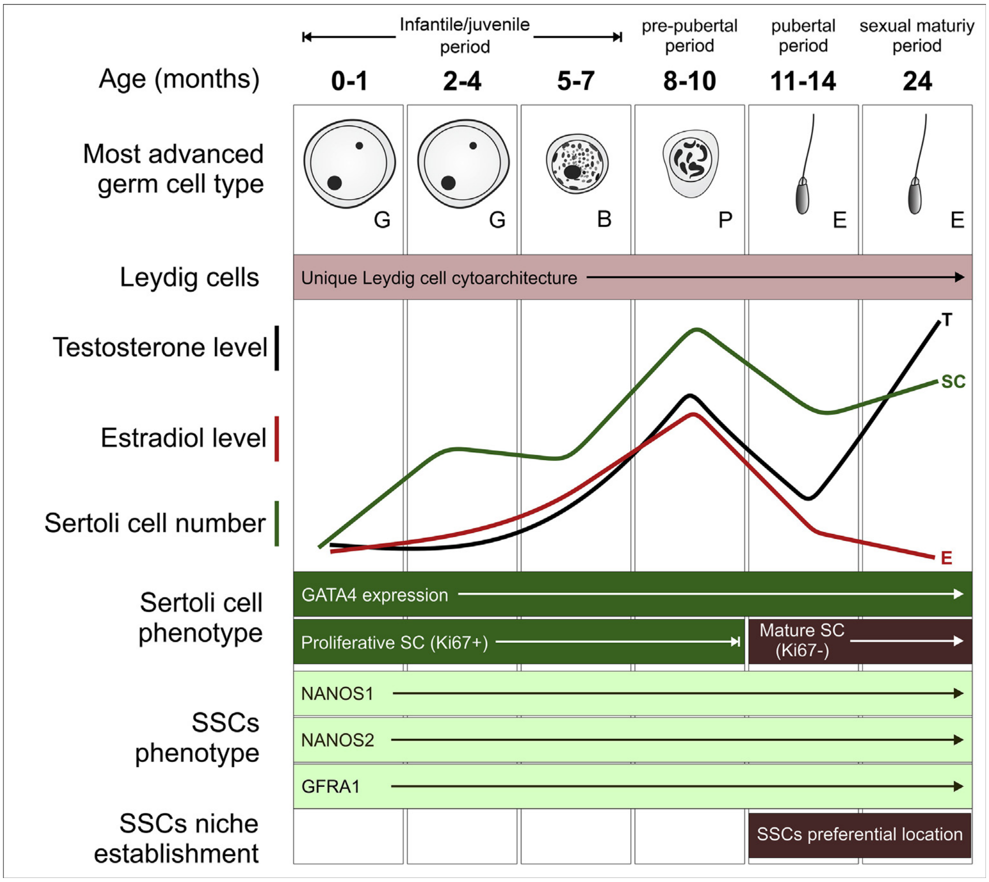

Fig. 8. Schematic representation of the main findings observed in the present study during postnatal testis development in the collared peccary. G = gonocyte; B = type B spermatogonia; P = pachytene spermatocyte; E = elongated spermatid; SC = Sertoli cell; SSCs = spermatogonial stem cells.

2011; França et al., 2016), herein we showed that along the postnatal development Sertoli cells express GATA-4, a zinc finger transcription factor often used as a functional marker of Sertoli cells. Because estradiol and testosterone presented high levels at the time of intensive Sertoli cell proliferation, as have been demonstrated in the literature (Tan et al., 2005; Lucas et al., 2014; Yang et al., 2015), our findings suggest that Sertoli cell proliferation in peccaries may be regulated by these hormones.

There are several studies in the literature investigating the pattern of expression of different proteins in SSCs. In order to characterize the early germ cell population, i.e. gonocytes and SSCs, in the present study we have used three classical markers (Nanos1, Nanos2 and GFRA1). In all postnatal age periods evaluated, high prevalence (~90–100%) of labelled gonocytes and A_{und}/SSCs were observed for these markers. This pattern of labelling is consistent with the literature, and there is solid evidence that the glial derived neurotrophic factor (GDNF) signaling is important for Nanos2 expression, preventing the differentiation of SSCs (Sada et al., 2012; van den Driesche et al., 2014; Pui and Saga, 2018). Moreover, these three markers were successfully demonstrated for mice (Grisanti et al., 2009; Yoshida, 2012), pigs (Kuijk et al., 2009), equids (Costa et al., 2012), domestic cats (Powell et al., 2011; Silva et al., 2012), non-human primates (Hermann et al., 2009; Maki et al., 2009), and humans (Spinnler et al., 2010; He et al., 2012).

Specifically for gonocytes, the expression of Nanos1, Nanos2 and GFRA1 in mammals have already been described. Moreover, Nanos proteins are evolutionarily conserved and usually related to the survival of primordial germ cells. In mice, gonocytes and SSCs express Nanos2, which may play a role in the inhibition of entry of germ cells into meiosis (Suzuki et al., 2010; Barrios et al., 2010). On the other hand, since the fetal period, in both human and marmoset (Mitchell et al., 2008), it was shown that Nanos1 is expressed in most gonocytes. Regarding GFRA1, this marker is expressed in nearly all mouse gonocytes, being also present in bulls (Niedenberger et al., 2015; Cai et al., 2016), whereas domestic cat gonocytes do not express GFRA1 (Tiptanavattana et al., 2015). Therefore, in overall the findings herein observed for gonocytes are consistent with the mammalian literature.

The GFRA1 staining is a classical SSCs marker for vertebrates (Hofmann et al., 2005; Hermann et al., 2007; Kuijk et al., 2009; Spinnler et al., 2010; Campos-Junior et al., 2012; de Rooij, 2017), and is very useful for evaluating the SSCs biology and niche. In adult collared peccaries, previous studies from our laboratory have demonstrated that A_{und} (usually considered as SSCs) were more frequently observed in the regions of the seminiferous tubules adjacent to the intertubular compartments without Leydig cells. Based on these findings, it was suggested that, possibly via testosterone, Leydig cells would favour SSCs differentiation and thus their commitment to spermatogenesis. In the present investigation, our two-dimension analyses have confirmed and extended these findings, demonstrating that SSCs niche is established at puberty (11–14 months of age), when sperm release is first observed. In contrast, during pre-pubertal ages, A_{und}/SSCS showed a random location pattern. In this regard, in order to evaluate the relationship between SSCs niche formation and sperm production, further investigations are still necessary. To our knowledge, the present study is the first to demonstrate when the SSCs niche establishment occurs during testis development, opening therefore new venues for a better understanding of male fertility.

The main results observed in the present study are illustrated in Fig. 8. As summarized in this figure, we found that the unique collared peccary testis cytoarchitecture is already established at birth, being therefore established during fetal life. Regarding the early germ cells, the vast majority of gonocytes and SSCs presented labelling for the three markers used (Nanos1, Nanos2 and GFRA1). Puberty, characterized when sperm is first released into the tubular lumen, occurs at around one year of age. This key morphological event is preceded by high levels of estradiol and testosterone, the end of Sertoli cell proliferation, and is correlated with the establishment of A_{und}/SSCs preferential location. Therefore, our results reinforced and extended the importance of the collared peccary as an animal model to investigate testis function in mammals, particularly the aspects related to testis organogenesis, SSCs biology and the establishment of SSCs niche.

5. Declaration of interest

None.

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