

1 **Semen quality, testicular B-mode and Doppler ultrasound and serum testosterone**  
2 **concentrations in dogs with established infertility**

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21

22 Abstract:

23 Retrospective examination of breeding records enabled the identification of 10 dogs of  
24 normal fertility and 10 dogs with established infertility of at least 12 months duration.

25 Comparisons of testicular palpation, semen evaluation, testicular ultrasound  
26 examination, Doppler ultrasound measurement of testicular artery blood flow, and  
27 measurement of serum testosterone concentration were made between the two groups  
28 over weekly examinations performed on three occasions.

29 There were no differences in testicular volume ( $\text{cm}^3$ ) between the two groups (fertile  
30 right testis =  $10.77 \pm 1.66$ ; fertile left testis =  $12.17 \pm 2.22$ ); (infertile right testis =  $10.25$   
31  $\pm 3.33$ ; infertile left testis =  $11.37 \pm 3.30$ ), although the infertile dogs all had  
32 subjectively softer testes compared with the fertile dogs. Infertile dogs were either  
33 azoospermic or when they ejaculated had lower sperm concentration, sperm motility  
34 and percentage morphologically normal spermatozoa than fertile dogs. Furthermore,  
35 infertile dogs had reduced sperm membrane integrity measured via hypo-osmotic  
36 swelling test. Infertile dogs had significantly lower basal serum testosterone  
37 concentrations ( $1.40 \pm 0.62$  ng / mL) than fertile dogs ( $1.81 \pm 0.87$  ng / mL) ( $P < 0.05$ ).

38 There were subjective differences in testicular echogenicity in some of the infertile  
39 dogs, and important differences in testicular artery blood flow with lower peak systolic  
40 and end diastolic velocities measured in the distal supra-testicular artery, marginal  
41 testicular artery and intra-testicular artery of infertile dogs ( $P < 0.05$ ). Notably,  
42 resistance index and pulsatility index did not differ between infertile and fertile dogs.  
43 These findings demonstrate important differences between infertile and fertile dogs  
44 which may be detected within an expanded breeding soundness examination.

45

46 Keywords: ultrasonography, pulse-wave Doppler, dogs, testis, infertility.

47

48 1. Introduction

49 Conducting a breeding soundness examination (BSE) is a well-established method for  
50 evaluating the breeding potential of dogs [1]. The principle of the BSE is that it may  
51 detect features predictive of poor breeding or fertilizing potential but despite the wide  
52 recommendation for use of the BSE [2] there have been no comprehensive studies  
53 examining differences in BSE between fertile and infertile dogs. There are lamentably  
54 few investigations comparing even individual components of the BSE between fertile  
55 and infertile dogs; the most significant study was performed more than 20 years ago and  
56 compared only sperm morphology [3]. More recent and elaborate investigation, for  
57 example of sperm DNA peroxidate, has found no differences between infertile and  
58 fertile dogs [4].

59

60 The key aspects of a BSE include clinical examination of the reproductive tract,  
61 observation of libido, examination of semen quality, and some cases ultrasound  
62 examination of the reproductive tract, and endocrine testing [1,2]. More recently,  
63 measurement of testicular artery flow has been purported to be of some value [5] and  
64 may form part of an expanded BSE, although data are available from only a small  
65 number of individuals [5].

66

67 The study aim was to establish which aspects of an expanded breeding soundness  
68 examination were different between known fertile and known infertile dogs.

69

70 2. Materials and methods

71 2.1. Animals

72 This study was performed in the Laboratory of Carnivore Reproduction at the School of  
73 Veterinary Medicine, State University of Ceará and approved by the Animal Ethics  
74 Committee of the institution (protocol 12641034-8).

75

76 Animals were selected based on evaluation of detailed breeding records from private  
77 breeders who had meticulous records and two groups were identified. Fertile dogs  
78 comprised 10 dogs that had mated at least 4 bitches during the previous 12 months each  
79 achieving at least two normal pregnancies with a normal litter size for the breed [6].  
80 Infertile dogs comprised 10 dogs that had mated at least 4 bitches during the previous  
81 12 months with no resultant pregnancy. All bitches had been previously pregnant and in  
82 both groups were bred by natural mating at a time identified by vaginal cytology and  
83 measurement of plasma progesterone concentration.

84

85 The fertile dogs comprised Labrador (2), Rottweiler (4) and German Shepherd (4)  
86 breeds, ranging from 2 to 8 years old, weighing 33 to 42 kg (mean =  $4.5 \pm 1.9$  SD). The  
87 infertile dogs comprised Fila Brasileiro (2), Golden Retriever (2), Rottweiler (3) and  
88 German Shepherd (3) breeds, aged from 4 to 8 years old (mean =  $5.4 \pm 1.4$  SD),  
89 weighing 35 to 44 kg.

90 Veterinary clinical examination and complete blood count at the beginning of the study  
91 confirmed that all dogs were clinically normal and healthy. All dogs were fed a  
92 maintenance complete dry food with *ad libitum* water for the duration of the study.

93

## 94 2.2. Breeding soundness examination

95 Each dog was subject to all aspects of the BSE on 3 occasions at 7 day intervals. The  
96 fertile / infertile status of the dog was not known by the evaluator. At each examination

97 the scrotal contents were palpated and a subjective assessment of the testes consistency  
98 was made which was recorded as firm or soft. Ejaculates were then collected from each  
99 dog by digital manipulation and the second fraction of the ejaculate was immediately  
100 subjected to detailed examination. The second fraction volume was recorded and a  
101 subjective microscopic assessment of the percentage total sperm motility [7] was made  
102 at x400 magnification at room temperature. Sperm concentration was measured using a  
103 Neubauer chamber after dilution with formal-saline [8], and sperm morphology was  
104 evaluated at x1000 magnification on Rose-Bengal stained slides [3]. Membrane  
105 integrity was evaluated at x400 magnification using the hypo-osmotic swelling test  
106 (HOST) [9].

107

108 Ultrasound examinations were performed on the right and left testis of each dog with 7  
109 days intervals using a SonoAce PICO machine (Medison, Korea) with a linear array  
110 transducer with 5 to 9 MHz capability. Dogs were positioned in dorsal recumbency,  
111 acoustic gel was applied to the skin, and the transducer was positioned initially on the  
112 lateral surface of the testis. Longitudinal and transverse B-mode images were made  
113 (using the mediastinum as a reference point for measuring the testicular length and  
114 width) and testicular volume was calculated using the formula for an ellipsoid;  $V =$   
115 length x width x height x 0.5236. The appearance of the parenchyma of each testis was  
116 recorded subjectively as normal echogenicity, hypoechoic, or hyperechoic. In addition  
117 the presence of abnormal echogenic stippling was recorded as present or absent.

118

119 For the measurement of testicular artery flow in three separate regions, color Doppler  
120 ultrasound was used with the transducer initially placed at the neck of the scrotum (to  
121 identify the tortuous distal (looping) region of the supra-testicular artery [here termed

122 distal supra-testicular artery)) immediately cranial to the cranial pole of the testis. The  
123 transducer was then moved distally (to identify the marginal region in longitudinal  
124 section [here termed marginal testicular artery] and the relatively straight intra-testicular  
125 arteries within the testicular parenchyma [here termed intra-testicular arteries]). The  
126 proximal region of the supra-testicular artery was not studied because it was not  
127 possible to ensure consistency of position between dogs. Within each region the color  
128 gain was adjusted to reduce any excess color noise and the pulse wave Doppler gate was  
129 positioned within the lumen of the vessel. Three waves of a cardiac cycle were used to  
130 measure mean values for peak systolic velocity (PSV), end diastolic velocity (EDV),  
131 and these were used by the machine software to calculate resistance index (RI) and  
132 pulsatility index (PI). The operator and machine presets (depth 4.5- 5.5 cm, pulsed  
133 repetition frequency 2.5 kHz, wall filter 5 cm/s, sample gate 2.0 mm) were consistent  
134 for each region at all examinations. The angle between the Doppler beam and the long  
135 axis of the vessel was less than 60°, using angle corrections when necessary. However,  
136 in most cases, an angle of 0° was used.

137

138 Blood was collected from each dog weekly on 3 occasions at 9 a.m each day. After  
139 clotting serum was harvested and frozen at -80°C until evaluation for testosterone  
140 concentration using a commercially available radioimmunoassay kit (Total Testosterone  
141 Coat-a-Count ® Diagnostics Products Corporation, Los Angeles, CA, USA). The intra-  
142 and inter-assay coefficients of variation were 1.28% and 5.9%, respectively.

143

#### 144 2.5. Statistical analysis

145 Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene test).

146 A two-factor ANOVA was used to test for differences in testicular volume between

147 weeks, right and left testis volume, and to investigate differences between the fertile and  
148 infertile dogs.

149

150 Semen quality data were submitted to the Friedman test to compare values between the  
151 weeks of evaluation and to the Mann-Whitney test for comparison between fertile and  
152 infertile dogs.

153

154 Doppler ultrasound parameters were compared using a two-factor ANOVA to test for  
155 differences between regions of the testes, right and left testes and between fertile and  
156 infertile dogs, using weeks of evaluation as one of the factors.

157

158 Two-factor ANOVA was used to examine differences between weeks and groups for  
159 serum testosterone concentrations. A significance level of  $P < 0.05$  was used in all  
160 cases, and the results were expressed as the mean  $\pm$  standard deviation.

161

162 To investigate any relations between intra-testicular artery flow, coefficients of  
163 correlation were calculated between each of the flow measurements (PSV, EDV, RI, PI)  
164 and each of the semen quality measurements (total motility, sperm concentration,  
165 HOST, morphologically normal sperm) for all 20 dogs. The Pearson product-moment  
166 correlation coefficient was calculated and significant correlations were considered  
167 significant when  $P < 0.05$ .

168

169 3. Results

170 The testes of all fertile dogs were reported as being firm in texture, whereas the testes  
171 from all infertile dogs were reported as soft in texture. No other scrotal abnormalities  
172 were noted.

173

174 The second fraction of the ejaculate from the fertile dogs had a white opaque  
175 appearance whilst for the infertile dogs the ejaculates were colorless in 5 dogs (these  
176 samples were confirmed as azoospermic) and watery-white in the remaining 5 dogs.  
177 Semen quality did not differ between the weeks of evaluation, and none of the  
178 azoospermic dogs produced an ejaculate containing sperm. Total sperm motility, sperm  
179 concentration, percentage of swollen sperm in the HOST and the percentage of normal  
180 spermatozoa were higher in fertile compared with infertile dogs that produced sperm,  
181 although there were no differences in the ejaculate volume (Table 1).

182

183 Testicular volume did not differ between the weeks of evaluation for either group.  
184 Testicular volume ( $\text{cm}^3$ ) was not different between the fertile (right testis =  $10.77 \pm$   
185  $1.66$ ; left testis =  $12.17 \pm 2.22$ ) and infertile dogs (right testis =  $10.25 \pm 3.33$ ; left testis  
186 =  $11.37 \pm 3.30$ )  $\text{cm}^3$ , although for each group the left testes had a significantly greater  
187 volume ( $P < 0.05$ ).

188

189 Subjective scoring of testicular echogenicity of the fertile dogs showed that 8 had  
190 bilateral normal echogenicity testes, 1 had bilateral normal echogenicity testes with  
191 echogenic stippling in one testes, whilst 1 dog had bilateral hypochoic testes. For the  
192 infertile dogs, 2 had normal echogenicity testes, 4 had bilateral hypochoic testes, 2 had  
193 bilateral hyperechoic testes, and 2 had bilateral hyperechoic testes with echogenic  
194 stippling in one testis.



195

196 Color Doppler allowed identification of all regions of the testicular artery of the left and  
197 right testes of all dogs. The distal supra-testicular artery had a tortuous pattern along its  
198 entire length, and although it was possible to visualize the artery in both groups, it  
199 subjectively appeared less tortuous and it was more difficult to capture the color  
200 Doppler signal in infertile dogs. The marginal testicular artery had a linear pattern and  
201 was observed along the entire length of the testis and did not appear different between  
202 either group. The intra-testicular arteries were visible throughout the testicular  
203 parenchyma of both groups, following a linear pattern directed towards the mediastinum  
204 testis.

205

206 When visualized by pulse-wave Doppler, the waveforms of the testicular artery blood  
207 flow in fertile dogs, within the supra-testicular region, were biphasic with a diastolic  
208 notch followed by a diastolic peak in 4 dogs and monophasic with systolic peaks,  
209 decreasing diastolic flow and low vascular resistance in 6 dogs. Testicular artery blood  
210 flow within the marginal and intra-testicular region was monophasic for all dogs. For  
211 the infertile dogs the waveforms had a more venous-like waveform appearance in the 3  
212 regions, with lower velocities than for the fertile dogs. This flow pattern was  
213 differentiated from venous flow which could also be identified.

214 Images for Color and pulse-wave Doppler for fertile and infertile dogs are provided in  
215 the supplemental material.

216

217 Doppler measurements did not differ between the weeks of evaluation or between the  
218 right and left testes for either group. Similarly, for either group there were no regional

219 differences in PSV, EDV, RI or PI, although each of these parameters was numerically  
220 greater in the distal supra-testicular region (Table 2).

221

222 Correlations between the Doppler arterial flow measurements of the intra-testicular  
223 arteries and semen quality for all 20 dogs showed that there was a significant negative  
224 correlation between RI and total motility ( $r = -0.30$ ;  $p = 0.05$ ), and between PI and total  
225 sperm motility ( $r = -0.37$ ;  $p = 0.01$ ). There were no other significant correlations.

226

227 Serum testosterone concentrations did not differ between the weeks in either the fertile  
228 and infertile dogs. Serum testosterone concentrations were significantly higher in the  
229 fertile dogs ( $1.81 \pm 0.87$  ng / mL) compared with the infertile dogs ( $1.40 \pm 0.62$  ng /  
230 mL).

231

232

#### 233 4. Discussion

234 The central principle of the breeding soundness examination is that particular  
235 components may be useful for the differentiation of normal from abnormal males. In  
236 this study a group of dogs of known fertile status were compared with a group that had  
237 failed to achieve any pregnancies over the preceding 12 months.

238

239 It was interesting that ultrasound-measured testicular volume did not differ between the  
240 fertile and infertile dogs, similar to observations previously made in the dog [10] and in  
241 men [11], llamas and alpacas [12]. It is clear that testicular volume alone is not a  
242 reliable parameter for evaluating dogs with a history of infertility, however softening of  
243 the testes detected by palpation was reported in all infertile but none of the fertile males

244 in the present study, demonstrating that this can be a useful component of the BSE. The  
245 relation between testis tone and semen quality has previously been remarked upon [3]  
246 but this is the first report that evidences softening of the testes as a significant feature of  
247 infertility.

248

249 Evaluation of a semen sample is an important aspect of the BSE [13]. In this study  
250 semen quality measurements from the fertile dogs were similar to those previously  
251 reported [7,14]. Interestingly, but not unexpectedly, 5 of the infertile dogs were  
252 azoospermic, and for the remaining infertile dogs which produced sperm there were  
253 lower values total sperm motility, sperm concentration, morphologically normal sperm  
254 and sperm membrane integrity evaluated using the hypo-osmotic swelling test,  
255 compared with the fertile dogs. Interestingly, whilst the HOST has been adequately  
256 described in dogs [9,15] there has been limited study of hypo-osmotic swelling of sperm  
257 in known infertile dogs. Our work is a useful addition to the literature in this area,  
258 especially since the validity of the HOST has recently been questioned [16].

259

260 In the present study, B-mode ultrasound imaging of the testicular parenchyma of the  
261 fertile dogs showed a subjective appearance similar to that previously reported [10],  
262 although interestingly one fertile dog had echogenic stippling present in one testis, and  
263 another had hypoechoic testes; both features that have been reported as abnormal  
264 [10,17]. Importantly, two infertile dogs had normal echogenicity testes, 4 had bilateral  
265 hypochoic testes, 2 had bilateral hyperechoic testes, and 2 had bilateral hyperechoic  
266 testes with echogenic stippling in one testis. It is clear that subjective assessment of  
267 testicular architecture is difficult to relate to fertile status, since both normal appearing

268 and hypoechoic testes were seen in both fertile and infertile dogs. Hyperechoic testes  
269 were only seen in infertile dogs.

270

271 Using color Doppler, it was possible to identify the distal supra-testicular, marginal and  
272 intra-testicular artery regions of the testicular artery, similar to that previously reported  
273 in dogs [18,19]. Blood flow was measured in the three regions at each examination, and  
274 the distal supra-testicular artery was easiest to identify having a tortuous pattern also  
275 observed in men [20], stallions [21] and in dogs [5,18-19,22-23]. The marginal region  
276 had an appearance similar to that previously reported [5,18-19,23], whilst the intra-  
277 testicular arteries had a linear pattern directed towards the mediastinum, unlike a report  
278 documenting that flow could not be measured in these vessels [22]. In men, studies have  
279 shown that the intra-testicular arteries were better visualized oblique to the longitudinal  
280 and transverse planes [20], which is similar to the imaging plane used in this study.

281

282 Pulse-wave Doppler detected two different normal waveforms in the fertile dogs,  
283 similar to previous studies in men [20], camelids [12] and dogs [18,23]. Previous work  
284 has demonstrated regional differences in PSV, EDV, RI or PI with highest values  
285 present within the distal supra-testicular artery [24, 25]. Similar trends were present in  
286 both fertile and infertile dogs in the present study although regional differences were not  
287 statistically significant.

288

289 In the infertile dogs, waveforms had low peak systolic velocities and appeared more  
290 venous-like; PSV and EDV were significantly lower in all regions of the testicular  
291 artery in the infertile dogs compared with the fertile dogs. RI and PI did not differ  
292 between fertile and infertile dogs in any region. The finding that infertile dogs had

293 lower PSV and EDV but that RI and PI were not different to fertile dogs is interesting,  
294 since reduced blood flow with no change in the vascular bed resistance can only be  
295 mediated by multiple factors. Potentially infertile dogs had smaller diameter and less  
296 tortuous vessels, which may allow for reduced flow with no change in RI [26].  
297 The magnitude of decreased PSV and EDV in the testicular artery of infertile dogs was  
298 small and may be difficult to document in an individual clinical case. Nevertheless, the  
299 features of low testicular artery flow noted in the infertile dogs was similar to that seen  
300 in infertile llamas and alpacas [12].  
301  
302 Measurement of testosterone may be useful in a breeding soundness examination,  
303 although generally, frequent samples are needed to account for normal diurnal variation  
304 [27]. In this, study serum testosterone concentrations were similar to those previously  
305 reported in dogs [27], and interestingly there were significantly lower concentrations in  
306 infertile dogs although these remained in the normal range. Recent studies in man have  
307 shown that although diurnal variation of testosterone occurs, if samples are collected at  
308 the same time of the day, large variations can be overcome and single samples may be  
309 diagnostically useful [28]. From the present study, although differences would be  
310 difficult to detect and interpret in clinical practice, it might be postulated that a useful  
311 assessment of Leydig cell function can be achieved by a single basal testosterone  
312 measurement.  
313  
314 This study provides comprehensive evidence that components of a breeding soundness  
315 examination can be related to fertility in dogs. In particular detection of testicular  
316 softening, changes in some seminal characteristics, increased testicular echogenicity,  
317 reduced testicular artery blood flow, and decreased serum testosterone concentrations

318 are associated with infertility, and should form important components of an expanded  
319 BSE.

320

321

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