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Sexual maturation of the Mongolian gerbil (*Meriones unguiculatus*): a histological, hormonal and spermatic evaluation

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Abstract. This study determined the phases of sexual development of the male Mongolian gerbil (*Meriones unguiculatus*) based on an integrative analysis of testicular morphology, hormonal data and sperm parameters. Male gerbils were analysed at 1, 7, 14, 21, 28, 35, 42, 50, 60, 70, 90, 100 and 120 days of age. Body, testicular and epididymal weights increased up to Day 70, 60 and 90, respectively. The impuberal phase, characterised by the presence of gonocytes, extended until Day 14. The prepubertal period lasted until Day 42, when puberty was achieved and a drastic increase in serum testosterone levels, mature adult Leydig cells and elongated spermatids was observed. Gerbils at 60 days of age showed a remarkable number of spermatozoa in the testis, epididymidis caput/corpus and cauda, and at Day 70 the maximum daily sperm production was reached. However, the gerbil may be considered sexually mature only from Day 90 onward, when sperm reserves become stable. The total transit time of spermatozoa along the epididymis of sexually mature gerbils was 11 days, with 1 day in the caput/corpus and 10 days in the cauda. These data cover a lacuna regarding the reproductive parameters of this rodent and provide foundations for its use in testicular toxicology studies.

Additional keywords: daily sperm production, epididymal sperm transit time, puberty, sperm motility.

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Introduction

The Mongolian gerbil (*Meriones unguiculatus*), also known as the Mongolian squirrel, is a murine rodent of the Gerbillinae subfamily found in arid regions of China and Mongolia. The gerbil was introduced as a laboratory rodent in the 1960s (Schwentker 1963; Rich 1968) and, in recent decades, it has assumed an important role in biological and biomedical experiments alongside other classic species such as the rat (*Rattus norvegicus*), the mouse (*Mus musculus*) and the hamster (*Mesocricetus auratus*). This rodent has been widely used as an experimental model in different areas of scientific research such as immunology (Jeffers *et al.* 1984; Nawa *et al.* 1994; Wiedemann *et al.* 2009), cell culture (Moritomo *et al.* 1991) and neurophysiology (Moller *et al.* 1979; Cao *et al.* 2005).

In the last decade there has been an emphasis concerning gerbil reproductive biology. Several descriptive or experimental studies have focussed on the prostate (dos Santos *et al.* 2003; Corradi *et al.* 2004; Santos *et al.* 2006; Góes *et al.* 2007; Rochel *et al.* 2007; Taboga *et al.* 2009; Fochi *et al.* 2013) or epididymis (Domeniconi *et al.* 2006, 2007), as well as the sex-steroid milieu (Blottner *et al.* 2000; Siegford *et al.* 2003; Juana *et al.* 2010). A general description of the postnatal development of the testes in the gerbil was made by Ninomiya and Nakamura (1987). Segatelli *et al.* (2000, 2002) showed that the seminiferous epithelium cycle of this small rodent includes 12 stages; the relative frequency of each stage and the duration of whole spermatogenesis (47.5 days) were estimated using ^{3}H-thymidine injection and autoradiography (Segatelli *et al.* 2004). Regarding the immature testis, in previous reports we have described the neonatal differentiation of gonocytes (Pinto *et al.* 2010*a*) and characterised Leydig cell populations (Pinto *et al.* 2010*b*). Thus, although the gerbil can be considered an excellent model for the study of issues related to the male reproductive system, a serious lacuna still persists concerning

the phases of testis development before adult age and sperm parameters, mainly sperm reserves and epididymal transit time. A detailed description of Mongolian gerbil sexual development supported by testicular histology, steroidogenesis and sperm production and reserve data are crucial to comprehension of its reproductive function and application in toxicological and developmental studies. Therefore, this study assessed the stages of sexual development of the Mongolian gerbil by means of an integrated evaluation of the morphological and physiological changes in the testis as well as some sperm parameters.

Materials and methods

Animals

Mongolian gerbils were kept in the Animal Breeding Center of São Paulo State University (UNESP), Institute of Biosciences, Humanities and Exact Sciences (IBILCE; São Paulo, Brazil) under controlled temperature (23–25°C), humidity (40–60%) and luminosity (12 h light : 12 h dark cycle). All animals were given free access to water and rodent feed (Labina; Purina, Paulínia, Brazil) *ad libitum*. Experimental procedures were performed in accordance with the National Council for Control of Animal Experimentation (CONCEA) and approved by the Ethical Committee for Animal Research of the Bioscience Institute/UNESP (Protocol CEEA no. 31/07). Male gerbils were used at the following ages: 1, 7, 14, 21, 28, 35, 42, 50, 60, 70, 90, 100 and 120 days of age. The offspring were obtained by mating female gerbils (90 days of age) in oestrus with male gerbils of the same age, in a ratio of 1 : 1. The births were evaluated daily in the morning and the birth date was considered to be Day 0. Only one male pup from every litter was used for each age, i.e. $n = 5$ for ages 1–50 days and $n = 15$ for the remaining ages. Gerbils were weighed, anesthetised with ketamine (800 μL kg^{-1}) and xylazine (200 μL kg^{-1}) and killed by CO_2 inhalation. Immediately after death the animals were decapitated for collection of blood and the testes and epididymis were removed and weighed.

Histological procedure

Histological analyses of the testes were performed on five animals per age. The left testes were fixed in Bouin's fluid for 6 h, washed for several days in 70% ethanol and processed for embedding in Paraplast (Merck, Darmstadt, Germany). Paraffin sections were stained with haematoxylin–eosin (HE) and used for immunocytochemistry. The right testes were fixed in 2.5% glutaraldehyde, 1% tannic acid, 3.5% sucrose and 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C. After 1 h in this solution, the testes were cut into smaller fragments and fixed for 1 h more in the same solution. Testicular fragments were post-fixed in 1% osmium tetroxide in cacodylate buffer for 2 h and embedded in araldite 502 (Electron Microscopy Sciences, Hatfield, PA, USA). One micron-thick sections were stained with a solution of 1% toluidine blue and 1% borax in water for light microscopic analyses. The analyses were performed using an Olympus BX60 photomicroscope (Olympus, Hamburg, Germany) and the images were digitalised using the software Image-Pro Plus 6.0 for Windows (Media Cybernetics, Bethesda, MD, USA).

Stages of testicular development

Histological analyses of the testes were performed in paraffin sections stained with HE to determine the different stages of postnatal testicular development: impuberal, prepubertal, pubertal and adult, according to Courot *et al.* (1970). These phases were determined based on the analysis of characteristics of the seminiferous cords/tubules regarding the presence of gonocytes, spermatogonia, primary spermatocytes, elongated spermatids and spermatozoa, as well as the lumen formation process. The gonocytes and germ cells at different stages of differentiation were identified based on the descriptions of Pinto *et al.* (2010*a*) and Segatelli *et al.* (2002), respectively.

The presence of mature adult Leydig cells (ALC) was also examined using combined analysis of thick sections and immunocytochemistry for the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD), according to previously published descriptions (Chamindrani Mendis-Handagama and Ariyaratne 2001). To detect 17β-HSD immunoreactivity, the sections were deparaffinised and rehydrated, then antigen retrieval was performed in citrate buffer, pH 6.0, at 97°C for 45 min. Blocking of endogenous peroxidases was obtained by covering the slides with 3% H_2O_2 in methanol for 20 min. The tissue sections were treated with Background Sniper solution (Biocare Medical, Concord, CA, USA) for 15 min to block non-specific protein linkage. Sequentially, sections were incubated overnight at 4°C with primary rabbit IgG anti-human 17β-HSD antibody (sc-32872; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 100 in 3% bovine serum albumin (BSA). The sections were then incubated with a biotinylated anti-rabbit antibody followed by the avidin–biotin complex ABC kit (Santa Cruz Biotechnology) for 45 min at 37°C. The immunoreaction was revealed with diaminobenzidine (DAB) for 3 min and the sections were counterstained with haematoxylin. The washes were accomplished with 0.05% Tween 20 in phosphate-buffered saline (PBS), pH 7.6. The negative control was obtained by omission of the primary antibody.

Serum hormone levels

After blood sample collection, the serum was separated by centrifugation (1200*g* at 4°C) for 20 min and stored at −20°C for subsequent hormone assays. The serum testosterone and oestradiol levels were determined with automatic equipment (VITROS ECi-Johnson and Johnson Ultra-Sensitive Quimioluminescent analysis) using specific reagents supplied by Johnson and Johnson (Langhorne, PA, USA). Eight animals were used from each group and the test was performed in triplicate. The sensitivity of the method was 1–1500 pg mL^{-1} for testosterone and 0.1–3814 ng mL^{-1} for oestrogen.

Sperm counts

Ten animals at Days 60, 70, 90, 100 and 120 were used for sperm count analyses. The right testis and epididymis were removed, weighed and immediately frozen for use. The testis sperm number, daily sperm production (DSP), sperm number and transit time in the epididymis were estimated. Homogenisation-resistant testicular spermatids and spermatozoa in the caput/corpus and cauda epididymidis were also estimated as described

previously (Robb *et al.* 1978; Fernandes *et al.* 2007), with adaptations as described below.

The testis was decapsulated, weighed and homogenised in 4 mL Saline-Triton-Merthiolate solution (STM solution; 0.9% NaCl containing 0.05% Triton X-100 and 0.01% Merthiolate), followed by sonication for 30 s. The samples were diluted 10 times and an aliquot was transferred to a Neubauer chamber (Laboroptik Ltd, Lancing, UK). Homogenisation-resistant spermatids were counted in quadruplicate for each animal to estimate the total number of spermatids per testis. The variation between the quadruplicates was less than 20%. To calculate DSP, the total number of homogenisation-resistant spermatids was divided by a time divisor. The time divisor is the number of days of the seminiferous cycle in which the homogenisation-resistant spermatids are present in the seminiferous epithelium (Amann 1970, 2008). Based on previous publications (Segatelli *et al.* 2000) showing that gerbil spermatids complete their morphological differentiation and the nucleus completes its condensation and takes on its definitive shape, together with our observations of spermatids in homogenised testicular parenchyma, we concluded that the nuclei resistant to homogenisation in testicular homogenates were those in Step 13, 14 and 15 spermatids, found in Stages I to VI of the cycle of the seminiferous epithelium in the gerbil (Segatelli *et al.* 2004). The duration of these stages is 5.81 days (Segatelli *et al.* 2004), thus, the time divisor of 5.81 was used to estimate DSP in gerbil. Then, the DSP per gram of testis was calculated in order to determine the spermatogenic efficiency.

The gerbil epididymis exhibits a particular shape: the caput and cauda regions are voluminous, composed of a coiled epididymidal duct, whereas the corpus is very slender and contains the uncoiled epididymidal duct (Domeniconi *et al.* 2007). For sperm counts, this organ was separated into two segments: one containing the caput/corpus and the other the cauda. Each segment was weighed and homogenised in an amount of STM solution according to its weight (1 mL of STM for each 200 mg of caput/corpus and 1 mL of STM for each 100 mg of cauda) followed by sonication for 30 s. The samples were diluted 20 times and an aliquot was transferred to a Neubauer chamber. Spermatozoa were counted in quadruplicate per animal. The sperm transit time along the epididymis was determined by dividing the number of spermatozoa in each portion by the DSP. The values are expressed as 10^6 per organ and $10^6\ g^{-1}$ of organ.

Sperm motility

Immediately after euthanasia, the cauda of the left epididymis was collected. Spermatozoa were obtained with the aid of a needle by means of rinsing with 1.0 mL of modified human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) at 34°C. A Makler counting chamber (Sefi-Medical, Haifa, Israel) warmed to 34°C was loaded with a small aliquot of sperm solution (10 µL). Sperm motility was assessed by visual estimation (100 spermatozoa per animal, in duplicate) under a phase-contrast microscope (Olympus BX60) at 200× magnification. Spermatozoa were classified as immotile, motile without progression or motile with progressive movement.

Statistical analysis

Parametric data were initially analysed by analysis of variance (one-way ANOVA) and subsequently by Tukey's test. For non-parametric data, the Kruskal–Wallis test followed by Dunn's test was used. Both tests are for multiple comparisons with significance levels of 5% ($P \leq 0.05$). All statistical evaluations were performed using the software Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Biometric data

Body weight of gerbils increased until Day 70 and remained stable until Day 120, with the most notable increase occurring between Days 35 and 42 (23.3 g ± 2.4 to 39.5 g ± 4.6; Fig. 1*a*). On the other hand, testicular weight increased until Day 60 and remained unchanged after this age. This increase was more outstanding from Day 35 to 60, thus testicular weight increased

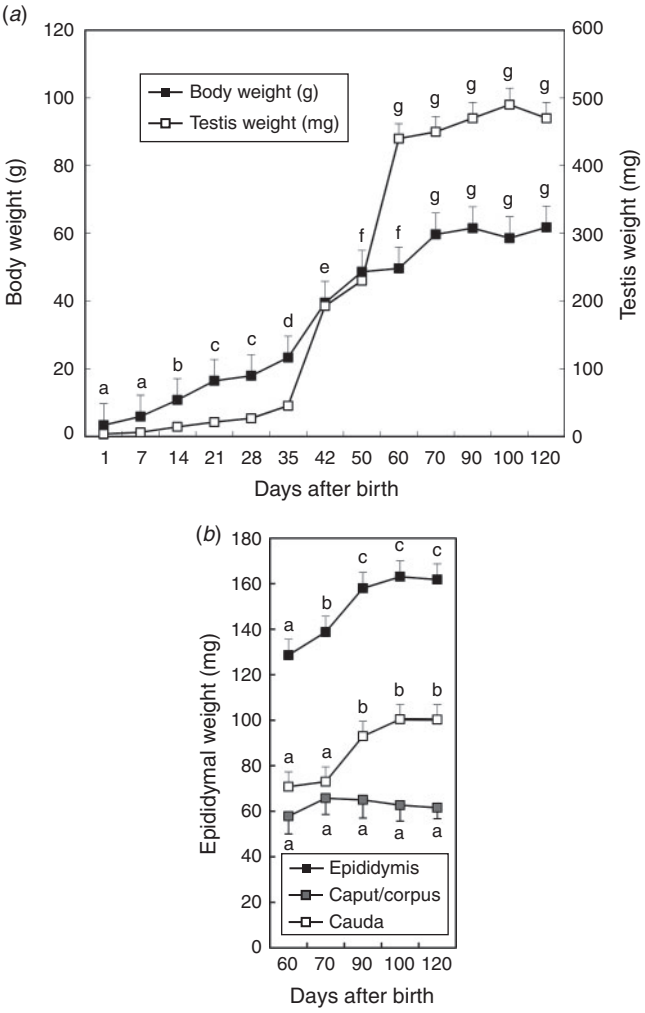

Fig. 1. (*a*) Body (g), testis (mg) and (*b*) epididymal (mg) weight of gerbils. Values represent the mean ± s.e.m. a, b, c, d, e, f, g = statistically significant difference between groups with different letters.

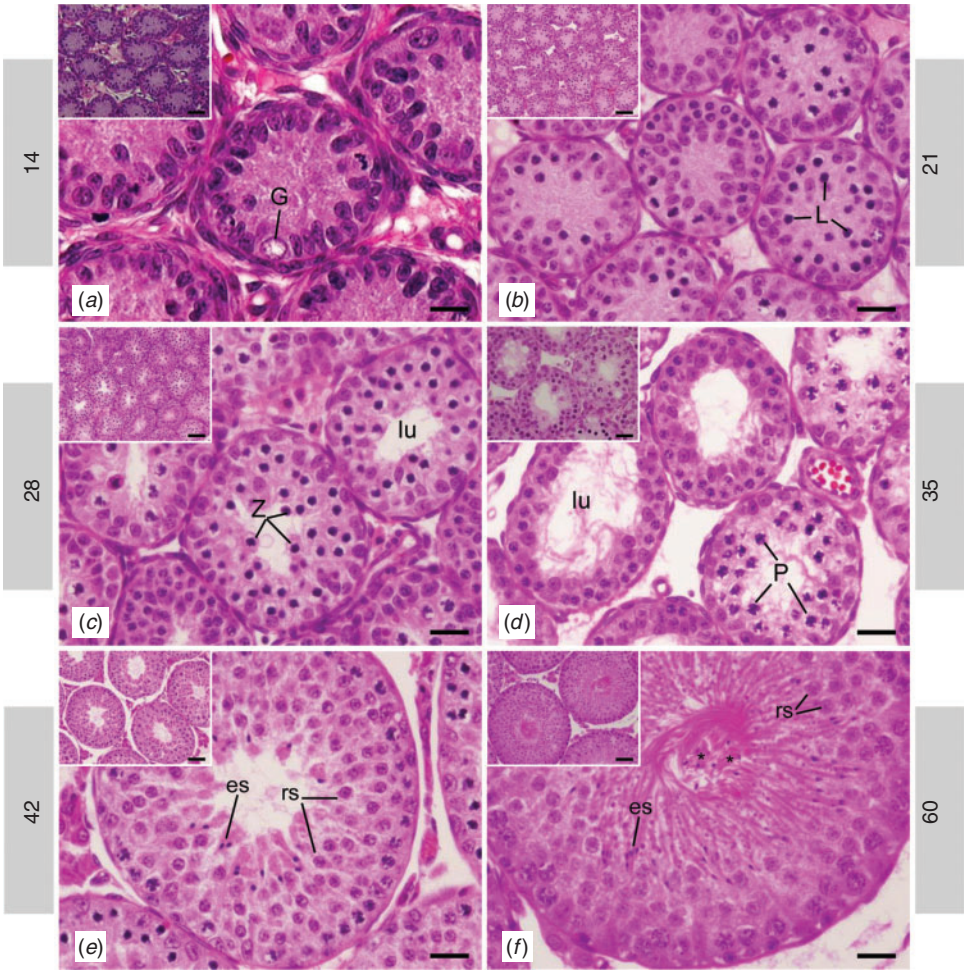

Fig. 2. Histological paraffin sections of gerbil testis at different stages of postnatal development stained with HE, showing the seminiferous tubules (inset) or detail of the seminiferous epithelium. (*a*) 14, (*b*) 21, (*c*) 28, (*d*) 35, (*e*) 42, (*f*) 60 days of age. G, gonocyte; L, spermatocyte in leptotene; Z, spermatocyte in zygotene; P, spermatocyte in pachytene; rs, round spermatid; es, elongated spermatid; * free spermatozoon. Bar = 20 μm; inset bar = 50 μm.

~75% between Days 35 and 42 and doubled between Days 50 and 60 (Fig. 1*a*). The epididymal weight increased between Days 60 and 90 and stabilised thereafter. This increase in the epididymis was mainly due to the increased weight of the cauda, since the caput/corpus weight did not change (Fig. 1*b*).

Stages of testicular development

Histological analysis of the testis demonstrated that the impuberal stage, characterised by the predominant presence of gonocytes and the absence of a lumen in the seminiferous cords, extended until Day 14 (Fig. 2*a*). Until Day 7 the majority of gonocytes were localised in the central region of the seminiferous cords, but at Day 14 90% of total gonocytes were observed at the base of the seminiferous cords and from Day 21 onward gonocytes were not visible. The prepubertal period occurred between Days 14 and 42. At Day 21, leptotene spermatocytes were observed (Fig. 2*b*), demonstrating that meiosis had already started. In the subsequent week (Day 28), it was possible to visualise spermatocytes in the zygotene stage and tubules with a lumen (Fig. 2*c*). The expansion of the lumen and pachytene spermatocytes were observed at Day 35 (Fig. 2*d*). At Day 42 the tubular diameter increased remarkably and elongated spermatids were already found (Fig. 2*e*), as well as mature ALC (Fig. 3*a*, *b*), indicating the onset of puberty. Mature ALC exhibited a flattened polyhedral shape and cytoplasm without lipid droplets (Fig. 3*a*) and positivity for the enzyme 17β-HSD (Fig. 3*b*). The presence of free spermatozoa in the testis of gerbils at Day 60 indicates full spermatogenesis (Fig. 2*f*).

Serum hormone levels

Serum testosterone levels did not change in gerbils between Days 14 and 35 and exhibited a marked increase from Day 35 to 50 (46.3 ± 9.9 vs 660.85 ± 20.5) and an additional increase from Day 70 to 90, stabilising thereafter (Fig. 4). Serum oestrogen levels showed no significant variation from Day 21 to 35, increased by ~27% at Day 42 and remained stable thereafter (Fig. 4).

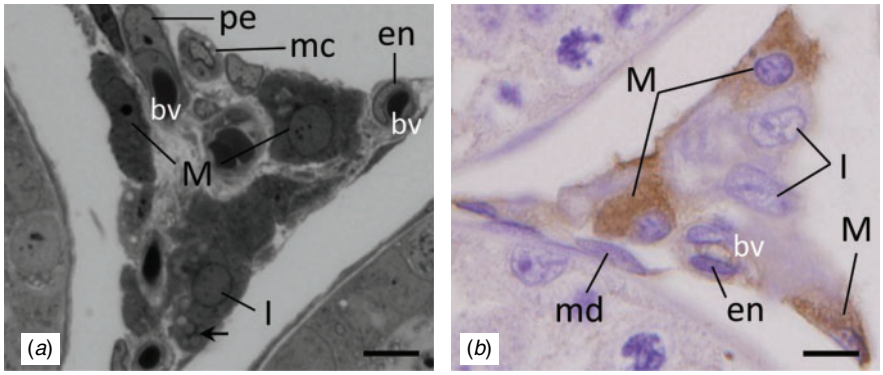

Fig. 3. Thick section stained with toluidine blue and immunolocalisation of the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) in the interstitial tissue of gerbil at 42 days of age. The immature adult Leydig cells (I) were recognised by the presence of lipid droplets (arrow) and the absence of labelling for 17β-HSD. In mature adult Leydig cells (M), the opposite was noted, i.e. the lack of lipid droplets and immunoreactivity for 17β-HSD. md, myoid cell; mc, macrophages; en, endothelial cells; pe, pericytes; bv, blood vessels. Bar = 20 μm.

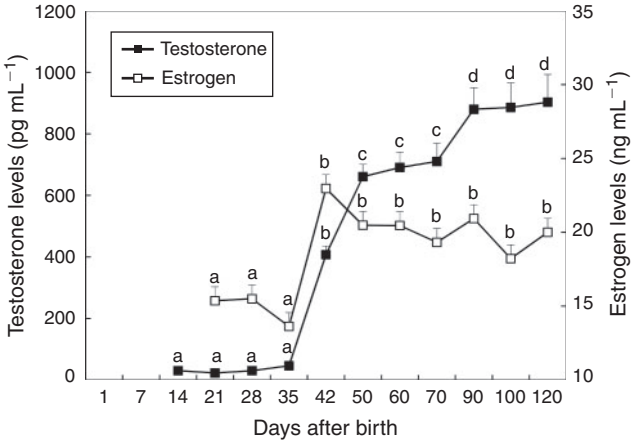

Fig. 4. Serum levels of testosterone and oestrogen in gerbils at 14 to 120 days of age. Values represent the mean ± s.e.m. a, b, c, d = statistically significant differences between groups with different letters.

Sperm parameters

The sperm parameters of gerbils at Days 60 to 120 are presented in Table 1. The counts revealed that both the testis and epididymis of gerbils at Day 60 already had a reasonable number of spermatozoa. Sperm number in the testis and DSP increased in animals until Day 70 and then remained stable after this age. The same pattern was observed for spermatogenic efficiency. The sperm counts per gram of tissue stabilised at Day 100 for the epididymidis caput/corpus and at Day 90 for the cauda. The transit time from Day 90 onward was ~1 day in the epididymidis caput/corpus and ~10 days in the cauda.

Sperm motility

From Day 60 onward the percentage of spermatozoa that were motile with progressive movement, motile without progression or immotile stabilised at ~56%, ~22% and ~22%, respectively (Table 2).

Discussion

There has been increasing interest in different aspects of the reproductive organs of the Mongolian gerbil, particularly the prostate (dos Santos *et al.* 2003; Corradi *et al.* 2004; Santos *et al.* 2006; Góes *et al.* 2007; Rochel *et al.* 2007; Taboga *et al.* 2009; Fochi *et al.* 2013) and epididymis (Domeniconi *et al.* 2006, 2007). However, the experimental and toxicological knowledge on this rodent is still incipient, partly due to the lack of information on its sexual maturation and rates of sperm production and reserves. This paper fulfills this deficiency by presenting a detailed overview of gerbil testicular development based on morphological aspects, the synthesis of steroids, sperm counts, epididymal transit time and sperm motility.

Ninomiya and Nakamura (1987) found that spermatogenesis in the gerbil commences at ~2 weeks of age, when spermatogonial mitoses are first observed. The present data, as well as a previous analysis of neonatal testis development (Pinto *et al.* 2010*a*), confirmed the findings of Ninomiya and Nakamura (1987) concerning the onset of spermatogenesis. Considering that the duration of spermatogenesis in the gerbil is 47.5 days (Segatelli *et al.* 2004) and that, at Day 60 spermatozoa were already observed in the epididymis, it can be concluded that, in fact, spermatogenesis begins at around 14 days of age. According to Ninomiya and Nakamura (1987), the testes of animals at 7 weeks of age exhibited few seminiferous tubules with spermatozoa, but at 10 weeks, the majority of tubules presented spermatozoa. Furthermore, they also verified that spermatozoa first appeared in the epididymis at 10 weeks with a remarkable increase at 12 weeks. We first observed spermatozoa in the epididymis earlier (~9 weeks or Day 60) than Ninomiya and Nakamura (1987), which may be due to differences between the laboratory lineages employed in these studies.

With respect to the levels of sex steroids, it is known that the production of androgens in rodents in the fetal and neonatal periods is due to the fetal Leydig cell population (O'Shaughnessy *et al.* 2006); these cells are very common in the gerbil testis up to Day 35 (Pinto *et al.* 2010*b*). Serum testosterone

Table 1. Sperm count parameters

Sperm parameters of Mongolian gerbils at 60 to 120 days of age. Values represent the mean ± s.e.m. [a,b,c]Values within rows with different superscript letters differ significantly ($P \leq 0.05$)

Parameter	Gerbil age				
	60 days	70 days	90 days	100 days	120 days
Spermatid number (10^6 per testis)	55.1 ± 3.9^{a}	66.3 ± 8.5^{b}	68.1 ± 17.9^{b}	70.2 ± 6.8^{b}	70.4 ± 3.2^{b}
Spermatid number (10^6 g^{-1} testis)	120.0 ± 11.6^{a}	139.8 ± 16.2^{b}	142.4 ± 31.3^{b}	146.0 ± 13.3^{b}	153.4 ± 24.1^{b}
DSP (10^6 per gerbil)	9.5 ± 0.7^{a}	11.5 ± 1.5^{b}	11.7 ± 3.1^{b}	12.1 ± 1.7^{b}	12.1 ± 0.6^{b}
DSP (10^6 g^{-1} testis)	20.6 ± 2.0^{a}	24.1 ± 2.8^{b}	24.5 ± 5.4^{b}	25.1 ± 2.3^{b}	26.4 ± 4.1^{b}
Caput/corpus epididymidal sperm number (10^6 per caput/corpus)[A]	8.2 ± 1.5^{a}	9.2 ± 0.8^{a}	$9.7 \pm 1.2^{a,b}$	10.2 ± 2.1^{b}	12.2 ± 3.0^{c}
Caput/corpus epididymidal sperm number (10^6 g^{-1} caput/corpus)[A]	138.4 ± 24.0^{a}	140.3 ± 15.2^{a}	$147.0 \pm 36.8^{a,b}$	166.8 ± 14.4^{c}	196.8 ± 46.4^{c}
Cauda epididymidal sperm number (10^6per cauda)[A]	55.3 ± 5.9^{a}	60.2 ± 4.6^{a}	118.0 ± 17.8^{b}	120.4 ± 19.5^{b}	119.6 ± 15.4^{b}
Cauda epididymidal sperm number (10^6 g^{-1} cauda)[A]	835.0 ± 130.6^{a}	825.6 ± 66.7^{a}	1173.5 ± 290.5^{b}	1129.0 ± 102.9^{b}	1159.0 ± 257.1^{b}
Sperm reserves (10^6 per gerbil)[B]	127.0^{a}	138.8^{a}	255.4^{b}	261.2^{b}	263.6^{b}
Epididymal sperm transit time (days)[A]					
Caput/corpus	0.7 ± 0.2^{a}	1.0 ± 0.1^{a}	0.9 ± 0.2^{a}	0.9 ± 0.2^{a}	1.0 ± 0.3^{a}
Cauda	4.8 ± 0.8^{a}	6.4 ± 0.7^{a}	10.1 ± 2.6^{b}	10.0 ± 1.1^{b}	9.8 ± 3.6^{b}
Total	5.5 ± 1.0^{a}	7.4 ± 0.8^{a}	11 ± 2.8^{b}	10.9 ± 1.3^{b}	10.8 ± 3.9^{b}

[A]Per epididymis.
[B]Data for one epididymis multiplied by 2.

Table 2. Sperm motility

Sperm motility of Mongolian gerbils at 60 to 120 days of age. Values represent the mean ± s.e.m.

Age	Motile with progressive movement (%)	Motile without progression (%)	Immotile (%)
60 days	56.29 ± 4.3	22.00 ± 3.7	21.71 ± 2.8
70 days	57.43 ± 3.0	21.71 ± 3.6	20.86 ± 3.8
90 days	56.57 ± 4.9	20.71 ± 3.9	22.71 ± 4.2
100 days	55.57 ± 5.6	20.86 ± 4.5	23.57 ± 4.8
120 days	57.71 ± 4.6	20.14 ± 3.1	22.14 ± 4.4

levels were not different from Day 14 to 35. Even though newly formed ALC were observed at Day 28 (Pinto *et al.* 2010*b*), studies in the rat indicate a smaller secretory capacity compared with mature ALC (Eckstein *et al.* 1987). The abrupt increase in serum testosterone at Day 42 was coincident with the appearance of mature ALC at this age.

DSP is the number of spermatozoa produced per day by a testis or the two testes of an individual (Amann 1970, 2008). The DSP is a quantitative indicator of success in spermatogenesis and, when expressed per gram of testicular parenchyma (DSP g^{-1}), it reflects the efficiency of sperm production, which is quite useful for comparisons of experimental conditions. DSP per testis and per gram of testis can be estimated by quantitative testicular histology (Amann 1970; França 1992) or by the method used here based on homogenates of testicular parenchyma and counts of homogenisation-resistant spermatids (Amann 1970; Robb *et al.* 1978). The second method is simplest and probably the most accurate. This method requires a time divisor to convert the number of counted cells per unit volume or mass of testis to the number of sperm cells produced each day (Amann 1970). As previously mentioned in the Methods section, the time divisor refers to the number of seminiferous cycle days in which the homogenisation-resistant spermatids are present in the seminiferous epithelium (Amann 1970, 2008). In this study the nuclei resistant to homogenisation in testicular homogenates were those in Step 13, 14 and 15 spermatids, found in Stages I to VI of the cycle of the seminiferous epithelium in the gerbil; the duration of these stages is 5.81 days and thus the time divisor was 5.81 days. In the rat, homogenisation-resistant spermatids are Step 17–19 spermatids and the time divisor is 6.1 days (Clermont *et al.* 1959), while in the mouse, the homogenisation-resistant spermatids are Step 14–16 spermatids and the time divisor is 4.84 days (Oakberg 1956). Thus, the procedures adapted here can be widely accepted for DSP estimation and the time divisor of gerbil is similar to those used in other laboratory rodents.

To our knowledge, this is the first report on sperm number and DSP determination in Mongolian gerbil by the method of counting homogenisation-resistant spermatids (Robb *et al.* 1978). Blottner *et al.* (2000) have determined the sperm number per testis and per gram of testis of sexually mature gerbils by another method (haemocytometer) and the numbers observed in our study are consistent with the values reported by these authors. It was observed that the DSP in the sexually mature gerbil is 12 ± 0.6 (10^6 per testis per day) with a spermatogenic efficiency of 26 ± 4.1 (10^6 g^{-1} of testis). Previous studies have

demonstrated that the DSP per testis of gerbils is 18 ± 3 and spermatogenic efficiency is 33 ± 5 (Segatelli *et al.* 2004). The difference between our data and those of Segatelli *et al.* (2004) can be explained by the different methods used. Segatelli *et al.* (2004) used the method of quantitative testicular histology proposed by França (1992), while we used the method of homogenisation-resistant spermatids described by Robb *et al.* (1978), as previously discussed. Generally, there is an inverse relationship between the length of the spermatogenic cycle and spermatogenic efficiency. Species in which the spermatogenic cycle length is shorter show a higher spermatogenic efficiency, while species that present a longer spermatogenic cycle have a lower spermatogenic efficiency (França *et al.* 2005). This is the case for several South American rodents, in which the relationship (spermatogenic cycle length/spermatogenic efficiency) occurs, like the spiny rat (8.6 days/82×10^6; Cordeiro-Júnior *et al.* 2010), agouti (9.5 days/52×10^6), paca (11.5 days/39×10^6; Costa *et al.* 2010) and capybara (11.9 days/10×10^6; Paula *et al.* 1999). The duration of the cycle in rats is 12.9 days (Leblond and Clermont 1952) and their spermatogenic efficiency is $24 \times 10^6\,g^{-1}$ of testis (Robb *et al.* 1978), while in mice the duration is 8.6 days (Oakberg 1956) and the spermatogenic efficiency is $47 \times 10^6\,g^{-1}$ of testis (França *et al.* 2005). Although the cycle of the gerbils has an intermediate length between rats and mice, 10.6 days, their spermatogenic efficiency ($26 \pm 4.1 \times 10^6$) resembles that of rat and differs from those observed for South American rodents.

The data on sperm counts and transit time in the epididymis of the gerbil presented here are novel. These data show that the total number of spermatozoa observed in the caput/corpus of the epididymis of adult gerbils was smaller when compared with the rat ($\sim$10 vs $\sim 140 \times 10^6$ per organ; Robb *et al.* 1978). However, if we consider the sperm number per gram of caput–corpus, the difference is smaller ($\sim$166 vs $\sim 250 \times 10^6\,g^{-1}$; Robb *et al.* 1978). Previous studies reported that few spermatozoa were seen in this region of the epididymis (Domeniconi *et al.* 2007). Regarding the cauda epididymidis, the total number of spermatozoa observed in the mature gerbil ($\sim 120 \times 10^6$ per organ) was somewhat lower than in the rat ($\sim 200 \times 10^6$/organ; Robb *et al.* 1978); however, contrary to what occurs in the caput/corpus when we consider the sperm number per gram of cauda, the sperm number was considerably higher in the gerbil ($\sim$1130 vs $\sim 460 \times 10^6\,g^{-1}$; Robb *et al.* 1978). This indicates the sperm storage capacity in the cauda epididymidis of the gerbil and indicates that it is almost three times higher than that in the rat, when expressed per gram of tissue.

The sperm transit time along the epididymis was determined by the ratio between sperm reserves and DSP, as suggested by Robb *et al.* (1978). Thus, it was found that the transit time of spermatozoa through the epididymis of sexually mature gerbil is 11 days. The sperm transit time along the epididymis for most mammalian species varies between 9 and 11 days (Amann *et al.* 1976; França *et al.* 2005). Among rodents there is wide variation in this value; for example, for hamster it is 15 days and for mouse it is 5.5 days (Amann *et al.* 1976; França *et al.* 2005). The gerbil is similar to most species in relation to total transit time (11 days), which is close to the rat, for which this time varies between 8 and 10 days, depending on the lineage (Robb *et al.* 1978; França *et al.* 2005). In general, the time required for sperm maturation within the caput and corpus ranges from 2 to 5 days (Amann *et al.* 1993; França *et al.* 2005). However, an intriguing finding was the rapid sperm transit time along the caput/corpus of the epididymis ($\sim$1 day) and the longer one in the cauda ($\sim$10 days). This may be explained by the particular structure of corpus segment in this species that appeared as a slender and straight segment of the epididymidal duct connecting caput and cauda epididymidis (Domeniconi *et al.* 2007). Moreover, it was reported that the functionality of corpus in this species might be lower compared with the other two segments, since histoenzymatic reactions showed that this region has lower reactivity to the enzymes related to metabolism of this organ such as succinate dehydrogenase (SDH), ATPase, acid and alkaline phosphatases previously assessed in the corpus epididymidis by Domeniconi *et al.* (2006). Additionally, Domeniconi *et al.* (2007) reported the absence of clear cells in the epithelium of this region. On the other hand, the cauda epithelium was characterised by a large number of these cells intercalated between the principal cells and an expressive presence of dark–narrow cells (Domeniconi *et al.* 2007). It is known that the clear and narrow cells are related to secretory activities responsible for acidification of the luminal fluid (Hermo and Robaire 2002). Intraluminal acidification maintains sperm quiescence in the epididymidis duct, preventing premature activation of acrosomal enzymes (Verma 2001). Although the data of Domeniconi *et al.* (2007) support the role of the cauda segment in sperm storage, as classically known for most mammals, the high sperm number per gram and transit time in this segment, added to the atypical corpus structure, may indicate that a portion of sperm maturation in the gerbil occurs in the proximal cauda.

Sperm maturation in the epididymis consists of a wide spectrum of physiological and biochemical alterations that improve its capacity for fertilisation (Hermo and Robaire 2002). Indeed, the analysis of sperm motility is one of the most important parameters used in the evaluation of sperm quality (Bonde *et al.* 1998; Winkle *et al.* 2009; Fernandez *et al.* 2011). Animals at Day 60 presented similar percentages of motility compared with sexually mature animals, which is evidence that, although the DSP and sperm reserves have not yet attained their maximum, the animals of this age already show rates of sperm motility typical of adulthood.

Various stages of testicular development precede testicular maturity in mammals: impuberal, prepubertal, pubertal and sexual maturity (Courot *et al.* 1970). Based on histological and hormonal analysis and sperm parameters, the testicular development phases of gerbil were established. It is known that the spectrum of sperm parameters is very wide and assessing of sperm function characteristics relevant to fertility would require the application of modern technologies and techniques beyond microscopy (Petrunkina *et al.* 2007; Petrunkina and Harrison 2011). However, this study evaluated only some sperm parameters such as sperm counts, epididymal transit time and sperm motility. The gerbil's testes from birth to 14 days of age show seminiferous cords with a predominant presence of gonocytes and the absence of a lumen, signs that characterise the impuberal stage. In the prepubertal phase (Days 14 to 42) the disappearance of gonocytes occurs along with proliferation of germ cells in the seminiferous epithelium. Prepubertal animals exhibit testicular

cords in the process of lumen formation, which does not occur homogeneously and synchronously along the testis. Puberty is defined as the age at which a male individual achieves reproductive capacity for the first time (Robb *et al.* 1978). Animals at Day 60 showed remarkable sperm production and reserves. Although these analyses were not performed on animals younger than 60 days of age, the characteristics proposed by Courot *et al.* (1970) that indicate the onset of puberty, such as tubular diameter increases, the appearance of elongated spermatids and mature ALC, in addition to a drastic increase in serum testosterone levels, were already observed in animals at Day 42. Additionally, published data for the rat indicate that, at Day 50, a small amount of sperm production and reserves can be observed (Robb *et al.* 1978). Thus, our data indicate that puberty begins in the gerbil at around 42 days of age. Previously, the onset of puberty in gerbils was determined by anatomical and hormonal measures (Siegford *et al.* 2003). Our findings confirm the observations of Siegford *et al.* (2003) that puberty in male gerbil begins between Days 43 and 48.

It is known that the maximum reproductive capacity of a male in terms of sperm production or epididymal sperm reserves is not attained until the testes reach adult size (Amann 1970). In the case of the gerbil, the adult testis weight was stabilised at Day 60; however, the first DSP maximum occurred at Day 70 ($11.5 \pm 1.5 \times 10^6$) and the maximum sperm reserve at Day 90 (255.4×10^6 255.gerbil). Thus, the gerbil may be considered sexually mature only at 90 days of age. Therefore, male gerbils at 60 days of age are able to reproduce; however, if sexually mature males are required for physiological studies, only gerbils of at least 90 days of age should be used. This evidence indicates that the gerbil differs from the mouse, which becomes sexually mature at ~45 days of age (Kilborn *et al.* 2002) and resembles the rat, which reaches sexual maturity at around 100 days of age (Robb *et al.* 1978).

The data presented here demonstrate a substantial refinement in the stages of sexual development, in addition to establishing sperm parameters (DSP, spermatogenic efficiency, sperm reserve, sperm transit time along the epididymis and sperm motility) for the Mongolian gerbil (*Meriones unguiculatus*). Thus, we determined that the impuberal phase extends until Day 14, the same period when spermatogenesis begins. The prepubertal period occurs between Days 14 and 42; puberty is attained at ~42 days of age and sexual maturity occurs at Day 90. Furthermore, we found that, in terms of laboratory rodents, the gerbil has a similar reproductive profile with the rat, except for higher a sperm number per gram and transit time in the cauda epididymidis. This range of information provides new foundations for future investigations involving the reproductive biology of this rodent, which has become an important experimental model in reproductive biology research.

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