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Feline testicular ultrasonogram differentiates pre vs. postpubertal and

normal vs. disrupted spermatogenesis

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25

26   **Abstract**

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28   The aims of this study were: to ultrasonograhically 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

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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 ultrasonograhically 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].

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96   **2. Materials and methods**

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98   **2.1 Experiment I**

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100   **2.1.1. Animals**

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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 m^2 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 mm^2) 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 μm 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**

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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 μg/kg

171   SC once a week for 4 consecutive weeks. Acyline [acetyl-D2Nal-D4ClPhe-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±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, coincidently 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.

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278

279 **Acknowledgements**

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375   **Figure legends**

376

377   Fig. 1: Testicular (mean ±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 ±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 μg/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	p	Heterogeneity	p
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
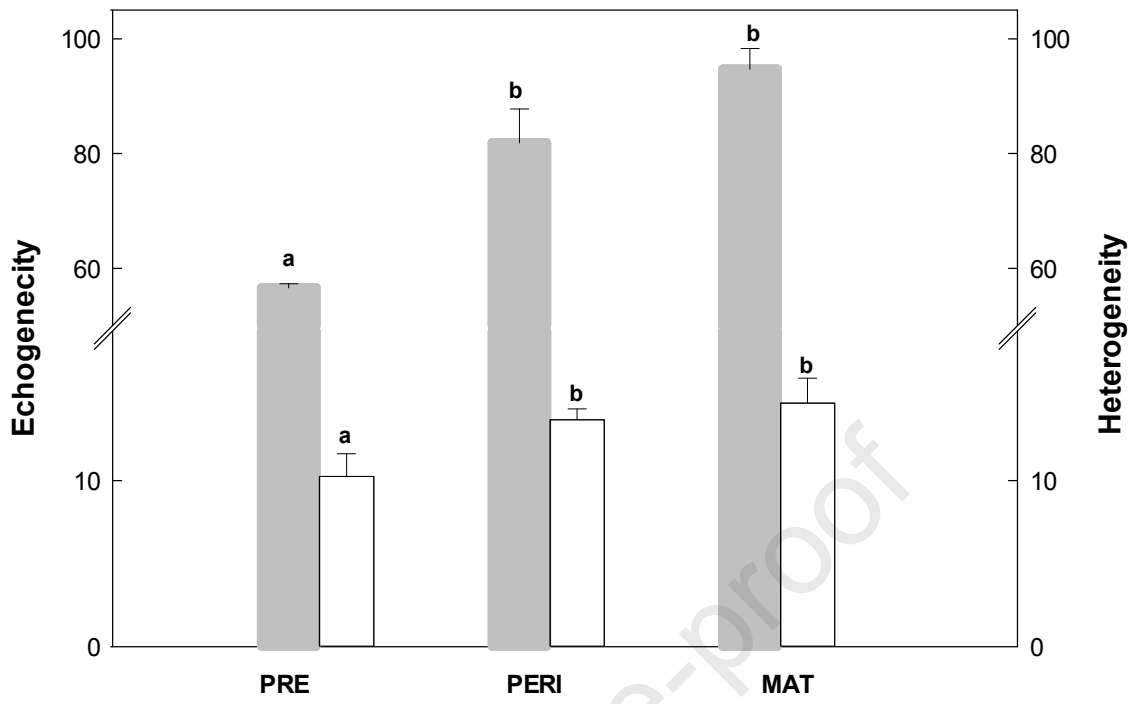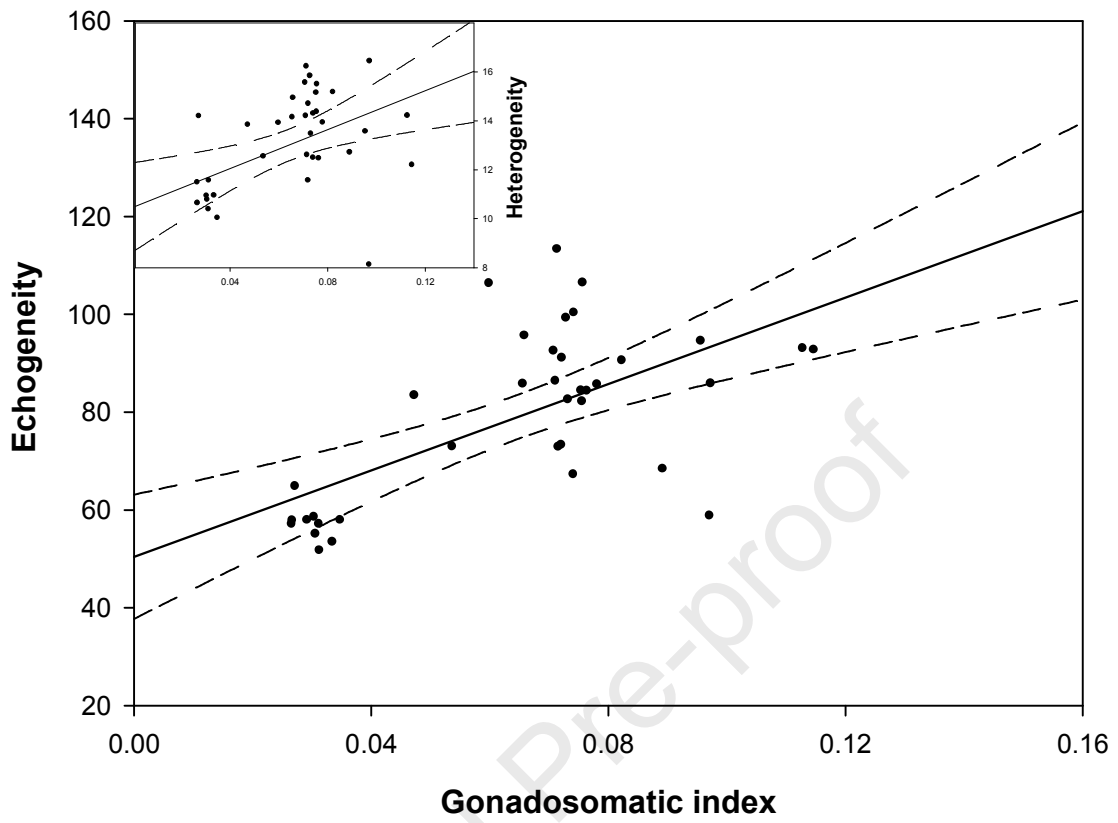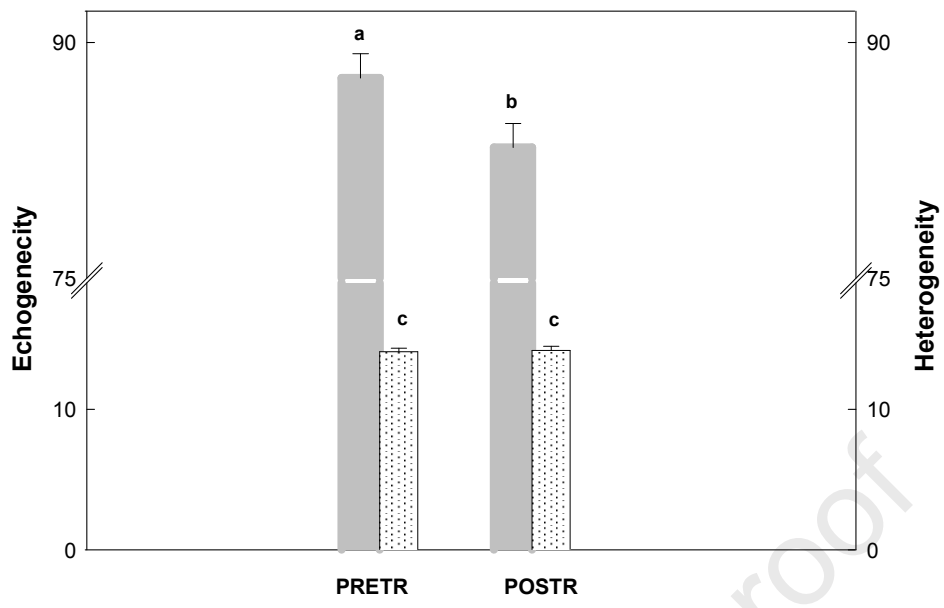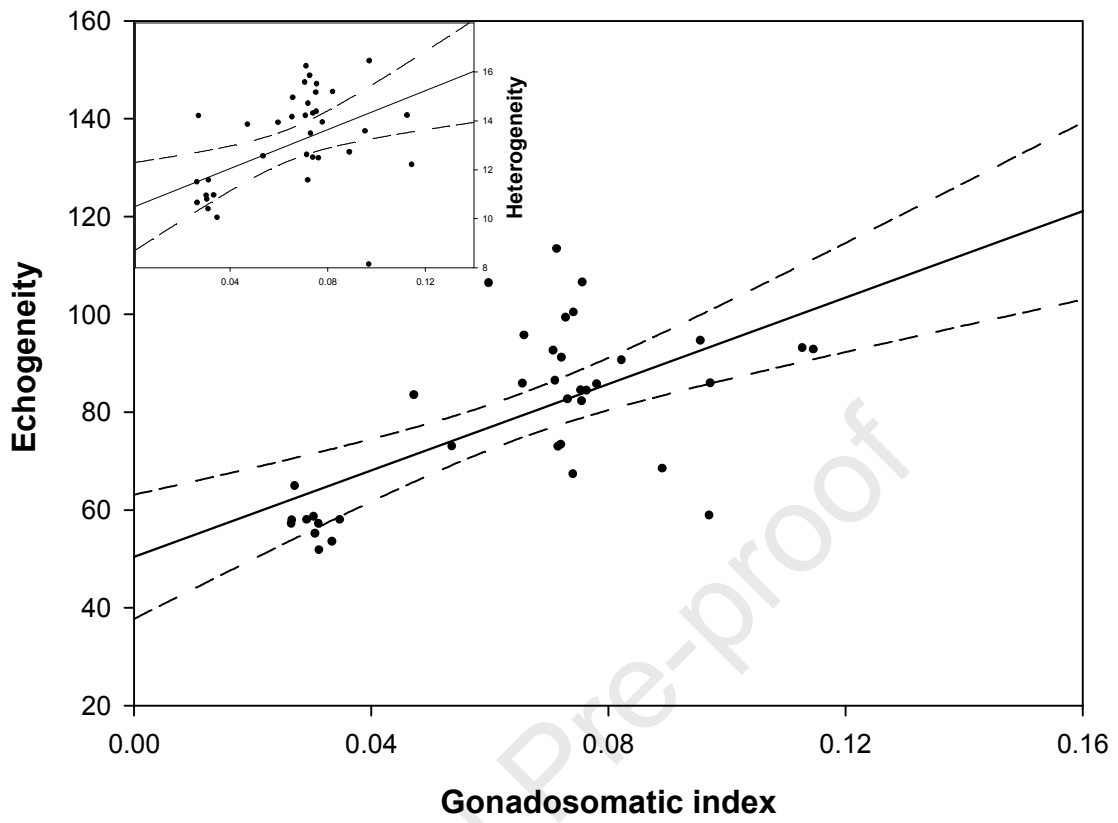
Highlights

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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