

Progression of Reproductive Changes Accompanying Testicular Dysfunction in Aging Thoroughbred Stallions: Case Studies

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Regular breeding soundness examinations in aging stallions are warranted to detect early changes that might predict onset of declining fertility. Management changes could then be instituted that might improve pregnancy rates during this period and until testicular dysfunction becomes so pronounced that a stallion must be retired for infertility. Authors' addresses: Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843-4475 (Blanchard, Brinsko, Varner, Love), and Equine Medical Associates, PSC, Lexington, KY 40583-3166 (Morehead); e-mail: tblanchard@cvm.tamu.edu. *Corresponding and presenting author. © 2013 AAEP.

1. Introduction

Superior stallions can command a sizeable book of mares to be bred each year throughout their reproductive lifespan. Many stallions achieve good pregnancy rates when bred beyond 20 years of age, but eventually fertility begins to decline. A myriad of causes can contribute to declining fertility in aging stallions, including testicular dysfunction.¹ As the ability to produce sufficient numbers of normal, motile sperm in ejaculates declines, so do pregnancy rates until the stallion eventually becomes so subfertile that it is no longer commercially feasible to continue breeding the horse. The purpose of this study was to evaluate our findings in three aging Thoroughbred stallions over this period of declining fertility to determine when testicular dysfunction

began as well as to characterize the progression of the disorder. These data have been used to formulate changes in breeding management for aging stallions with testicular dysfunction.²

2. Materials and Methods

Stallions, Semen Collection, and Testicular Measurement
 Three aging Thoroughbred stallions were evaluated for breeding soundness over several years as their fertility declined. Stallions were usually evaluated at least once yearly (typically between November and January): Stallion 1 from 20 through 24 years of age; Stallion 2 from 18 through 27 years of age; and Stallion 3 from 18 through 24 years of age. Stallions were mated several times to mares in estrus to stabilize extragonadal sperm reserves before

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semen was collected. After 1 to 2 days of sexual rest, semen collections (two to four ejaculates over a 1- or 2-day period) were accomplished with the use of an artificial vagina^a equipped with an in-line nylon micromesh filter^b to permit collection of gel-free semen.¹ Before semen collection, each stallion was exposed to a mare in estrus to stimulate sexual interest. The erect penis was rinsed thoroughly with water and cotton immediately before semen collection and was dried with paper towels. Each stallion was then allowed to mount a mare in estrus, and the penis was deflected into the artificial vagina. After semen collection, the gel-free semen was transported to an adjacent laboratory and placed in an incubator (37°C) before processing (see "Semen Evaluation"). Daily sperm output (DSO) was estimated as previously described.³

Testicular measurements were obtained by means of transcutaneous ultrasonography, and the length, width, and height of each testis was used to calculate testicular volume. Total testicular volume was represented by adding the volume of the left and right testis. Predicted DSO was calculated on the basis of total testicular volume.⁴

Semen Evaluation

Volume of gel-free semen for each ejaculate was measured in a graduated cylinder. Sperm concentration was determined with the use of an Equine Densimeter,^c unless concentration was dilute (ie, $<100 \times 10^6/\text{mL}$), in which case hemacytometer counts were used to determine sperm concentration.¹ The total sperm number was determined by the product of semen volume \times concentration. Gel-free semen was extended in one of two extenders^{d,e} at a 9:1 ratio (extender:semen), and placed in an incubator for 15 minutes. Sperm motility (total/progressive; percentage) was assessed subjectively by one experienced examiner with the use of a phase-contrast microscope with a warming stage at $\times 250$ magnification.¹

Aliquots of raw semen were mixed with buffered formol saline and stored until evaluated for sperm morphologic characteristics with the use of a differential interference contrast microscope at $\times 1250$ magnification.¹ One hundred sperm were evaluated per specimen, and all morphologic features (normal, proximal droplet, distal droplet, abnormal head, bent midpiece, swollen or irregular midpiece, bent or broken tail, detached head, coiled tail, abnormal acrosome, or premature germ cell) were recorded and expressed as percentages.⁵

Determination of Spermatogenic Efficiency

Spermatogenic efficiency was calculated by as a percentage, on the basis of predicted sperm output ([actual DSO/predicted DSO from testicular measurements] $\times 100$).⁶

Sperm Chromatin Structure Assay

One-milliliter aliquots of raw semen from the last ejaculate collected at each examination period were

pipetted into 1.2-mL cryovials, placed on dry ice, and shipped frozen to the laboratory for storage at -80°C until assayed for sperm DNA integrity by the Sperm Chromatin Structure Assay as previously described.⁷ Values recorded for each assay were mean α -t and %COMP α -t.

Hormonal Assays

When assays were performed to determine circulating hormone concentrations, jugular blood was collected into 7-mL heparinized tubes once hourly for 4 hours in the morning. Tubes were centrifuged, and equal volumes of plasma were pipetted from each hourly sampling into 2-mL cryovials that were frozen and shipped to a commercial endocrine laboratory on dry ice. Radioimmunoassays were performed to determine circulating concentrations of testosterone, total estrogens, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) and, in some instances, inhibin.

On some occasions (when resting testosterone concentration was low), a gonadotropin (GnRH; 100 μg , IV) stimulation test was performed. Jugular blood samples were collected immediately before GnRH administration and at 15, 30, 60, and 120 minutes after GnRH administration for assay of testosterone concentrations.⁸

Pregnancy Rates

Pregnancy rates per cycle (PR/C) and per season (SPR) were determined from analysis of breeding records.

Normalized Data

Additionally, PR/C, spermatogenic efficiency, percentage of the maximal testicular volume (Max volume; the volume obtained on the first year each stallion's testes were measured), and total number of progressively motile, morphologically normal (PMMN) sperm at DSO were normalized to the last year each stallion bred mares (year of retirement because of infertility during that breeding season) and averaged among the three stallions.

3. Results

Pregnancy rates and findings from breeding soundness examinations over a 5-year period of declining fertility for Stallions 1 through 3 are presented in Tables 1, 2, and 3, respectively. Fig. 1 provides results of the data normalized to the last year at stud and averaged among stallions.

Early in the course of progression of testicular dysfunction in these aging stallions, a period of decreasing sperm output occurred before a remarkable change in testicular size. The diminished sperm output resulted from lowered spermatogenic efficiency. Pregnancy rates often were not markedly affected early in this period. Before testicular size was noticeably decreased, sperm output, spermatogenic efficiency, and PR/C declined dramatically. Eventually, SPR declined as well. Although these

Table 1. Breeding Soundness Examination Findings for Stallion 1 Between 20 and 24 Years of Age

Age, y	Book	PR/C	SPR	TTV	DSO	Effic	%MN	%PM	Mean	COMP	Test	Est	LH	FSH	Inh	Test Res
19	79	55%	86%	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
20	73	30%	67%	382	5.80	73%	43%	40%	NE	NE	NE	NE	NE	NE	NE	NE
21	63	31%	70%	380	3.48	43%	45%	30%	241	16%	NE	NE	NE	NE	NE	NE
22	53	21%	45%	312	2.09	32%	50%	25%	239	16%	650	275	2.0	2.1	NE	1300
23	40	25%	48%	308	2.06	32%	25%	15%	257	31%	784	156	11.0	10.2	NE	1126
24	20	7%	15%	260	1.74	32%	25%	15%	286	31%	650	156	NE	NE	NE	980

NE indicates not examined; Book, number of mares bred in season; PR/C, pregnancy rate per cycle, %; SPR, seasonal pregnancy rate, %; TTV, total testicular volume, cc; DSO, daily sperm output, $\times 10^9$; Effic, spermatogenic efficiency, %; %MN, percentage of morphologically normal sperm; %PM, percentage of progressively motile sperm, SCSA values; Mean, Mean- α ; COMP, %COMP- α (percentage of cells outside the main population); Test, circulating testosterone concentration, pg/mL (normal range, 500–2000); Est, circulating total estrogens concentration, pg/mL (normal range, 150–400); LH, circulating luteinizing hormone concentration, ng/mL (normal range, 0.5–5.0); FSH, circulating follicle-stimulating hormone concentration, ng/mL (normal range, 0.5–15.0); Inh, circulating inhibin concentration, ng/mL (normal range, 2.2–3.4); Test Res, highest concentration of testosterone within 2 hours of GnRH administration, pg/mL (normal increase $\geq 100\%$ within 2 hours).

changes were associated with a decrease in percentage of morphologically normal and progressively motile sperm in ejaculates, sperm chromatin structure assays did not reveal prominent changes in sperm DNA integrity. It was only late in the course of progression of testicular dysfunction (last 2 years of breeding) that sperm DNA integrity began to deteriorate. Baseline hormonal concentrations usually did not differ from reported normal ranges until after spermatogenic efficiency had declined to $\leq 40\%$, DSO approximated ≤ 2 billion, total number PMMN sperm at DSO was < 500 million, and the average PR/C diminished to $\leq 33\%$. Sperm motility and morphology values typically but not always deteriorated from those found when each horse was still achieving normal pregnancy rates. Low total estrogen concentration was sometimes noted in the last 1 to 3 years of breeding (ie, two of three stallions). We also noted a low circulating concentration of testosterone in two stallions during this same time period (ie, 116–403 pg/mL), and some-

times testosterone did not achieve the lower limit of reported normal circulating concentration (ie, ≥ 500 pg/mL) within 2 hours of GnRH administration in one of three stallions. Total testicular volume during the last year at stud decreased to approximately 50% of the previous size when the stallions were several years younger. At this late stage of testicular dysfunction, variations from normal endocrine values were more likely to include low estrogen, testosterone and inhibin concentrations, and, in some instances, high FSH and LH concentrations.

4. Discussion

These findings support the premise that age-related testicular dysfunction follows a progressive pattern of deterioration. First, a decline in sperm output, including total number of normal motile sperm, is observed in ejaculates. This may be detected during pre-season breeding soundness examination but sometimes is found later during the breeding sea-

Table 2. Breeding Soundness Examination Findings for Stallion 2 Between 18 and 27 Years of Age

Age, y	Book	PR/C	SPR	TTV	DSO	Effic	%MN	%PM	Mean	COMP	Test	Est	LH	FSH	Inh	Test Res
17	88	70%	97%	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
18	103	68%	88%	514	8.22	75%	47%	45%	232	18%	NE	NE	NE	NE	NE	NE
19	108	67%	85%	508	8.53	78%	45%	45%	250	20%	NE	NE	NE	NE	NE	NE
21	112	55%	84%	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
22	92	52%	86%	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
23	81	48%	83%	459	3.41	35%	49%	55%	234	9%	460	276	0.05	0.05	1.96	750
24	76	57%	88%	442	3.77	40%	43%	50%	231	15%	593	235	0.5	0.08	NE	645
25	78	34%	68%	360	1.73	23%	41%	30%	252	20%	274	231	1.8	0.8	4.19	494
26	61	19%	44%	346	1.50	21%	47%	25%	295	19%	NE	NE	NE	NE	NE	NE
27	26	0%	0%	299	1.00	17%	42%	25%	275	42%	116	128	2.8	0.8	3.99	370

NE indicates not examined; Book, number of mares bred in season; PR/C, pregnancy rate per cycle, %; SPR, seasonal pregnancy rate, %; TTV, total testicular volume, cc; DSO, daily sperm output, $\times 10^9$; Effic, spermatogenic efficiency, %; %MN, percentage of morphologically normal sperm; %PM, percentage of progressively motile sperm, SCSA values; Mean, Mean- α ; COMP, %COMP- α (percentage of cells outside the main population); Test, circulating testosterone concentration, pg/mL (normal range, 500–2000); Est, circulating total estrogens concentration, pg/mL (normal range, 150–400); LH, circulating luteinizing hormone concentration, ng/mL (normal range, 0.5–5.0); FSH, circulating follicle-stimulating hormone concentration, ng/mL (normal range, 0.5–15.0); Inh, circulating inhibin concentration, ng/mL (normal range, 2.2–3.4); Test Res, highest concentration of testosterone within 2 hours of GnRH administration, pg/mL (normal increase $\geq 100\%$ within 2 hours).

Table 3. Breeding Soundness Examination Findings for Stallion 3 Between 18 and 24 Years of Age

Age, y	Book	PR/C	SPR	TTV	DSO	Effic	%MN	%PM	Mean	COMP	Test	Est	LH	FSH	Inh	Test Res
17	94	67%	92%	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
18	93	57%	84%	331	6.98	100%	42%	55%	NE	NE	NE	NE	NE	NE	NE	NE
19	79	49%	84%	330	6.20	93%	40%	50%	230	7%	NE	NE	NE	NE	NE	NE
20	93	54%	87%	331	5.84	87%	40%	50%	225	6%	NE	NE	NE	NE	NE	NE
21	57	55%	84%	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
22	69	44%	80%	220	2.43	54%	43%	45%	233	16%	535	170	0.5	0.4	NE	NE
23	74	48%	80%	174	1.90	40%	40%	45%	217	21%	334	NE	13.1	23.4	1.77	NE
24	19	0%	0%	91	0.65	35%	25%	15%	278	31%	403	96	3.0	0.8	NE	610

NE indicates not examined; Book, number of mares bred in season; PR/C, pregnancy rate per cycle, %; SPR, seasonal pregnancy rate, %; TTV, total testicular volume, cc; DSO, daily sperm output, $\times 10^9$; Effic, spermatogenic efficiency, %; %MN, percentage of morphologically normal sperm; %PM, percentage of progressively motile sperm, SCSA values; Mean, Mean- α t; COMP, %COMP- α t (percentage of cells outside the main population); Test, circulating testosterone concentration, pg/mL (normal range, 500–2000); Est, circulating total estrogens concentration, pg/mL (normal range, 150–400); LH, circulating luteinizing hormone concentration, ng/mL (normal range, 0.5–5.0); FSH, circulating follicle-stimulating hormone concentration, ng/mL (normal range, 0.5–15.0); Inh, circulating inhibin concentration, ng/mL (normal range, 2.2–3.4); Test Res, highest concentration of testosterone within 2 hours of GnRH administration, pg/mL (normal increase $\geq 100\%$ within 2 hours).

son, when pregnancy rates are noted to be lower than expected. As illustrated in Figure 1, the decline in output of normal motile sperm and the decline in spermatogenic efficiency precedes a decrease in testicular size. Decreased spermatogenic efficiency has been associated with an increased rate of germ cell degeneration during spermatogenesis.^{9,10} As spermatogenic efficiency declines, a higher percentage of morphologically abnormal sperm and fewer motile sperm are often present in the ejaculate.¹⁰ Thus, when testicular size began decreasing dramatically in the latter 2 to 3 years before forced retirement, sperm output dropped precipitously despite an apparent plateau in spermatogenic efficiency.

Our findings in these three stallions confirm that testicular dysfunction can become quite pronounced before sperm DNA integrity becomes severely compromised, as has been reported previously.¹⁰ We also noted an increase in mean- α t in two stallions

during their latter 2 years before forced retirement. Whereas the %COMP α -t measures the proportion of cells outside the main population of typically normal cells, an elevated mean- α t suggests that a greater proportion of sperm in the main population of the ejaculate has DNA of compromised integrity.

Others have described that aging stallions with more mild testicular dysfunction may not have significant changes in circulating hormone concentrations.^{11–15} As testicular dysfunction worsens, endocrine changes appear. Roser¹⁵ hypothesized that stallions with declining fertility usually first exhibit increasing circulating concentrations of FSH, followed later by decreasing concentrations of estradiol and inhibin, and, with further progression of testicular dysfunction, circulating concentrations of testosterone decline.¹⁵ Besides determining baseline circulating concentrations of the above-mentioned hormones, Roser also recommends GnRH and human chorionic gonadotropin (hCG) stimulation tests to identify stal-

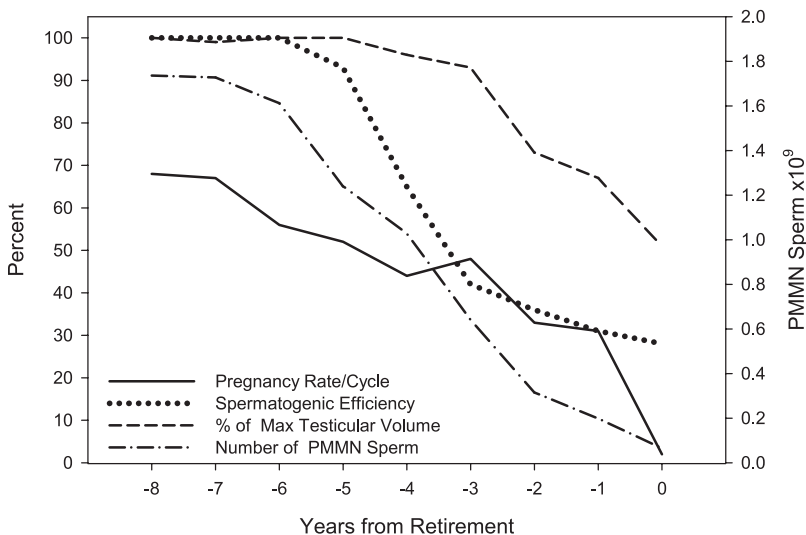


Fig. 1. Pregnancy rate per cycle (%), spermatogenic efficiency (%), percentage of initial (Max) testicular volume recorded at first examination, and total number of progressively motile morphologically normal (PMMN) sperm in ejaculates at daily sperm output (DSO) in three aging Thoroughbred stallions. Data are normalized to year of retirement because of infertility for each stallion and averaged among stallions.

lions with poor testosterone response.¹⁵ Although global conclusions for all stallions cannot be drawn from only three stallions in this study, two were noted to have low concentrations of testosterone (or testosterone and estrogens) as the earliest detected variation from normal hormone concentrations.

Summary

Regular breeding soundness examinations in aging stallions are warranted to detect early changes that might predict onset of declining fertility. Management changes (eg, decreasing mating frequency, reinforcement breeding, mating closer to ovulation, and/or planned multiple matings per estrus, as previously described²) could then be instituted that might improve pregnancy rates during this period and until testicular dysfunction becomes so pronounced that a stallion must be retired for infertility.

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^aMissouri-model artificial vagina, Nasco, Ft Atkinson, WI 53538.

^bDisposable Nylon Mesh Gel Filters, Animal Reproduction Systems, Chino, CA 91710.

^cEquine Densimeter, Animal Reproduction Systems, Chino, CA 91710.

^dEZ Mixin-CST, Animal Reproduction Systems, Chino, CA 91710.

^eINRA96, IMV Technologies, L'Aigle, France.

Fear of disease progression is a situationspecific and fully discernible (reportable) emotion based on personal experience of a life-threatening disease. This article presents the results of a study of cancer patientsâ€™ coping behavior according to the levels of fear of disease progression experienced.Â reproductive-system cancer (Monasypova, 2012). As a rule, in cases of illness, customary inferences of the integrity and firmness of one's self are prone to alteration, one's customary system of senses and values is destabilized, and one's behavior changes as well (Nikolayeva, 1987; Tkhostov, 2002).