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Age-Related and Seasonal Variation in the Sertoli Cell Population, Daily Sperm Production and Serum Concentrations of Follicle-Stimulating Hormone, Luteinizing Hormone and Testosterone in Stallions[1]

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ABSTRACT

Testes and blood samples were obtained from 201 stallions aged 6 months to 20 years in either December-January (nonbreeding season) or June-July (breeding season) to study the effect of age and season on reproductive parameters. Seasonal differences in the Sertoli cell population of adult (4–20 years old) horses were characterized by a 36% larger number of Sertoli cells in the breeding season than in the nonbreeding season. Seasonal elevation in the Sertoli cell population was associated with an increase in testicular weight and daily sperm production per testis (DSP/testis). Concentrations of luteinizing hormone (LH) and testosterone in serum varied with season. Although follicle-stimulating hormone (FSH) concentrations also tended to be higher in the breeding season, this trend was not statistically significant ($P<0.08$). Sertoli cell numbers averaged over both seasons, like testicular weights, increased with age until 4–5 years of age, but were stabilized thereafter. This age-related difference was also associated with increased concentrations of FSH, LH and testosterone, and with increased DSP/testis. The Sertoli cell population was capable of increasing in the adult horse by fluctuating its size with season. The number of elongated spermatids per Sertoli cell over both seasons increased with age up to 4–5 years of age and was stabilized thereafter. Thus, seasonal and/or age-related differences in DSP/testis were associated with significant elevations in serum concentrations of FSH, LH and testosterone, testicular weights, numbers of elongated spermatids per Sertoli cell and elevation of the Sertoli cell population.

INTRODUCTION

Sertoli cell population is thought to be stable in the adult testis (Steinberger and Steinberger, 1977). When differences in numbers of Sertoli cells per cross section were detected without mitotic activity or degeneration of Sertoli cell nuclei following hypophysectomy, it was felt that these differences reflected shrinkage of the seminiferous tubules (Clermont and Morgentaler, 1955). Indeed, this assumption lead to the development of the Sertoli cell-correction factor (Clermont and Morgentaler, 1955) which has been used in quantitive expressions of spermatogenesis in various species including man and horses (Clermont and Morgentaler, 1955; Oakberg, 1959; Lino, 1971; Rowley and Heller, 1971; Berndtson, 1977, Berndtson et al., 1979). While this assumption was supported by the failure to observe mitotic figures in rat Sertoli cells after 15 days of age (Clermont and Perey, 1957) and by the failure of adult rat Sertoli cells to display mitotic figures or to incorporate [^{3}H] thymidine in culture (Steinberger and Steinberger, 1977), conflicting data exist. For instance, the increased number of Sertoli cells per tubular cross section could not be explained by tubular shrinkage alone in irradiated mice (Nebel and Murphy, 1960). Using autoradiography, Nagy (1972) found that rat Sertoli cells continued their division process much longer than their mitotic indices indicated. The total number of Sertoli cells per testis increased beyond 20 days in rat testes lacking germ cells (Bergh, 1981). Likewise, although the numbers of animals were small and findings were not statistically signifi-

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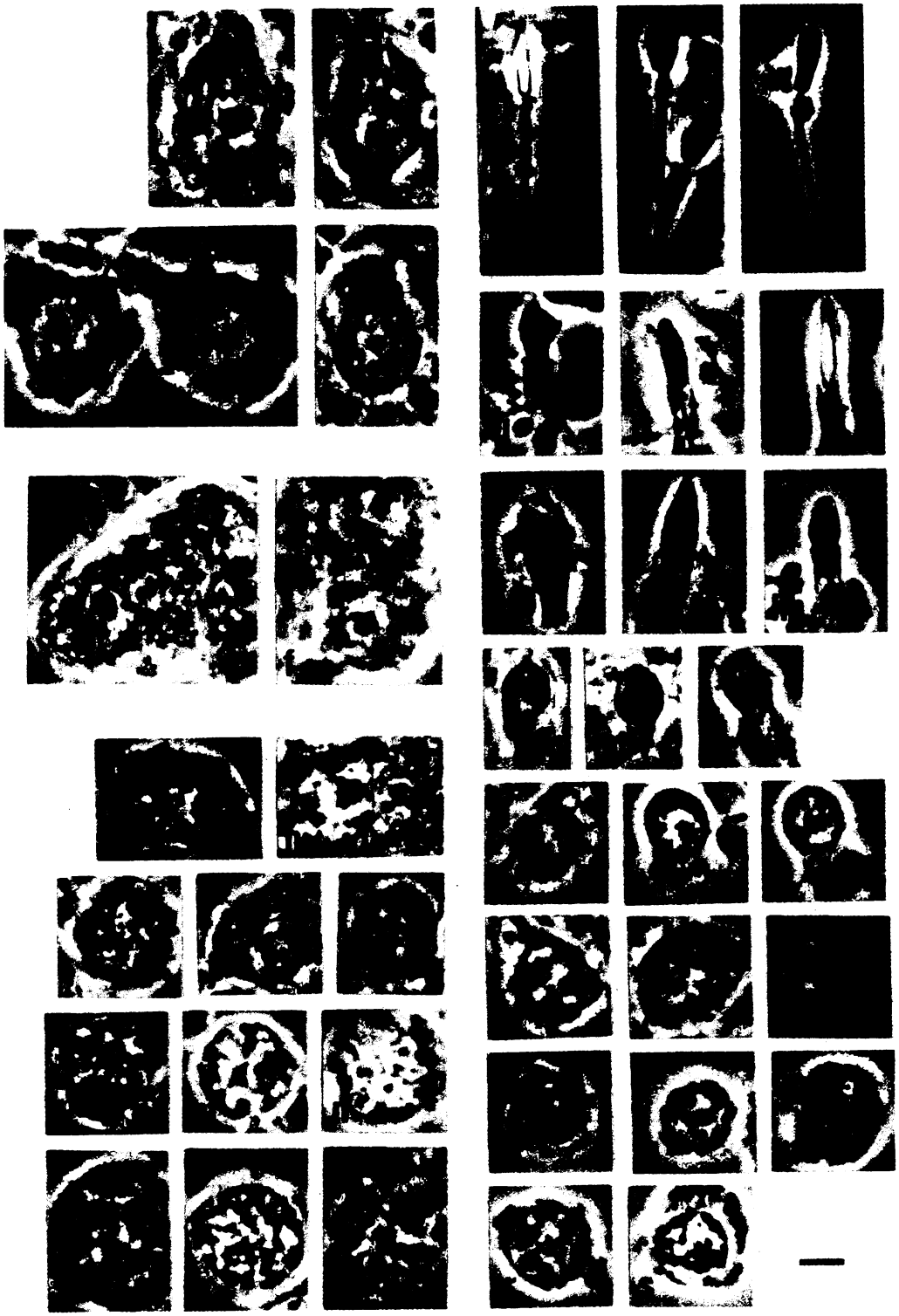

FIG. 1. Phase-contrast microscopic view of typical nuclear morphology of Leydig cells, Sertoli cells and germinal cells in homogenates of glutaraldehyde-fixed horse testes. These views represents selected focal planes obtained by focusing up and down on each nucleus.

a-d). Sertoli cells often retained some granular cytoplasm. Nuclei were identified by their large size, overall pear shape, and irregular, indented nuclear envelopes. Especially the tapered end had indentation which appeared as dark lines and may have extended deep into the nucleoplasm or along half of the long axis of the nucleus. In addition to size and shape, the chromatin pattern is important to distinguish Sertoli cell nuclei from other testicular cells. The nucleus is predominately euchromatic with no large chromatin clumps other than the nucleolus and with almost no heterochromatin clumps directly attached to the nuclear envelope. Single, large, irregular-shaped nucleoli were deeply embedded in the nucleoplasm away from the surface of nuclei. When viewed from the tapered end of the nucleus, large, lateral indentations from detached infringing germ cells can be seen (*c*), while the nucleoplasm appears identical to that from nuclei viewed from the side (*c*). Sertoli cells from horses <1 year were somewhat smaller (*d*) than those from older horses and often had smaller, double nucleoli.

e and f). Leydig cells remained clustered and were not evenly dispersed as were Sertoli and germinal cells. Granular cytoplasm was usually still attached to the large, highly euchromatic, spherical nucleus with one or more large nucleoli.

g and h). Type A spermatogonia had mostly ovoid nuclei with fine and moderately fine chromatin granules and two or more large nucleoli. The nuclei were the second largest among germinal cells.

i-k). Type B spermatogonia had spherical nuclei which were somewhat smaller than nuclei of Type A spermatogonia (*g and h*) and which had several smaller nucleoli each. Like Type A spermatogonia, the nuclear boundaries are well defined.

l-n). Nuclei of early primary spermatocytes contained moderately coarse chromatin granules which were separated by lighter nucleoplasm. Often the lighter nucleoplasm created an apparent notch in which coarse chromatin was displaced in the spherical nucleus. Nuclear boundaries are not well defined.

o-q). The spherical nuclei of late primary spermatocytes were the largest nuceli of the germinal cells and had a homogenous mixture of fine (lighter) and coarse chromatin clumps. While two or more larger chromatin granules were present, no distinct single large nucleolus was formed. Often when cytoplasm was attached to these late primary spermatocytes, a large, dark, spherical Golgi apparatus was visible (*o*). The nuclear boundaries were better defined than were younger primary spermatocytes (*l-n*).

r and s). Nuclei of secondary spermatocytes were considerably smaller than primary spermatocytes (*l-q*) or spermatogonia (*g-k*). These spherical nuclei had fine chromatin with few coarse chromatin granules found anywhere in the nucleoplasm and often attached to the nuclear envelope.

t-v). Nuclei of the youngest spermatids were spherical and contained coarse and fine granules not unlike the nucleoplasm of secondary spermatocytes. Nuclei of eary spermatids, like nuclei of all spermatids with spherical nuclei (*t-bb*), are the smallest germinal cell nuclei. Newly formed spermatid nuclei were the smallest (*t*) and still had dispersed chromatin. Other early spermatids may be characterized by a large, single, centrally located nucleolus and the lack of an acrosomic structure. However, late in Stage VI, the acrosomic vesicle appears in the cytoplasm (*v*). These spermatids are the younger generation of spermatids found in Stages V-VI and would be classified as early Sa spermatids.

w-y). Spherical nuclei of more developed spermatid found in Stages VII and VIII show further signs of acrosomic development the spherical acrosomic vesicle and granule present (w) or the flattened acrosomic vesicles attached to the nucleus (x). The formation and extension of the acrosomic cap over the nucleus and the first distinct appearance of the tail attached to the side of the nucleus adjacent to the developing acrosome. (y) characterize later development of Sa spermatids.

z-bb). The latest spermatids with spherical nuclei found the latter part of Stage VII and in Stage I are characterized by the acrosomic cap and developing tail. Nuclei of these Sb_1 spermatids are essentially spherical and are similar in size to earlier spermatids (*v - y*).

cc-ee). Nuclei of spermatids undergoing elongation found in Stage II were characterized by the first appearance of the manchette which extends from the middle of the nucleus, not covered by the developing acrosome, to a short distance down the tail. The developing tail now has a distinct annulus just below its connection with the nucleus. These are Sb_2 spermatids.

ff-bb). Spermatid nuclei found in Stages III - IV are characterized by their elongated shape but the chromatin has not yet fully condensed. The manchette extends further down the developing tail, and the dense acrosomic granule is still concentrated at the anterior tip of the nucleus. These are Sc spermatids.

ii-kk). Spermatid nuclei of Sd_1 type found in Stages V and VI still have the manchette which now encloses only about a fourth of the nucleus and extends down the developing tail. The nucleus has undergone condensation of its chromatin and has taken on the shape of typical equine sperm.

ll-nn). Sd_2 spermatids found in Stages VII and VIII have undergone further chromatin condensation and tail development. The annulus has migrated to its most distal location and mitochondria have surrounded the middle piece from the nucleus to the annulus. The cytoplasmic droplet located at the proximal position next to where the tail attaches to the head can be seen on tails of the most advanced Sd_2 spermatids (*nn*). Bar length is 5 μm.

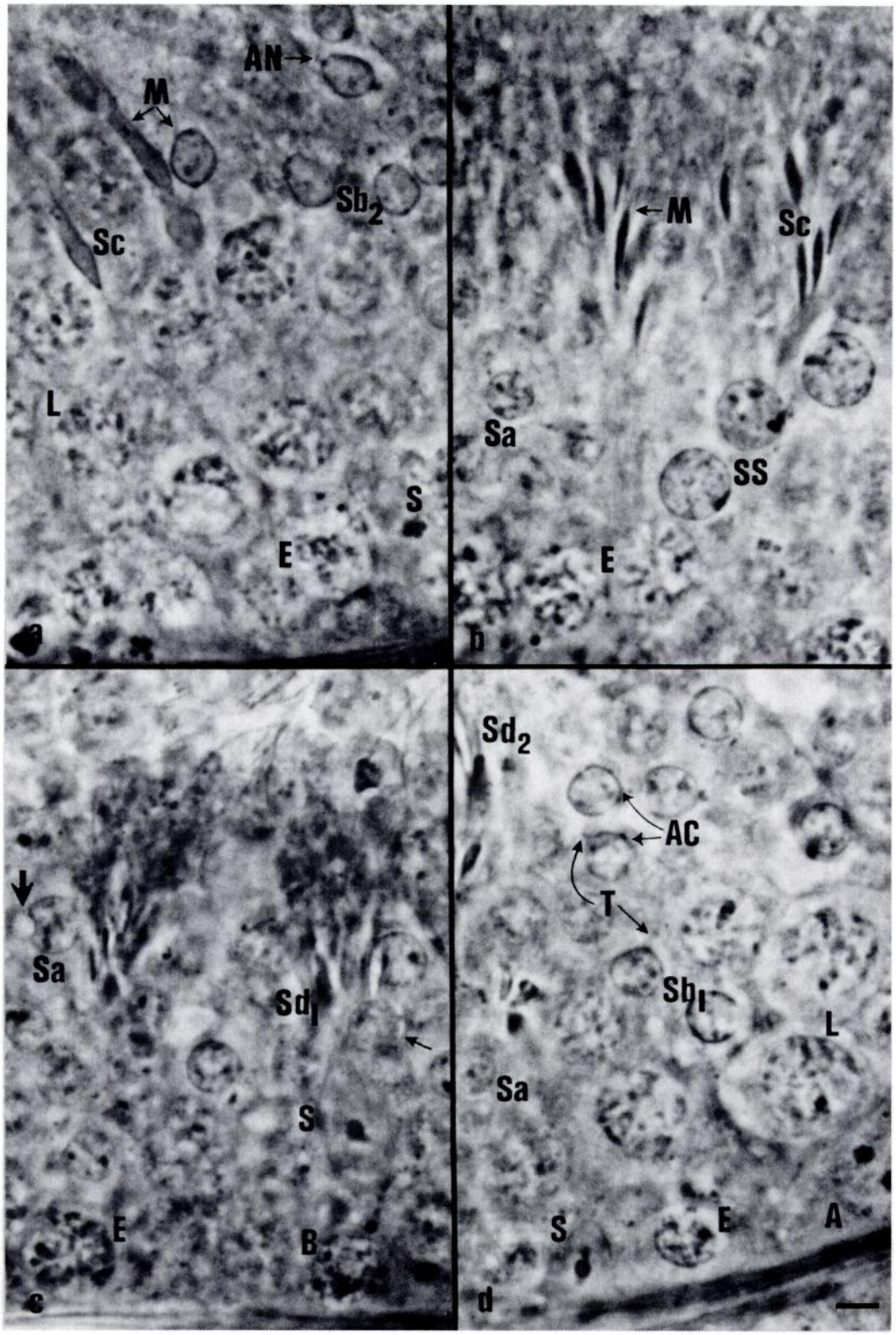

FIG. 2. Phase-contrast micrographs of Sertoli cells and germinal cells found in various stages of the cycle of the seminiferous epithelium (Swierstra et al., 1974) viewed in situ in unstained, 20-μm Epon sections.

a). *Transition Between Stages II and III.* Elongating nuclei of Sb_2 spermatids (Sb_2) with their newly formed manchette (M) and annulus (AN) around their developing tails are found in Stage II (*right side*). Stage III (*left side*) is characterized by a more distinct manchette (*M*) and completion of nuclear elongation with slight chromatin darkening and condensation in Sc spermatids (*Sc*). In both stages, nuclei of Sertoli cells (*S*), early (*E*) and late (*L*) primary spermatocytes are present.

b). *Transition Between Stages IV and V.* The presence of secondary spermatocytes (*SS*) denotes Stage IV (*right side*), while newly formed Sa spermatids (*Sa*) produced by the second meiotic division characterize Stage V. A generation of primary spermatocytes (*E*) and one or two generations of spermatids are found in these stages. Sc spermatids (*Sc*) of Stage IV give rise to Sd_1 spermatids of Stage V. The nuclei of spermatids are darkened by chromatin condensation. The manchette (*M*) is still present in Stage V and will persist into Stage VI.

c). *Stage VI of the Cycle.* In Stage VI the spermatids (Sd_1) have not yet begun to migrate toward the lumen as in Stage VII and VIII, but Type B (*B*) spermatogonia first appear and will persist into Stage VII. Acrosomic development of Sa spermatids (*Sa*) is evident with a small spherical acrosomic vesicle (*large arrow*) or flattened acrosomic vesicle (*small arrow*) in contact with the nucleus. One generation of primary spermatocytes (*E*) is present in Stage VI. A Sertoli cell nucleus (*S*) with its indented nuclear envelope and large nucleolus is shown.

d). *Transition Between Stages VIII and I.* Resulting from the division of Type B spermatogonia in Stage VII, a newly formed, early (*E*) generation of primary spermatocytes first appear in Stage VIII (*left side*). The early generation of primary spermatocytes of Stage VII become the later generation of primary spermatocytes (*L*) present in both Stages VIII and I. In Stage VIII, the most mature Sd_2 spermatids (Sd_2) which first appeared in Stage VII are present and are released by spermiation. A generation of spermatids, late Sa (*Sa*) in Stage VIII and Sb_1 (Sb_1) in Stage I are present. The Sb_1 spermatids have a dark acrosomic cap (*AC*) over one pole of the nucleus and a developing tail (*T*) attached to the side or other pole. Nucleus of a Type A spermatogonium (*A*) with its two nucleoli and a Sertoli cell nucleus (*S*) are indicated. These latter two cell types characterize all stages of the cycle. Bar equals 5 μm.

cant, Hochereau-de Reviers and Lincoln (1978) found that the number of Sertoli cells per testis increased by approximately 36% in adult red deer between its nonbreeding and breeding seasons.

Failure to detect significant differences in Sertoli cell numbers in adults may reflect the lack of determination of the total number of Sertoli cells per testis in a sufficient number of animals under conditions which significantly alter testicular weight without total cessation of sperm production, such as occurs with age and season in horses (Johnson and Neaves, 1981). Since stallions experience several seasonal and age-related changes, equine testes should be a good model for possible changes in Sertoli cell numbers. Seasonal differences include serum luteinizing hormone levels (LH) (Thompson et al., 1977), serum testosterone levels (Berndtson et al., 1974; Wiesner and Kirkpatrick, 1975), sexual behavior, seminal volume and sperm output (Skinner and Bowen, 1968; Pickett et al., 1970; Thompson et al., 1977). Age-related differences have included sexual behavior, testicular weight and size, seminal volume, sperm output, testicular composition and daily sperm production (DSP) (Horie and Nishikawa, 1955; Skinner and Bowen, 1968, Amann et al., 1979, Squires et al., 1979, Thompson et al., 1979; Johnson and Neaves, 1981). Age-related differences have also included increases in the Leydig cell population in 2- to 20-year-old stallions with a threefold increase in Leydig cell numbers per testis and a fivefold increase in Leydig cell volume per testis (Johnson and Neaves, 1981).

Recently in our laboratory, a new method of enumerating human testicular cells (Johnson et al., 1981) including the Sertoli cells (Johnson and Neaves, 1982) was applied to 20 testes from 4- to 5-year-old horses, and it was found that the Sertoli cell population increased by approximately 48% ($P<0.05$) between the onset and middle of the natural breeding season (2.74 ± 0.29 vs. $4.06 \pm 0.54 \times 10^9$). However, the number of Sertoli cells per g parenchyma was unchanged with season (25.5 ± 3.3 vs. $22.8 \pm 2.2 \times 10^6$). This finding was supported by the fact that total nuclear volume of Sertoli cells per testis (determined histometrically from the percentage Sertoli cell nuclei in parenchyma based on 2500 counts/testis × parenchymal weight) was significantly larger in the breeding season (1.80 ± 0.09 vs. 3.20 ± 0.52 ml), while the average size of a Sertoli cell nucleus (20 nuclei per testis measured for width and height under Nomarski optics in 20-μm sections by the method of Johnson and Neaves, 1982) was constant (11.35 ± 0.12 vs. 11.65 ± 0.19 μm average

maximum diameter). Further, when Sertoli cell nuclei containing nucleoli were counted per cross section in 20 tubular cross sections per testis, no difference (4.1 ± 0.2 vs. 4.0 ± 0.1; $P>0.05$) was found; however, the length of tubules in the same horses was greater (1.9 ± 0.1 vs. 2.9 ± 0.3 km; $P<0.01$) in the middle of the breeding season (Johnson and Neaves, 1981). Given age-related differences in testicular weight (Amann et al., 1979; Johnson and Neaves, 1981) and our finding of consistant numbers of Sertoli cells/g, it is anticipated that Sertoli cell numbers will also differ with age.

To evaluate the age-related and seasonal variation in the Sertoli cell population and corresponding variations in other reproductive parameters, testes and blood from 201 horses age 6 months to 20 years were obtained either in the nonbreeding or breeding season. Testicular weight, concentrations of LH, follicle-stimulating hormone (FSH), and testosterone, DSP/testis, and the Sertoli cell population increased with age. Testicular weight, serum LH and testosterone, DSP/testis, DSP/g, and the Sertoli cell population increased during the breeding season. These findings are in contrast to the current theory that the Sertoli cell population is stable in adult mammalian testes (Steinberger and Steinberger, 1977) and indicate that Sertoli cell numbers increase in adult stallions by fluctuating with season.

MATERIALS AND METHODS

Specimens and Hormone Assays

Pairs of testes and single blood samples from 201 horses, 6 months to 20 years of age, were obtained from a commercial slaughterhouse in the nonbreeding (December and January) or breeding season (June and July). Ages of horses were determined by tooth replacement and wear. Within 15 min of death, a single blood sample was obtained during exsanguination of the horse, and testes were obtained. Both were placed on ice and returned to the laboratory where testes were freed from fascia and the epididymis and weighed. Parenchymal weight was determined by subtracting the weight of the tunica albuginea from the whole testicular weight. Slices of parenchyma (1 to 3 g) were fixed in 2% glutaraldehyde in 100 mM cacodylate buffer and subsequently stored at 5°C. Blood samples, allowed to clot during their transport on ice, were centrifuged for 10 min at 10,000 × *g*. Sera were kept frozen until evaluated by radioimmunoassay for FSH, LH and testosterone (Thompson et al., 1979, 1983a,b). Three testosterone concentrations were omitted on the basis of extremely high values (>2 standard deviations from the mean). For statistical analysis, undetectable hormone concentrations were assigned the minimal detectable concentration for each hormone.

Preparation of Homogenates and Identification of Cell Nuclei

Testes (122) from all horses >4 years of age and from 15 or 17 randomly selected horses from each of the younger age groups for each season were evaluated for the DSP and number of Sertoli cells. Fixed testicular parenchyma was blotted on a piece of dry dental wax to remove surface buffer without drawing fluid from the depths of the tissue. Once blotted and weighed, approximately 0.4 g of tissue was homogenized in a Waring Blender for 6 min in 100 ml of fluid containing 150 mM NaCl, 0.05% (v/v) Triton X-100, and 3.8 mM NaN_3 (Amann and Lambiase, Jr., 1969). As noted in previous studies on humans (Johnson et al., 1981; Johnson and Neaves, 1982), this procedure produced a cell suspension (Fig. 1) from which various types of germinal or Sertoli cell nuclei could be enumerated by phase-contrast cytometry. The identification of Sertoli cell nuclei in the homogenate was based on observation of different focal planes of each nucleus in which nuclear size, nuclear shape, nuclear indentations, chromatin features, relationship to occasionally attached germinal cells, and a cytoplasmic shape were considered (Figs. 1 and 2). The identity of germinal cells and Sertoli cells was verified by the comparison of whole nuclei of these cells found in the homogenate to these cell nuclei in situ viewed in 20-μm Epon sections by phase-contrast microscopy (Fig. 2). Thick (20 μm) sections which contained the whole nucleus of a cell proved important in classification of spermatid nuclei into different types using the classification that Clermont (1963) used for humans and important in identification of the stages of the cycle in which these nuceli appear. These findings are given in the legends (Figs. 1 and 2).

Calculation of DSP and Numbers of Sertoli Cells

Elongated spermatid nuclei (Fig. 1) characteristic of Stages III-VIII (Fig. 2; Swierstra et al., 1974) were evaluated in the homogenate. Daily sperm production per gram parenchyma (DSP/g) was calculated for each horse by dividing the number of elongated spermatid nuclei enumerated in the homogenate by the product of the weight of tissue homogenized times the 8.2-day life span of these spermatids (Swierstra et al., 1974). The number of Sertoli cells/g was calculated by dividing the number of Sertoli cell nuclei in the homogenate by the weight of tissue homogenized.

In preliminary studies (20 testes from 4- to 5-year-old horses), DSP/g based on elongated spermatid nuclei in homogenates of fixed testicular parenchyma (16.3 ± 1.2 and 18.0 ± 2.2 × 10^6 for the onset and middle of the breeding season) compared favorably with those obtained for the conventional (Amann et al., 1976) homogenates of unfixed testes (18.8 ± 1.6 × 10^6 over both seasons for the same testes; Johnson and Neaves, 1981). Likewise, the numbers of Sertoli cells per g parenchyma determined from homogenates of fixed testes and by histometric analysis of the same 20 testes was similar for the onset (25.5 ± 3.3 vs. 21.1 ± 2.1 × 10^6) and middle of the breeding season (22.8 ± 2.2 vs. 20.8 ± 1.9 × 10^6). Assuming that the average volume of the Sertoli cell nucleus could be approximated by using the formula for a sphere and the

average diameter (height and width measured in 20-μm Epon section with Normarski optics), the number of Sertoli cells/g was calculated histometrically by dividing the total volume of Sertoli cell nuclei in the testis by the product of the volume of a single Sertoli cell nucleus and parenchymal weight. This histometric approach, independent of stage of the cycle, not only yielded similar relative values for the number of Sertoli cells per g between seasons but also yielded absolute values similar to the homogenate method. Precision of duplicate estimates made on separate pieces of fixed tissue was estimated at 5.9% average coefficient of variation for DSP/g and was 18.6% average coefficient of variation for Sertoli cell numbers/g. Each estimate of either cell type was based on four separate cytometer chamber counts, because two additional counts did not improve the precision of Sertoli cell numbers. A two-way analysis of variance (Sokal and Rohlf, 1969) was employed to analyze the variation due to age-related and seasonal effects. Means within age groups were compared by Student-Newman-Keuls procedure. Correlation coefficients were tested for significance (Sokal and Rohlf, 1969).

RESULTS

When testes from horses <2 to 20 years of age were compared over both seasons, the right, left and paired testicular weights (Table 1) as well as parenchymal weight (Table 2) were higher in the older horses (Table 3). Likewise, these same measures over all ages were significantly higher in the breeding season, and there was a significant age × season interaction for testicular and parenchymal weights (Tables 1–3). Less seasonal differences in weights were observed in younger horses (Tables 1 and 2). Older horses had significantly elevated concentrations of FSH, LH and testosterone when both seasons were compared (Tables 1 and 3). LH and testosterone concentrations over all ages were significantly elevated in the breeding season. Concentrations of FSH also tended ($P<0.08$) to be elevated in the breeding season (Tables 1 and 3). There was an age × season interaction for concentration of LH (Tables 1 and 3).

Glutaraldehyde fixation of horse testes prior to homogenization rendered nuclei of Sertoli cells and germinal cells resistant to homogenization while a testicular cell suspension was produced (Fig. 1). To observe identifiable features on many nuclei, different focal planes within a nucleus must be observed by up and down focus. Since phase-contrast cytometry yields number per unit volume, the problem of estimating the average volume of a single nucleus of cells with nonspherical shape such as the Sertoli cell nucleus was eliminated. Nuclei

TABLE 1. Age-related and seasonal differences in testicular weights and serum concentrations of FSH, LH and testosterone.

Season	Age (years)	Number of horses	Testicular weights (g)			Hormone concentration		
			Right	Left	Paired	FSH (ng/ml)	LH (ng/ml)	Testosterone (pg/ml)
Nonbreeding	<2	19	41.0 ± 6.5[a]	50.3 ± 6.3	91.4 ± 12.6	19.1 ± 4.6	1.8 ± 0.3	41.7 ± 8.2
	2–3	40	60.7 ± 6.1	83.9 ± 7.0	144.6 ± 11.7	16.3 ± 2.0	2.9 ± 0.4	91.0 ± 14.3
	4–5	16	125.0 ± 14.3	144.8 ± 11.3	269.8 ± 24.1	35.9 ± 10.4	21.8 ± 7.0	196.3 ± 58.0
	6–12	9	122.8 ± 16.1	119.8 ± 15.9	242.6 ± 30.6	29.0 ± 6.4	16.7 ± 6.2	301.6 ± 84.2
	13–20	7	148.0 ± 15.1	119.0 ± 25.6	267.0 ± 32.2	96.9 ± 35.4	17.7 ± 6.1	216.6 ± 51.9
Breeding	<2	30	43.4 ± 6.4	50.3 ± 5.9	92.7 ± 12.1	29.1 ± 3.2	2.7 ± 0.3	67.3 ± 11.8
	2–3	52	80.3 ± 5.8	99.5 ± 4.3	179.8 ± 9.6	34.3 ± 3.1	4.1 ± 0.7	108.3 ± 19.5
	4–5	11	149.3 ± 18.2	152.6 ± 17.2	301.8 ± 35.2	38.8 ± 3.5	16.6 ± 5.3	192.2 ± 47.4
	6–12	8	201.6 ± 22.3	202.2 ± 21.6	403.8 ± 43.8	55.5 ± 7.0	42.1 ± 13.6	286.6 ± 103.2
	13–20	9	174.2 ± 14.4	201.2 ± 12.7	375.3 ± 20.3	85.0 ± 19.9	49.3 ± 13.6	451.7 ± 87.6

[a]Mean ± standard error of the mean.

TABLE 2. Age-related and seasonal differences in parenchymal weight, daily sperm production, the Sertoli cell population and the number of elongated spermatids per Sertoli cell.

Season	Age (years)	Number of horses	Parenchymal weight per/testis(g)	Daily sperm production per g ($\times 10^6$)	Number of Sertoli cells per g ($\times 10^6$)	Number of elongated spermatids per Sertoli cell
Nonbreeding	<2	15	31.4 ± 5.7[a]	4.2 ± 1.0	36.9 ± 3.1	0.9 ± 0.3
	2–3	15	61.8 ± 9.4	11.5 ± 1.8	35.8 ± 3.1	2.8 ± 0.5
	4–5	16	116.9 ± 11.1	15.5 ± 1.3	24.1 ± 1.8	5.5 ± 0.6
	6–12	9	103.7 ± 14.0	15.0 ± 1.4	28.9 ± 2.7	4.6 ± 0.6
	13–20	7	112.0 ± 14.4	12.8 ± 2.7	18.6 ± 2.8	6.9 ± 1.7
Breeding	<2	15	37.9 ± 6.0	5.8 ± 1.9	32.1 ± 3.6	1.4 ± 0.5
	2–3	17	75.6 ± 8.3	14.6 ± 2.1	38.3 ± 2.9	3.5 ± 0.6
	4–5	11	131.3 ± 15.3	17.7 ± 1.3	28.9 ± 2.1	5.4 ± 0.6
	6–12	8	173.6 ± 18.8	19.7 ± 1.0	19.7 ± 1.9	8.7 ± 0.9
	13–20	9	155.8 ± 8.4	19.3 ± 0.7	23.1 ± 1.6	7.0 ± 0.4

[a]Mean ± standard error of the mean.

of Sertoli cells and elongated spermatids were enumerated directly by phase-contrast cytometry in these suspensions. Identity of Sertoli cell nuclei and germinal cell nuclei was verified when nuclei found in the homogenate were compared with nuclei observed in situ in 20-μm Epon, unstained histologic sections viewed under phase-contrast microscopy (Fig. 2). Detailed descriptions given in the legends (Figs. 1 and 2) and not repeated here are important aids in correct identification of different cell types in the homogenates.

When homogenates from 122 horses representing five age groups and two seasons were evaluated, a significant age-related decrease ($P<0.01$) in the number of Sertoli cells/g and increase in DSP/g and a significant seasonal increase in DSP/g were found (Tables 2 and 3). With an age-related and seasonal increase ($P<0.01$) in parenchymal weight (Table 2), both the number of Sertoli cells per testis (Fig. 3) and DSP/testis (Fig. 4) were increased ($P<0.01$) by both age and by season. There was a significant interaction between age and season on DSP/testis as larger seasonal differences existed between older horses (Table 3). However, no interaction was found between age and season for DSP/g, the number of Sertoli cells/g, or number of Sertoli cells/testis (Tables 1–3; Fig. 4). Not only did the number of Sertoli cells/testis increase in the breeding season and with age, but there were cyclic variations over age due to the seasonal effect (Fig. 3). The number of Sertoli cells/g and number of Sertoli cells/testis were significantly correlated ($P<0.01$) with circulating concentrations of LH ($r=-0.33$, $r=0.26$) and testosterone ($r=-0.32$, $r=0.32$), but only Sertoli cells/g was significantly ($P<0.01$) correlated ($r=-0.32$) with FSH concentrations. DSP/g and DSP/testis were significantly correlated ($P<0.01$) with circulating concentrations of LH ($r=0.27$, $r=0.50$) and testosterone ($r=0.32$, $r=0.50$) but were not correlated ($P>0.05$) with FSH concentrations.

The number of elongated spermatids per Sertoli cell was significantly higher in older horses and seasonal differences were noted (Tables 2 and 3). There was a significant interaction between season and age as larger seasonal differences were found in older horses than in younger horses. The increase in the number of elongated spermatids per Sertoli cell reflects two facts: *1*) the population of germinal cells increases its numbers with age up to 4–5 years, and *2*) individual Sertoli cells of 4- to

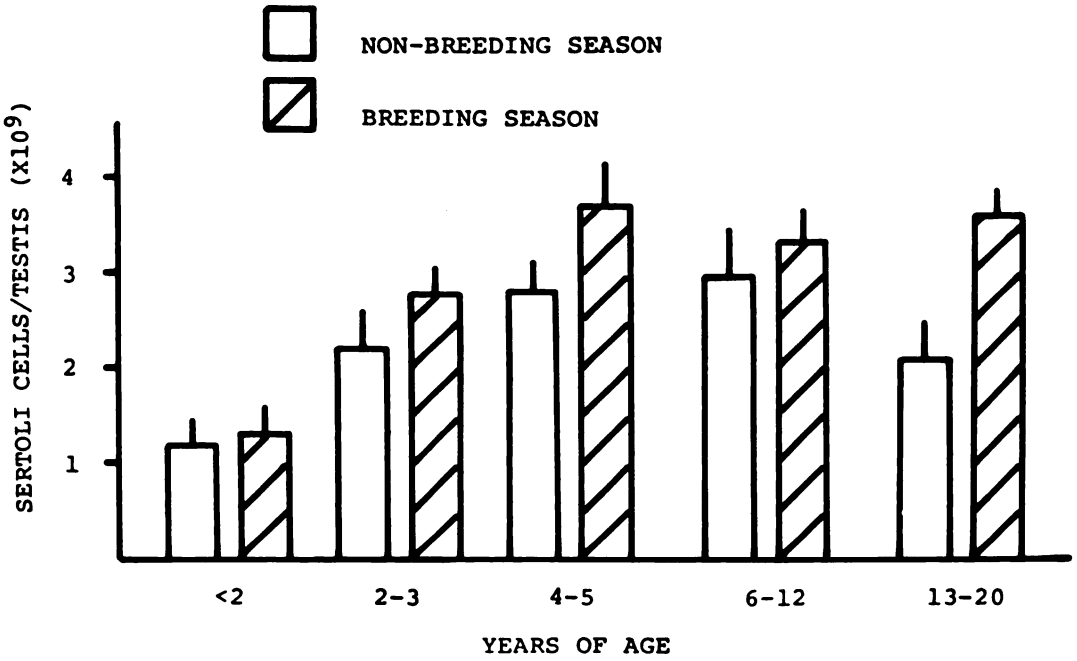

FIG. 3. Age-related and seasonal differences in the number of Sertoli cells per testis.

TABLE 3. Significance levels for each parameter analyzed by a two-way analysis of variance and significance of differences among means for age groups.

Parameter	Main effect		Age × season interaction	Differences among means from age groups[a]				
	Age	Season		<2	2–3	4–5	6–12	13–20
Testicular weight								
Right	0.01	0.01	0.05	A	B	C	C	C
Left	0.01	0.01	0.01	A	B	C	C	C
Paired	0.01	0.01	0.01	A	B	C	C	C
Parenchymal weight	0.01	0.01	0.05	A	B	C	C	C
Hormone levels								
FSH	0.01	NS[b]	NS	A	A	A	A	B
LH	0.01	0.01	0.01	A	A	B	C	C
Testosterone	0.01	0.05	NS	A	A	B	C	C
Daily sperm production								
Per g parenchyma	0.01	0.01	NS	A	B	B	B	B
Per testis	0.01	0.01	0.01	A	B	C	C	C
Number of Sertoli cells								
Per g parenchyma	0.01	NS	NS	A	A	B	B	B
Per testis	0.01	0.01	NS	A	B	B	B	B
Elongated spermatid nuclei/Sertoli cell	0.01	0.05	0.05	A	B	C	C	C

[a]Means with the same letter are not significantly (P>0.05) different.

[b]*NS*=P>0.05.

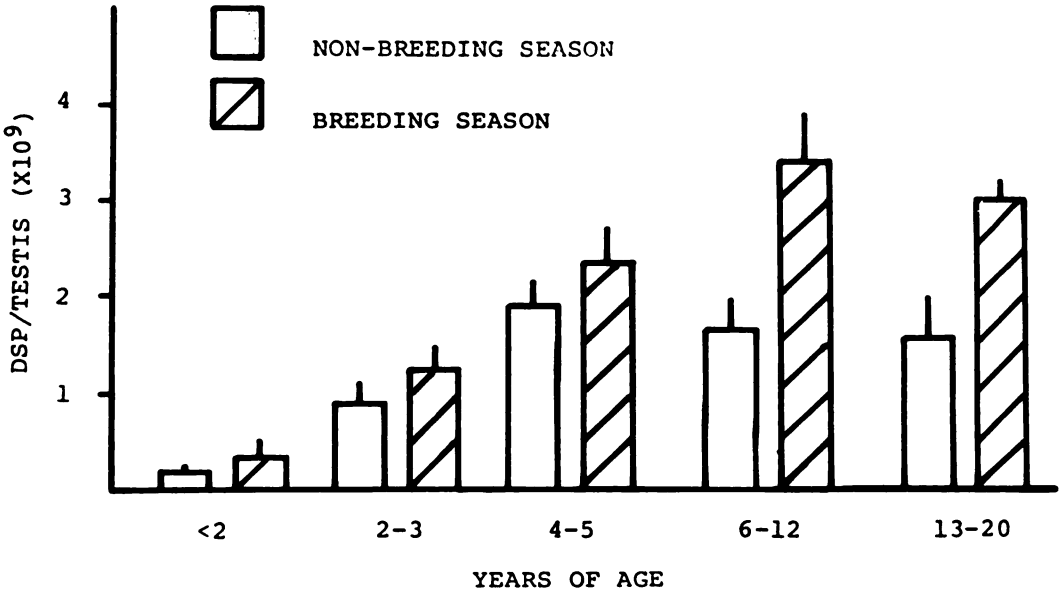

FIG. 4. Age-related and seasonal differences in daily sperm production per testis (DSP/testis).

20-year-old horses are able to accommodate a larger number of elongated spermatids (Tables 2 and 3). The number of elongated spermatids per Sertoli cell was significantly ($P<0.01$) correlated with circulating concentrations of LH ($r=0.42$), testosterone ($r=0.49$) and FSH ($r=0.25$).

DISCUSSION

The present study supports our preliminary finding of significant seasonal variation in the Sertoli cell population in adult horse testes. The number of Sertoli cell/g parenchyma (Table 2) for 4- to 5-year-old horses was similar to our preliminary data determined for 4- to 5-year-old horses by direct enumeration of Sertoli cells in homogenates of fixed testes (approx. 24×10^6/g). Likewise, human testes have 25×10^6 Sertoli cells/g (histometric) or 26×10^6/g (homogenate method; Johnson and Neaves, 1982), red deer testes have approximately 7 to 14×10^6/g (Hochereau-de Riviers and Lincoln, 1978), rat testes lacking germ cells have approximately 23×10^6/testis (Hochereau-de Reviers and Courot, 1978) or approximately 30×10^6/testis (Bergh, 1981), and normal rat testes have approximately 54×10^6/testis (Hochereau-de Reviers and Courot, 1978) or approximately 22×10^6/testis (Wing and Christensen, 1982). While the ram (approx. 6×10^9) and bull (approx. 7×10^9) had more Sertoli cell/testis (Hochereau-de Reviers and Courot, 1978) than did the horse (approx. 3×10^9), the Sertoli cell population of the horse is capable of increasing in the adult by fluctuating with season (Fig. 3; Table 3).

In addition to the seasonally breeding red deer (Hochereau-de Reviers and Lincoln, 1978), increases in the Sertoli cell population have been reported to occur in adult animals during recovery from X-irradiation (Nebel and Murphy, 1960), artificial cryptorchidism (Clegg, 1963), and during FSH replacement treatment following hypophysectomy (Murphy, 1965). However, these findings were not given credence because of lack of statistical significance (Hochereau-de Reviers and Lincoln, 1978) or have not received wide acceptance because differences could possibly be confounded with shrinkage of seminiferous tubules (Steinberger and Steinberger, 1977). The same criticism would not apply to our estimate of number of Sertoli cells/testis determined by the homogenate method (Johnson and Neaves, 1982; Table 2). Even if the length of seminiferous tubules shortened, the tubular volume density was reduced, or Sertoli cell nuclei increased their volume density causing the number of Sertoli cell/g to increase, a proportional reduction in parenchymal weight would

result. Thus, the estimated number of Sertoli cells/testis (number/g times parenchymal weight) would be unaffected by tubular shrinkage. Furthermore, the diameter and volume density of tubules in adult horses do not vary with season (Johnson and Neaves, 1981), nor do the volume density and size of Sertoli cell nuclei (see *Introduction*). Likewise, the number of Sertoli cells/g was not altered by season, but the number of Sertoli cells/testis was significantly larger in the breeding season (Tables 2 and 3; Fig. 3).

The source (proliferation or differentiation) of the additional Sertoli cells found in the breeding season and the fate (cell death or de-differentiation) of the Sertoli cells following the breeding season is not known at this time. However, in testes obtained in the onset of the breeding season, we have observed mitotic figures located at the base of seminiferous epithelium in Stages II and III. In contrast, mitotic activity of both A and B spermatogonia is thought to occur between Stages V and VIII (Swierstra et al., 1974). Intense efforts to find mitotic figures in Sertoli cells may have been hampered by the apparent short time (approx. 2 months) required for the Sertoli cell population (see *Introduction*) and seminiferous tubular length (Johnson and Neaves, 1981) to increase significantly. It may be necessary to evaluate Sertoli cell mitotic activity and/or the presence of precurser cells within the length of seminiferous tubules as well as in the transitional zones located between seminiferous tubules and the rete testis (Amann et al., 1977) each month between the two seasons to gain insight as to the possible source and fate of these additional Sertoli cells.

Age-related increases in LH and testosterone (Tables 1 and 3) are in agreement with age-related increases in Leydig cell volume/testis, volume/g parenchyma, and the number/testis (Johnson and Neaves, 1981). Seasonal elevation of LH and testosterone (Tables 1 and 3) may be important in the seasonal increase in total Leydig cell volume per testis, the diameter and volume of a single Leydig cell nucleus, and the total Leydig cell nuclear volume per testis (Johnson and Neaves, 1981). Seasonal changes in concentration of LH were very dramatic in stallions sampled throughout the year (Thompson et al., 1977) and were linked to changes in photoperiod. Our study revealed a seasonal trend ($P<0.08$) in FSH concentrations as well (Tables 1 and 3). While the volume density of seminiferous tubules did not vary with season or age, larger tubular volume and length (Johnson and Neaves, 1981) and higher levels of sperm output (Thompson et al., 1977) and sperm production were observed in the breeding season (Fig. 4). Thus, it is reasonable to assume that differences in hormone concentrations (LH, testosterone, FSH), Leydig cell structure, seminiferous tubules and sperm production rates are interrelated. The number of Sertoli cells/g and DSP/testis were significantly ($P<0.01$) correlated with LH, FSH and testosterone concentrations.

Sperm production efficiency (DSP/g) was lowest in testes from horses <2 years of age. The mean values tended to be lower in horses 2–3 years old than in those 4–20 years old (Table 2); however, this difference was not significant (Table 3). There were 5 horses 8–12 months of age that were randomly selected from the <2 group over both seasons whose DSP/g was $<1.3 \times 10^6$. The trend of less sperm production efficiency for 2- to 3-year-old horses supports the previous finding that 2- to 3-year-old horses had not reached maximum sperm production efficiency (Johnson and Neaves, 1981) and is consistent with aging studies on the rat (Robb et al., 1978). We would conclude from the seasonal increase in DSP/g and the age × season interaction for the number of spermatids with elongated nuclei/Sertoli cell (Tables 2 and 3), that testes in the breeding season are more adapted for high levels of sperm production. However, testes from horses in the nonbreeding season did continue to produce sperm, albeit at a lower rate (Table 2).

Since Sertoli cells are intimately associated with germinal cells and the size of the Sertoli cell population has been shown to be significantly correlated to the number of type A spermatogonia in adult male rats, rams, and bulls (Hochereau-de Reviers and Courot, 1978), it would seem reasonable for seasonal fluctuations in the Sertoli cell population to be involved in seasonal changes in DSP/testis of horses. An elevated Sertoli cell population in the breeding season would accommodate a larger number of germinal cells than would normally be supported during the nonbreeding season when sperm output (Thompson et al., 1977) and sperm production (Table 2) continued at a lower rate. Indeed, unlike other seasonal breeders which drastically alter the number of germinal cells per Sertoli cell (i.e., only Sertoli

cells and younger germinal cells remain in the nonbreeding season; Neaves, 1973), the adult horse may have a limited capability to alter the number of germinal cells per Sertoli cell and maintaining a continued production of sperm throughout the year. However, adult horse testes do alter the ratio of germ cells to Sertoli cells on a limited basis (Tables 2 and 3). Given this limited capability to alter the ratio of germ cells per Sertoli cell, another mechanism by which the adult horse testis may alter its weight and total sperm production with season would be to fluctuate its Sertoli cell population as well.

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