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Credit Author Statement

F. D´Francisco- Formal analysis -Writing original draft.

C. Lapuente- Methodology.

M. Lopez Merlo- Methodology.. C. Barbeito Software and Supervision;

C Gobello Conceptualization;; Funding acquisition; Investigation; Supervision;

Writing - review & editing

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**Feline testicular ultrasonogram differentiates pre vs. postpubertal and
normal vs. disrupted spermatogenesis**

F. D´Francisco^{a,b}, C. Lapuente^{a,b}, M. Lopez Merlo^{a,b}, C. Barbeito^b, C. Gobello^{a,b}

^aCenter of Reproductive Physiology, Faculty of Veterinary Sciences, National
University of La Plata & ^bCONICET, Argentina.

Corresponding Author:

Prof. Cristina Gobello, MV, DVM, DECAR

Center of Reproductive Physiology

Faculty of Veterinary Sciences,

National University of La Plata

60 y 118. La Plata CC 296 (B 1900 AVW), Argentina

E mail: cgobello@fcv.unlp.edu.ar – cristinagobello@gmail.com

Tel: 54-221-155239561 - 54-221-4825372

Fax: 54-221-425-7980 (Attn Dr Gobello)

26

Abstract

27

28 The aims of this study were: to ultrasonographically describe and compare testicular
29 parenchyma echogenicity and heterogeneity using digital image analysis in: I)
30 prepubertal (PREP), peripubertal (PERI) and mature (MAT) cats; II) Normal and
31 abnormal mature felids. Secondary, the relationships between histomorphological and
32 ultrasonographic attributes of the testes were also determined. I) Fourteen, PREP, PERI
33 and MAT male cats were ultrasonographically examined and then castrated. II) Seven
34 adult cats were ultrasonographically examined before and after a GnRH antagonist
35 administration and then castrated. All the testes were grossly and histomorphometrically
36 assessed. In the frozen digital images of the longitudinal ultrasound sections, 3 regions
37 of interest (ROI, 1 mm²) were selected. Within each ROI the echogenicity and the
38 heterogeneity of the testicular parenchyma were digitally analyzed. In experiment I,
39 testicular volume (0.15 ± 0.0 vs. 0.49 ± 0.1 vs. 1.65 ± 0.1 ; $P < 0.01$) and gonadosomatic
40 index (0.04 ± 0.0 vs. 0.05 ± 0.0 vs. 0.08 ± 0.0 ; $P < 0.01$), echogenicity (56.54 ± 0.75 vs.
41 81.87 ± 5.88 vs. 94.67 ± 3.62 ; $P < 0.01$) and heterogeneity (10.2420 ± 1.3740 vs. 13.65 ± 0.65
42 vs. 14.67 ± 1.49 ; $P < 0.01$) augmented throughout PRE, PERI, and MAT. In experiment
43 II, testicular volume (1.00 ± 0.09 vs. 0.85 ± 0.09 ; $P < 0.05$), echogenicity (87.74 ± 1.53
44 vs. 83.32 ± 1.54 ; $P < 0.01$) but not heterogeneity (14.09 ± 0.26 vs. 14.19 ± 0.29 ; $P > 0.05$)
45 decreased in the post GnRH antagonist abnormal testes. For both experiments, testicular
46 volume, seminiferous tubular diameter, percentage of spermatids as the most mature
47 cell type, and luminal/intertubular ratio were highly correlated ($P < 0.01$) with their
48 echotextural attributes. Computer-assisted image analysis of B mode ultrasonogram
49 appears as a good indicator of pubertal development and mild alterations of
50 spermatogenesis in felids.

51 **Keywords:** Puberty- Andrology – Ecography –Subfertility- Histology- Germinal
52 epithelium

53

54

55 **1. Introduction**

56

57 Domestic cats have been increasing in popularity as pets over their canine
58 counterparts. Several explanations for this shift have been postulated including that cats
59 cost less to keep, and are more feasible in urban settings. Domestic felids have also been
60 extensively used as models for human diseases and for assisted reproductive
61 technologies in wild endangered felids [1]. With the increasing interest in breeding cats
62 the requests to determine puberty and to investigate cases of reproductive failure have
63 become more frequent.

64 Sexual immaturity and testicular lesions, are causes of sub and infertility in
65 domestic cats [2,3]. Both puberty and mild testicular lesions are difficult to clinically
66 diagnose in this species. Although the most accurate test for diagnosis is testicular
67 biopsy, this “gold standard” procedure is invasive and, therefore, rarely performed in
68 practice. Thus, sensitive, non-invasive diagnostic methods are still required to
69 contribute to the breeding soundness examination in this species

70 Two-dimensional ultrasonography is a non invasive diagnostic technique that has
71 been widely used in the andrological exam of most mammalian species. Although,
72 testicular parenchyma is commonly described as a tissue of medium heterogeneity and
73 homogeneous echotexture [4] these appreciations are subjective. The present
74 availability of high-frequency and resolution equipments has permitted the accurate
75 evaluation of the cellular composition of a tissue. Additionally, digital image analysis

76 supports ultrasound findings to be a quantifiable tool. Thus, echogenicity i.e. pixel
77 intensity is described in terms of numerical values which range from 0 (absolute black)
78 to 255 (absolute white) and heterogeneity as pixel standard deviation of echogenicity,
79 which reflects the existence of interspersed hyper- and hypoechoic areas in a tissue [5].
80 These quantitative evaluations have been mainly carried out in farm animals testes [5-7]
81 and there are also some reports in horses and dogs [8,9]. Although, fine tuning of this
82 technique would contribute to the reproductive performance of this species, to date, no
83 computer-assisted image analysis of testicular ultrasonograms appear to have been
84 published in domestic cats.

85 Thus the aims of this study were twofold: to ultrasonographically describe and compare
86 testicular parenchyma echogenicity and heterogeneity using digital image analysis in: I)
87 prepubertal, peripubertal and postpubertal cats and II) Normal and abnormal mature
88 feline testes. Secondary, the relationships between histomorphological and
89 ultrasonographic attributes of the gonads were also determined. In Experiment II, a
90 pharmacological model of spermatogenic impairment was used. For this purpose,
91 acyline, a potent gonadotrophin releasing hormone (GnRH) antagonist was selected as
92 spermatogenic disruptor, based on previously described deleterious effects on feline
93 spermatogenesis [10].

94

95

96 **2. Materials and methods**

97

98 **2.1 Experiment I**

99

100 **2.1.1. Animals**

101

102 Fourteen, 5 to 36 months of age, 2.5 to 4.7 kg body weight, prepubertal (PREP),
103 peripubertal (PERI) and mature (MAT) healthy, crossbred male cats that were born in
104 our institutional cat colony were included in this study. The animals were kept free in
105 enriched 5 m² rooms with 14 hours of light/10 dark per day, fed *premium* commercial
106 food and given water *ad libitum*. This study was reviewed and approved by the Animal
107 Care and Use Committee of the Veterinary School of the National University of La
108 Plata and all experiments were conducted under the guidelines established in The Guide
109 for The Care and Use of Laboratory Animals, USA.

110

111 **2.1.2. Ultrasonographic evaluations**

112

113 Ultrasonographic evaluations were undertaken by a single experienced evaluator
114 using a real time B-mode ultrasound machine (Toshiba Nemio XG, Japan) with a 14
115 MHz lineal transducer in both testicles of each animal. All machine settings were
116 established at the first examination according to best image quality and remained
117 unaltered for all remaining examinations (Gain: 100, focal depth: 2 cm). Acoustic gel
118 was applied to the transducer and coupled directly to the clipped scrotum with minimum
119 pressure to obtain the images. The testes were imaged in the longitudinal and transverse
120 planes and dimensions were obtained from frozen images, using the ultrasound
121 callipers. Total testicular volume was also calculated as described by Linn et al. (2009
122 [11]). In the frozen digital images (jpeg of 640 x 480 pixels) of the longitudinal
123 sections, 3 regions of interest (ROI, 1 mm²) were selected, between the central
124 mediastinum and the testicular capsule (12 ROI/cat). Within each ROI the echogenicity

125 and the heterogeneity of the testicular parenchyma were analyzed using Image J
126 software (National Institutes of Health, Bethesda, Maryland, USA).

127

128 **2.1.3. Orchidectomies**

129

130 A routine open castration was performed [12]. For the surgery, the animals were
131 pre-medicated with atropine sulfate, (Atropine Sulfate, John Martin; 0.04 mg/kg,
132 subcutaneously), acepromazine maleate (Acedan, Holiday; 0.03 mg/ kg
133 subcutaneously), and butorphanol (Torbutol Plus, Fort Dodge; 0.2 mg/kg,
134 intramuscularly). Anesthesia was induced with sodium thiopental (Pentovet TM,
135 Richmond; 8 mg/kg, intravenous). After the males were endotracheally intubated,
136 anesthesia was maintained with isoflurane and oxygen in a closed system. After surgery
137 ketoprofen (Ketofen, Fort Dodge; 1 mg/kg) was injected subcutaneously (once) and
138 then orally every 24 hours for 4 additional days. After orchidectomy all the cats were
139 placed for adoption.

140

141 **2.1.4. Gross, seminal and histological examination**

142

143 Immediately after surgical removal, the testes were weighed (g). Gonadosomatic
144 index (%; [13]) was also calculated. The testes were sectioned longitudinally, placed in
145 Bouin's fixative for 24 h and then changed to alcohol 70 and processed routinely with
146 paraffin embedding. After processing, 5 μ m serial sections were cut, mounted on slides,
147 dried, deparaffinized in xylene, rehydrated in graded ethanol solutions and stained with
148 hematoxylin and eosin. All histological determinations were made by a single operator
149 without knowledge of the age at which the animals were castrated.

150 Histological images were obtained from a microscope (Olympus BX50, Tokyo, Japan);
151 10X or 40X through an attached digital RGB video camera (Omax A35180U3, China)
152 and digitalized in a 24 bit true color TIFF format. Ten round tubular profiles per testis
153 were evaluated for mean tubular diameter (μm), mean germinal epithelium height (μm),
154 as well as the identification of the most developed germ cell found in the seminiferous
155 tubules of each cat. The percentages of tubular cross sections containing different
156 identified most mature cell were recorded. The proportion of the tubular/intertubular
157 and luminal/intertubular compartments were also calculated. Images were analyzed by
158 planimetry (Image Pro Plus v6.0-Media Cybernetics, Silver Spring, MA, USA).

159 Peripubertal state (PERI) was defined when tubular diameter was 100 -150 μm
160 and a multilayer germinal epithelium (up to the spermatide or spermatozoa stage) was
161 present in more than 50% of the tubules. Lower and higher values were considered pre
162 (PREP) and mature (MAT) states, respectively [14].

163

164 **2.2. Experiment II**

165

166 **2.2.1. Animals and pharmacological protocol**

167

168 Seven, 1 to 3 years old, 3.2 to 4.8 kg, cross-bred fertile cats from our
169 institutional cat colony were administered acyline (Contraception & Reproductive
170 Health Branch Center for Population Research, NIH, Bethesda, MD, USA) 330 $\mu\text{g}/\text{kg}$
171 SC once a week for 4 consecutive weeks. Acyline [acetyl-D₂Nal-D₄CIPhe-D₃PalSer-
172 Aph(ac)-DAph(Ac)-Leu-Lys(lpr)-Pro-D-Ala-Nh₂] was provided in a lyophilized
173 powder which was suspended in sterile distilled water (concentration, 2 mg/mL).The
174 antagonist dose and frequency of administration were selected according to our previous

175 studies [10,15]. The animals were kept under the experimental conditions described for
176 Experiment I. This study was reviewed and approved by the Animal Care and Use
177 Committee of the Veterinary School of the National University of La Plata and all
178 experiments were conducted under the guidelines established in The Guide for The Care
179 and Use of Laboratory Animals, USA.

180

181 **2.2.2. Follow up**

182

183 Testicular ultrasound evaluations were carried out one week before treatment
184 (PRETR) and one week after the last antagonist administration (POSTR). Then all the
185 cats were submitted to orchidectomies. Immediately after surgical removal, the testes
186 were grossly and histologically assessed. All ultrasonographic and histological
187 procedures were carried out as explained for Experiment I. The castrated cats were also
188 placed for adoption.

189

190 **2.3. Statistical analysis**

191

192 Normality of obtained data of both experiments was confirmed by Shapiro-Wilk
193 test. Gross testicular parameters and ROIs echogenicity and heterogeneity values of the
194 3 groups (PREP vs. PERI vs. MAT) of Experiment I and the 2 groups (PRETR vs.
195 POSTR) of Experiment II were compared by one way ANOVA followed by Tukey test
196 and paired Student's t test, respectively. For both experiments, echotextural parameters
197 were correlated with gross and microscopic attributes of the testes by Pearson
198 correlation test. Descriptive statistic was expressed as mean \pm SEM and the level of
199 significance was set at $P < 0.05$ (SPSS, Inc., Chicago, IL, USA).

200

201 3. Results

202

203 In Experiment I, 6, 2 and 6 cats were histomorphometrically classified as PREP,
204 PERI, and MAT, respectively. Testicular volume (0.15 ± 0.0 vs. 0.49 ± 0.1 vs. 1.65 ± 0.1 ;
205 $P < 0.01$) and gonadosomatic index (0.04 ± 0.0 vs. 0.05 ± 0.0 vs. 0.08 ± 0.0 ; $P < 0.01$)
206 increased with reproductive development, showing differences among the 3 groups.
207 Both echogenicity ($P < 0.01$) and heterogeneity ($P < 0.01$) also augmented throughout
208 PREP, PERI, and MAT (Fig. 1).

209 In Experiment II, acyline caused mild histological spermatogenic impairment with a
210 diminution of 34% and 18% of normal germinal height and tubular diameter,
211 respectively when compared to what has been reported for normal testes in this species
212 [13]. In this experiment, testicular volume (cc; 1.00 ± 0.09 vs. 0.85 ± 0.1 ; $P < 0.05$) and
213 parenchyma echogenicity ($P = 0.01$) but not heterogeneity ($P > 0.05$; **Fig. 2**) decreased
214 in POSTR. Correlations between gross (also **Fig. 3**) and histomorphological testicular
215 parameters with echotextural attributes of both experiments are shown in **Table 1**.

216

217 4. Discussion

218

219 Male cats are particularly difficult to andrologically examine not only because of
220 their comparatively reduced body size but also for their innate tendency to experience
221 stress requiring sedation or even anesthesia for minor maneuvers. Thus, in this study,
222 computer-assisted image analysis of B mode ultrasonogram was evaluated as a non-
223 invasive technique to contribute to the male cat breeding soundness examination.

224 As expected, in the growing cats of Experiment I, testicular volume and
225 gonadosomatic index increased up to what has been previously described as adult
226 values [13]. In line with reports in humans [16], ruminants [5,17-19] and horses [9], in
227 these cats, computer-assisted analysis of testicular ultrasonograms could differentiate
228 pre from postpubertal state.

229 In domestic felids, the same as in other mammals, the onset of spermatogenesis
230 during peripubertal development is accompanied by changes in testicular microstructure
231 [14]. An increase of seminiferous tubule diameter and lumen as well as of the epithelial
232 height, due to the appearance of more mature spermatogenic cell types, occur [19].
233 Thus, the increase in echogenicity is associated with these testicular changes leading to
234 the onset of pubertal spermatogenesis [5,17,20]. Furthermore in agreement with a
235 similar study in ram lambs, the seminiferous tubular diameter of the present study had
236 the strongest correlation with testicular echotexture [5].

237 To investigate the echotextural characteristics of adult cats with impaired
238 spermatogenesis, a suitable pharmacological model was used for Experiment II. As
239 previously reported [15], in these cats, the GnRH antagonist treatment reduced testicular
240 volume causing mild seminiferous tubular deterioration which could mimic subfertility
241 cases. In spite of these mild histomorphometric testicular changes, computer-assisted
242 image analysis of B mode ultrasonogram could differentiate abnormal from normal
243 spermatogenesis by a significant decrease in pixel intensity. Although it can be assumed
244 than more severe lesions, e.g. testicular degeneration or atrophy, could be quantitatively
245 more evident, the reproductive importance to these end stage lesions is much less
246 clinically relevant. Similarly, in bulls echogenicity decreased in the first 2–3 weeks after
247 scrotal insulation, coincidentally with the diminution in sperm motility and normal
248 morphology [21]. In stallions, retained abdominal testes have also lower and less

249 heterogeneous echogenicity than scrotal testes [9]. In domestic dogs, poor seminal
250 quality has been associated with decreased parenchyma echogenicity [22] and future
251 sperm motility was positively related to gonadal echogenicity [23].

252 Testicular spermatogenic cells are known to affect the grey-scale appearance of
253 scrotal ultrasonograms [5] and changes in the composition of tubular cell population
254 affect echotexture. Thus, in both experiments, the cytological and echotextural
255 correlations shifted from negative with the less mature cell types -i.e. spermatogonias -
256 to positive with the more mature cell types - i.e. elongated spermatids- in
257 spermatogenesis evidencing a marked increase in echogenicity.

258 It was concluded that, in domestic cats, testicular parenchyma variations were
259 accompanied by changes in their echotexture, thus, computer-assisted image analysis of
260 B mode ultrasonogram appears as a good indicator of pubertal development and mild
261 alterations of spermatogenesis. Further research in a larger number of animals needs to
262 be carried out before computerized image analysis of ultrasonograms may be widely
263 used in clinical settings.

264

265 **Author statement**

266

267 Florencia D'Francisco: Analysis and initial interpretation of data, Writing-
268 Original draft preparation. Cristina Gobello: Conceptualization, Methodology, Writing-
269 Reviewing and Editing, General supervision, Funding. Mariana Lopez Merlo:
270 Acquisition and interpretation of echographical data. Camila Lapuente: Investigation:
271 Claudio Barbeito: Acquisition and interpretation of histological data.

272

273 **Declaration of competing interest**

274

275 The authors declare that there is no conflict of interest that could be perceived as
276 prejudicing the impartiality of the research reported.

277

278

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280

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287 Argentina.

288

289

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291

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375 **Figure legends**

376

377 **Fig. 1:** Testicular (mean \pm SEM) echogenicity (solid; $P < 0.01$) and heterogeneity
378 (scattered; $P < 0.01$) of prepubertal (PREP; $n=6$), peripubertal ($n=2$; PERI), and mature
379 (MAT; $n=6$) male cats. Different letters above the columns show differences of $P < 0.01$.

380

381 **Fig. 2:** Testicular (mean \pm SEM) echogenicity (solid; $P < 0.01$) and heterogeneity
382 (scattered; $P > 0.05$) of 7 adult male cats before (PRE) and after (POSTR) 4 weekly
383 administrations of sc acyline (330 μ g/kg) SC. Different letters above the columns show
384 differences of $P = 0.01$.

385

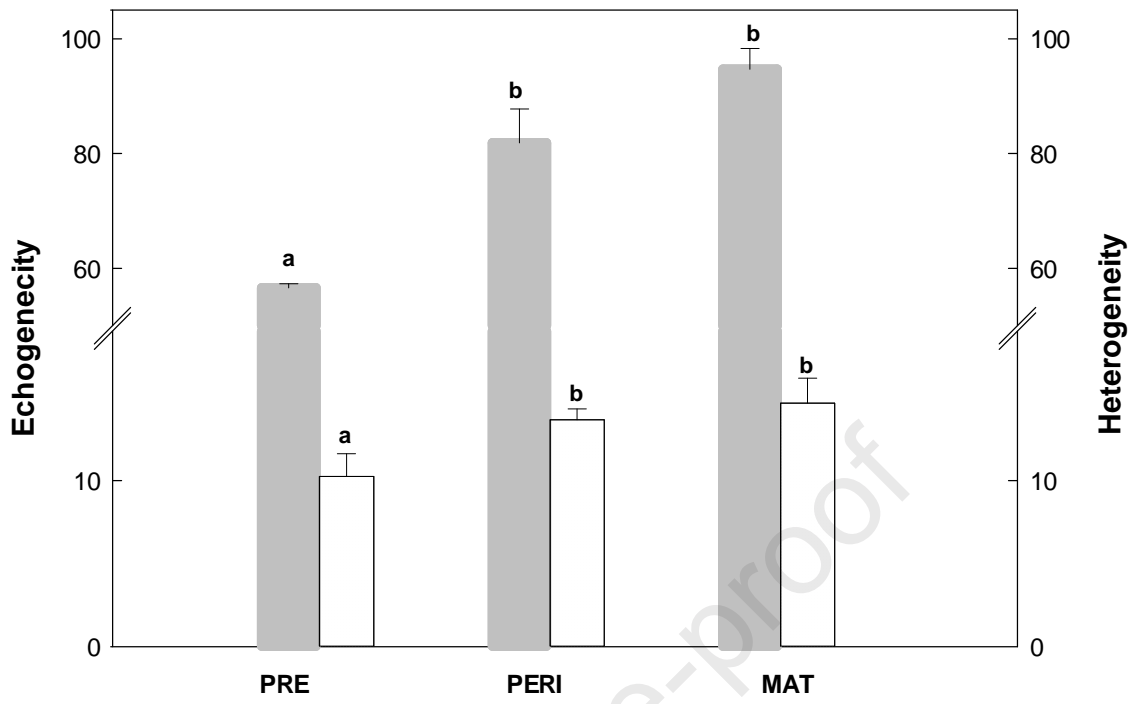
386 **Fig. 3:** Correlations between gonadosomatic index and echogenicity (**A**; $P < 0.01$) and
387 heterogeneity (**B**; $P < 0.01$) of cats of **Fig 1 and 2**. See also **Table 1**.

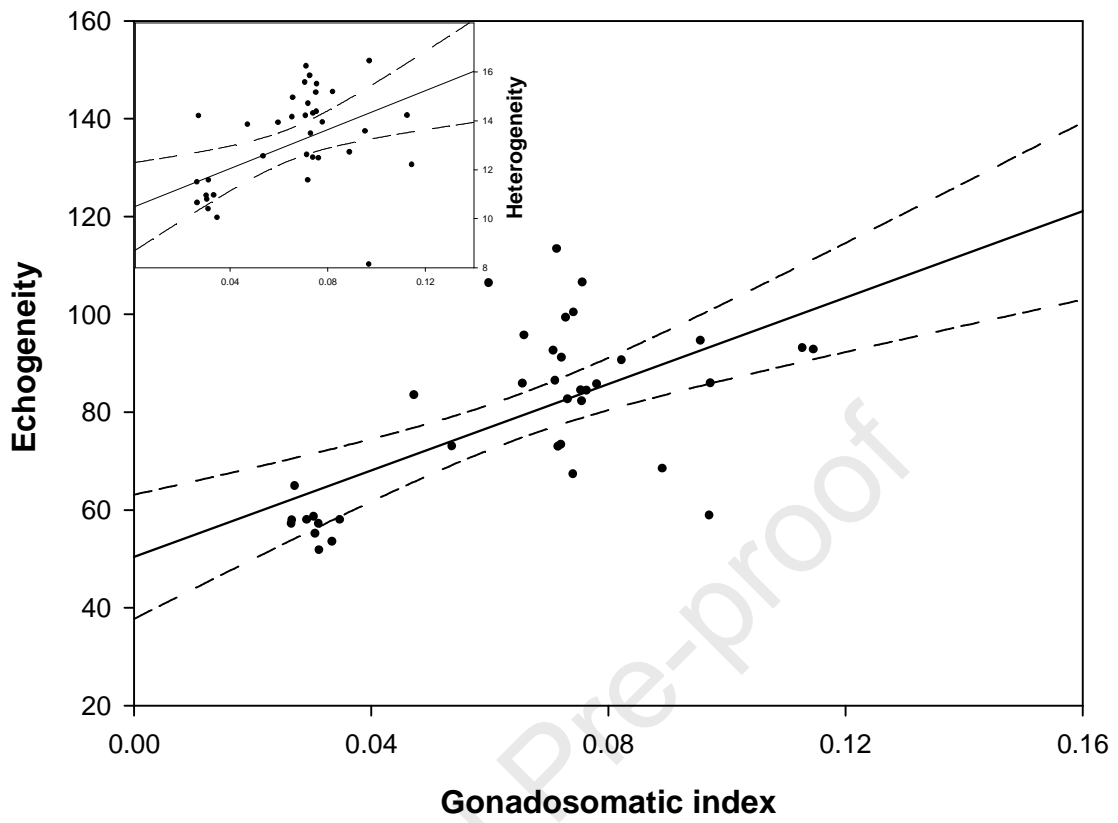
388

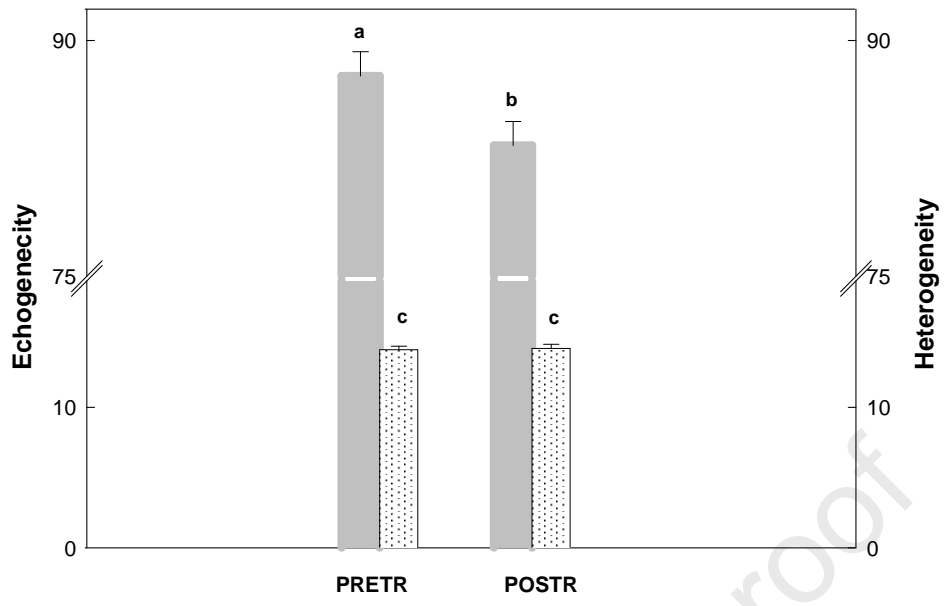
389 **Table 1:** Correlations between testicular gross and histomorphological with
390 echotextural attributes of cats of **Fig 1 and 2**.

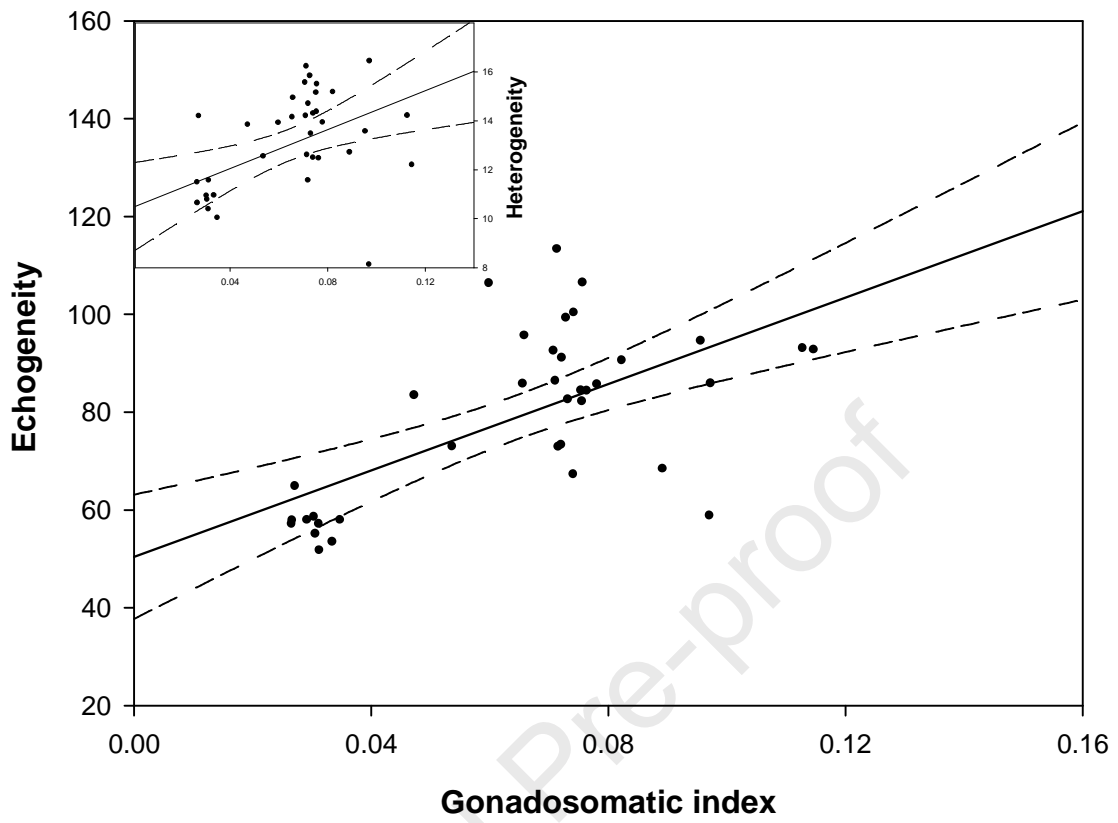
391

Gross & microscopic parameters	Echogenicity	<i>P</i>	Heterogeneity	<i>P</i>
Testicular volume (cc ³)	0.58	< 0.01	0.69	< 0.01
Gonadosomatic index	0.63	< 0.01	0.46	< 0.01
Tubular diameter (μm)	0.69	< 0.01	0.71	< 0.01
Epithelium height (μm)	0.08	> 0.1	0.22	> 0.1
Spermatogonia (%)	-0.75	< 0.01	-0.78	< 0.01
Primary spermatocytes (%)	0.14	> 0.1	0.12	> 0.1
Round spermatids (%)	0.41	0.01	0.43	0.01
Elongated spermatids (%)	0.62	< 0.01	0.69	< 0.01
Spermatozoa (%)	0.39	< 0.05	0.23	> 0.1
Tubular/intertubular ratio	0.38	< 0.05	0.23	> 0.1
Luminal/intertubular ratio	0.54	< 0.01	0.42	0.01









Highlights

In domestic felids, increasing testicular echogenicity and heterogeneity characterize pubertal attainment.

In mature cats, decreasing testicular echogenicity indicates spermatogenic impairment.

Testicular volume and gonadosomatic index correlate with echogenicity and heterogeneity.

Testicular echogenicity and heterogeneity correlate with tubular diameter, percentage of elongated spermatids and luminal/intertubular ratio.

Testicular echogenicity correlates with tubular/intertubular ratio.

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